Research Article

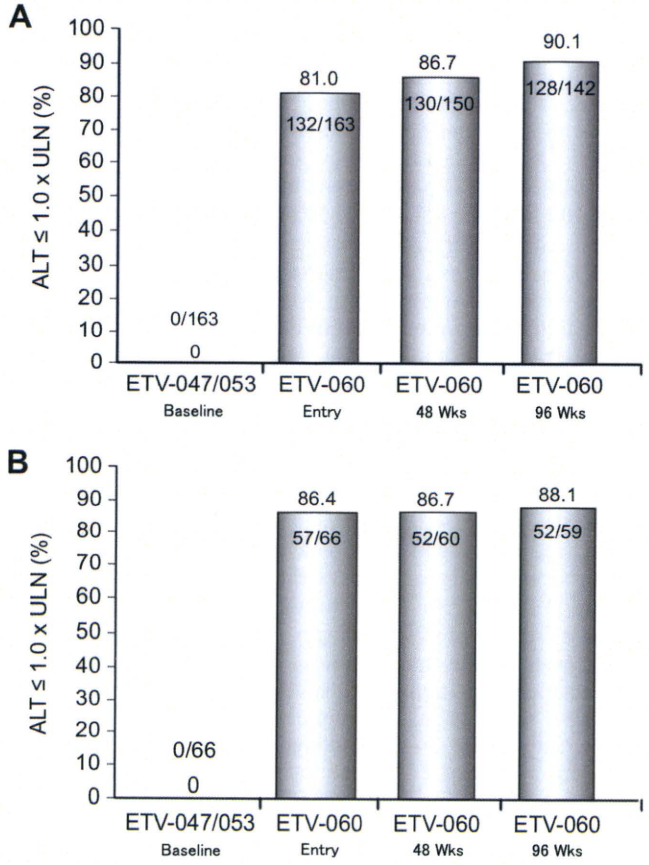

Fig. 3. Proportions of patients with normal ALT (ALT ⩽1.0× ULN) over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and sixty-three patients in the entecavir nucleoside-naïve long-term treatment cohort and 66 patients in the entecavir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

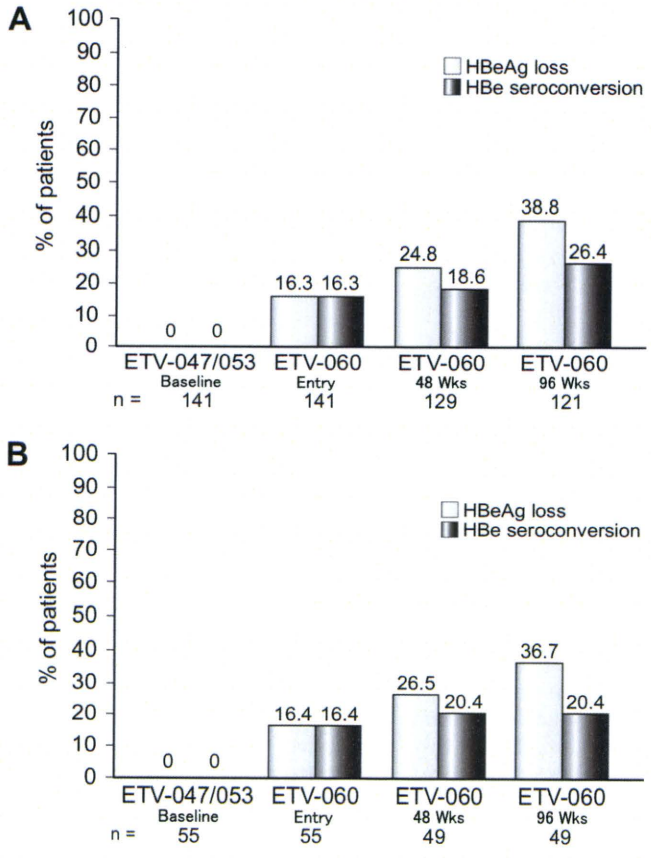

Fig. 4. Proportions of patients with HBeAg loss and HBe seroconversion over time in (A) the entecavir nucleoside-naïve long-term treatment cohort and (B) the entecavir 0.5 mg cohort. One hundred and forty-one patients in the entecavir nucleoside-naïve long-term treatment cohort and 55 patients in the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

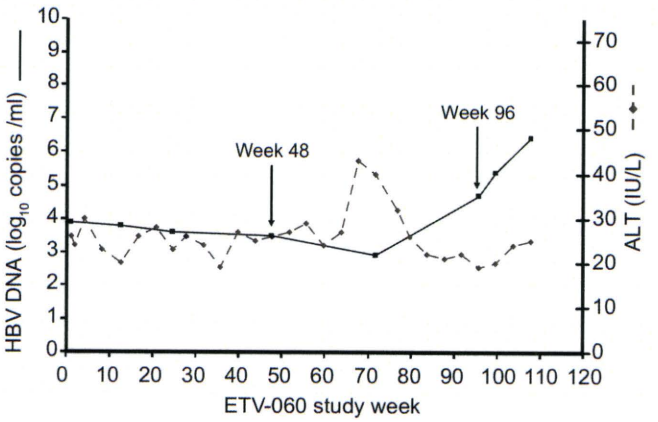

Fig. 5. On-treatment HBV-DNA and ALT profiles for the patient in the 0.5 mg entecavir cohort who developed entecavir resistance during treatment in ETV-060. Following virological breakthrough at week 96, genotypic resistance analysis revealed the presence of entecavir resistance (rt L180M, rt S202G,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

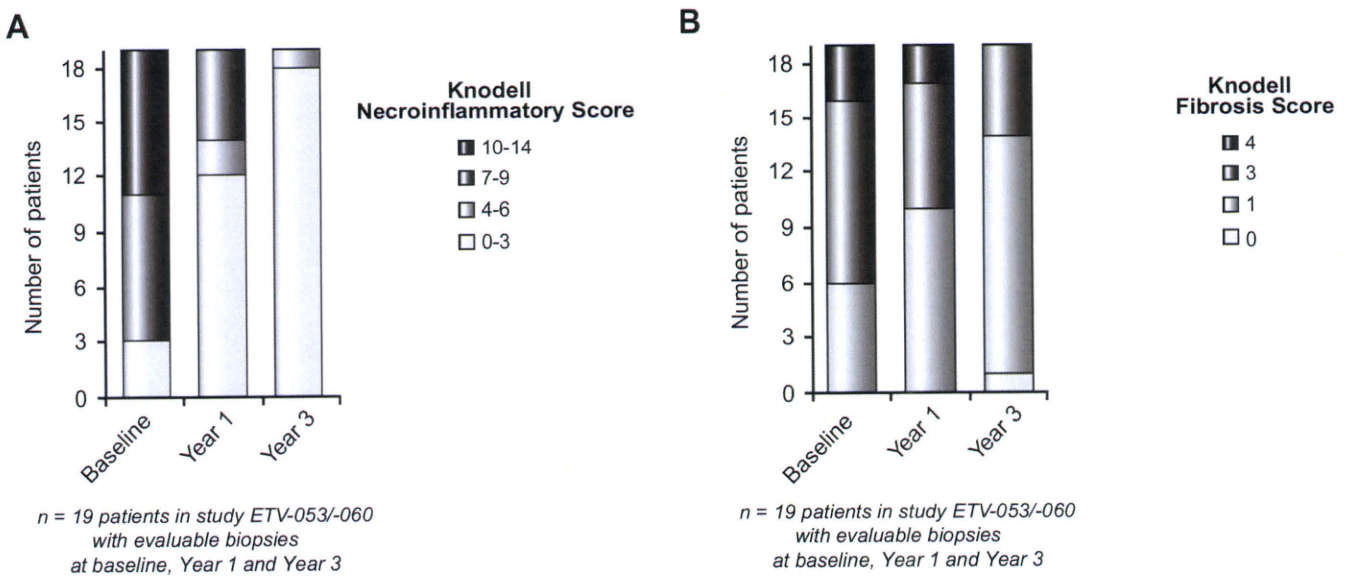

Fig. 6. (A) Distribution of Knodell necroinflammatory scores at pre-treatment baseline, year 1 (48 weeks), and year 3 (148 weeks), for 19 patients in the *entecavir 0.5 mg cohort* with evaluable liver biopsies at all three time points. (B) Distribution of Knodell fibrosis scores at pre-treatment baseline,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

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Impact of *IL-28B* SNPs on control of hepatitis C virus infection: a genome-wide association study

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Fumio Imazeki

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Japan
Tel.: +81 432 262 086
Fax: +81 432 262 083
imazekif@faculty.chiba-u.jp

Osamu Yokosuka

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Japan
Tel.: +81 432 262 086
Fax: +81 432 262 083
yokosukao@faculty.chiba-u.jp

Masao Omata

Author for correspondence:
Yamanashi Prefectural Central Hospital, Japan
Tel.: +81 552 537 111
Fax: +81 552 538 011
momata-tky@umin.ac.jp

"...four independent studies have clearly shown a close association of *IL-28B* single nucleotide polymorphisms with treatment response to PEG-IFN plus ribavirin, with consistent results among patients of different ethnic origin."

