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Investigating an Outbreak of Acute Viral Hepatitis Caused by Hepatitis E Virus Variants in Karachi, South Pakistan

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Hepatitis E is a classic water-borne disease in developing countries. Detection of anti-HEV IgM and IgG antibodies, in addition to HEV RNA are useful epidemiological markers in diagnosis of hepatitis E. This study was conducted to investigate an outbreak of acute viral hepatitis in South-Pakistan. Anti-HEV IgM and IgG were assessed comparatively with serological kits manufactured by Abbott, Cosmic, TGH, and Wantai, selecting HEV RNA as reference assay. Molecular evolutionary analysis was performed by phylogeny and HEV spread time analysis by Bayesian Coalescent Theory approach. Of the 89 patients, 24 (26.9%) did not have acute hepatitis viral marker. Of the remaining 65 cases, 4 (6.1%) were positive for anti-HAV IgM, one (1.5%) for anti-HBc IgM, 2 (3%) for HCV, 53 (81.5%) for anti-HEV IgM, and 5 (7.7%) were hepatitis-negative. The Wantai test was 100% sensitive and specific followed by Cosmic (98.1% and 100%), TGH (98.1% and 97.2%) and Abbott (79.2% and 83.3%). Two HEV variant strains were detected by phylogeny responsible for this acute hepatitis outbreak. Estimates on demographic history of HEV showed that HEV in Pakistan has remained at a steady nonexpanding phase from around 1970 to the year 2005, in which it expanded explosively with the emergence of new HEV variants. In conclusion, the limited sensitivity of available assay (Abbott anti-HEV EIA) may be a concern in HEV diagnosis in Pakistan. This study cautions that the dissemination of the variant strains to other areas of Pakistan may lead to explosive HEV outbreaks. ***J. Med. Virol. 83:622–629, 2011.*** © 2011 Wiley-Liss, Inc.

KEY WORDS: acute viral hepatitis outbreak; serological diagnosis; HEV variants; phylogeny; molecular clock; bayesian coalescent analysis

INTRODUCTION

Hepatitis E is an important public health concern in many developing countries particularly in Asia, Africa, and Latin America. Hepatitis E virus (HEV) has been responsible for a substantial proportion of sporadic (non-epidemic) cases of acute hepatitis as well as large water-borne epidemics related to poor hygiene and sanitation [Emerson and Purcell, 2003; Kar et al., 2008]. Although the disease is self-limiting and no chronic sequelae or carrier state has been documented in the general population, but it can acquire chronic/

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carrier state in immune compromised patients [Kamar et al., 2008].

HEV is a non-enveloped virus and its genome is a single-stranded, positive-sense RNA, which is capped and polyadenylated [Tam et al., 1991; Kabrane-Lazizi et al., 1999]. The genome is approximately 7.2 kb and contains three open reading frames (ORF1–3) [Tam et al., 1991]. ORF1 encodes non-structural proteins including the helicase and RNA-dependent RNA polymerase [Agrawal et al., 2001]. ORF2 and ORF3 overlap, and the ORF2 and ORF3 proteins are translated from a single bicistronic subgenomic RNA [Graff et al., 2006]. The ORF2 protein is the viral capsid protein, whilst the ORF3 protein is essential for virion egress from infected cells [Yamada et al., 2009].

HEV have been classified into four genotypes and regardless of the genotype of the virus or the country of origin, it appears to have one single serotype [Emerson and Purcell, 2003]. Most of the enzyme immunoassays (EIAs) for HEV infection are based on either recombinant HEV proteins or synthetic peptides. Serological detection of antibodies to the virus even of different origins is possible by relying on major epitopes derived from the ORFs of the virus and is the established procedure for the diagnosis of acute HEV infection [Seriwatana et al., 2002]. However, epidemiological studies require the detection of HEV RNA by PCR in addition to anti-HEV IgM and IgG antibodies, especially in outbreak investigations.

HEV along with hepatitis A virus (HAV) infections are the most common cause of viral hepatitis in Pakistan in the general population [Hamid et al., 1996, 2002; Rab et al., 1997]. HAV infection has been shown to cause severe illness in adult patients with underlying chronic liver disease (CLD). However, vaccination against HAV has reduced substantially this risk. HEV-induced acute hepatitis may also be fulminant [Hamid et al., 1996; Peron et al., 2007] and like HAV, HEV could be expected to cause a severe illness in patients with underlying CLD. Characteristically, in pregnant women, the illness is particularly severe and carries a high case fatality rate (15–25%) [Hamid et al., 1996; Bhatia et al., 2008].

A number of reports revealed different outbreaks of acute viral hepatitis in Pakistan associated primarily with HEV [Malik Iftikhar Ahmed, 1996; Rab et al., 1997]. In this study, the samples were collected from Karachi, the industrial capital and the largest metropolitan city in South-Pakistan. The aims of this study were to investigate: (1) the etiologies of acute viral hepatitis (2) serological diagnosis of HEV and (3) the demographic history of HEV in Pakistan.

MATERIALS AND METHODS

Patients

Between December 1, 2007, and July 31, 2008, the consecutive patients (n = 89) attending the outpatient and inpatient clinics of The Aga Khan University (AKU), who presented symptoms associated with acute liver disease, were included in the study. The most common symptoms included jaundice, fatigue, weakness, nausea, and a loss of appetite and in some cases bleeding, ascites and liver encephalopathy.

Serological Diagnosis

All patients were screened by serological tools for hepatitis A–D virus infections. HBsAg, anti-HCV, anti-HDV, and anti-HAV IgM were tested in each sample by Abbott enzyme immunoassays (EIAs) (Abbott Laboratories, Chicago, IL). Four different serological assays, that is, Abbott (Abbott Laboratories), Cosmic (Cosmic Viragent, Tokyo, Japan), TGH (Toshiba General Hospital, Tokyo, Japan) and Wantai (Wantai Co. Ltd, Beijing, China) were evaluated comparatively for the detection of anti-HEV. HEV RNA detection by reverse transcriptase PCR (RT-PCR) was selected as the reference assay to analyze the specificity and sensitivity of each test. To rule out the ambiguity in HEV RNA detection, samples were also tested for anti-HEV IgA, as this test is very sensitive in nature for detection of acute HEV infections and may be a choice marker for differentiating acute infections [Mitsui et al., 2005; Takahashi et al., 2005; Elkady et al., 2007]. A case of HEV infection was considered, if it is positive for both HEV RNA and anti-HEV IgA, where "early acute infection" was defined if a case is negative for anti-HEV IgG, while "acute infection" was defined if it is positive for anti-HEV IgG. The sensitivity of an anti-HEV IgM assay was defined as the percentage of anti-HEV IgM positivity from among the HEV cases showing the profiles of early acute or acute infection. The specificity of an anti-HEV IgM assay was defined as the percentage of negative samples among those negative to HEV RNA showing the profiles of only previous infection or not infected. Anti-HBc IgM in samples was tested by ELISA (Fujirebio, Inc., Tokyo, Japan) and anti-HIV using the Jinedia HIV-1/2 Mix PA kit (Bio-Rad, Fujirebio, Inc., Tokyo, Japan). Biochemical markers such as alanine-amino transferase (ALT), aspartate-aminotransferase (AST), alkaline phosphatase (ALP) and bilurubin levels were measured in all samples.

Informed consent was obtained at the time of blood sampling from each patient included in the study. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethical committee of the institution.

Detection of HEV RNA and HEV Genotyping

Total RNA was extracted from the serum samples using the SepaGene RV-R Nucleic acid extraction kit (Sanko Junyaku Co., Ltd, Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA were reverse transcribed to complementary DNA using SuperScript II RNase H_Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described

previously [Ohno et al., 1997]. Screening of HEV RNA was carried out with a specific set of screening primers targeting a partial nucleotide sequence of ORF1 region of the HEV genome, as described previously [Takahashi et al., 2003]. The sequences spanning 326 nt in ORF1 and 247 nt in ORF2 of HEV RNA corresponding to nt 125–450 and nt 6050–6296 of the Nepali isolate; GenBank accession number AF015830 was obtained for HEV genotyping. A sequence of 868 nt in the RNA-dependent RNA polymerase region (corresponding to nt 3918–4785 of AF051830) was also obtained for HEV RNA positive cases for molecular evolutionary analysis.

Detection of HBV DNA

HBV DNA was extracted by a QIAamp DNA Blood Mini Kit (Qiagen, Inc., Hilden, Germany) from 100 ml of each HBsAg positive serum. Partial core and S regions were amplified in order to detect HBV DNA in the samples using the primers described previously [Sugauchi et al., 2001]. The detection limit for this study was 100 copies/ml [Tanaka et al., 2004].

Sequencing and Phylogenetic Analysis

The amplicons obtained were sequenced directly with Prism Big Dye (Applied Biosystems, Foster City, CA) in an ABI 3100 DNA automated sequencer. The sequences for phylogenetic analysis were retrieved from DDBJ/EMBL/GenBank. Alignments were performed using CLUSTALW (http://clustalw.ddbj.nig.ac.jp/top-e.html) and neighbor-joining trees were constructed with 6-Parametric method and bootstrapped 1,000 times to confirm the reliability of the phylogenetic tree [Shin et al., 2008].

