

Comparative Genotype Analysis of Hepatitis A Virus: Two One-Year Studies in South Korea in 2002 and 2011

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Hepatitis A virus (HAV) positive stool samples were collected from acute hepatitis A patients during the two study periods of 2002 and 2011 in Seoul, South Korea, and their genetic variability was determined. From a total of 79 specimens, the nucleotide sequences of the VP1 and 2A junction were successfully amplified in 27 (34.2%) samples and subjected to sequence analysis. Genetically, there was a dramatic change in HAV subgenotypes from IA to IIIA during the past ten years. Sequence analysis identified that most strains belonged to genotype I, which is the main genotype globally. The subgenotype IA (93.3%, n=13/14) was the major subgenotype in 2002, whereas the subgenotype IIIA (69.2%, n=9/13) was predominant in 2011. Interestingly, a IIIA strain was identified from a patient who had a history of travel to India in 2002. The finding presented provides new insight into the genetic shift of circulating HAVs in South Korea.

Key Words: Hepatitis A virus; Genetic variation; VP1/2A junction

INTRODUCTION

Hepatitis A virus (HAV) is the most general cause of acute infectious hepatitis, with about 1.5 million people infected annually worldwide (1). HAV generally spreads through the fecal and oral transmission. Development in living standards and socio-economic growth are associated with a reduce in the incidence of HAV infection in developing countries over the past decades. In provinces where HAV is prevalent, children may transmit the virus

even though they lack evident clinical features, allowing the virus reservoirs to spread from developing countries into developed nations (2).

HAV is a sole member of the family Picornaviridae and is a member of the genus Hepatovirus (3). The virus genome is a 7.5 kb positive strand of RNA with a single poly protein that is divided into three functional regions named P1, P2, and P3. P1 encodes the capsid proteins (VP1-VP4), whereas P2 and P3 encode non-structural proteins necessary for virus replication (4). HAV has been categorized into seven major genotypes (I-VII) according to the sequence of

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the VP1/2A junction (5). Genotypes I, II, III, and VII are known to be human strains, whereas genotypes IV, V, and VI are exclusively simian in origin (6). Genotypes I and III are the most common worldwide and are further divided into subgenotypes IA, IB, IIIA, and IIIB. Comparative analysis of this region was suggested as a molecular epidemiologic marker to investigate the relatedness of individual strains with regard to parameters such as geographical or infectious origin.

Although a vaccination program is being implemented in South Korea and most adults had immunity to HAV until 20 years ago (7), there has been a significant recent increase in HAV infection (8~11). The greater part of children and young adults were not infected with HAV at early ages, making morbidity most likely upon infection. Several recurring epidemic HAV infection have occurred, and the infection is now recognized as a public health trouble (12). The number of adult cases of acute hepatitis A has progressively increased during the last ten years, reflecting the changing epidemiology of HAV according to rapid improvements in socioeconomic status (8, 13). In this study, HAV fecal specimens obtained from patients in the years 2002 and 2011 in Seoul, South Korea were analyzed with respect to the phylogenetic relatedness of their genotypes, and endemic patterns were determined by comparison with other geographically defined isolates.

MATERIALS AND METHODS

Ethics statement

Cases of acute hepatitis A in South Korea diagnosed at the Department of Internal Medicine at the Catholic University Hospital in 2002 and at the Department of Internal Medicine at Chung-Ang University Hospital in 2011 were assessed. All participants provided written informed consent. For all cases, collected samples were analyzed under protocols approved by the Chung-Ang University College of Medicine IRB (Protocol #2009-13).

Stool specimens

Among available 79 samples, 34 fecal specimens were

collected from patients diagnosed with an acute form of hepatitis A at the Catholic University School of Medicine in 2002 and 45 samples were collected from Chung-Ang University Hospital in 2011. Acute hepatitis A was defined by the presence of specific clinical symptoms accompanied by detection of IgM anti-HAV in serum samples using a commercially available assay according to the manufacturer instructions (Diasorin, Saluggia, Italy). All specimens were diluted ten-fold with PBS (pH 7.4) and clarified by centrifugation at 10,000 g for 10 min. The supernatants were stored at -20°C before use.