Hepatitis C virus (HCV) infection is a problem worldwide affecting approximately 170 million individuals. HCV causes chronic hepatitis leading to cirrhosis and hepatocellular carcinoma (HCC). The incidence of HCC caused by HCV is increasing in European countries and the USA, with HCV infection a leading cause of liver transplantation in industrialized countries [1]. More than 20 years have passed since the discovery of HCV by Houghton and colleagues [2]. In the last decade, the efficacy of therapy for chronic hepatitis C has improved. Interferon (IFN) treatment enables the eradication of HCV in chronically infected patients. In genotype 1-naive patients with a high viral load, the ratio of sustained virological response (SVR), defined as undetectable HCV RNA 6 months after IFN therapy, has gone from approximately 10% with standard IFN for 6 months to approximately 50% with a combination of pegylated IFN (PEG-IFN) and ribavirin (RBV) for 1 year, which is the current standard therapy [3].

Efficacy of IFN treatment is determined by a number of factors associated with the virus, host and IFN [4]. Genetic variance could influence the difference in treatment response; however, to date, few single nucleotide polymorphisms (SNPs) have been identified. Recently it has become possible to examine the association of genetic variation with observable traits in the analysis of around 500,000 SNPs across a whole genome, although not all known SNPs (currently in excess of 25 million SNPs) can be analyzed.

Between August 2009 and January 2010, four research groups independently identified SNPs in the *IL-28B* region as associated with response to PEG-IFN plus RBV treatment among HCV-infected individuals of European, African and Asian ancestry [5–8]. Ge *et al.* identified rs12979860 (located ~3 kb upstream of *IL-28B*) as the variant most strongly associated with SVR [5]. In their study, patients of European ancestry showed an association of the CC genotype with a twofold (95% CI: 1.8–2.3) greater rate of SVR than the TT genotype. The rate of SVR was found to be similar in people of African–American ancestry with a threefold (95% CI: 1.9–4.7) greater rate of SVR and in Hispanics a twofold (95% CI: 1.4–3.2) greater rate of SVR. The frequency of the CC genotype was 39, 16 and 35% in European–Americans, African–Americans and Hispanics, respectively, indicating that the genome frequency is quite different among these populations, which might explain the distinct response rates to PEG-IFN and RBV among them [5].

Suppiah *et al.*, Tanaka *et al.* and Rauch *et al.* found the strongest association with rs8099917 (located ~8 kb upstream of *IL-28B*), which is in linkage disequilibrium with rs12979860 [6–8]. Suppiah *et al.* and Rauch *et al.* demonstrated *IL-28B* polymorphisms in European cohorts,

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which was associated with an effect on treatment response (odds ratio [OR]: 2.0; 95% CI: 1.6–2.5) and on treatment failure (OR: 5.2; 95% CI: 2.9–9.3), respectively [6,7]. In the study by Suppiah *et al.*, SVR was achieved in 55.9% of 442 patients with the TT genotype, in 36.4% of 357 with the GT genotype and 30.6% of 49 with the GG genotype [6].

Tanaka *et al.* identified *IL-28B* SNP rs8099917 to be associated with SVR in Japanese patients (OR: 12.1; 95% CI: 6.5–22.4), which is a more profound effect than in European cohorts [8]. SVR was achieved in 63.8% of 196 patients with the TT genotype, in 13.3% of 113 with the GT genotype and none of the five patients with the GG genotype. They also showed that the rs8099917 G allele was the most significant factor for predicting nonvirological response (OR: 37.7; 95% CI: 16.7–83.9) after adjusting for confounding factors, suggesting that Japanese patients with the G allele must wait to receive new antiviral therapy.

"The presence of *IL-28B* minor alleles, leading to an unfavorable treatment response, was reported to be associated with lower expression of *IL-28* mRNA in peripheral blood cells."

Approximately 70% of infected individuals develop chronic infection, but the mechanism of persistence remains to be elucidated. The *IL-28B* SNP mentioned previously was also reported to be associated with spontaneous clearance of HCV [7,9]. Thomas *et al.* compared the rs12979860 variation in HCV cohorts of individuals who spontaneously cleared the virus or had persistent infection, and showed that patients with the CC genotype were associated with better treatment response and were three times more likely to clear HCV relative to patients with the CT and TT genotypes of European and African ancestry [9]. Furthermore, they analyzed the rs12979860 C allele frequency by genotyping 2371 individuals from 51 populations worldwide and showed a striking global pattern of allele frequencies: highest frequency (>90%) in East Asia and Oceania, lowest frequency (<50%) in Africa, and intermediate frequency in Europe [9]. This global difference of allele frequency might explain the different frequency in viral clearance and treatment response among these populations.

In the study by Rauch *et al.*, the frequencies of the rs8099917 TT, GT and GG genotypes were 0.78, 0.21 and 0.01 among patients with spontaneous clearance, 0.68, 0.29 and 0.03 among chronically infected patients with SVR, and 0.42, 0.51 and 0.07 among those without SVR, respectively, suggesting that the minor G allele is associated with both persistence of infection and treatment failure [7].

The *IL-29*, *IL-28A* and *IL-28B* genes are located on chromosome 19 and encode IFN-λ1, -λ2, and -λ3, respectively, a new family of IFN-related cytokines recently described [10,11]. IFN-λ interacts with a transmembrane receptor, IFNλR1/IL-10R2, and activates downstream JAK–STAT and MAPK pathways to induce potent antiviral responses [12]. The receptor of IFN-α/β is IFNαR1/IFNαR2 and is different from that of IFN-λ, but the downstream signaling pathway is common. Marcello *et al.*, however, reported that the kinetics of IFN-λ-mediated STAT activation and induction of potential effector genes were distinct from those of IFN-α [13] and that these two proteins, IFN-λ and IFN-α, might have complementary roles in the suppression of HCV. The presence of *IL-28B* minor alleles, leading to an unfavorable treatment response, was reported to be associated with lower expression of *IL-28* mRNA in peripheral blood cells [6,8]. Therefore, a treatment regimen of both IFN-α and IFN-λ might be more promising than the current IFN-α therapy. Pagliaccetti *et al.* reported a cooperative activity of IFN-λ1 and IFN-α or IFN-γ in inhibiting HCV replication and inducing antiviral gene expression [14]. A Phase Ib clinical trial of IL-29 (IFN-λ1) has reported antiviral effects against HCV and low toxicity [15]. A Phase II trial is planned.

The efficacy of recently developed antiviral drugs such as NS3/4A protease inhibitor or NS5A polymerase inhibitor will have no relation to the genetic variant, but these new drugs should be administered in combination with PEG-IFN plus RBV because resistance to them can easily develop. Therefore information on this genetic variant could also be useful for future therapy. For example, patients with a major allele could be treated with standard PEG-IFN plus RBV while patients with a minor allele could be treated with a new drug in combination with PEG-IFN plus RBV.

In conclusion, four independent studies have clearly shown a close association of *IL-28B* SNPs with treatment response to PEG-IFN plus RBV, with consistent results among patients of different ethnic origin. This will open a window for genotype-based personalized medicine for patients with chronic hepatitis C. We should keep in mind, however, that treatment response is predicted by many factors likely to be unrelated to *IL-28B* SNPs, such as age, gender, viral genotype, fibrosis and compliance.

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The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

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Original Article

Internal ribosomal entry-site activities of clinical isolate-derived hepatitis A virus and inhibitory effects of amantadine

Tatsuo Kanda, Fumio Imazeki, Shingo Nakamoto, Kohichiroh Okitsu, Keiichi Fujiwara and Osamu Yokosuka

Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, Chiba, Japan

Aim: Little is known about specific naturally-occurring internal ribosomal entry site (IRES) activities of hepatitis A virus (HAV). We examined these activities using the bicistronic reporter assay and the effects of antiviral amantadine against their activities.

Methods: Six HAV IRES clones from three patients with fulminant hepatitis and three with self-limited acute hepatitis were obtained. The activities of their IRES were analyzed using bicistronic reporter assay in hepatocyte- and non-hepatocyte-derived cell lines, and the potential efficaciousness of the amantadine was examined.

Results: One clone from fulminant hepatitis had a deletion in domains III–IV of HAV IRES had higher IRES activities than HM175 in HLE and Huh-7 cells. In Huh-7 cells, amantadine is effective for inhibiting HAV IRES activities, and especially fulminant hepatitis-derived ones.

Conclusion: HAV IRES derived from clinical isolates have various activities. Bicistronic reporter assay using clinical isolates may be another useful tool for testing antiviral activities like those of amantadine and the new acridines and hydrazones recently reported.

