several possible explanations can be offered. First, those authors examined the localization of EGFP-G3BP within 48 h postinfection, and we observed it at later times (Fig. 4). Second, they used only EGFP-tagged G3BP instead of endogenous G3BP1. Third, they used a Jc1FLAG2 (p7-nsGluc2A) clone, and an HCV-JFH1 clone could markedly induce the recruitment of the core protein to LDs compared to that of Jc1. Also, Jangra et al. failed to observe the recruitment of DDX6 to LDs at 2 days after infection with HJ3-5 virus (16). Accordingly, we also observed that most of the DDX6 still formed intact P bodies at earlier times (12 h or 24 h postinfection). Importantly, we observed the recruitment of DDX6 to LDs 48 h later (Fig. 4). Furthermore, those authors did not show the ringlike structure formation of the HJ3-5 core protein around LDs, unlike the JFH1 core protein that we used in this study. The interaction of the HCV core protein with DDX6 may explain the recruitment of P-body components to LDs. However, we do not yet know whether the P-body function(s) can be performed on LDs. At least, HCV infection did not affect the translation of several host mRNAs with 5′ caps and 3′ poly(A) tails despite the disruption of P-body formation at 72 h postinfection (Fig. 6), suggesting that HCV does not affect P-body function and that HCV recruits functional P bodies to LDs.

We need to address the potential role of stress granule components, such as PABP1, in HCV replication/translation, since the HCV genome does not harbor the 3′ poly(A) tail. Intriguingly, we have found that the accumulation of HCV RNA was significantly suppressed in PABP1 knockdown RSc cells (Fig. 7F). In this regard, Tingting et al. demonstrated previously that G3BP1 and PABP1 as well as DDX1 were identified as the HCV 3′-UTR RNA-binding proteins by proteomic analysis and that G3BP1 was required for HCV RNA replication (35). Yi et al. also reported that G3BP1 was associated with HCV NS5B and that G3BP1 was required for HCV RNA replication (42). We observed a moderate effect of siG3BP1 on HCV RNA replication (Fig. 7F). In contrast, the accumulation of HCV RNA was significantly suppressed in ATX2 and Lsm1 knockdown cells as well as in PABP1 knockdown cells (Fig. 7F), suggesting that ATX2, Lsm1, and PABP1 are required for HCV replication.

Taking these results together, this study has demonstrated for the first time that HCV hijacks P-body and stress granule components around LDs. This hijacking may regulate HCV RNA replication and translation. Indeed, we have found that the accumulation of genome-length HCV-O (genotype 1b) (14) RNA was markedly suppressed in DDX6 knockdown O cells (data not shown). More importantly, these P-body and stress granule components may be involved in the maintenance of the HCV RNA genome without 5′ cap and 3′ poly(A) tail structures in the cytoplasm for long periods, since the hijacking of P-body and stress granule components by HCV occurred at later times.

ACKNOWLEDGMENTS

We thank D. Trono for the lentiviral vector system, T. Wakita for HCV-JFH1, and K. T. Jeang for pHA-DDX3. We also thank T. Nakamura and K. Takeshita for their technical assistance.

This work was supported by a grant-in-aid for scientific research (C) from the Japan Society for the Promotion of Science (JSPS); by a grant-in-aid for research on hepatitis from the Ministry of Health, Labor, and Welfare of Japan; and by the Viral Hepatitis Research Foundation of Japan. M.K. was supported by a research fellowship from the JSPS for young scientists.

REFERENCES

1. **Anderson, P., and N. Kedersha.** 2007. Stress granules: the Tao of RNA triage. Trends Biochem. Sci. **33:**141–150.
2. **Ariumi, Y., et al.** 2003. Distinct nuclear body components, PML and SMRT, regulate the *trans*-acting function of HTLV-1 Tax oncoprotein. Oncogene **22:**1611–1619.
3. **Ariumi, Y., et al.** 2007. DDX3 DEAD-box RNA helicase is required for hepatitis C virus RNA replication. J. Virol. **81:**13922–13926.
4. **Ariumi, Y., et al.** 2008. The DNA damage sensors ataxia-telangiectasia mutated kinase and checkpoint kinase 2 are required for hepatitis C virus RNA replication. J. Virol. **82:**9639–9646.
5. **Ariumi, Y., et al.** 2011. The ESCRT system is required for hepatitis C virus production. PLoS One **6:**e14517.
6. **Beckham, C. J., and R. Parker.** 2008. P bodies, stress granules, and viral life cycles. Cell Host Microbe **3:**206–212.
7. **Bridge, A. J., S. Pebernard, A. Ducraux, A. L. Nicoulaz, and R. Iggo.** 2003. Induction of an interferon response by RNAi vectors in mammalian cells. Nat. Genet. **34:**263–264.
8. **Brummelkamp, T. R., R. Bernard, and R. Agami.** 2002. A system for stable expression of short interfering RNAs in mammalian cells. Science **296:**550–553.
9. **Chable-Bessia, C., et al.** 2009. Suppression of HIV-1 replication by microRNA effectors. Retrovirology **6:**26.
10. **Cristea, I. M., et al.** 2010. Host factors associated with the Sindbis virus RNA-dependent RNA polymerase: role for G3BP1 and G3BP2 in virus replication. J. Virol. **84:**6720–6732.
11. **Emara, M. M., and M. A. Brinton.** 2007. Interaction of TIA-1/TIAR with West Nile and dengue virus products in infected cells interferes with stress granule formation and processing body assembly. Proc. Natl. Acad. Sci. U. S. A. **104:**9041–9046.
12. **Hijikata, M., N. Kato, Y. Ootsuyama, M. Nakagawa, and K. Shimotohno.** 1991. Gene mapping of the putative structural region of the hepatitis C virus genome by in vitro processing analysis. Proc. Natl. Acad. Sci. U. S. A. **88:**5547–5551.
13. **Hijikata, M., et al.** 1993. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. **90:**10773–10777.
14. **Ikeda, M., et al.** 2005. Efficient replication of a full-length hepatitis C virus genome, strain O, in cell culture, and development of a luciferase reporter system. Biochem. Biophys. Res. Commun. **329:**1350–1359.
15. **Jangra, R. K., M. Yi, and S. M. Lemon.** 2010. Regulation of hepatitis C virus translation and infectious virus production by the microRNA miR-122. J. Virol. **84:**6615–6625.
16. **Jangra, R. K., M. Yi, and S. M. Lemon.** 2010. DDX6 (Rck/p54) is required for efficient hepatitis C virus replication but not IRES-directed translation. J. Virol. **84:**6810–6824.
17. **Ji, H., et al.** 2008. MicroRNA-122 stimulates translation of hepatitis C virus RNA. EMBO J. **27:**3300–3310.
18. **Jones, C. T., et al.** 2010. Real-time imaging of hepatitis C virus infection using a fluorescent cell-based reporter system. Nat. Biotechnol. **28:**167–171.
19. **Jopling, C. L., M. Yi, A. M. Lancaster, S. M. Lemon, and P. Sarnow.** 2005. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. Science **309:**1577–1581.
20. **Jopling, C. L., S. Schütz, and P. Sarnow.** 2008. Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. Cell Host Microbe **4:**77–85.
21. **Kato, N., et al.** 1990. Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis. Proc. Natl. Acad. Sci. U. S. A. **87:**9524–9528.
22. **Kedersha, N., and P. Anderson.** 2007. Mammalian stress granules and processing bodies. Methods Enzymol. **431:**61–81.
23. **Kuroki, M., et al.** 2009. Arsenic trioxide inhibits hepatitis C virus RNA replication through modulation of the glutathione redox system and oxidative stress. J. Virol. **83:**2338–2348.
24. **Kushima, Y., T. Wakita, and M. Hijikata.** 2010. A disulfide-bonded dimer of the core protein of hepatitis C virus is important for virus-like particle production. J. Virol. **84:**9118–9127.
25. **Mamiya, N., and H. J. Worman.** 1999. Hepatitis C virus core protein binds to a DEAD box RNA helicase. J. Biol. Chem. **274:**15751–15756.
26. **Miyanari, Y., et al.** 2007. The lipid droplet is an important organelle for hepatitis C virus production. Nat. Cell Biol. **9:**1089–1097.
27. **Naldini, L., et al.** 1996. In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector. Science **272:**263–267.
28. **Nonhoff, U., et al.** 2007. Ataxin-2 interacts with the DEAD/H-box RNA helicase DDX6 and interferes with P-bodies and stress granules. Mol. Biol. Cell **18:**1385–1396.
29. **Owsianka, A. M., and A. H. Patel.** 1999. Hepatitis C virus core protein interacts with a human DEAD box protein DDX3. Virology **257:**330–340.

Downloaded from http://jvi.asm.org/ on January 28, 2012 by Okayama University

30. **Parker, R., and U. Sheth.** 2007. P bodies and the control of mRNA translation and degradation. Mol. Cell **25:**635–646.
31. **Randall, G., et al.** 2007. Cellular cofactors affecting hepatitis C virus infection and replication. Proc. Natl. Acad. Sci. U. S. A. **104:**12884–12889.
32. **Rocak, S., and P. Linder.** 2004. DEAD-box proteins: the driving forces behind RNA metabolism. Nat. Rev. Mol. Cell Biol. **5:**232–241.
33. **Scheller, N., et al.** 2009. Translation and replication of hepatitis C virus genomic RNA depends on ancient cellular proteins that control mRNA fates. Proc. Natl. Acad. Sci. U. S. A. **106:**13517–13522.
34. **Smith, R. W., and N. K. Gray.** 2010. Poly(A)-binding protein (PABP): a common viral target. Biochem. J. **426:**1–11.
35. **Tingting, P., F. Caiyun, Y. Zhigang, Y. Pengyuan, and Y. Zhenghong.** 2006. Subproteomic analysis of the cellular proteins associated with the 3′ untranslated region of the hepatitis C virus genome in human liver cells. Biochem. Biophys. Res. Commun. **347:**683–691.
36. **Tourrière, H., et al.** 2003. The RasGAP-associated endoribonuclease G3BP assembles stress granules. J. Cell Biol. **160:**823–831.
37. **Wakita, T., et al.** 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. **11:**791–796.
38. **Weston, A., and J. Sommerville.** 2006. Xp54 and related (DDX6-like) RNA helicase: roles in messenger RNP assembly, translation regulation and RNA degradation. Nucleic Acids Res. **34:**3082–3094.
39. **White, J. P., A. M. Cardenas, W. E. Marissen, and R. E. Lloyd.** 2007. Inhibition of cytoplasmic mRNA stress granule formation by a viral proteinase. Cell Host Microbe **2:**295–305.
40. **Wilson, J. A., C. Zhang, A. Huys, and C. D. Richardson.** 2011. Human Ago2 is required for efficient miR-122 regulation of HCV RNA accumulation and translation. J. Virol. **85:**2342–2350.
41. **Yedavalli, V. S., C. Neuveut, Y. H. Chi, L. Kleiman, and K. T. Jeang.** 2004. Requirement of DDX3 DAED box RNA helicase for HIV-1 Rev-RRE export function. Cell **119:**381–392.
42. **Yi, Z., et al.** 2006. Subproteomic study of hepatitis C virus replicon reveals Ras-GTPase-activating protein binding protein 1 as potential HCV RC component. Biophys. Biochem. Res. Commun. **350:**174–178.
43. **You, L. R., et al.** 1999. Hepatitis C virus core protein interacts with cellular putative RNA helicase. J. Virol. **73:**2841–2853.
44. **Zufferey, R., D. Nagy, R. J. Mandel, L. Naldini, and D. Trono.** 1997. Multiply attenuated lentiviral vector achieves efficient gene delivery in vivo. Nat. Biotechnol. **15:**871–875.

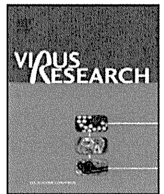
Downloaded from http://jvi.asm.org/ on January 28, 2012 by Okayama University

Virus Research 158 (2011) 209–215

Contents lists available at ScienceDirect

Virus Research

journal homepage: www.elsevier.com/locate/virusres



Cross-species transmission of gibbon and orangutan hepatitis B virus to uPA/SCID mice with human hepatocytes

Pattaratida Sa-nguanmoo[a,1], Yasuhito Tanaka[b,1], Parntep Ratanakorn[c], Masaya Sugiyama[d], Shuko Murakami[b], Sunchai Payungporn[e], Angkana Sommanustweechai[f], Masashi Mizokami[d], Yong Poovorawan[a,*]

[a] Center of Excellence in Clinical Virology, Department of Pediatrics, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[b] Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Kawasumi, Mizuho, Nagoya 467-8601, Japan
[c] Faculty of Veterinary Science, Mahidol University, Nakhon-Pathom 73170, Thailand
[d] The Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Kounodai, Ichikawa 272-8516, Japan
[e] Department of Biochemistry, Faculty of Medicine, Chulalongkorn University, Bangkok 10330, Thailand
[f] Dusit Zoo, Zoological Park Organization, Bangkok 10300, Thailand

ARTICLE INFO

Article history:
Received 10 February 2011
Received in revised form 31 March 2011
Accepted 1 April 2011
Available online 12 April 2011

Keywords:
Non-human primate
Cross-species transmission
Chimeric mouse
Phylogenetic analysis
HBV cccDNA
Immunofluorescence method

ABSTRACT

To investigate the potential of cross-species transmission of non-human primate HBV to humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, in which the mouse liver has been engrafted with human hepatocytes, were inoculated with non-human primate HBV. HBV-DNA positive serum samples from a gibbon or orangutan were inoculated into 6 chimeric mice. HBV-DNA, hepatitis B surface antigen (HBsAg), and HB core-related antigen in sera and HBV cccDNA in liver were detectable in 2 of 3 mice each from the gibbon and orangutan. Likewise, applying immunofluorescence HBV core protein was only found in human hepatocytes expressing human albumin. The HBV sequences from mouse sera were identical to those from orangutan and gibbon sera determined prior to inoculation. In conclusion, human hepatocytes have been infected with gibbon/orangutan HBV.
© 2011 Elsevier B.V. All rights reserved.