Primer design

Based on the genomic map (13), primers for HAV genotyping were chosen from the VP1/2A junction region. The primers were designed by aligning sequences of six HAV strains (GenBank accession numbers AB020567, K02990, X75214, X15464, M59810, and D00924) from NCBI GenBank using Primer 3 software (14) with default settings. The primers used to amplify the VP1/2A junction region were HAV-2741F [5'-TATTGATTAYAAAACCTG-CYCTTGG-3'; positions 2741-2764] and HAV-3433R [5'-GTCCATTTCTCATCATTCAATCT-3'; positions 3411-3433]. Internal oligonucleotides HAV-2895F [5'-TCTAT-TCAGATTGCM AATTAYAATC-3'; positions 2895-2919] and HAV-3376R [5'-TCAAGAGTCCACACACTTCT-3'; positions 3357-3376] were used as nested PCR and sequencing primers. Base positions are from GenBank accession number AB020567.

RNA extraction for genotyping

HAV RNA was extracted using Trizol reagent (Gibco BRL Life Technologies, Grand Island, NY, USA). In brief, 0.3 ml of supernatant from a fecal suspension in PBS was mixed with 0.7 ml Trizol reagent and 0.2 ml chloroform/isoamylalcohol (24:1). After centrifugation at 12,000 g for 10 min, the RNA in the aqueous solution was precipitated by adding an equal volume of isopropanol. The RNA precipitate was collected by centrifugation at 12,000 g for 10 min, washed with 70% ethanol, and dissolved in 20 µl RNase-free water.

Reverse transcription-polymerase chain reaction

Nested RT-PCR was performed in three steps. First, 5 μ l extracted RNA was denatured with 20 μ M reverse primer (HAV-3433R) and 5 μ l ddH₂O in 70°C for 10 min. Then, cDNA was synthesized with the denatured RNA in a reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 2.5 mM MgCl₂, 2.5 mM dNTPs, and 2.5 units AMV reverse transcriptase (Promega Corporation, Madison, WI, USA) at 42°C for 60 min. The first PCR was performed to amplify the flank region of the VP1/2A junction. Five microliters of cDNA was added to a PCR reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 0.01% gelatin, 1.5 mM MgCl₂, 0.2 mM of each dNTPs, 20 μ M each primer (HAV-2741F and HAV-3433R) and 2.5 units Taq polymerase (Roche Diagnostics, Indianapolis, IN, USA). The PCR conditions consisted of one cycle of denaturation at 98°C for 1 min, followed by 30 cycles at 95°C for 1 min, 45°C for 1 min, and 72°C for 1 min 30 sec, and one final extension at 72°C for 10 min in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA, USA). Nested PCR was performed using 5 μ l of the first amplification product as a template and the primer set HAV-2895F and HAV-3376R. The reaction mixture was the same as for the first amplification and the thermal cycling profile consisted of one cycle of 95°C for 7 min, 30 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. Both first and nested PCR products were analyzed by electrophoresis in 2% SeaKem LE agarose gels (FMC Bioproducts, Rockland, ME, USA) with ethidium bromide staining. The results were viewed using a GelDoc XR image-analysis system (BioRad Laboratories Inc, Hercules, CA, USA).

Nucleotide sequencing and phylogenetic analysis of the VP1/2A junction region

The amplified cDNA (482 bp) was purified using the QIAquick purification kit (Qiagen GmbH, Westburg, Germany) and sequenced using the BigDye terminator cycle sequencing kit and an ABI PRISM 3730 automated DNA sequencer (Applied Biosystems). HAV-2895F was

used as a sequencing primer for analysis of the 168-bp VP1/2A junction region. The resultant sequences were aligned using the CLUSTAL_X 1.81 program (15) with parameters set against the corresponding sequences of 41 other geographically defined HAV strains from the NCBI GenBank database. A rooted tree was constructed using the neighbor-joining algorithms (15) from the PHYLIP suite of programs (16). Evolutionary distance matrices were generated by the neighbor-joining method (17) and tree topology was evaluated using a bootstrap analysis of the neighbor-joining dataset with the SEQBOOT and CONSENSE programs from the PHYLIP package.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in GenBank under the accession numbers EU186418-EU186431 and JQ066751-JQ066763.