The nucleotide sequence data reported in this paper appear in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession numbers AB513496–AB513616.

Molecular Evolutionary Analyses

A reconstructed tree was built on the ORF1 sequence of 868 nucleotides of partial RNA polymerase region (RDRP) of HEV genome by Mega using Pakistan isolates and all the available reference sequences retrieved from databases (DDBJ/EMBL/GenBank). To estimate virus effective population sizes through time, a coalescent-based approach was used which is implemented in a Bayesian Markov Chain Monte Carlo (MCMC) inference framework in the program BEAST [Lemey et al., 2009]. All possible combinations of the relaxed [Drummond et al., 2006] and strict molecular clock models with Bayesian skyline [Drummond et al., 2005], constant, exponential, and logistic growth coalescent models were analyzed. The Bayes Factors (BF) were estimated for each pair of models, as implemented in Tracer v1.4 and suggested previously [Suchard et al., 2001]. MCMC sampling was performed for at least 1×10^7 generations, sampling a tree every 1,000 generations. HKY model of nucleotide substitution was used with gamma distribution, five rate categories and a substitution rate of 0.84×10^{-3} as described by Tanaka et al. [2006]. The program Tracer (http://tree.bio.ed.ac.uk) was used to check for convergence and to determine whether appropriate mixing of the posterior target distribution had been achieved (effective sample size >100).

RESULTS

Etiology of Acute Hepatitis

The time lag between the start of symptoms of acute infection and the collection of samples in this study was 6.9 ± 4.7 days. Of the 89 patients, 24 (26.9%) had chronic HBV infection with no detectable acute viral marker. Of the remaining 65 cases, 4 (6.1%) were positive for anti-HAV IgM, 1 (1.5%) was positive for anti-HBc IgM, 2 (3%) were positive for HCV, 53 (81.5%) were positive for HEV RNA, and 5 (7.7%) were hepatitis-negative (Table IA). Serological assays (Anti-HEV IgM and IgG) of Abbott, Cosmic, TGH, and Wantai were evaluated comparatively selecting HEV RNA detection by PCR as a reference. The Wantai test was 100% sensitive and specific followed by Cosmic (98.1% and 100%), TGH (98.1% and 97.2%) and Abbott (79.2%

TABLE IA. Base Line and Clinical Characteristics of 65 Patients From Pakistan With Acute Liver Diseases

Features	Total	AHA	AHB	AHE	HCV	NABCDE
Age (years)	30.9 ± 14.5	26.5 ± 11.4	19	29.4 ± 11.3	32.6 ± 9.4	31.8 ± 23
Gender (male)	43	2	0	38	2	3
ALT, IU/L (mean ± SD)	1,630.6 ± 1,687.5	1,677.7 ± 870.5	2,140	2,146.4 ± 1,570.4	819.3 ± 683.5	2,301.8 ± 3,171.8
AST, IU/L (mean ± SD)	1,955 ± 1,986.3	2,087.3 ± 977.6	2,140	1,893.4 ± 1,900.3	651.6 ± 531	2,645 ± 3,609.5
ALP, IU/L (mean ± SD)	171.8 ± 106.4	150 ± 60.3	133	160.9 ± 70.4	106.3 ± 17	333.8 ± 270
GGT, IU/L (mean ± SD)	119.8 ± 160.1	112 ± 58	182	103.9 ± 95	55 ± 17.4	431.8 ± 531.9
Total Bil. mg/dl (mean ± SD)	11.4 ± 14.1	9.3 ± 3.2	3	11.3 ± 10.2	12.5 ± 12.8	3.6 ± 3
Anti-HAV IgM, n (%)	4 (6.1)	4 (100)	0	0	0	0
Anti-HEV IgM, n (%)	53 (84.1)	0	0	53 (100)	0	0
Anti-HEV IgG, n (%)	52 (82.5)	1 (25)	0	51 (96.2)	1 (50)	5 (100)
HEV RNA, n (%)	53 (84.1)	0	0	53 (100)	0	0
Anti-HBc IgM, n (%)	1 (1.5)	0	1 (1.5)	0	0	0
HBV DNA, n (%)	11 (17.4)	0	1 (100)	10 (18.9)	0	0
Total	65	4	1	53	2	5

TABLE IB. Comparison of Sensitivity and Specificity of Anti-HEV IgM Assays in 89 Suspected Acute Liver Disease Patients

Tests	Sensitivity% (+/tested)	Specificity% (−/tested)
Abbott	79.2 (42/53)	83.3 (30/36)
Cosmic	98.1 (52/53)	100 (36/36)
TGH	98.1 (52/53)	97.2 (35/36)
Wantai	100 (53/53)	100 (36/36)

and 83.3%) (Table IB). The results of anti-HEV IgA testing for subset of samples (n = 17) with non-specific or not-sensitive IgM results were in complete agreement with the results of HEV RNA detection by PCR (data not shown). There were two cases (3%) with early acute infection, which were detectable by all kits except Abbott. Five patients (7.7%) were negative for all hepatitis viral markers. Underlying HBV infection with detectable HBV DNA was observed in 10 (15.4%) of acute hepatitis E patients. The baseline and clinical features of these 65 patients with acute liver disease are summarized in Table IA.

Phylogenetic Analysis

In order to analyze the genomic heterogeneity of HEV, phylogenetic trees were constructed in two partial ORF1 parts and in ORF2 of the HEV genome against all sequences available in the database. All sequences corresponded to genotype 1 clustering in two groups referred from now "variant 1" and "variant 2." Phylogenetic trees based on alignment of the ORF1 sequences suggested that the strains were related more closely to the strains from Nepal and India. The topology of the tree obtained either the 5′ terminal end of ORF1 or in RNA dependent RNA polymerase (RDRP) region of ORF1 was similar to that obtained in the 3′terminal sequence of the ORF2 (Fig. 1A–C). Phylogenetic tree in RDRP region distinctly separates the both clusters with high bootstrap values (Fig. 1C). The consistent high

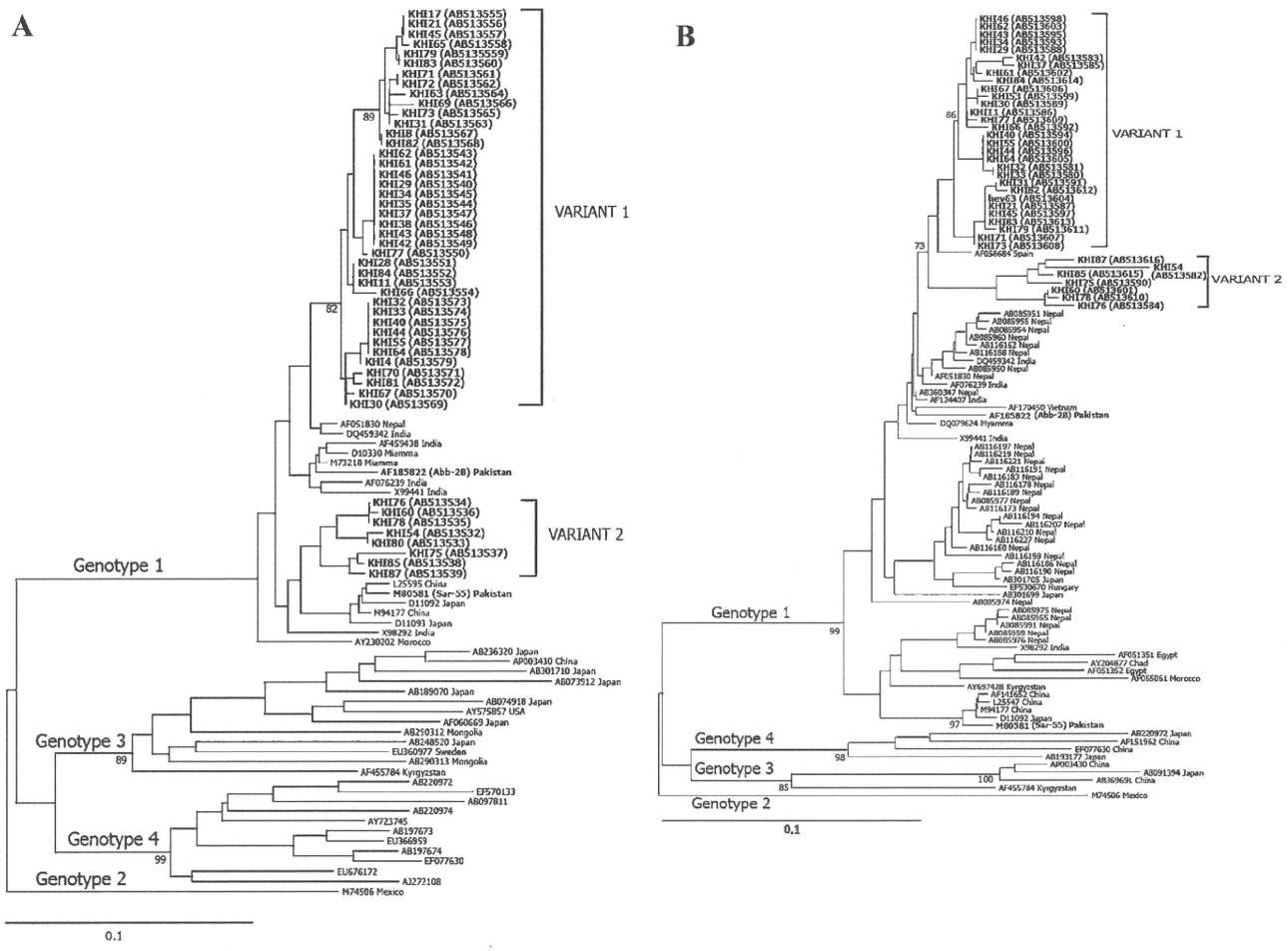

Fig. 1. A–C: Phylogenetic trees constructed (A: ORF1 region 326nt) (B: ORF2, 247nt) (C: ORF1-RDRP region, 868 nt) of HEV genome. Pakistan isolates are in bold letters, aligned with all the available reference sequences retrieved from databases (DDBJ/EMBL/Gene Bank). Variant strains isolated in this study are represented in bold letters showing isolate name (database reference number). The numbers in the tree indicate bootstrap reliability by the interior branch test. Exceptional strains are indicated according to their area of origin.