1. Introduction

Hepatitis B is caused by hepatitis B virus (HBV), a hepatotropic virus of the family *Hepadnaviridae*. This family comprises two genera, Avihepadnavirus and Orthohepadnavirus which can infect birds and mammals, respectively (Mason et al., 2005). As for humans, approximately 350 million chronic carriers have been infected by HBV worldwide (Lavanchy, 2004) and 15–40 percent have developed liver cirrhosis and hepatocellular carcinoma (Lee, 1997; McQuillan et al., 1989; Sharma et al., 2005). In addition to humans, HBV also infects higher non-human primates (apes) such as orangutans (*Pongo pygmaeus*), gibbons (*Hylobates* sp. and *Nomascus* sp.), gorillas (*Gorilla gorilla*), and chimpanzees (*Pan troglodytes*) (Grethe et al., 2000; MacDonald et al., 2000; Makuwa et al., 2003; Noppornpanth et al., 2003; Sall et al., 2005; Sa-nguanmoo et al., 2008; Starkman et al., 2003; Warren et al., 1998). In comparison with human HBV, non-human primate HBVs contain a 33 nucleotide deletion in the *PreS1* gene and all non-human primate HBVs cluster within their respective group separate from each human HBV genotype (Grethe et al., 2000; Kramvis et al., 2005; Robertson, 2001; Takahashi et al., 2000).

Several experiments have been conducted to study cross-species transmission of human HBV to non-human primates. Human HBsAg positive sera were intravenously inoculated into chimpanzees. In all experiments, inoculated chimpanzees displayed HBsAg in their sera (Kim et al., 2008; Tabor et al., 1980). In 1977, Bancroft et al. inoculated pooled saliva collected from 5 human carriers into gibbons. Gibbons which received subcutaneous injections of the pooled saliva developed serological markers of HBV infection. In contrast, gibbons infected via either the nasal or oral route did not show evidence of HBV infection (Bancroft et al., 1977). However, the negative results in this study are probably attributable to the lack of a sufficiently sensitive test available at that time. Alter et al. transmitted semen and saliva of carrier patients to chimpanzees. Chimpanzees developed HBsAg and elevated ALT after inoculation (Alter et al., 1977). In 1980, Scott et al. inoculated semen donated by HBsAg and HBeAg positive patients

* Corresponding author. Tel.: +66 2 2564909; fax: +66 2 2564929.
E-mail address: Yong.P@chula.ac.th (Y. Poovorawan).
[1] These authors contributed equally to this work.

0168-1702/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2011.04.002

into gibbons via the subcutaneous and vaginal route. Moreover, saliva of carrier patients was pooled and inoculated into gibbons via the subcutaneous and oral route. The results showed that semen and saliva from carrier patients cause asymptomatic disease in gibbons when transmitted via the subcutaneous or vaginal route, yet not via the oral route (Scott et al., 1980).

In addition to these experiments, Mimms et al. performed studies by infecting a chimpanzee with gibbon HBV. The HBV-DNA sequence from this chimpanzee was similar to that of gibbon HBV (Mimms et al., 1993). In conclusion, human HBV can be transmitted to non-human primates and cross-species transmission of non-human primate HBV can occur among various non-human primate species. However, cross-species transmission of non-human primate HBV to humans has not yet been supported by scientific evidence. To avoid performing experiments in humans, severe combined immunodeficiency mice transgenic for urokinase-type plasminogen activator, with the liver replaced with human hepatocytes (chimeric mice) serve as a suitable model for studies on human liver-specific pathogens such as HCV and HBV, human hepatic metabolism of pharmaceutical agents, and human hepatic toxicity of candidate anti-proliferative agents (Kneteman and Mercer, 2005). The mice present evidence that more fully characterizes the repopulation of the mouse liver with human hepatocytes (Meuleman et al., 2005). Histological studies have revealed that chimeric mice show evidence of human hepatocyte replace integration with infiltration into mouse liver. Moreover, human albumin and 21 other human specific proteins can be detected in mouse sera (Dandri et al., 2001; Mercer et al., 2001). Subsequently, these mice were used to support woodchuck and human hepatocyte culture and were supported infection with woodchuck hepatitis virus (WHV) and HBV (Meuleman et al., 2005; Petersen et al., 1998; Tabuchi et al., 2008).

The aim of this study has been to demonstrate that non-human primate HBV can be replicated in human hepatocytes in order to consider preventive measures in case of potential HBV transmission from non-human primates to humans.

2. Materials and methods

The study was approved by the Faculty of Veterinary Science, Animal Care and Use Committee, Mahidol University. All experiments were performed in a biosafety level 2 laboratory.

2.1. Gibbon and orangutan HBsAg-positive serum

To study cross-species transmission of non-human primate HBV to humans, the HBsAg and HBV-DNA positive sera of white-cheeked gibbon (*Nomascus leucogenys*) and orangutan (*P. pygmaeus*) were collected from Dusit zoo, Bangkok and Khao Pratub Chang Wildlife Breeding Center, Ratchaburi, Thailand, respectively. These sera constitute the stored surplus sera from a previous study (Sa-nguanmoo et al., 2008).

2.2. Chimeric mice inoculation

Twelve-week-old SCID mice transgenic for urokinase-type plasminogen activator with human hepatocytes (PhoenixBio Co, Ltd., Hiroshima, Japan) were used in this study (Tateno et al., 2004). Real-time PCR was employed to detect non-human primate HBV DNA concentration in gibbon and orangutan serum. This detection method has been shown elsewhere (Abe et al., 1999).

The minimum infectious dose of pre-acute and late acute HBV for HBV transmission to chimeric mice with human hepatocyte repopulation is approximately 10^0 and 10^2 copies (Tabuchi et al., 2008). In this study, 10^4 gibbon or orangutan HBV genome equivalents were intravenously inoculated into 3 chimeric mice of each group. However, none of the chimeric mice showed evidence of HBV markers until week 4 after inoculation. Then, all chimeric mice were re-inoculated with 10^5 genome equivalents.

2.3. Serum collection and HBV DNA extraction

Twenty microliter serum samples were collected once a week after inoculation. HBV DNA was extracted from 5 μl mouse sera by using the QIAamp® DNA Mini kit (QIAGEN, QIAGEN Sciences Inc., MD) following the manufacturer's recommendation.

2.4. HBV DNA quantitative method

HBV DNA quantity was determined by real-time PCR (ABI 7500 Fast Real-time PCR, Applied Biosystems, Foster City, CA). To that end, the small S region was amplified as previously described (Abe et al., 1999). Briefly, 5 μl of DNA were subjected to quantitative HBV DNA analysis by ABI 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA). The reaction mixture comprised 12.5 μl TaqMan® Universal PCR MasterMix (Applied Biosystems, Foster City, CA), 0.5 μl of 10 μM forward primer (HBSF2: 5′-CTTCATCCTGCTGCTATGCCT-3′), 0.5 μl of 10 μM reverse primer (HBSR2: 5′-AAAGCCCAGGATGATGGGAT-3′), 0.5 μl of 10 μM probe (HBSP2G: FAM-ATGTTGCC CGTTTGTCCTCTAATTCCAG-TAMRA) and 6 μl distilled water. The real-time PCR was performed under the following conditions: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 30 s, and 4 °C for the holding step. The HBV viral load in unknown samples was calculated by comparison with the standard curve. The detection limit in this study was 1000 copies/ml due to the small sample volume.

2.5. DNA extraction from mouse liver tissue and cccDNA detection in liver and sera of infected chimeric SCID mice

Mouse liver tissues from one HBV-DNA positive mouse each from the gibbon and orangutan HBV inoculation group were collected at week 15 after inoculation. To extract DNA from mouse liver tissue, 25 mg of liver tissue were extracted by using the DNeasy® Blood & Tissue kit (QIAGEN, QIAGEN Sciences Inc., MD) and eluted in 200 μl of elution buffer. HBV cccDNA was detected by conventional PCR (GeneAmp® PCR System 9700, Applied Biosystems, Foster City, CA). Primer sequences have been previously published (Suzuki et al., 2009). Partially double-stranded HBV DNA could not be amplified by these primers. The details have been previously described (Mason et al., 1998). Briefly, 5 μl of DNA were subjected to amplification by GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The reaction mixture comprised 1 U of Ampli Taq Gold® (Applied Biosystems, Foster City, CA), 2.5 μl of 10× PCR buffer containing 15 mM $MgCl_2$, 2 μl of GeneAmp® dNTP Mix (Applied Biosystems, Washington, UK), 1 μl of 10 μM forward primer (cccF2: 5′-CGTCTGTGCCTTCTCATCTGA-3′), 1 μl of 10 μM reverse primer (cccR4: 5′-GCACAGCTTGGAGGCTTGAA-3′), and 13.3 μl distilled water. The PCR was performed under the following conditions: 96 °C for 10 min, followed by 45 cycles of 95 °C for 30 s, 60 °C for 30 s, and 72 °C for 45 s, and 4 °C for the holding step.

2.6. Entire genome sequencing and phylogenetic analysis

Mouse serum samples positive for HBV DNA were subjected to further studies by sequencing the entire genome sequences. To amplify the entire genome, 1 μl of DNA re-suspended solution was used as template for round I PCR. The entire genome was distinguished into two segments (fragment A and fragment B). Fragment A was amplified by 10 μM forward primer (HBV17F-SARU: 5′-CAAACTCTGCAAGATCCCAGAG-3′) and 10 μM reverse

primer (HBV1799R-SARU 5′-GACCAATTTATGCCTACAGCCTC-3′). Fragment B was amplified by 10 μM forward primer (HBV1595F-SARU: 5′-CTTCACCTCTGCACGTTGCATGG-3′) and 10 μM reverse primer (HBV262R-SARU: 5′-CCACCACGAGTCTAGACTCTGTGG-3′). Both fragment A and fragment B used the same reaction mixture as follows: 5 μl of 2.5 mM dNTP, 2 μl of 10 μM forward primer, 2 μl of 10 μM forward primer, 0.33 μl of LA-Taq (TaKaRa BIO INC, Shiga, Japan), and 29.67 μl distilled water. The amplification method was performed on GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). The thermal cycle was continued as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 15 min (final extension).

For the second round PCR, 2 μl of round I PCR was used as template. Round I PCR product of fragment A was nested by HBV47F-SARU forward primer (5′-CTGTATTTTCCTGC-TGGTGGCTCCAG-3′) and HBV1760R-SARU reverse primer (5′-TAACCTCGTCTCCGCCCCAAACTC-3′). The first round I PCR product of fragment B was nested by HBV1608F-SARU forward primer (5′-GCATGGAGACCACCGTGAACG-3′) and HBV201R-SARU reverse primer (5′-TGTAACACGAGCAGGGGTCCTAGG-3′). Both fragment A and fragment B used reaction mixtures as round I PCR except increasing in the first round PCR template to 2 μl and adjusting distilled water to 28.67 μl. The amplification program was performed as follows: 95 °C for 2 min (pre-denaturation) and followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 2 min, and 72 °C for 20 min (final extension).

The second round PCR products were segregated by electrophoresis on 1% agarose gel stained with ethidium bromide. The bands of PCR products were purified using the QIAquick Gel Extraction kit (QIAGEN GmbH, Hilden, Germany). Purified products were further analyzed by ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA).

The genome was sequenced using the 8 primer sets previously published (Sugauchi et al., 2001). Cycle sequencing was performed using the BigDye Terminator 3.1V cycle sequencing kit (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. The conditions for sequencing were programmed into the GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA) as previously reported (Sugauchi et al., 2001). Nucleotide sequences were edited and assembled using SEQMAN 4.00 (LASERGENE program package, DNASTAR, DNASTAR Inc., Madison, WI). All complete HBV genomes isolated from mouse sera were compared to nucleotide sequences available at the GenBank database by using the Blast program (NCBI, Bethesda, MD). Moreover, the HBV sequences obtained from mouse sera were compared with gibbon and orangutan HBV strains determined prior to inoculation and also compared with other non-human primate HBVs and each human genotype from the GenBank database (NCBI, Bethesda, MD). Genetic comparison was performed by Clustal X program version 2.0.10 (European Bioinformatics Institute, Cambridge, UK). Subsequently, the phylogenetic tree was constructed using the Tamura – 3 parameter neighbor-joining method by Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0 (The Biodesign Institute, Tempe, AZ).

2.7. HBsAg, HBcrAg, and human albumin measurement in mouse sera

Mouse sera were diluted (1:10) and subjected to chemiluminescence enzyme immunoassay (CLEIA) (Fujirebio Diagnostic, Inc., Tokyo, Japan) to detect HBV surface antigen (HBsAg) and HBV core – related antigen: – the antigen which includes both the HBV precore/core proteins (HBcrAg) (Kimura et al., 2005; Shinkai et al., 2006). HBcrAg measurement by this assay implies detection of precore/core proteins, including core protein and HBeAg (Kimura et al., 2002, 2005; Rokuhara et al., 2003; Wong et al., 2007). HBcrAg also showed a good correlation with HBV DNA levels in Asian patients (Kimura et al., 2002; Rokuhara et al., 2003, 2005) and intrahepatic parameters, including fibrosis scores, intrahepatic HBV, cccDNA and nuclear HBcAg (Wong et al., 2007). To expose the core protein and HBeAg, the diluted serum was fist incubated with the solution that contains sodium dodecylsulfate. Subsequently, the lysate was added to the plate coated with primary antibody to HBcAg and HBeAg. After incubation, the plate was washed to discard excess primary antibody and the second antibody labeled with alkaline phosphatase was added. Upon addition of substrate solution, the incubated reaction was measured by chemiluminescent enzyme immunoassay (CLEIA). Fully automated analysis was performed using the Lumipulse® System (Fujirebio Diagnostic, Inc., Tokyo, Japan). Human serum albumin (h-Alb) levels were determined applying a commercial enzyme linked immunosorbent (ELISA) test kit (Bethyl Laboratories Inc., Montgomery, TX).

