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Accepted for publication 27 September 2009

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EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Long-term use of entecavir in nucleoside-naïve Japanese patients with chronic hepatitis B infection☆

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Background & Aims: To evaluate the long-term efficacy of entecavir in nucleoside-naïve chronic hepatitis B patients.

Methods: One hundred and sixty-seven patients treated with entecavir 0.01 mg, 0.1 mg or 0.5 mg for 24–52 weeks in Phase II studies entered rollover study ETV-060 and received entecavir 0.5 mg daily. Responses were evaluated among patients with available samples.

Results: After 96 weeks in ETV-060 (120–148 weeks total entecavir treatment time), 88% (127/144) of patients had HBV-DNA <400 copies/ml; 90.1% (128/142) had alanine aminotransferase (ALT) ⩽1× the upper limit of normal (ULN) among those with abnormal baseline ALT; and 26% (32/121) achieved HBe seroconversion among those HBeAg(+) at baseline. A subset of 66 patients received entecavir 0.5 mg (approved dose) from Phase II baseline: at week 96 in ETV-060, 83% (48/58) had HBV-DNA <400 copies/ml, 88% (52/59) had ALT ⩽1× ULN, and 20% (10/49) achieved HBe seroconversion. Twenty-one out of 66 patients had paired baseline and on-treatment biopsies: 100% (21/21) and 57% (12/21) demonstrated histologic improvement, and improvement in fibrosis, respectively, over 3 years. The 3-year cumulative probability of resistance was 3.3% for all patients and 1.7% for the 0.5 mg subset.

Conclusions: Long-term entecavir for nucleoside-naïve patients resulted in high rates of virological, biochemical, and histological response, with minimal resistance.

Keywords: Entecavir; Nucleoside-naïve; Long-term treatment; Japanese; Chronic hepatitis B.

Received 22 April 2009; received in revised form 1 December 2009; accepted 9 December 2009; available online 24 March 2010

☆ The work was carried out at: Sapporo Kosei General Hospital, Hokkaido, Japan; Iwate Medical University, Iwate, Japan; Tohoku University Hospital, Miyagi, Japan; Saitama Medical School, Saitama, Japan; Graduate School of Medicine, Chiba University, Chiba, Japan; Keio University Hospital, Tokyo, Japan; International Medical Center of Japan, Tokyo, Japan; Nihon University School of Medicine, Tokyo, Japan; Toranomon Hospital, Tokyo, Japan; Niigata University Medical and Dental Hospital, Niigata, Japan; University of Yamanashi Hospital, Yamanashi, Japan; Shinsyu University School of Medicine, Nagano, Japan; Graduate School of Medicine, Nagoya University, Aichi, Japan; Graduate School of Medical Science, Nagoya City University, Aichi, Japan; Social Insurance Central General Hospital, Aichi, Japan; Gifu Municipal Hospital, Gifu, Japan; Ogaki Municipal Hospital, Gifu, Japan; Kyoto Prefectural University of Medicine, Kyoto, Japan; Osaka University Graduate School of Medicine, Osaka, Japan; Osaka Koseinenkin Hospital, Osaka, Japan; National Hospital Organization, Osaka National Hospital, Osaka, Japan; Osaka Rosai Hospital, Osaka, Japan; National Hospital Organization, Osaka Minami Medical Center, Osaka, Japan; Akashi Municipal Hospital, Hyogo, Japan; Okayama University Graduate School of Medicine, Dentistry, and Pharmaceutical Sciences, Okayama, Japan; Kawasaki Hospital, Okayama, Japan; Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; Yamaguchi University Hospital, Yamaguchi, Japan; Ehime University Hospital, Ehime, Japan; Fukuoka University Hospital, Fukuoka, Japan; Kurume University School of Medicine, Fukuoka, Japan; Nagasaki University Hospital of Medicine and Dentistry, Nagasaki, Japan; National Hospital Organization, Nagasaki Medical Center, Nagasaki, Japan; Oita University Faculty of Medicine, Oita, Japan; Kumamoto University Hospital, Kumamoto, Japan; Faculty of Medicine, University of Miyazaki, Miyazaki, Japan; Inazumi Memorial Hospital, Hokkaido, Japan; Okayama Saiseikai General Hospital, Okayama, Japan; Kagawa Prefectural Central Hospital, Kagawa, Japan; Musashino Red Cross Hospital, Tokyo, Japan; Kurashiki Central Hospital, Okayama, Japan; Tsuyama Central Hospital, Okayama, Japan; Hiroshima City Hospital, Hiroshima, Japan; Fukuyama City Hospital, Hiroshima, Japan; Mitoyo General Hospital, Kagawa, Japan.

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Abbreviations: CHB, chronic hepatitis B; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBeAg, hepatitis B e antigen; ALT, alanine aminotransferase; HAI, histologic activity index; ULN, upper limit of normal; PCR, polymerase chain reaction; ITT, intention-to-treat.

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Journal of Hepatology **2010** vol. 52 | 791–799

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Introduction

Chronic hepatitis B (CHB) affects 350–400 million people worldwide [1]. The prevalence is highest in the Asia–Pacific region, where 75% of all chronically infected individuals live, and up to 25% of CHB patients die of liver cirrhosis, hepatic decompensation or hepatocellular carcinoma (HCC) [2]. In Japan, the prevalence of CHB ranges from 0.8% to 4%, with geographic variation within the country [2–5]. The vast majority of CHB patients in Japan are infected with hepatitis B virus (HBV) of genotype C [6,7]. Infection with genotype C virus has been associated with delayed HBe seroconversion, more advanced liver disease, and increased probability of HCC development [8–11].

Recent studies have shown that CHB patients with moderate or elevated serum HBV-DNA are at the highest risk of developing long-term complications, including cirrhosis and HCC [11,12–14]. Yuen et al. showed that among Asian patients with CHB, disease progression was also seen in patients with persistently detectable viraemia and normal or minimally elevated levels of alanine aminotransferase (ALT), including patients who had achieved HBe seroconversion [12]. Consistent with these findings, current CHB treatment recommendations emphasize the importance of prolonged maximal HBV-DNA suppression and the avoidance of resistance [15–17].

Medications currently used for CHB include interferons (conventional and pegylated), lamivudine, adefovir, telbivudine, and entecavir. The interferons are efficacious in a subgroup of patients with genotype A infection, low baseline viral load and elevated baseline ALT but are often associated with treatment-limiting adverse events [18–20]. Lamivudine is well tolerated and initially efficacious, but the emergence of resistance in approximately 70% of patients after 4–5 years limits its benefit during long-term therapy [21,22]. Adefovir treatment is frequently associated with suboptimal HBV-DNA suppression and a cumulative probability of resistance of 29% at 5 years among HBeAg(−) patients, and resistance appears to be higher in the HBeAg(+) population [23–25]. Treatment with telbivudine leads to virological breakthrough, with resistance in 21.6% of HBeAg(+) and 8.6% of HBeAg(−) patients after only 2 years [26].

Entecavir has been shown to be highly effective at suppressing HBV-DNA replication to undetectable levels and normalizing ALT in Phase II studies of nucleoside-naïve CHB patients in Japan and in multinational studies [27–30]. Treatment for 24 weeks in the Japanese study ETV-047 showed that entecavir 0.5 mg daily resulted in superior viral load reduction compared with lamivudine 100 mg daily [28]. In the Japanese study ETV-053, treatment with entecavir 0.5 mg daily for 52 weeks resulted in significant histological improvement as well as viral load reduction and ALT normalization [27]. Immediately after completion of treatment in study ETV-047 or ETV-053, patients were eligible to enrol in rollover study ETV-060 and receive entecavir 0.5 mg daily. We present the long-term efficacy, safety, and resistance results for patients treated with entecavir in Phase II studies who rolled over into study ETV-060, for a total entecavir treatment time of up to 3 years (120–148 weeks). A subset of patients received the approved dose of entecavir (0.5 mg daily) continuously from Phase II baseline, and results for that cohort are also presented.

Patients and methods

Study design

Study ETV-060 was a rollover study designed to provide open-label entecavir to patients who completed previous entecavir therapy in Phase II studies ETV-047 or ETV-053 in Japan. In study ETV-047, 137 nucleoside-naïve patients were randomized to a range of daily doses of entecavir (0.01 mg [$n = 35$], 0.1 mg [$n = 34$], 0.5 mg [$n = 34$]) or lamivudine 100 mg [$n = 34$] for 24 weeks [34]). In study ETV-053, 66 nucleoside-naïve patients were randomized to entecavir 0.1 mg ($n = 32$) or entecavir 0.5 mg ($n = 34$) daily for 52 weeks [27]. Patients who completed 24 weeks of entecavir treatment in study ETV-047 ($n = 101$) or 52 weeks of entecavir treatment in study ETV-053 ($n = 66$) were enrolled in ETV-060 and received entecavir 0.5 mg daily in an open-label fashion. After 96 weeks of treatment in study ETV-060, patients could discontinue the study and were eligible to receive commercially available entecavir, which was approved by Japanese health authorities while study ETV-060 was ongoing. The current analysis describes results for patients who completed 96 weeks in study ETV-060 for a total entecavir treatment time of 120 weeks (patients from –047) or 148 weeks (patients from –053) (Fig. 1). Patients began dosing in ETV-060 immediately after completion of the previous study with no treatment gap or interruption.

During study ETV-060, clinical and laboratory assessments (serum chemistries, haematology, prothrombin time/INR, urinalysis) were made at baseline, at weeks 2 and 4, and every 4 weeks thereafter during dosing. Assessments of HBV-DNA by PCR assay and HBV serologies were performed at baseline, weeks 12 and 24, and subsequently every 24 weeks during dosing. Baseline liver biopsies in study ETV-053 were performed within 6 weeks of initiation of study therapy; or if a liver biopsy had been previously obtained within 52 weeks before initiation of protocol therapy, it was used as the baseline specimen for histological evaluation. Liver biopsies were evaluated using the Knodell Histologic Activity Index (HAI) and Knodell fibrosis scores and the New Inuyama classifications [31].

The study was conducted in compliance with the ethical principles of the Declaration of Helsinki, Good Clinical Practice guidelines, and Articles/Notifications of the Ministry of Health, Labour and Welfare in Japan. Written informed consent was obtained from all patients.

Study population

Inclusion criteria for studies ETV-047 and ETV-053 have been described previously [27,28]. Eligible patients were adults with CHB infection, compensated liver disease, and no more than 12 weeks prior treatment with anti-HBV nucleoside analogues. Patients could be HBeAg(+) or (−), and were required to have elevated ALT (1.25–10× the upper limit of normal [ULN] in ETV-047 and 1.3–10× ULN in ETV-053 at screening) and active viral replication (HBV-DNA $\geq 10^5$ copies/ml by PCR assay at screening in ETV-053 and $\geq 10^{7.6}$ copies/ml for patients in ETV-047). Patients were excluded from studies –047 and –053 if they had cirrhosis or evidence of liver decompensation, other forms of liver disease or suspected hepatic tumours, HIV infection or treatment with immunosuppressive therapy or interferon within 24 weeks prior to initiation of study medication. Pregnant and nursing women were also excluded.

