positivity rate was 20.2%, 10.7%,4.0%, and 2.8–7%, respectively, while in urban and rural Malaysia, it was 2% and 44–50%, respectively [27]. Compared to these data, the rate of anti-HEV IgG positivity detected in this study is slightly higher than average. There might be possible selection bias because we could not perform the random sampling. However, the screening for hepatitis virus infections does not conducted among the general population in Cambodia. On the other hand, the sensitivity of detection of anti-HEV IgG differs according to the assay. We detected by the kit used the purified recombinant HEV ORF2 protein in EIA same as the method described previously [28]. The results of the detection of anti-HEV IgG using this recombinant HEV ORF2 protein showed that the positivity of anti-HEV IgG was as high as 98% among the totally 57 acute hepatitis samples with positive for HEV RNA (56/57 samples: anti-HEV IgM/IgG +/+, HEV RNA +) [28–30](unpublished data). Furthermore, the sensitivity of the assay was not depend on genotype, and it also showed the high positivity among the swine [31]. Therefore, we considered that the sensitivity of detection of anti-HEV IgG by our used assay was high.

Our present results demonstrate that men have a significantly higher rate of HEV infection than women; similar association has been observed in Japan among blood donors [25], and reported by the National Epidemiological Surveillance of Infectious Diseases [32]. Moreover, similar correlation has been shown in the United Kingdom [33, 34], the United States [35], and China [36]. The reason for such an association is not clear; given that HEV infection is primarily transmitted via the oral route, it can be hypothesized that game meat consumption and other food preferences may be involved.

Higher prevalence of anti-HEV IgG in older age groups indicates age-related risk. Even after the adjustment for other factors, older people demonstrate significantly higher rates of anti-HEV IgG positivity, if the 7–19-year-old population group is taken as baseline. In Vietnam [26] and Indonesia [37], it has been reported that the rate of anti-HEV positivity rises with age. Conceivable reason for the correlation of anti-HEV IgG positivity with age is because currently occurring new infections cause the number of people with a prior history of HEV infection to increase with age. Although the period when HEV RNA is detectable in the blood after HEV infection is brief constituting 28.3 days [38], two HEV RNA-positive participants (0.23%: 2/868 [95%CI: 0–0.55%]) were identified in our cross-sectional study of 868 people. Assuming that HEV RNA detection period is about four weeks, HEV incidence is estimated at 3.00/100 person-years (0–7.2/100 person-years). Among rural Chinese population, the rate of anti-HEV IgG positivity was 38%, with the incidence of 2.8/10,000 person-years [36], and in rural Bangladesh regarded as HEV endemic region, the prevalence of anti-HEV total Ig was 22.5% and the incidence was 63.9/1,000 person-years [39]. Compared to these data, HEV incidence determined in our study can be regarded as high, indicating that infection control measures are required.

HEV infection is often foodborne in developed countries, including Japan and Europe [4, 6]; in developing countries, contaminated water is considered to be a major cause [40–42]. Thus, there has been an outbreak of HEV infection in southwestern Vietnam along the Hau river, adjacent to Cambodia [43], and HEV has been detected in the water of Siem Reap River, Cambodia [8], indicating a possibility that HEV outbreak could also occur in Cambodia. In turn, HAV positive rate among the participants in this study was 88.8% (767/864; [86.7–90.9%]): adults (18 years or older) had 99.6% (552/554; [99.1–100.0%]) and minors (17 years or younger) had 69.4% (215/310; [64.2–74.5%]), indicating age-related effect.

In Cambodia, nearly everyone is assumed to have HAV exposure by the time they reach adulthood. As with HEV infection, HAV is transmitted via the fecal-oral route, and by adulthood, nearly everyone is HAV-infected via contaminated food or water, indicating serious issues with health management. To prevent HEV infection in Cambodia, proper water hygiene

is regarded as the first necessary measure. Multivariate analysis also showed that house workers had a significantly higher rate of anti-HEV IgG positivity than farmers, office workers, students, and craftsmen, which suggested possible problems with water hygiene for cooking or washing the cloth, or handling with raw stuff [44].

The HEV isolate fully sequenced in this study is most closely related to the strains isolated from swine in Guangxi and Guangdong, both in southern China. The cause of HEV infection for the participants with detected HEV RNA (including the CVS-Sie10 isolate) is unknown; however, HEV is a zoonotic pathogen that infects pigs, wild boars, and other animals, and causal relationship between consumption of contaminated meat and hepatitis E onset has been confirmed [29]. In Japan, the majority of foodborne infections are presumed to be related to meat consumption [32], which can be also true in Cambodia; therefore, it is possible that the consumption of undercooked meat or drinking water contaminated by animal waste may be the cause of HEV infection for the participants in this study.

In Japan, HEV infection by blood transfusion has been reported in Hokkaido [45, 46], which is regarded as a region with increasing danger of HEV infection; therefore, it is the only place in the world where donor blood is screened for HEV RNA. In recent years, HEV infection through blood transfusion has been regarded as a growing problem also in the West, and the pros and cons of HEV blood screening have been considered. In this study, we identified HEV genotype 4 characterized with high post-infection aggravation rate, and also found that new HEV infections occur very frequently in Cambodia, raising concerns about HEV infection through blood transfusion and suggesting that it may be necessary to enhance the safety of blood supply.

The present study is the first to detect HEV genotype 4 in human blood in Cambodia and to report sequencing of genotype 4 full-length genome. Our survey revealed high HEV prevalence among Cambodian general population, including frequent cases of early HEV infection, suggesting that measures to prevent HEV infection, such as improving water and food safety and spreading health and hygiene education in school, are urgently required.

Supporting Information

S1 Table. Results of the questionnaire. This table shows the answers of eight questions. Question 3 and 4 were not asked to elementary school students.
(DOCX)

Author Contributions

Conceived and designed the experiments: HY OL SS SH SHD JT. Performed the experiments: HY KT KK MA. Analyzed the data: HY KT TA KK JT. Contributed reagents/materials/analysis tools: NG JT. Wrote the paper: HY KT JT. Survey for blood sampling: HY OL SS CC SH SHD MF TA NG JT. Coordinate the survey between Japan and Cambodia: SS JT.

References

1. WHO. Hepatitis E (Fact sheet No. 280): World Health Organization; 2014 [updated June cited 2014 December 24]. Available from: http://www.who.int/mediacentre/factsheets/fs280/en/.
2. WHO. Prevention and Control of Viral Hepatitis Infection: Framework for Global Action: World Health Organization; 2012 [cited 2014 December 24]. Available from: http://www.who.int/csr/disease/hepatitis/GHP_Framework_En.pdf.
3. Takahashi K, Terada S, Kokuryu H, Arai M, Mishiro S. A wild boar-derived hepatitis E virus isolate presumably representing so far unidentified "genotype 5". Kanzo. 2010; 51(9):536–538.
4. Dalton HR, Bendall R, Ijaz S, Banks M. Hepatitis E: an emerging infection in developed countries. Lancet Infect Dis. 2008 Nov; 8(11):698–709. doi: 10.1016/S1473-3099(08)70255-X PMID: 18992406

5. Mizuo H, Yazaki Y, Sugawara K, Tsuda F, Takahashi M, Nishizawa T, et al. Possible risk factors for the transmission of hepatitis E virus and for the severe form of hepatitis E acquired locally in Hokkaido, Japan. J Med Virol. 2005 Jul; 76(3):341–349. PMID: 15902701

6. Takahashi M, Okamoto H. Features of hepatitis E virus infection in humans and animals in Japan. Hepatol Res. 2014 Jan; 44(1):43–58. doi: 10.1111/hepr.12175 PMID: 23721425

7. Okamoto H. Genetic variability and evolution of hepatitis E virus. Virus Res. 2007 Aug; 127(2):216–228. PMID: 17363102

8. Kitajima M, Matsubara K, Sour S, Haramoto E, Katayama H, Ohgaki S. First detection of genotype 3 hepatitis E virus RNA in river water in Cambodia. Trans R Soc Trop Med Hyg. 2009 Sep; 103(9):955–957. doi: 10.1016/j.trstmh.2009.04.004 PMID: 19446859

9. Caron M, Enouf V, Than SC, Dellamonica L, Buisson Y, Nicand E. Identification of genotype 1 hepatitis E virus in samples from swine in Cambodia. J Clin Microbiol. 2006 Sep; 44(9):3440–3442. PMID: 16954296

10. Buchy P, Monchy D, An TT, Srey CT, Tri DV, Son S, et al. [Prevalence of hepatitis A, B, C and E virus markers among patients with elevated levels of Alanine aminotransferase and Aspartate aminotransferase in Phnom Penh (Cambodia) and Nha Trang (Central Vietnam)]. PMID: Bull Soc Pathol Exot. 2004 Aug; 97(3):165–171.

11. Kasper MR, Blair PJ, Touch S, Sokhal B, Yasuda CY, Williams M, et al. Infectious etiologies of acute febrile illness among patients seeking health care in south-central Cambodia. Am J Trop Med Hyg. 2012 Feb; 86(2):246–253. doi: 10.4269/ajtmh.2012.11-0409 PMID: 22302857

12. Yamada H, Fujimoto M, Svay S, Lim O, Hok S, Goto N, et al. Seroprevalence, genotypic distribution and potential risk factors of hepatitis B and C virus infections among adults in Siem Reap, Cambodia. Hepatol Res. 2015 Apr; 45(4):480–487. doi: 10.1111/hepr.12367 PMID: 24905888

13. Genaral Population Census of Cambodia 2008: National Institute of Statistics, Ministry of Planning Phnom Penh, Cambodia; [cited 2014 Jan 11]. Available from: http://camnut.weebly.com/uploads/2/0/3/8/20389289/2009_census_2008.pdf.