2.8. Immunohistofluorescence assay

To detect HBcAg and human albumin, thick mouse liver tissue was prepared by cutting the frozen mouse liver with a Leica CM1900 Cryostat-microtome (Meyer Instruments, Inc., Houston, TX) and mounting the slices on glass slides. Histological analysis was performed by immunofluorescence assay as previously reported (Sugiyama et al., 2006). Briefly, mouse liver tissue was blocked by DakoCytomation antibody diluent (Dako North America, Inc., Carpinteria, CA) for 10 min at room temperature. After drying by air, the tissue was incubated in the dark with 50 μg/ml of polyclonal rabbit anti-hepatitis B virus core antigen (HBcAg) (Dako North America, Inc., Carpinteria, CA) for 1 h at 37 °C. After washing 5 times with 1× phosphate buffered saline (PBS) (GIBCO, Invitrogen Corporation, Carlsbad, CA) the tissue was incubated with 50 μg/ml of Cy3® goat anti-rabbit IgG (H + L) (Invitrogen Molecular Probes, Eugene, OR) or 5 μg/ml of goat anti-human albumin FITC (Bethyl Laboratories, Inc., Montgomery, TX) in the dark at 37 °C for 1 h. After washing 5 times with 1× PBS, the tissue was mounted by VECTASHIELD mounting medium with DAPI (Vector Laboratories, Inc., Birmingham, CA). The stained mouse tissue was examined under a Nikon Microscope ECLIPSE E800 (Nikon Instruments, Inc., Melville, NY).

3. Results

3.1. Serum HBV DNA, HBsAg, HBcrAg and human albumin level quantitation

Upon first inoculation with serum containing 10^4 copies of gibbon or orangutan HBV, none of the mice could be infected. Then, chimeric mice were re-inoculated with 10^5 copies. One mouse died before re-inoculation. After re-inoculation, mouse sera were collected once a week. Samples were subjected to quantitative HBV DNA analysis by real-time PCR while HBsAg and HBcrAg were quantitatively determined by CLIEA. Four of 5 mice could be infected with gibbon or orangutan HBV. Two mice each from the gibbon and orangutan groups showed levels of HBV DNA, HBsAg, and HBcrAg with the remaining mouse not displaying any of these markers. In detail, HBV DNA and HBcrAg could be detected in serum samples from two mice of the gibbon group (code 101 and 103) and two mice of the orangutan group (code 201 and 202) 4 weeks after inoculation. HBsAg was present in the orangutan group 4 weeks and in the gibbon groups 6 weeks after inoculation, respectively.

In this experiment, the expected HBV markers HBV DNA, HBsAg and HBcrAg could be detected in mouse serum around 4–5 weeks after inoculation. This finding matched previous studies that had

inoculated human HBV genotypes A2, C2, B1 and J into chimeric SCID mice (Sugiyama et al., 2009; Tatematsu et al., 2009). The time appearance and progression of non-human primate HBV markers presented as same as with human HBV markers (Ganem and Prince, 2004). Human albumin (h-Alb) was measured by ELISA as a quality control. Serum h-Alb levels prior to inoculation of all mice in this study exceeded 7 mg/ml indicating a human hepatocyte replacement index (RI) of over 70 percent (PhoenixBio Co, Ltd., Hiroshima, Japan) and were stable during the experiment (Fig. 1). Mean alanine aminotransferase (ALT) levels were around 200 IU/L in the uPA/SCID mouse sera. After non-human primate HBV inoculation, ALT levels slightly increased in this study (data not shown).

3.2. Intrahepatic cccDNA detection in liver tissue and mouse sera

Using the specific primers that amplify only cccDNA (Suzuki et al., 2009), HBV cccDNA was detected in mouse liver tissue from those mice that had been infected with gibbon and orangutan HBV (Fig. 2A). Moreover, cccDNA was found in the sera of mice infected with gibbon HBV (Fig. 2B).

3.3. Phylogenetic analysis of the entire HBV genome from mouse sera

HBV-DNA from all four mice was amplified and subjected to sequencing of the entire genome. The sequences from mouse sera were identical to HBV from gibbon or orangutan serum determined prior to inoculation (gibbon code GD14, GenBank ID: HQ603061; orangutan code OS25, GenBank ID: EU155824) (Fig. 3). Comparison between the complete HBV sequences from mouse sera and gibbon or orangutan sera prior to inoculation showed 99.9% and 100% similarity, respectively.

3.4. HBcAg and human albumin detection in mouse liver tissue

The mouse liver was also tested for HBcAg by staining with polyclonal rabbit anti-HBcAg and goat anti-rabbit IgG labeled with Cy3 (Fig. 4A). To locate the human hepatocyte area in chimeric mouse liver, the tissue was examined for human albumin. The same mouse liver tissue was stained with goat anti-human albumin conjugated with FITC (Fig. 4B). The study confirmed that HBcAg was found in the same area of human hepatocytes (Fig. 4C).

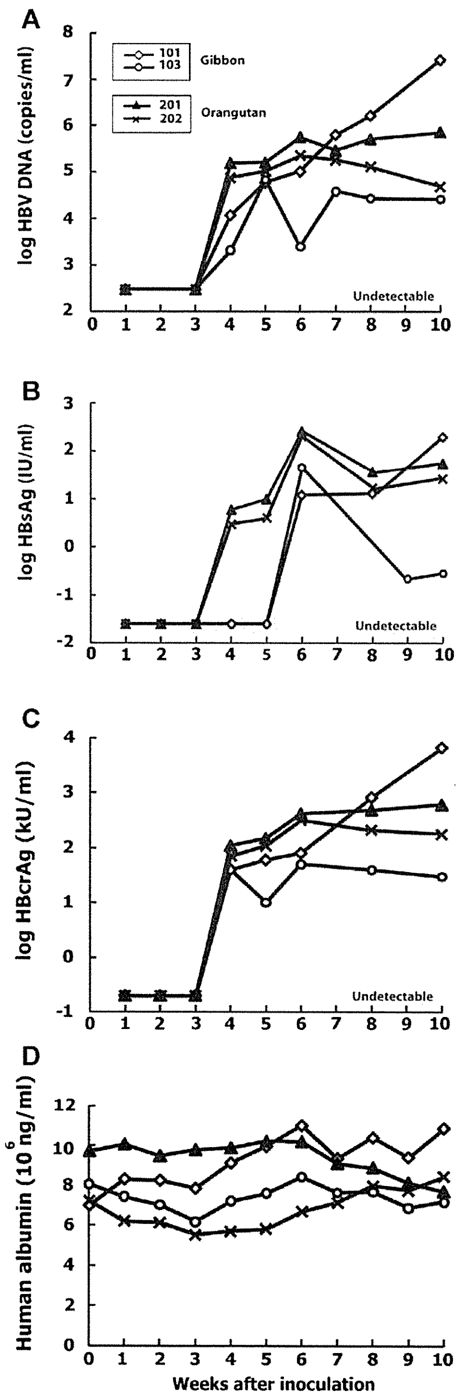


Fig. 1. HBV DNA, HBsAg, HBcrAg, and human albumin concentration in inoculated mouse sera on secondary inoculation. (A) Serum HBV DNA level. Gray zone indicates an area below the minimum sensitivity of real-time PCR (<10^3 copies/ml) (B) HBsAg concentration. The limitation of the test is 0.05 IU/ml. (C) HBcrAg level with the limited sensitivity at 1 kU/ml and (D) h-Alb concentration.

4. Discussion

In a previous study, Hu et al. (2000) constructed a phylogenetic tree and found that the *S* gene sequence from two chimpanzees clustered with human HBV genotypes A and C which could suggest possible virus transmission from human to chimpanzee. Currently, there is no evidence indicating natural infection of humans with non-human primate HBV (Noppornpanth et al., 2003). However, non-human primate HBV would be transmitted to humans because the respective HBV genomes are largely similar.

In this study, cross-species transmission was performed using chimeric mice containing human hepatocytes. The results showed that HBV-DNA, HBsAg and HBcrAg can be detected in sera of mice inoculated with HBV-DNA positive sera from orangutan or gibbon carriers. Detection of HBV cccDNA in liver as well as immune staining data have provided the evidence that gibbon and orangutan HBV can be replicated in human hepatocytes of the chimeric mice sero-positive for HBV DNA. HBsAg and HBV DNA concentrations could increase over time following inoculation. Interestingly, based on phylogenetic analysis, all strains of HBV sequences obtained from mouse sera inoculated with gibbon or orangutan HBV carrier sera grouped with HBV from gibbon and orangutan sera determined prior to inoculation. Nucleotide comparison between HBV in mouse sera and the HBV strain used for inoculation showed 100% identity.

HBV infection depends on the infectious doses of HBV inoculums and host factors. In our experiment, one SCID mouse with human hapatocytes could not be infected with non-human primate HBV. This mouse lacks T- and B-lymphocytes as a protection from viral infection but still, it remains clear from viral infection. Some

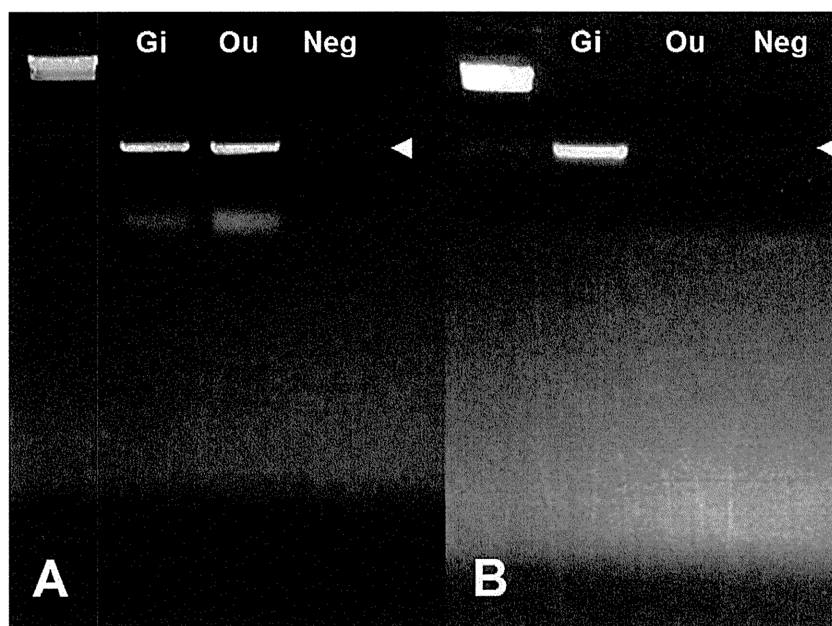


Fig. 2. CccDNA detection in liver (A) and sera (B) of mice that infected with gibbon HBV (Gi) or orangutan HBV (Ou). Neg represents negative PCR control (lacking DNA template). Arrow represents the target cccDNA PCR product.

researchers have attributed this to innate immunity of SCID mice (Lin et al., 1998). SCID mice have a normal innate immune system such as monocytes and macrophages (Ansell and Bancroft, 1989) which probably plays an important role in these mice. Moreover, infection of human hepatocytes with non-human primate HBV may be difficult due to the higher infectious dose required. Moreover, research on the early step of non-human primate HBV attachment to human hepatocytes has not been performed and the pathway of non-human primate HBV infection is still unclear. In comparison with human HBV, it might not be easy for non-human primate HBV to infect human hepatocytes.

Notably, a previous study has reported a new human HBV genotype (HBV-J) isolated from a Japanese patient with hepatocellular carcinoma (Tatematsu et al., 2009). The first HBV strain of interspecies HBV genotype J was closely related to gibbon and orangutan HBV strains and had a deletion of 33 nucleotides at the *preS1* region identical to non-human primate strains. Interestingly, this patient used to live in Borneo—a gibbon and orangutan habitat and hence, an endemic area (Tatematsu et al., 2009). He may have been infected with non-human primate HBV either by close contact or by eating raw meat of non-human primate HBV carriers (personal communication). However, infection of humans with non-human

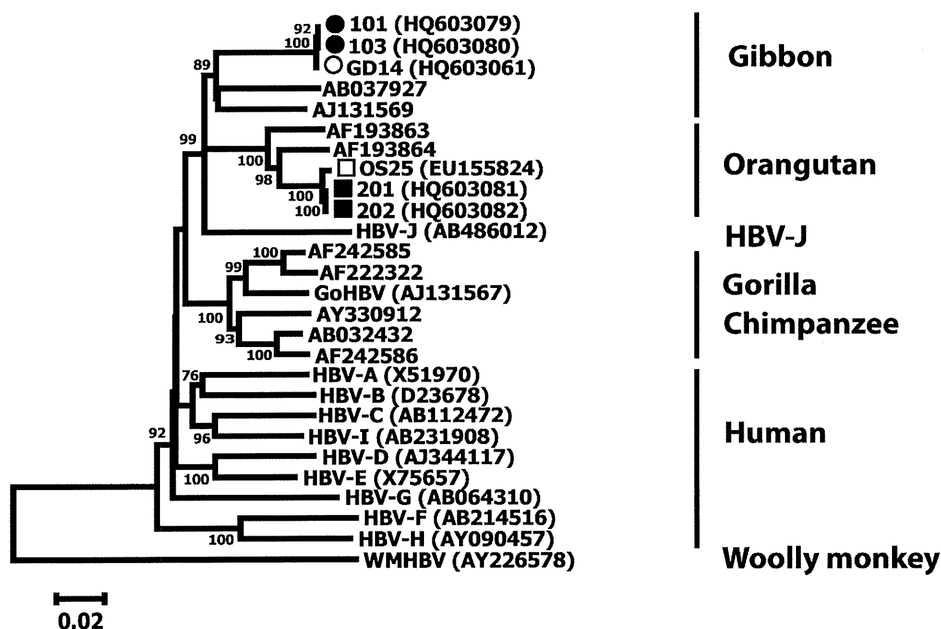


Fig. 3. Phylogenetic analysis of the entire HBV sequence obtained from mouse sera and available sequences of non-human primate HBV strains from GenBank database. Support of each branch as determined from 1000 bootstrap samples. Only 75% bootstrap values are indicated at each node. The scale bar at the bottom represents the genetic distance. Non-human primate HBV sequences obtained from our study are indicated by symbol (gibbon, ○; orangutan, □). HBV sequences obtained from mouse sera were ● and ■ for mice inoculated with gibbon and orangutan sera, respectively.