RESULTS

Description of HAV patients

In 2002 we analyzed 14 patients, comprised of 8 (57.1%) males and 6 (42.9%) females, with a mean age of 29.1 years (range 14 to 46 years). In 2011 we analyzed 11 (64.7%) male patients and 6 female (35.3%) patients aged from 26 to 42 years. The patients were hospitalized mainly with symptoms of fatigue, nausea, and right-upper-quadrant (RUQ) discomfort, although two cases presented with manifestations of jaundice. All of the patients were sporadic cases. The mean peak alanine aminotransferase (ALT) level was 4,134 IU/ml (range, 970~7,020 IU/ml) and the mean peak bilirubin was 5.3 mg/dl (range, 1.1~10.1 mg/dl). One patient (a 26-year-old female) was diagnosed with fulminant hepatic failure. She became comatose within two days following admission, with a factor V level 7% of normal. She underwent emergency orthotopic liver transplantation. Another patient (a 30-year-old male) had experienced jaundice without liver failure for two months following admission. The other patients recovered spontaneously, with a marked decline in ALT activity and clotting factor normalization within four weeks following admission.

Genotyping and phylogenetic analysis

The HAV genotype was identified in 14 (41.2%) of 34 specimens collected in 2002 and in 13 (15.5%) of 84 specimens in 2011. A 482-bp fragment of the VP1 and 2A regions was successfully amplified by RT-PCR and subjected to sequence analysis in a total of 27 strains, CAU 02-01, CAU 02-03, CAU 02-04, CAU 02-06, CAU 02-08, CAU 02-09, CAU 02-10, CAU 02-11, CAU 02-12, CAU 02-16, CAU 02-18, CAU02-21, CAU 02-22, CAU02-23, CAU 11-01, CAU 11-02, CAU 11-07, CAU 11-12, CAU 11-13, CAU 11-17, CAU 11-46, CAU 11-62, CAU 11-76, CAU 11-79, CAU 11-83, CAU 11-84, and CAU 11-117. The nucleotide sequences of the 168-bp VP1/2A junction region were compared with those of 4 Korean isolates and 34 foreign strains in the GenBank database to determine the genotype and epidemiological transmission. Nucleotide sequence alignment followed by phylogenetic analyses indicated that 16 strains could be classified into subgenotype IA, 1 isolate was exclusively grouped into subgenotype IB, and the remaining 10 sequences were categorized into subgenotype IIIA.

Phylogenetic analysis of 2002 strains

Thirteen of the 14 strains isolated in 2002 were subgenotype IA. These strains were further divided into three clusters using the neighbor-joining algorithms and supported by high bootstrap values (Fig. 1a). CAU 02-08, CAU 02-09, and CAU 02-23 belonged to the cluster of mixed Japanese and Chinese strains whereas CAU 02-04, CAU 02-07, and CAU 02-21 were related to the Japanese cluster. However, the other seven CAU isolates, CAU 02-01, CAU 02-03, CAU 02-06, CAU 02-10, CAU 02-11, CAU 02-12, and CAU 02-18, appeared to belong to a new cluster together with six Korean isolates of KU98 serious strains that were previously identified in 1998 and represented a Korean native cluster based on 94.0% shared nucleotide identity. The nucleotide sequences of these strains had 95.8~99.4% nucleotide identity similarity with MS-1, the subgenotype IA prototype strain detected in the USA in 1964, but showed relatively low nucleotide identities

(91.0~88.0%) with HM-175, the prototype subgenotype IB detected in China in 1989.

Among these strains, the nucleotide sequences of CAU 02-11 and CAU 02-12, which were isolated from a family outbreak between brothers, were identical and showed no nucleotide variability with CAU 02-10. This cluster also included three Japanese strains, A68, A159, and FH-1. Korean isolates close to the USA cluster were not found. Russian strains, 1406, RUS_17_03, Kular-1982-2101, and RM 238 belonging to genotype I showed a closer relationship to strains in the Korean cluster than those in the Chinese, Japanese, and USA clusters, with >94.6% nucleotide identity.

Interestingly, CAU 02-16 appeared in a subgenotype IIIA lineage that was clearly separated from the other Korean strains. This strain was phylogenetically closest to India 90 detected in 1990, with 97.6% nucleotide identity, and prototype strain PA21 detected from Panama in 1980, with 97.4% nucleotide identity. It is notable that this is the first imported genotype IIIA strain identified in this country and that the patient had a history of travel to India before symptoms manifested.