Key words: amantadine, fulminant hepatitis, hepatitis A virus, hepatocyte, internal ribosomal entry site

INTRODUCTION

HEPATITIS A VIRUS (HAV) is a member of the genus *Hepatovirus* in the *Picornaviridae* family. HAV is a positive-sensed single-stranded RNA genome of approximately 7.5 kb in length. The genome codes a large open reading frame (ORF), which is flanked by 5′ non-translated region (5′NTR) and 3′NTR. The downstream part of 5′NTR represents the internal ribosomal entry site (IRES), which mediates cap-independent translation initiation.[1,2] HAV causes acute hepatitis and occasionally leads to severe fulminant hepatitis with fatal outcomes in unvaccinated individuals. Almost 3500 acute hepatitis cases were reported in 2006, representing an estimated 32 000 HAV cases annually in the USA.[3] HAV has dramatically affected rates of the disease in the USA. There continued to be missed opportunities for testing and/or vaccination, and so adherence to recommended HAV vaccination is still low.[4] This highlights the urgent need for a new therapeutic option other than vaccine.[5–10]

Picornavirus translation is initiated in a cap-independent fashion by a mechanism involving the binding of the 40S ribosomal subunit at a site located hundreds of bases downstream of the 5′ end of the RNA, which has been termed IRES. Although the details of translation initiation by internal entry are unknown, it likely involves the interaction of a set of *trans*-acting cellular translation initiation factors with the *cis*-acting IRES, resulting in the binding of the 40S ribosomal subunit to the RNA.[11] HAV IRES spans a region from nt. 161 to the first initiator, AUG, located at nt. 734, and encompasses most of 5′NTR of the viral mRNA.[12] In

Correspondence: Associate Professor Tatsuo Kanda, Department of Medicine and Clinical Oncology, Graduate School of Medicine, Chiba University, 1-8-1 Inohana, Chuo-ku, Chiba 260-8677, Japan. Email: kandat-cib@umin.ac.jp

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HAV genomes, the nucleotide sequence of 5′NTR is more conserved than those of other sites,[13,14] and 5′NTR is predicted to fold into a complex secondary/tertiary structure characterized by six major domains designated I–VI.[15] Domain VI contains the initiation codon. We previously showed that RNA interference targeting various domains of HAV IRES could suppress HAV translation and replication,[6] indicating that some HAV IRES domains might be used as a universal, effective target for specific inhibition of HAV infection.[6] HAV IRES could represent an appropriate target for antiviral drug development.

Amantadine is a tricyclic symmetric amine for use both as an antiviral and an anti-parkinsonian drug. Amantadine inhibits cell-culture-grown HAV IRES-mediated translation in human hepatoma cells,[7] supporting the observation that amantadine could suppress HAV replication in cell culture.[9,16–18] We do not know whether amantadine could suppress clinical isolates from hepatitis A patients.

Here, we examined the HAV IRES activities of clinical isolates from fulminant hepatitis and self-limited acute hepatitis patients in a number of cell lines and tested the effects of amantadine on their IRES-mediated translation by reporter assay. As translation of fulminant hepatitis-derived IRES varies, but it is still efficiently suppressed by amantadine, the approaches described here might open new strategies for useful therapeutic options in cases of fulminant hepatitis A.

METHODS

Cell lines and reagents

HUMAN HEPATOMA CELL lines Huh-7, HepG2 and HLE, the human cervical carcinoma cell line HeLa, and African green monkey kidney cell lines BSC-1 and CV-1 were purchased from Health Science Research Resources Bank (Japanese Collection of Research Bioresources, Osaka, Japan) and maintained in Dulbecco's minimum essential medium (Gibco BRL, Gaithersburg, MD, USA). Amantadine hydrochloride was purchased from Sigma-Aldrich (St Louis, MO, USA).

Bicistronic reporter plasmids

The simian virus (SV)40 promoter plasmid pSV40-HAV-HM175-IRES encodes in a bicistronic fashion the *Renilla reniformis* luciferase (Rluc), the HAV IRES derived from pHM175 (kindly provided by S. U. Emerson, National Institutes of Health, Bethesda, MD, USA),[19] followed by the firefly luciferase (Fluc). It was prepared by polymerase chain reaction (PCR)-based subcloning the IRES (nt. 139–854) of HAV strain HM175[19] and Rluc into pGL3-promoter Vector (Promega, Madison, WI, USA) (Fig. 1a, upper part). Plasmids pSV40-HAV-F1-IRES, pSV40-HAV-F2-IRES, pSV40-HAV-F3-IRES, pSV40-HAV-A1-IRES, pSV40-HAV-A2-IRES and pSV40-HAV-A3-IRES replaced HAV-F1-IRES, HAV-F2-IRES, HAV-F3-IRES, HAV-A1-IRES, HAV-A2-IRES and HAV-A3-IRES, respectively, into HAV-HM175 of the plasmid pSV40-HAV-HM175-IRES. F1–F3 and A1–A3 are derived from fulminant hepatitis and self-limited acute hepatitis, respectively.[20,21] The sequences of plasmids were confirmed by directly sequencing using ABI 377 (Applied Biosystems, Urayasu, Japan).

Transfection and *in vitro* reporter assays

Approximately 1.0×10^5 cells per well were placed in a six-well plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. Cells were transfected with 0.4 μg of pSV40-HAV-IRES using Effectene Transfection Reagent (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Six hours after transfection, the cells were washed once with phosphate buffered saline (PBS), and culture media with or without drugs were added. Forty-eight hours after transfection, cells were harvested using reporter lysis buffer (Toyo Ink, Tokyo, Japan), and luciferase activity was determined by luminometer (AB-2200-R; ATTO, Tokyo, Japan).[7] To control for variations in transcription, IRES activity was assessed by measuring the ratio of *Renilla* and firefly luciferases. All samples were run in triplicate.

Data analysis

The sequences reported in this study have been deposited in GenBank under accession numbers AB513790 to AB513795 for F1 to A3. Sequence analyses were performed using GENETYX ver. 9 (GENETYX, Tokyo, Japan). Data were expressed as mean ± standard deviation. Statistical analysis was done using Student's *t*-test. $P < 0.05$ was considered significant.

RESULTS AND DISCUSSION

Naturally occurring HAV IRES

HEPATITIS A VIRUS strains associated with human disease show genetic divergence.[22,23] First, we cloned the sequences of HAV derived from clinical isolates and made each bicistronic vector. PCR products were derived from serum samples of patients with fulminant hepatitis, in whom prothrombin time had

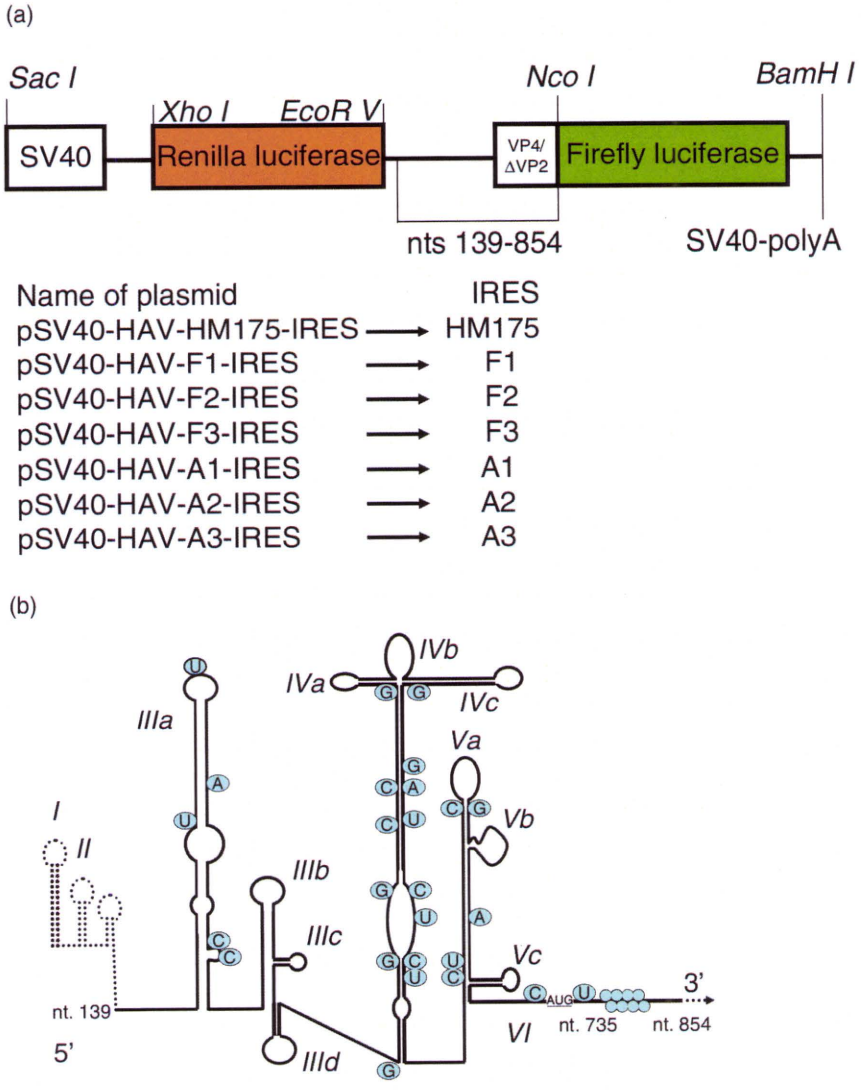

Figure 1 Bicistronic reporter constructs used in this study. (a) pSV40-HAV-HM175-IRES was described previously.[6,7] It encodes the *Renilla* luciferase genes, the internal ribosome entry site (IRES) of hepatitis A virus (HAV) HM175, and the firefly luciferase gene under the control of the simian virus 40 promoter (SV40). pSV40-HAV-F1-IRES, pSV40-HAV-F2-IRES, and pSV40-HAV-F3-IRES encode the IRES from fulminant hepatitis F1, F2, and F3, respectively, instead of the IRES of HM175. pSV40-HAV-A1-IRES, pSV40-HAV-A2-IRES and pSV40-HAV-A3-IRES encode the IRES from self-limited acute hepatitis A1, A2 and A3, respectively, instead of the IRES of HM175. (b–g) Secondary structure and mutations in the HAV IRES constructs used in this study.[6,7,11] Major structural domains are labeled I–VI; blue circles indicate mutations and red-dashed lines were deleted parts, compared with HM175 clone. HAV IRES constructs used in this study include the black line parts. F1 (b), F2 (c) and F3 (d) and A1 (e), A2 (f) and A3 (g) were derived from fulminant and acute self-limited hepatitis, respectively.