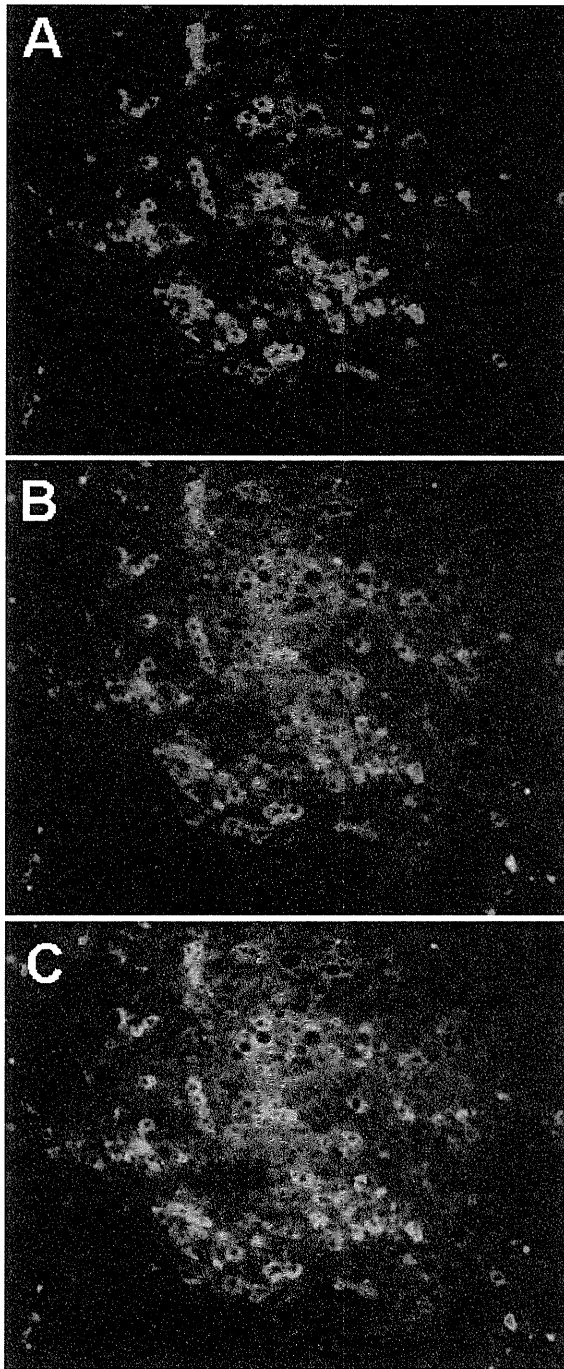


Fig. 4. Immunohistofluorescence of SCID mice infected with gibbon HBsAg-positive serum. Mouse liver tissue incubated for HBcAg (A), human albumin (B), and co-localization of HBcAg and human albumin (C).

primate HBV by eating raw meat or close contact with non-human primate HBV carriers would be hypothesis.

Yet, it has been reported that chimeric SCID mice with human hepatocytes can be infected by inoculation with HBV positive chimpanzee sera (Tabuchi et al., 2008) similar to what has been found in this study. In that previous study, human hepatocyte transplanted chimeric mice were used to study the HBV infectious titer in sera of pre-acute and late acute phase patients. These mice were inoculated with HBV infected chimpanzee sera. The chimeric mice also displayed HBV infection markers such as HBsAg, anti-HBc and anti-HBs as has been shown in this research. But the HBV in chimpanzee sera used to inoculate chimeric mice was human HBV, in contrast to the non-human primate HBV used in this study. Thus, this study is the first scientific evidence to prove and confirm that non-human primate such as gibbon and orangutan HBV can infect and replicate in human hepatocytes. Moreover, this finding can support the discovery of the HBV-J genotype which was found in the human and the assumption that humans can be infected with non-human primate HBV strains is still hypothesis.

Even though uPA-SCID mice with human hepatocytes constitute a useful animal model to study cross-species transmission, this model does not mirror the humoral and cellular immune response of the natural host. In real life, humans may be infected with non-human primate HBV and may clear this virus by their immune system. However, the results of this study indicated that human hepatocytes of chimeric mice have been infected with HBV from gibbon, orangutan and also with human HBV from infected chimpanzee sera as previously reported (Tabuchi et al., 2008). Previous studies have demonstrated cross-species transmission of human HBV to non-human primates, of non-human primate HBV to other species of non-human primates, and this study has demonstrated that non-human primate HBV can replicate in human hepatocytes. As non-human primates represent various virus reservoirs, not only of HBV but also lymphocryptovirus (LCV), Epstein-Barr virus (EBV), or simian foamy virus (SFV), people in close contact with animal HBV carriers should be aware and protect themselves from animal bites or exposure to infected blood or body fluids of non-human primates.

Acknowledgements

This research was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science, and Technology and a grant-in-aid from the Ministry of Health, Labour, and Welfare of Japan, Takeda Science Foundation, the Department of Virology & liver Unit, Nagoya City University Graduate School of Medical Sciences, Japan, the exchange program fellowship under the program "Strategic Scholarship for Frontier Research Network" of Thailand's Commission on Higher Education, the Center of Excellence in Clinical Virology, Chulalongkorn University, the Chulalongkorn University Graduate Scholarship to Commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, the Postdoctoral Fellowship, the National Research University Project of CHE (HR1155A) and Ratchadaphiseksomphot Endowment Fund.

We would like to thank the veterinary doctors and staff of the Dusit zoo, Krabok Koo and Khao Pratubchang Wildlife Breeding Center for sera and taking care of all those non-human primates. We also would like to thank Ms. Kanako Tatematsu, Ms. Hatsue Naganuma, and the entire staff of the Center of Excellence in Clinical Virology, Faculty of Medicine, Chulalongkorn University, Thailand and the Department of Virology & Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya City University, Japan for their assistance. Finally, we would like to thank Ms. P. Hirsch for reviewing the manuscript.

References

Abe, A., Inoue, K., Tanaka, T., Kato, J., Kajiyama, N., Kawaguchi, R., Tanaka, S., Yoshiba, M., Kohara, M., 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J. Clin. Microbiol. 37, 2899–2903.

Alter, H.J., Purcell, R.H., Gerin, J.L., London, W.T., Kaplan, P.M., McAuliffe, V.J., Wagner, J., Holland, P.V., 1977. Transmission of hepatitis B to chimpanzees by hepatitis B surface antigen-positive saliva and semen. Infect. Immun. 16, 928–933.

Ansell, J.D., Bancroft, G.J., 1989. The biology of the SCID mutation. Immunol. Today 10, 322–325.

Bancroft, W.H., Snitbhan, R., Scott, R.M., Tingpalapong, M., Watson, W.T., Tanticharoenyos, P., Karwacki, J.J., Srimarut, S., 1977. Transmission of hepatitis B virus to gibbons by exposure to human saliva containing hepatitis B surface antigen. J. Infect. Dis. 135, 79–85.

Dandri, M., Burda, M.R., Török, E., Pollok, J.M., Iwanska, A., Sommer, G., Rogiers, X., Rogler, C.E., Gupta, S., Will, H., Greten, H., Petersen, J., 2001. Repopulation of

mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. Hepatology 33, 981–988.
Ganem, D., Prince, A.M., 2004. Hepatitis B virus infection—natural history and clinical consequences. N. Engl. J. Med. 350, 1118–1129.
Grethe, S., Heckel, J.O., Rietschel, W., Hufert, F.T., 2000. Molecular epidemiology of hepatitis B virus variants in nonhuman primates. J. Virol. 74, 5377–5381.
Hu, X., Margolis, H.S., Purcell, R.H., Ebert, J., Robertson, B.H., 2000. Identification of hepatitis B virus indigenous to chimpanzees. Proc. Natl. Acad. Sci. U.S.A. 97, 1661–1664.
Kim, S.H., Kim, S.H., Oh, H.K., Ryu, C.J., Park, S.Y., Hong, H.J., 2008. In vivo hepatitis B virus-neutralizing activity of an anti-HBsAg humanized antibody in chimpanzees. Exp. Mol. Med. 40, 145–149.
Kimura, T., Ohno, N., Terada, N., Rokuhara, A., Matsumoto, A., Yagi, S., Tanaka, E., Kiyosawa, K., Ohno, S., Maki, N., 2005. Hepatitis B virus DNA-negative dane particles lack core protein but contain a 22-kDa precore without C-terminal arginine-rich domain. J. Biol. Chem. 280, 21713–21719.
Kimura, T., Rokuhara, A., Sakamoto, Y., Yagi, S., Tanaka, E., Kiyosawa, K., Maki, N., 2002. Sensitive enzyme immunoassay for hepatitis B virus core-related antigens and their correlation to virus load. J. Clin. Microbiol. 40, 439–445.
Kneteman, N.M., Mercer, D.F., 2005. Mice with chimeric human livers: who says supermodels have to be tall? Hepatology 41, 703–706.
Kramvis, A., Kew, M., François, G., 2005. Hepatitis B virus genotypes. Vaccine 23, 2409–2423.
Lavanchy, D., 2004. Hepatitis B virus epidemiology, disease burden, treatment, and current and emerging prevention and control measures. J. Viral. Hepat. 11, 97–107.
Lee, W.M., 1997. Hepatitis B virus infection. N. Engl. J. Med. 337, 1733–1745.
Lin, Y.L., Liao, C.L., Chen, L.K., Yeh, C.T., Liu, C.I., Ma, S.H., Huang, Y.Y., Huang, Y.L., Kao, C.L., King, C.C., 1998. Study of Dengue virus infection in SCID mice engrafted with human K562 cells. J. Virol. 72, 9729–9737.
MacDonald, D.M., Holmes, E.C., Lewis, J.C., Simmonds, P., 2000. Detection of hepatitis B virus infection in wild-born chimpanzees (*Pan troglodytes verus*): phylogenetic relationships with human and other primate genotypes. J. Virol. 74, 4253–4257.
Makuwa, M., Souquière, S., Telfer, P., Leroy, E., Bourry, O., Rouquet, P., Clifford, S., Wickings, E.J., Roques, P., Simon, F., 2003. Occurrence of hepatitis viruses in wild-born non-human primates: a 3 year (1998–2001) epidemiological survey in Gabon. J. Med. Primatol. 32, 307–314.
Mason, A.L., Xu, L., Guo, L., Kuhns, M., Perrillo, R.P., 1998. Molecular basis for persistent hepatitis B virus infection in the liver after clearance of serum hepatitis B surface antigen. Hepatology 27, 1736–1742.
Mason, W.S., Burrell, C.J., Casey, J., Gerlich, W.H., Howard, C.R., Kann, M., Lanford, R., Newbold, J., Schaefer, S., Taylor, J.M., Will, H., 2005. The DNA and RNA transcribing viruses. In: Fauquet, C.M., Mayo, M.A., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy. Eighth Report of the International Committee on Taxonomy of Viruses. Elsevier/Academic Press, London, pp. 371–382.
McQuillan, G.M., Townsend, T.R., Fields, H.A., Carroll, M., Leahy, M., Polk, B.F., 1989. Seroepidemiology of hepatitis B virus infection in the United States. 1976 to 1980. Am. J. Med. 87, 5S–10S.
Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. Nat. Med. 7, 927–933.
Meuleman, P., Libbrecht, L., De Vos, R., de Hemptinne, B., Gevaert, K., Vandekerckhove, J., Roskams, T., Leroux-Roels, G., 2005. Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. Hepatology 41, 847–856.
Mimms, L.T., Solomon, L.R., Ebert, J.W., Fields, H., 1993. Unique preS sequence in a gibbon-derived hepatitis B virus variant. Biochem. Biophys. Res. Commun. 195, 186–191.
Noppornpanth, S., Haagmans, B.L., Bhattarakosol, P., Ratanakorn, P., Niesters, H.G., Osterhaus, A.D., Poovorawan, Y., 2003. Molecular epidemiology of gibbon hepatitis B virus transmission. J. Gen. Virol. 84, 147–155.
Petersen, J., Dandri, M., Gupta, S., Rogler, C.E., 1998. Liver repopulation with xenogenic hepatocytes in B and T cell-deficient mice leads to chronic hepadnavirus infection and clonal growth of hepatocellular carcinoma. Proc. Natl. Acad. Sci. U.S.A. 95, 310–315.
Robertson, B.H., 2001. Viral hepatitis and primates: historical and molecular analysis of human and nonhuman primate hepatitis A, B, and the GB-related viruses. J. Viral. Hepat. 8, 233–242.
Rokuhara, A., Sun, X., Tanaka, E., Kimura, T., Matsumoto, A., Yao, D., Yin, L., Wang, N., Maki, N., Kiyosawa, K., 2005. Hepatitis B virus core and core-related antigen quantitation in Chinese patients with chronic genotype B and C hepatitis B virus infection. J. Gastroenterol. Hepatol. 20, 1726–1730.
Rokuhara, A., Tanaka, E., Matsumoto, A., Kimura, T., Yamaura, T., Orii, K., Sun, X., Yagi, S., Maki, N., Kiyosawa, K., 2003. Clinical evaluation of a new enzyme immunoassay for hepatitis B virus core-related antigen; a marker distinct from viral DNA for monitoring lamivudine treatment. J. Viral. Hepat. 10, 324–330.
Sall, A.A., Starkman, S., Reynes, J.M., Lay, S., Nhim, T., Hunt, M., Marx, N., Simmonds, P., 2005. Frequent infection of *Hylobates pileatus* (pileated gibbon) with species-associated variants of hepatitis B virus in Cambodia. J. Gen. Virol. 86, 333–337.
Sa-nguanmoo, P., Thongmee, C., Ratanakorn, P., Pattanarangsan, R., Boonyarittichaikij, R., Chodapisitkul, S., Theamboonlers, A., Tangkijvanich, P., Poovorawan, Y., 2008. Prevalence, whole genome characterization and phylogenetic analysis of hepatitis B virus in captive orangutan and gibbon. J. Med. Primatol. 37, 277–289.
Scott, R.M., Snitbhan, R., Bancroft, W.H., Alter, H.J., Tingpalapong, M., 1980. Experimental transmission of hepatitis B virus by semen and saliva. J. Infect. Dis. 142, 67–71.
Sharma, S.K., Saini, N., Chwla, Y., 2005. Hepatitis B virus: inactive carriers. Virol. J. 2, 82.
Shinkai, N., Tanaka, Y., Orito, E., Ito, K., Ohno, T., Hirashima, N., Hasegawa, I., Sugauchi, F., Ueda, R., Mizokami, M., 2006. Measurement of hepatitis B virus core-related antigen as predicting factor for relapse after cessation of lamivudine therapy for chronic hepatitis B virus infection. Hepatol. Res. 36, 272–276.
Starkman, S.E., MacDonald, D.M., Lewis, J.C., Holmes, E.C., Simmonds, P., 2003. Geographic and species association of hepatitis B virus genotypes in non-human primates. Virology 314, 381–393.
Sugauchi, F., Mizokami, M., Orito, E., Ohno, T., Kato, H., Suzuki, S., Kimura, Y., Ueda, R., Butterworth, L.A., Cooksley, W.G., 2001. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: complete genome sequence and phylogenetic relatedness. J. Gen. Virol. 82, 883–892.
Sugiyama, M., Tanaka, Y., Kato, T., Orito, E., Ito, K., Acharya, S.K., Gish, R.G., Kramvis, A., Shimada, T., Izumi, M., Kaito, M., Miyakawa, Y., Mizokami, M., 2006. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. Hepatology 44, 915–924.
Sugiyama, M., Tanaka, Y., Kurbanov, F., Maruyama, I., Shimada, T., Takahashi, S., Shirai, T., Hino, K., Sakaida, I., Mizokami, M., 2009. Direct cytopathic effects of particular hepatitis B virus genotypes in severe combined immunodeficiency transgenic with urokinase-type plasminogen activator mouse with human hepatocytes. Gastroenterology 136, 652–662.
Suzuki, F., Miyakoshi, H., Kobayashi, M., Kumada, H., 2009. Correlation between serum hepatitis B virus core-related antigen and intrahepatic covalently closed circular DNA in chronic hepatitis B patients. J. Med. Virol. 81, 27–33.
Tabor, E., FrÖsner, G., Deinhardt, F., Gerety, R.J., 1980. Hepatitis B e antigen and antibody: detection by radioimmunoassay in chimpanzees during experimental hepatitis B. J. Med. Virol. 6, 91–99.
Tabuchi, A., Tanaka, J., Katayama, K., Mizui, M., Matsukura, H., Yugi, H., Shimada, T., Miyakawa, Y., Yoshizawa, H., 2008. Titration of hepatitis B virus infectivity in the sera of pre-acute and late acute phases of HBV infection: transmission experiments to chimeric mice with human liver repopulated hepatocytes. J. Med. Virol. 80, 2064–2068.
Takahashi, K., Brotman, B., Usuda, S., Mishiro, S., Prince, A.M., 2000. Full-genome sequence analyses of hepatitis B virus (HBV) strains recovered from chimpanzees infected in the wild: implications for an origin of HBV. Virology 267, 58–64.
Tatematsu, K., Tanaka, Y., Kurbanov, F., Sugauchi, F., Mano, S., Maeshiro, T., Nakayoshi, T., Wakuta, M., Miyakawa, Y., Mizokami, M., 2009. A genetic variant of hepatitis B virus divergent from known human and ape genotypes isolated from a Japanese patient and provisionally assigned to new genotype J. J. Virol. 83, 10538–10547.
Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., Yoshizato, K., 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am. J. Pathol. 165, 901–912.
Warren, K.S., Niphuis, H., Heriyanto, Verschoor, E.J., Swan, R.A., Heeney, J.L., 1998. Seroprevalence of specific viral infections in confiscated orangutans (*Pongo pygmaeus*). J. Med. Primatol. 27, 33–37.
Wong, D.K., Tanaka, Y., Lai, C.L., Mizokami, M., Fung, J., Yuen, M.F., 2007. Hepatitis B virus core-related antigens as markers for monitoring chronic hepatitis B infection. J. Clin. Microbiol. 45, 3942–3947.