Efficacy analyses

Efficacy end points included proportions of patients achieving the following: HBV-DNA <400 copies/ml, ALT normalization (ALT $\leq$1.0× ULN) among patients with abnormal ALT at baseline, and HBeAg loss and HBe seroconversion among patients HBeAg(+) at baseline. Histological end points are presented for the cohort that received entecavir 0.5 mg daily from Phase II baseline and include improvement in Knodell HAI and Knodell fibrosis scores among patients with evaluable biopsy pairs. Histological improvement was defined as a $\geq$2-point decrease in the Knodell necroinflammatory score and no worsening of fibrosis (worsening: $\geq$1-point increase in the Knodell fibrosis score). Improvement in fibrosis was defined as a $\geq$1-point decrease in the Knodell fibrosis score. Histological results were also assessed by the New Inuyama classification [31].

Safety analyses

Safety analyses included the incidence of adverse events, serious adverse events, laboratory abnormalities and discontinuations due to adverse events on treatment during ETV-060, including data for patients treated beyond 96 weeks. On-treatment ALT flares were defined as ALT >2× baseline and >10× ULN.

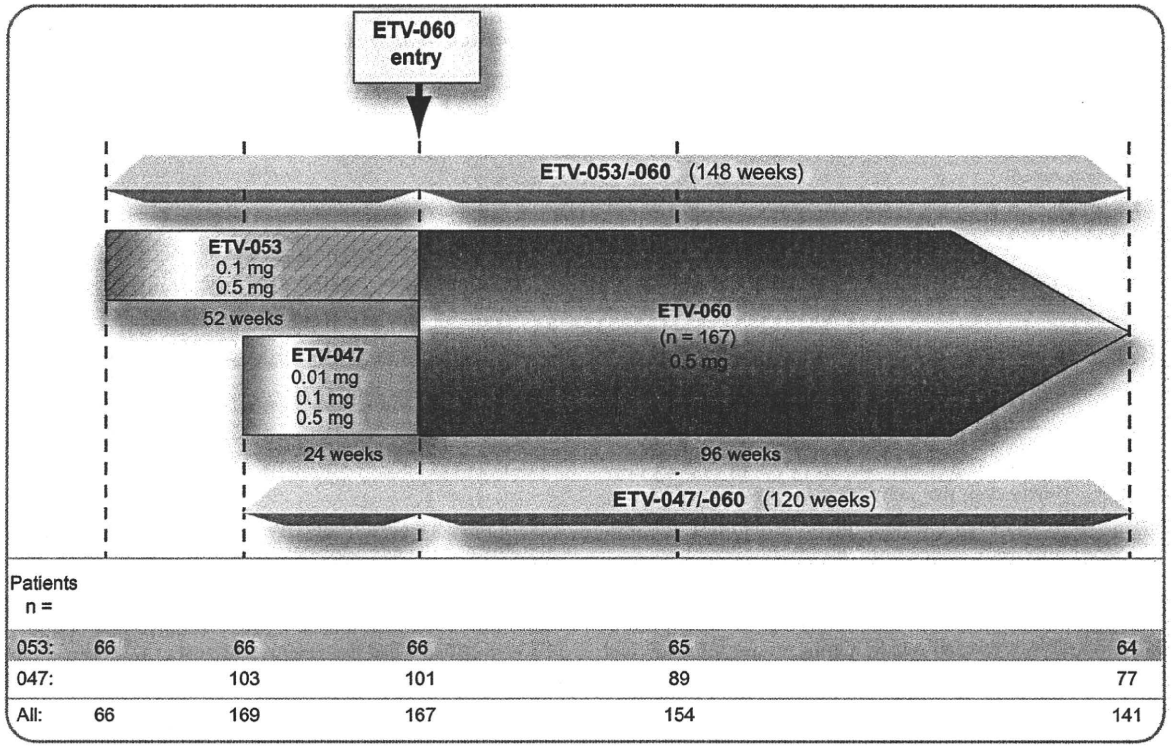

Fig. 1. Entecavir nucleoside-naïve long-term treatment cohort. One hundred and one patients completed 24 weeks of entecavir 0.01 mg, 0.1 mg or 0.5 mg treatment daily in study ETV-047, and 66 patients completed 52 weeks of entecavir 0.1 mg or 0.5 mg treatment daily in study ETV-053. Of these, 167 were enrolled in study ETV-060 with no interruption or gap in treatment. One hundred and forty-four patients remained on entecavir 0.5 mg daily through 96 weeks in study ETV-060 (for a total entecavir treatment time of 120–148 weeks).

Resistance monitoring

During treatment, HBV polymerase/reverse transcriptase substitutions were analyzed for all patients who had HBV-DNA ⩾400 copies/ml at weeks 100 and 120 (from Phase II [pre-treatment] baseline) for patients originating in study ETV-047, and at weeks 100 and 148 for patients originating in study ETV-053. Samples from all patients, who experienced virological breakthrough during ETV-060 (increase in HBV-DNA of ⩾1 $\log_{10}$ copies/ml from nadir in two consecutive measurements), were also analyzed for HBV polymerase/reverse transcriptase substitutions.

Assay methods

Serum HBV-DNA was determined by Roche Amplicor™ PCR assay (LOQ = 400 copies/ml; Roche Diagnostics K.K., Tokyo, Japan) in a central laboratory. Clinical laboratory tests, PCR assays for HBV-DNA, and serological tests were performed at SRL, Inc. (Tokyo, Japan), the central clinical laboratory designated by the trial sponsor. Genotypic analysis of HBV strains was performed using a PCR-based restriction fragment length polymorphism assay (SRL, Inc., Tokyo, Japan). On-treatment testing for resistance was carried out using a direct-sequencing PCR method.

Statistical analysis

Analyses of efficacy and safety end points were based on patients who received at least one dose of study medication in study ETV-060. Only descriptive summaries were performed. Parameters represented by continuous variables were summarized by the mean, median, standard deviation, minimum, and maximum. Analyses of HBV-DNA as a continuous parameter were applied after $\log_{10}$ transformation. In the analysis of binary end points, patients with missing on-treatment measurements were treated as missing (non-completer = missing). An additional sensitivity analysis using the last observation carried forward method was conducted for the end point of HBV-DNA <400 copies/ml at week 96. In this analysis, the last observed HBV-DNA levels were carried forward for patients without week 96 measurements, i.e., patients who either discontinued prior to week 96 or who were still on study but had a missing HBV-DNA measurement at week 96.

Results

One hundred and sixty-seven patients were treated with entecavir in Phase II studies ETV-047 or -053 and entered ETV-060 (Fig. 1). Twenty-three patients discontinued treatment during ETV-060 for the following reasons: adverse event (6), protocol violation (2), withdrawal of consent (4), pregnancy (1), loss to follow-up (4), insufficient effect (1), and complete response (4) or stability of disease condition (1) in the judgement of the investigator. Table 1 shows the baseline (pre-treatment) demographics and disease characteristics for all treated patients ($n = 167$); the cohort of patients who received the approved dose of entecavir (0.5 mg daily) from Phase II baseline through the end of treatment ($n = 66$); and the subset of patients who received 0.5 mg entecavir and had biopsies at baseline, week 48, and weeks 144–148. Among all treated patients, 72% were male, and the mean age was 43 years. Mean HBV-DNA was 7.88 $\log_{10}$ copies/

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Table 1. Baseline demographics and disease characteristics of the entecavir nucleoside-naïve long-term treatment cohort ($n = 167$), the entecavir 0.5 mg cohort ($n = 66$), and the subset of patients from the entecavir 0.5 mg cohort with evaluable liver biopsies at baseline, week 48, and week 144–148 ($n = 19$). Patients were treated with different doses of entecavir in Phase II studies ETV-047 and ETV-053, and subsequently received 0.5 mg daily in rollover study ETV-060. This table describes characteristics at pre-treatment (Phase II) baseline.

Characteristic	Long-term treatment cohort $n = 167$	Entecavir 0.5 mg cohort $n = 66$	Entecavir 0.5 mg cohort with long-term liver biopsy $n = 19$
Male, *n* (%)	120 (71.9)	48 (72.7)	15 (78.9)
Age (years), mean ± SD	42.5 ± 11.0	43.2 ± 10.5	43.8 ± 10.3
Weight (kg), mean ± SD	65.9 ± 3.5	65.5 ± 12.2	66.8 ± 13.2
Ethnicity Japanese, *n* (%)	167 (100)	66 (100)	19 (100)
HBV-DNA, mean ± SD			
Log_{10} copies/ml by PCR	7.88 ± 1.01	8.03 ± 0.93	7.61 ± 0.95
HBeAg – positive, *n* (%)	141 (84.4)	55 (83.3)	13 (68.4)
ALT (IU/L), mean ± SD	151.2 ± 130.8	142.2 ± 87.7	140.5 ± 68.5
Abnormal ALT (ALT >1.0× ULN), *n* (%)	163 (97.6)	64 (97.0)	19 (100)
HBV genotype, *n* (%)			
A	4 (2.4)	1 (1.5)	0
B	5 (3.0)	1 (1.5)	0
C	154 (92.2)	63 (95.5)	19 (100)
Others	4 (2.4)	1 (1.5)	0

ml, mean ALT was 151 IU/l, and 84% (141/167) of patients were HBeAg(+). Ninety-two per cent (154/167) of patients were infected with HBV genotype C. Baseline demographics and disease characteristics were similar for all patient cohorts.

Virological response

Mean HBV-DNA levels fell rapidly during studies ETV-047 and ETV-053 [27,28]. For the cohort that entered ETV-060 from the two Phase II studies ($n = 167$), HBV-DNA fell from a mean of 7.88 log copies/ml at pre-treatment baseline to a mean of 3.41 log_{10} copies/ml at ETV-060 baseline. Viral load was further suppressed during treatment in ETV-060 and was maintained at low levels through 96 weeks (120–148 weeks total entecavir treatment time). Forty-nine per cent (82/167) of patients in the cohort had HBV-DNA <400 copies/ml at ETV-060 entry (Fig. 2A). By week 96 of the study, this proportion had increased to 88% (127/144). Of the 82 patients with HBV-DNA <400 copies/ml at ETV-060 entry, 81 patients (99%) maintained this response to the end of treatment. Eighty-five patients had HBV-DNA >400 copies/ml at ETV-060 entry; 62 (73%) achieved HBV-DNA <400 copies/ml during treatment in ETV-060, and 23 (27%) maintained >400 copies/ml at end of treatment. Among the 23 patients who discontinued treatment during ETV-060, 14 had HBV-DNA <400 copies/ml at the last on-treatment measurement. A sensitivity analysis using the last observation carried forward method was conducted based on the intention-to-treat (ITT) population. The last observed HBV-DNA levels for all subjects who either were still on study but had a missing PCR test at week 96 or discontinued prior to week 96 were carried forward; this maintained the total number of subjects in this cohort intact ($n = 167$). When the HBV-DNA end point was re-calculated using this method, 85% (142/167) of patients had HBV-DNA <300 copies/ml at week 96.

Biochemical response

Almost all patients (97.6%; 163/167) in the Phase II studies had abnormal ALT (ALT >1.0× ULN) at pre-treatment baseline (Table 1 and Fig. 3A). At the time of entry into study ETV-060, 81.0% (132/163) of those patients demonstrated normalized ALT levels (Fig. 3A). By ETV-060 week 48, that proportion had risen to 86.7%, and by week 96 (120–148 weeks total entecavir treatment time), the rate of ALT normalization was 90.1%.