14. Imai M, Kondo M, Sudo K, Saito T, Sato H, Takebe Y, et al. [Simple method for typing human immunodeficiency virus type I (B, E) by PCR and chronological change of distribution of subtype (B to E) in Japan]. Kansenshogaku Zasshi. 1997 Sep; 71(9):918–923. PMID: 9339629

15. Takahashi K, Kang JH, Ohnishi S, Hino K, Mishiro S. Genetic heterogeneity of hepatitis E virus recovered from Japanese patients with acute sporadic hepatitis. J Infect Dis. 2002 May 1; 185(9):1342–1345. PMID: 12001054

16. Felsenstein J. Confidence-Limits on Phylogenies—an Approach Using the Bootstrap. Evolution. 1985; 39(4):783–791.

17. Gojobori T, Ishii K, Nei M. Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. J Mol Evol. 1982; 18(6):414–423. PMID: 7175958

18. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987 Jul; 4(4):406–425. PMID: 3447015

19. Abe T, Aikawa T, Akahane Y, Arai M, Asahina Y, Atarashi Y, et al. Demographic, epidemiological, and virological characteristics of hepatitis E virus infections in Japan based on 254 human cases collected nationwide. Kanzo. 2006; 47(8):384–391.

20. Okamoto H, Takahashi M, Nishizawa T. Features of hepatitis E virus infection in Japan. Intern Med. 2003 Nov; 42(11):1065–1071. PMID: 14686743

21. Peron JM, Bureau C, Poirson H, Mansuy JM, Alric L, Selves J, et al. Fulminant liver failure from acute autochthonous hepatitis E in France: description of seven patients with acute hepatitis E and encephalopathy. J Viral Hepat. 2007 May; 14(5):298–303. PMID: 17439518

22. Dalton HR, Bendall RP, Keane FE, Tedder RS, Ijaz S. Persistent carriage of hepatitis E virus in patients with HIV infection. N Engl J Med. 2009 Sep 3; 361(10):1025–1027. doi: 10.1056/NEJMc0903778 PMID: 19726781

23. Kamar N, Selves J, Mansuy JM, Ouezzani L, Peron JM, Guitard J, et al. Hepatitis E virus and chronic hepatitis in organ-transplant recipients. N Engl J Med. 2008 Feb 21; 358(8):811–817. doi: 10.1056/NEJMoa0706992 PMID: 18287603

24. Haagsma EB, van den Berg AP, Porte RJ, Benne CA, Vennema H, Reimerink JH, et al. Chronic hepatitis E virus infection in liver transplant recipients. Liver Transpl. 2008 Apr; 14(4):547–553. doi: 10.1002/lt.21480 PMID: 18383084

25. Takeda H, Matsubayashi K, Sakata H, Sato S, Kato T, Hino S, et al. A nationwide survey for prevalence of hepatitis E virus antibody in qualified blood donors in Japan. Vox Sang. 2010 Nov; 99(4):307–313. doi: 10.1111/j.1423-0410.2010.01362.x PMID: 20576022

26. Hau CH, Hien TT, Tien NT, Khiem HB, Sac PK, Nhung VT, et al. Prevalence of enteric hepatitis A and E viruses in the Mekong River delta region of Vietnam. Am J Trop Med Hyg. 1999 Feb; 60(2):277–280. PMID: 10072151

27. Worm HC, van der Poel WH, Brandstatter G. Hepatitis E: an overview. Microbes Infect. 2002 May; 4 (6):657–666. PMID: 12048035

28. Mizuo H, Suzuki K, Takikawa Y, Sugai Y, Tokita H, Akahane Y, et al. Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. J Clin Microbiol. 2002 Sep; 40 (9):3209–3218. PMID: 12202555

29. Tei S, Kitajima N, Takahashi K, Mishiro S. Zoonotic transmission of hepatitis E virus from deer to human beings. Lancet. 2003 Aug 2; 362(9381):371–373. PMID: 12907011

30. Shrestha A, Lama TK, Karki S, Sigdel DR, Rai U, Rauniyar SK, et al. Hepatitis E epidemic, Biratnagar, Nepal, 2014. Emerg Infect Dis. 2015 Apr; 21(4):711–713. doi: 10.3201/eid2104.141512 PMID: 25811975

31. Takahashi M, Nishizawa T, Miyajima H, Gotanda Y, Iita T, Tsuda F, et al. Swine hepatitis E virus strains in Japan form four phylogenetic clusters comparable with those of Japanese isolates of human hepatitis E virus. J Gen Virol. 2003 Apr; 84(Pt 4):851–862. PMID: 12655086

32. National Institute of Infectious Diseases and Tuberculosis and Infectious Diseases Control Division MoH, Labour and Welfare. Infectious Agents Surveillance Report. 2014; 35(1):1–14.

33. Dalton HR, Stableforth W, Hazeldine S, Thurairajah P, Ramnarace R, Warshow U, et al. Autochthonous hepatitis E in Southwest England: a comparison with hepatitis A. Eur J Clin Microbiol Infect Dis. 2008 Jul; 27(7):579–585. doi: 10.1007/s10096-008-0480-z PMID: 18299907

34. Lewis HC, Boisson S, Ijaz S, Hewitt K, Ngui SL, Boxall E, et al. Hepatitis E in England and Wales. Emerg Infect Dis. 2008 Jan; 14(1):165–167. doi: 10.3201/eid1401.070307 PMID: 18258100

35. Kuniholm MH, Purcell RH, McQuillan GM, Engle RE, Wasley A, Nelson KE. Epidemiology of hepatitis E virus in the United States: results from the Third National Health and Nutrition Examination Survey, 1988–1994. J Infect Dis. 2009 Jul 1; 200(1):48–56. doi: 10.1086/599319 PMID: 19473098

36. Zhu FC, Huang SJ, Wu T, Zhang XF, Wang ZZ, Ai X, et al. Epidemiology of zoonotic hepatitis E: a community-based surveillance study in a rural population in China. PLoS One. 2014; 9(1):e87154. doi: 10.1371/journal.pone.0087154 PMID: 24498033

37. Corwin A, Putri MP, Winarno J, Lubis I, Suparmanto S, Sumardiati A, et al. Epidemic and sporadic hepatitis E virus transmission in West Kalimantan (Borneo), Indonesia. Am J Trop Med Hyg. 1997 Jul; 57 (1):62–65. PMID: 9242320

38. Takahashi M, Tanaka T, Azuma M, Kusano E, Aikawa T, Shibayama T, et al. Prolonged fecal shedding of hepatitis E virus (HEV) during sporadic acute hepatitis E: evaluation of infectivity of HEV in fecal specimens in a cell culture system. J Clin Microbiol. 2007 Nov; 45(11):3671–3679. PMID: 17728471

39. Labrique AB, Zaman K, Hossain Z, Saha P, Yunus M, Hossain A, et al. Epidemiology and risk factors of incident hepatitis E virus infections in rural Bangladesh. Am J Epidemiol. 2010 Oct 15; 172(8):952–961. doi: 10.1093/aje/kwq225 PMID: 20801864

40. Naik SR, Aggarwal R, Salunke PN, Mehrotra NN. A large waterborne viral hepatitis E epidemic in Kanpur, India. Bull World Health Organ. 1992; 70(5):597–604. PMID: 1464145

41. Bile K, Isse A, Mohamud O, Allebeck P, Nilsson L, Norder H, et al. Contrasting roles of rivers and wells as sources of drinking water on attack and fatality rates in a hepatitis E epidemic in Somalia. Am J Trop Med Hyg. 1994 Oct; 51(4):466–474. PMID: 7943574

42. Rab MA, Bile MK, Mubarik MM, Asghar H, Sami Z, Siddiqi S, et al. Water-borne hepatitis E virus epidemic in Islamabad, Pakistan: a common source outbreak traced to the malfunction of a modern water treatment plant. Am J Trop Med Hyg. 1997 Aug; 57(2):151–157. PMID: 9288807

43. Corwin AL, Khiem HB, Clayson ET, Pham KS, Vo TT, Vu TY, et al. A waterborne outbreak of hepatitis E virus transmission in southwestern Vietnam. Am J Trop Med Hyg. 1996 Jun; 54(6):559–562. PMID: 8686771

44. Inoue G, Michitaka K, Takahashi K, Abe N, Oka K, Nunoi H, et al. A case of acute hepatitis E developed in a housewife who had cooked and eaten wild boar meat a month before. Kanzo. 2006; 47(10):459–464.