Hepatology Research

Hepatology Research 2011; 41: 838–845 doi: 10.1111/j.1872-034X.2011.00843.x

Original Article

Clarification of interspousal hepatitis C virus infection in acute hepatitis C patients by molecular evolutionary analyses: Consideration on sexual and non-sexual transmission between spouses

Ikuo Nakamura,[1,2] Yasuhito Tanaka,[3] Kaori Ochiai,[2] Fuminori Moriyasu,[1] Masashi Mizokami[4] and Michio Imawari[5]

[1]*Department of Gastroenterology, Tokyo Medical University, Tokyo,* [2]*Division of Gastroenterology and Hepatology, Saitama Medical Center, Jichi Medical University, Saitama,* [3]*Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya,* [4]*Research Center for Hepatitis and Immunology, National Center for Global Health and Medicine, Ichikawa, and* [5]*Division of Gastroenterology, Department of Medicine, Showa University, School of Medicine, Tokyo, Japan*

Aim: Previous studies evaluating the possibilities of interspousal sexual transmission of hepatitis C virus (HCV) have yielded many conflicting results. The aim of this study was to clarify the source of HCV infection in acute hepatitis C patients using phylogenetic analyses of nucleotide sequences of HCV E1 region.

Methods: Four acute hepatitis C patients were hospitalized in 2002–2007. The diagnosis was based on medical records, laboratory tests including HCV markers, and ultrasonographic examination of the liver. In each spouse of four patients, serum HCV antibody was assayed. In the subjects whose serum HCV antibody was positive, additional tests on HCV viral load and genotype were carried out. Then phylogenetic analyses of nucleotide sequences of partial HCV E1 region (440 nucleotides) of the patients and their spouses were performed.

Results: Hepatitis C virus antibody changed from negative to positive in the course of hospitalization and HCV RNA could be detected in every patient. Therefore they were diagnosed as acute hepatitis caused by HCV infection. In every spouse of four patients, HCV antibody and HCV RNA were positive. Three of four couples had the identical genotype and homogeneity of nucleotide sequences of HCV E1 region in three couples ranged from 97.9% to 100%. The results of phylogenic analyses suggested that interspousal HCV infection occurred in the three couples.

Conclusion: In conclusion, interspousal infection might be one of the important sources of acute HCV infection in Japan. The usefulness of phylogenetic analysis of nucleotide sequences of HCV E1 region for clarifying interspousal HCV infection was validated.

Key words: hepatitis C virus, interspousal infection, sexual transmission

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection is one of the major causes of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC). The prevalence of HCV infection is estimated to be 170 million persons throughout the world[1] and two million in Japan. Transmission of HCV through parenteral exposures, including blood transfusion, transfusion of blood products, occupational injury with a needle contaminated with blood, intravenous drug abuse, and tattooing is well documented.[2] However, a large proportion of patients who had not been exposed to any obvious risk factor were found to be infected with HCV.[2] Additionally, household contact as a route of HCV infection has been reported.[3–5] Therefore, the healthcare setting such as shared use of personal hygiene items included

Correspondence: Dr Ikuo Nakamura, Department of Gastroenterology, Tokyo Medical University, 6-7-1, Nishishinjuku, Shinjuku-ku, Tokyo 160-0023, Japan. Email: ikuonaka@tokyo-med.ac.jp
Received 27 April 2011; revision 18 May 2011; accepted 18 May 2011.

© 2011 The Japan Society of Hepatology

toothbrushes, razor blades and nail clippers might be the risk factor of HCV transmission.

So far, the importance of interspousal HCV infection including sexual and non-sexual transmission in acute and chronic hepatitis C patients was controversial and has not been elucidated.[6–8] The aim of this study was to clarify the source of HCV infection in acute hepatitis C patients using phylogenetic analyses of nucleotide sequences of HCV E1 region. In the present study, we investigated the possibility of interspousal HCV infection of four acute hepatitis C patients.

METHODS

Patients

THE STUDY WAS performed at the Division of Gastroenterology and Hepatology, Saitama Medical Center, Jichi Medical University. It was approved by the Ethical Committee of the institute and written informed consent was obtained from all subjects. All procedures in this study were conducted in accordance with the Helsinki Declaration. Four acute hepatitis C patients were hospitalized in 2002–2007. The diagnosis of acute hepatitis C was based on medical records, laboratory tests including HCV markers, and ultrasonographic examination of the liver. Especially, when HCV Ab in serum changes from negative to positive during the course of hepatitis, HCV infection could be definitely diagnosed as acute infection. In each spouse of four patients, serum anti-HCV antibody (HCV Ab) was assayed. In the subjects whose serum HCV Ab was positive, additional tests on HCV viral load and genotype were carried out. Then phylogenetic analyses of nucleotide sequences of partial HCV E1 region (440 nucleotides) of the patients and their spouses were performed.

Methods

Hepatitis C virus Ab in serum was estimated by third-generation enzyme-linked immunosorbent assay (ELISA) (Abbott Laboratories, North Chicago, IL, USA). HCV RNA in serum was quantified by the Ampicor-HCV Monitor Assay (Roche Diagnostics, Tokyo, Japan). The serum was subjected to genotyping by reverse transcription-polymerase chain reaction (RT-PCR) using universal and HCV-specific primers derived from the core region of HCV.[9]

Nucleic acids were extracted from the serum samples using a SepaGene RV-R Nucleic acid extracting kit (Sanko Junyaku Co., Ltd, Tokyo, Japan) in accordance with the manufacturer's protocol. Viral RNA was reverse-transcribed to cDNA using SuperScript II RNase H-Reverse Transcriptase (Invirogen Corp., Carlsbad, CA, USA) and random hexamer primer (Takara Shuzo Co. Ltd, Tokyo, Japan) as described previously. A sequence spanning 440 nucleotides (nt) in the partial E1 region was amplified by PCR with primers described previously[10] (HCVE1-s1 [840–865]: GCAACAGGGAAC CTTCCTGGTTGCTC and 45AT-as2 [1305–1327]:GA CCARTTCATCATCATRTCCCA [R = A or G]). PCR products were directly sequenced with Prism Big Dye (Applied Biosystems, Foster City, CA, USA) in an ABI 3100 DNA automated sequencer. To reduce the number of artificial substitutions arising in PCR, PLATINUM Pfx DNA Polymerase (Invirogen Corp.) with very high fidelity was used. The sequences determined were used to confirm HCV genotypes and to construct phylogenetic trees. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 1000 times. Overall mean genetic distances in all nucleotides positions, synonymous and nonsynonymous positions were estimated by MEGA version 2.1.[11]

RESULTS

THE CLINICAL CHARACTERISTICS of the patients studied are shown in Table 1. Three patients were males and one patient was female. The ages of the patients ranged from 33 to 73 years old. The peak values of serum alanine aminotransferase (ALT) in four patients ranged from 1054 U/L to 4560 U/L. The peak values of total bilirubin in serum of the patients were between 3.6 mg/dL and 23.8 mg/dL. HCV Ab in serum was estimated to be negative before or just after admission to the hospital in every patient. Then HCV Ab changed to positive between at admission and 5 weeks after admission to the hospital in each patient. Furthermore, HCV RNA could be detected in every patient. Therefore, they were diagnosed as acute hepatitis caused by HCV infection. The genotypes of HCV were 1a in one patient (Patient A[Pt-A]), 1b in two patients (Pt-B and Pt-C), and 2a in one patient (Pt-D). The peak values of serum HCV load in four patients ranged from 160 KIU/mL to above 5000 KIU/mL. None of the four patients had a history of blood transfusion, intravenous drug use, tattoo or acupuncture.

The clinical characteristics of the spouses of patients studied are shown in Table 2. One patient was male and three patients were females and the ages ranged from 26 to 75 years old. By interview of family history, each spouse of the patients was revealed to be suffered from

© 2011 The Japan Society of Hepatology

Table 1 Clinical characteristics of patients studied

	Patient A	Patient B	Patient C	Patient D
Gender	Male	Male	Female	Male
Age at onset (years)	33	56	73	46
Peak value of ALT (U/L)	3000	1606	1054	4560
Peak value of T.bil (mg/dL)	6.9	23.8	5.2	3.6
HCV Ab	(−) → (+)	(−) → (+)	(−) → (+)	(−) → (+)
Time point of positive conversion (after admission to the hospital)	2 weeks	At admission	4 weeks	5 weeks
Peak value of HCV load (KIU/mL)	200	160	above 5000	above 5000
HCV genotype	1a	1b	1b	2a
Blood transfusion	(−)	(−)	(−)	(−)
Intravenous drug use	(−)	(−)	(−)	(−)
Tattoo	(−)	(−)	(−)	(−)
Acupuncture	(−)	(−)	(−)	(−)

ALT, alanine aminotransferase; HCV Ab, anti-HCV antibody; Tbil, total bilirubin.

chronic hepatitis C. Between 2002 and 2007, we took care of four acute hepatitis C patients in the hospital and HCV Ab in serum was estimated in each spouse of four patients. HCV Ab was positive in serum of every spouse of the acute hepatitis C patients between 2002 and 2007 in the hospital (4/4:100%) (Table 2). The values of serum HCV load in the spouses of four patients ranged from 730 KIU/mL to 4900 KIU/mL. The genotype of HCV in the spouse of Pt-A (Sp-A) was 1a. The genotype of HCV in the spouse of Pt-B (Sp-B) and in the spouse of Pt-C (Sp-C) was 1b. Therefore, HCV genotypes were identical in three couples. However, genotype of HCV in the spouse of Pt-D (Sp-D) was 1b, which was different from the genotype of Pt-D.

The sequences of partial HCV E1 region of the couple with HCV of genotype 1a (Pt-A/Sp-A) are shown in Figure 1a. The homogeneity of two nucleotide sequences was 99.1% and two isolates were essentially identical. The nucleotide sequences of partial HCV E1 region of the two couples with HCV of genotype 1b (Pt-B/Sp-B and Pt-C/Sp-C) were shown in Figure 1b. The homogeneity of the sequences of couple B was 100%, and that of couple C was 97.9%. Therefore, it was confirmed that two HCV strains of couple B and those of couple C were essentially identical, respectively. The results of phylogenetic analyses depending on the above sequences were shown in Figure 2. Two strains of couple A (Pt-A and Sp-A) clustered discretely from other genotype 1a strains and control sequences. Furthermore, two strains of couple B (Pt-B and Sp-B) clustered discretely from other genotype 1b strains and control sequences. In addition, two strains of couple C (Pt-C and Sp-C) clustered discretely from other genotype 1b strains and control sequences. The results of the phylogenetic analyses shown in Figure 2 suggested interspousal infection of HCV in couple A, B and C. On the other hand, interspousal infection of couple D with HCV of different genotype (2a and 1b) was denied.

Table 2 Clinical characteristics of spouses of patients studied

	Spouse of Patient A	Spouse of Patient B	Spouse of Patient C	Spouse of Patient D
Gender	Female	Female	Male	Female
Age (years)	26	54	75	43
HCV Ab	(+)	(+)	(+)	(+)
HCV load (KIU/mL)	730	above 850	4900	3800
HCV genotype	1a	1b	1b	1b
HCV genotype between spouses	identical	identical	identical	different

HCV Ab, anti-hepatitis C virus antibody.