Serological response

One hundred and forty-one patients (84%) were HBeAg(+) at pre-treatment baseline (Table 1 and Fig. 4A). At the time of entry into study ETV-060, 16.3% (23/141) of those patients had lost HBeAg and undergone HBe seroconversion (Fig. 4A). By week 96 of ETV-060 (120–148 weeks total entecavir treatment time), 38.8% (47/121) of patients had lost HBeAg, and 26.4% (32/121) had undergone HBe seroconversion. Among patients who underwent HBe seroconversion in ETV-060, the majority had achieved HBV-DNA suppression (<400 copies/ml) during treatment in study ETV-047 or ETV-053. One patient lost HBsAg and one patient underwent HBs seroconversion during treatment in study ETV-060.

Resistance

One hundred and sixty-four out of 167 patients were monitored for resistance through the end of treatment in ETV-060 (three patients refused consent for resistance testing). Five patients developed genotypic resistance to entecavir, which emerged during the third year of treatment, for a 3-year cumulative probability of resistance of 3.3%. Four of these five patients had received the lower (non-approved) doses of entecavir (0.01 mg or 0.1 mg) during the Phase II studies prior to ETV-060. Of the five patients with resistance, one patient had achieved HBV-DNA levels <400 copies/ml prior to developing resistance, and four patients experienced virological breakthrough. Fig. 5 provides HBV-DNA and ALT profiles for the patient who received continuous treatment with the approved 0.5 mg dose. This patient had detectable levels of HBV-DNA after 48 weeks of entecavir treatment in ETV-060. Genotypic resistance testing did not reveal any mutations associated with resistance to entecavir. The patient experienced virological breakthrough at week 96, which was associated with development of entecavir resistance (rt L180M, rt S202G, rt M204V).

Safety

Mean exposure to entecavir during study ETV-060 was 103.9 weeks (range: 5.1–140.6 weeks). Adverse events were reported for 99% (166/167) of patients, and most were mild to moderate in severity (Table 2). The most common clinical adverse event was nasopharyngitis (16.1%). Increased serum lactic acid

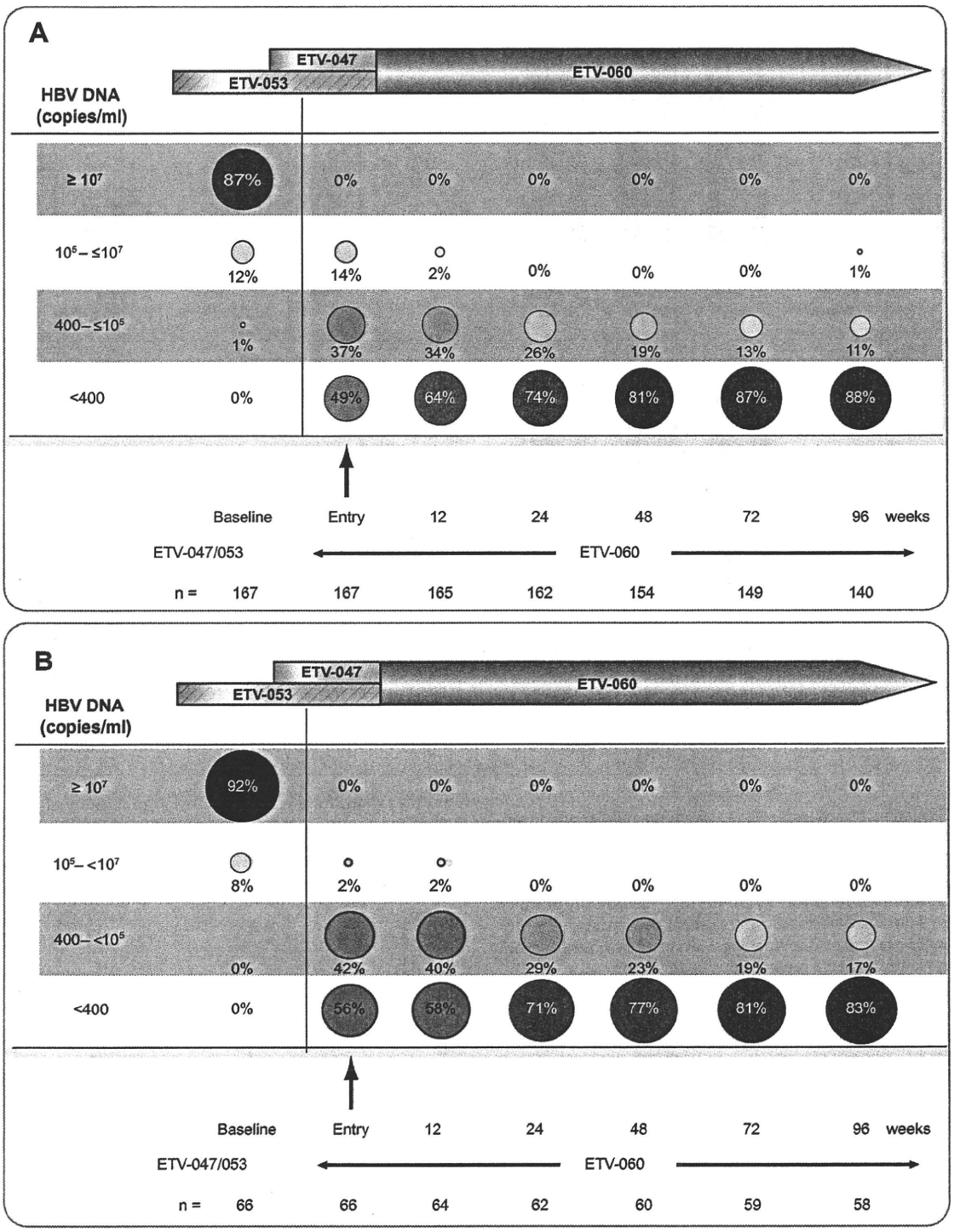

Fig. 2. Distribution of HBV-DNA levels over 96 weeks of treatment in rollover study ETV-060 (total entecavir treatment time, 120–148 weeks) for (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort.

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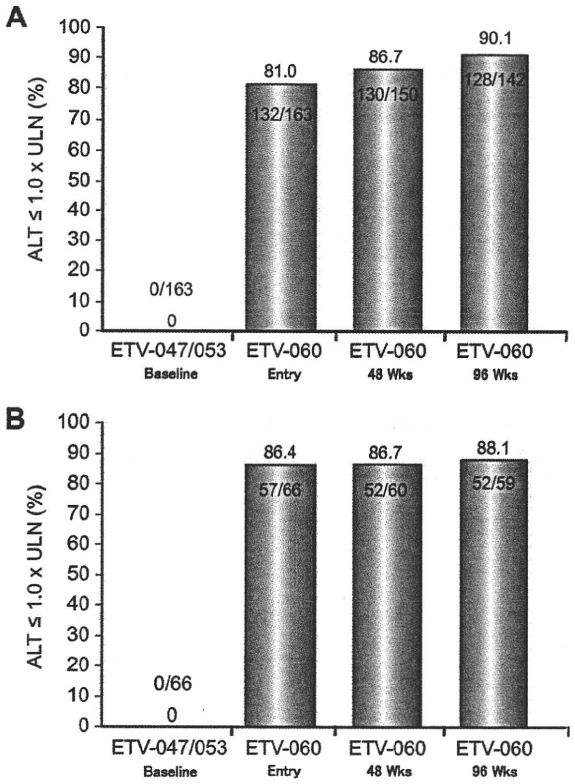

Fig. 3. Proportions of patients with normal ALT (ALT ⩽1.0× ULN) over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and sixty-three patients in the entecavir nucleoside-naïve long-term treatment cohort and 66 patients in the entecavir 0.5 mg cohort had abnormal ALT (>1.0× ULN) at pre-treatment baseline.

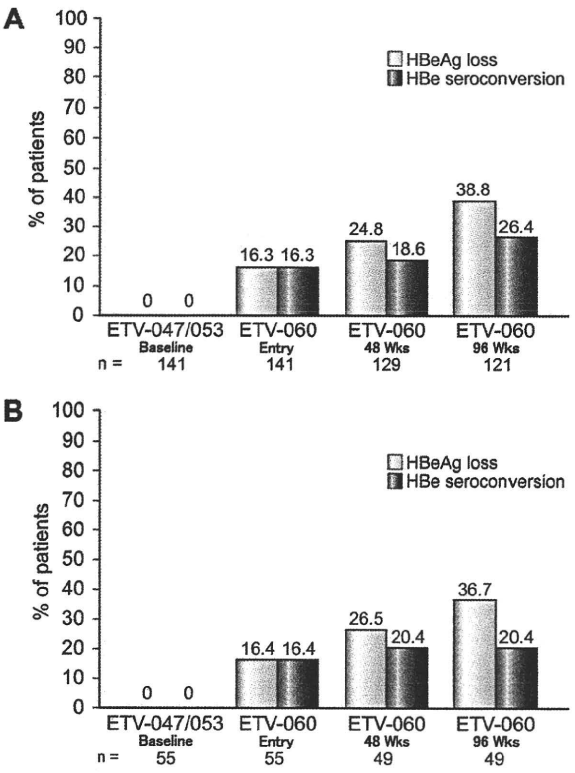

Fig. 4. Proportions of patients with HBeAg loss and HBe seroconversion over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and forty-one patients in the entecavir nucleoside-naïve long-term treatment cohort and 55 patients in the entecavir 0.5 mg cohort were HBeAg(+) at pre-treatment baseline.

(44.3%) and increased lipase (32.3%) were the most common laboratory adverse events. The most common Grade 3–4 adverse event (clinical or laboratory) was increased lipase, which occurred in 6% of patients. The frequency of clinical or laboratory serious adverse events was 13.7% (22/167), the majority of which resolved on continued entecavir treatment. Five patients (3%) discontinued treatment due to adverse events. There were no ALT flares. No deaths were reported during the study.

Entecavir 0.5 mg cohort

A subset of 66 patients (66/167) received the approved dose of entecavir (0.5 mg daily) from Phase II baseline through to the end of ETV-060. For this subset, among patients with available samples, 83% (48/58) had HBV-DNA <400 copies/ml by week 96 (Fig. 2B). When this end point was re-calculated using the last observation carried forward analysis, 80% (53/66) achieved HBV-DNA <400 copies/ml. By week 96 in ETV-060, 88% (52/59) of patients in the 0.5 mg cohort had ALT ⩽1.0× ULN (Fig. 3B), 37% (18/49) had lost HBeAg, and 20% (10/49) achieved HBe seroconversion (Fig. 4B). The mean change in HBV-DNA from pre-treatment baseline through to the end of ETV-060 was -5.19 $\log_{10}$ copies/ml. Resistance emerged in only one patient in this cohort, for a cumulative 3-year probability of resistance of 1.7%.

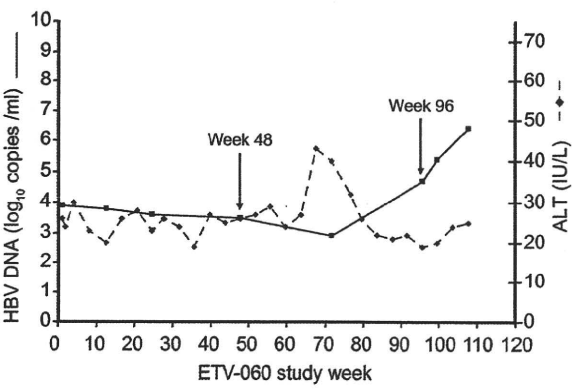

Fig. 5. On-treatment HBV-DNA and ALT profiles for the patient in the 0.5 mg entecavir cohort who developed entecavir resistance during treatment in ETV-060. Following virological breakthrough at week 96, genotypic resistance analysis revealed the presence of entecavir resistance (rt L180M, rt S202G, rt

Table 2. Summary of safety during ETV-060: entecavir nucleoside-naïve long-term treatment cohort.

