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# Inter-Genotypic Recombinant Hepatitis C Virus Strains in Japan Noted by Discrepancies Between Immunoassay and Sequencing

**Hiromi Hoshino,[1] Kunihiko Hino,[1] Hiroshi Miyakawa,[2] Kazuaki Takahashi,[3] Sheikh Mohammad Fazle Akbar,[3]* and Shunji Mishiro[3]**

[1]*Delta Clinic, Tokorozawa, Japan*
[2]*Teikyo University School of Medicine University Hospital, Mizonokuchi, Kawasaki, Japan*
[3]*Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan*

Genetic recombination plays a significant role in the survival and evolution of hepatitis C virus (HCV), but methodological limitations have hindered the exploration of genetic recombination. HCV serotypes were evaluated in 104 patients with chronic hepatitis C when they initially presented in hospitals. Subsequently, HCV genotypes were analyzed using primers for core gene and NS5B gene. Near-complete nucleotide sequences of eight HCV isolates from two suspected patients with 2b/1b recombinant HCV were analyzed by amplification of nine overlapping regions of HCV-specific oligonucleotide primers at different time points: (i) at the first admission; (ii) before and (iii) after interferon therapy; and (iv) after development of hepatocellular carcinoma. The nucleotide sequence of eight HCV isolates obtained was 9,321–9,471 nucleotides in length, comprising a single ORF (polyprotein of 3,014 amino acids.) and segregated into discordant genotypes of 2b and 1b HCV with a recombination junction in NS2. This study highlights the need for more precise characterization of HCV in clinical samples where there is a discrepancy between immunoassays and sequencing. It also demonstrates the circulation of novel inter-genotypic recombinant HCV in Japan, because the cross over point of 2b/1b recombinant HCV in eight clinical isolates of these two patients differed from previously reported HCV recombinant from the Philippines and Japan. ***J. Med. Virol. 84: 1018–1024, 2012.*** © 2012 Wiley Periodicals, Inc.

**KEY WORDS:** HCV recombination; Japan; serogroup; genotype



## INTRODUCTION

Hepatitis C virus (HCV) is a member of the Flaviviridae family and it is a major cause agent of post-transfusion hepatitis and parenterally transmitted, sporadic non-A, non-B hepatitis throughout the world [Moradpour et al., 2007]. Considerable advances have been made in the fields of epidemiology and virology during the last two decades, and there have been many investigations on the mode of HCV transmission and therapeutic approaches [Neumann et al., 1998; Di Bisceglie and Hoofnagle, 2002; Seeff, 2002]. However, major concerns remain about the development of prophylactic vaccines and the treatment of non-responder patients, which may be attributable to limited understanding of HCV genetic diversity.

HCV is an RNA virus and it exploits all the known mechanisms of genetic variation to ensure its survival [Choo et al., 1991; Enomoto et al., 1996; Holmes et al., 1999; Chung et al., 2010]. The polymerase enzyme of most RNA viruses, including HCV, lacks proofreading ability, so it is unable to correct errors during viral replication and this leads to genomic mutations. In addition, HCV exists in a host as a heterogeneous population of viruses with closely related genomes, a condition referred to as a quasispecies. These alterations in the HCV genome allow the virus to escape the host immune response and these changes in the viral

*Correspondence to: Sheikh Mohammad Fazle Akbar, MD, PhD, Principal Investigator, Toshiba General Hospital, Higashi Oi 6-3-22, Shinagawa, Tokyo 140-8522, Japan.
E-mail: sheikh.akbar@po.toshiba.co.jp

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genome also explain: (i) the susceptibility of patients infected with HCV to the development of persistent infection; (ii) the difficulty in developing a prophylactic vaccine against HCV; and (iii) low response of some patients with HCV to antiviral drug therapy [Farci et al., 2002; Chambers et al., 2005]. In addition to genomic mutations and quasispecies, genetic recombination seems to play a significant role in the evolution of RNA viruses. Recombination has been reported in other members of the Flaviviridae family (pestiviruses, flaviviruses, hepaciviruses, and Japanese encephalitis viruses) and a natural intergenotypic recombinant (2k/1b) of HCV was identified for the first time in Saint Petersburg, Russia [Kalinina et al., 2002]. A few further cases of chronic hepatitis C with recombinant HCV have been reported from other countries [Kalinina et al., 2002; Colina et al., 2004; Kageyama et al., 2006; Gao et al., 2007; Morel et al., 2011].

However, little is known about recombinant HCV in Japan, where about two million people are infected with HCV. HCV serotypes and genotypes are checked in Japanese patients with HCV to provide insights into disease pathogenesis and predict the prognosis of antiviral therapy. HCV recombination was not reported in Japan until 2010, which may indicate that HCV recombination is either absent or a rare event in Japan. Regular clinical practice suggested that this may actually reflect an inherent limitation of the HCV characterization procedure in Japan. The HCV serotype is defined using NS4-based immune assays that are not suitable for the identification of HCV recombinants. Likewise, HCV genotyping is based on the assessment of nucleotide sequences in a single subgenomic region and it also fails to consider HCV recombination. Full HCV genome sequencing or the assessment of HCV genotypes in multiple regions are not conducted in regular clinical practice.

Hoshino et al. [2010] have reported a novel 2b/1b HCV recombination in a Japanese patient infected with HCV, which provided credible evidence of the discrepancies in HCV serotyping and HCV genotyping in regular clinical practice. Immune assay indicated that the patient was infected with HCV from serogroup 1. This was supported by HCV genotyping using primers for the NS5B region of HCV. However, when primers for the HCV core region were used to assess the genotype, the patient was found to have HCV genotype 2b.

On the basis of this preliminary observation, an extended study was conducted with serogrouping and genotyping of HCV from 104 patients with chronic hepatitis C. HCV recombinants were detected in two patients with chronic hepatitis C. Sera were available from these patients at different time points during the last 14–18 years. Both received antiviral therapy and one recently developed hepatocellular carcinoma (HCC). Analyses of HCV recombination in eight HCV isolates from these two index patients have provided important insights into HCV virology in Japan.

## MATERIALS AND METHODS

This study enrolled 104 patients with chronic hepatitis C who presented at Delta Clinic, Tokorozawa, Saitama, Japan. The diagnosis of chronic hepatitis C was made on the basis of the clinical, biochemical, and virological profiles of patients. All expressed HCV RNA in their sera. None had a concurrent infection with hepatitis B virus or human immune-deficiency virus-1. Two patients with HCV recombinants were marked as index patients. The clinical profiles of the two patients with intergenotypic HCV recombinants are shown in Figure 1. Informed consent was obtained from patients after explaining the nature and purpose of the study.

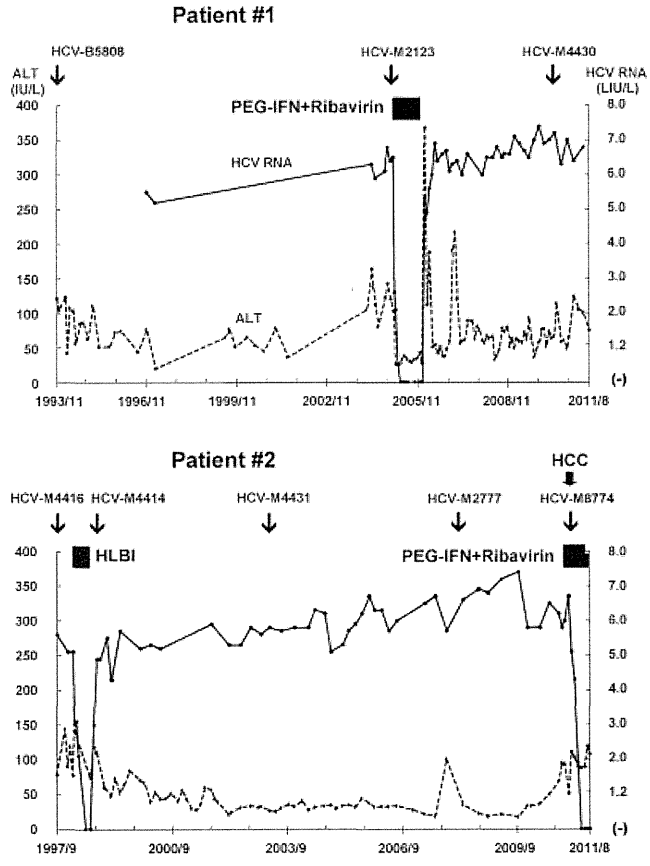


Fig. 1. Clinical profiles of two index patients with recombinant hepatitis C virus (HCV) 2b/1b. Both patients received antiviral therapy, but a sustained response was not achieved in either patients. Three sera samples were available from Patient #1, as shown by arrows; (1) during first appearance at the hospital in 1993 (MCV-B5808), (2) before therapy with peg-interferon (IFN) and ribavirin in 2005 (HCV-M2123), and (3) in 2010 (HCV-M4430). Five sera samples were collected from Patient #2; (1) before treatment with human lymphoblastoid interferon (HBLI) in 1997 (HCV-M4416), (2) after interferon therapy in 1998 (HCV-M4414), (3) in 2003 (HCV-MM4431), (4) before treatment with peg-IFN and ribavirin in 2007 (HCV-M2777), and (5) in 2011, after development of hepatocellular carcinoma (HCV-M8774). Treatment with antiviral drugs is shown as a black bar. HCC in Patient #2 indicates the time of diagnosis of hepatocellular carcinoma.

### Serotyping of HCV

The serotypes of HCV isolates from all patients with chronic hepatitis C were determined using a commercial ELISA with NS4-based immunoassays, according to the manufacturer's instructions (Immucheck F-HCV Gr. Sysmex Corporation, Kobe, Japan).

### RNA Extraction, cDNA Synthesis, Amplification, and Sequencing

Isolation of RNA, genotyping of HCV, and sequencing of the HCV genome were conducted according to previously described methods, but with some modifications [Takahashi et al., 2009]. Nucleic acids were extracted from serum using a QIAamp MinElute Virus Spin Kit (Qiagen, Hilden, Germany). One of the primers were specific for 2b/1b HCV recombinant (Primer C, Table I). HCV-RNA genomes were reverse transcribed and cDNA was amplified using PCR with primers specific for nine overlapping regions of the HCV genome (Table I). Reverse transcription and first-round PCR were conducted using a PrimeScript II High Fidelity One Step RT-PCR Kit (Takara Bio Inc, Shiga, Japan), while second-round PCR was conducted with PrimeSTAR GXL DNA Polymerase (Takara Bio Inc). The 5′-terminal sequences were amplified using a SMARTer RACE cDNA Amplification Kit (Clontech Laboratories Inc., Mountain View, CA).

Final products were sequenced using a 3100 DNA Sequencer with a BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). Genetic analyses of HCV sequences were conducted using the neighbor-joining method with the program Genetyx-Mac Version 13 (Genetyx Corporation, Tokyo, Japan).