decreased to less than 40% with hepatic encephalopathy of grade II or more within 8 weeks after the onset of disease,[24] and with self-limited acute hepatitis in whom prothrombin time had not decreased below 40%. We sequenced these isolates, showing the differences of these sequences in Figure 1(b–g). Of note, one was derived from fulminant hepatitis F3 with a sequence deletion from nt. 233–380 in the HAV IRES region (Fig. 1d) corresponding to a portion of domain III to a part of domain IV. This F3 nucleotide sequence matched 75.60–77.93% of those of cell culture-derived clone HM175 or other clinical isolates in this study. The

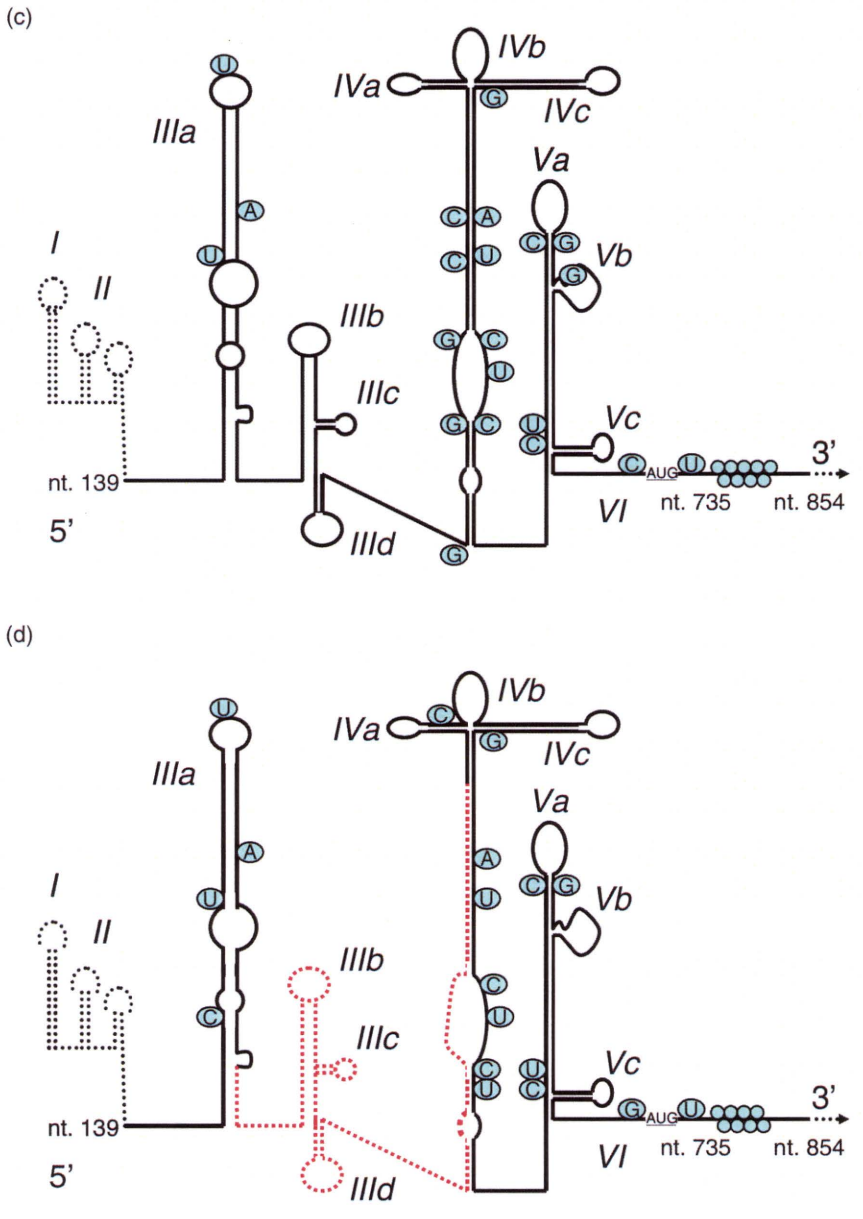

Figure 1 *Continued.*

nucleotide sequences of other clinical isolates matched 95.25–95.81% of that of HM175. The ones other than F3 matched 98.18–99.30% with each other (data not shown).

HAV IRES activities from clinical isolates vary in human hepatocytes

Several mutational studies of HAV IRES were previously reported.[25,26] Our major concern before starting this study was whether HAV IRES activities were correlated to the severities of the clinical manifestations of hepatitis A, as HAV genome replication is directly dependent on IRES-mediated translation. Then, we examined how these IRES activities behaved in hepatocytes and non-hepatocytes. It is thought that HAV mainly replicates in the liver where it induces inflammation,[1,2] so we initially examined translation efficiencies of HAV IRES in the following human hepatoma cells: Huh-7, HepG2 and HLE cells (Fig. 2a–c). The IRES activities (firefly luciferase/*Renilla* luciferase: cap-independent/cap-

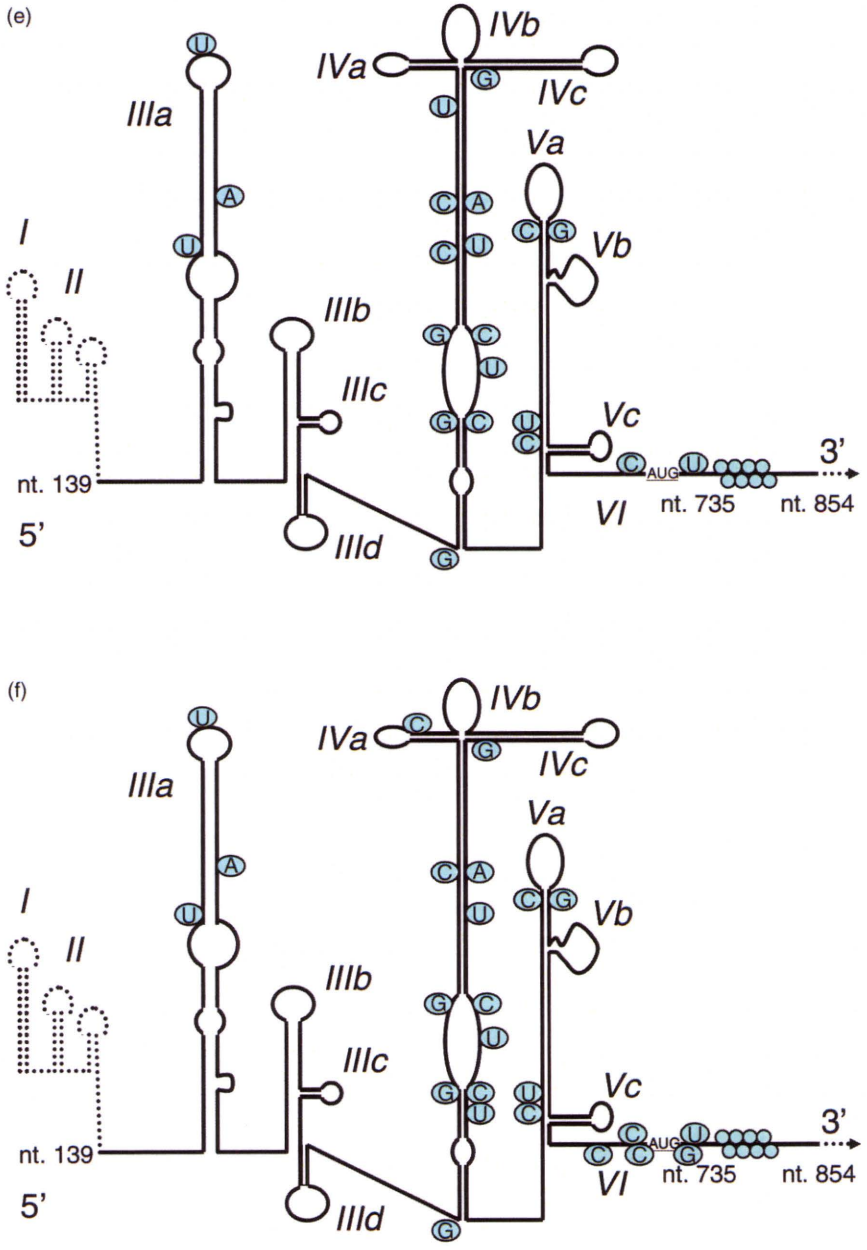

Figure 1 *Continued.*

dependent translation efficiencies) of HM175 were used as control. The HM175 strain of HAV was originally recovered from stool of a patient with hepatitis A in Melbourne, Australia. It is well-known that plasmid pHM175 was isolated directly from primary African green monkey kidney cells infected with this virus and *in vitro* transcribed RNA from this clone were cell-culture grown.[13,19] In Huh-7 cells, in which HAV can replicate and which are commonly used for HAV research,[27] the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.05-, 0.39-, 3.76-, 0.048-, 2.19- and 0.6-fold, respectively, of that of HM175 (Fig. 2a). In HepG2, the IRES activities of F1, F2, F3, A1, A2 and A3 were 1.29-, 0.33-, 3.68-, 0.12-, 8.91- and 4.23-fold, respectively, of that of HM175 (Fig. 2b). In HLE, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.85-, 1.35-, 5.17-, 0.55-, 29.06- and 4.31-