On-treatment	Number of patients (%) ETV-060 $n = 167$
Any adverse event	166 (99.4)
Clinical adverse events	161 (96.4)
Clinical serious adverse events	22 (13.7)
Grade 3–4 clinical adverse events	8 (4.8)
Most frequent clinical adverse events*	
Nasopharyngitis	102 (61.1)
Headache	34 (20.4)
Diarrhoea	26 (15.6)
Laboratory adverse events	160 (95.8)
Laboratory serious adverse events	0
Grade 3–4 clinical adverse events	16 (9.6)
ALT increased	10 (6)
ALT flare†	0
Discontinuations due to adverse events	5 (3.0)
Deaths	0

* Occurring in at least 15% of patients.
† ALT >2× baseline and >10× ULN.

Twenty-one (21/66) patients in the 0.5 mg cohort, all originating from study ETV-053, had paired evaluable liver biopsies at pre-treatment (Phase II) baseline and either week 100 or week 148 (ETV-060 weeks 48 or 96, respectively). Nineteen (19/21) patients had evaluable biopsies at three time points: baseline, week 48, and week 148. Among this latter subset, 89% (17/19) had HBV-DNA <400 copies/ml at week 148. Histological improvement was observed in 100% (19/19) of these patients from baseline through week 148. There was a marked improvement in the distribution of Knodell necroinflammatory scores with increasing treatment time (Fig. 6A). The two patients who had repeat biopsies at week 100 (but not at week 148) also demonstrated histological improvement from baseline through to week 100. The mean Knodell necroinflammatory score improved from 8.95 at baseline to 1.89 at week 148, and 95% of patients (18/19) exhibited minimal necroinflammation (Knodell NI score ⩽3 points) at week 148 (Fig. 6A). Improvements in Knodell fibrosis scores were demonstrated in 63% (12/19) of patients with evaluable biopsies at baseline, week 48, and week 148 (Fig. 6B). Ten patients in this cohort had advanced fibrosis (Knodell fibrosis score = 3), and three patients had cirrhosis (Knodell fibrosis score = 4) at pre-treatment baseline, and 11 out of these 13 patients (85%) showed improvement at week 148. Among 21 patients with biopsies at baseline and either week 100 or week 148, 12/21 (57%) demonstrated an improvement in Knodell fibrosis scores, and 9/21 showed no change. The mean Knodell fibrosis score improved from 2.53 at baseline to 1.47 at week 148. Assessment of liver histology by the New Inuyama classification system confirmed the results obtained using the Knodell classification system (data not shown).

Discussion

The current long-term study of entecavir presents results for a cohort of patients treated continuously for 3 years. The strengths of this study include its focus on a well-defined cohort followed closely over 3 years, as well as the long-term follow-up liver biopsies on a subset of that cohort enabling a direct assessment of the effect of entecavir therapy on liver disease progression. These results show that long-term treatment with entecavir is well tolerated and achieves histological improvement, durable HBV-DNA suppression, and minimal resistance. Of 167 patients in the cohort, 86% (144) completed 96 weeks in the follow-up study for a total of 2.5–3 years of entecavir therapy, and only one patient discontinued treatment due to resistance emergence. In both global long-term studies of entecavir and in the present study, continuation of therapy beyond 2 years resulted in approximately 90% of patients achieving or maintaining HBV-DNA levels below the PCR assay limit of detection of 300–400 copies/ml [32]. These results were consistent with the results of a sensitivity analysis (last observation carried forward), in which 85% of patients achieved HBV-DNA <400 copies/ml on their last HBV-DNA observation. This method accounts for patient drop-out

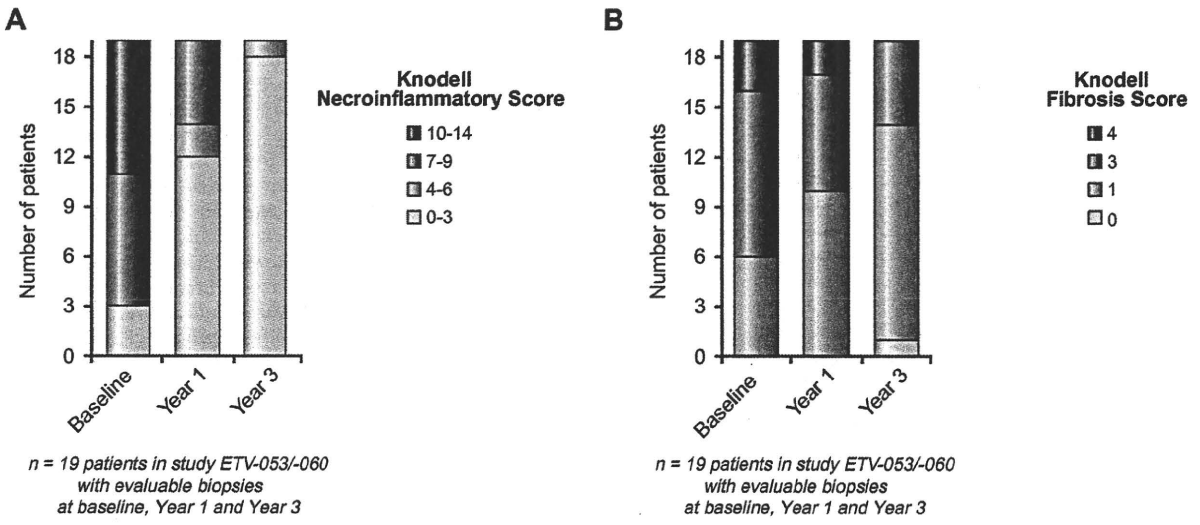

Fig. 6. (A) Distribution of Knodell necroinflammatory scores at pre-treatment baseline, year 1 (48 weeks), and year 3 (148 weeks), for 19 patients in the *entecavir 0.5 mg cohort* with evaluable liver biopsies at all three time points. (B) Distribution of Knodell fibrosis scores at pre-treatment baseline, year 1 (48 weeks), and year 3 (148 weeks), for 19 patients in the *entecavir 0.5 mg cohort* with evaluable liver biopsies at all three time points.

and missing samples, both of which are common occurrences in long-term studies. However, the interpretation of this sensitivity analysis should be approached cautiously, as it assumes: (1) that subjects who discontinued treatment without achieving HBV-DNA <300 copies/ml would not have achieved it with longer treatment; (2) and that patients who achieved this end point prior to discontinuing would have maintained it over time.

The degree of viral suppression reported in this study is higher than that reported for a cohort of HBeAg(+) patients treated with lamivudine for 3 years [33] and higher than that reported for cohorts of HBeAg(+) or HBeAg(−) patients treated with adefovir for 3 years [23,24]. In the current study, 84% of patients were HBeAg(+), and mean baseline HBV-DNA was 7.88 $\log_{10}$ copies/ml, 1 log higher than the baseline viral load in the adefovir study of HBeAg(−) patients. The rate of HBe seroconversion following entecavir treatment for 3 years in this study (26%), is somewhat lower than previously reported for patients treated with adefovir or lamivudine for 3 years (40% and 43%, respectively) [24,33]. This may be related to the large proportion (92%) of HBV genotype C patients enrolled in this study, which has previously been associated with delayed HBe seroconversion [10,34].

The results of long-term epidemiological-outcome studies have demonstrated that CHB patients with persistently detectable HBV-DNA are at highest risk of liver disease progression [12–14]. This suggests that long-term suppression of HBV-DNA should help minimize CHB complications. Liaw et al. demonstrated the value of antiviral therapy in a landmark study of CHB patients with cirrhosis or advanced fibrosis treated with long-term lamivudine [35]. Lamivudine-treated patients experienced lower rates of liver disease progression and HCC compared to those who received placebo, but the benefits were reduced by the emergence of lamivudine resistance.

High rates of histological improvement and improvement in fibrosis were observed in the current study among patients who received entecavir 0.5 mg from baseline. This improvement in liver histology is likely related to effective viral suppression. Long-term suppression of HBV-DNA is a key objective of CHB therapy, with the ultimate aim of preventing or reversing liver disease progression [15,36]. In previous studies, maintenance of virological suppression has been associated with improved liver histology among patients treated with nucleoside antivirals. Dienstag et al. showed that long-term treatment with lamivudine resulted in histological improvement, including reversal of fibrosis and cirrhosis; however, those benefits were lost when lamivudine resistance emerged [37]. Mommeja-Marin et al. showed statistically significant correlations between viral load suppression and histological improvement among HBeAg(+) patients treated with nucleoside analogues [38]. Hadziyannis et al. showed that 5 years of adefovir therapy for a cohort of HBeAg(−) patients resulted in virological suppression along with improvements in necroinflammation and fibrosis [23]. The current study demonstrates that continued entecavir treatment beyond 1 year results in increasing proportions of patients achieving HBV-DNA reduction to <400 copies/ml and further improvements in necroinflammation and fibrosis. At 3 years, all patients in the entecavir 0.5 mg cohort with evaluable biopsy pairs demonstrated histological improvement, and most (57%) showed improvement in fibrosis, including 85% (11/13) of those who had advanced fibrosis or cirrhosis at baseline.

The potent HBV-DNA suppression achieved in the current study, in combination with entecavir's high genetic barrier to resistance, likely contributed to the observed low rate of resistance emergence: 3-year cumulative probability of resistance of 3.3% for all patients and 1.7% for patients who received the approved dose of entecavir (0.5 mg) throughout the treatment period. The rate of 1.7% for patients treated continuously with the approved dose is consistent with that reported in entecavir global studies, in which the cumulative probability of resistance in nucleoside-naïve patients was 1.2% through 5 years [39]. The current study differs from the global studies in its focus on a well-defined cohort who were followed continuously with no dose interruption. In comparison with the consistently low rate of entecavir resistance observed among nucleoside-naïve patients, adefovir resistance emerged at rates of 20% among HBeAg(+) patients treated for 5 years (median of 235 weeks) and 29% among HBeAg(−) patients treated for 5 years [23,24].

In summary, the long-term data presented in the current report demonstrate that continuous entecavir therapy for 3 years is well tolerated in Japanese patients and provides durable clinical benefit. The high antiviral potency and low rate of resistance emergence shown in the current study support entecavir as an appropriate choice of first-line therapy for nucleoside-naïve chronic hepatitis B.

Conflicts of interest

Hiroki Ishikawa, Nobuyuki Masaki and Taku Seriu are employees of Bristol-Myers Squibb. Masao Omata is Member of Advisory Board for Bristol-Myers Squibb.

The other authors have nothing to disclose.

Acknowledgements

This work was sponsored by Bristol-Myers Squibb. Taku Seriu, Hiroki Ishikawa and Nobuyuki Masaki are employees of Bristol-Myers Squibb. Masao Omata serves as an advisor for Bristol-Myers Squibb. In addition to the authors, other study investigators included Joji Toyota, Chitomi Hasebe, Teruaki Kawanishi, Kazuyuki Suzuki, Yoshiyuki Ueno, Satoshi Mochida, Namiki Izumi, Hidetsugu Saito, Naohiko Masaki, Keiko Tatemoto, Yoshiyuki Arakawa, Hiromitsu Kumada, Yasunobu Matsuda, Shunichi Okada, Eiji Tanaka, Yoshiaki Katano, Etsuro Orito, Shinichi Kakumu, Noboru Hirashima, Eiichi Tomita, Takashi Kumada, Takeshi Okanoue, Kazihiro Katayama, Michio Kato, Harumasa Yoshihara, Taizo Hijioka, Kosaku Sakaguchi, Gotaro Yamada, Keisuke Hino, Norio Horiike, Shotaro Sakisaka, Ryukichi Kumashiro, Keisuke Hamasaki, Hiroshi Yatsuhashi, Masataka Seike, Yutaka Sasaki, Katsuhiro Hayashi, Hiroshi Ikeda, Masanori Miyake, Yasuyuki Araki, Kozo Fujio and Masaharu Ando. The study coordinating committee included Kazuyuki Suzuki, Hiromitsu Kumada, Takeshi Okanoue, Yasushi Shiratori, Michio Sata and Hirohito Tsubouchi. The biopsy reading committee included Fumio Imazeki, Mitsuhiko Moriyama and Masayoshi Kage.