## RESULTS

HCV serotypes and HCV genotypes were in complete agreement in 101 of the 104 chronic hepatitis C patients used in this cohort. However, a discrepancy between the HCV serogroup and HCV genotype was detected in three patients. One of them belonged to HCV serogroup 1 with HCV genotype 2, but HCV recombination was not detected in this patient. On the other hand, two patients with chronic hepatitis C

TABLE I. HCV-Specific Oligonucleotide Primers Used in This Study

| Primer—specificity | Stage—polarity | Nucleotide sequence (5′–3′) | Nt position[a] |
|---|---|---|---|
| Primer set A—2b | 1st—sense | CCATGAATCACTCCCCTGTG | 30–49 |
| | 2nd—sense | GAACTACTGTCTTCACGCAG | 52–71 |
| | 1st—antisense | ARGCTGTCATTGCARTTGAG | 1,620–1,639 |
| | 2nd—antisense | GCGGTCCGGTTTATGTGCCA | 1,599–1,618 |
| Primer set B—2b | 1st—sense | GCATGGCATGGGACATGATG | 1,291–1,310 |
| | 2nd—sense | CCAACTCTYACCATGATCCT | 1,323–1,342 |
| | 1st—antisense | AGGAGARTCTTGTATGCTGG | 2,850–2,869 |
| | 2nd—antisense | GTAAGAGTRAAGATGGATAT | 2,829–2,848 |
| Primer set C—2b/1b | 1st—sense | TGGATGCTCATYATACTGGG | 2,559–2,578 |
| | 2nd—sense | GAGGCRGCGCTTGAGAAGCT | 2,586–2,605 |
| | 1st—antisense | GTAGCGGTGGCGAGYACGAC | 4,383–4,402 |
| | 2nd—antisense | CCAGCCGTCTCCGCTTGGTCC | 4,352–4,372 |
| Primer set D—1b | 1st—sense | GGCGCCTAYGACATCATAAT | 4,275–4,294 |
| | 2nd—sense | TGTGATGARTGCCACTCAAC | 4,296–4,315 |
| | 1st—antisense | GGTGGTGAGCGGGCTGGTGA | 5,743–5,762 |
| | 2nd—antisense | CGCTGATGAARTTCCACATG | 5,645–5,664 |
| Primer set E—1b | 1st—sense | TGGAARTGTCTCATACGGCT | 5,175–5,194 |
| | 2nd—sense | ATCATGGCATGCATGTCGGC | 5,286–5,305 |
| | 1st—antisense | AYGCCCGTCACGTAGTGGAA | 6,648–6,667 |
| | 2nd—antisense | GTGGTGTAYGCGTTGATGGG | 6,534–6,553 |
| Primer set F—1b | 1st—sense | TGGGACTGGATATGCACGGT | 6,294–6,313 |
| | 2nd—sense | AAGACCTGGCTCCAGTCCAA | 6,327–6,346 |
| | 1st—antisense | ACTGTGGACGCCTTCGCCTT | 7,824–7,843 |
| | 2nd—antisense | TCCTTGAGCACGTCCCGGTA | 7,800–7,819 |
| Primer set G—1b | 1st—sense | TCCATGCCCCCCCTTGAGGG | 7,512–7,531 |
| | 2nd—sense | AGCGACGGGTCTTGGTCTAC | 7,554–7,573 |
| | 1st—antisense | CATGAAGCCACCCTATTGAT | 9,054–9,073 |
| | 2nd—antisense | TGGAGAGTAACTATGGAGTGA | 9,027–9,047 |
| Primer set H—1b | 1st—sense | GAGGCTATGACTAGGTACTC | 8,631–8,650 |
| | 2nd—sense | AGACAGCTAGACACACTCCA | 8,803–8,822 |
| | 1st—antisense | ATGGCCTATTGGCCTGGAGTG | 9,400–9,420 |
| | 2nd—antisense | CTATTGGCCTGGAGTGKTTA | 9,396–9,415 |
| Primer set I—1b | 1st—sense | CTCCATAGTTACTCTCCAGG | 9,030–9,049 |
| | 2nd—sense | TTGCGAGTCTGGAGACATCGG | 9,099–9,119 |
| | 1st—antisense | ATGATCTGCAGAGAGGCCAG | X-tail |
| | 2nd—antisense | CACGGACCTTTCACAGCTAG | X-tail |

[a]Analyzed according to genome sequence of HCV-M2123(AB558135).

showed HCV recombination of 2b/1b and these patients were regarded as index patients. Their clinical features of two patients with HCV recombinants are shown in Figure 1. Patient #1 was born in 1962 and works as a health care worker. In 1988, she experienced a needle prick accident at the age of 26 and expressed HCV antibody in her sera with elevated levels of serum alanine aminotransferase (ALT). Sera were collected from this patient on three occasions: (i) in 1993, when she first attended hospital; (ii) in 2005, prior to interferon therapy; and (iii) in 2011, to assess the present status of her illness (Fig. 1). Patient #2 is a 72-year-old male with a history of blood transfusion during an operation in 1963. A regular health check in 1996 showed that he was expressing HCV antibody in his sera. Sera were collected from this patient for characterization of HCV on five occasions: (i) in 1997, when first attending the hospital before interferon therapy; (ii) in 1998, after interferon therapy; (iii) in 2003, when HCV RNA was high and ALT remained elevated after antiviral therapy; (iv) in 2010, before starting a second regime of antiviral therapy; and (v) in 2011, when he developed HCC.

### Analyses of HCV Sequences of Patient #1 and Patient #2

Serotyping of patient sera using NS4-based immunoassays indicated that both index patients were infected with HCV serogroup 1. Three HCV isolates, that is, HCV-B5808, HCV-M2123, and HCV-M4430, were retrieved from sera collected from index Patient #1 over the last 18 years (1993–2011; Fig. 1). Five HCV isolates, that is, HCV-M4416, HCV-M4414, HCV-M4431, HCV-M2777, and HCV-M8774, were isolated from index Patient #2 over the last 14 years (1997–2011; Fig. 1). To gain better insight, the near-complete genomic structure of all eight HCV isolates (three from Patient #1 and five from Patient #2) was determined by amplifying nine overlapping regions of the HCV genome (Table I). Using these primers, near-complete nucleotide sequences of HCV except the 3′-tail were obtained. The nucleotide (nt) sequence of HCV-B5808 (HCV isolate of Patient #1) obtained length of 9,471 nt, comprising of 5′UTR (nt 1–341), open reading frame (nt 342–9,383 for 3014aa), 3′UTR (nt 9,384–9,423). The rest seven HCV isolates retrieved from patient #1 and patient #2 had nt sequences of 9,321–9,471 nt with a polyprotein of 3,014 amino acids. Figure 2 shows that all eight HCV isolates (DDBL/EMBL/GeneBank accession numbers AB5558135 and AB677527–AB677533) from these two patients were related closely to 2b HCV isolates of 5′UTR-NS2 region sequence of known HCV 2b isolates. However, all HCV isolates from the two index patients were similar to reported 1b HCV isolates based on the NS3-3′UTR region (Fig. 2). This suggested that there might be inter-genotypic recombination of 2b and 1b in these two patients. Both patients received antiviral therapy and one developed HCC. The HCV recombinant 2b/1b persisted in all HCV isolates over a period of 14–18 years.

### 2b/1b Recombinant HCV Found in Eight Isolates Represents a Novel HCV Recombinant

The recombination cross-over point in these HCV isolates was assessed using data from the full genome sequences. The HCV sequences of the NS2 and NS3 regions are in Figure 2. Analysis of the HCV sequences in these regions indicated that the cross-over point was located in NS2 (marked with an arrowhead in Fig. 2). The recombination cross-over point was similar in all eight isolates in the two index patients with chronic hepatitis C. In addition to preliminary report in 2010 of HCV recombinant from Japan [Hoshino et al., 2010], HCV recombinants have been reported from two Asian countries, that is, the Philippines in 2006 [Kageyama et al., 2006] and Japan in 2011 [Yokoyama et al., 2011]. The cross-over points of other recombinant HCV isolates from the Philippines and Japan were located in the NS3 region. Thus, the recombinant HCV isolates found in the current study appear to be novel HCV recombinants in Japan.

### Phylogenetic Tree of HCV Recombination in Eight Isolates From Two Index Patients

Figure 3 shows the phylogenetic tree constructed for the full genome sequences of eight HCV isolates from two index patients. Full genome sequencing showed that the 2b/1b HCV recombinant persisted in both patients and all isolates collected at different time points over the last 18 and 14 years from Patient #1 and Patient #2, respectively.

The rate of genetic change in HCV during the course of the chronic infections was comparatively low in these patients. HCV isolate M4430 was isolated from the sera of Patient #1 17 years 6 months after collecting sera containing HCV isolate B5508. HCV isolate M8774 was collected 14 years after isolating M4416 from Patient #2. However, only $0.94 \times 10^{-3}$ base substitutions per site per year were detected in Patient #1. In the case of Patient #2, the frequency of base substitutions was $0.41 \times 10^{-3}$ per site per year. This is comparatively lower than the prevalence of HCV genetic drift during natural chronic infections [Okamoto et al., 1992; Ogata et al., 1991].

## DISCUSSION

Japan has about 2 million people with HCV infections and most belong to HCV genotype 1. Although several million people throughout Asia are infected with HCV, HCV recombinants have only been identified rarely from Asian countries. A 2b/1b recombinant HCV strain was identified in the Philippines [Kageyama et al., 2006] and Hoshino et al. [2010] subsequently reported the first case of a HCV recombinant from Japan based on the analysis of a partial HCV genomic sequence. Yokoyama et al. [2011]