Phylogenetic analysis of 2011 strains

Nine strains, CAU11-17, CAU11-79, CAU11-1, CAU 11-7, CAU11-12, CAU11-83, CAU11-62, CAU 11-13, and CAU11-84 belonged to subgenotype IIIA, the predominant genotype (Fig. 1b). These subgenotype IIIA strains showed 97.6~95.8% nucleotide identities to India 90 from subgenotype IIIA and 96.4~94.6% nucleotide identities to PA21 from subgenotype IIIA. These IIIA strains also shared 87.5~86.9% nucleotide identities with KRM003 in subgenotype IIIB. Another three strains, namely CAU11-46, CAU11-02, and CAU11-76, clustered into subgenotype IA and were further divided into three different lineages designated as B, C, and E, which contained Belarus strains (Oct5724 and Nov5735), Korean strains (KU98-7, KU98-156, and KU98-13), and Japanese strains (A-12, M-118, and A414), respectively. CAU11-117 was classified into subgenotype IB.

2002

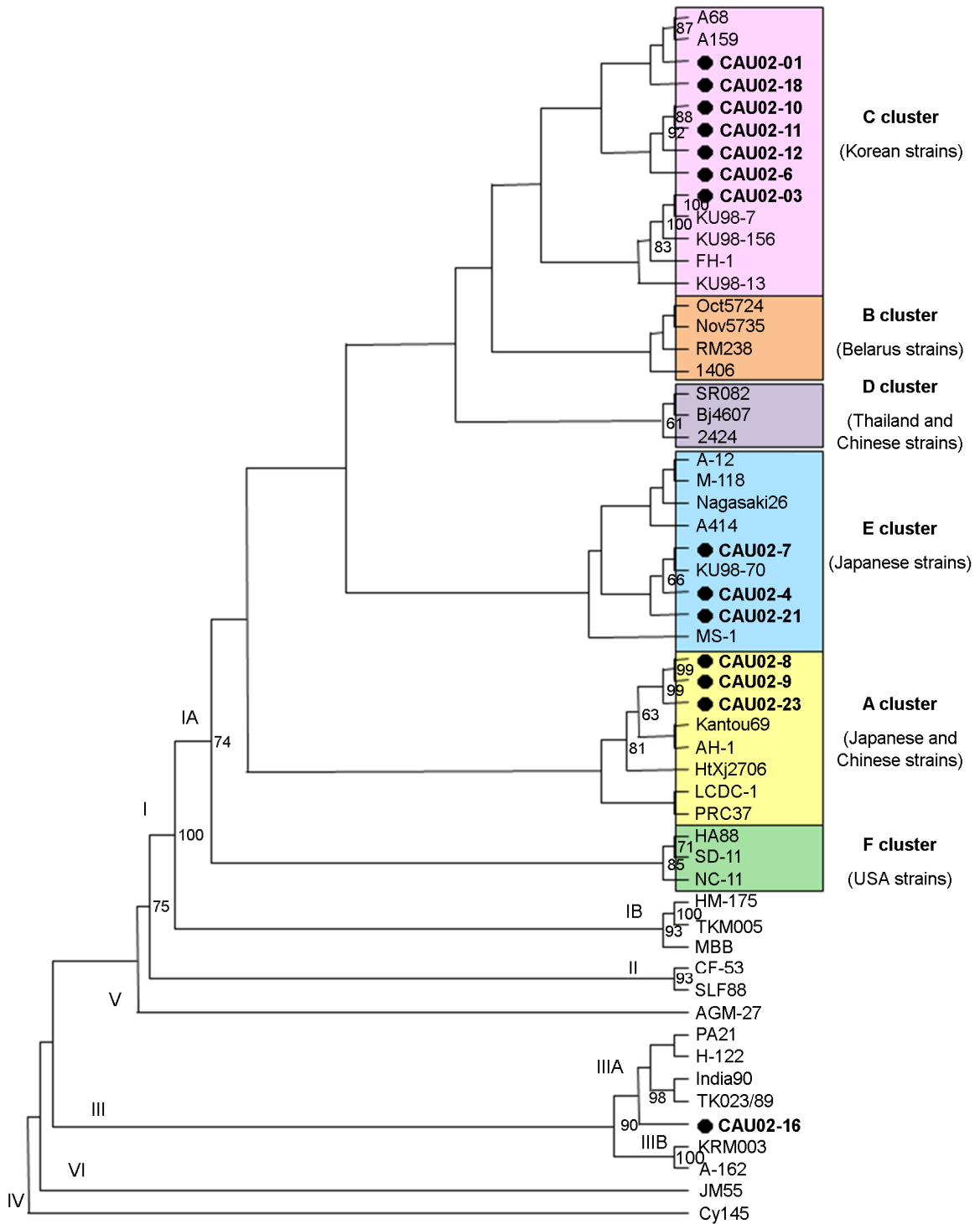


Figure 1a.