45. Matsubayashi K, Nagaoka Y, Sakata H, Sato S, Fukai K, Kato T, et al. Transfusion-transmitted hepatitis E caused by apparently indigenous hepatitis E virus strain in Hokkaido, Japan. Transfusion. 2004 Jun; 44(6):934–940. PMID: 15157263

46. Matsubayashi K, Kang JH, Sakata H, Takahashi K, Shindo M, Kato M, et al. A case of transfusion-transmitted hepatitis E caused by blood from a donor infected with hepatitis E virus via zoonotic foodborne route. Transfusion. 2008 Jul; 48(7):1368–1375. doi: 10.1111/j.1537-2995.2008.01722.x PMID: 18651907

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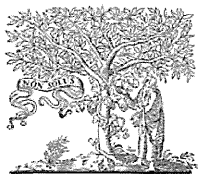

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Effect of tenofovir disoproxil fumarate on drug-resistant HBV clones

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KEYWORDS
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Drug resistance

Summary *Background & aims:* Tenofovir disoproxil fumarate (TDF) has been approved for chronic hepatitis B treatment, and favorable susceptibility of hepatitis B virus (HBV) has been indicated. However, differences in TDF susceptibility among HBV genotypes and drug-resistant strains are unclear. In this study, TDF susceptibilities between genotypes A and C were evaluated *in vitro* and *in vivo* using several drug-resistant HBV clones.
Methods: HBV expression plasmids were constructed from sera of HBV carriers, and drug-resistant substitutions were introduced by site-directed mutagenesis. TDF susceptibility was evaluated by changes of core-associated HBV replication intermediates *in vitro* or by change of serum HBV DNA in human hepatocyte chimeric mice carrying each HBV clone *in vivo*.
Results: TDF susceptibilities of lamivudine-resistant clones (rtL180M/M204V) and lamivudine plus entecavir-resistant clones (rtL180M/S202G/M204V) were similar to wild type clones

Abbreviation: HBV, hepatitis B virus; NAs, nucleot(s)ide analogues; ETV, entecavir; LMV, lamivudine; ADV, adefovir dipivoxil; TDF, tenofovir disoproxil fumarate.

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in vitro. However, lamivudine plus adefovir-resistant clones (rtA181T/N236T) acquired tolerance to TDF, and the rtN236T mutation was considered to be a causal substitution for TDF resistance. Furthermore, genotypic differences in TDF susceptibility were also observed between genotypes A and C *in vitro*, and the differences could be confirmed *in vivo* ($p = 0.023$).
Conclusions: The present study indicates that TDF susceptibility varies among HBV genotypes and drug-resistant HBV clones.

Introduction

Hepatitis B virus (HBV) infection is a serious global health problem. Some chronically infected individuals develop active hepatitis, liver cirrhosis, and hepatocellular carcinoma, and more than 780,000 people die every year from advanced liver diseases.[1] The ultimate goal of anti-viral treatments of chronic hepatitis B is complete elimination of HBV from infected hepatocytes. However, once HBV infects human hepatocytes, HBV genomes are carried into the nucleus where some form covalently closed circular DNA (cccDNA), a minichromosome with histone and non-histone proteins.[2–7] Chronic hepatitis B patients are generally treated either with interferon, which facilitates the immune response in achieving seroconversion, or with nucleot(s)ide analogues (NAs), which suppress viral replication and prevent the progression of liver disease by combating inflammation.[8–10] However, neither of these currently available anti-viral therapies affect the HBV cccDNA pool, and immune modulators may also be required in order to eliminate the virus completely because of these characteristics of the HBV life cycle.

NAs have strong potency to inhibit reverse transcription or DNA synthesis during HBV replication, and serum HBV DNA can be decreased below the detection limit.[11] However, because of the difficulty of eliminating HBV cccDNA, long-term NA administration is necessary. Prolonged NA treatment can suppress hepatitis and prevent progression of liver fibrosis, but it sometimes leads to the emergence of drug resistance.[12–15] Known drug-resistant HBV strains have amino acid substitutions in the reverse transcriptase (RT) domain of the HBV polymerase gene, including the YMDD (tyrosine–methionine–aspartate–aspartate) motif.[11,13–17] The HBV polymerase contains four different functional regions: a priming region, a spacer region, a catalytic region, and a carboxy terminal region,[18] but the crystal structure of HBV polymerase has not been solved. The RT domain belongs to a catalytic region that can synthesize DNA using RNA as a template RNA and plays an important role in the activity of the polymerase.[19–21] NAs induce anti-viral activity by interacting with the RT domain of HBV polymerase. Even a single amino acid substitution could induce conformational changes in the polymerase and reduce the binding affinity between NAs and HBV polymerase.[7,22] Thus, it is important to understand the molecular mechanisms of NA resistance to improve anti-viral therapy.

Since NAs that rarely induce resistance, such as entecavir (ETV), have been approved worldwide, the emergence of drug-resistant viral mutants or breakthrough hepatitis has been substantially reduced but remains.[15] Although lamivudine (LMV)-refractory chronic hepatitis B patients have been treated with LMV plus adefovir dipivoxil (ADV) combination therapy, the frequency of emergence of LMV plus ADV-resistant HBV was found to be 1.6% during up to 3 years of combination therapy[23] and, recently, multi-drug-resistant HBV strains resistant to ETV plus ADV or all three NAs have resulted in viral breakthrough or breakthrough hepatitis.[24] Therefore, strategies for multi-drug-resistant HBV are urgently needed. To date, tenofovir disoproxil fumarate (TDF) has been approved for the treatment of chronic hepatitis B in several countries and is expected to become a mainstay therapeutic agent against multi-drug-resistant HBV because of the lower incidence of drug resistance and the high susceptibility of HBV.[25–28] However, it is unclear whether such multi-drug-resistant HBV strains have enough sensitivity to TDF.

HBV strains are classified into eight genotypes (A–H), which have distinct geographical distributions. HBV genotypes have been reported to vary with respect to clinical characteristics and susceptibility to NAs, and incidence of drug resistance and HBe seroconversion also varies among genotypes.[29–31] Although HBV genotype A was considered as a risk factor for development of resistance to LMV,[32,33] it was unknown how HBV genotypes influence the sensitivity to each type of NA therapy.

HBV can transiently infect only a small range of mammals, such as chimpanzees and tree shrews, and small animals such as mice and rats cannot be infected. Because of this characteristic, until recently it has been difficult to establish a small animal model to analyze the mechanisms of HBV infection and replication *in vivo*. Human hepatocyte chimeric mice, in which mouse hepatocytes are largely replaced by implanted human hepatocytes, provides a small animal model able to support HBV infection.[34,35] Using this chimeric mouse model and a cell culture system in which HBV can replicate and produce viral particles, sensitivity to NAs could be evaluated using variable HBV clones containing drug-resistant mutations.[13,14,36] In the present study, we evaluated TDF susceptibilities *in vitro* and *in vivo* of several drug-resistant HBV strains derived from two genotypes and considered countermeasures for several drug-resistant strains of HBV. The results may improve understanding of the differences in TDF susceptibility among HBV genotypes and drug-resistant HBV strains and to develop therapeutic strategies for chronic hepatitis B.

Materials and methods

Patients

Serum samples were obtained from two hepatitis B patients. The serum containing HBV genotype A was obtained

from an acute hepatitis B patient with high serum ALT levels (>3000 IU/L) without anti-viral treatment. The serum was positive for HBe antigen, and the HBV DNA level was >8.8 log copies/ml. The serum containing HBV genotype C was obtained from a chronic hepatitis B patient after 2 years of lamivudine treatment. Lamivudine-resistant HBV (L180M/M204V) had appeared in the serum. The serum was negative for HBe antigen, and the HBV DNA level was 8.1 log copies/ml.

Construction of HBV expression plasmids

HBV DNA was extracted from the sera containing HBV genotype A (A2) or C (C2), and 1.4-fold length HBV DNA genomes were cloned into a plasmid vector, pTRE2 (BD Biosciences, Franklin Lakes, NJ).[36] The cloned plasmid containing wild type HBV genotype A was designated A-WT, and the plasmid containing genotype C was designated C-WT. Subsequently, both plasmids were modified using a commercially available site-directed mutagenesis kit (QuickChange Site-Directed Mutagenesis Kit, Stratagene, La Jolla, CA),[14] and seven drug-resistant clones were generated (Fig. 1). The name and substitutions of the four plasmids are defined as follows: A- or C-A181T: Genotype A or C of LMV-resistant HBV clones carrying an amino acid substitution at rtA181T; A- or C-L180M/M204V: Genotype A or C of LMV-resistant HBV clone with rtL180M plus rtM204V; A- or C-N236T: Genotype A or C of ADV-resistant HBV clones with rtN236T; A- or C-A181T/N236T: Genotype A or C of LMV/ADV-resistant HBV clones with rtA181T plus rtN236T; A- or C-L180M/M204V/N236T: Genotype A or C of LMV/ADV-resistant HBV clone with rtL180M plus rtM204V plus rtN236T; A- or C-L180M/S202G/M204V: Genotype A or C of LMV/ETV-resistant HBV clone with rtL180M plus rtS202G plus rtM204V; A- or C-L180M/S202G/M204V/N236T: Genotype A or C of ETV/ADV-resistant HBV clone with rtL180M plus rtS202G plus rtM204V plus N236T. The scheme of all plasmids is shown in Fig. 1.

Susceptibilities to nucleotide analogues

HepG2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum at 37 °C in 5% CO_2. Cells were seeded to semi-confluence in 6-well tissue culture plates. 2 μg/well of the HBV expression plasmid was transiently transfected into HepG2 cells using TransIT-LT1 (Mirus, Madison, WI) according to the instruction provided by the manufacturer. Transfected cells were treated once with various concentrations of TDF 24 h after transfection. The culture medium containing TDF was refreshed 48 h after TDF treatment, and cells were collected to quantify HBV replication intermediates 72 h after TDF treatment.[37] All experiments were performed in triplicate, and the experiment was performed three times independently.

Analysis of replicative intermediate of HBV by Southern blot hybridization and quantitation

The harvested cells were lysed with 250 μl of lysis buffer (10 mM Tris—HCl [pH 7.4], 140 mM NaCl, and 0.5% [vol/vol] NP-40) followed by centrifugation for 2 min at 15,000 × g.