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ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Aug. 2010, p. 3205–3211 Vol. 54, No. 8
0066-4804/10/$12.00 doi:10.1128/AAC.01372-09

Importance of Serum Concentration of Adefovir for Lamivudine-Adefovir Combination Therapy in Patients with Lamivudine-Resistant Chronic Hepatitis B[▽]

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Received 29 September 2009/Returned for modification 21 February 2010/Accepted 1 May 2010

Lamivudine (LMV)-adefovir pivoxil (ADV) combination therapy suppresses the replication of LMV-resistant hepatitis B virus (HBV), although its efficacy in suppressing HBV varies among patients. This study analyzed the clinical, virological, and pharmaceutical factors that influence the effect of the combination therapy. Patients negative for hepatitis B virus e antigen (HBeAg) and with low HBV DNA titers immediately prior to the combination therapy effectively cleared serum HBV DNA ($P = 0.0348$ and $P = 0.0310$, respectively). The maximum concentration of ADV in serum (ADV C_{max}) was higher in patients who showed HBV DNA clearance ($P = 0.0392$), and the cumulative clearance rates of HBV DNA were significantly higher in patients with ADV C_{max} equal to or greater than 24 ng/ml ($P = 0.0284$). HBeAg negativity and lower HBV DNA at the start of the combination therapy and higher ADV C_{max} were found to be independent factors for serum HBV DNA clearance. Serum creatinine increased significantly during the combination therapy, and the ADV C_{max} was higher in patients with low creatinine clearance rates. In conclusion, higher serum concentrations of ADV are associated with a good response to therapy based on clearance of HBV DNA in serum. However, care should be taken to prevent worsening of renal function due to high ADV serum concentrations.

Hepatitis B virus (HBV) infection is a serious global health problem. The risk of chronic HBV infection in immunocompetent adults is generally less than 5% but increases significantly in young children and immunocompromised adults (15, 36). Chronically infected individuals often develop chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma, and more than 500,000 people die every year from advanced liver diseases (6). Complete elimination of the virus is difficult, and patients are generally treated with interferon and nucleoside/nucleotide analogues, which suppress viral replication and prevent the progression of liver disease by combating inflammation (11, 23, 33). However, the emergence of drug-resistant viral mutants and hepatitis flare-up (breakthrough hepatitis) is a serious concern for such suppressive therapies (7, 9, 20, 32).

Lamivudine (LMV) is the first approved nucleoside analogue that terminates viral DNA synthesis by inhibiting chain elongation (31). Serum HBV DNA levels decrease soon after commencement of LMV therapy. However, long-term therapy frequently results in the emergence of drug-resistant HBV mutants (8, 24). In one study, the rate of LMV resistance increased from 24% in patients treated for 1 year to 70% after 4 years of treatment (21). LMV resistance is usually associated with amino acid substitutions in the YMDD motif of the viral reverse transcriptase (RT) (rtM204V/I/S) (4, 5, 19, 26). Additional substitutions, rtL180M and rtV173L, then further enhance the mutated transcriptase activity (1, 12, 26). The emergence of resistant mutants also often results in viral breakthrough and subsequent breakthrough hepatitis (21). The nucleotide analogue adefovir dipivoxil (ADV) potently suppresses the replication of both wild-type and LMV-resistant HBV both *in vitro* and *in vivo* (17, 25, 27, 37). LMV-ADV combination therapy is therefore recommended as a standard therapy for breakthrough hepatitis in Japan. Although both the combination therapy and ADV monotherapy are reported to be efficacious in patients with LMV-resistant HBV (28), the combination therapy carries a lower risk of emerging LMV-ADV double-resistant mutants (13, 14, 18). Recently, muta-

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▽ Published ahead of print on 24 May 2010.

TABLE 1. Baseline characteristics of 53 patients who received LMV-ADV combination therapy

Characteristic[a]	Value		
	VR (n = 39)	Non-VR (n = 14)	P value
Sex (male/female)	31/8	8/6	NS[c]
CH/LC	24/15	8/6	NS
HBV genotype C	39	14	
At start of LMV monotherapy			
Age (yr)	54[b] (31–70)	52[b] (27–66)	NS
HBV DNA (log copies/ml)	6.7[b] (2.6–8.5)	6.7 (3.9–8.4)	NS
HBeAg (+/−)	14/25	10/4	0.0236
Duration of LMV monotherapy (wk)	96[b] (0–166)	69[b] (0–213)	NS
At start of LMV plus ADV combination therapy			
Age (yr)	56[b] (32–73)	54[b] (27–69)	NS
BMI (kg/cm^2)	22.3[b] (15.6–27.3)	22.2[b] (18.6–26.2)	NS
Breakthrough hepatitis (+/−)	25/14	8/6	NS
HBV DNA (log copies/ml)	5.6[b] (2.6–8.7)	7.2[b] (4.4–8.0)	0.0310
HBeAg (+/−)	15/24	10/4	0.0348
ALT (IU/liter)	44[b] (12–654)	39[b] (18–310)	NS
Cr (mg/dl)	0.74[b] (0.49–1.28)	0.73[b] (0.45–1.05)	NS
CL_{CR} (ml/min/1.73 m^2)	114.3[b] (56.7–163.1)	101.4[b] (74.9–180.7)	NS
Duration of combination therapy (wk)	186[b] (68–311)	168[b] (58–276)	NS

[a] CH, chronic hepatitis; LC, liver cirrhosis; ALT, alanine transaminase; Cr, creatinine; +, positive; −, negative.
[b] Median value.
[c] NS, not significant.

tions conferring resistance to both LMV and ADV (through a combination of rtA181T/V and rtI233V or rtA181T/V and rtN236T) have been reported (2, 30), although the incidence of these mutations remains lower than the incidence associated with monotherapy.

We recently observed that some patients on LMV-ADV combination therapy who developed LMV resistance showed a poor response to long-term combination therapy. Decrease of serum HBV DNA levels in these patients leveled off, and HBV DNA levels sometimes remained higher than 4 log copies/ml.

The present study investigated those factors that affect the virological response to LMV-ADV combination therapy. We considered the nucleotide and amino acid sequences of HBV reverse transcriptase virological factors and the LMV/ADV concentrations pharmacological factors and then correlated the results with the clinical data of the patients.

MATERIALS AND METHODS

Patients. Between July 2003 and May 2009, 59 consecutive patients with chronic hepatitis or cirrhosis due to LMV-resistant HBV infection were treated with LMV-ADV combination therapy at Hiroshima University Hospital. Of these, 53 patients who received the combination therapy for more than 48 weeks were analyzed in this study. Patients began to receive the combination therapy based on the following criteria: (i) increase in serum HBV DNA levels of ≥1 log copy/ml in comparison with the nadir level during LMV monotherapy with or without breakthrough hepatitis, (ii) detection of mutations in the HBV RT domain related to LMV resistance by direct sequence analysis before the combination therapy, and (iii) serum creatinine levels of <1.5 mg/dl. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Hiroshima University Hospital ethics committee. Written informed consent was obtained from each patient. Patients coinfected with hepatitis C virus or human immunodeficiency virus were excluded from the study. In addition, patients were not administered drugs that affected serum concentrations of LMV and ADV.

The 53 patients were divided into two groups according to virological response: virological responders (VR) and non-VR. Since cessation of the combination therapy in LMV-resistant chronic hepatitis B patients is likely to lead to severe acute exacerbation, response to the therapy was assessed under extended combination therapy. VR were defined by sustained negative serum HBV DNA (<2.6 log copies/ml by the Amplicor HBV Monitor test [Roche Diagnostics, Basel, Switzerland]) for at least 12 weeks, while non-VR showed sustained positive HBV DNA tests until the final observation. In cases of cessation of the combination therapy, the point of discontinuation was defined as the final observation point. Table 1 details the clinical and virological features of the two groups.

The patients were administered daily oral doses of 10 mg ADV and 100 mg LMV. Sera were collected from the patients every month during the combination therapy and stored at −80°C until they were used. Serum HBV DNA, liver function, complete blood count, and serum creatinine were measured every month.

Sequence analysis of the HBV polymerase RT domain. HBV DNA was extracted from 100 μl of stored serum samples using the Smitest R&D (Genome Science Laboratories, Tokyo, Japan) and dissolved in 20 μl of sterile water. The extracted DNA was then amplified by nested PCR using 1 μl of DNA as a template for the first PCR. PCR was performed in 25 μl of reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM each deoxynucleoside triphosphate (dNTP), 20 pmol of each primer, and 1.25 units of LA *Taq* (Takara Bio Inc., Shiga, Japan) with the buffer supplied by the manufacturer. The first PCR products were diluted 10-fold, and 1 μl was used as a template for the second PCR. The primers used in this study were S2F (nucleotides [nt] 3189 to 3215; 5′-CAGGGATCCT CAGGCCATGCAGTGGAAC-3′) and X2R1 (nt 1606 to 1625; 5′-GTTCACG GTGGTCTCCATGC-3′) for the first PCR, and B2 (nt 65 to 84; 5′-GGCTCM AGTTCMGGAACAGT-3′) (where M is A or C) and X2R1 for the second PCR. The PCR protocol was as follows: initial denaturation at 94°C for 2 min and 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min, and final extension at 72°C for 7 min. After amplification, the final PCR products were gel purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany) and sequenced using the dye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA). Sequence analysis was performed on an ABI Prism 3100 Avant Genetic Analyzer (Applied Biosystems).

Measurement of serum concentrations of LMV and ADV. Serum concentrations of LMV and ADV were measured at the last time of observation in 39 of 53 patients who received the combination therapy. Blood sampling for trough values of LMV and ADV was performed at least 24 h after the drugs were taken. Subsequent blood sampling was performed 1 and 2 h after both of the drugs were taken for concentration measurement by liquid chromatography-tandem mass spectrometry (LC–MS-MS) analysis, using an LC-20A system (Shimadzu, Japan) and a Chromolith Performance RP-18e high-performance liquid chromatography (HPLC) column (Waters) for chromatography and an API4000 system

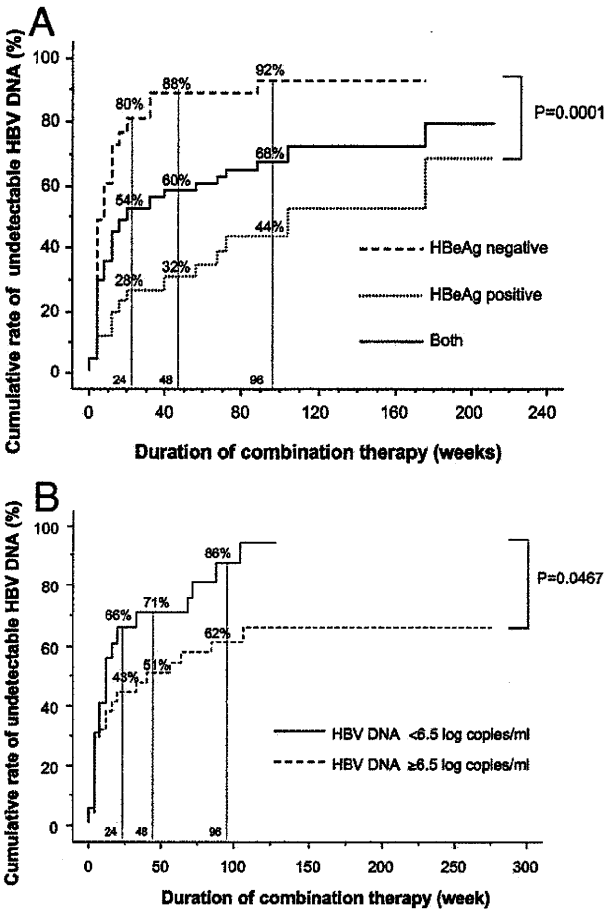

FIG. 1. Cumulative HBV DNA clearance rates in patients treated with lamivudine plus adefovir. Patients were assessed for HBeAg status (A) and HBV DNA levels (B).