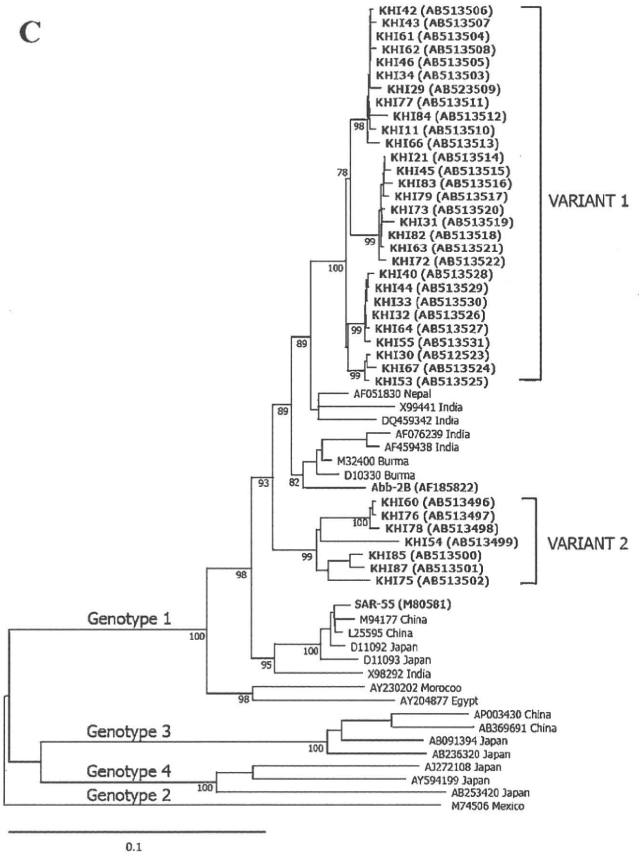

Fig. 1. (*Continued*)

bootstrap values within the clusters describe the homology of the strains with each other, a characteristic of epidemic.

Sequence Analysis in 5′Terminus of ORF1, RDRP Region of ORF1, and 3′Terminus of ORF2

The comparison of the ORF1 and ORF2 nucleotide sequences obtained in this study with all previously published strains indicated that the HEV variant 1 was more closely related to Nepalese strain (96–97% identity), followed by Burma (94–96% identity), Indian (93–95% identity), and previous Pakistani strains (90–95% identity; Table II). The HEV variant 2 of this study indicated an almost similar degree of identity (94–95%) with Nepalese, Burma, Indian, and previous Pakistani strains in ORF1 but differed substantially in ORF2 with only 89–91% of identity. These sequences were divergent significantly from Mexican, Chinese, and Japanese strains of HEV genotype 2, 3, 4 strains, respectively (80–86% identity). Interestingly, the amino acid sequence comparisons did not show any significant difference from other Asian strains (96–100% identity) (Table II). Cumulatively these results indicate that the new variant strains although have much nucleotide variations in different regions of HEV genome from previous Asian strains but these nucleotide variations are more or less synonymous ones.

Historical Analysis of HEV Population

Evolutionary analysis was performed in BEAST under a range of molecular clock and coalescent model combinations [Magiorkinis et al., 2009; Pybus et al., 2009], using the sequences in the most conserved RDRP region of HEV genome. In each case, the best fitting model was the relaxed molecular clock model plus the Bayesian skyline demographic model. The Bayesian skyline plot summarizes the spread and epidemic growth of HEV in Pakistan (Fig. 2). It shows clearly that HEV genotype 1 was in a steady nonexpanding phase from around 1970 (the lower 95% credible interval of the tMRCA) to the year 2005, in which it expanded explosively with the emergence of new HEV variant strain. The growth phase is preceded by a reduction in the effective number of infections (Fig. 2). However, this reduction is not significant given the size of the estimated confidence limits.

DISCUSSION

The IgM class of antibody to the virus is considered as the specific marker for differentiating the acute from the convalescent phase of an infection [Ma et al., 2009]. Consecutive patients (n = 89) presenting with symptoms

TABLE II. Nucleotide (nt.) and Deduced Amino Acid (a.a.) Sequence Identity of Selected HEV Strains and Pakistan New HEV Variants

		Percentage identity nt. (a.a)			
		ORF1, 326 nt		ORF2, 247 nt	
HEV strain	Genotype	Variant 1	Variant 2	Variant 1	Variant 2
Pakistan, M80581	1	92 (100)	95 (99)	90 (100)	90 (96)
Pakistan, AF185822	1	93 (100)	95 (98)	95 (100)	90 (96)
India, X99441	1	93 (98)	94 (96)	95 (100)	89 (96)
Nepal, AF051830	1	96 (100)	95 (98)	97 (100)	91 (96)
Burma, D10330	1	94 (99)	95 (97)	96 (100)	91 (96)
Mexico, M74506	2	80 (94)	83 (93)	86 (85)	85 (88)
China, AP003430	3	81 (91)	82 (88)	85 (98)	87 (94)
Japan, AJ272108	4	81 (92)	80 (90)	82 (96)	80 (93)

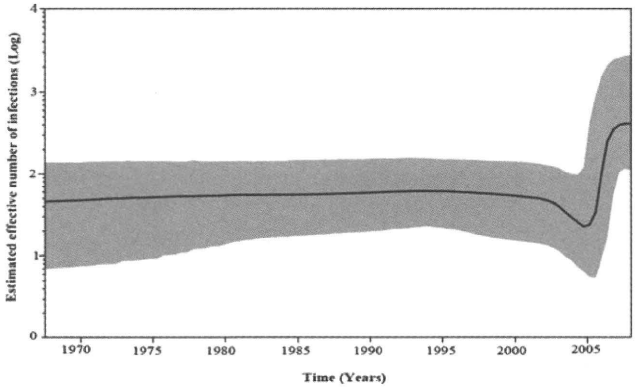

Fig. 2. Hepatitis E population dynamics in Pakistan based on relaxed-clock analysis of ORF1-RDRP region. The dark line in Bayesian skyline plot shows the estimated effective population size through time. The gray area represents the 95% highest posterior density confidence intervals for this estimate.

of acute liver diseases were tested to differentiate HAV, HBV, and HEV acute infections. Of the 89 patients, 24 (26.9%) did not have detectable acute viral marker however were detected with chronic HBV infection. HEV appeared to be the major etiological agent detected positive in 81.5% of the remaining 65 cases with acute viral hepatitis.

Although it has been understood that HEV outbreaks in developing nations are waterborne and occur in areas with poor sanitation, the only available and rapid methods in most of these countries is serological testing for HEV (anti-HEV IgM/IgG). Reliable serological tests are thus indispensible in such situations and the periodic evaluation of already available assays with new ones is therefore necessary not only for the management of hepatitis E but also for studying the epidemiology of disease. Given the findings that the viremia in acute HEV infection may prolong for a period of $\geq$2 weeks from the onset of illness [Clayson et al., 1995; Myint et al., 2006] and in the absence of reference serological assay, HEV RT-PCR was selected as the reference assay for HEV detection in this study. Among the four EIAs for detecting IgM anti-HEV, the Wantai assay showed 100% precision. The Cosmic assay showed 99.7% concordance with the Wantai IgM assay, while Abbott IgM assay was the least suitable assay in detection of these HEV variants. The difference in the detection among these assays may be attributed to the difference in the strain-specific antigenic domains included in these tests [Ma et al., 2009]. However, the difference in detections among these tests was observed for variant-1 only. To rule out the ambiguity in detection of HEV RNA, anti-HEV IgA was tested in the subset of samples with non-specific or not-sensitive results. IgA is the class of antibody, which is elicited during the acute phase of viral infections including HEV [Elkady et al., 2007], which was found to be detectable for a longer time of infection even at the stage when HEV RNA is no longer detectable [Mitsui et al., 2005; Takahashi et al., 2005]. Results of anti-HEV IgA showed 100% concordance with HEV RNA detection. One of the limitations of this study was that the performance of all serological assays was assessed for symptomatic infections. Cases of asymptomatic HEV infection in subclinical forms of infection exist and they are thought to exceed icteric infections in HEV outbreak [Clayson et al., 1995; Myint et al., 2006]. Further studies in the anti-HEV testing therefore could be vital in investigations to identify all infected persons rather than just those with clinical disease.