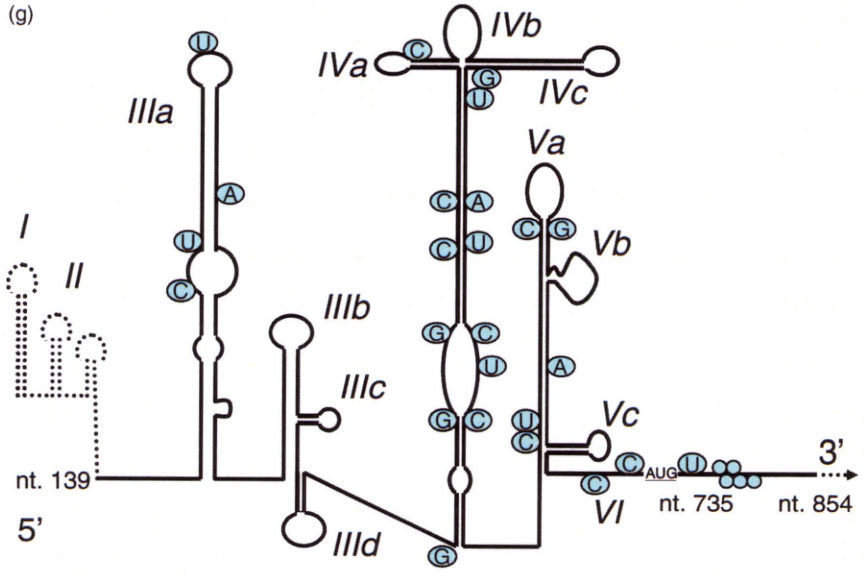

Figure 1 *Continued.*

fold, respectively, of that of HM175 (Fig. 2c). Compared with HM175, F3, A2 and A3 tended to have higher IRES activities in liver-derived cell lines, F1 had similar IRES activity to HM175, and F2 and A1 tended to have lower IRES activities than HM175.

F3 has a large deletion in domains III–IV (Fig. 1d). In A2, several nucleotide mutations around the AUG codon in domain VI of IRES were seen. These mutations possibly affected their IRES activities. A3 IRES activities in Huh-7, HepG2 and HLE were 0.6-, 4.23- and 4.31-fold, respectively. A2 showed very high activity in HLE compared to other IRES. These results may have been influenced by certain cellular factors.

HM175 HAV IRES activities not lower than those of clinical isolates in non-hepatocyte-derived cell lines

In HeLa, another permissive cell line[28] for HAV, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.33-, 0.31-, 1.0-, 0.11-, 0.87- and 0.32-fold, respectively, of that of HM175 (Fig. 2d).

Acute hepatitis A occasionally presents the manifestation of acute renal failure during the course of the disease.[10] We thus examined HAV IRES activities in African green monkey kidney cell lines BSC-1 and CV-1, as they were reported to be permissive for HAV replication,[29,30] and then we examined the HAV IRES activities in these two cell lines. In BSC-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.11-, 0.10-, 0.73-, 0.02-, 0.48- and 0.86-fold, respectively, of that of HM175 (Fig. 2e). In CV-1, the IRES activities of F1, F2, F3, A1, A2 and A3 were 0.04-, 0.20-, 0.46-, 0.05-, 0.76- and 0.62-fold, respectively, of that of HM175 (Fig. 2f). IRES activities of F3, A2 and A3, which tended to be higher in hepatocytes (Fig. 2a–c), did not differ much from that of HM175 in these non-hepatic cells (Fig. 2d–f). The IRES activities of HM175 in Huh-7, HepG2, HLE, HeLa, BSC-1 and CV-1 were 43.3, 469, 18.2, 618, 1814 and 1097, respectively.

Amantadine has inhibitory effect on clinical isolate-derived HAV-IRES-mediated translations

Amantadine has potential as an antiviral agent against HAV.[9,16–18] We previously reported that amantadine has an inhibitory effect on HAV HM175 IRES-mediated translation.[7] However, the effects of amantadine on clinical isolates from hepatitis A patients were still unknown. Concentrations of 1–100 μg/mL of amantadine were non-cytotoxic to hepatocytes.[7] Huh-7 cells were treated with 100 μg/mL of amantadine or PBS 24 h after transfection of reporter plasmids. Forty-eight hours after transfection, dual-reporter assay was performed for the evaluation of cap-dependent and cap-independent translation initiation (Fig. 3). In A2 isolates, IRES activity in the presence of amantadine was 0.93-fold that in its absence. However, amantadine at 100 μg/mL was effective against all fulminant hepatitis-derived IRES

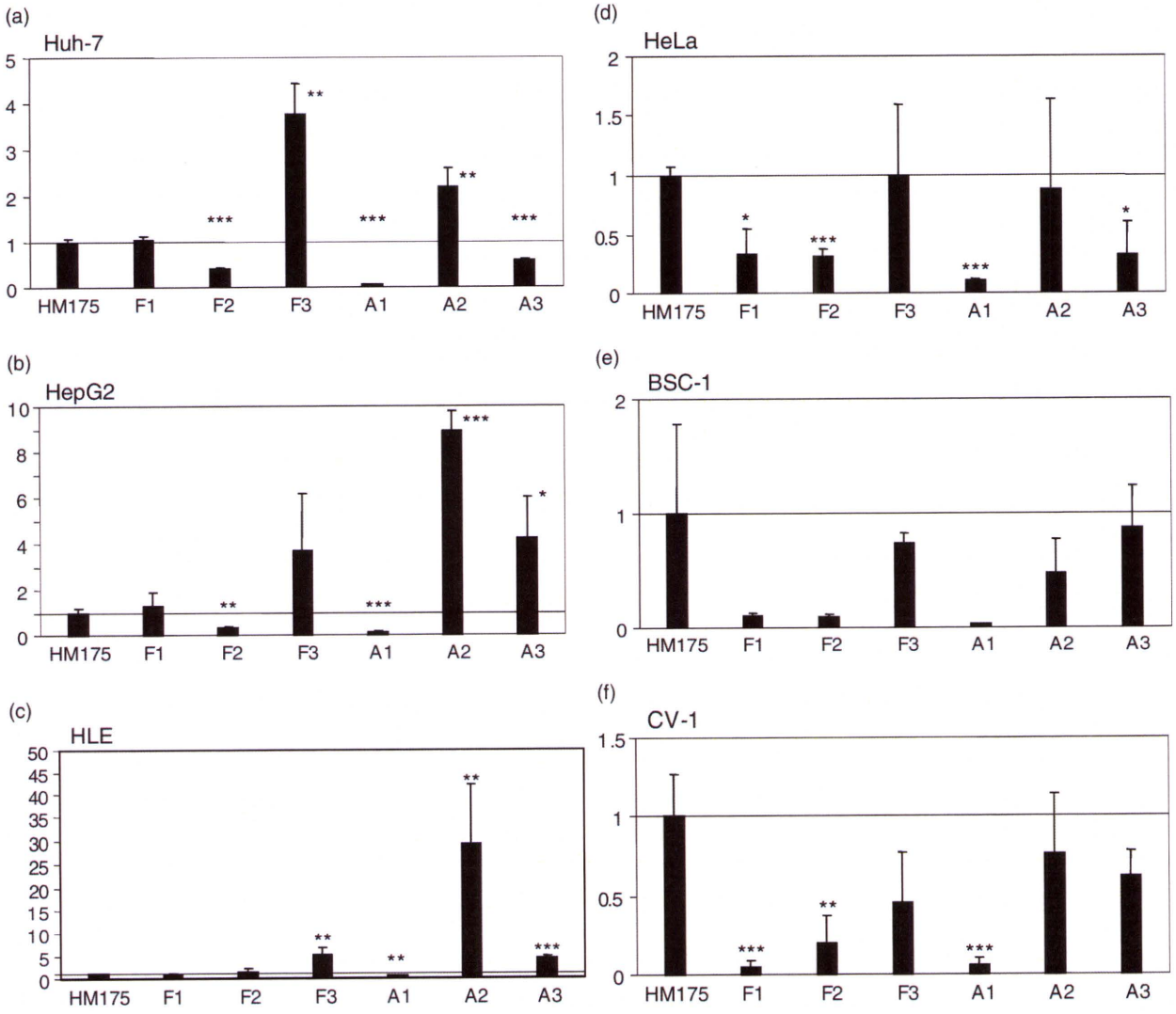

Figure 2 Clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities in hepatocytes and non-hepatocytes. Plasmids of pSV40-HAV-IRES were transfected into hepatocytes: Huh-7 (a), HepG2 (b), HLE (c) and non-hepatocytes: HeLa (d), BSC-1 (e) and CV-1 (f). Forty-eight hours after transfection, dual-luciferase assays were performed. The IRES activities (firefly luciferase/*Renilla* luciferase) of pSV40-HAV-HM175-IRES were set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. $^{*}P < 0.05$ vs HM-175; $^{**}P < 0.01$ vs HM-175; $^{***}P < 0.005$ vs HM-175.

activities (Fig. 3). Future studies will reveal whether fulminant hepatitis-derived HAV is more sensitive to amantadine than HAV from self-limited acute hepatitis.