(MDS Sciex, Canada) for mass detection and analysis. The instrument was operated in electrospray-positive-ionization mode, and the signal was detected by multiple-reaction monitoring. We defined the highest concentration for the three time points as the maximum concentration of LMV (LMV C_{max}) or ADV (ADV C_{max}) in serum. The AUC_{0-2} (the area under the drug concentration-time curve at 0 to 2 h) of LMV and ADV was calculated by the trapezoidal rule.

Statistical analysis of clinical data. The background characteristics and serum concentrations were compared using the chi-square test and the Mann-Whitney U test. The cumulative probability of undetectable HBV DNA was analyzed by the Kaplan-Meier method, and differences between the curves were tested by the log rank test. P values of less than 0.05 were considered statistically significant.

RESULTS

Effects of LMV-ADV combination therapy. Table 1 details the clinical characteristics of 39 VR and 14 non-VR subjects. Serum HBV DNA in 39 VR decreased to continuously undetectable levels, while serum HBV DNA in 14 non-VR decreased to plateau levels but never became undetectable by the final observation. A larger proportion of VR than non-VR were HBeAg negative prior to the start of LMV monotherapy. Similarly, a larger proportion of VR than non-VR patients were HBeAg negative and had lower serum HBV DNA immediately prior to the combination therapy. The cumulative clearance rates of HBV DNA were significantly higher in HBeAg-negative patients and in those with lower HBV DNA levels (<6.5 log copies/ml) just before the combination therapy than in patients positive for HBeAg or with HBV DNA levels equal to or greater than 6.5 log copies/ml (Fig. 1A and B). Out of 25 patients who were HBeAg positive immediately prior to combination therapy, none had seroconverted to anti-HBe after completing the combination therapy, and none of the total 53 showed viral breakthrough or breakthrough hepatitis during the combination therapy.

Genotyping of LMV- and ADV-resistant mutants. The nucleotide and amino acid sequences were determined for the RT domain in 47 of the 53 patients by the direct-sequencing method at the time just before HBV DNA clearance or at the nadir of HBV DNA levels after initiation of the combination therapy. Negative amplification of HBV DNA because of low HBV DNA values precluded such analysis in the remaining 6 patients. As shown in Table 2, the amino acid substitutions rtS85A and A181T, previously reported to confer ADV resistance (16, 40), were detected in 2 patients and 1 patient, respectively. The 2 patients with an rtS85A mutation also had YMDD motif mutations (Table 2), and their HBV DNA levels decreased gradually to undetectable levels at 62 and 177 weeks after the beginning of combination therapy, respectively. In contrast, HBV levels in the patient with a unique rtA181T mutation did not decrease to undetectable levels following 58 weeks of combination therapy until the patient was successfully treated with entecavir (ETV) monotherapy (reference 39 and data not shown).

Virological response to the combination therapy according to serum concentrations of LMV and ADV. To further explore the poor response of non-VR to therapy, drug concentration analysis was then undertaken in 29 VR and 10 non-VR, and the C_{max} and AUC_{0-2} values of LMV and ADV were compared. ADV C_{max} was significantly higher in VR than in non-VR (Fig. 2A), although the difference for ADV AUC_{0-2} was not statistically significant (Fig. 2B). The median values of ADV C_{max} and ADV AUC_{0-2} were 24 ng/ml and 37 ng · h/ml, respectively. The cumulative HBV DNA clearance rates were significantly higher in patients with high ADV C_{max} values (≥24 ng/ml) (Fig. 2C), and most of these patients belonged to

TABLE 2. Amino acid sequence substitutions in the HBV RT domain

Substitution[a]	No. of patients with substitution[a]	
	VR (n = 33)	Non-VR (n = 14)
rtM204 M		
Alone	7	3
+rtA181T	0	1
rtM204V/I		
Alone	19	10
+rtV214A/E	2	0
+rtQ215H	2	0
+rtV84I	1	0
+rtS85A	1	0
+rtS85A + rtV214E	1	0

[a] Two known ADV-resistant amino acid substitutions (A181T and S85A) are underlined.

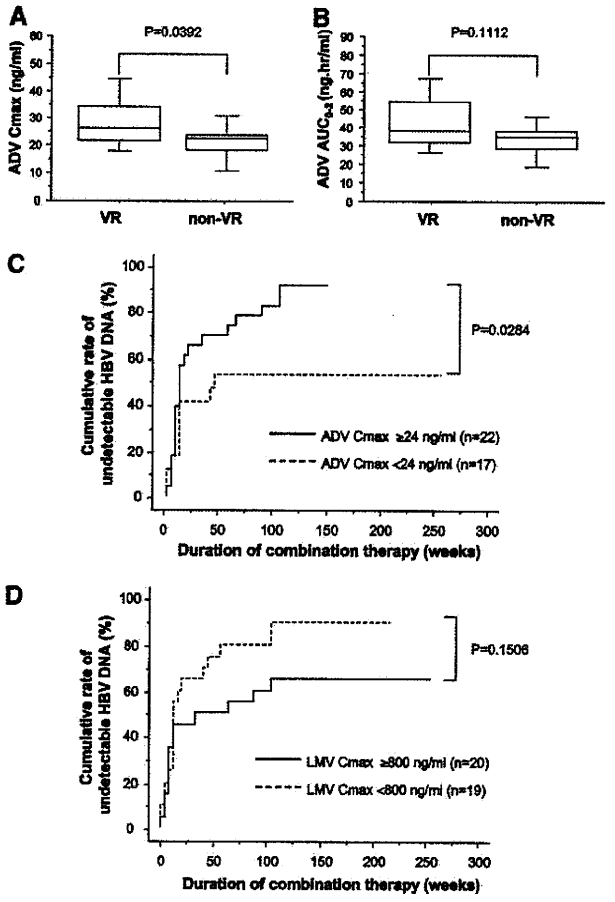

FIG. 2. Serum concentrations of ADV and effects of combination therapy. (A and B) Effects of the combination therapy based on ADV C_{max} (A) and AUC_{0-2} (B) determinations. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (C and D) Cumulative clearance rates of HBV DNA by ADV C_{max} (C) and LMV C_{max} (D).

the VR group (Table 3). However, the C_{max} and AUC_{0-2} of LMV were not significantly different between the VR and non-VR groups, and there was no difference in HBV clearance rates between patients with high or low C_{max} or AUC_{0-2} of

TABLE 3. Serum concentration of ADV and efficacy of LMV-ADV combination therapy

Parameter and value	No. (%) with value		*P* value
	VR	Non-VR	
AUC_{0-2} (ng · h/ml)			
≥37	15 (52)	3 (30)	0.2071
<37	14 (48)	7 (70)	
Total	29	10	
C_{max} (ng/ml)			
≥24	20 (69)	2 (20)	0.0097
<24	9 (31)	8 (80)	
Total	29	10	

TABLE 4. Multivariate analysis of factors associated with HBV DNA clearance in LMV-ADV combination therapy

Factor[a]	Category	*P* value	Odds ratio	95% CI
HBeAg	1 (positive)	0.0170	1	1.475–25.129
	2 (negative)		7.194	
HBV DNA (log copies/ml)	1 (≥6.5)	0.0485	1	1.178–22.367
	2 (<6.5)		4.185	
ADV C_{max} (ng/ml)	1 (<24)	0.0019	1	2.833–99.836
	2 (≥24)		16.818	

[a] At the start of LMV-ADV combination therapy. Factors: gender, age, background liver status, HBeAg, HBV DNA, ALT, Cr, RT mutation, ADV C_{max}, and ADV AUC_{0-2}.

LMV (Fig. 2D and data not shown). The AUC_{0-2} and C_{max} levels of both LMV and ADV did not correlate with the body mass index (BMI) (data not shown).

Analysis of independent predictive factors for VR. To analyze predictive factors for achieving VR, multivariate analysis was conducted. When factors appearing in Tables 1, 2, and 3 were analyzed simultaneously, higher ADV C_{max} and HBeAg negativity and lower HBV DNA at the start of the combination therapy were found to be independent factors for VR (Table 4). ADV C_{max}, in particular, was a strong determinant factor for VR (odds ratio, 16.818; 95% confidence interval [CI], 2.833 to 99.836).

Renal function and serum concentrations of the drugs. LMV and ADV are excreted from the kidney. Serum creatinine levels increased in 17 (32.1%) of 53 patients during the combination therapy, while the median serum creatinine levels increased significantly from 0.74 mg/dl at baseline to 0.86 mg/dl at the end of the observation period in 53 patients treated with LMV and ADV (Fig. 3A). The dose of ADV was reduced in 6 (11.3%) of the 53 patients to 5 mg/day or 10 mg every 2 days, and ADV administration was stopped in 3 (5.7%) patients due to elevated serum creatinine levels (≥1.5 mg/dl). The HBV DNA titers of 6 patients who reduced the dose of ADV never had a flare-up after the reduction. Five of the 6 patients belonged to the VR and one to the non-VR group. Serum creatinine levels returned to pretherapy values in all patients who reduced or stopped treatment with ADV. Next, we investigated whether the drug concentration was related to renal function. The C_{max} and AUC_{0-2} values of LMV and ADV were compared between patients whose creatinine clearance rates (CL_{CR}) were normal and those whose rates were low. As shown in Fig. 3B and C, both C_{max} and AUC_{0-2} of ADV were significantly higher in patients with CL_{CR} of <80 ml/min/1.73 m^2. In contrast, there was no relationship found between CL_{CR} and C_{max}/AUC_{0-2} of LMV (Fig. 3D and E).