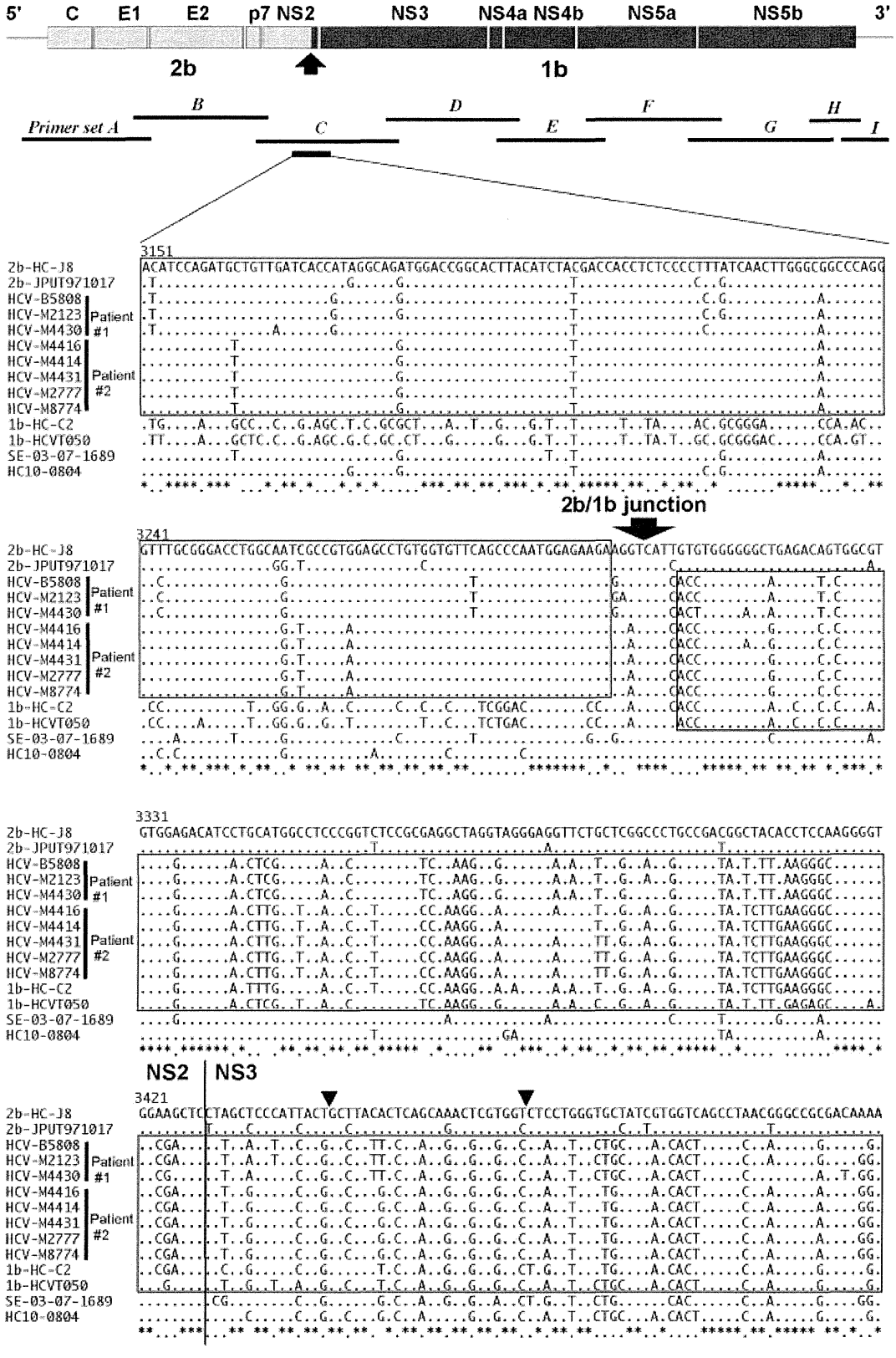


Fig. 2. Alignment of the nucleotide sequences of eight HCV isolates from two index patients with recombinant HCV 2b/1b (three from Patient #1 and five from Patient #2). Four reference sequences (2b-HC-J8, 2b-JPUT971017, 1b-HC-C2 and 1b-HCVT050 and HCV2b/1b) are also shown in this figure. Nucleotide sequences of recombinant HCV strains from the Philippines (SE-03-07-1689) and Japan (HC-10-0804) are shown along with eight clinical isolates from the two patients. The black arrow indicates the possible cross-over point from 2b to 1b in the NS2 region of the eight HCV isolates from the two index patients. The cross-over points in the NS3 region of the HCV recombinants from the Philippines and Japan are shown as black triangles.

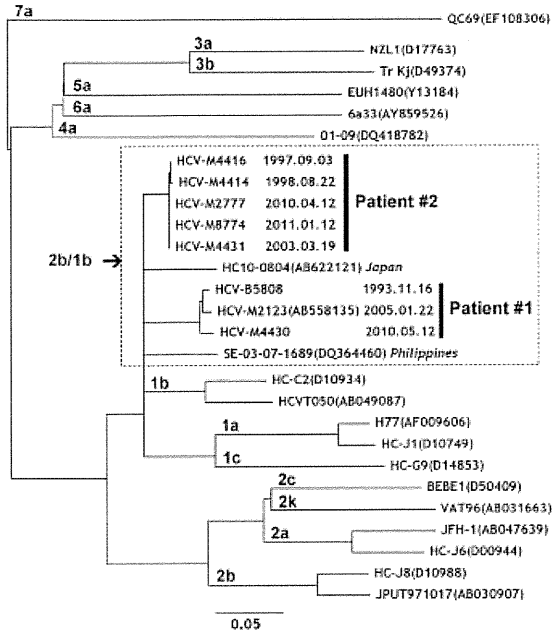


Fig. 3. Rooted neighbor-joining tree showing the phylogenetic relationship among the HCV isolates and prototype strains. All eight isolates from the two index patients (Patient #1 and Patient #2) with chronic hepatitis C are shown in bold. Representative HCV strains of genotype 7a, 3a, 3b, 5a, 6a, 4a, 1b, 1a, 1c, 2c, 2k, 2a, and 2b from different geographical regions are also included. The HCV 2b/1b recombinants reported from the Philippines [Kageyama et al., 2006] and Japan [Yokoyama et al., 2011] are shown for comparison.

reported a child from Japan with a 2b/1b recombinant. It appears that the rate of HCV recombination may be low in Japan, but further study is required.

The current study identified two chronic hepatitis C patients who were infected with recombinant HCV with the 2b/1b genotype. It is noteworthy that the HCV recombinants described in this study are completely different from those reported from the Philippines [Kageyama et al., 2006] and Japan [Yokoyama et al., 2011]. The estimated cross-over points of the recombinant HCV strains from the Philippines and Japan differed from those of the two index patients in the current study (Fig. 2). The recombination cross-over point in previous studies was located in the NS3 region of the HCV genome, whereas it was located in the NS2 region of both patients. Recombinant HCV strains with inter-genotypic junctions in between core and NS4 region have been detected in this study because all reported HCV recombinants have inter-genotypic junctions around NS2-NS3 region of HCV genome and this study was intended to identify inter-genotypic recombinant HCV strains noticed by discrepancies between immunoassays and sequencing. Further studies accomplished with primers capable of detecting inter-genotypic recombinants in other regions of HCV genome may provide further insights about HCV recombinants. Data from the current study and published data suggest that HCV recombinants are a comparatively rare event in HCV evolution, but the nature and properties of HCV recombination merit further studies to develop better insights into HCV virology.

Several points of this study are worthy of mention. This study assessed HCV recombination at different points during HCV infection, that is, during its natural course, before and after antiviral therapy, and after the development of HCC. The analysis of eight HCV isolates retrieved from these two patients at different time points over 14–18 years showed that the 2b/1b HCV recombinants persisted in patients throughout chronic HCV infection, before and after antiviral therapy, and after the development of HCC. The clinical impact of this observation needs to be clarified in future. In addition, the genetic drift of the HCV genome over 14–18 years of chronic HCV infection with recombinant HCV appeared to be low compared to the rate of nucleotide change in normal chronic HCV infections [Ogata et al., 1991; Okamoto et al., 1992].

Another point of profound clinical importance is the discrepancy between HCV immunoassays and HCV genotyping. Also, the surrogacy of HCV genotyping based on the estimation of a single subgenomic HCV region genome appears questionable [Dixit et al., 1995]. The HCV genotype is related to the progression of liver diseases and the response to antiviral therapy in patients with chronic hepatitis C [Shirakawa et al., 2008; Ghany et al., 2009]. This and other studies of HCV recombinants indicate that it would be premature to predict treatment outcome based on current clinical practices that assess either the serogroup or the genotype of only the NS5B or core region. It is tempting to propose that HCV genotype assessments should be conducted for all patients with chronic hepatitis C with genotype 2b who are non-responders to antiviral therapy. In addition to clinical factors, HCV recombination may be another factor determining non-responsiveness to antiviral therapy in patients with chronic hepatitis C.

Both patients with HCV recombinants had a history of possible HCV infection, that is, one patient had a history of needle prick while the second had a blood transfusion. Both patients were natives of Japan and their clinical history provided no clues regarding their infection from outside Japan. The cross over point of HCV recombinant reported by Yokoyama et al. [2011] from Japan also differed from that of HCV isolates shown in this study. Thus, the HCV recombinants described in this study appear to be novel recombinants with novel cross-over points in the NS2 region of the HCV genome.

In conclusion, it appears that different forms of HCV recombination are circulating in Japan. HCV recombination may not be as rare as believed previously. Attention should be given to assessing HCV genotyping using multiple primers. HCV may try to evade host immunity via genotypic recombination, so the real implications of these observations remain to be clarified in the context of vaccine development

and the management of antiviral therapy in non-responders in the future.

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Hepatology Research

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***Original Article***

# Three cases of acute or fulminant hepatitis E caused by ingestion of pork meat and entrails in Hokkaido, Japan: Zoonotic food-borne transmission of hepatitis E virus and public health concerns

Kencho Miyashita,[1,3] Jong-Hon Kang,[2] Akiyoshi Saga,[3] Kazuaki Takahashi,[5] Tsuyoshi Shimamura,[4] Atsushi Yasumoto,[1] Hiraku Fukushima,[1] Susumu Sogabe,[1] Kouhei Konishi,[1] Takumi Uchida,[1] Akira Fujinaga,[1] Takeshi Matsui,[2] Yasuo Sakurai,[2] Kunihiko Tsuji,[2] Hiroyuki Maguchi,[2] Masahiko Taniguchi,[4] Natsumi Abe,[5] Sheikh Mohammad Fazle Akbar,[5] Masahiro Arai[5] and Shunji Mishiro[5]

[1]*Department of Gastroenterology, Abashiri Kosei General Hospital, Abashiri,* [2]*Center for Gastroenterology, Teine Keijinkai Hospital,* [3]*Department of Gastroenterology, Aiiku Hospital,* [4]*Department of General Surgery, Graduate School of Medicine, Hokkaido University, Sapporo, and* [5]*Department of Medical Sciences, Toshiba General Hospital, Tokyo, Japan*

*Aim:* In developed countries including Japan, the transmission route of indigenous hepatitis E virus (HEV) infection is obscure. Accordingly, public health implications of indigenous HEV infection have not been well addressed. The aim of this study was to clarify the route of transmission of a small outbreak of acute hepatitis E and assess the public health implications of indigenous zoonotic HEV transmission.

*Methods:* Three patients with non-A, B and C acute hepatitis, two of whom presented in a critical condition, were assessed for HEV infection using polymerase chain reaction and their route of infection; the genome sequences of the infecting HEV were also analyzed. A phylogenetic tree based on the full, or near full, HEV RNA sequences were constructed by neighbor-joining method.

*Results:* All three patients ingested grilled pork meat and entrails at the same barbecue restaurant in Abashiri, Hokkaido, Japan. When comparing partial to entire, or nearly entire, nucleotide sequences of HEV detected in these patients, they were 99.9–100% identical to each other. These genotype 4 isolates had great resemblance to the genome sequences of the isolates from the mini-outbreak in 2004 in Kitami, a city adjacent to Abashiri. These Kitami/Abashiri strains were segregated into a single cluster on the phylogenetic tree of HEV genotype 4 indigenous to Japan.

*Conclusion:* Indigenous HEV transmission via a zoonotic food-borne route has been demonstrated in Kitami and Abashiri via pork meat and entrails contaminated with virulent HEV strains. Because a similar outbreak can recur in the future, infection sources and distribution routes should be clarified rapidly for public health.

**Key words:** fulminant hepatitis, genotype 4, hepatitis E, Kitami/Abashiri strains, zoonosis

## INTRODUCTION

EVIDENCE NOW SHOWS that the hepatitis E virus (HEV) infection is no longer confined to developing countries. HEV transmission routes specific to industrialized societies have been eagerly investigated.[1–5] These studies have shown a zoonotic transmission of HEV from ingestion of the meat of deer, wild boars and pigs in industrialized countries.[3,6–9] It is unclear, however, whether these instances of zoonotic HEV transmission have occurred randomly. Mini-outbreaks of HEV infection may inevitably repeat themselves even in developed countries, especially if the society concerned has a history of zoonotic transmission specific to the regional livestock cultivation and distribution industries.