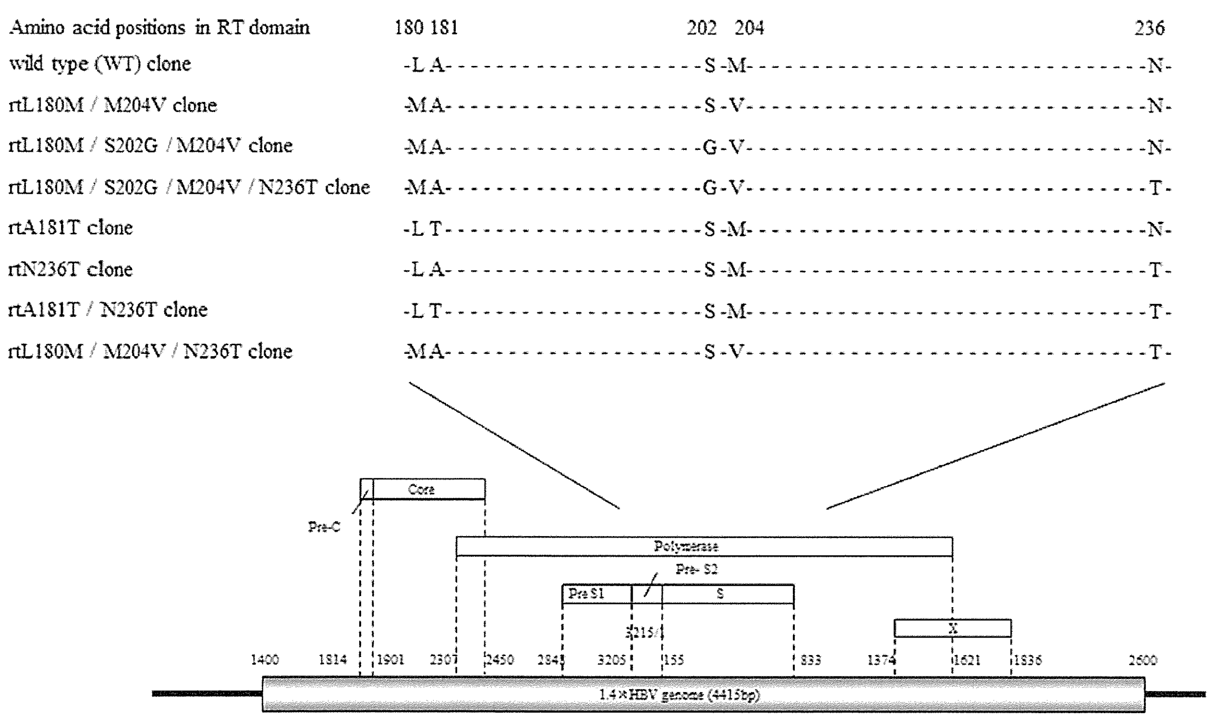

Figure 1 *Construction of used HBV expression plasmids.* Wild type genotype A and C plasmids contained a 1.4-genome-length HBV genome. Resistant mutant clones were generated by introducing point mutations in the RT domain of HBV genomes based on these wild type plasmids.

The core-associated HBV genome was immunoprecipitated by mouse anti-HBV core monoclonal antibody 2A21 (Institute of Immunology, Tokyo, Japan) and subjected to Southern blot analysis after sodium dodecyl sulfate–proteinase K digestion followed by phenol extraction and ethanol precipitation. The DNA was detected with a full-length HBV DNA probe labeled by the DIG DNA labeling and detection kit (Roche Diagnostics, Basel, Switzerland) according to the instructions provided by the manufacturer. Quantitative analysis was performed by real-time PCR with SYBR green using the 7300 Real-Time PCR System (Applied Biosystems).[14] The HBV-specific primers used for amplification were 5′-TTTGGGGCATGGACATTGAC-3′ and 5′-GAGTGCTGTATGGTGAGGTG-3′. The amplification conditions included initial denaturation at 95 °C for 10 min, followed by 45 cycles of denaturation at 95 °C for 15 s, annealing at 58 °C for 5 s and extension at 72 °C for 6 s. The lower detection limit of this assay was 300 copies. According to our previous research, HBV replication intermediate titers were correlated with densities in Southern blot analysis.[13,14,37]

To evaluate the transcriptional activities of transfected plasmids, intracellular core-associated HBV DNA plus RNA, which reflects total pre-genomic RNA produced from transfected plasmids, was measured. Intracellular core-associated HBV nucleic acids were collected by immunoprecipitation with anti-HBc antibody and purified by the method described above. Then, purified HBV nucleic acids were reverse-transcribed as previously described and the aliquots of the nucleic acid solutions were then used for measurement of HBV DNA.[38,39]

Determination of IC_{50} and HBV replication capacities

TDF susceptibilities were evaluated by IC_{50} because reductions of core-associated replication intermediate were considered to reflect suppression of reverse transcription activity. IC_{50} was calculated by GraphPad prism 6 software (GraphPad Software, Inc. http://www.graphpad.com) with non-linear regression analysis using best-fit values for dose–response curves.[13,14] IC_{50} rates, which indicated the ratio of drug concentrations for 50% reduction of HBV production based on wild type clones, were expressed as the fold changes of IC_{50} values from wild type in each genotype. Viral replication activity, which was regarded as representing HBV replication capacities, was measured by the relative mean intracellular core-associated HBV DNA titers based on wild type in each genotype. The statistical significance of dose–response curves was examined using the extra-sum-of-squares F test.

Evaluation of TDF susceptibilities *in vivo*

The $uPA^{+/+}/SCID^{+/+}$ mice were prepared, and the human hepatocytes were transplanted as described previously.[13,14,35,36] The experiments were performed in accordance with the guidelines of the local committee for animal experiments at Hiroshima University. Six mice were inoculated with 1×10^8 copies of culture supernatants containing HBV particles. Mouse sera were collected weekly, and HBV DNA titers were measured within a few days. The aliquots of mouse serum samples were stored at −80 °C. After HBV DNA titers in the mouse sera reached plateau, the mice were treated with 90 mg/kg body weight/day of TDF for 3 weeks. Based on FDA (Food and Drug Administration) recommendations for dosage calculation for animal testing, the optimal dose of lamivudine for our mouse model was determined to be 30 mg/kg/day. The optimal dose of TDF (90 mg/kg/day) was also determined based on clinical doses of LMV and TDF. HBV DNA was extracted from 10 μl of mouse sera by the SMITEST R&D (Genome Science Laboratories, Tokyo) and dissolved in 20 μl of sterile water. HBV DNA was quantified by real-time PCR as described previously.[14,36] As replication activity differed between HBV genotypes A and C, TDF susceptibilities were compared by presumed suppressed HBV DNA between HBV genotype A and C. Presumed suppressed HBV DNA titers refers to the numbers of reduced HBV particles and is estimated by calculating the area of reduced HBV DNA titers based on the hypothesis that HBV production continued with the same speed before and during TDF treatment. The decreased levels of HBV DNA in mouse sera were evaluated as follows. Appropriate curves of the changes of HBV DNA levels in each mouse during TDF treatment were drawn by a one phase decay model in each mouse using GraphPad prism 6 software (GraphPad Software Inc., CA, USA) and expressed using the following mathematical formula (Supplemental Fig. 2);

$$Y = (Y_0 - plateau)e^{-KX} + plateau,$$

where X denotes week after TDF, and Y_0, K and *plateau* are parameters. The presumed TDF susceptibilities *in vivo* were represented by area (copies.week), which was calculated by integrating the suppressed titers of HBV DNA (copies) compared with the titers before treatment over the treatment duration (week) as follows (Supplemental Fig. 2)

$$\int_0^3 (Y_0 - Y)dX = 3Y_0 - \int_0^3 \{(Y_0 - plateau)e^{-KX} + plateau\}dX = 3(Y_0 + plateau) - \frac{(1 - e^{-3K})(Y_0 - plateau)}{K}.$$

The differences in susceptibility were evaluated by Welch's t-test.

Results

Susceptibility of HBV wild type to TDF

To analyze the sensitivity of HBV wild type to TDF treatment, *in vitro* analysis was performed using A-WT and C-WT clones. HBV replication intermediates decreased dose-dependently, and both HBV wild type clones were considered to have high sensitivities to TDF (Fig. 2A and B and Table 1). To compare the sensitivities between HBV genotypes, IC_{50} values were calculated based on the reduction rates of HBV replication intermediates. A small but significant difference was observed in IC_{50} values between wild type genotypes A and C (A-WT; 0.5080 μM, C-WT; 0.2534 μM, $p < 0.01$), and HBV genotype C appears to be