DISCUSSION

The poor response of chronic HBV infection to nucleotide/nucleoside therapy is commonly attributed to amino acid substitutions in the RT domain of HBV polymerase. Several RT amino acid mutations that induce resistance to ADV have been reported, although the incidence is much lower than that reported for LMV. The HBV polymerase RT domain substi-

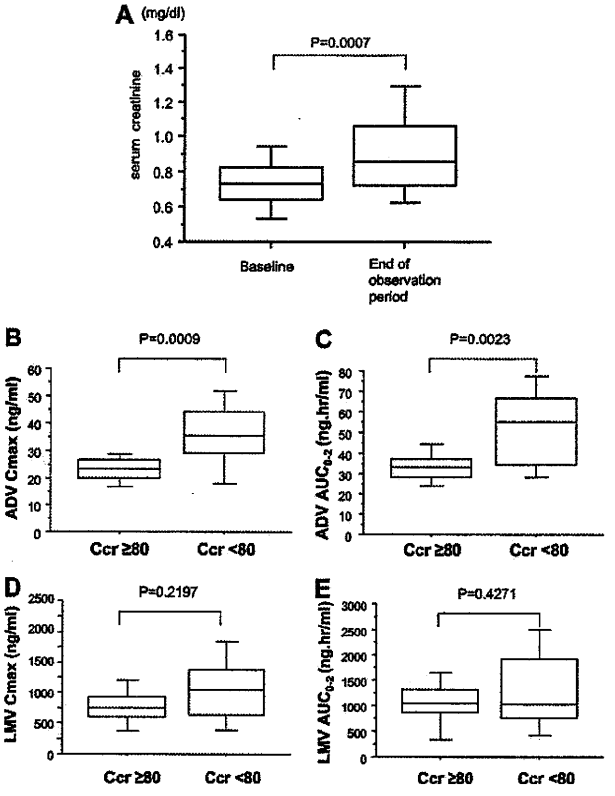

FIG. 3. (A) Comparison of serum creatinine concentrations just before the start of the combination therapy and at the end of the observation period. Renal function and concentrations of LMV and ADV are shown. (B to E) The ADV C_{max} (B), LMV C_{max} (D), and AUC_{0-2} of ADV (C) and LMV (E) were compared between patients with high (≥80 ml/min/1.73 m^2) and low (<80 ml/min/1.73 m^2) CL_{CR}. In these box-and-whisker plots, the lines within the boxes represent median values; the upper and lower lines of the boxes represent the 25th and 75th percentiles, respectively; and the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively.

tutions rtV84, rtS85, rtA181, rtV214, rtQ215, rtI233, rtN236, and rtP237 are associated with ADV resistance (16, 40), and rtA181 and rtQ215 mutations are associated with cross-resistance to LMV and ADV (23, 35). To study the incidence and the effects of amino acid substitutions in the RT domain of HBV polymerase in patients receiving combination therapy, this study initially analyzed serum samples for amino acid sequences in the region. We identified the previously reported A181T and S85A substitutions, as well as substitutions at rt84, rt214, and rt215 that might confer resistance to ADV. However, all these mutations, except for A181T, were found in VR.

These results are consistent with a previous report that most of these mutations confer only limited resistance to ADV therapy (16). In contrast, one of 14 patients who failed to clear HBV DNA in the present study had an apparent ADV resistance mutation. This unique mutation, A181T, which disrupts a stop codon in the HBs gene, is reported to be involved in resistance against both LMV and ADV (39). Therefore, it became apparent in the present series of experiments that only one of 14 patients responded poorly to the combination therapy due to the emergence of a resistant viral clone.

However, none of the remaining 13 patients had amino acid substitutions known to induce resistance to ADV. This is consistent with a recent report by Lampertico et al. (22) citing 11% of patients who failed to clear serum HBV DNA despite 3 years of combination therapy. In addition, none of these patients had a known ADV-resistant strain of HBV. Yatsuji et al (40) also reported 6 of 132 patients with transiently fluctuating HBV DNA levels (from <2.6 to 3.1 log copies/ml) and wild-type genotypes for rtA181 and rtN236.

To further explore the poor response to combination therapy, the concentration of ADV was investigated with respect to the drug's efficacy. Although it is noted that ADV is converted to the diphosphate derivative in hepatocytes by adenylate kinase and inhibits viral DNA polymerase (3, 29), the detailed metabolic pathway remains unclear. According to experimental data from GlaxoSmithKline K.K., when chronic hepatitis B patients were administered oral doses of 10 mg ADV and 100 mg LMV, the ADV C_{max} and AUC_{0-24} were 20.1 ± 3.3 ng/ml and 231.5 ± 33.7 ng · h/ml, respectively (AUC_{0-2} data not shown). The reported 50% inhibitory concentration (IC_{50}) of ADV is 0.36 to 0.39 μM, equal to 180.5 to 195.6 ng/ml (38, 39) and much higher than the values obtained in this study (ADV C_{max}, 5.1 to 54.6 ng/ml). This difference might come from the fact that the concentration of orally administered ADV should be higher in portal blood but lower in the peripheral blood. At any rate, there have been no reports detailing effective serum concentrations of ADV. In this study, the ADV C_{max} was higher in VR, and cumulative clearance rates of HBV DNA were higher in patients with higher ADV C_{max} values. The reason for the lack of association between the efficacy of the combination therapy and the ADV AUC_{0-2} remains unclear and might be related to different absorption profiles or metabolic profiles for the drugs or lack of power due to the small number of patients analyzed. However, these results indicate that poor response to the combination therapy arises at least in part from a low serum concentration of ADV. Because 90.9% (20/22) of patients with ADV C_{max} values equal to or greater than 24 ng/ml could clear serum HBV DNA, it is expected that non-VR with ADV C_{max} values below 24 ng/ml can achieve VR by boosting the serum level of ADV. Therefore, it might be recommended to raise the serum level of ADV to over 24 ng/ml in such cases. Two choices are considered for boosting the serum concentration of ADV: increasing the dose of ADV or using drugs that affect the serum concentration of ADV, such as an inhibitor of organic anion transporters (10, 34).

Meanwhile, renal dysfunction sometimes occurs as a side effect of ADV, and serum creatinine levels actually increased in patients administered the combination therapy; 11.3% of patients had to reduce the dose of ADV, and 5.7% of patients had to discontinue ADV due to elevated serum creatinine levels. Furthermore, the serum concentration of ADV was higher in patients with low CL_{CR}. This finding suggests a possible worsening of renal dysfunction in patients treated with ADV due to the generation of a vicious cause-effect circle (a higher ADV concentration worsens renal function). Although we did not investigate the safety range of ADV concentrations

in this study, and the upper limit of the range is not known, it is considered important that adequate and precise doses of ADV should be prescribed to patients, especially those with impaired renal function, instead of simply increasing the serum concentration of ADV. This study suggests that monitoring the serum ADV concentration would be useful to fine tune the appropriate drug dosage.

ACKNOWLEDGMENTS

This work was carried out at the Research Center for Molecular Medicine, Faculty of Medicine, Hiroshima University, and the Analysis Center of Life Science, Hiroshima University. We thank Rie Akiyama, Sachi Tanaka, and Miyuki Matsushita for their excellent technical assistance and Yoshiko Nakata and Aya Furukawa for secretarial assistance.

This study was supported in part by a Grant-in-Aid for Scientific Research from the Japanese Ministry of Labor and Health and Welfare.

We have no conflicts of interest.

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Research Article

EASL EUROPEAN ASSOCIATION FOR THE STUDY OF THE LIVER | JOURNAL OF HEPATOLOGY

Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients

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Background & Aims: A common genetic variation at the IL28 locus has been found to affect the response of peg-interferon and ribavirin combination therapy against chronic hepatitis C virus (HCV) infection. An allele associated with a favorable response (rs8099917 T), which is the major allele in the majority of Asian, American, and European populations, has also been found to be associated with spontaneous eradication of the virus.
Methods: As no studies have yet analyzed the effect of the polymorphism on biochemical and inflammatory changes in chronic infection, we analyzed a cohort of patients with chronic hepatitis C ($n = 364$) for the effect of the IL28 polymorphism on viral, biochemical, and histological findings.
Results: We found that the proportion of HCV wild type core amino acids 70 and 91 was significantly greater ($p = 1.21 \times 10^{-4}$ and 0.034) and levels of gamma-GTP significantly lower ($p = 0.001$) in patients homozygous for the IL28 major allele. We also found that inflammation activity and fibrosis of the liver were significantly more severe in patients homozygous for the IL28 major allele ($p = 0.025$ and 0.036, respectively). Although the higher gamma-GTP levels were also associated with higher inflammatory activity and fibrosis, multivariate analysis showed that only the IL28 allele polymorphism, sex, alcohol consumption, and liver fibrosis were independently associated with gamma-GTP levels ($p = 0.001$, 0.0003, 0.0013, and 0.0348, respectively).
Conclusions: These results suggest that different cytokine profiles induced by the IL28 polymorphism resulted in different biochemical and inflammatory conditions during chronic HCV infection and contribute to the progression of liver diseases.

Keywords: IL28; SNP; Histological activity; Inflammation; gamma-GTP.
Received 23 November 2009; received in revised form 9 March 2010; accepted 29 March 2010; available online 31 May 2010
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Abbreviations: HCV, hepatitis C virus; SNP, single nucleotide polymorphism; ISDR, interferon sensitivity determining region; BMI, body mass index.

Introduction

Hepatitis C virus infection is one of the major causative agents of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [1]. The best current therapeutic regimen is pegylated interferon and ribavirin combination therapy [2,3]. Although the eradication rate of the virus has been improved by extending the treatment period from the standard 48 to 72 weeks for genotype 1b infected patients, active viral replication still remains in nearly half of these patients [4].

Recent studies have identified both host and viral factors predictive of interferon therapy. Among the viral factors, a forty amino acid stretch in the NS5 region has been found to be predictive of response to interferon monotherapy [5,6]. More recently, Akuta et al. identified amino acid substitutions in the core region (core aa70 and 91) that are predictive for the effect of interferon and ribavirin combination therapy [7,8].

Among the host factors, many common polymorphisms in the human genome, including single nucleotide polymorphisms (SNP), have been identified [9–13]. We recently reported that a SNP in the MAPKAPK3 gene is associated with response to interferon therapy [14]. More recently, three groups of researchers found that several SNPs in the IL28 locus are related to the effectiveness of combination therapy [15–17]. We also performed a genome wide association study and confirmed that variation at the IL28 locus is related to the effectiveness of combination therapy (Chayama K, personal communication).

Research Article

These viral and host factors must influence the natural course of viral infection. Host immune cells produce interferon and other cytokines in response to viral infection. For RNA viruses such as HCV, cellular sensors such as RIG-I detect the double stranded RNA and activate a pathway to produce cytokines, including alpha and beta interferons that trigger an antiviral response to eradicate the virus [18]. Genetic polymorphism of genes involved in innate immunity is likely to influence the strength and nature of this defense. In fact, a polymorphism in the IL28 locus has been reported to correlate with spontaneous eradication of HCV [19]. However, little is known about how these factors affect the course of chronic infection of the virus. In this study, we focused on histological findings in the liver. We also analyzed viral and biochemical factors in patients chronically infected with HCV. We found that histological aspects of the liver (fibrosis and activity), HCV core amino acid substitutions, and gamma-GTP are associated with the polymorphism.

Materials and methods

Study subjects

We analyzed a cohort of 364 consecutive adult patients with chronic hepatitis C virus infection who visited Hiroshima University hospital and received liver biopsies between December 2002 and November 2008 and who agreed to provide blood samples for the human genome study. All patients included in the study had positive HCV viremia in serum for more than six months, assessed using a commercial quantitative polymerase chain reaction (PCR) assay (COBAS Amplicor HCV Monitor Test, v2.0; Roche Diagnostics, Branchburg, NJ). Patients with decompensated liver disease were excluded, as were patients co-infected with hepatitis B virus, or human immunodeficiency virus and patients with apparent auto-immune hepatitis and alcoholic liver disease. All patients provided written informed consent for the genomic analysis. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the ethical committees of Hiroshima University and Riken. The patient profiles are listed in Table 1. Using criteria reported by Desmet et al. [20], liver biopsy samples were evaluated by two pathologists. To verify consistency and accuracy, one of the pathologists independently re-evaluated samples analyzed by the other, and both pathologists were blind with respect to the IL28 polymorphism. We excluded insufficient or inconclusive biopsy samples, including those that were less than 10 mm^2 in size and containing less than 10 portal tracts. The amount of alcohol consumed was calculated according to the frequency of consumption and the alcohol concentration of beverages consumed. We estimated alcohol concentrations as follows: 5% for beer, 17% for sake, 25% for Japanese vodka, and 43% for whiskey; 1 ml of alcohol was considered equivalent to 0.886 g. The amount of alcohol consumed was divided into three categories: none, light (0–20 g/day), moderate (21–50 g/day). Heavy drinkers (more than 50 g/day) were excluded from the study.