© 2011 The Japan Society of Hepatology

```
(a)
CONS. 1a    1 TTTCTCTATCTTCCTTCTGGCCCTGCTCTCTTGCCTGACTGTGCCCGCTTCAGCCTACCAAGTGCGCAACTCCACGGGGC  80
Pt-A        1 ...T.................T..........................G..............A.........T....C.  80
Sp-A        1 ...T.................T..........................G..............A.........T....C.  80

CONS. 1a   81 TTTACCATGTCACCAATGATTGCCCTAACTCGAGTATTGTGTACGAGGCGGCCGATGCCATCCTGCACACTCCGGGGTGT 160
Pt-A       81 ..............................C.....................A....T...........A..TT..T........ 160
Sp-A       81 .............T.........T.C.....................A....T...........A..TT.......... 160

CONS. 1a  161 GTCCCTTGCGTTCGCGAGGGTAACGCCTCGAGGTGTTGGGTGGCGGTGACCCCCACGGTGGCCACCAGGGACGGCAAACT 240
Pt-A      161 ........................A.......A.........C.....G.......A........T.....T........ 240
Sp-A      161 ........................A.......A.........C.....G.......A........T.....T........ 240

CONS. 1a  241 CCCCACAACGCAGCTTCGACGTCACATCGATCTGCTTGTCGGGAGCGCCACCCTCTGTTCGGCCCTCTACGTGGGGGACC 320
Pt-A      241 ....G...................T.....C.......................T..C........T..T.........T 320
Sp-A      241 ....G...................T.....C.......................T..C........T..T.........T 320

CONS. 1a  321 TGTGCGGGTCTGTCTTTCTTGTCGGTCAACTGTTCACCTTCTCTCCCAGGCGCCACTGGACGACGCAAGGCTGCAATTGT 400
Pt-A      321 ................C....................T.....C......A.A........A..C....A......C... 400
Sp-A      321 ................C....................T.....C......A.A........A..C....A......C... 400

CONS. 1a  401 TCTATCTATCCCGGCCATATAACGGGTCACCGCATGGCA                                         439
Pt-A      401 ...G.G..C........C.....................                                         439
Sp-A      401 ...G....C........C.....................                                         439

(b)
CONS. 1b    1 TTTCTCTATCTTCCTCTTGGCTCTGCTGTCCTGTTTGACCATCCCAGCTTCCGCTTATGAAGTGCGCAACGTGTCCGGGGTGTACCATGTCACGAACGAC 100
Pt-B        1 ............................................................G..................A.A.................. 100
Sp-B        1 ............................................................G..................A.A.................. 100
Pt-C        1 .......................................................................C.......A.A.................. 100
Sp-C        1 ..............................T................Y.......................C.......A.A.................. 100

CONS. 1b  101 TGCTCCAACTCAAGCATTGTGTATGAGGCAGCGGACATGATCATGCATACCCCCGGGTGCGTGCCCTGCGTTCGGGAGAACAACTCCTCCCGTTGCTGGG 200
Pt-B      101 ............................................................A....................................... 200
Sp-B      101 ............................................................A....................................... 200
Pt-C      101 ....................................T............G............................GG..TA................ 200
Sp-C      101 ....................................T............G............................G....W................ 200

CONS. 1b  201 TAGCGCTCACTCCCACGCTCGCGGCCAGGAACACCAGCGTCCCCACTACGACAATACGACGCCACGTCGATTTGCTCGTTGGGGCGGCTGCTTTCTGCTC 300
Pt-B      201 ...................A...........TG.....A.T.........G..........G..T................................... 300
Sp-B      201 ...................A...........TG.....A.T.........G..........G..T................................... 300
Pt-C      201 ...................T............T................A....................C.....T...............C....... 300
Sp-C      201 ...................T............T................R....................C.....Y...............C....... 300

CONS. 1b  301 CGCTATGTACGTGGGGGATCTCTGCGGATCTGTTTTCCTCGTCTCCCAGCTGTTCACCTTCTCGCCTCGCCGGCATGAGACAGTACAGGACTGCAATTGC 400
Pt-B      301 T................................................................................T.................. 400
Sp-B      301 T................................................................................T.................. 400
Pt-C      301 .....................T................................T..........................G..............C..T 400
Sp-C      301 .....................T.........................................................G..............C... 400

CONS. 1b  401 TCAATCTATCCCGGCCACGTATCAGGTCACCGCATGGCT                                                             439
Pt-B      401 ........C..T.....TT.G........T.........                                                             439
Sp-B      401 ........C..T.....TT.G........T.........                                                             439
Pt-C      401 .......................................                                                             439
Sp-C      401 .......................................                                                             439
```

Figure 1 Nucleotide sequences of hepatitis C virus (HCV) E1 region of patients and the spouses. (a) Nucleotide sequences of HCV E1 region of Patient A [Pt-A] and the spouse [Sp-A] (Genotype 1a). (b) Nucleotide sequences of HCV E1 region of Pt-B/Sp-B and Pt-C/Sp-C (Genotype 1b). The nucleotide that is not identical between spouses is shown by the box.

© 2011 The Japan Society of Hepatology

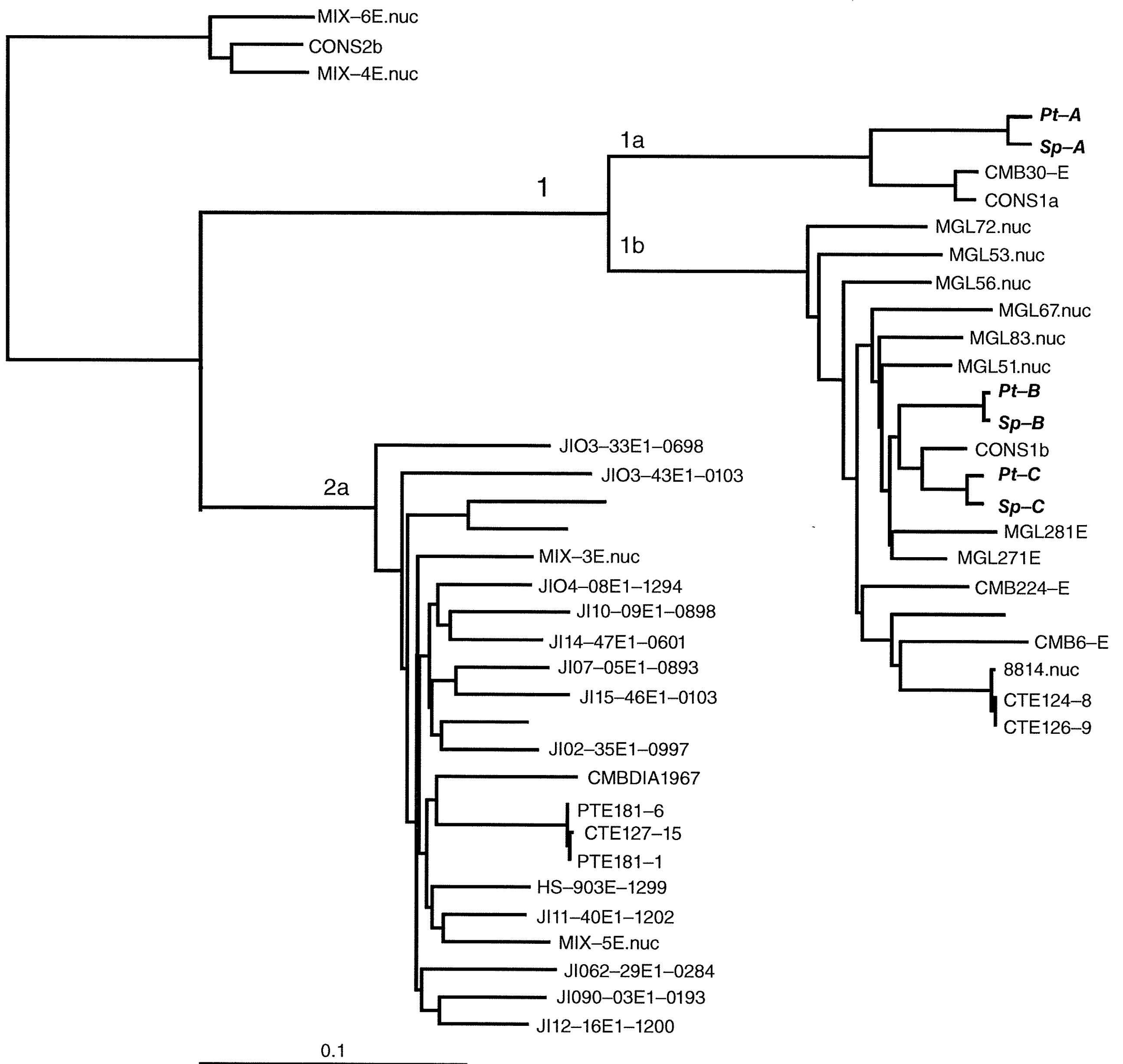


Figure 2 Phylogenetic tree based on the nucleotide sequences of hepatitis C virus (HCV) E1 region from acute hepatitis C patients and their spouses. The distance scale represents the number of nucleotide substitution per position.

DISCUSSION

IN THIS STUDY, four patients with acute hepatitis C were reported. In some cases, the differential diagnosis of acute hepatitis C and acute exacerbation of HCV carrier might be difficult. In our study, HCV Ab in serum was estimated to be negative before or just after the admission to the hospital in every patient. Then, HCV Ab changed from negative to positive in the course of hospitalization and HCV RNA could be detected in every patient. Therefore, they were diagnosed as acute hepatitis caused by HCV infection. Additionally, HCV Ab and HCV RNA in serum were positive in every spouse of four patients. Three of four couples had HCV RNA with the identical genotype. Homogeneiety of nucleotide sequences of HCV E1 region in three couples

© 2011 The Japan Society of Hepatology

ranged from 97.9% to 100%. Furthermore, the results of phylogenetic analyses suggested that interspousal HCV infection occurred in the three couples. As previously reported,[12,13] the usefulness of phylogenetic analysis of nucleotide sequences of HCV E1 region for clarifying interspousal HCV infection was validated.

To clarify the route of HCV transmission in acute hepatitis C patient, interview of the patient on past history such as surgical operation, blood transfusion, transfusion of blood product, and needle stick exposure, life history such as intravenous drug use, tattoo, acupuncture, and homosexual and heterosexual contact, and family history including that of spouse was thought to be valuable. In addition, examination of HCV Ab in serum of spouse or sexual partner of the patient might be useful. In our study, every spouse of four patients of acute hepatitis C was revealed to be HCV Ab positive and HCV RNA positive in serum. Furthermore, interspousal HCV infection in three couples was strongly suggested by phylogenetic analyses depending on the nucleotide sequences of HCV E1 region.

Hepatitis C virus transmission between spouses occurred by either sexual or non-sexual routes. Non-sexual transmission of HCV between spouses was established in health care setting such as shared use of personal hygiene items included toothbrushes, razor blades and nail clippers. The frequency and importance of sexual transmission of HCV have been controversial and it is difficult to define the related risk adequately.[14–38] In fact, multiple risk factors promote HCV acquisition and may be present in a single individual.[39]

On the frequency of intrafamilial and interspousal HCV infection, Ackerman *et al.* reported review by the meta-analysis depending on the data of 67 papers published up to 2000.[6] In uncontrolled studies (54 publications: 50 full papers and four letters to the editor), the pooled prevalence of HCV Ab among 4250 stable sexual contacts of patients with HCV-related chronic liver disease (CLD) was shown to be 13.48%. Also, the calculated pooled prevalence of HCV Ab in the non-sexual contacts of the different subgroups ranged from 1.14% in siblings, 2.67% in offspring and household contacts of pediatric CLD patients to 10.96% in parents of the same patient population. Furthermore, in controlled studies (13 publications: 11 full papers and two letters to the editor), the pooled prevalence of HCV Ab among 175 siblings and household contacts of patients with CLD was 4.0% compared with 0% among 109 contacts of anti-HCV-negative controls (odds ratio [OR] 9.75).

On the importance of sexual transmission of HCV, Tohme *et al.* analyzed 80 qualifying reports regarding the evidence for or against sexual transmission published between 1995 and 2009, based on the study design, representativeness of the study population, and the methods used for case ascertainment.[8] They reported that there was no increased risk of sexual transmission of HCV among heterosexual couples in regular relationships and that this risk increased among persons with multiple sexual partners (adjusted odds ratio [aOR] 2.2–2.9). In addition, they reported that there appeared to be a real increased risk for women coinfected with human immunodeficiency virus (HIV) or other sexually transmitted infections (aOR 3.3–3.9).

For differential diagnosis of sexual transmission and non-sexual transmission of HCV between spouses, interview of hepatitis C patients on several points such as duration of marriage, the rate of sexual transmission, mucosal trauma/genital ulcerative disease, and health care setting such as shared use of personal hygiene items included toothbrushes, razor blades and nail clippers was considered to be essential. It was suggested that sexual exposure to HCV in the spouse of HCV carrier should be considered only after an accurate exclusion of other routes of interspousal spread of the infection. In the present study, there was no risk of HCV infection in the healthcare setting such as shared use of personal hygiene items included toothbrushes, razor blades and nail clippers in the couple A, B and C. In addition, bleeding or laceration during sexual intercourse had occurred in three couples.

There was a report of a case in that interspousal HCV transmission occurred by non-sexual route.[7] That was a case of acute hepatitis C progressing to chronic hepatitis over a follow-up of 4 years in a 44-year-old woman having a long-standing monogamous relationship with an HCV infected partner. The infection followed the accidental percutaneous exposure to her husband's contaminated blood containing a high viral load by needle stick injury with the tip of the needle used by her husband to measure capillary glycemia. The importance of interview on several factors related to non-sexual HCV transmission in both healthcare setting and outside healthcare setting was confirmed.

The possibility of sexual transmission of HCV infection is supported by the isolation of HCV RNA from semen and uterus cervical smears in some studies.[40–44] The prevalence of HCV RNA in body fluid was reported to be 29% (6/21) in seminal plasma, and 31% (5/16) in uterus cervical smears.[44] The presence of HCV in semen and cervical smears might establish sexual transmission

© 2011 The Japan Society of Hepatology

of HCV. Actually the case of acute hepatitis C, who acquired HCV during an accidental or opportunistic vaginal sexual liaison was reported.[13] The case was in the absence of ongoing co-habitation, in the absence of bleeding or laceration during intercourse. She was a 62-year-old woman and acquired acute hepatitis C virus infection after heterosexual contact with a known HCV positive man. There were no known other sexual or non-sexual risk factors for HCV acquisition. Phylogenetic analysis confirmed the case and index were infected with identical genotype 3a strains, consistent with heterosexual transmission in the absence of specific risk factors.