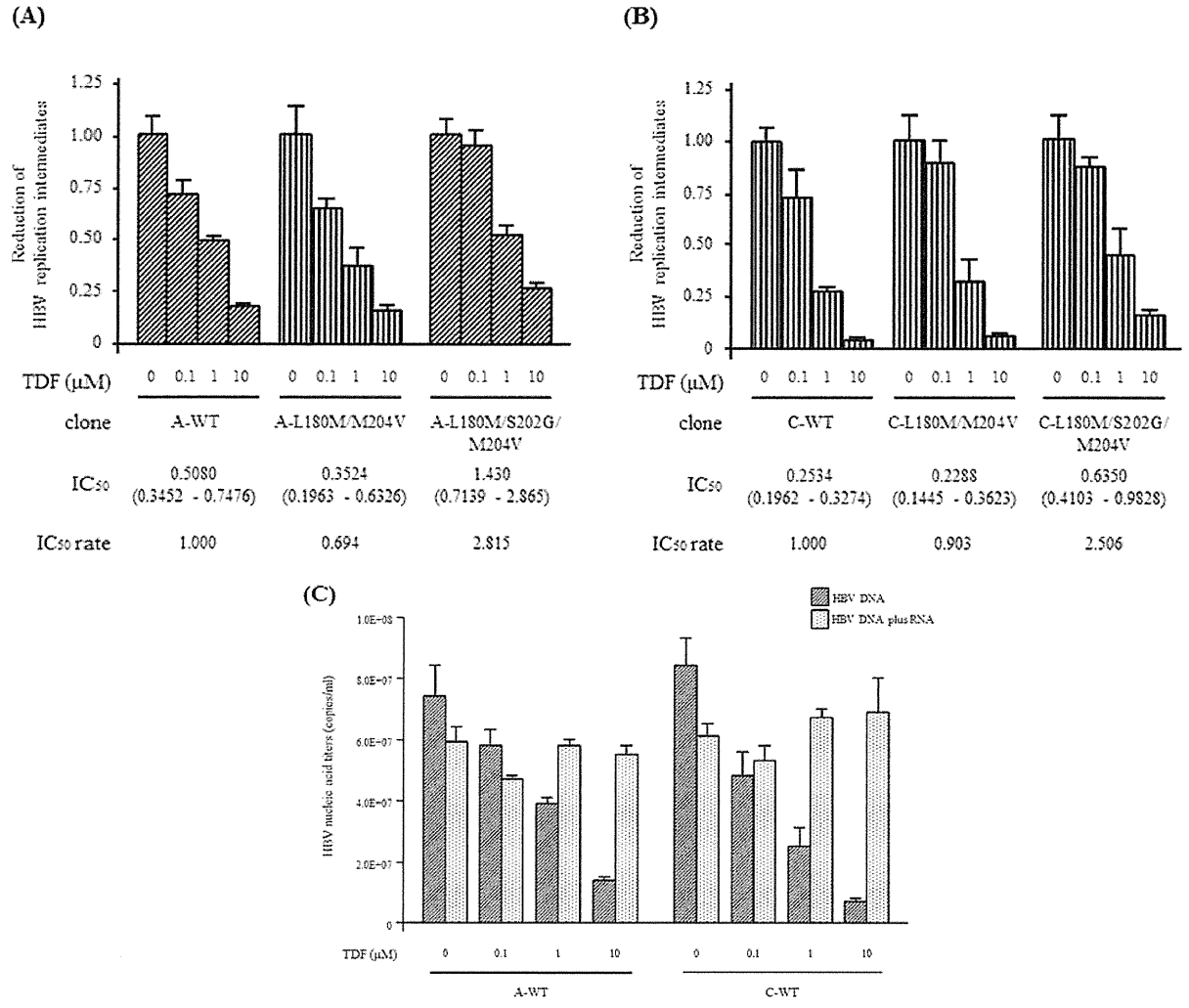

Figure 2 *Analysis of TDF susceptibility in WT, LR and ER plasmid.* (A, B) 72 h after TDF treatment, TDF susceptibilities were evaluated by measuring HBV core-associated replication intermediates by real-time PCR (A: HBV genotype A, B: HBV genotype C). (C) Comparison of HBV DNA and HBV DNA plus RNA titers in core-associated replication intermediates between genotype A and C *in vitro*. HBV DNA and HBV DNA plus RNA titers in HBV core-associated replication intermediates were measured by real-time PCR without or with reverse transcription, respectively. Experiments were performed in triplicate. Values are expressed as the mean plus standard deviation. IC_{50} was calculated with non-linear regression analysis of dose—response curves. IC_{50} rates are expressed as fold changes of IC_{50} values from wild type in each genotype.

Table 1 Comparison of IC_{50} values from WT clone genotype A and C *in vitro*.

Clones	Genotype A			Genotype C			Fold (A/C)	*p* value compared with A vs. C
	IC_{50} (μM)	Fold (/WT)	P value compared with WT	IC_{50} (μM)	Fold (/WT)	*p* value compared with WT		
WT	0.5080	–	–	0.2534	–	–	2.00	<0.01
rtL180M/M204V	0.3524	0.69	0.32	0.2288	0.90	0.52	1.54	0.15
rtL180M/S202G/M204V	1.430	2.81	<0.01	0.6350	2.51	<0.01	2.25	0.07
rtA181T	0.7771	1.53	0.34	0.4533	1.79	<0.01	1.71	0.12
rtN236T	2.691	5.30	<0.01	1.073	4.23	<0.01	2.51	0.02
rtA181T/N236T	2.473	4.87	<0.01	1.138	4.49	<0.01	2.17	0.02

more susceptible to TDF than genotype A. However, the difference in susceptibility might be influenced by differences in the replicative abilities of HBV clones. As core-associated HBV DNA plus RNA titers reflect viral transcriptional activity of the transfected HBV expression plasmids, changes in intracellular core-associated HBV DNA and HBV DNA plus RNA titers were measured. As shown in Fig. 2C, intracellular core-associated HBV DNA plus RNA titers were similar between genotypes A and C, and the titers did not decrease by TDF treatment. This suggests that HBV genotype A and C clones had equivalent transcriptional activities, and the difference in TDF sensitivity might be influenced by the suppression of reverse transcription.

Analysis of LMV or LMV plus ETV-resistant HBV

To analyze TDF susceptibility in LMV- or LMV/ETV-resistance HBV strains, we performed *in vitro* analyses using rtL180M/M204V and rtL180M/S202G/M204V clones. As shown in Fig. 2A and B, TDF reduced HBV replication intermediates produced from both genotype A and C clones in a dose dependent manner. Although reductions of replication intermediates were similar between genotypes A and C, IC_{50} values of HBV genotype A were 1.54–2.25-fold higher than those of HBV genotype C in both LMV- and ETV-resistant clones (Table 1). These results suggest that TDF treatment might be effective for LMV- and ETV-resistant HBV strains, but sufficient HBV DNA reduction could not be obtained in patients infected with ETV-resistant HBV genotype A strains.

Analysis of LMV plus ADV-resistant HBV

To analyze TDF susceptibility to LMV plus ADV resistance, we constructed LMV plus ADV-resistant clones containing two amino acid substitutions at rtA181 and rtN236 in both genotypes A and C. Furthermore, to compare the impact of these substitutions, we also constructed mutants containing single substitutions at rtA181T (LMV-resistant or LMV/ADV-resistant) or rtN236T (ADV-resistant) in the HBV RT domain. As shown in Fig. 3A and B and Table 1, the susceptibility of rtA181T clones was similar to wild type in both HBV genotypes (IC_{50} of A-A181T; 0.7771 μM, C-A181T; 0.4533 μM, each $p > 0.05$), but susceptibilities of rtN236T clones were significantly decreased (IC_{50} of A-N236T; 2.691 μM, C-N236T; 1.073 μM, $p < 0.01$ and $p = 0.02$, respectively). Furthermore, IC_{50} values of LMV plus ADV-resistant clones (rtA181T/N236T) were further increased in comparison with those of wild type in both genotype A (2.473 μM, $p < 0.01$) and C (1.138 μM, $p < 0.01$). Although IC_{50} of A-N236T was higher than that of A-A181T/N236T, there was no significance between IC_{50} of A-N236T and that of A-A181T/N236T. These results indicated that rtA181T plus rtN236T substitutions might yield intermediate resistance to TDF treatment and that TDF susceptibility of genotype A clones was lower than that of genotype C. It might be difficult to obtain sufficient viral suppression in patients who are infected with LMV plus ADV-resistant genotype A strains.

Association between HBV replication and drug resistance

To compare replication activity among HBV expression plasmids, Southern blot analysis was performed using isolated core-associated HBV DNA. Compared to wild type, HBV replication activity decreased synergistically when drug-resistant substitutions were introduced into the HBV genome (Supplemental Fig. 1). To analyze the association between drug resistance and HBV replication activity, correlations between IC_{50} rate and viral replication activity were evaluated. The IC_{50} value fold change was calculated based on the IC_{50} value of wild type and designated the IC_{50} rate. As it was difficult to evaluate the amount of DNA by Southern blot densities, viral replication activity was calculated based on the amount of core-associated intracellular HBV replication intermediates. As shown in Fig. 4A, LMV-resistant mutations (A-A181T or A-L180M/M204V) and ETV-resistant mutations (A-L180M/S202G/M204V) decreased their viral replication activities, but IC_{50} rates were not significantly different from the A-WT clone. However, the additional substitution rtN236T significantly increased IC_{50} rates in every clone (A-A181T vs. A-A181T/N236T, A-L180M/M204V vs. A-L180M/M204V/N236T, and A-L180M/S202G/M204V vs. A-L180M/S202G/M204V/N236T). Similar results were observed except for rtA181T clone using HBV genotype C clones (Fig. 4B). Notably, the rtA181T/N236T substitution, which is sometimes observed in patients with LMV plus ADV resistance, increased both the IC_{50} rate and viral replication activity in both HBV genotype A and C.