Table 1. Characteristics of patients.

Characteristics of patients	
Age [median (range)]	59 (20–82)
Sex (male/female)	212/152
BMI [median (range)]	23 (16–39)
Alcohol consumption (Unavailable/none/0–20 g/day/21–50 g/day)	64/110/65/125
Hb [median (range)] mg/dl	14 (8–18)
Platelet [median(range)] × $10^4/mm^3$	14 (4–41)
ALT [median (range)]IU/L	62 (2–611)[c]
gamma-GTP [median (range)] IU/L	50 (7–680)
Genotype (1b/2a or 2b/1b + 2b/undertermined)	260/84/1/19
Fibrosis (F0/F1/F2/F3/F4)	4/116/141/66/37
Activity (A0/A1/A2/A3)	1/102/206/51
Virus titer [median (range)]kIU/l	1400 (<0.5–26,000)
Core 70[a] (wild/mutant/undertermined)	120/77/167
Core 91[a] (wild/mutant/undertermined)	107/88/169
ISDR[b] mutation (0/1/>2/undertermined)	58/70/48/188

[a] Hepatitis C virus core amino acid 70R and 91L are presented as wild type. Substituted amino acids are considered mutants.
[b] Interferon sensitivity determining region. Number of amino acids substituted from the prototype genotype 1b sequence were calculated.
[c] ALT levels of two patients remained around 2 IU/L even though AST and gamma-GTP levels were comparable to other chronic hepatitis C patients (peaking above 100 IU/L and returning to normal following SVR), probably due to deficiency of the ALT enzyme. These values were omitted from analysis of ALT.

Genotyping

Genotyping of some of the samples was performed as part of a genome wide association study using the Illumina HumanHap610-Quad Genotyping BeadChip (Illumina, Inc., CA) at Riken Yokohama Institute. Genotyping of the remaining samples was performed using TaqMan assay or Invader assay as described previously [21,22].

Analysis of amino acid sequences in the core and ISDR region

HCV RNA was extracted from 100 µl serum samples by SepaGene RV-R (Sanko Junyaku Co., Tokyo, Japan) and dissolved in 20 µl of H_2O. The RNA was then reverse transcribed with random primers and MMLV reverse transcriptase (Takara Shuzo, Tokyo, Japan). The resultant cDNA was then amplified by nested PCR. PCR was performed in 25 µl of reaction mixture containing 2.5 mM $MgCl_2$, 0.4 mM of each dNTP, 20 pmol of each primer and 1.25 U of LA Taq (Takara Bio Inc.) with a buffer supplied by the manufacturer. One microliter of 10×-diluted products from the first PCR was used as a template for the second PCR. The PCR primer sequences are listed in Table 2. The PCR protocol involved initial denaturation at 95 °C for 5 min, 35 cycles of denaturation for 30 s at 94 °C, annealing of primers for 1 min at 57 °C and extension for 1 min at 72 °C, followed by final extension at 72 °C for 7 min. The amplified DNA fragments were separated onto a 2% agarose gel and purified with the QIAquick gel extraction kit (Qiagen, Hilden, Germany). Nucleotide sequences were determined using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems Inc., CA).The obtained nucleotide and amino acid sequences were compared with the prototype sequence of genotype 1b HCV-J (GenBank Accession Number D90208) [23]. Amino acids at positions 70 and 91 of the core region that were identical to the prototype (arginine and leucine, respectively) were considered wild type.

Statistical analysis

χ^2 and Mann–Whitney U-tests were applied to detect significant associations. Simple and multiple regression analyses were used to examine the association between serum gamma-GTP levels and the values of other markers. When the data were not normally distributed, Box-Cox power transformation was performed to remove skewness, followed by linear regression analyses. All of the statistical analyses were two sided, and $p < 0.05$ was considered significant. All statistical analysis was performed using the PASW Statistics 18 program (SPSS Inc., IL).

Results

IL28 locus genotypes and viral and biochemical markers

We compared viral and biochemical markers with IL28 genotypes. First we analyzed the relationship between IL28 genotypes

Table 2. Primers used in this study.

Core region	
Outer forward	5′-GCC ATA GTG GTC TGC GGA AC-3′
Outer reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
Inner forward	5′-GCT AGC CGA GTA GTG TT-3′
Inner reverse	5′-GGA GCA GTC CTT CGT GAC ATG-3′
ISDR[a]	
Outer forward	5′-TTC CAC TAC GTG ACG GGC AT-3′
Outer reverse	5′-CCC GTC CAT GTG TAG GAC AT-3′
Inner forward	5′-GGG TCA CAG CTC CCA TGT GAG CC-3′
Inner reverse	5′-GAG GGT TGT AAT CCG GGC GTG C-3′

[a] Interferon sensitivity determining region.

Table 3. Amino acid substitutions in the core region of HCV and IL28 genotype.

SNP	Allele (1/2)	Genotype			p value[a]	OR
		11	12	22		(95% CI)[b]
rs8099917	T/G					
	Core aa70					
	Wild	2	17	101	1.21E-04	0.30
	Non-wild	3	28	46		(0.14–0.55)
	Core aa91					
	Wild	3	18	86	0.034	0.50
	Non-wild	2	27	59		(0.26–0.95)
	ISDR					
	0–1	2	37	89	0.120	1.90
	>2	2	7	39		(0.84–4.3)
	HCV genotype					
	1	6	63	190	0.443	0.81
	2	1	25	58		(0.47–1.4)

[a] p value by χ^2 test for the minor allele dominant model.
[b] Odds ratio for the minor allele in a dominant model.

Table 4. Factors associated with higher gamma-GTP levels.

Variable	Simple		Multiple	
	Estimate	p	Estimate	p
Age	−0.00004	0.899436		
Sex (male vs. female)	0.04647	5E-09	0.033	0.0003
BMI	−0.00257	0.044003		
Activity (A2–4 vs. A0–1)	−0.02518	0.004103	−0.015	0.1415
Fibrosis (F2–4 vs.F0–1)	−0.03	0.000382	−0.021	0.0348
Alcohol consumption	−0.03962	6.81E-06	−0.029	0.0013
IL28 genotype (2/2 vs. 1/2, 1/1)	0.02641	0.003522	0.03	0.001
HCV genotype (1 vs. 2)	−0.0068	0.471293		
Log virus titer (Log IU/ml)	0.00032	0.748826		
Core aa70 (wild vs. others)	−0.01589	0.117424		
Core aa91 (wild vs. others)	−0.01422	0.162341		
ISDR (0–1 vs. ⩾2)	0.00253	0.824685		

Simple and multiple regression analyses were used to examine the association between serum gamma-GTP and the values of other markers. All of the statistical analyses were two sided, and p <0.05 was considered significant.

and substitutions in the HCV core protein amino acids 70 and 91, as well as the HCV genotype and the number of amino acid substitutions in the ISDR. As shown in Table 3, there are significant associations between amino acid substitutions in the core region and the genotype of the rs8099917 SNP at the IL28B locus. In particular, patients homozygous for the major IL28 allele were significantly associated with wild type core amino acid 70 (OR = 0.30; p = 1.21E-04). A similar trend is seen with core amino acid 91 substitutions (OR = 0.50; p = 0.034). Patients with more than one amino acid substitution in the ISDR region also tended to occur in patients homozygous for the major allele, although the difference was not statistically significant (Table 3). There was no correlation between the HCV genotype and the IL28 allele.

We further examined the relationship between IL28 and biochemical markers such as ALT, gamma-GTP, total cholesterol, HDL cholesterol, serum iron, and HCV RNA levels. Only the gamma-GTP level was significantly associated with the IL28 genotype. As shown in Fig. 1A, the gamma-GTP levels were lowest in the IL28 major allele homozygotes and highest in minor allele homozygotes. As drinking alcohol is known to elevate gamma-GTP levels, we examined the effect of alcohol intake in our cohort. As shown in Fig. 1B, there was an association between alcohol and gamma-GTP levels. As we found that the gamma-GTP level is higher in patients with core amino acid 70 substitutions (Fig. 1C), we performed multivariate analysis to examine what factors contribute to higher levels of gamma-GTP. As shown in Table 4, a simple regression analysis revealed that serum gamma-GTP levels were associated with sex, BMI, inflammation activity, liver fibrosis, alcohol consumption, and IL28 genotype, whereas in multiple regression analysis, sex, liver fibrosis, alcohol consumption, and IL28 genotype remained positively associated with serum gamma-GTP levels.

Histological findings and polymorphism in the IL28 locus

We then analyzed the relationship between the IL28 locus polymorphisms and histological findings. We divided patients into mild fibrosis (F0 and F1) and severe fibrosis (F2–4) as well as lower activity (A0 and A1) and higher activity (A2 and A3) and compared these factors against IL28 genotypes. As shown in Table 5, both inflammatory activity and fibrosis were significantly associated with IL28 genotype. Inflammation was more active (A2–3) in patients homozygous for IL28 major alleles

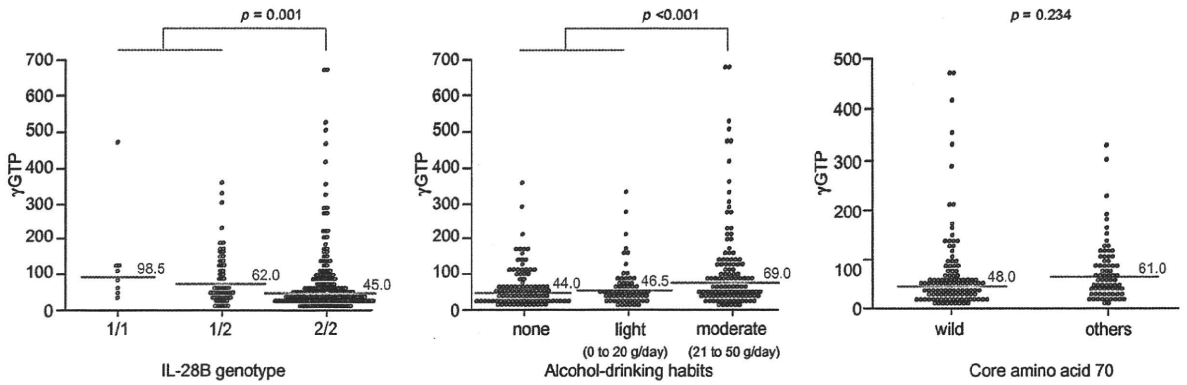

Fig. 1. gamma-GTP levels and IL28 genotype, alcohol intake, and core amino acid substitutions. gamma-GTP levels according to (A) IL28 genotypes, (B) alcohol consumption, and (C) core amino acid 70 substitutions are shown. Horizontal bars represent the median. Mann–Whitney U-test was used to compare gamma-GTP levels.