Clinical manifestations among hepatitis A patients in the present study varied from self-limited acute hepatitis to severe fulminant hepatitis. It was reported that chronic liver diseases and older patients influenced the severity of hepatitis A, and the host immune response may vary at the cellular level in such patients. An absence of obvious correlation between genotypes and clinical status has been reported.[31] In this study, the fulminant hepatitis F3 clone had a relatively higher IRES activity in hepatocytes. We previously demonstrated that inhibition of IRES activities by small interfering RNA (siRNA) leads to the suppression of HAV viral replication in cell culture. Fujiwara *et al.*[32] reported that higher viral load in patients with fulminant and severe hepatitis A may be associated with the pathogenesis of disease severity. Further studies are needed.

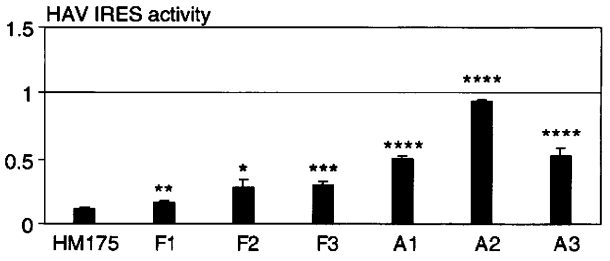

Figure 3 The effects of amantadine against clinical isolate-derived hepatitis A virus (HAV) internal ribosome entry site (IRES) activities. Twenty-four hours after transfection of reporter plasmids, Huh-7 cells were treated with 100 μg/mL of amantadine or phosphate buffered saline (PBS). The effects of amantadine against IRES activities (firefly luciferase/*Renilla* luciferase) are shown. Each value of IRES activity treated with PBS was set at 1. F1, F2 and F3 and A1, A2 and A3 were derived from fulminant and acute self-limited hepatitis, respectively. $^{*}P < 0.05$ vs HM-175; $^{**}P < 0.01$ vs HM-175; $^{***}P < 0.005$ vs HM-175; $^{****}P < 0.001$ vs HM-175.

It was reported that an antibiotic resistance titration assay (ARTA) is useful for HAV neutralization including virus-receptor interaction.[33,34] We also tested the effects of amantadine against HAV IRES of clinical isolates and confirmed the effects against fulminant hepatitis clones. Recently, it was reported that new acridines and hydrazones derived from cyclic β-diketone have stronger antiviral activities against HAV than amantadine.[9] Bicistronic reporter assay using clinical isolates may be another useful tool for testing antiviral activities like those of these drugs.

In conclusion, HAV IRES activities from clinical isolates vary in relation to different cell lines. For the development of antiviral agents against HAV, it seems important to investigate these effects on clinical isolates.

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Mechanism of Entecavir Resistance of Hepatitis B Virus with Viral Breakthrough as Determined by Long-Term Clinical Assessment and Molecular Docking Simulation▽†

Motokazu Mukaide,[1,2] Yasuhito Tanaka,[1]* Tadasu Shin-I,[1] Man-Fung Yuen,[3] Fuat Kurbanov,[1] Osamu Yokosuka,[4] Michio Sata,[5] Yoshiyasu Karino,[6] Gotaro Yamada,[7] Kohsaku Sakaguchi,[8] Etsuro Orito,[9] Manami Inoue,[10] Sumbella Baqai,[11] Ching-Lung Lai,[3] and Masashi Mizokami[12]

Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan[1]; SRL, Inc., Tokyo, Japan[2]; Department of Medicine, University of Hong Kong, Queen Mary Hospital, Hong Kong[3]; Department of Medicine and Clinical Oncology, K1, Graduate School of Medicine, Chiba University, Chiba, Japan[4]; Department of Internal Medicine, Kurume University School of Medicine, Kurume, Japan[5]; Department of Gastroenterology, Sapporo Kosei Hospital, Sapporo, Japan[6]; Department of Internal Medicine, Center for Liver Diseases, Kawasaki Hospital, Kawasaki Medical School, Okayama, Japan[7]; Department of Gastroenterology and Hepatology, Okayama University Graduate School of Medicine, Dentistry and Pharmaceutical Sciences, Okayama, Japan[8]; Department of Gastroenterology, Nagoya Daini Red Cross Hospital, Nagoya, Japan[9]; Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo, Japan[10]; Department of Internal Medicine, Alameda County Medical Center, Oakland, California[11]; and Research Center for Hepatitis and Immunology, International Medical Center of Japan Konodai Hospital, Ichikawa, Japan[12]

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The mechanism by which entecavir resistance (ETVr) substitutions of hepatitis B virus (HBV) can induce breakthrough (BT) during ETV therapy is largely unknown. We conducted a cross-sectional study of 49 lamivudine (LVD)-refractory patients and 59 naïve patients with chronic hepatitis B. BT was observed in 26.8% of the LVD-refractory group during weeks 60 to 144 of ETV therapy. A line probe assay revealed ETVr substitutions only in the LVD-refractory group, i.e., in 4.9% of patients at baseline, increasing to 14.6%, 24.4%, and 44.8% at weeks 48, 96, and 144, respectively. Multivariate logistic regression analysis adjusted for age, gender, HBV DNA levels, and LVD resistance (LVDr) (L180M and M204V, but not M204I) indicated that T184 substitutions and S202G (not S202C) were a significant factor for BT (adjusted odds ratio [OR], 141.12, and 95% confidence interval [CI], 6.94 to 2,870.20; OR, 201.25, and 95% CI, 11.22 to 3608.65, respectively). Modeling of HBV reverse transcriptase (RT) by docking simulation indicated that a combination of LVDr and ETVr (T184L or S202G) was characterized by a change in the direction of the D205 residue and steric conflict in the binding pocket of ETV triphosphate (ETV-TP), by significantly longer minimal distances (2.2 Å and 2.1 Å), and by higher potential energy (−117 and −99.8 Kcal/mol) for ETV-TP compared with the wild type (1.3 Å; −178 Kcal/mol) and LVDr substitutions (1.5 Å; −141 Kcal/mol). Our data suggest that the low binding affinity of ETV-TP for the HBV RT, involving conformational change of the binding pocket of HBV RT by L180M, M204V plus T184L, and S202G, could induce BT.

Infection with hepatitis B virus (HBV) is extremely widespread and affects more than 350 million people worldwide. Chronic HBV infection leads to the development of complications, such as liver cirrhosis (LC) and hepatocellular carcinoma (HCC) (12). HBV has been classified into 8 geographically, genetically, and clinically diverse genotypes, designated alphabetically from A to H according to their order of discovery (14). Genotypes B and C are prevalent in Asia, and genotype C is associated with more serious liver disease, including LC and HCC, and a poorer response to interferon therapy than genotype B (5). The ultimate therapeutic goal when treating chronic HBV infection is to prevent the development of LC and HCC by eliminating or producing sustained suppression of HBV replication. However, lamivudine resistance (LVDr) was reported to occur in 24% of patients treated for 1 year and in 74% of those treated for 5 years (16, 26). The rate of adefovir resistance (ADVr) in nucleoside-naive hepatitis B e antigen (HBeAg)-negative patients has been reported to be 0% after 1 year, but after 5 years of treatment, the rate increases to 28% to 42% (13). Entecavir (ETV) has been shown to be more potent *in vitro* than either LVD or ADV. Results from clinical studies showed that the efficacy of ETV was superior to that of the direct comparator, LVD, in both nucleoside-naive and LVD-refractory patients (6, 11, 15, 18).

* Corresponding author. Mailing address: Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan. Phone: 81-52-853-8191. Fax: 81-52-842-0021. E-mail: ytanaka@med.nagoya-cu.ac.jp.

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TABLE 1. Patient characteristics of naïve and LVD-refractory patients

Characteristic	Value	
	Naïve (n = 59)[a]	LVD refractory (n = 41)[a]
Male/female no.	41/18	34/7
Mean age (yr)	46.5 ± 8.4	48.6 ± 8.3
HBeAg positive (%)	33 (55.9)	23 (56.1)
Mean ALT (U/liter)	118.9 ± 108.6	119.8 ± 99.0
Mean HBV DNA ($\log_{10}$ copies/ml)	6.7 ± 1.8	6.8 ± 1.0
Genotypes (no. A/B/C/D/E)	3/11/43/1/1	5/7/28/1/0

[a] Values are means ± standard deviations.

The persistence of LVDr substitutions in patients switched to ETV is worrisome, because LVDr was shown to enhance the risk of developing ETVr and treatment failure, defined as viral breakthrough (BT) (an increase in serum HBV DNA of at least 1 $\log_{10}$ copy/ml compared with the nadir value as observed during ETV therapy) (20). A recent *in vitro* study showed that LVDr (L180M and M204V) substitutions confer an ~8-fold reduction in susceptibility to ETV and that additional substitutions at residues T184, S202, and M250 are needed to confer high levels of ETVr and BT (2, 3).

These analyses, however, used a limited number of patient isolates and/or laboratory HBV clones, and there has been a paucity of community-based data derived from long-term trials regarding the clinical outcomes of ETVr variants in naïve or LVD-refractory patients. Therefore, the aim of this study was to evaluate the incidence of ETVr and BT by comparing outcomes following 3-year ETV treatment in treatment-naïve patients and LVD-refractory patients. ETVr was assessed by using a recently reported line probe assay (HBV DR v.3) (7). Importantly, as the mechanism by which ETVr substitutions can induce BT during ETV therapy is largely unknown, changes in the conformation of HBV reverse transcriptase (RT) arising from LVDr and ETVr substitutions were modeled by using 3-dimensional (3D) docking simulation.