*Correspondence: Dr Jong-Hon Kang, Center for Gastroenterology, Teine Keijinkai Hospital Maeda, 1-12 Teine-ku, Sapporo 006-8555, Japan. Email: kanjh@keijinkai.or.jp*
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A mini-outbreak of acute HEV infection in Abashiri, a city in the northern Japanese prefecture of Hokkaido, has occurred. In the course of the investigation for the transmission route of indigenous HEV, attention has been given to a similar mini-outbreak of hepatitis E occurring 2 years previously at Kitami, another city situated 50 km from Abashiri.[8,9] This finding has provided an opportunity to address the public health implications of sporadic community-acquired cases of HEV infection in Japan by comparing viral sequences of Abashiri's acute HEV patients with those of Kitami's patients.

In the present study, the route of transmission in a mini-outbreak of acute hepatitis E is clarified, and the public health implications of indigenous zoonotic HEV transmission in Japan is also investigated.

## METHODS

### Patients

THREE PATIENTS EXHIBITING the features of non-A, B and C acute hepatitis presented in Hokkaido, Japan. Although different physicians treated the patients at different institutions, all of them ate pork meat and entrails together at a barbecue party in a restaurant at Abashiri on 1 February 2006. The ingested pork meat and entrails were grilled by gas cookers during concurrent alcohol consumption. It was unclear whether they were thoroughly cooked or not.

The clinical profiles of the patients are shown in Table 1. Serum anti-hepatitis B core antigen immunoglobulin (Ig)M, hepatitis C virus RNA, and anti-hepatitis A IgM were not detected in any patient although patient

**Table 1** Characteristics and clinical features in patients with acute HEV infection

| Patient characteristics, laboratory data and outcome | Patient number | | |
|---|---|---|---|
| | 1 | 2 | 3 |
| Age (years)/sex | 53/male | 58/male | 56/male |
| Symptoms | Jaundice, malaise | Appetite loss | Fever |
| Estimated time of infection | 1 February | 1 February | 1 February |
| Time of onset | Fourth week of March | First week of March | First week of March |
| Underlying liver disease† | None | None | Inactive HBV carrier |
| Alcohol intake | 30 g/day, 33 years | 30 g/day, 20 years | 30 g/day, 30 years |
| Peak AST (IU/L) | 297 | 9045 | 3266 |
| Peak ALT (IU/L) | 929 | 5297 | 4468 |
| Peak total bilirubin (mg/dL) | 12.6 | 2.6 | 10.3 |
| Lowest prothrombin time (%), INR | 74.0, 1.25 | 38.0, 2.04 | 16.6, 4.82 |
| HEV RNA/genotype | +/4 | +/4 | +/4 |
| Anti-HEV IgG/OD‡ value | +/2.170 | +/2.878 | +/1.761 |
| Anti-HEV IgM/OD value | +/3.118 | +/2.878 | +/1.761 |
| Anti-HAV IgM | <0.8 | <0.8 | <0.8 |
| HBsAg (IU/mL) | 0.01 | 0.05 | 10.74 |
| Anti-HBc IgM | 1.7 | <0.09 | <0.09 |
| HBV DNA (log copies/mL) | <2.6 | n.t.§ | <2.6 |
| Anti-HCV (U) | <1.0 | <1.0 | <1.0 |
| HCV RNA (IU/mL) | <50 | <50 | <50 |
| Disease progression | Self-limited hepatitis | Acute severe hepatitis¶ | Fulminant hepatitis†† |
| Outcome | Survived | Survived | Survived |

Normal range: AST, aspartate aminotransferase (10–40 U/L); ALT, alanine aminotransferase (5–45 U/L); total bilirubin (0.2–1.0 mg/dL); prothrombin time (80–100%, 0.84–1.14); anti-HEV IgG, hepatitis E virus immunoglobulin G (<0.191); anti-HEV IgM (<0.447); anti-HAV, hepatitis A virus IgM (<0.8); HBsAg, hepatitis B surface antigen (<0.06); anti-HBc, hepatitis B core IgM (<0.9); HBV, hepatitis B virus DNA (<2.6 log copies/mL); anti-HCV, hepatitis C virus (<1.0); HCV RNA (<50 IU/mL).
†Underlying liver disease defined as disease which had been diagnosed before onset of hepatitis E.
‡"OD", optical density.
§"n.t.", not tested.
¶"acute severe hepatitis" defined as hepatitis with ≤40% in lowest prothrombin time without hepatic coma.
††"fulminant hepatitis" defined as hepatitis with hepatic coma within 8 weeks after onset.

3 had hepatitis B surface antigen. The clinical courses of these patients differed considerably with patient 1's showing a self-resolving course, patient 2's presenting with serious coagulopathy due to severe hepatitis and patient 3's developing fulminant hepatitis with recovery within 3 months after hospital admission.

Among the nearly 40 customers having eaten the grilled pork meat and entrails at the same restaurant in Abashiri on the same day, 11 of them (nine men and two women) voluntarily agreed to be checked for HEV. The biochemistry, anti-HEV IgM and IgG, and HEV RNA were checked using blood sample collections at 25–29 weeks after the dinner on 1 February 2006.

Informed consent was obtained from all patients and volunteers after explaining the nature and purpose of the study; approval for this study was obtained from the hospital's institutional review board. The study protocol conformed to guidelines provided in the Declaration of Helsinki for clinical trials.

### Detection of HEV-related antibodies

Serum anti-HEV IgG and IgM were determined by using a commercial enzyme-linked immunosorbent assay (ELISA) kit (Viragent HEV-Ab; Cosmic, Tokyo, Japan).

### Detection of the full-length sequences of HEV isolates

Detection and nucleotide sequencing of the serum HEV RNA were performed by methods described previously.[10,11] Briefly, the nucleic acids were extracted from the serum with commercial kits (Smitest EX-R&D; Genome Science, Fukushima, Japan). The nucleotide sequences of HEV were reverse transcribed to cDNA and amplified by polymerase chain reaction (PCR) in 17 overlapping regions with 20-mer primers deduced from the nucleotide sequences of HEV deposited in the international DNA Data Bank of Japan (DDBJ)/GenBank/European Molecular Biology Laboratory (EMBL) database. Reverse transcription was performed, and the first and second round of PCR was carried out in the presence of Platinum *Taq* DNA Polymerase High Fidelity (Invitrogen, Carlsbad, CA, USA). The final products were sequenced in a 377 DNA sequencer. The sequences rich in G-C were amplified, and those not amplifiable by the above PCR methods were subjected to PCR with primers deduced from adjacent 5′- and 3′-sequences. The 5′- and 3′-terminal sequences were amplified with 5′-Full RACE Core Set (TaKaRa Bio, Shiga, Japan) and Oligo (dt) 20 primer (Invitrogen), respectively.

### Phylogenetic analyses of HEV isolates

A phylogenetic tree based on the full, or nearly full, HEV RNA sequence was constructed by the neighbor-joining method. Analyses were performed with the use of computer software (GENETYX-MAC ver. 13.0; Genetyx, Tokyo, Japan).

## RESULTS

### Diagnosis of acute HEV infection in 4 patients

SERUM HEV RNA was detected in all three patients in the early phases of hepatitis. Given the clinical presentations, absence of markers of acute hepatitis A, B and C, and the presence of HEV RNA and anti-HEV, these patients were diagnosed with acute hepatitis E (Table 1) infection. In addition, among the 11 volunteers who attended the same barbecue party, a 51-year-old man who was a colleague of patient 3 showed an elevation of both serum anti-HEV IgG and IgM; he, however, had normal alanine aminotransferase levels and no HEV RNA by PCR for which he was, therefore, diagnosed with an asymptomatic HEV infection. None of the four patients above dined together again after 1 February 2006.

### Similarity of nucleotide sequences of the HEV in three patients with acute hepatitis E in Abashiri

Full-length sequences in 7255 nucleotides were determined for genotype 4 HEV from patient 2 (JMM-Aba06C) and patient 3 (JKO-Aba-FH06C). Also, partial nucleotide sequences containing 432 nucleotides in the replicase region and 1136 nucleotides in the ORF2 region of HEV were analyzed for patient 1 (JKU-Aba06). Comparison of the full-length sequence between JMM-Aba06C and JKO-Aba-FH06C revealed only six nucleotide differences out of 7255 nucleotides with a sequence homology of 99.92% (Table 2). In addition, when these three isolates were compared among each other for 1568 nucleotide sequences, JKU-Aba06 (patient 1) showed 100% identicalness with JKO-Aba-FH06C (patient 3) and 99.94% with JMM-Aba06C (patient 2). The GenBank/EMBL/DDBJ accession numbers for the nucleotide sequences of HEV isolates were AB291967–70.

**Table 2** Nucleotide sequence analyses for the hepatitis E virus strains from the mini-outbreaks in Kitami and Abashiri

| Isolate names/regions | Overlap match (%) where mini-outbreak occurred | | | | |
|---|---|---|---|---|---|
| | **JKU-Aba06** | **JMM-ba06C** | **JKO-Aba-FH06** | HRC-HE14C | JTC-Kit-FH04L |
| **JKU-Aba06/Abashiri** | | | | | |
| **JMM-Aba06C/Abashiri** | 99.94 | | | | |
| **JKO-Aba-FH06C/Abashiri** | 100 | 99.92 | | | |
| HRC-HE14C/Kitami | 99.11 | 99.38 | 99.43 | | |
| JTC-Kit-FH04L/Kitami | 98.92 | 99.43 | 99.49 | **99.86** | |
| JST-Kit04C/Kitami | 99.11 | 99.38 | 99.43 | **99.99** | **99.99** |

Full, or nearly full, length nucleotide sequences were determined and compared to each other except for JKU-Aba06.
Among JKU-Aba06 and 5 other strains, 1568 nucleotide sequences containing 432 nucleotides in the replicase region and 1136 nucleotides in ORF2 were compared.
Bold letters represents the strains separated in Kitami and Abashiri regions in Hokkaido Prefecture, Japan.

## Close relationship of HEV genome between mini-outbreaks in Abashiri in 2006 and Kitami in 2004

To assess any relationship between HEV isolates of the present mini-outbreak in Abashiri in 2006 with those in Kitami in 2004, partial or entire genome sequences of Abashiri isolates such as JKU-Aba06, JMM-Aba06C and JKO-Aba-FH06C were compared with Kitami isolates HRC-HE14C, JTC-Kit-FH04 and JST-Kit04C.[8,9] JMM-Aba06C and JKO-Aba-FH06C showed 99.38–99.49% identicalness with HRC-HE14C, JTC-Kit-FH04L and JST-Kit04C (Table 2). These Abashiri isolates, JMM-Aba06C and JKO-Aba-FH06C, revealed merely 37 or 41 nucleotide differences from JTC-Kit-FH04L and 41 or 45 nucleotide differences with JST-Kit04C. The chronological intervals of blood sampling were 513–538 days in these two mini-outbreaks.