In the present study, interspousal HCV infection of HCV was strongly suggested in three patients of four acute hepatitis C patients by the result of phylogenetic analysis depending on nucleotide sequences of HCV E1 region. Furthermore, sexual and non-sexual transmission of HCV was discussed. In conclusion, interspousal infection might be one of the important sources of acute HCV infection in Japan. In addition, the usefulness of phylogenetic analysis of nucleotide sequences of HCV partial E1 region for clarifying interspousal HCV infection was validated.

ACKNOWLEDGMENTS

THIS STUDY WAS supported in part by grants from the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labor and Welfare of Japan.

REFERENCES

1 WHO. Global surveillance and control of hepatitis C. Report of WHO consultation organized with the Viral Hepatitis Prevention Board, Antwerp, Belgium. *J Viral Hepat* 1999; **6**: 35–47.
2 Tibbs CJ. Methods of transmission of hepatitis C. *J Viral Hepat* 1995; **2**: 113–19.
3 Alter MJ. Transmission of hepatitis C virus – route, dose and titer. *N Engl J Med* 1994; **330**: 784–6.
4 Seeff LB, Alter HJ. Spousal transmission of the hepatitis C virus. *Ann Intern Med* 1994; **120**: 807–9.
5 McCashland TM, Schafer DF. Hepatitis C. Sexually exposed? *Am J Gastroenterol* 1996; **91**: 2069–70.
6 Ackerman Z, Ackerma E, Paltiel O. Intrafamilial transmission of hepatitis C virus: a systematic review. *J Viral Hepat* 2000; **7**: 93–103.
7 Orlando R, Lirussi F. Hepatitis C virus infection: sexual or non-sexual transmission between spouses? A case report and review of the literature. *Infection* 2007; **35**: 465–8.
8 Tohme RA, Holmberg SD. Is sexual contact a major mode of hepatitis C virus transmission? *Hepatology* 2010; **52**: 1497–505.
9 Ohno T, Mizokami M. Genotyping by type-specific primers that can type HCV types 1–6. *Methods Mol Med* 1999; **19**: 159–64.
10 Tanaka Y, Hanada K, Mizokami M *et al.* A comparison of the molecular clock of hepatitis C virus in the United States and Japan predicts that hepatocellular carcinoma incidence in the United States will increase over the next two decades. *Proc Natl Acad Sci U S A* 2002; **99**: 15584–9.
11 Kumar S, Tamura K, Nei M. MEGA: Molecular Evolutionary Genetics Analysis software for microcomputers. *Comput Appl Biosci* 1994; **10**: 189–91.
12 Nakayama H, Sugai H, Ikeya S *et al.* Molecular investigation of interspousal transmission of hepatitis C virus in two Japanese patients who acquired acute hepatitis C after 40 or 42 years of marriage. *J Med Virol* 2005; **75**: 258–66.
13 Nguyen O, Shappeard V, Douglas MW *et al.* Acute hepatitis C infection with evidence of heterosexual transmission. *J Clin Virol* 2010; **49**: 65–8.
14 Alter MJ, Hadler SC, Judson FN *et al.* Risk factors for acute non-A non-B hepatitis in the United States and association with hepatitis C virus infection. *JAMA* 1990; **264**: 2231–5.
15 Fried MW, Shindo M, Fong TL *et al.* Absence of hepatitis C viral RNA from saliva and semen of patients with chronic hepatitis C. *Gastroenterology* 1992; **102**: 1306–8.
16 Kotwal GJ, Rustgi VK, Baroudy BM. Detection of hepatitis C virus-specific antigens in semen from non-A. Non-B hepatitis patients. *Dig Dis Sci* 1992; **37**: 641–4.
17 Akahane Y, Kojima M, Sugai Y *et al.* Hepatitis C virus infection in spouses of patients with type C chronic liver disease. *Ann Intern Med* 1994; **120**: 748–52.
18 Chang TT, Liou TC, Young XZ *et al.* Intrafamilial transmission of hepatitis C virus: the important role of inapparent transmission. *J Med Virol* 1994; **42**: 91–6.
19 Kao JH, Hwang YT, Chen PJ *et al.* Transmission of hepatitis C between spouses: the importance role of exposure duration. *Am J Gastroenterol* 1996; **5**: 2087–90.
20 Caporaso N, Ascione A, Stroffolini T *et al.* Spread of hepatitis C virus infection within families. *J Viral Hepat* 1998; **5**: 67–73.
21 Guadagnino V, Stroffolini T, Foca A *et al.* Hepatitis C virus infection in family setting. *Eur J Epidemiol* 1998; **14**: 229–32.
22 Van Damme P, Vellinga A. Epidemiology of hepatitis B and C in Europe. *Acta Gastroenterol Belg* 1998; **61**: 175–82.
23 Neumayr G, Propst A, Schwaighofer H *et al.* Lack of evidence for the heterosexual transmission of hepatitis C. *Q J Med* 1999; **2**: 505–8.
24 Sun CA, Chen HC, Lu CF *et al.* Transmission of hepatitis C virus in Taiwan: prevalence and risk factors based on a nationwide survey. *J Med Virol* 1999; **59**: 290–6.
25 Wejstal R. Sexual transdon of hepatitis C virus. *J Hepatol* 1999; **31**: 92–5.

© 2011 The Japan Society of Hepatology

26 Kao JH, Liu CJ, Chen PJ *et al*. Low incidence of hrepatitis C virus transmission between spouses: a prospective study. *J Gastroenterol Hepatol* 2000; **15**: 391–5.
27 Gross JB. Hepatitis C: a sexually transmitted disease? *Am J Gastroenterol* 2001; **96**: 3051–3.
28 Stroffolini T, Lorenzoni U, Menniti-Ippolito F *et al*. Hepatitis C virus infection in spouses: Sexual transmission or common exposure to the same risk factors? *Am J Gastroenterol* 2001; **96**: 3138–41.
29 Yee LJ, Weiss HL, Langner RG *et al*. Risk factors for acquisition of hepatitis C virus infection: a case series and potential implications for disease surveillance. *BMC Infect Dis* 2001; **1**: 8–13.
30 Alter MJ. Prevention of spread of hepatitis C. *Hepatology* 2002; **36**: 593–8.
31 Mazoff CD. Re: J Gross (editorial) Hepatitis C: a sexually transmitted disease? *Am J Gastroenterol* 2002; **97**: 1256–7.
32 Memon MI, Memon MA. Hepatitis C: an epidemiological review. *J Viral Hepat* 2002; **9**: 84–100.
33 Sulkowski MS, Stuart CR, Thomas DL. Needlestick transmission of hepatitis C. *JAMA* 2002; **287**: 2406–13.
34 Henderson DK. Managing occupational risks for hepatitis C transmission in the health care setting. *Clin Microbiol Rev* 2003; **316**: 546–68.
35 Marincovich B, Castilla J, Del Romero J *et al*. Absence of hepatitis C virus transmission in a prospective cohort of heterosexual serodiscordant couples. *Sex Transm Dis* 2003; **79**: 160–2.
36 Goldberg D, Anderson E. Hepatitis C: who is at risk and how do we identify them? *J Viral Hepat* 2004; **11**: 12–18.
37 Vandelli C, Renzo F, Romanò L *et al*. Lack of evidence of sexual transmission of hepatitis C among monogamous couples: results of a 10-year prospective follow-up study. *Am J Gastroenterol* 2004; **99**: 855–9.
38 Tahan V, Karaca C, Yildrim B *et al*. Sexual transmission of HCV between spouses. *Am J Gastroenterol* 2005; **100**: 821–4.
39 Karaca C, Cakaloglu Y, Denir K *et al*. Risk factors for the transmission of hepatitis C virus infection in the Turkish population. *Dig Dis Sci* 2006; **51**: 365–9.
40 Leruez-Ville M, Kunstmann JM, De Almeida M *et al*. Detection of hepatitis C virus in the semen of infected men. *Lancet* 2000; **356**: 42–3.
41 Manavi M, Watkins-Riedel T, Kucera E *et al*. Evidence of hepatitis C virus in cervical smears. *J Infect* 1999; **38**: 60–1.
42 Nyamathi A, Robbins WA, Fahey JL *et al*. Prevalence and predictors of hepatitis C virus RNA in the semen of homeless men. *Biol Res Nurs* 2002; **4**: 22–30.
43 Blackard JT, Smeaton L, Hiasa Y *et al*. Detection of hepatitis C virus (HCV) in serum and peripheral-blood mononuclear cells from HCV-monoinfected and HIV/HCV-coinfected persons. *J Infect Dis* 2005; **192**: 258–65.
44 Farias A, Re V, Mengarelli S *et al*. Detection of hepatitis C virus (HCV) in body fluids from HCV monoinfected and HCV/HIV coinfected patients. *Hepato-Gastroenterology* 2010; **57**: 300–4.

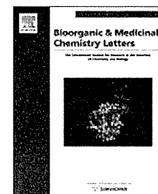
© 2011 The Japan Society of Hepatology

Bioorganic & Medicinal Chemistry Letters 21 (2011) 6328–6331

Contents lists available at SciVerse ScienceDirect

Bioorganic & Medicinal Chemistry Letters

journal homepage: www.elsevier.com/locate/bmcl



Antiviral activity of novel 2′-fluoro-6′-methylene-carbocyclic adenosine against wild-type and drug-resistant hepatitis B virus mutants

Jianing Wang[a], Uma S. Singh[a], Ravindra K. Rawal[a], Massaya Sugiyama[b], Jakyung Yoo[a], Ashok K. Jha[a], Melissa Scroggin[a], Zhuhui Huang[c], Michael G. Murray[c], Rajgopal Govindarajan[a], Yasuhito Tanaka[b], Brent Korba[d], Chung K. Chu[a,*]

[a] The University of Georgia, College of Pharmacy, Athens, GA 30602, USA
[b] Nagoya City University, Graduate School of Medical Sciences, Nagoya 467-8601, Japan
[c] Southern Research Institute, Frederick, MD 21701, USA
[d] Georgetown University Medical Center, Washington, DC 20057, USA

ARTICLE INFO

Article history:
Received 29 June 2011
Revised 25 August 2011
Accepted 26 August 2011
Available online 1 September 2011

Keywords:
Carbocyclic nucleoside
anti-HBV
Drug-resistant

ABSTRACT

Novel 2′-fluoro-6′-methylene-carbocyclic adenosine (**9**) was synthesized and evaluated its anti-HBV activity. The titled compound demonstrated significant antiviral activity against wild-type as well as lamivudine, adefovir and double lamivudine/entecavir resistant mutants. Molecular modeling study indicate that the 2′-fluoro moiety by a hydrogen bond, as well as the van der Waals interaction of the carbocyclic ring with the phenylalanine moiety of the polymerase promote the positive binding, even in the drug resistant mutants.

Published by Elsevier Ltd.

Chronic hepatitis B virus (HBV) infection is one of the leading causes of morbidity and mortality worldwide. Chronic infection with HBV occurs in approximately 350 million of the world population, including 1.7 million in the USA.[1] HBV infection can persist for the life of the host, often leading to severe consequences such as liver failure, cirrhosis and eventually hepatocellular carcinoma, resulting in annually 0.5–1.2 million deaths worldwide.[2] HBV is an incomplete double-stranded DNA virus. Its DNA replication is unique because it includes a reverse transcription step. The HBV DNA polymerase/reverse transcriptase is an essential and multifunctional enzyme, which operates as a DNA polymerase/reverse transcriptase, an RNAse H, through coordinating the assembly of viral nucleocapsids, as well as catalyzing the generation of DNA primers.[3] Nucleoside analogues can suppress HBV replication by inhibiting the viral polymerase/reverse transcriptase. The pivotal role of nucleoside/nucleotide analogues such as lamivudine, adefovir, telbivudine, entecavir, clevudine, and tenofovir has been demonstrated by their therapeutic efficacy in clinical practice. However, long-term therapy with these drugs is often associated with viral resistance, which significantly compromises the clinical application of these agents. For example, the extensive use of lamivudine resulted in the emergence of mutants that are resistant to the anti-HBV activity; 24% after a 1-year therapy, increasing to over 70% after 4 years of therapy. Adefovir has been used for the patients, who develop lamivudine-resistant mutants, however, a significant number of patients (29% after 5 years of use) also develop the adefovir resistant mutant (N236T).

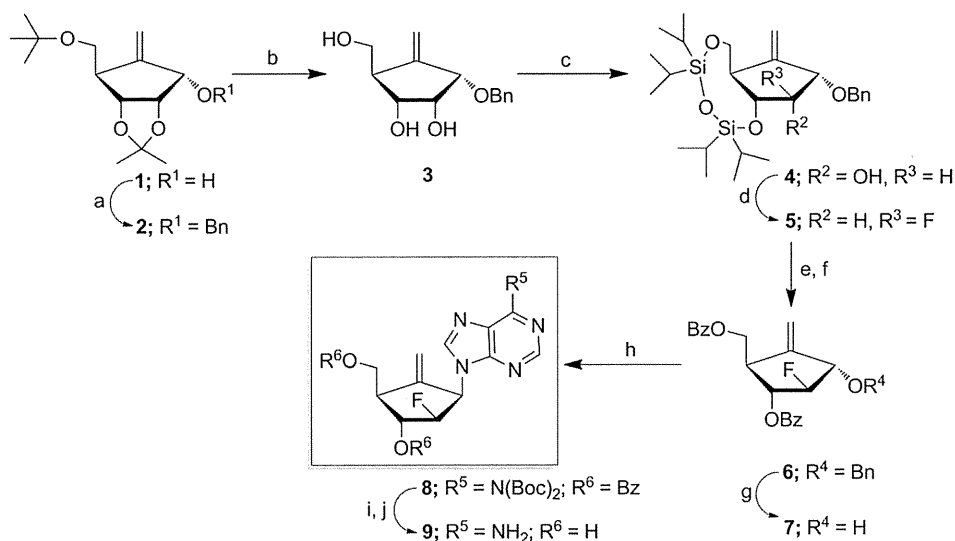
Entecavir is a carbocyclic 2′-deoxyguanosine analog that demonstrates potent anti-HBV activity[4] and is recommended for patients with the wild-type strain as well as for those patients harboring lamivudine-resistant strains.[5] However, a recent study by Tanaka and his co-workers suggest that the viral breakthrough was observed in the lamivudine-refractory group in 4.9% of patients at baseline and increase to 14.6%, 24% and 44.8% at weeks 48, 96 and 144, respectively.[6]

In view of the fact that currently adefovir and entecavir are the most prescribed drugs for the treatment of chronic HBV infection, it is critical to discover the agents that do not confer cross-resistance with the adefovir and lamivudine/entecavir-mutants for the future treatment of drug resistant patients. In this report we try to demonstrate that our newly discovered compound **9** may potentially play a significant role for that purpose.