MATERIALS AND METHODS

Study design. We conducted a cross-sectional study of 100 patients (Tables 1 and 2), 45 of whom were from Japan, 25 from the United States, and 30 from Hong Kong. The patients were subdivided into two groups; treatment-naïve (n = 59) and LVD-refractory (n = 41) patients, whose gender, age, HBeAg status, and mean HBV DNA levels are summarized in Table 1. The patients received 0.5 mg or 1.0 mg ETV. The 1.0-mg ETV once-daily (QD) dosage has been approved for use in LVD-refractory patients, and only patients treated with 1.0 mg per day were included in resistance assessments. The study protocol conformed to the 1975 Declaration of Helsinki and was approved by the Ethics Committees of the institutions, and written informed consent was obtained from each participant.

Screening for drug-resistant substitutions. Simultaneous detection of wild-type HBV and drug-induced substitutions was performed using HBV DR v.3 and v.2 (Innogenetics, Ghent, Belgium) according to the manufacture's protocol. HBV DR v.3 and v.2 were developed for detection of ETVr-specific substitutions (T184SCGA/ILFM, S202G/C/I, and M250V/I/L), TDFr-specific substitutions (A194T), and newly reported ADVr (I233V) substitutions, as well as LVDr (L80V/I, V/G173L, L180M, and M204V/I) and ADVr (A181T/V and N236T) substitutions. The HBV DR assay consistently detected ETVr-specific substitutions present in ≥5% of the virus population when the HBV DNA concentration was ≥4 $\log_{10}$ copies/ml (7). The AUTOLiPA (Innogenetics, Ghent, Belgium) was used for the automated test procedure. An 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (HBV DR v.3 and v.2). PCR products were directly sequenced.

Statistical analyses. The statistical significance of observed differences was assessed using the chi-square test and the Mann-Whitney U test, where appropriate. In the 67 patients (38 naïve and 29 LVD refractory) with 3 years of ETV treatment (Fig. 1), the logistic regression model was used to assess the factors associated with BT. STATA 10 (Statacorp LP, TX) and the Statistical Program for Social Sciences (SPSS 12.0 for Windows; SPSS Inc., Chicago, IL) were used for all analyses.

HBV polymerase sequencing. HBV DNA was extracted from serum samples using a Qiagen QIAamp DNA blood minikit (Qiagen GmbH, Germany), and an 867-bp-long fragment of the polymerase gene (domains A to F) was amplified using biotinylated PCR primers (INNO-LiPA). PCR products were directly sequenced. Nucleotide mixtures were reliably detected when they were mixed at a ratio of approximately 25% or greater.

Three-dimensional-structure-based docking simulation methods. The amino acid sequence of HBV RT was retrieved from GenBank (gene Pol product of accession no. X75665), and the 323rd to 697th residues, which correspond to the finger, palm, and thumb domains, were extracted. The sequence and that of HIV RT, retrieved from the Protein Data Bank (accession no. 1RTD), were aligned using BLASTP (1), and then the resulting alignment was modified manually to obtain a match of the RT-specific motifs in both sequences. The main-chain structure of HBV RT was built from the alignment and the 3D structure of HIV RT (accession no. 1RTD) (8) by the use of the "nest" module (17) in the JACKAL package (19), where global energy minimization was done to find the most stable backbone structure. The loop and secondary-structure regions were then refined (24), after which the side chain structure was refined by the use of the "scap" module in the package (23). The 3D structures of HBV RT containing three sets of substitutions, L180M plus M204V, L180M plus S202G plus M204V, and L180M plus T184L plus M204V, were also designed in the same manner.

The binding site of ETV was searched on the wild-type HBV RT molecule by docking simulation. First, the structure of ETV triphosphate (ETV-TP) was designed by a small-molecule-editing function in the SYBYL 8.0 package (Tripos Inc., St. Louis, MO). Then, the possible binding sites of the ligand were searched from the surface of the protein by the use of the "Surflex-Dock" (9) module in the package. Here, the docking candidate area was restricted to the surfaces of the residues that were within 3 Å from L180, T184, S202, Y203, M204, D205, or D206. The binding potential was estimated from the GOLD score calculated by

TABLE 2. Three-year assessment (HBeAg loss, ALT normalization, and HBV-DNA 2.6) of naïve and LVD-refractory patients

Parameter	Value for follow-up week:					
	Naïve (n = 59)[a]			LVD refractory (n = 41)[a]		
	48	96	144	48	96	144
Follow-up [n (%)]	59 (100)	39 (66.1)	38 (64.4)[b]	41 (100)	40 (97.6)[c]	26 (63.4)[d]
HBeAg loss [n (%)]	5 (15.2)	7 (24.1)	9 (32.1)	4 (17.4)	7 (31.8)	4 (22.2)
ALT normalization [n (%)]	24 (40.7)	25 (64.1)	27 (71.1)	16 (39.0)	20 (50.0)	13 (50.0)
HBV DNA loss [n (%)]	24 (40.7)	28 (71.8)	28 (73.7)	15 (36.6)	19 (47.5)	12 (46.2)

[a] Values are means ± standard deviations.

[b] One naïve patient (J44) stopped ETV therapy at week 80 (ALT, 119 U/liter, and HBV DNA, 7.6 $\log_{10}$ copies/ml at baseline; ALT, 17, and HBV DNA, <2.6 at week 80) due to severe headache during therapy. Twenty patients in Hong Kong stopped ETV therapy between weeks 48 and 72.

[c] One LVD-refractory patient (J37) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 96.

[d] Two patients (J33 and J40) switched from ETV therapy to LVD plus adefovir due to BTH with ETVr before week 144. Twelve patients in the United States were treated with ETV for <120 weeks.

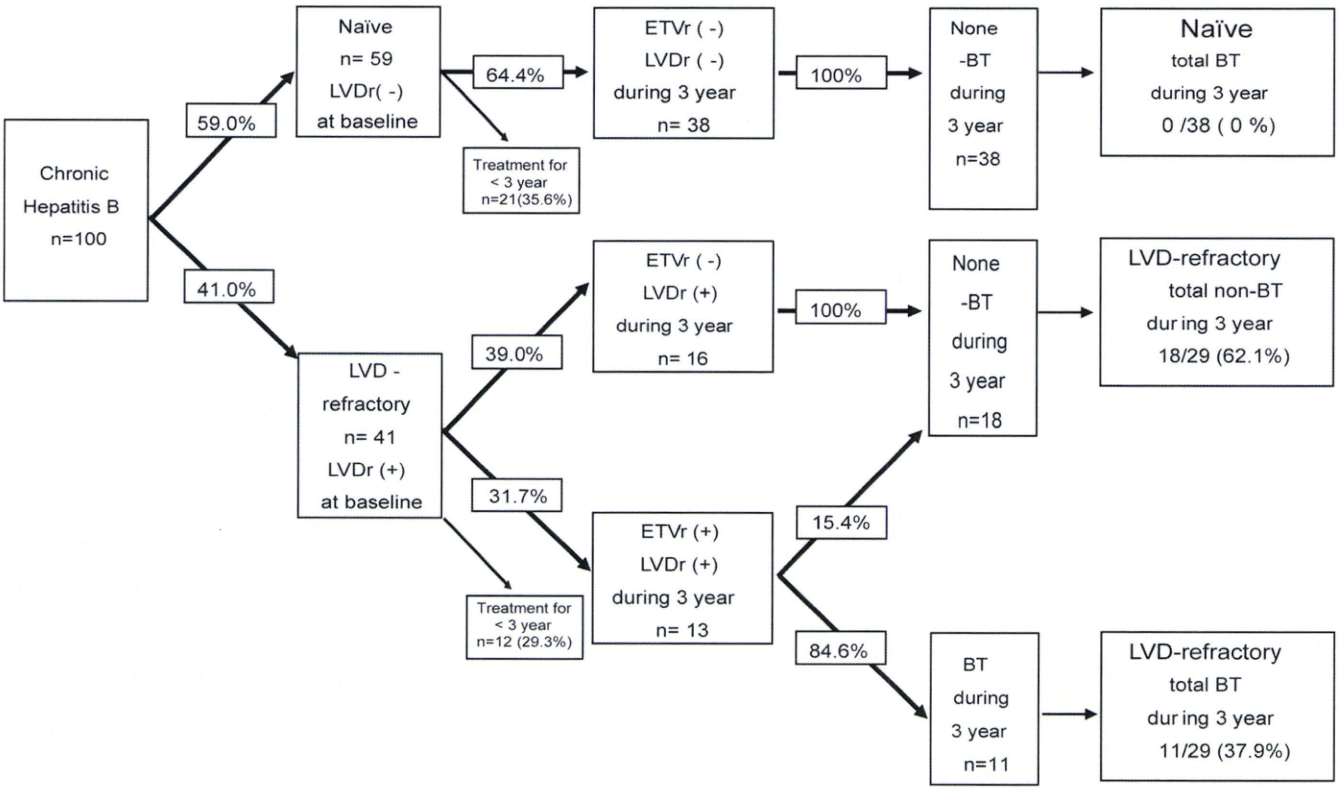


FIG. 1. Flowchart of 100 naïve/LVD-refractory patients during ETV therapy.

the "CScore" module (4) in the package. The score was evaluated based on hydrogen bond energy, the internal energy of molecules, and complex energy between ligand and protein. The minimal distance between their molecular surfaces was also calculated.