## Persistence of similar HEV genome at Kitami and Abashiri

In addition to the mini-outbreak of acute hepatitis E at Kitami in 2004, sporadic cases of the same disease were also reported around Kitami and Abashiri. A comparison of the entire, or nearly entire, nucleotide sequences of the HEV genomes of the seven isolates separated in Kitami and Abashiri and one isolate identified in Monbetsu (70 km from Kitami and 90 km from Abashiri) was made (Table 3). Two HEV strains, HE-JA36 and HE-JA41, were isolated from the patients in Kitami, and HE-JA28 was separated from the patient in Monbetsu (H. Okamoto, Jichi Medical School, Tochigi, Japan, pers. comm.). The data demonstrated 99.3–99.9% homology among these eight isolates. The constructed phylogenetic tree based on the full-length genome analysis confirmed that strains obtained in Kitami and Abashiri belonged to genotype 4 indigenous to Japan. A single cluster segregated from adjacent strains separated in other parts of Hokkaido, Japan (Fig. 1b) was gathered. The Kitami/Abashiri strains showed only 84–88.3% sequence homology with HEV genotype 4 isolates detected from other regions in Japan (Fig. 1b, Table 3). The genome sequence of case 1 was not included in this phylogenetic tree because only partial sequences of the HEV genome were done.

## Absence of nucleotide substitution at nucleotide 1816 and 3148 in Kitami/Abashiri strains responsible for fulminant hepatitis

Because nucleotide substitution at nucleotide 1816 and 3148 in genotype 4 HEV was reported to be significantly associated with fulminant hepatitis,[12] nucleotide sequences were compared in JKO-Aba-FH06C and JTC-Kit-FH04L. By analyzing full-length genome sequences, both T at nt 1816 and C at nt 3148 were observed in JKO-Aba-FH06C and JTC-Kit-FH04L.

## DISCUSSION

TO DATE, SPORADIC indigenous hepatitis E infections have been reported throughout the industrialized world.[20–24] The transmission routes of HEV in these countries remain obscure although a zoonotic foodborne route has been shown in several instances ascertained molecularly.[3,7,9] Autochthonous HEV infection, therefore, seems to be a growing public health concern even in developed countries.

Hepatitis E virus isolates identified from the three symptomatic acute hepatitis E patients who had eaten

**Table 3** Comparison of entire-length nucleotide sequences in Kitami/Abashiri strains with those in other HEV isolates

| Genotype | Isolate name | Accession no. | Host | Diagnosis | Collection date (year/month/day) | Habitat (city, prefecture, country) | Nucleotide length | Nucleotide JKO-Aba-FH06C | Identity (%) JMM-Aba06C | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| **4** | **JKO-Aba-FH06C** | **Current study** | **Human** | **FH** | **2006/3/10** | **Abashiri, Hokkaido, Japan** | **7255** | | **99.9** | **Current study** |
| **4** | **JMM-Aba06C** | **Current study** | **Human** | **AH** | **2006/3/9** | **Abashiri, Hokkaido, Japan** | **7255** | **99.9** | | **Current study** |
| **4** | **HE-JA36** | **AB220977** | **Human** | **AH** | **2004/1/6** | **Kitami, Hokkaido, Japan** | **7266** | **99.5** | **99.4** | **Inoue *et al.*[12]** |
| **4** | **HRC-HE14C** | **AB291965** | **Human** | **Blood Donor** | **2004/9/20** | **Kitami, Hokkaido, Japan** | **7255** | **99.4** | **99.4** | **Matsubayashi *et al.*[9]** |
| **4** | **JST-KitAas04C.** | **AB291966** | **Human** | **AH** | **2004/10/12** | **Kitami, Hokkaido, Japan** | **7255** | **99.4** | **99.3** | **Matsubayashi *et al.*[9]** |
| **4** | **JTC-Kit-FH04L** | **AB291959** | **Human** | **FH** | **2004/9/24** | **Kitami, Hokkaido, Japan** | **7209** | **99.4** | **99.3** | **Matsubayashi *et al.*[9]** |
| **4** | **HE-JA28** | **AB220976** | **Human** | **AH** | **2002/12/13** | **Monbetsu, Hokkaido, Japan** | **7266** | **99.4** | **99.4** | **Inoue *et al.*[12]** |
| **4** | **HE-JA41** | **AB220979** | **Human** | **AH** | **2004/8/17** | **Kitami, Hokkaido, Japan** | **7265** | **99.4** | **99.3** | **Inoue *et al.*[12]** |
| 4 | JSM-Sap95 | AB161717 | Human | AH | 1995/3/28 | Sapporo, Hokkaido, Japan | 7202 | 98.3 | 98.2 | Takahashi *et al.*[13] |
| 4 | HE-JF4 | AB220972 | Human | FH | 2002/10/2 | Sapporo, Hokkaido, Japan | 7271 | 97.6 | 97.5 | Inoue *et al.*[12] |
| 4 | JKK-Sap | AB074917 | Human | AHS | 2000/11/10 | Sapporo, Hokkaido, Japan | 7235 | 97.6 | 97.5 | Takahashi *et al.*[11] |
| 4 | JTS-Sap02 | AB161718 | Human | AH | 2002/9/14 | Sapporo, Hokkaido, Japan | 7202 | 97.5 | 97.5 | Takahashi *et al.*[11] |
| 4 | JYW-Sap02 | AB161719 | Human | AH | 2002/8/30 | Sapporo, Hokkaido, Japan | 7202 | 97.5 | 97.5 | Takahashi *et al.*[11] |
| 4 | HE-JF5 | AB220973 | Human | FH | 2002/12/2 | Sapporo, Hokkaido, Japan | 7270 | 97.5 | 97.5 | Inoue *et al.*[12] |
| 4 | HE-JA19 | AB220975 | Human | AH | 2002/12/24 | Sapporo, Hokkaido, Japan | 7262 | 97.3 | 97.3 | Inoue *et al.*[12] |
| 4 | HE-JA37 | AB220978 | Human | AH | 2004/1/30 | Sapporo, Hokkaido, Japan | 7281 | 97.1 | 97 | Inoue *et al.*[12] |
| 4 | HE-JA1 | AB097812 | Human | AH | 1997/12/6 | Sapporo, Hokkaido, Japan | 7258 | 88.4 | 88.4 | Nishizawa *et al.*[14] |
| 4 | HE-JF3 | AB220971 | Human | FH | 1998/6/19 | Mito, Ibaraki, Japan | 7262 | 88.3 | 88.2 | Inoue *et al.*[12] |
| 4 | swJ13-1 | AB097811 | Swine | | | Hokkaido, Japan | 7258 | 88.3 | 88.3 | Nishizawa *et al.*[14] |
| 4 | JSN-Sap-FH02C | AB20239 | Human | FH | 2002/3/21 | Sapporo, Hokkaido, Japan | 7251 | 88.1 | 88.1 | Takahashi *et al.*[15] |
| 4 | JYN-Sap01C | AB193177 | Human | AH | 2001/12/28 | Sapporo, Hokkaido, Japan | 7256 | 88.1 | 88.1 | Takahashi *et al.*[15] |
| 4 | HE-JK4 | AB099347 | Human | AH | 2002/4/25 | Tochigi, Japan | 7250 | 88.1 | 88 | Kuno *et al.*[16] |
| 4 | JYN-Nii02L | AB193178 | Human | AH | 2002/4/30 | Niigata, Japan | 7154 | 88.1 | 88 | Takahashi *et al.*[11] |
| 4 | JAK-Sai | AB074915 | Human | AH | | Saitama, Japan | 7236 | 88.1 | 88 | Takahashi *et al.*[13] |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 4 | JSF-Tot03C | AB193176 | Human | FH | 2003/3/12 | Tottori, Japan | 7251 | 88 | 88 | Takahashi *et al.*[15] |
| 4 | swJB-H7 | AB481227 | Swine | | | Japan | 7253 | 87.8 | 87.8 | |
| 4 | HE-JI4 | AB080575 | Human | AH | 1905/6/22 | Tochigi, Japan | 7186 | 87.4 | 87.3 | Takahashi *et al.*[17] |
| 4 | HE-JA2 | AB220974 | Human | AH | 1998/9/4 | Sapporo, Hokkaido, Japan | 7268 | 86.7 | 86.6 | Inoue *et al.*[12] |
| 4 | CCC220 | AB108537 | Human | AH | 2000/6 | Changchun, Jilin, China | 7193 | 85.4 | 85.4 | Liu *et al.*[18] |
| 4 | E087-SAP04C | AB369688 | Human | AH | | Sapporo, Hokkaido, Japan | 7227 | 85.2 | 85.2 | |
| 4 | IND-SW-00–01 | AY723745 | Swine | | | India | 7262 | 84.6 | 84.6 | |
| 4 | E067-SIJ05C | AB369690 | Human | AH | | Shinjuku, Tokyo, Japan | 7236 | 84.5 | 84.4 | |
| 4 | swCH31 | DQ450072 | Swine | | | China | 7248 | 84.3 | 84.3 | |
| 4 | SH-SW-zs1 | EF570133 | Swine | | | China | 7293 | 84.3 | 84.2 | |
| 4 | swCH25 | AY594199 | Swine | | | Xinjian, China | 7270 | 84.2 | 84.2 | |
| 4 | T1 | AJ272108 | Human | | | Beijing, China | 7232 | 84.2 | 84.2 | |
| 4 | KNIH-hHEV4 | FJ763142 | Human | AH | | South Korea | 7260 | 84.1 | 84.1 | |
| 4 | swCH189 | FJ610232 | Swine | | | Gansu, China | 7284 | 84.1 | 84.1 | |
| 4 | swGX40 | EU676178 | Swine | | | China | 7267 | 84 | 84 | |
| 4 | JYI-ChiSai01C | AB197674 | Human | AH | 2001/4/12 | Shanghai, China | 7260 | 84 | 84.4 | Koike *et al.*[19] |
| 4 | JKO-ChiSai98C | AB197673 | Human | AH | 1998/10/27 | Xian, Shaanxi, China | 7257 | 84 | 83.9 | Koike *et al.*[19] |
| 4 | HEVN2 | AB253420 | Human | | | Okinawa, Japan | 7253 | 84 | 84 | |
| 4 | Ch-S-1 | EF077630 | Human | | | China | 7261 | 83.9 | 83.8 | |
| 4 | swGX32 | EU366959 | Swine | | | China | 7281 | 83.8 | 83.8 | |
| 4 | DQ1 | DQ279091 | Swine | | | China | 7234 | 83.8 | 83.7 | |
| 1 | B1 | M73218 | Human | | | Burma | 7207 | 75.4 | 75.3 | |
| 3 | US1 | AF060668 | Human | | | USA | 7202 | 75.3 | 75.3 | Schlauder *et al.*[20] |
| 2 | M1 | M74506 | Human | | | Mexico | 7180 | 73.9 | 73.9 | |

Bold letters represents the strains separated in Kitami and Abashiri regions in Hokkaido Prefecture, Japan.
AH, acute hepatitis; ASH, acute severe hepatitis; FH, fulminant hepatitis.