HEV strains belong to a single serotype and display considerable genetic diversity according to the time and place of isolation [Lu et al., 2006]. In order to gain insight into the genetic variability and mode of evolution of HEV in Pakistan, the HEV strains were sequenced in two parts of ORF1 (5′end of ORF1 and RDRP region) and the hyper-variable region of the ORF2. The data were consistent for all three parts, showing two independent and distinct phylogenetic clusters of HEV within genotype 1. These new strains were related more closely to the strains from Nepal, India, and Burma rather than previous Pakistan strains [Bryan et al., 2002; Shrestha et al., 2004; He, 2006], indicating that different epidemiological strains may be circulating in different regions of Pakistan. The other explanation may be that HEV has been in continuous spread in Pakistan and it had continued evolution in infected individuals in Pakistan, leading to the observed genomic variability. The fact that no significant amino acid substitutions were observed indicates that genomic mutations of HEV may occur naturally in infected individuals without significant immunological pressure from the host and that selective forces that do not allow amino acid substitutions may be involved in observed pattern of divergence [Shrestha et al., 2004].

To estimate the virus affecting the population through time, a coalescent-based approach was used which is implemented in a Bayesian Markov Chain Monte Carlo (MCMC) inference framework in the program BEAST [Drummond et al., 2006; Lemey et al., 2009; Pybus et al., 2009]. BEAST incorporates uncertainty in the phylogeny by integrating across tree topologies to estimate relative genetic diversity, an indicator of effective population size under a neutral evolutionary process. Estimates show that HEV genotype 1 was in a steady nonexpanding phase in Pakistan, from around 1970 (the lower 95% credible interval of the tMRCA) to the year 2005, in which it spread explosively with the emergence of new HEV variant strains. The growth phase is preceded by a dip in the effective number of infections. This could be due to a combination of epidemiological processes-stochastic or spatial effects that result in delay of exponential growth of emerging population due to reduced mean growth rates [Lande, 1998; May et al., 2001; Pybus et al., 2003]. However, this dip is not significant given the size of the estimated confidence limits. HEV has been reported in Pakistan as early as the 1950s and 1960s to the early 1990s during which HEV was found to be single main cause of at least 70% of acute hepatitis cases mainly affecting the adult population [reviewed by Malik Iftikhar Ahmed, 1996]. In

1994, a massive outbreak of HEV (December 1993–March 1994) occurred in Islamabad, the capital of Pakistan [Iqbal et al., 1989]. The epidemic was also reported in Lahore in December of the same year, when 283 cases were admitted to the army hospital at the same time [Malik Iftikhar Ahmed, 1996].

The epidemic history of these newly emerged Pakistan strains of HEV in the year 2005 is evidenced by the gastroenteritis outbreak which occurred in Karachi, Pakistan in summer (June–September) 2005 (http://www.dawn.com/2005/05/26/local10.htm), associated with sewage-contaminated water supply. Tens of thousands of people were affected by this gastroenteritis outbreak, which masked apparently the HEV outbreak and the newly emerged strain remained undetected and continued to spread in the community in an unrecognized form causing acute hepatitis.

In South Asia both HAV and HEV are considered as the most common causes of acute hepatitis. However, HEV has been the leading etiological agent responsible for large-scale epidemics in Pakistan [Malik Iftikhar Ahmed, 1996; Rab et al., 1997]. In this study, HEV variants were found responsible for acute hepatitis outbreak in Karachi, South-Pakistan. Limited sensitivity of available serological assay in Pakistan (Abbott anti-HEV EIA) may be a concern in diagnosing early acute infections and outbreak investigations. This study cautions that HEV variant strains have great epidemic potential and their dissemination to other areas in the country may lead to explosive HEV outbreaks.

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Analysis of 5′ Nontranslated Region of Hepatitis A Viral RNA Genotype I from South Korea: Comparison with Disease Severities

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Abstract

The aim of the study was to analyze genotype I hepatitis A virus (HAV) 5′ nontranslated region (NTR) sequences from a recent outbreak in South Korea and compare them with reported sequences from Japan. We collected a total of 54 acute hepatitis A patients' sera from HAV genotype I [27 severe disease (prothrombin time INR≥1.50) and 27 mild hepatitis (prothrombin time INR <1.00)], performed nested RT-PCR of 5′ NTR of HAV directly sequenced from PCR products (~300 bp), and compared them with each other. We could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)], all of which were subgenotype IA. Sequence analysis revealed that sequences of severe disease had 93.6%–99.0% homology and of self-limited disease 94.3%–98.6% homology, compared to subgenotype IA HAV GBM wild-type IA sequence. In this study, confirmation of the 5′NTR sequence differences between severe disease and mild disease was not carried out. Comparison with Japanese HAV A10 revealed ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively, although the nucleotide sequences in this study showed high homology (93.6%–100%). In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to Japanese sequences previously reported from Japan, and this region would be considered one of the antiviral targets. Further studies will be needed.

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Introduction

Although hepatitis A vaccination is highly effective, providing herd protection and decreasing mortality and morbidity related to the hepatitis A virus (HAV) [1–3], HAV is still a common cause of hepatitis reportedly leading to occasional lethal acute liver failure in many countries of the world [4–7]. Recently, a rise in the frequency of hepatitis A outbreaks was observed in South Korea, which lies adjacent to Japan, while the number of adult hepatitis A cases in Japan has been progressively decreasing during the last several years. There is a concern regarding a possible HAV epidemic in Japan in the near future, as universal vaccination against hepatitis A is not performed in this country.

HAV is a member of the genus *Hepatovirus* in the *Picornaviridae* family, and has a positive-sense single-stranded RNA genome approximately 7.5 kb in length [8]. The genome codes a large open reading frame (ORF), which is flanked by 5′ nontranslated 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 and is important for HAV replication [9,10]. 5′NTR of HAV is also known as one of the most highly conserved in the HAV genome sequences, making this region one of the likely candidates for antiviral targets [9,11]. It was reported that nucleotide variations in the central portion of 5′NTR of HAV may influence the severity of hepatitis A [12].

Human HAV strains can be grouped into four genotypes (I, II, III and IV) and unique simian strains belong to three additional genotypes (IV, V and VI). Between each of these genotypes, the nucleotide sequence varies by 15–20% of the base positions in the P1 region [13]. Genotype I is the most abundant type worldwide, and genotype IA in particular has been reported from North America, Korea, China, Japan and Thailand [14].

The aim of this study is to characterize the recent HAV genotype I 5′NTR sequences in Korea, to compare them with those reported from Japan and to clarify this region as a target candidate for anti-HAV drugs.

Materials and Methods

Patients

Fifty-four patients infected with HAV subgenotypes IA and IB were included in this study. Serum samples were collected at four

hospitals located in the Seongnam city area, near Seoul, South Korea. Our study was approved by the Seoul National University Bundang Hospital Institutional Review Board (IRB), and we obtained written informed consent from every patient enrolled during Sep 2008 to Aug 2008. We collected serum or plasma samples immediately after hospital admission, and they were stored at −70°C. The 54 patients comprised 27 with severe disease, defined as prolonged prothrombin time [international normalized ratio (INR) > or = 1.5] and 27 with mild disease: self-limited acute hepatitis in this study (Table S1A & S1B).

Primers for PCR and Direct Sequencing

For amplification of HAV sequences and bidirectional direct sequencing of the amplified segments, we prepared several primers for PCR and sequencing as previously described [12]. These primers were prepared with the sequence reported by Cohen et al [8].

Detection of Hepatitis A Virus RNA in Serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method. Reverse transcription was performed with HAV genome specific antisense primer (5′-AGTACCTCA-GAGGCAAACAC-3′) as previously described [12].

In the first round PCR, 1 μl of 20 μl of the cDNA solution was used. The first round PCR was performed with 50 μl of reaction mixture containing 25 pmol of outer antisense primer (5′-AGTACCTCAGAGGCAAACAC-3′) and sense primer (5′-TCTTGGAAGTCCATGGTGAG-3′), 200 μM of each dNTP, 50 mM KCl, 10 mM Tris HCL (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 units of Ex Taq polymerase (Takara Bio Inc., Ohtsu, Shiga, Japan). Amplification conditions consisted of 35 cycles of 95°C for one minute, 50°C for one minute, and 72°C for one minute, and 1 μl of the first round product was used for the second round of PCR with the same PCR mixture, except 1.0 μM of inner sense primer (5′-GGGACTTGATACCT-CACCGC-3′) and antisense primer (5′-CCACATAAGGCCC-CAAAGAA-3′) were used. Amplification conditions for the second round were the same as those for the first round. The second-round PCR products (6 μl) were analyzed by 8% polyacrylamide gel electrophoresis, stained with SyBr green (Takara), and visualized by UV transillumination. In all experiments, the negative samples showed negative results for HAV RNA. HAV genotypes were determined by previously described methods based on the VP1-P2A region [14].