Difference in TDF susceptibility between HBV genotypes A and C

As shown in Table 1, every strain derived from genotype A had lower TDF susceptibility than genotype C *in vitro*. To verify the difference of susceptibilities between HBV genotype A and C, *in vivo* analyses were also performed using human hepatocyte chimeric mice. After HBV DNA titers in mouse sera reached more than 8 log copies/ml, the mice were treated with 90 mg/kg/day of TDF for 3 weeks. As shown in Fig. 5A and B, HBV DNA titers were reduced in all mice by TDF treatment, and the reduction of HBV DNA titers after 3 weeks of TDF treatment was similar between HBV genotypes A and C ($p = 0.99$, each −0.5 log copies/ml/week, data not shown). However, in comparison with Fig. 5A and B, HBV DNA titers in mice infected with C-WT clone increased more rapidly and reached higher levels than those with the A-WT clone ($p < 0.0001$, genotype A; +0.13 log copies/ml/week, genotype C; +0.58 log copies/ml/week, data not shown). Taking into account this difference in viral growth, appropriate curves of the changes of HBV DNA levels were evaluated by the real values of HBV DNA titers using the one phase decay model (Supplemental Table 1). Comparing the presumed TDF susceptibilities between A-WT and C-WT, A-WT was significantly more weakly suppressed than C-WT after 3 weeks of TDF treatment ($p = 0.023$) (Fig. 5C).

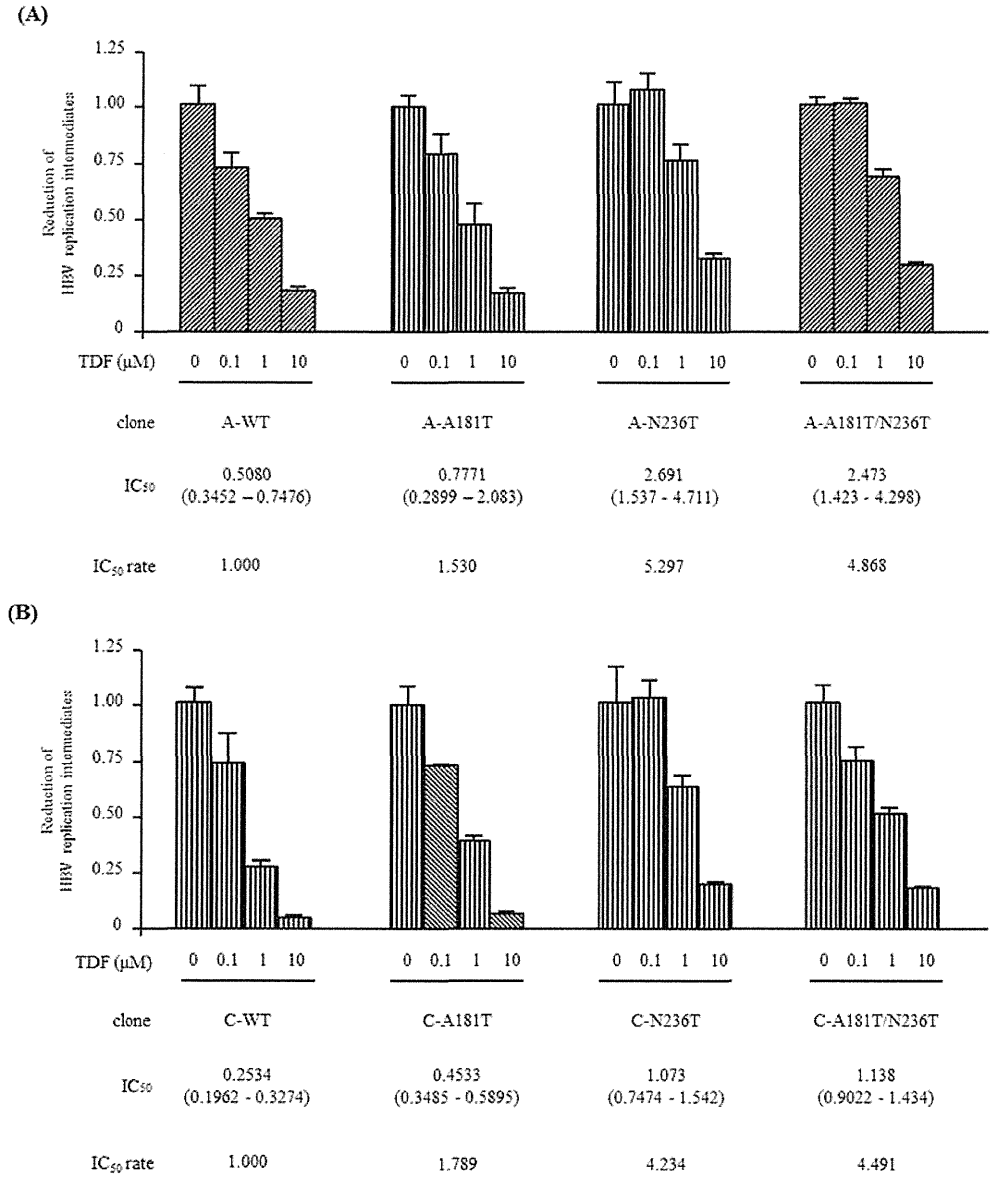

Figure 3 *Analysis of TDF susceptibilities in LMV and/or ADV-resistant HBV clones.* (A, B) 72 h after TDF treatment, TDF susceptibilities of several HBV clones related with LMV and/or ADV resistance were evaluated by measuring HBV core-associated replication intermediates by real-time PCR (A: HBV genotype A, B: HBV genotype C). Experiments were performed in triplicate. Values are expressed as the mean plus standard deviation. IC_{50} was calculated with non-linear regression analysis of dose–response curves. IC_{50} rates are expressed as the fold changes of IC_{50} values from wild type in each genotype.

Discussion

To date, five NAs have been approved for treatment of chronic hepatitis B by the US Food and Drug Administration.[15] NAs strongly inhibit HBV replication and inhibit liver inflammation and improve liver fibrosis in chronic hepatitis B. However, prolonged NA treatment increases the incidence of drug-resistant HBV mutants, which may result in viral breakthrough or breakthrough hepatitis.[40,41] Therefore, it is important to establish strategies for managing drug resistance. According to previous reports, various drug-resistant mutations have been identified in the HBV RT domain, with most substitutions localized in the palm region.[11,12,15,17] We have also identified a substitution, rtA181T, in the palm region of HBV polymerase and demonstrated that the substitution was associated with resistance to LMV treatment using *in vitro* and *in vivo* HBV replication models.[14] In the present study, several drug-resistant HBV clones containing amino acid substitutions in the HBV RT domain (e.g. rtL180M plus rtM204V and rtA181T for LMV-resistant clones, rtN236T for ADV-resistant clone and rtL180M plus rtS202G plus rtM204V for ETV-resistant clone) were constructed, and TDF susceptibility was evaluated using these *in vitro* and *in vivo* HBV replication models.

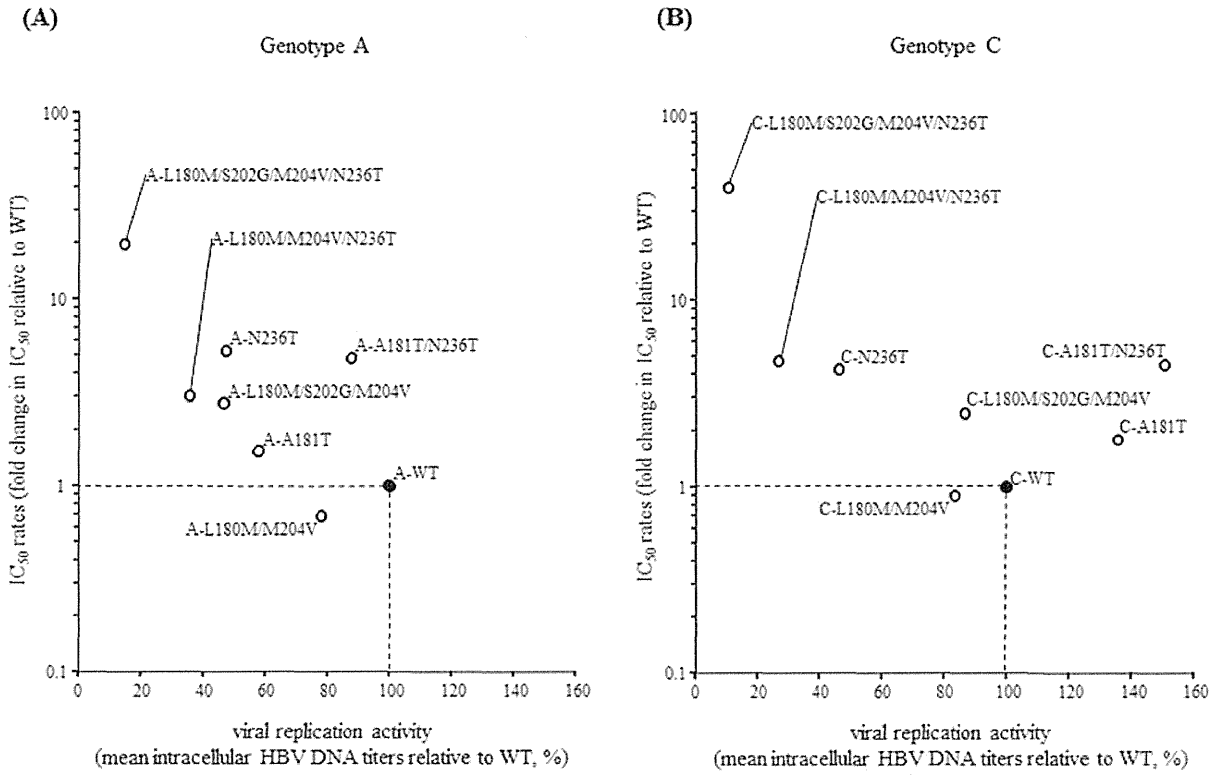

Figure 4 *IC_{50} rate versus viral replication activity of resistant mutant HBV clones.* (A, B) Each HBV clone was 2-dimensionally arranged in accordance with IC_{50} rate and viral replication activity. IC_{50} rates were expressed as the fold changes of IC_{50} values from those of wild type. Viral replication activities were represented as the relative mean HBV DNA ratios from wild type intracellular core-associated replication intermediates (A: HBV genotype A, B: HBV genotype C).