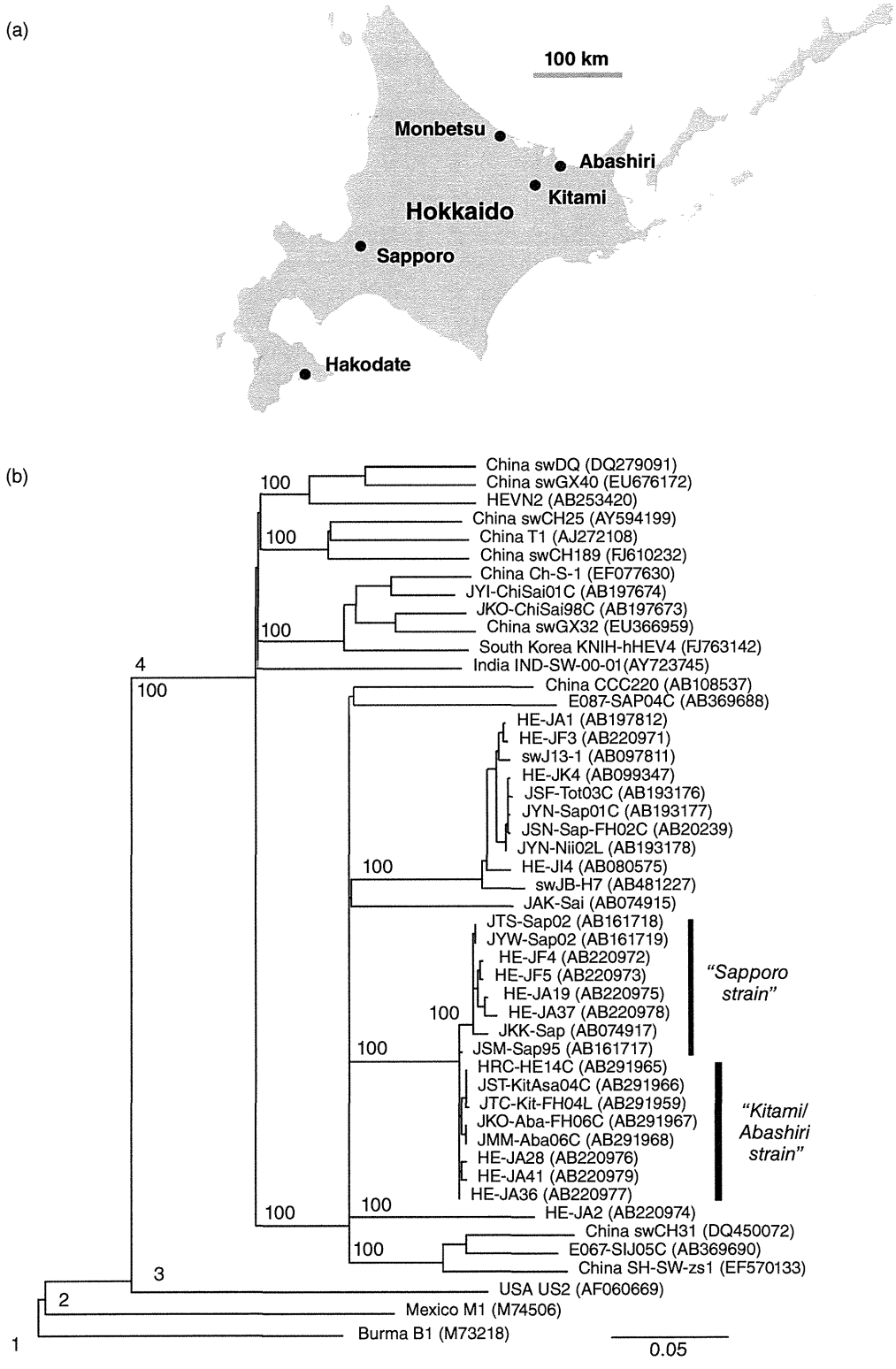

**Figure 1** (a) Three symptomatic and one asymptomatic patient with acute infection of hepatitis E virus (HEV) attended the same barbecue restaurant in Abashiri, Hokkaido, Japan. Among them, two were admitted in Sapporo and one in Abashiri. (b) Phylogenetic tree based on the full, or nearly full, length nucleotide sequence of HEV genotype 4 by neighbor-joining method. Two of the three isolates, JKO-Aba-FH06 and JMM-Aba06C, analyzed in this study were segregated into a unique cluster ("Kitami/Abashiri strains") distinct from others in genotype 4 indigenous to Japan.

grilled pork meat and entrails at the same party had extremely high sequence homology among the HEV isolates in genomic analyses (Table 2), suggesting a zoonotic transmission of indigenous Japanese HEV strains from pigs to humans in Japan.

In addition, this study demonstrates the persistent presence of virulent strains of HEV in the Kitami/Abashiri region of Hokkaido, Japan. The HEV strain in the Kitami cases of 2004 shows high sequence homology with the Abashiri cases of 2006, as described above. Among the strains, JKO-Aba-FH06C, isolated from patient 3 in Abashiri, and JTC-Kit-FH04L, isolated from Kitami in 2004, demonstrate the strongest resemblance in full-length sequence of nucleotides (Table 2).

The isolates identified from the Kitami and Abashiri patients have also been segregated into a single cluster on the phylogenetic tree of HEV genotype 4 (Fig. 1). They show subtle, but distinct, differences in nucleotide identity from other strains in Hokkaido Prefecture, such as JSM-Sap95 and JKK-Sap[11] isolated at Sapporo (Fig. 1, Table 3). Given that the causative strains in the above Abashiri cases have a close resemblance to those in Kitami in 2004 and that the two areas are contiguous to each other, these HEV instances may share the same channels of distribution and production of pork meat.

The public health impact of the above findings demonstrates significance because the HEV strains of Kitami/Abashiri belong to genotype 4, which is associated with development of fulminant acute hepatitis E.[24] In fact, one of the three HEV patients during the mini-outbreak at Kitami in 2004 has died of fulminant hepatitis.[8,9] Among the six total hepatitis E patients in Kitami and Abashiri, two patients have developed fulminant hepatitis while one has presented with severe hepatitis. Given these facts, suspicion arises that the Kitami/Abashiri strains may be associated with disease progression. In the meantime, another case of fulminant hepatitis due to infection with HEV genotype 4 has been reported in Hakodate, Hokkaido, approximately 400 km from Abashiri and Kitami;[25] the nucleotide sequence of the HEV isolate has shown extreme homology with the Kitami/Abashiri strains. These facts indicate that the Kitami/Abashiri strains of HEV may be spreading to different parts of Hokkaido Prefecture, and further expansion of this strain to other islands of the Japanese archipelago may be possible.

The association between the genomic distinction and fulminant hepatitis in genotype 4 of HEV remains unclear. Because the T at nucleotide 1816 and C at nucleotide 3148 have remained in the JTC-Kit-FH04L and JKO-Aba-FH06C responsible for fulminant hepatitis, no apparent correlation exists between the nucleotide substitutions C1816 and U3148[12] and disease progression in patients infected with the Kitami/Abashiri strains.

In order to prevent HEV infection caused by ingestion of pork meat and entrails, the regional community may need further instruction on proper food handling and cooking techniques. Individual indigenous sources and routes of infection should be clarified. Moreover, administrative precautionary measures must be taken for protecting the livestock and distribution industries as well as regional consumers' health.

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# Novel Cell Culture-Adapted Genotype 2a Hepatitis C Virus Infectious Clone

**Tomoko Date,[a] Takanobu Kato,[a] Junko Kato,[b] Hitoshi Takahashi,[a]* Kenichi Morikawa,[a,c]* Daisuke Akazawa,[a,d] Asako Murayama,[a] Keiko Tanaka-Kaneko,[e] Tetsutaro Sata,[e]* Yasuhito Tanaka,[f] Masashi Mizokami,[g] and Takaji Wakita[a]**

Department of Virology II, National Institute of Infectious Diseases, Tokyo,[a] Institute of Geriatrics, Tokyo Women's Medical University, Tokyo,[b] Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo,[c] Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa,[d] Department of Pathology, National Institute of Infectious Diseases, Tokyo,[e] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya,[f] and The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Chiba,[g] Japan

**Although the recently developed infectious hepatitis C virus system that uses the JFH-1 clone enables the study of whole HCV viral life cycles, limited particular HCV strains have been available with the system. In this study, we isolated another genotype 2a HCV cDNA, the JFH-2 strain, from a patient with fulminant hepatitis. JFH-2 subgenomic replicons were constructed. HuH-7 cells transfected with *in vitro* transcribed replicon RNAs were cultured with G418, and selected colonies were isolated and expanded. From sequencing analysis of the replicon genome, several mutations were found. Some of the mutations enhanced JFH-2 replication; the 2217AS mutation in the NS5A interferon sensitivity-determining region exhibited the strongest adaptive effect. Interestingly, a full-length chimeric or wild-type JFH-2 genome with the adaptive mutation could replicate in Huh-7.5.1 cells and produce infectious virus after extensive passages of the virus genome-replicating cells. Virus infection efficiency was sufficient for autonomous virus propagation in cultured cells. Additional mutations were identified in the infectious virus genome. Interestingly, full-length viral RNA synthesized from the cDNA clone with these adaptive mutations was infectious for cultured cells. This approach may be applicable for the establishment of new infectious HCV clones.**

Hepatitis C virus (HCV) is a principal agent in posttransfusion and sporadic acute hepatitis (6, 19). HCV belongs to the *Flaviviridae* family and *Hepacivirus* genus. Infection with HCV leads to chronic liver diseases, including cirrhosis and hepatocellular carcinoma (16). HCV is a major public health problem, infecting an estimated 170 million people worldwide (6, 16, 19). Current standard therapy for HCV-related chronic hepatitis is based on the combination of interferon (IFN) and ribavirin although virus eradication rates are limited to around 50% (7, 24, 30). Telaprevir and boceprevir were approved by the U.S. Food and Drug Administration in 2011 in combination with pegylated alpha interferon and ribavirin for the treatment of genotype 1 chronic hepatitis C (34, 35). Both agents inhibit the NS3-NS4A serine protease essential for replication of HCV (25, 36). It is important to develop more anti-HCV drugs with different modes of action to achieve greater efficacy and to avoid the emergence of drug-resistant viruses. To that end, a detailed understanding of the viral replication mechanism is needed to discover novel antiviral targets. An efficient virus culture system is indispensable for detailed analysis of HCV life cycles. In an important development, a subgenomic HCV RNA replicon system has been developed (22) to assess HCV replication in cultured cells. Furthermore, an efficient HCV culture system was established by using a JFH-1 strain virus isolated from a fulminant hepatitis patient (20, 38, 41). By transfection of *in vitro* transcribed full-length JFH-1 HCV RNA into HuH-7 cells, efficient JFH-1 RNA replication and infectious viral particle production were detected. However, this efficient virus production was not reproduced by other HCV strains, even when adaptive mutations were introduced to enhance the replication efficiency in cultured cells (29). Thus, other HCV strains that can replicate in cultured cells and produce infectious virus particles are needed. The J6CF strain is infectious to chimpanzees but does not replicate in cultured cells (26, 27, 40). We constructed chimeric replicon and virus constructs of the J6CF and JFH-1 strains to elucidate the difference in their molecular mechanisms (26, 27). We determined that the NS3 helicase and the NS5B to 3′X regions are important for the efficient replication of the JFH-1 strain and that several amino acid mutations in the C terminus of NS5B are pivotal for replication. However, we could not rescue the replication of other virus strains, such as Con1, with these mutations. This result indicates that different approaches are needed to create replication-competent virus strains in cultured cells.