Direct Sequencing of HAV cDNA Fragments

To prepare the sequence template (nucleotides 75-638 of 5′NTR of HAV), PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA), and then sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were analyzed using Applied Biosystems 3730xl (Life Technologies).

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this article will appear in GenBank nucleotide sequence databases with accession numbers AB571027 to AB571045.

Phylogenetic Analysis

To examine the heterogeneity of the viral sequences obtained, a phylogenetic tree was constructed using the neighbor joining methods. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 10,000 times. These analyses were conducted with the Genetyx-WIN program, version 10 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's t test and Welch's t test.

Results

Clinical Features of Patients with Acute Hepatitis A Genotype 1 in Korea

Characteristics of these patients at admission are summarized in Table S1. There were no differences in age and gender ratio between the severe and mild disease groups. Mean age of the severe and mild disease groups was 32.1±6.1 and 32.6±5.8 years, respectively. Male gender was dominant in both groups (male/female: 19/8 and 18/9 in the severe and mild disease groups, respectively). Almost all patients of both groups were subgenotype IA, with only two and one being subgenotype IB in the severe and mild disease groups, respectively.

Sequence Analysis of Korean Isolates

Although the VP1/2A region could be detected in the same serum or stool samples of the same patients, we could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)] by reverse-transcription-nested PCR. All these sequences were subgenotype IA. Then we performed further sequence analysis in these 19 patients by the methods of Fujiwara et al [12]. Japanese studies showed that fewer nucleotide variations were found between nucleotides 200 and 500 of 5′NTR in cases of fulminant hepatitis and severe acute hepatitis than in cases of self-limited acute hepatitis [12]. We thusly performed sequence analysis of the region between nucleotides 200 and 500.

Sequences between nucleotides 200 and 500 were then compared with the wild-type HAV GBM/WT RNA (X75215) [15]. The nucleotide sequence identities of 5′NTR from severe and mild cases ranged from 93.6% to 99.0% and from 94.3% to 98.6%, respectively, compared with wild-type HAV GBM sequence. The distribution of nucleotide variations is shown in Table S2A & S2B. Sequences from cases of severe and mild diseases were mostly similar. Although there was no statistical significance, ^{214}C, ^{220}T and ^{464}T were found in one case each of the mild disease group (Table S2B). On the other hand, ^{227}deletion of nucleotide and ^{382}A, respectively, were found in two and one cases of the severe disease group (Table S2A). The number of nucleotide substitutions is shown in Figure 1A & 1B. The average number of substitutions between nucleotides 200 and 500 was 10.8 (6.8) [mean (SD)] per case in severe disease and 6.8 (4.5) in mild disease. Differences between severe and mild cases were not statistically significant. We could not construct a phylogenetic tree using these sequences (data not shown).

Comparison to Japanese HAV Sequences Reported from 1984 to 1999

5′NTR of HAV possesses a secondary structure including stems and loops, functions as an IRES, and plays an important role in translation and replication of this virus [9,16]. There are six domains in IRES, which is located between nucleotides 151 and 734. Portions of domains III and IV are present between nucleotides 200 and 500. Domain III is located between nucleotides 99 and 323, and domain IV is located between nucleotides 324 and 586. The region between nucleotides 203 and

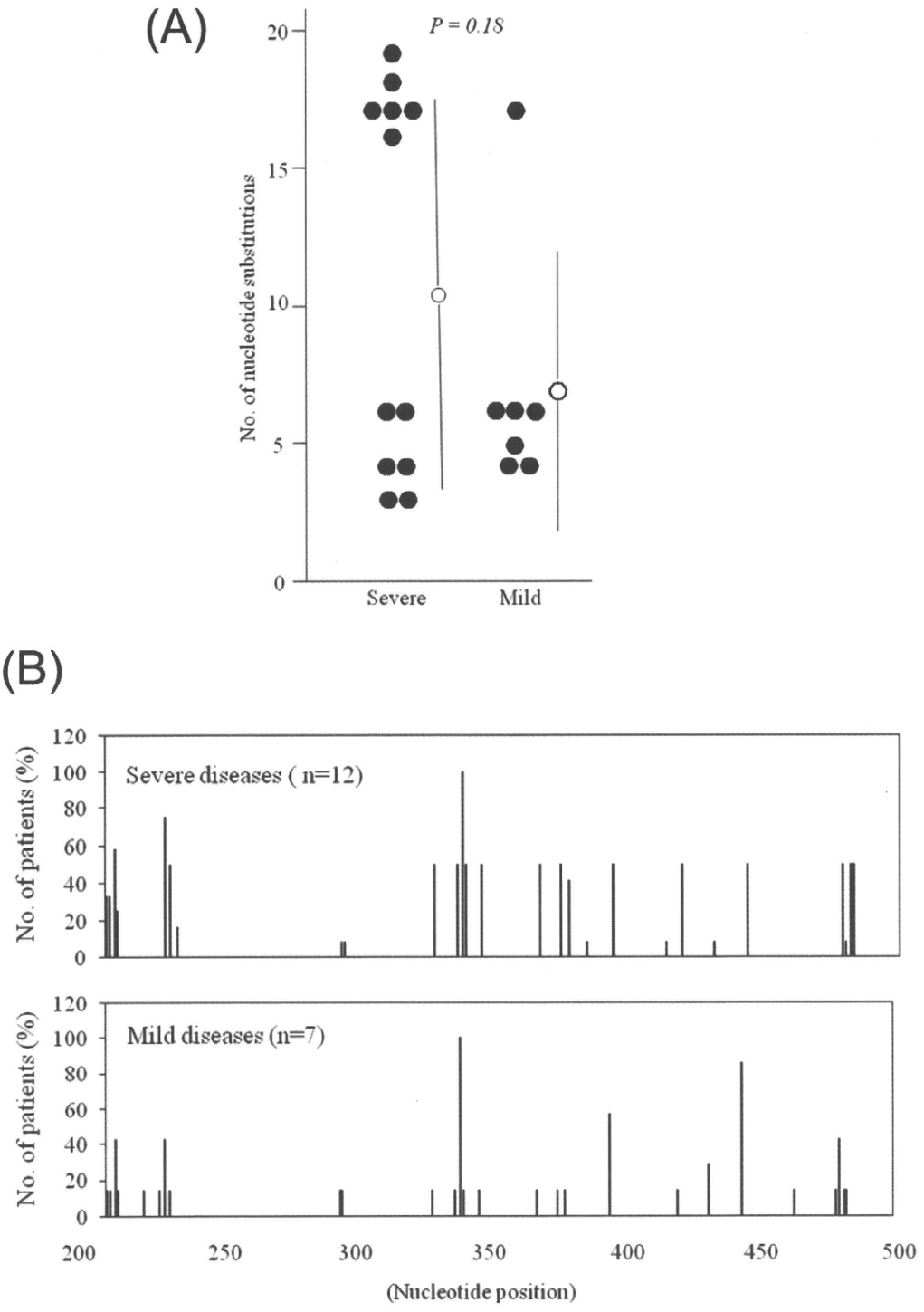

Figure 1. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV GBM. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500. Nucleotide sequences were compared with HAV GBM/WT RNA (X75215) [15]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
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250 is particularly pyrimidine-rich. To examine the homology with the HAV sequences from Japan reported by Fujiwara et al. [12], we compared the sequences from nucleotides 200 to 500 with A10 (AB045328) from Japan [12]. The nucleotide sequence identities of 5′NTR from severe and mild disease groups ranged from 94.3% to 99.6% and from 93.6% to 100%, respectively, compared with the HAV A10 sequence [12] (Table S3A & S3B). In the Korean group, we found ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively.