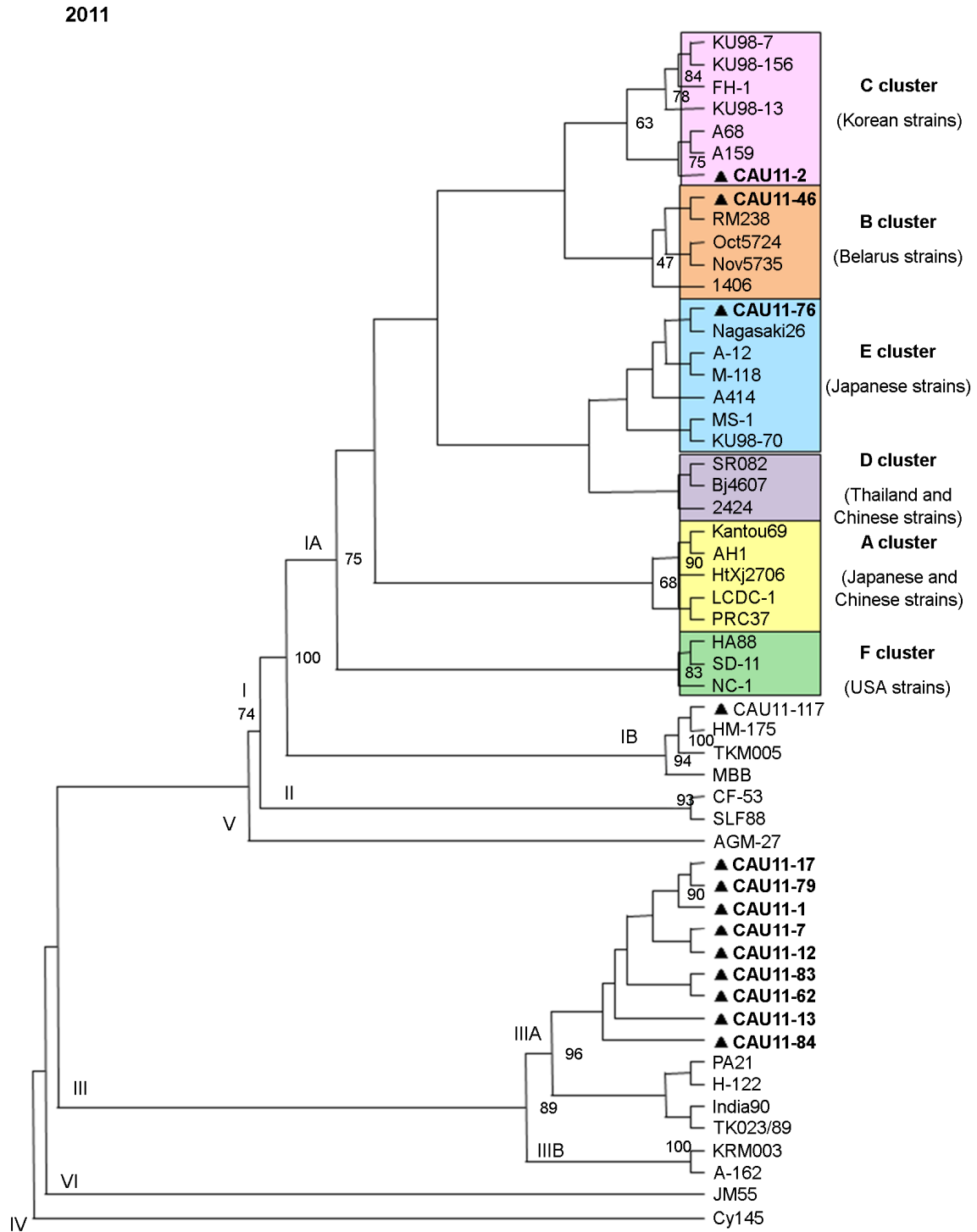


Figure 1b.