In the present study, we isolated HCV cDNA, named JFH-2, from a fulminant hepatitis patient. The replication efficiency of the JFH-2 clone in the subgenomic replicon assay was lower than that of JFH-1 although the introduction of adaptive mutations enhanced JFH-2 replication. Interestingly, the full-length chimeric or wild-type JFH-2 genome with adaptive mutations could replicate and produce infectious virus particles. The virus infection efficiency was sufficient for autonomous virus propagation in cultured cells.

## MATERIALS AND METHODS

**Cell culture system.** HuH-7, Huh-7.5.1 (a generous gift from Francis V. Chisari), and Huh7-25 cells were cultured in 5% $CO_2$ at 37°C in Dulbec-

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Address correspondence to Takaji Wakita, wakita@nih.go.jp.
* Present address: Hitoshi Takahashi, Influenza Virus Research Center, National Institute of Infectious Diseases, Musashimurayama, Tokyo, Japan; Kenichi Morikawa, Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, University of Lausanne, Lausanne, Switzerland; Tetsutaro Sata, Toyama Institute of Health, Toyama, Japan.

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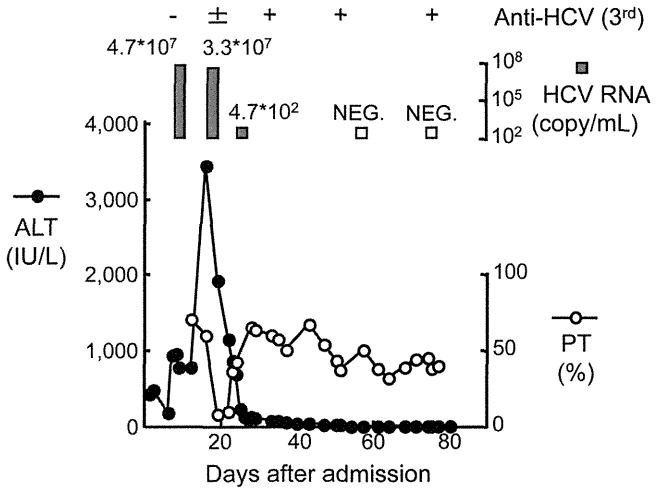


FIG 1 Clinical course of second fulminant hepatitis patient infected with JFH-2. The patient was admitted by reason of acute liver failure. Alanine aminotransferase (ALT) levels, prothrombin time (PT), HCV RNA, and anti-HCV antibodies were determined and followed in his serum.

co's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (DMEM-10) (3, 41).

**HCV clones.** The genotype 2a clone JFH-2 was isolated from a patient with fulminant hepatitis (15). Briefly, HCV cDNA was cloned from a fulminant hepatitis patient, a 62-year-old man who had a history of coronary artery bypass surgery without blood transfusion. One year after the surgery, he developed an acute auditory disorder and received a course of betamethasone therapy. After withdrawal of betamethasone, the patient developed fulminant hepatitis as diagnosed by acute liver failure associated with stage II encephalopathy and low prothrombin time. He experienced prolonged liver failure and died after 80 days. HCV RNA was detected in his serum only during the acute phase (Fig. 1). Total RNA was extracted from serum during the acute phase, and HCV cDNA covering the entire genome was amplified by reverse transcription-PCR (RT-PCR). All amplified products were purified and then cloned into pGEM-T EASY vectors (Promega, Madison, WI). PCR products and plasmids were sequenced by using specific primer sets (Table 1), BigDye Terminator Mix, and an automated DNA sequencer (models 310 and 377; PE Biosystems, Foster City, CA). The JFH-2 subgenomic replicon (SGR) clones, pSGR-JFH2.1 and pSGR-JFH2.2 (DDBJ/EMBL/GenBank accession numbers AB690456 and AB690457, respectively), were constructed according to the method for pSGR-JFH1 construction (11). Several mutations were introduced into the pSGR-JFH2.1 replicon construct, as reported previously (11). The reporter replicon constructs, pSGR-JFH2.1/Luc and pSGR-JFH2.1/Luc, were developed by rearrangement with pSGR-JFH2.1 and pSGR-JFH2.2 (accession numbers AB690458 and AB690459, respectively) as described previously (12). pJ6/JFH1 was previously obtained from pJFH1 by replacement with the 5′ untranslated region (UTR) to the p7 region (EcoRI-BclI) of the J6CF strain (a kind gift from Jens Bukh) (3, 40). A full-length HCV cDNA was constructed by using the 5′ end to NS2 of pJ6/JFH1 and NS3 to the 3′ end of pSGR-JFH2.1, and the resulting construct was named pJ6/JFH2 (accession number AB690460). Another full-length HCV construct, pJFH2 containing the full-length JFH-2 cDNA downstream of the T7 RNA promoter sequence, was also constructed by replacing the 5′ UTR to NS2 of pJ6/JFH2 with JFH2 sequences, as described previously (accession number AB690461) (1, 37, 38).

**Subgenomic replicon assay.** Subgenomic replicon RNA was synthesized as reported previously (11). Synthesized replicon RNA was adjusted to 10 μg with cellular RNA isolated from untransfected HuH-7 cells and then electroporated into naive HuH-7 cells as reported previously (11). G418 (1.0 mg/ml) was added to the culture medium, and the drug-resistant colonies were fixed with buffered formalin and stained with crystal violet or cloned and expanded for further analysis. Total RNA was extracted from the cloned G418-resistant cells by using Isogen reagent (Nip-

TABLE 1 Primer list used for cloning and sequencing of JFH-2 clone

| Forward primer | | Reverse primer | |
|---|---|---|---|
| Name | Sequence (5′→3′) | Name | Sequence (5′→3′) |
| 44S | CTGTGAGGAACTACTGTCTT | 1323R | GGTGACCAGTTCATCATCAT |
| 317S | GGGAGGTCTCGTAGACCGTG | 1440R | GCTCCCTGCATAGAGAAGTA |
| 844S | GGGTTAATTATGCAACAGGGAAC | 2367R | CATTCCGTGGTAGAGTGCA |
| 1141S | TGTCCGCCACGCTCTGCT | 2445R | TCCACGATGTTTTGGTGGAG |
| 1361S | CCCGAGGTCATCATAGACAT | 3568R | TGTTCCGAGGAAGGACTGAG |
| 2106S | CTGTTGTGCCCCACGGACTG | 3765R | TCAGCGTTCCGCGTGACCA |
| 2285S | AACTTCACTCGTGGGGATCG | 4706R | TTGCAGTCGATCACGGAGTC |
| 3211S | GGCACTTACATCTATGACCACCTC | 5331R | GAGGTCATGACCAGCACGTG |
| 3471S | TGGGCACCATAGTGGTGAG | 5563R | CTGCAGCAAGCCTTGGATCT |
| 3930S | TCGATTTCATCCCCGTTGAG | 5970R | TTCTCGCCAGACATGATCTT |
| 4278S | CCTATGACATCATCATATGCGATGAATGCC | 6152R | AGTGAGTAGGGGCGACGTGGTTTCCTCTGG |
| 4301S | CCTATGACATCATCATATGCGATG | 6505R | CCTGCCAGGTGTTCATGCAG |
| 4547S | AAGTGTGACGAGCTCGCGG | 6605R | GCATACTCTGAGGCCGCCAC |
| 5021S | TTTTGGGAGGCAGTTTTCAC | 6897R | GTGATGTGGGGCGGATCTGTTAGCATGGAC |
| 6383S | TGTCAAAAGGGGTACAAGGG | 7648R | TCCTCCTCGGAGCAAGTAGA |
| 6774S | TCCGGGATGAGGTCTCGTTC | 8913R | GCGTACTGGATGATGTTTCC |
| 6881S | ATTGATGTCCATGCTAACAG | 3X-54R | GCGGCTCACGGACCTTTCAC |
| 7198S | GGCTTGGGCACGGCCTGA | 3X-75R | TACGGCACTCTCTGCAGTCA |
| 7244S | ACCGCTTGTGGAATCGTGGA | | |
| 7657S | CGTGTGCTGCTCCATGTCAT | | |
| 7993S | CAGCTTGTCCGGGAGGGC | | |
| 8337S | TTTCGTATGATACCCGATGCTT | | |
| 8704S | CGCCCCTCCGGGTGACCCCCCCAGACCGGA | | |
| 9123S | CACGAACTGACGCGGGTGGC | | |

pon Gene, Tokyo, Japan), and the replicon RNA was quantitated by Northern blotting and real-time detection RT-PCR as reported previously (11, 37). The cDNAs of the HCV RNA replicon were synthesized and then amplified by PCR. The sequence of each replicon was determined.

Luciferase reporter replicons were analyzed as follows. Five micrograms of synthesized replicon RNA was transfected into HuH-7 cells by electroporation. Transfected cells were harvested serially at 4, 24, 48, 72, and 96 h after transfection. Luciferase activities were quantified by a Lumat LB9507 instrument (EG&G Berthold, Bad Wildbad, Germany) and a luciferase assay system (Promega). Assays were performed at least in triplicate, and the results were expressed as relative luciferase activity.

**Analysis of G418-resistant cells.** In RNA-transfected dishes, G418-resistant colonies were isolated by using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan) and expanded until 80% to 90% confluence in 10-cm diameter dishes. Expanded cells were analyzed as described previously (11).

**Northern blot analysis.** Four micrograms of isolated RNA samples was electrophoretically separated in a 1% agarose gel containing formaldehyde and transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK, Ltd., Buckinghamshire, England) and immobilized by a Stratalinker UV cross-linker (Stratagene, La Jolla, CA). Hybridization was performed with a [$\alpha$-$^{32}$P]dCTP-labeled DNA probe by using Rapid-Hyb Buffer (GE Healthcare UK, Ltd.). The NS3 to 3′X region of the JFH-1 sequence was used as a template of DNA probe synthesis with a Megaprime DNA Labeling System (GE Healthcare UK, Ltd.) (37).

**Western blot analysis of HCV proteins.** The protein samples were separated on a 10% polyacrylamide gel. After electrophoresis, the proteins were transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore Corp., Bedford, MA) with a semidry blotting apparatus (Biocraft, Tokyo, Japan). Transferred proteins were incubated with blocking buffer containing 5% nonfat dry milk (Snow brand, Sapporo, Japan) in phosphate-buffered saline. Anti-NS3 rabbit polyclonal antibody raised against recombinant NS3 protein and horseradish peroxidase-labeled goat anti-rabbit Ig (BioSource, Camarillo, CA) were used to detect HCV NS3 protein. The signals were detected with a chemiluminescence system (ECL Plus; GE Healthcare UK, Ltd.). The quantity and quality of the loaded samples were confirmed to be similar by Coomassie brilliant blue staining of the gel.

**RT-PCR and sequencing analysis.** The cDNAs of HCV RNA were synthesized from total cellular RNA isolated from replicon RNA-transfected cells or from the culture medium of full-length HCV RNA-transfected cells with antisense primer in the 3′X tail region. These cDNAs were subsequently amplified with DNA polymerase (TaKaRa LA Taq; TaKaRa Bio Inc.). The sequence of each amplified DNA was determined directly as described above.