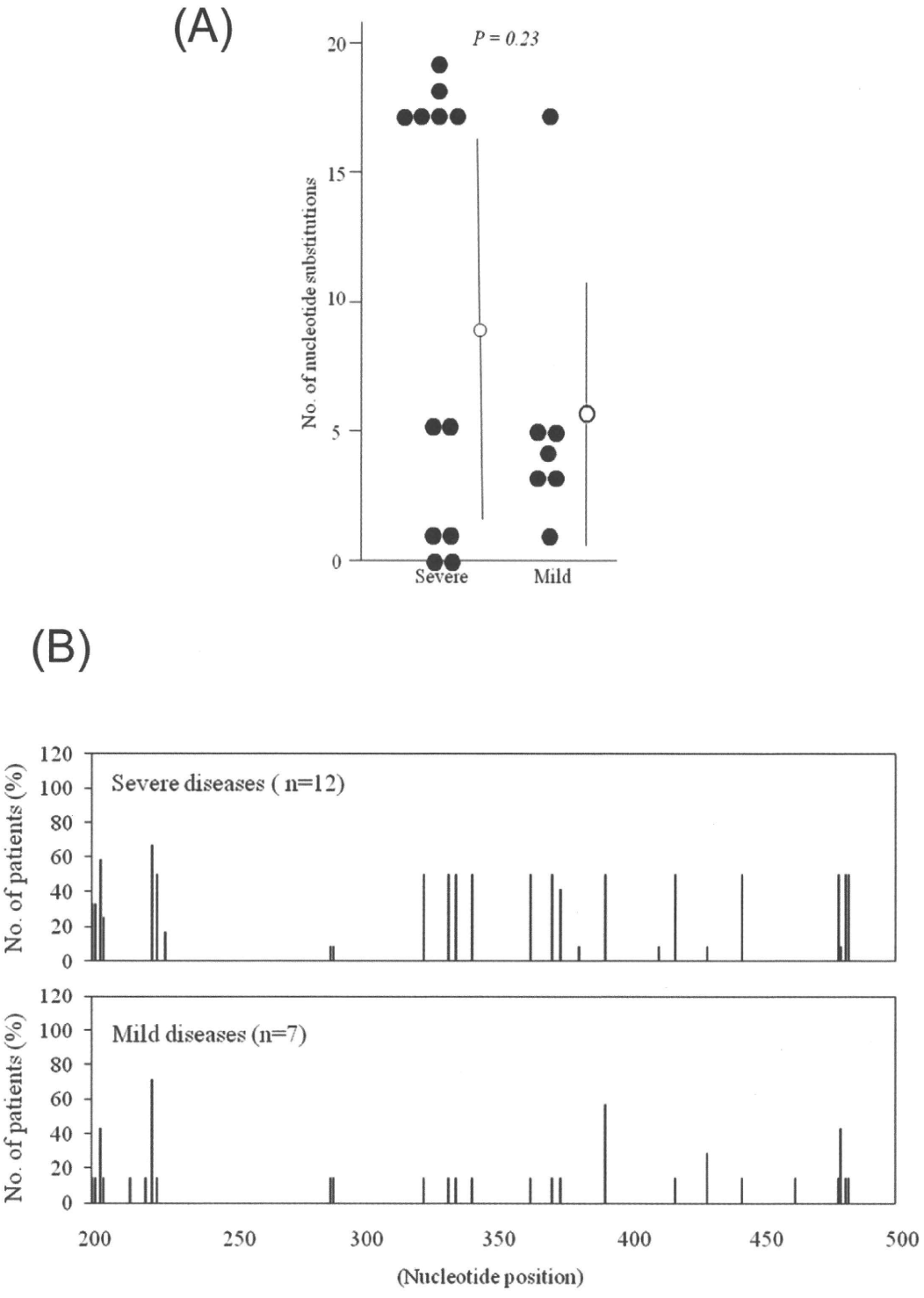

Figure 2. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV A10. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500 Nucleotide sequences were compared with A10 (AB045328) from Japan [12]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
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The number of nucleotide substitutions is shown in Figure 2A & 2B, with the average number between nucleotides 200 and 500 being 9.7 (8.2) [mean (SD)] per case in severe disease and 5.4 (5.2) in mild disease. Again, differences between severe and mild cases were not statistically significant.

Discussion

The number of adult hepatitis A cases has been progressively increasing during the last several years in Korea [6,14]. In Japan, on the other hand, the number of patients with sporadic type A

hepatitis has recently been on the decrease. In the 9 years from 1999 inclusive, 763, 381, 491, 502, 303, 139, 170, 320 and 157 hepatitis A cases were reported to the Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan (www.nih.go.jp). Japan lies adjacent to Korea, separated by the Sea of Japan. The two countries have some cultural similarities. In Japan, there is no universal vaccination program against hepatitis A and hepatitis B. These circumstances have raised concerns about a possible HAV epidemic in Japan. We then analyzed HAV genome sequences from Korea and compared them with the reported sequences from Japan over the past several years.

In the present study, as most of the HAV strains belonged to subgenotype IA in Korea [14], we chose only genotype I patients for analysis. Among 54 HAV IgM positive sera, 35.1% (n = 19) were positive for HAV RNA by nested RT-PCR for 5′NTR. All these strains belonged to subgenotype IA. We tried to perform phylogenetic tree analysis, but these 19 strains formed a single cluster to which almost all Japanese sequences reported by Fujiwara et al [12] belonged (data not shown). Fujiwara et al [12] found an association between the severity of hepatitis A and nucleotide variations in 5′NTR of Japanese HAV RNA. In the present study, we did not confirm 5′NTR sequence differences between severe disease and mild disease.

The age of HAV sequence-analyzed patients in the present study was 30.5±5.9 and 31.4±5.0 years, respectively, in severe and mild diseases. The gender of HAV sequence-analyzed patients was male-dominant (male/female: 8/4 and 6/1 in the severe disease and mild disease groups, respectively). In the study by Fujiwara et al [12], the patients were also male-dominant, but their age with fulminant hepatitis and severe acute hepatitis (43.1±14.4 year, $P = 0.010$ and 41.6±12.6, $P = 0.010$, respectively) was significantly higher than the age of severe-disease patients. On the other hand, the age of their patients with self-limited acute hepatitis was similar to that of our mild-disease patients. We defined patients with prothrombin time INR ≥1.50 as severe hepatitis in this study, whereas Fujiwara et al [12] defined patients with prothrombin time of less than 40% as severe hepatitis with (fulminant hepatitis) or without encephalopathy (severe acute hepatitis).

In Japan, similar to the situation in Korea [6], young adults seem not to have protective antibody against HAV, and so it appears that hepatitis A cases can be expected to increase in the near future.

A previous study showed that the 5′ border of IRES is located between nucleotides 151 and 257, while the 3′ border extends to the 3′ end of 5′NTR, between nucleotide 695 and the first initiation codon at 735 [17]. ^{222}C to G or T substitution was located on the loop structure at domain IIIa of HAV IRES. A previous Japanese study showed that nucleotide 225 substitutions occurred in 80% of the sequences around nucleotide position 222 [12]. ^{392}G to A substitution located at domain IV of HAV IRES was observed in 64.2% (9/14) of the Korean HAV sequences. Fujiwara et al [12] also reported that substitutions at nucleotide 391 were seen in 32% of Japanese HAV patients. It is possible that these substitutions were non-specific mutations.

In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to the Japanese sequences previously reported, and this region may represent a viable antiviral target. In Japan, as in Korea, the introduction of childhood vaccination and catch-up vaccination for adolescents and young adults should be considered.

Supporting Information

Table S1 Patient Characteristics. (A) Severe disease, (B) Mild disease.
(DOC)

Table S2 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for HAV GBM/WT RNA (X75215) [15] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

Table S3 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for A10 (AB045328) from Japan [12] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

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We dedicate this work in the memory of the late Dr. Koji Yano, Clinical Research Center, NHO Nagasaki Medical Center, Omura, Nagasaki, Japan.

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Author Contributions

Conceived and designed the experiments: TK SHJ FI OY. Performed the experiments: TK SHJ. Analyzed the data: TK SHJ KF. Contributed reagents/materials/analysis tools: TK SHJ FI KF OY. Wrote the paper: TK SHJ KF. Collected the samples: SHJ.

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VIROLOGY JOURNAL

SHORT REPORT Open Access

Inhibitory effects on HAV IRES-mediated translation and replication by a combination of amantadine and interferon-alpha

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Abstract

Hepatitis A virus (HAV) causes acute hepatitis and sometimes leads to fulminant hepatitis. Amantadine is a tricyclic symmetric amine that inhibits the replication of many DNA and RNA viruses. Amantadine was reported to suppress HAV replication, and the efficacy of amantadine was exhibited in its inhibition of the internal ribosomal entry site (IRES) activities of HAV. Interferon (IFN) also has an antiviral effect through the induction of IFN stimulated genes (ISG) and the degradation of viral RNA. To explore the mechanism of the suppression of HAV replication, we examined the effects of the combination of amantadine and IFN-alpha on HAV IRES-mediated translation, HAV replicon replication in human hepatoma cell lines, and HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. IFN-alpha seems to have no additive effect on HAV IRES-mediated translation inhibition by amantadine. However, suppressions of HAV replicon and HAV replication were stronger with the combination than with amantadine alone. In conclusion, amantadine, in combination of IFN-alpha, might have a beneficial effect in some patients with acute hepatitis A.

Short report

Hepatitis A virus (HAV), a member of the family Picornaviridae, causes acute hepatitis and occasionally fulminant hepatitis, a life-threatening disease. As the broad epidemiological picture of hepatitis A changes, the public health importance of this disease is being increasingly recognized [1]. It is a significant cause of morbidity worldwide, although the mortality rate due to hepatitis A is low (improved intensive care and transplantation have contributed to a reduction in deaths). Improved sanitation and living standards mean that fewer countries remain highly endemic, but the risk of HAV infection is present in countries lacking HAV immunity or where the endemicity of hepatitis A is low or intermediate [1]. In such situations, these outbreaks can prove to be long and difficult to control. Vaccination and informing the general public about good hygienic measures are important for the prevention of HAV infection, but new therapeutic options are also desirable.