Carbocyclic nucleosides are an interesting class of compounds in which the methylene group replaces the oxygen atom of a furanose ring. As a consequence, the glycosidic bond is resistant to nucleoside phosphorylase as well as nucleoside hydrolase, which makes the carbocyclic nucleosides more stable towards metabolic

* Corresponding author. Address: Department of Pharmaceutical and Biomedical Sciences, College of Pharmacy, The University of Georgia, Athens, GA 30602, USA. Tel.: +1 706 542 5379; fax: +1 706 542 5381.
E-mail addresses: DCHU@rx.uga.edu, DCHU@mail.rx.uga.edu (C.K. Chu).

0960-894X/$ - see front matter Published by Elsevier Ltd.
doi:10.1016/j.bmcl.2011.08.113



Scheme 1. Synthesis of target compound **9**. Reagents and conditions: (a) NaH, BnBr, DMF, 0 °C; (b) TFA/H_2O (2:1), 50 °C; (c) $TIDPSCl_2$/imidazole, DMF, 0 °C; (d) DAST, CH_2Cl_2, rt; (e) TBAF/AcOH, THF, rt; (f) BzCl, pyridine, rt; (g) BCl_3, CH_2Cl_2, −78 °C; (h) *N*,*N*-dibocprotected adenine, DIAD, Ph_3P, THF, 0 °C; (i) TFA, CH_2Cl_2, rt; (j) DIBAL-H, CH_2Cl_2, −78 °C.

degradation.[7] Due to these features, carbocyclic nucleosides have received much attention as potential chemotherapeutic agents.[8] Carbovir and entecavir are examples of results of these efforts.

It is also well known that incorporation of a fluorine atom at the 2′-position of nucleosides can increase the stability of the glycosyl bond towards chemical and metabolic degradation.[9,10] A fluorine substitution on the carbocyclic sugar moiety has been proven to be useful in producing effective antiviral agents as demonstrated by our group in 2′-fluoro-5-methyl-β-L-arabinofuranosyluracil (L-FMAU or clevudine)[11] as well as in clofarabine.[12]

In view of the 2′-F substitution[9,10] as well the introduction of an exocyclic double bond to carbocyclic nucleosides,[4] which have been beneficial for anti-HBV activity such as in entecavir, 2′-fluoro-6′-methylene-carbocyclic adenosine or (+)-9-[(1*R*,2*R*,3*R*,4*R*)-2-fluoro-3-hydroxy-4-(hydroxymethyl)-5-methylenecyclopentan-1-yl] adenine **9** was synthesized and evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir (double)-resistant mutants in vitro.

The synthesis of the target nucleoside **9** commenced with compound **7** as the key intermediate (Scheme 1). Compound **1** was synthesized according to the reported procedure from our group.[13] The allylic hydroxyl group of **1** was protected with a benzyl group and subsequent deprotection of the acetonide and the *t*-butyl group of compound **2** gave **3** in 86% yield. The 3, 5-hydroxy groups of **3** were selectively protected with 1,3-dichloro-1,1,2,2-tetraisopropyl disilazine to give **4** in 95% yield. Transformation of the 2-β-hydroxyl group to 2-α-fluoro was accomplished by treating the alcohol **4** with DAST to give 47% yield of compound **5**. However, debenzylation of **5** was unsuccessful under the Birch reduction or the Lewis acid (BCl_3) conditions. Therefore, the silyl group of **5** was removed by using tetrabutyl ammonium fluoride (TBAF/HOAc) to yield 82% of a diol, which was re-protected by benzoyl chloride in pyridine to give the fully protected intermediate **6** in 86% yield. The compound **6** was then treated with BCl_3 at −78 °C to obtain the key intermediate **7** in 76% yield. *N*,*N*-diboc protected adenine was synthesized according to the reported protocol in literature[14] and condensed with **7** to obtain **8** in 51% yield. The deprotection of the Boc group was carried out by TFA to afford 82% yield. Eventually, the treatment of DIBAL-H gave the target compound **9**[15] in 76% yield.

The synthesized nucleoside **9** was evaluated for its antiviral activity against wild-type HBV as well as adefovir, lamivudine and lamivudine/entecavir-drug resistant mutants in vitro,[16] and the results are summarized in Table 1. As the compound **9** is a derivative of an adenine analog, we directly compared its antiviral activity to that of adefovir instead of entecavir (a guanine analog) although the carbocyclic moiety is similar to that of entecavir. Furthermore, compound **9**, an adenine analogue, can interact with the thymidine moiety in the DNA template–primer site while entecavir interacts with the cytosine moiety at the same site in the active site. Thus, the base moiety is the major deciding factor, not the sugar moiety in determining the mode of action.

The target compound **9** demonstrated a significant antiviral in vitro activity against wild-type (WT) HBV with an EC_{50} value of 1.5 μM. The antiviral potency was similar to that of adefovir, while being 7-fold less potent than lamivudine. However, the concentration of the compound **9** required to inhibit 90% (EC_{90}) of wild-type HBV is 4.5 μM, which is 1.5-fold more potent than adefovir (EC_{50} 7.1 μM; Table 1).

The compound **9** also showed excellent activity against both lamivudine and adefovir resistant HBV mutants.[17] Particularly, the compound **9** showed a 4.5-fold enhanced potency of EC_{50} (1.7 μM) and a 7.8-fold more favorable EC_{90} (4.6 μM) against adefovir mutant rtN236T. For lamivudine mutants, rtM204V and rtM204I, the compound **9** showed an EC_{50} value of 1.8 versus 1.6 μM for adefovir, and 1.0 versus 1.9 μM for compound **9** and adefovir, respectively, while in the EC_{90} value, compound **9** demonstrated more favorable anti-HBV activity for both mutants, rtM204 V (4.7 vs 7.0 μM) and rtM204I (5.0 vs 8.0 μM). For mutant rtL180M, the antiviral activity of compound **9** was similar to that of lamivudine in the EC_{50} 2.1 versus 1.5 μM, while the compound **9** exhibited a 4.3-fold increased antiviral activity in the EC_{90} value (5.1 vs 22.0 μM).

Compound **9** was also evaluated against the lamivudine double mutant, rtL180M/rtM204V, and it exhibited the EC_{50} 2.2 μM that was equal to the adefovir, while the EC_{90} value of 5.5 μM of compound **9** was more effective than that of adefovir (8.5 μM). In addition, deamination studies with adenosine deaminase from calf thymus indicated that the compound **9** was completely stable.[18]

In preliminary studies, compound **9** was also evaluated against lamivudine/entecavir double resistant clone (L180M + S202I + M202V), in which compound **9** demonstrated significant anti-HBV activity (EC_{50} 0.67 μM) against the mutant. In the case of lamivudine and entecavir, there are significant decrease in their

Table 1
In vitro anti-HBV activity against adefovir, lamivudine and entecavir drug-resistant mutants in the intracellular HBV DNA replication assay[16,17]

Strains	Compound **9** (μM)				Adefovir (μM)			Lamivudine (μM)			Entecavir (μM)		
	EC_{50}[b]	EC_{90}[c]	CC_{50}[d,e]	Fold resistance[f] (EC_{90})	EC_{50}	EC_{90}	Fold resistance (EC_{90})	EC_{50}	EC_{90}	Fold resitance (EC_{90})	EC_{50}	EC_{90}	CC_{50}
Wild Type	1.5	4.5	>100	–	1.3	7.1	–	0.2	0.6	–	0.008	0.033	28
rtM204V	1.8	4.7	>100	1.0	1.6	7.0	1.0	>100	>100	>166	NT[h]	NT	NT
rtM204I	1.0	5.0	>100	1.1	1.9	8.0	1.1	>100	>100	>166	NT	NT	NT
rtL180M	2.1	5.1	>100	1.1	5.5	7.7	1.1	1.5	22.0	36.7	NT	NT	NT
rtLM/rtMV[a]	2.2	5.5	>100	1.2	2.1	8.5	1.2	>100	>100	>166	NT	NT	NT
rtN236T	1.7	4.6	>100	1.0	7.8	36.0	5.1	0.2	0.9	1.5	NT	NT	NT
rtLM/rtMV/rtSG[g]	0.67	NT	NT	–	NT	NT	–	>500[i]	NT	–	1.20[j]	NT	NT

[a] rtLM/rtMV = rtL180M/rtM204V double mutant.
[b] Effective concentration required to inhibit 50% of HBV-DNA.
[c] Concentration required to reduce infectious virus titer by 90%.
[d] The > sign indicates that the 50% inhibition was not reached at the highest concentration tested.
[e] The drug concentration required to reduce the cellular viability by 50% as assayed by an MTT assay.
[f] Fold resistance = (mutant EC_{90})/(wt EC_{90}).
[g] rtLM/rtMV/rtSG = rtL180M/rtM204V/rtS202G.
[h] NT = not tested.
[i] Ref. 19.
[j] Ref. 20.

antiviral potency (EC_{50} > 500 and 1.2 μM, respectively) as shown in Table 1.[19,20]

It was of interest to know how the compound **9** demonstrated the favorable anti-HBV activity in comparison to that of adefovir. Therefore, molecular modeling studies were conducted to obtain the insight of the molecular mechanism of compound **9** by using the Schrodinger suite.[21] The homology model of HBV RT was constructed based on the published X-ray crystal structure of HIV reverse transcriptase (PDB code: 1RTD),[22] which was previously used for molecular mechanism studies of several anti-HBV nucleosides.[23] In the homology model of HBV polymerase, the relative position of α-, β- and γ-phosphates of compound **9** with respect to the catalytic triad were assumed to occupy the similar position to the dNTP in the crystal structure of the HIV-1 RT-DNA-dNTP complex. The molecular docking[24] of compound **9** shows that the triphosphate forms all the network of hydrogen bonds with the active site residues, S85, A86, A87, R41, K32 (Fig. 1a). The γ-phosphate of compound **9** retains a critical H-bonding with the OH of S85 with connection of hydrogen bonds between S85 and N236. Generally, the N236T mutant loses the hydrogen bond to S85, which results in destabilization of the S85 to γ-phosphate interaction, thus causes resistance. However, compound **9** (as its triphosphate) maintains a critical H-bonding with S85 (Fig. 1b) similar to that as observed in wild type HBV (Fig. 1a).

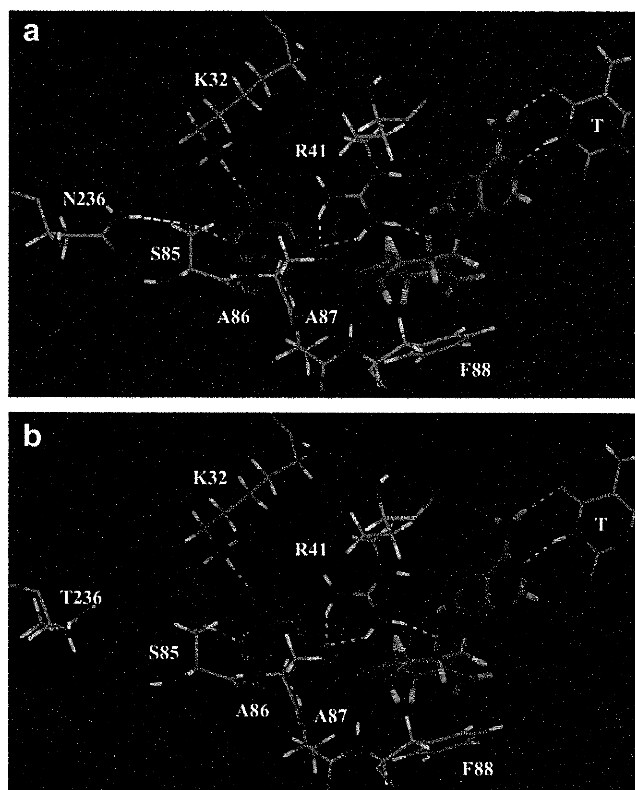


Figure 1. Binding mode and van der Waals interaction of compound **9** (a) in wild-type HBV and (b) in N236T adefovir mutant HBV. Yellow dotted lines are hydrogen bonding interactions (<2.5 Å).

The carbocyclic ring with an exocyclic alkene of compound **9** occupies the hydrophobic pocket (residues F88, L180 and M204) and makes the favorable van der Waals interaction with F88 (Fig. 1a and b). The 2′-fluorine substituent in the carbocyclic ring of compound **9** appears to promote an additional binding with R41 as shown in Figure 1a and b, which corroborates with the antiviral activity of compound **9** shown in Table 1. Overall, the modeling studies can qualitatively explain the favorable anti-HBV activity of the newly discovered compound **9** in WT (Fig. 1a) as well as against adefovir resistant mutant, N236T (Fig. 1b). These modeling studies are qualitative, and therefore, more quantitative calculation is warranted in the future.

In summary, a novel carbocyclic adenosine derivative **9** was synthesized, and evaluated for its anti-HBV activity. From these studies, the target nucleoside demonstrated significant anti-HBV activity against both the wild-type as well as the major nucleoside-resistant HBV mutants (adefovir and lamivudine), including the lamivudine/entecavir double mutant. In view of these promising anti-HBV activities, further biological and biochemical studies of the nucleoside **9** is warranted to assess the full potential as an anti-HBV agent.

Acknowledgments

This research was supported by the U.S. Public Health Service Grant AI-25899 (C.K.C.), NO1-AI-30047 (Z.H. & M.G.M.), NOI-AI-30046 (B.K.) from the National Institute of Allergy and Infectious Diseases, NIH and grant from the National Research Foundation of Korea Grant (JKY) (NRF-2009-352-E00065).