Figure 1. Phylogenetic trees based on 168-bp HAV VPI/2A junction region nucleotide sequences show genetic relationships between 4 Korean isolates and 37 HAV strains obtained from the GenBank database. The shaded boxes indicate the 16 genotype I CAU isolates and the unfilled boxes indicate geographically related clusters. The numbers at the nodes indicate the level of bootstrap support (%) based on neighbor-joining analysis of 1,000 re-sampled datasets; only values above 50% are given. Hepatitis A virus Cy145 (L07732) was used as the out-group. (a) 2002 data; (b) 2011 data

DISCUSSION

Recently, hepatitis A has become increasingly important because its incidence is dramatically increasing in adolescents and adults in South Korea. This reflects the lack of anti-HAV antibody in this age group as a result of the low chance of HAV infection over the last ten years (8, 13, 18). Considering this changing epidemiology, a policy for hepatitis A vaccination in these populations in addition to children might be warranted. This circumstance has resulted in the need for surveillance study of HAV infection. However, only limited information is available about the HAV genotypes circulating in this country with respect to the relatedness of domestic isolates from different geographical areas (7).

In order to comprehend the genetic variability of the HAVs in South Korea, the nucleotide sequences of recent HAV isolates were compared with isolates identified ten years previously. This comparison revealed a remarkable change in circulating HAV in South Korea from subgenotype IA to IIIA over the ten-year period from 2002-2011. There was a considerable increase in HAV genetic variability during the second period of evaluation (2011), suggesting comparable circulation of two dissimilar HAV genotypes or a prospective antigenic drift of HAV in the Korean population.

HAV genotyping approaches have made it possible to differentiate between the strains involved in outbreaks of sporadic cases on the basis of nucleotide variability (1). Based on field studies, it was believed that different transmission pathways were involved in the outbreaks and that many distinct but closely related HAV strains were circulating in these areas and were separately responsible for the spread of disease (12). On the other hand, among the 27 (22.9%) strains sequenced in this study, indistinguishable or closely related HAV strains were identified in fecal samples collected during different outbreaks in South Korea, which indicates a common outbreak source.

In the cluster study, the subgenotype IA cluster 'C' consists of three previously identified KU98 isolates and represents

Korean native strains based on greater than 94.0% nucleotide identity. Japanese strains are known to make up a unique cluster or to be mixed with Chinese strains. However, three Japanese strains, A68, A159, and FH-1, were located in the Korean cluster, and interestingly these strains were also isolated between 2001 and 2002 in China. Therefore these Japanese strains might be more closely related to Korean isolates than to Japanese strains.

Phylogenetic analysis of the 2011 data showed a dominant subgenotype IIIA that was first isolated from Panamanian owl monkeys in 1980 and subsequently found in humans in Sweden, the United States, Nepal, Sri Lanka, India, and Malaysia, (19). To identify this as an imported virus, knowledge of the travel experience of patients is crucial. To our knowledge, the first identified genotype IIIA strain in this country (CAU 02-16) was found to be more closely related to the Indian 90 strain than the prototype strain PA21, and the patient had a history of travel to India before the symptoms manifested. Since the incubation period for HAV ranges from 10 to 50 days, it is possible that this strain was introduced from India.

As a result of improved sanitation standards, HAV strains have constantly and rapidly changed in Korea (20, 21). Recent outbreaks of HAV in South Korea showed characteristic epidemiological characteristics that might be different from those of many other developed nations, where the role of young children in the transmission of HAV has been the most significant factor. More than 60% of Korean children younger than ten have protective immunity, probably due to vaccination over the past 5~15 years (22). Therefore, the recent and unexpected raise in subgenotype IIIA might be due to autochthonous circulation among pre-disposed young adults, possibly via food-borne transmission (1).

There was no significant difference in the clinical features of patients with genotype IA and those with genotype IIIA at the hospital admission. However, the presence of two cases with genotype IIIA infection who had fulminant hepatic failure and sustained jaundice, respectively, suggests that genotype IIIA infection may have more severe clinical outcomes in young adults than genotype IA infection. We

conclude that a change in circulating HAV subgenotypes from genotype IA to IIIA has occurred over the past decade in South Korea during the recent community-wide epidemic of hepatitis A.

The restrictions of this study include incomplete assessment of the risk factors for HAV infection and insufficient explanations of the etiology of the observed alteration in circulating HAV genotypes. HAV genotype IIIA infection in young adults is known to be associated with more severe clinical features at the hospital admission than HAV genotype IA infection (23). Further studies on the immunological differences underlying these diverse clinical features based on the different genotypes are needed.

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