Amantadine, a tricyclic symmetric amine, inhibits HAV replication *in vitro* [2]. We previously reported that amantadine inhibits hepatitis A virus internal ribosomal entry site (IRES)-mediated translation in human hepatoma cells [2]. Interferons (IFNs) also exhibit antiviral effects against HAV infection [2,3]. In the present study, we examined the effects of amantadine with or without IFN-alpha, on HAV IRES activities, HAV subgenomic replicon replication and HAV replication *in vitro* as a proof of concept for the development of a more effective treatment to control HAV infection.

First, we evaluated the cytotoxicity of amantadine and IFN-alpha by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay. Amantadine concentrations in a range of 1 - 125 μg/mL and those of 1 - 150 μg/mL for 12-h incubation were non-toxic for Huh7 cells and for HuhT7 cells, respectively (Figures 1A and 1B). Amantadine could be incubated for a short time, e.g., 12 h, with the cells, and then the dose of amantadine could be

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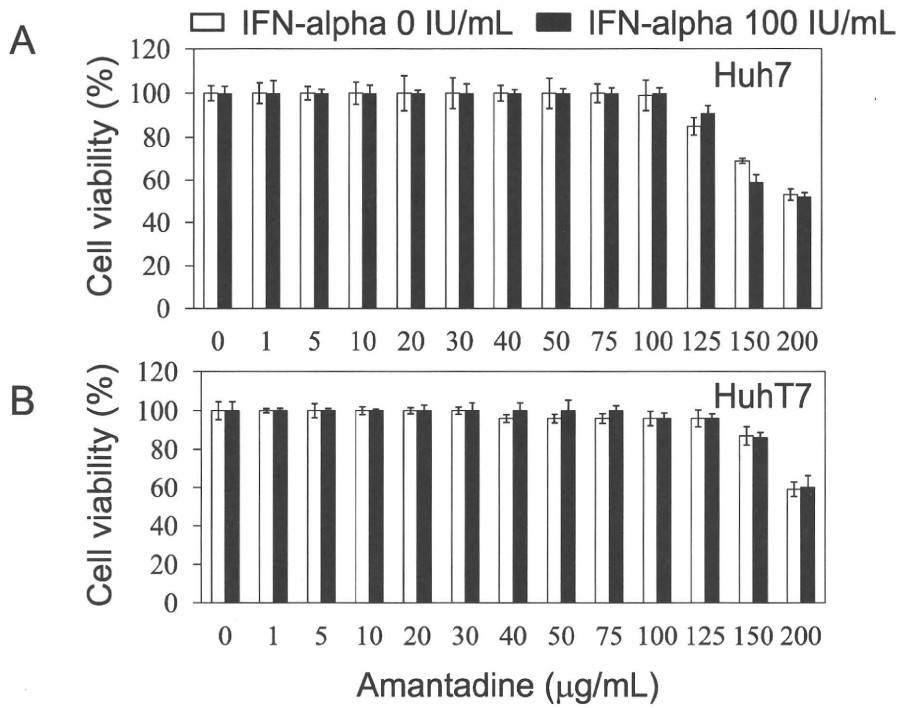

Figure 1 Effects of amantadine on cell growth and viability. MTS assays of cells 12 h after treatment with amantadine with or without 100 U/mL interferon (IFN)-alpha. (A) Huh7 cells. (B) HuhT7 cells. Data are expressed as mean ± SD.

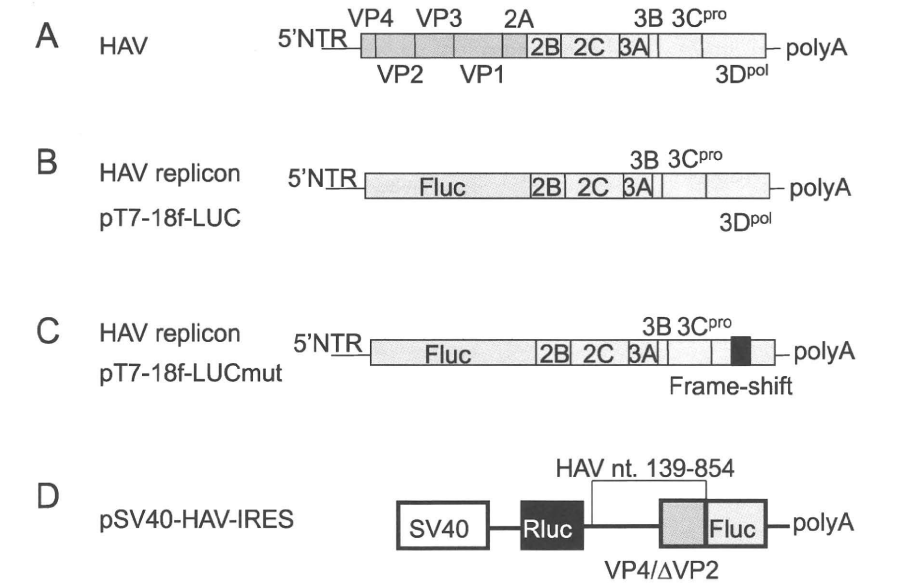

Figure 2 Structures of reporter constructs used in this study. (A) Structure of HAV genome. **(B)** Structure of the replication-competent HAV replicon (HAV replicon) pT7-18f-LUC, which contains an open-reading frame of firefly luciferase (Fluc) flanked by the first four amino acids of HAV polyprotein and by 12 C-terminal amino acids of VP1. This segment is followed by P2 and P3 domains of HAV polyprotein (HAV strain HM175 18f) [9,10]. **(C)** Structure of replication-incompetent HAV replicon (mut) (mut-HAV replicon) pT7-18f-LUCmut, which contains a frame-shift mutation in the polymerase 3 D [9,10]. **(D)** Bicistronic reporter constructs: pSV40-HAV IRES was described previously [2,4]. It encodes the Renilla luciferase genes (Rluc), the internal ribosomal entry site (IRES) HAV HM175, and the firefly luciferase gene (Fluc) under the control of the simian virus 40 promoter (SV40).

increased to higher than 100 μg/mL. With the combination of amantadine and 100 IU/mL IFN-alpha, we did not observe increased cytotoxicity compared with amantadine alone.

We previously reported that the introduction of siRNA targeted against the 5'NTR region of HAV HM175 inhibits HAV IRES-mediated translation and HAV replication [4]. Interestingly, amantadine and IFN also inhibited HAV IRES-mediated translation and HAV replication [2,3,5-8]. Accordingly, we planned to identify more effective strategies for suppressing HAV IRES-mediated translation and HAV replication. IRES is an attractive target for antivirals because HAV IRES is located in the 5'NTR region, the most conserved region among HAV strains. In the present study, we evaluated the HAV antiviral activity of amantadine and IFN-alpha. We initially examined the effects of this combination on HAV IRES-mediated translation using a luciferase reporter assay. Huh7 cells were transfected with pSV40-HAV IRES reporter vector, encoding SV40 promoter driven-*Renilla reniformis* and firefly luciferase, separated by HAV-IRES (Figure 2) [2], and treated with amantadine and/or IFN-alpha. Inhibition of luciferase activity at different levels was observed with amantadine with or without 100 IU/mL IFN-alpha (Figure 3A). Although the strongest suppression was noted with the combination of 10 μg/mL amantadine and 100 IU/mL IFN-alpha, IFN-alpha showed no additive effect on the translation inhibition by 50-100 μg/mL amantadine. This finding prompted us to examine whether IFN-alpha has additive suppression of HAV replicon replication by amantadine. We have reported that RNA replication of HAV can be analyzed in a DNA-based replicon system using HuhT7 cells that stably express T7-RNA polymerase in the cytoplasm (Figure 1) [9-11]. The luciferase activities determined after transfection of replicon DNA are a direct measure of RNA translation and replication. This is because replication in positive-stranded RNA viruses can be easily assessed with a viral replicon carrying the luciferase gene in place of viral structural genes. Moreover, luciferase activity due to translation or translation and replication can be evaluated when the transfection of a replication-competent replicon (HAV replicon) is compared with that of a replication-incompetent replicon (mut) (mut-HAV replicon) [8].