RESULTS

Clinical efficacy. The clinical backgrounds and the percentages of LVD-naïve and LVD-refractory patients who achieved HBeAg loss, alanine aminotransferase (ALT) normalization, and non-PCR-detectable HBV DNA levels (<2.6 $\log_{10}$ copies/ml) during the ETV treatment course are summarized in Table 2. There were no significant differences in clinical data at entry between the 2 groups. The rates of HBeAg loss, ALT normalization, and HBV DNA loss were significantly higher in naïve patients than in LVD-refractory patients.

Detection of substitutions responsible for ETV resistance in naive and LVD-refractory patients during treatment for 144 weeks. The characteristics of patients who had ETVr substitutions detected by HBV DR v.3 are summarized in Table 3. The percentage of the typical LVDr (L180M, M204V, and M204I) or ETVr observed in naïve patients was 0% (0/38) during the 144-week treatment period.

Among the patients examined at entry prior to treatment with ETV, in 41 LVD-refractory patients, M204V (30/41; 72.4%), M204I (24/41; 58.5%), L180M (38/41; 92.7%), L80V (6/41; 14.6%), L80I (18/41; 43.9%), and V173L (4/41; 9.76%) substitutions were detected. In the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively (Fig. 2). In the 29 patients treated with ETV for 3 years, T184SCGA, T184ILMF, S202G, and S202C were found in 5 (17.2%), 4 (13.8%), 9 (31.0%), and 1 (3.4%), respectively. Neither S202I nor M250V/I/L substitutions were detected in this population.

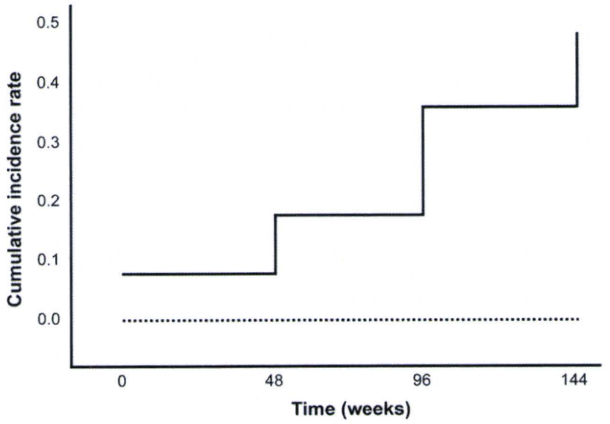
A comparative summary of the ETVr substitutions, detected by HBV DR v.3 and direct sequencing, during week −8 (8 weeks before the start of treatment) and week 144 is presented in Table 4. HBV DR v.3 revealed ETVr substitutions earlier (up to 48 weeks) than did direct sequencing. In addition, HBV DR v.3 allowed the detection of mixed quasispecies containing different substitutions.

Viral BT during the 144 weeks on treatment. The rates of BT among 59 naive and 41 LVD-refractory patients treated with ETV for 144 weeks are summarized in Fig. 1. There were no cases of BT in the LVD-naïve group during the 144-week treatment period, whereas in the LVD-refractory group treated with 1.0 mg ETV, 11 of 13 patients with genotypic ETVr had evidence of BT after 60 to 144 weeks of treatment, followed by 7 breakthrough hepatitis (BTH) (defined as a flare up of ALT) patients (median interval, 11.4 weeks after BT). The LVDr substitutions (L180M and M204V/I) were detected in all of the BT patients in specimens obtained at baseline (Table 3). Among the 11 patients with BT, 8 (72.7%) had an additional S202G substitution and 7 (63.6%) had a T184SCGA or T184ILMF substitution, indicating that the T184 and/or S202 substitution emerged before BT during ETV treatment (Table 3 and Fig. 2). Seven patients with BTH had LVDr

TABLE 3. Characteristics of ETVr detected by HBV DR v3 among 13 patients at week 0, 24, 48, 72, 96, and 144

Case	Age (yr)	Sex	HBV genotype	ALT (IU/liter)			HBV DNA (copies/ml)			Wk			No. of wks ETVr to BT	LVDr at baseline	ETVr at baseline	ETVr emerged during therapy
				Baseline	Nadir	Peak	Baseline	Nadir	Peak	ETVr	BT	BTH				
J20	34	M	C	82	22	131	7.6	4.5	7.4	<92[a]	92	100	<22	M180, V204	ND[f]	ILMF184, G202
J27	61	M	C	79	38	150	7.4	3.9	7	<128[b]	128	144	<32	M180, V204	ND	SCGA184
J30	43	M	C	95	21	108	7.6	4.6	6.8	48	76	92	28	I80, M180, V/I204	ND	G202
J33	43	M	C	69	21	199	7.6	5.1	7.1	96	116	128	18	I80, L173, M180, VI204	ND	(ILFM184),[c] G202
J37[d]	48	M	C	465	43	76	7.6	4.7	6	48	60	60	12	I80, M180, V204, I204	ND	G202
J39	59	M	C	35	22	82	7	4.6	8	72	108	128	36	M180, V204	ND	SCGA/ILFM184, G202
J40	28	M	C	149	15	398	5.3	3.6	6.6	−8	64	72	72	M180, V204	(G+C) 202	G202
J22	44	M	C	240	17	24	7.6	3.5	5.6	<140[e]	144	NO	<44	M180, I204	ND	G202
J28	45	M	B	43	15	23	6.9	5.5	6.5	0	144	NO	144	M180, V204	SCGA184, V233	SCGA184, V233
U72	47	F	C	29	25	44	7.5	3.8	5	<144	144	NO	<48	L + V80, M180, V204	ND	SCGA184, (S+G)202
H55	47	F	C	102	19	20	6.1	3	4.2	48	88	NO	40	V + I80, M180, V204	ND	ILFM184
J19	57	M	B	233	29	43	7.4	2.8	3.3	24	NO	NO	NO	V + 180, M180, I204	ND	G202
U42	52	M	A	135	36	50	>7.6	5.3	5.8	<80	NO	NO	NO	M180, V204	ND	SCGA184

[a] Not tested during weeks 72 and 92.
[b] Not tested during weeks 96 and 128.
[c] ILFM184 was detected at week 144.
[d] Switch to ADV/LVD at week 60.
[e] Not tested during weeks 96 and 140.
[f] ND, not detected.



LVD-refractory patients

Weeks	0	48	96	144
No.	41	41	41	29*
ETVr	2 (4.9%)	6 (14.6%)	10 (24.4%)	13 (44.8%)
T184SCGA	1	1	3	5
T184ILFM	0	1	4	4
S202G	1	4	7	9
S202C	1	1	1	1
S202I	0	0	0	0
M250VIL	0	0	0	0

*Twelve patients in the US have ETV treatment for <120 weeks.

FIG. 2. Kaplan-Meier plot and tabulated data for time to ETVr and cumulative ETVr patterns over 144 weeks. Pretreatment variables (solid line, LVD refractory; broken line, naïve) were analyzed in relation to the occurrence of ETVr. A previous LVD treatment was associated with a more rapid occurrence of ETVr (Breslow analysis; $P < 0.001$). Among the patients examined at entry prior to treatment with ETV, for the 41 LVD-refractory patients, the cumulative ETVr substitutions were detected in 2/41 (4.9%) at baseline and increased to 6/41 (14.6%), 10/41 (24.4%), and 13/29 (44.8%) at weeks 48, 96, and 144, respectively. Neither the S202I nor the M250V/I/L substitution was detected in this population.

(100% for both M180 and V204) at baseline and ETVr substitutions (S202G, 85.7%; T184SCGA/ILFM, 57.1%) during 3-year ETV treatment. Representative cases with BTH during ETV therapy are shown in the supplemental material.

Pretreatment status (LVD refractory or naïve) was analyzed in relation to the occurrence of ETVr, BT, and BTH during the 144-week course of ETV therapy. The log rank analysis of pretreatment variables showed that prior refractoriness to LVD was associated with more rapid occurrence of ETVr (Fig. 2), BT, and BTH ($P \leq 0.001$, $P < 0.001$, and $P = 0.0039$, respectively). Additionally, among the 67 patients receiving 3-year ETV treatment, BT occurred in 10 of 52 (19.2%) patients with HBV genotype C and 1 of 8 (12.5%) with genotype B, whereas no BT was observed in patients with genotypes A, D, and E. No significant association between BT and HBV genotypes was found.

Baseline characteristics and factors associated with viral breakthrough during 3-year ETV therapy. When non-BT and BT groups within the 67 patients treated with ETV for 144 weeks were compared, no significant baseline differences were observed in mean age, gender, serum ALT levels, HBV DNA, or HBeAg status (Table 1), while 2-$\log_{10}$-unit reductions in HBV DNA levels or undetectable (<2.6) HBV DNA levels at the end of year 1 were significantly higher in the non-BT group (Table 5). Interestingly, the proportion of patients refractory to LVD with both the L180M and M204V substitutions at baseline ($P < 0.001$) and the incidence of S202G or T184SCGA/ILMF substitutions during the 3-year ETV treatment ($P < 0.001$) were significantly higher in the BT group.

None of the BT cases reached undetectable HBV DNA levels at the end of the first year of ETV treatment (BT, 0%, versus non-BT, 58.9%), but all were refractory to LVD (BT, 100%, versus non-BT, 32.1%) and had the L180M substitution