**Full-length HCV RNA transfection.** Full-length HCV RNA was synthesized from pJ6/JFH2, pJFH2, and the derivatives of these constructs with adaptive mutations, as described previously (13, 37, 38). Synthesized HCV RNA (10 $\mu$g) was transfected into Huh-7.5.1 or Huh7-25 cells. HCV core protein levels in the culture medium were measured by immunoassay (31). HCV RNA levels in the culture medium were quantified as described above. Infectivity of culture supernatants was determined by measuring the focus formation efficiency (13, 41). In some experiments, HCV core protein levels in the transfected cells were determined as described previously (37, 38). To examine virus secretion and infectivity after long-term culture, the transfected cells were serially passaged. Virus infection was neutralized by using mouse anti-CD81 monoclonal antibody (clone JS-81; BD Pharmingen, Franklin Lakes, NJ) and anti-HCV human IgG purified from HCV carrier serum (a gift from H. Yoshizawa and J. Tanaka, Hiroshima University).

**Density gradient analysis.** Culture medium derived from the transfected or infected cells was harvested for density gradient analysis. Cleared culture medium was layered onto a stepwise sucrose gradient (60% [wt/vol] to 10%) and centrifuged for 16 h in an SW41 rotor (Beckman, Palo Alto, CA) at 200,000 × *g* at 4°C. After centrifugation, 18 fractions were harvested from the bottom of the tubes. The HCV core protein, HCV RNA levels, and infectivity in each fraction were determined as described above.

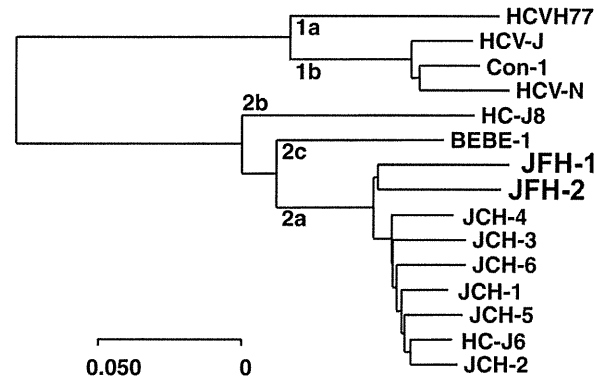


FIG 2 Phylogenetic analysis of JFH-2. Phylogenetic tree of the NS3 to NS5B amino acid sequences of HCV including the JFH-2 strain and genotype 2 strains for which the entire genome has been reported (JFH-1, accession number AB047639; HC-J6, D00944; HC-J8, D10988; and BEBE1, D50409) and representative genotype 1 strains for which the entire genome has been reported (H77, AF009606; HCV-Con1, AJ238799; HCV-J, D90208, and HCV-N, AF139594). This phylogenetic tree was drawn by using Kimura's two-parameter method.

**Electron microscopy.** To visualize HCV particles, we adsorbed the density gradient-purified virus samples onto carbon-coated grids for 1 min. Then, the grids were stained with 1% uranyl acetate for 1 min and examined under an H-7650 transmission electron microscope (Hitachi High-Technologies Co., Tokyo, Japan) (32). Immunogold labeling was performed with an antibody directed against E2 (AP33; a kind gift from Genentech, South San Francisco, CA) diluted 1:50 in blocking solution and secondary antibody coupled to 10-nm gold particles.

**Human hepatocyte chimeric mouse experiments.** Human hepatocytes were transplanted into urokinase-type plasminogen activator-transgenic SCID mice(uPA$^{+/+}$ SCID$^{+/+}$) as described previously (33). All mice received hepatocyte transplants from the same donor. Human hepatocyte chimeric mice, in which liver cells were largely (>90%) replaced with human hepatocytes, were used to reduce the potential influence by mouse-derived mRNA. Human albumin levels in the sera of mice were monitored to evaluate the replacement ratio of the human hepatocytes in the mouse liver. The mice were obtained from Phoenix Bio Co., Ltd. (Hiroshima, Japan). Four mice were divided into two groups. Each group of mice was inoculated with $1 \times 10^6$ RNA copies of either purified J6/JFH2/AS HCV particles or JFH-2 patient serum. The HCV RNA titer in inoculated mouse serum was monitored by real-time detection RT-PCR each week after inoculation.

## RESULTS

**HCV clone from a fulminant hepatitis patient.** HCV cDNA was isolated from a fulminant hepatitis patient as described in Materials and Methods (clone JFH-2) (15). HCV RNA was detected by RT-PCR in the patient's serum during the acute phase (Fig. 1). All viral markers of the other hepatitis viruses were negative. By the phylogenetic analysis, the JFH-2 clone was clustered into genotype 2a (Fig. 2). JFH-2 exhibits 87.6%, 89.0%, and 88.9% nucleotide homology with JFH-1, J6CF, and JCH-1, respectively, and 90.6%, 91.8%, and 91.8% amino acid homology with JFH-1, J6CF, and JCH-1, respectively (Table 2). The JFH-1 strain is cell culture replication-competent, but the J6CF and JCH-1 strains are incompetent. However, the homology data for nucleotide and amino acid sequences are very similar in both the structural and nonstructural regions. We also mapped the

TABLE 2 Percent homology between JFH-2 and other genotype 2a strains

| Region | JFH-2 nucleotide profile | | | | JFH-2 amino acid profie | | | |
|---|---|---|---|---|---|---|---|---|
| | Lenght (nt)[a] | % Identity vs strain: | | | Length (aa)[b] | % Amino acid identity vs strain: | | |
| | | JFH-1 | J6CF | JCH-1 | | JFH-1 | J6CF | JCH-1 |
| Entire genome | 9683 | 87.60 | 88.98 | 88.88 | 3033 | 90.64 | 91.79 | 91.79 |
| UTR[c] | 576 | 96.35 | 98.61 | 96.88 | NA[d] | | | |
| Structural | 2439 | 86.14 | 87.90 | 86.51 | 813 | 89.30 | 89.54 | 88.56 |
| Nonstructural | 6663 | 87.44 | 88.59 | 89.12 | 2220 | 91.13 | 92.61 | 92.97 |
| 5′ UTR | 340 | 98.82 | 99.71 | 99.71 | NA | | | |
| Core | 573 | 91.80 | 93.02 | 91.97 | 191 | 92.15 | 95.29 | 92.15 |
| E1 | 576 | 87.50 | 88.89 | 89.06 | 192 | 90.10 | 92.19 | 89.58 |
| E2-p7 | 1290 | 83.02 | 85.19 | 82.95 | 430 | 87.67 | 85.81 | 86.51 |
| NS2 | 651 | 84.18 | 85.87 | 89.09 | 217 | 87.56 | 88.02 | 91.24 |
| NS3 | 1893 | 87.64 | 88.54 | 89.33 | 631 | 92.87 | 94.61 | 94.45 |
| NS4A | 162 | 88.27 | 88.27 | 88.27 | 54 | 96.30 | 92.59 | 94.44 |
| NS4B | 783 | 89.91 | 90.04 | 89.14 | 261 | 96.93 | 97.32 | 96.55 |
| NS5A | 1398 | 83.48 | 85.98 | 85.48 | 466 | 82.83 | 86.70 | 86.48 |
| NS5B | 1776 | 90.37 | 91.10 | 91.84 | 591 | 94.08 | 94.75 | 95.43 |
| 3′ UTR | 236 | 92.80 | 97.03 | 92.80 | NA | | | |

[a] nt, nucleotides.
[b] aa, amino acids.
[c] UTR, 5′ UTR plus 3′ UTR.
[d] NA, not applicable.

positions of different amino acid sequences of each strain (Fig. 3A). The E2 and NS5A regions are more variable than other regions (Fig. 3A and Table 2); however, it is difficult to find particular mutation positions or regions specific for the JFH-2 strain.

**Subgenomic replicon analysis of the JFH-2 clone.** Interestingly, some parts of the viral cDNA sequences in the JFH-2 viral genome were a mixture of different sequences, especially in the NS3 region. By the cloning analysis, we found two major se-

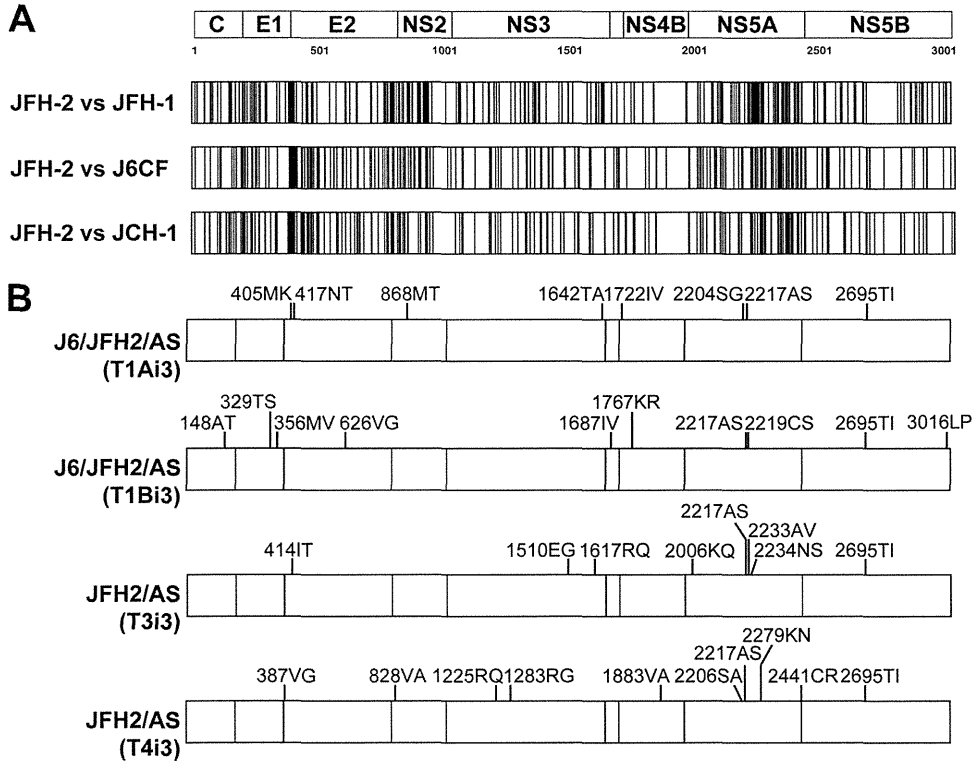


FIG 3 Maps of amino acid sequences among genotype 2a HCV strains and mutations found in the cell culture-adapted viruses. (A) Amino acid sequences of the entire open reading frame (3,033 amino acids) of JFH-1, JFH-2, J6CF (accession numbers AB047639, AB690461, and AF177036, respectively), and JCH-1 strains were compared. The positions of different sequences are indicated by vertical lines. (B) Virus genome sequences were determined in the T1Ai3 and T1Bi3 culture media of the J6/JFH2/AS virus-inoculated cells or T3i3 and T4i3 culture media of the JFH2/AS virus-inoculated cells, as described in the text. Amino acid mutations are indicated with their positions and residues.