To further determine the effects of the combination of amantadine and IFN-alpha on HAV replication, we transfected the HAV replicon or mut-HAV replicon into HuhT7 cells, and the drugs were added 24 h later. Reporter assays were performed 48 or 72 h after transfection. The transfection efficacy of HAV replicon was estimated as 20-30% in our systems. Luciferase activity was normalized with respect to the protein concentration of cell

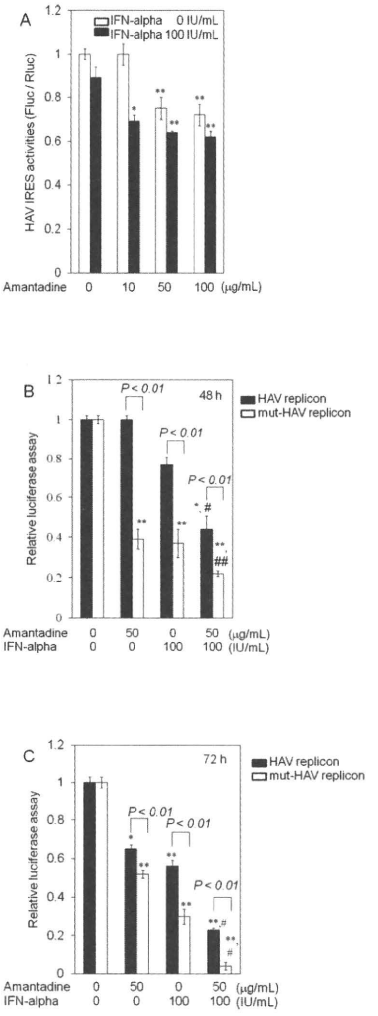

Figure 3 (A) Effects of amantadine with or without interferon on the hepatitis A virus (HAV) internal ribosomal entry site (IRES) activities in Huh7 cells. Approximately 2 × 10^5 cells were seeded on a 6-well tissue culture plate (Iwaki Glass, Tokyo, Japan) 24 h prior to transfection. pSV40-HAV-IRES (0.3 μg) was transfected into Huh7 cells using the Effectene transfection reagent (Qiagen, Tokyo, Japan). 24 h after transfection, amantadine and/or IFN in various concentrations was added to cells. 48 h after transfection, cell extracts were prepared, and luciferase assays were performed using the Dual Luciferase assay system (Toyo Ink, Tokyo, Japan) according to the manufacturer's instructions [2]. For controlling the variations in transcription, IRES activity was assessed by measuring the ratio of *Renilla* and firefly luciferases. All samples were run in triplicate. *Renilla* and firefly luciferase activities were measured as relative light units using a luminescencer (JNRII-AB-2300; ATTO, Tokyo, Japan). **(B, C) Effects of amantadine with or without interferon on the HAV subgenomic replicon replication in HuhT7 cells. (B)** 48 h after transfection and **(C)** 72 h after transfection. Black columns, replication-competent HAV replicon; white columns, replication-incompetent HAV replicon (mut). Relative luciferase activities without any treatments were set at 1. Data are expressed as mean (columns) ± SD (vertical lines). *$P < 0.05$ and **$P < 0.01$, compared with untreated control by Student's t test. #$P < 0.01$ and ## $P < 0.05$, compared with amantadine alone or IFN-alpha alone by Student's t test.

lysates. In this DNA-based system, 48 h after transfection, the replication rates of the HAV replicon were 100%, 77%, and 44% compared to those of control when treated with amantadine alone, IFN alone, and their combination, respectively (Figure 3B). On the other hand, since the mut-HAV replicon cannot replicate, the luciferase activity (39%, 37%, and 22% compared to those of control for the same test conditions, respectively) is due to translation of the viral RNA and not replication. Amantadine alone showed 52% at 72 h, higher than 37% at 48 h, supporting the notion that amantadine might suppress translation of the viral RNA. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. These findings support our observation of the suppression of HAV IRES-mediated translation by amantadine and IFN-alpha. Suppression effects at 48 h after transfection by the combination of amantadine and IFN-alpha against HAV replication were stronger than those by amantadine or IFN-alpha monotreatment. IFN-alpha was more effective than amantadine against the HAV replicon ($P = 0.0027$) (Figure 3B).

Seventy-two hours after transfection, the replication rates of the HAV replicon were 65%, 56%, and 23% compared to those of control when treated with amantadine alone, IFN-alpha alone, and their combination, respectively (Figure 3C). The replication rates of the mut-HAV replicon were 52%, 30%, and 4% of those of control, respectively. IFN-alpha was more effective than amantadine against the replication of HAV replicon or mut-HAV replicon ($P < 0.001$ or $P < 0.001$). Suppression effects of the combination of amantadine and IFN-alpha at 72 h post-transfection were stronger than those of amantadine or IFN-alpha monotreatment. Suppression effects of these treatments were stronger in the mut-HAV replicon than in the HAV replicon. Moreover, it is important to note that the effects of this combination were observed at earlier time points (Figure 3C).

Next, we performed an infectivity assay using the virus to investigate the effects of combination of amantadine and IFN-alpha on tissue culture-adapted HAV strain KRM003 (genotype IIIB, accession no. L20536) propagation in African green monkey kidney GL37 cells [12-14]. GL37 cell monolayers in 96-well culture plates were infected with HAV at a multiplicity of infection (MOI) of 5 or 50 for 1 h at 37°C in a CO_2 incubator. Without removing the inoculum, drug-containing media were added to appropriate wells. The final concentrations of amantadine, IFN-alpha, and their combination were 50 μg/ml, 100 IU/ml and 50 μg/ml of amantadine and 100 IU/ml of IFN-alpha, respectively. After incubation for 72 h, infected cells were evaluated with ELISA. Suppression of HAV replication by the combination of amantadine and IFN-alpha was stronger than those of amantadine alone, IFN-alpha alone, and untreated control (Figure 4).

IFNs are proteins induced by lymphocytes and other cells including hepatocytes in response to viruses such as HAV. In virus-infected cells, dsRNA activates antiviral interferon pathways and the production of IFN type I. The secreted IFN type I induces a positive feedback loop that results in the expression of interferon-stimulated genes (ISGs), including RNase L and protein kinase R (PKR) [15]. Our study supports the fact that the administration of IFN-alpha suppresses HAV replication through HAV IRES mediated-translation and other mechanisms and that, on the other hand, amantadine suppresses HAV replication mainly through HAV IRES mediated-translation.

There are several reports concerning HAV suppressing intracellular dsRNA-induced retinoic acid-inducible gene I (RIG-I)-mediated IFN regulatory factor 3 (IRF-3) activation to block induction of IFN [16,17]. Yang et al. reported that HAV proteins interact with mitochondrial antiviral signaling protein, an essential component of virus-activated signaling pathways that induce protective IFN responses [18]. However, in this study, the

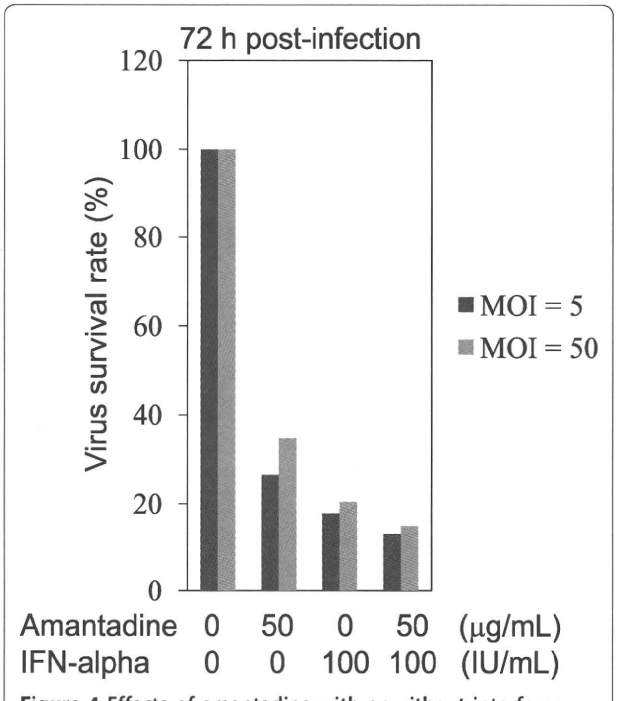

Figure 4 Effects of amantadine with or without interferon on HAV KRM003 genotype IIIB strain replication in African green monkey kidney cell GL37. GL37 cell monolayers in 96-well culture plates were infected with HAV [at a multiplicity of infection (MOI) of 5 or 40] for 1 h at 37°C in a CO_2 incubator. Amantadine and/or IFN was added to cells. After the incubation for 72 h, infected cells were evaluated with ELISA. The rate of virus survival was measured using this equation: Virus survival rate (%) = 100 × Absorbance with drug/Absorbance without drug.

administration of exogenous IFN-alpha could suppress HAV replication, although endogenous IFNs produced by cells also may play an important role in inhibiting viral replication. Further studies will be needed.

Amantadine inhibits the replication of many DNA and RNA viruses and is also used as a drug for the treatment of Parkinson's disease [2]. It is known that the M2 protein of influenza A virus is a target of amantadine [19]. Furthermore, it has been reported to inhibit HAV IRES-mediated translation and replication by our group and other researchers [2,3,5-8].

Therefore, we examined the possibilities of the combination of amantadine and IFN-alpha against HAV because these two drugs were previously reported to be effective against HAV [2,3,5-8]. To our knowledge, this is the first study demonstrating that a combination of amantadine and IFN-alpha can suppress HAV replication more effectively than amantadine or IFN-alpha alone.

Abbreviations

HAV: hepatitis A virus; **IRES**: internal ribosomal entry site; **IFN**: interferon; **MTS**: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt.

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Authors' contributions

LY, Tatsuo Kanda, FI and OY conceived and designed the study. LY, Tomoko Kiyohara and Tatsuo Kanda performed the experiments. LY, Tomoko Kiyohara, Tatsuo Kanda and FI analyzed data and wrote the manuscript. Tomoko Kiyohara, KI and TW contributed to experiments using a whole HAV virus. Tomoko Kiyohara, Tatsuo Kanda and VG contributed to the interpretation of the interpretation of the results and took part to the critical revision of the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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Original Article

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

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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

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