Although it is rare to develop resistance to TDF with prolonged treatment,[25–27] some reports have demonstrated substitutions conferring resistance to TDF treatment.[42,43] However, these results have been controversial. One report indicated that a rtA194T substitution might be a TDF-resistant substitution, but another report demonstrated high sensitivity in rtA194T mutant clones. To verify these controversial results, we analyzed TDF susceptibility using rtA194T clones derived from HBV genotypes A and C. As favorable reductions of HBV replication intermediates were observed in both genotypes (data not shown), it is possible that the rtA194T substitution might not confer resistance. On the other hand, another substitution at rtN236T has been reported to reduce efficacy of TDF.[42,43] As shown in Fig. 3A and B, TDF sensitivity using rtN236T-substituted clones obtained 2–4 fold resistance compared with wild type in both genotypes A and C. Interestingly, additional rtN236T substitutions reduced HBV replication in several HBV resistant clones such as rtL180M/M204V clones or rtL180M/S202G/M204V clones (Fig. 4A and B). Because HBV replication activities of rtL180M/M204V/N236T mutants or rtL180M/S202G/M204V/N236T were significantly suppressed, these mutants have not been reported as multi-drug-resistant clinically. Although rtP177G and rtF249A substitutions were also indicated to be TDF-resistant substitution *in vitro*,[44] these substitutions might not become serious problems clinically because of the significant reduction of HBV replication activities. However, in analyses using another resistance mutant, rtA181T/N236T, HBV replication activities were not suppressed by adding a rtN236T substitution, indicating that serum HBV DNA could not be reduced sufficiently in patients who carried the rtA181T/N236T clone. There are some supportive reports about rtA181T/N236T mutants by *in vitro* analyses,[42] and lower cumulative HBV DNA reduction rate of patients with ADV-resistant HBV than that of patients without ADV-resistant HBV was also indicated in a clinical study.[45]

It is known that clinical events, such as progression of liver fibrosis, risk of HCC, and interferon susceptibility and the incidence of lamivudine resistance differ among HBV genotypes.[41,46,47] Although differences among HBV genotypes are considered to be associated with NA sensitivity,[32,41,48,49] it has not been clear whether the susceptibilities of nucleotide analogues differ among HBV genotypes. Therefore, it is important to understand genotypic differences in TDF sensitivity in order to establish new therapeutic strategies for chronic hepatitis B. As shown in Fig. 2A and B, favorable reductions of HBV replication intermediates were observed by TDF treatment dose-dependently in *in vitro* analysis using HBV wild type, LMV-resistant (rtL180M/M204V or rtA181T) or ETV-resistant clones (rtL180M/S202G/M204V). However, in comparison with genotype A and C clones, IC_{50} values of HBV genotype A clones were higher than those of genotype C clones (Table 1). To verify the genotypic differences in TDF

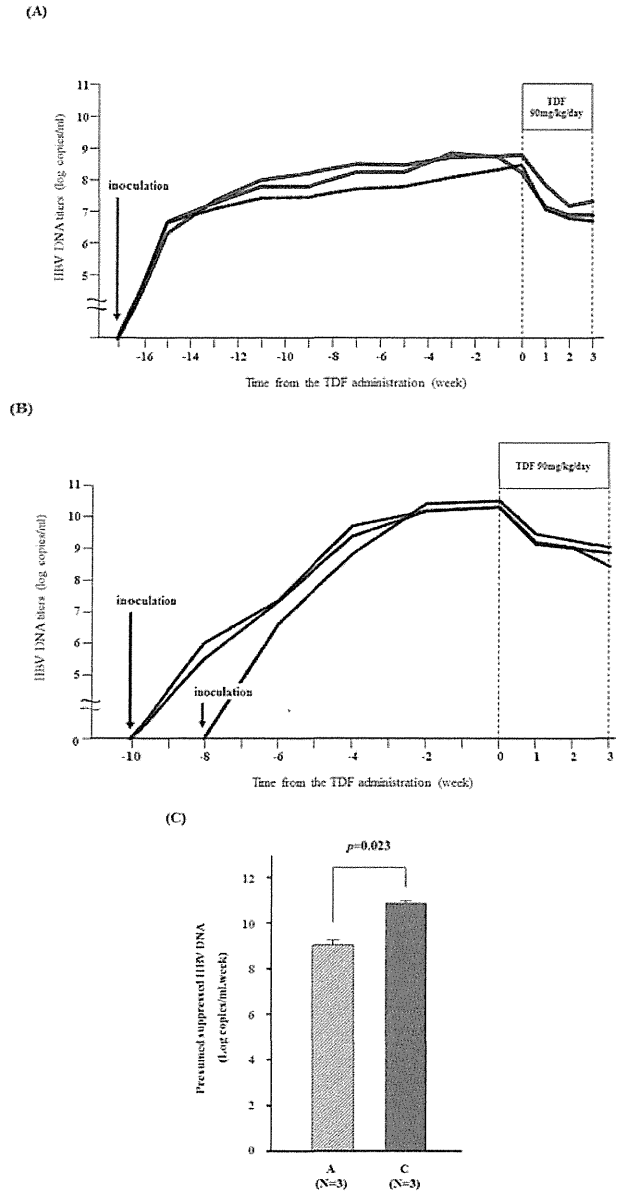

Figure 5 *Comparison of TDF susceptibilities between genotypes A and C using human hepatocyte chimeric mice.* (A, B) Each of 3 human hepatocyte chimeric mice were inoculated with A-WT or C-WT HBV particles and treated with 90 mg/kg body weight/day of TDF for 3 weeks. Changes in HBV DNA titers in mouse sera were followed. (A; A-WT, B; C-WT) (C) Comparing the presumed TDF susceptibilities between A-WT and C-WT, A-WT was suppressed significantly more weakly than C-WT after 3 weeks of TDF treatment ($p = 0.023$). Values are expressed as mean plus standard deviation and were evaluated by Welch's t-test.

susceptibility, *in vivo* analysis was also performed using human hepatocyte chimeric mice. According to a previous study, viral growth is different between HBV genotypes A and C in chimpanzee,[50] and a significant difference in viral growth was also observed in the present *in vivo* study (Fig. 5) because of the influences of several factors, such as viral fitness in hepatocytes and formation of cccDNA. Therefore, TDF susceptibilities were evaluated by the calculation of the area (copies.week) by integrating the suppressed titers of HBV DNA (copies) over treatment duration (week) (Supplemental Fig. 2). As shown in Fig. 5, the presumed TDF susceptibility of the C-WT clone was significantly higher than the A-WT clone in spite of its relatively high viral growth. In accordance with these *in vitro* and *in vivo* analyses, HBV genotype A might have weaker tolerance to TDF treatment, and the incidence of TDF resistance might be higher in patients infected with HBV genotype A than in genotype C.

In conclusion, we analyzed TDF susceptibility of several drug-resistant HBV strains using *in vitro* and *in vivo* models. We found that TDF was effective for LMV- or ETV-resistant HBV clones, but the susceptibility was intermediately decreased by adding a rtN236T substitution in the RT domain like the LMV plus ADV-resistant HBV clone. Furthermore, HBV clones generated from genotype A had lower sensitivities to TDF than those from genotype C *in vitro* and *in vivo*. These results might yield valuable information for constructing a suitable strategy for chronic hepatitis B on the grounds of HBV genotypic differences.

Competing interests

None to declare.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jinf.2015.09.038.

References

1. WHO. *Hepatitis B fact sheet.* 2014. http://www.who.int/mediacentre/factsheets/fs204/en/.
2. Belloni L, Allweiss L, Guerrieri F, Pediconi N, Volz T, Pollicino T, et al. IFN-alpha inhibits HBV transcription and replication in cell culture and in humanized mice by targeting the epigenetic regulation of the nuclear cccDNA minichromosome. *J Clin Invest* 2012 Feb 1;**122**(2):529–37. http://www.

ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22251702.
3. Belloni L, Pollicino T, De Nicola F, Guerrieri F, Raffa G, Fanciulli M, et al. Nuclear HBx binds the HBV minichromosome and modifies the epigenetic regulation of cccDNA function. *Proc Natl Acad Sci U S A* 2009 Nov 24;**106**(47):19975–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19906987.
4. Brechot C, Gozuacik D, Murakami Y, Paterlini-Brechot P. Molecular bases for the development of hepatitis B virus (HBV)-related hepatocellular carcinoma (HCC). *Semin Cancer Biol* 2000 Jun;**10**(3):211–31. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10936070.
5. Murakami Y, Saigo K, Takashima H, Minami M, Okanoue T, Brechot C, et al. Large scaled analysis of hepatitis B virus (HBV) DNA integration in HBV related hepatocellular carcinomas. *Gut* 2005 Aug;**54**(8):1162–8. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16009689.
6. Nagaya T, Nakamura T, Tokino T, Tsurimoto T, Imai M, Mayumi T, et al. The mode of hepatitis B virus DNA integration in chromosomes of human hepatocellular carcinoma. *Genes Dev* 1987 Oct;**1**(8):773–82. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2828171.
7. Yaginuma K, Kobayashi H, Kobayashi M, Morishima T, Matsuyama K, Koike K. Multiple integration site of hepatitis B virus DNA in hepatocellular carcinoma and chronic active hepatitis tissues from children. *J Virol* 1987 Jun;**61**(6):1808–13. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=3033312.
8. Conjeevaram HS, Lok AS. Management of chronic hepatitis B. *J Hepatol* 2003;**38**(Suppl. 1):S90–103. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12591188.
9. Lee YS, Suh DJ, Lim YS, Jung SW, Kim KM, Lee HC, et al. Increased risk of adefovir resistance in patients with lamivudine-resistant chronic hepatitis B after 48 weeks of adefovir dipivoxil monotherapy. *Hepatology* 2006 Jun;**43**(6):1385–91. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16729316.
10. Suzuki Y, Kumada H, Ikeda K, Chayama K, Arase Y, Saitoh S, et al. Histological changes in liver biopsies after one year of lamivudine treatment in patients with chronic hepatitis B infection. *J Hepatol* 1999 May;**30**(5):743–8. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10365796.
11. Ghany M, Liang TJ. Drug targets and molecular mechanisms of drug resistance in chronic hepatitis B. *Gastroenterology* 2007 Apr;**132**(4):1574–85. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17408658.
12. Lok AS, Zoulim F, Locarnini S, Bartholomeusz A, Ghany MG, Pawlotsky JM, et al. Antiviral drug-resistant HBV: standardization of nomenclature and assays and recommendations for management. *Hepatology* 2007 Jul;**46**(1):254–65. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17596850.
13. Yatsuji H, Hiraga N, Mori N, Hatakeyama T, Tsuge M, Imamura M, et al. Successful treatment of an entecavir-resistant hepatitis B virus variant. *J Med Virol* 2007 Dec;**79**(12):1811–7. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17935165.
14. Yatsuji H, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Emergence of a novel lamivudine-resistant hepatitis B virus variant with a substitution outside the YMDD motif. *Antimicrob Agents Chemother* 2006 Nov;**50**(11):3867–74. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16982790.
15. Zoulim F, Locarnini S. Hepatitis B virus resistance to nucleos(t)ide analogues. *Gastroenterology* 2009 Nov;**137**(5):1593–608. e1–2, http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19737565.
16. Ghany MG, Doo EC. Antiviral resistance and hepatitis B therapy. *Hepatology* 2009 May;**49**(Suppl. 5):S174–84. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19399794.
17. Locarnini S. Primary resistance, multidrug resistance, and cross-resistance pathways in HBV as a consequence of treatment failure. *Hepatol Int* 2008 Jun;**2**(2):147–51. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19669299.
18. Seeger C, Mason WS. Hepatitis B virus biology. *Microbiol Mol Biol Rev* 2000 Mar;**64**(1):51–68. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=10704474.
19. Davis MG, Wilson JE, VanDraanen NA, Miller WH, Freeman GA, Daluge SM, et al. DNA polymerase activity of hepatitis B virus particles: differential inhibition by L-enantiomers of nucleotide analogs. *Antiviral Res* 1996 May;**30**(2–3):133–45. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=8783805.
20. Jacobo-Molina A, Ding J, Nanni RG, Clark Jr AD, Lu X, Tantillo C, et al. Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA. *Proc Natl Acad Sci U S A* 1993 Jul 1;**90**(13):6320–4. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=7687065.
21. Poch O, Sauvaget I, Delarue M, Tordo N. Identification of four conserved motifs among the RNA-dependent polymerase encoding elements. *Embo J* 1989 Dec 1;**8**(12):3867–74. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=2555175.
22. Yang HI, Yeh SH, Chen PJ, Iloeje UH, Jen CL, Su J, et al. Associations between hepatitis B virus genotype and mutants and the risk of hepatocellular carcinoma. *J Natl Cancer Inst* 2008 Aug 20;**100**(16):1134–43. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18695135.
23. Yatsuji H, Suzuki F, Sezaki H, Akuta N, Suzuki Y, Kawamura Y, et al. Low risk of adefovir resistance in lamivudine-resistant chronic hepatitis B patients treated with adefovir plus lamivudine combination therapy: two-year follow-up. *J Hepatol* 2008 Jun;**48**(6):923–31. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18433925.
24. Liu Y, Wang C, Zhong Y, Chen L, Li X, Ji D, et al. Evolution and suppression of HBV strains with multidrug resistance to lamivudine, adefovir dipivoxil and entecavir in a patient with chronic hepatitis B. *Antivir Ther* 2010;**15**(8):1185–90. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21149926.
25. Gordon SC, Krastev Z, Horban A, Petersen J, Sperl J, Dinh P, et al. Efficacy of tenofovir disoproxil fumarate at 240 weeks in patients with chronic hepatitis B with high baseline viral load. *Hepatology* 2013 Aug;**58**(2):505–13. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23364953.
26. Kitrinos KM, Corsa A, Liu Y, Flaherty J, Snow-Lampart A, Marcellin P, et al. No detectable resistance to tenofovir disoproxil fumarate after 6 years of therapy in patients with chronic hepatitis B. *Hepatology* 2014 Feb;**59**(2):434–42.

http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23939953.
27. Snow-Lampart A, Chappell B, Curtis M, Zhu Y, Myrick F, Schawalder J, et al. No resistance to tenofovir disoproxil fumarate detected after up to 144 weeks of therapy in patients monoinfected with chronic hepatitis B virus. *Hepatology* 2011 Mar;**53**(3):763–73. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=21374657.
28. van Bommel F, de Man RA, Wedemeyer H, Deterding K, Petersen J, Buggisch P, et al. Long-term efficacy of tenofovir monotherapy for hepatitis B virus-monoinfected patients after failure of nucleoside/nucleotide analogues. *Hepatology* 2010 Jan;**51**(1):73–80. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19998272.
29. Chien RN, Yeh CT, Tsai SL, Chu CM, Liaw YF. Determinants for sustained HBeAg response to lamivudine therapy. *Hepatology* 2003 Nov;**38**(5):1267–73. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=14578866.
30. Damerow H, Yuen L, Wiegand J, Walker C, Bock CT, Locarnini S, et al. Mutation pattern of lamivudine resistance in relation to hepatitis B genotypes: hepatitis B genotypes differ in their lamivudine resistance associated mutation pattern. *J Med Virol* 2010 Nov;**82**(11):1850–8. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=20872711.
31. Sugiyama M, Tanaka Y, Kato T, Orito E, Ito K, Acharya SK, et al. Influence of hepatitis B virus genotypes on the intra- and extracellular expression of viral DNA and antigens. *Hepatology* 2006 Oct;**44**(4):915–24. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17006908.
32. Palumbo E. Hepatitis B genotypes and response to antiviral therapy: a review. *Am J Ther* 2007 May–Jun;**14**(3):306–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17515708.
33. Suzuki Y, Kobayashi M, Ikeda K, Suzuki F, Arfase Y, Akuta N, et al. Persistence of acute infection with hepatitis B virus genotype A and treatment in Japan. *J Med Virol* 2005 May;**76**(1):33–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15779048.
34. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 2001 Aug;**7**(8):927–33. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=11479625.
35. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 2004 Sep;**165**(3):901–12. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15331414.
36. Tsuge M, Hiraga N, Takaishi H, Noguchi C, Oga H, Imamura M, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 2005 Nov;**42**(5):1046–54. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16250045.
37. Noguchi C, Ishino H, Tsuge M, Fujimoto Y, Imamura M, Takahashi S, et al. G to A hypermutation of hepatitis B virus. *Hepatology* 2005 Mar;**41**(3):626–33. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=15726649.
38. Hatakeyama T, Noguchi C, Hiraga N, Mori N, Tsuge M, Imamura M, et al. Serum HBV RNA is a predictor of early emergence of the YMDD mutant in patients treated with lamivudine. *Hepatology* 2007 May;**45**(5):1179–86. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=17465002.
39. Tsuge M, Murakami E, Imamura M, Abe H, Miki D, Hiraga N, et al. Serum HBV RNA and HBeAg are useful markers for the safe discontinuation of nucleotide analogue treatments in chronic hepatitis B patients. *J Gastroenterol* 2013 Oct;**48**(10):1188–204. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23397114.
40. Angus P, Vaughan R, Xiong S, Yang H, Delaney W, Gibbs C, et al. Resistance to adefovir dipivoxil therapy associated with the selection of a novel mutation in the HBV polymerase. *Gastroenterology* 2003 Aug;**125**(2):292–7. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12891527.
41. Kobayashi M, Suzuki F, Akuta N, Suzuki Y, Arase Y, Ikeda K, et al. Response to long-term lamivudine treatment in patients infected with hepatitis B virus genotypes A, B, and C. *J Med Virol* 2006 Oct;**78**(10):1276–83. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16927289.
42. WEt Delaney, Ray AS, Yang H, Qi X, Xiong S, Zhu Y, et al. Intracellular metabolism and in vitro activity of tenofovir against hepatitis B virus. *Antimicrob Agents Chemother* 2006 Jul;**50**(7):2471–7. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16801428.
43. Sheldon J, Camino N, Rodes B, Bartholomeusz A, Kuiper M, Tacke F, et al. Selection of hepatitis B virus polymerase mutations in HIV-coinfected patients treated with tenofovir. *Antivir Ther* 2005;**10**(6):727–34. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=16218172.
44. Qin B, Budeus B, Cao L, Wu C, Wang Y, Zhang X, et al. The amino acid substitutions rtP177G and rtF249A in the reverse transcriptase domain of hepatitis B virus polymerase reduce the susceptibility to tenofovir. *Antiviral Res* 2013 Feb;**97**(2):93–100. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23261845.
45. Cho HJ, Kim SS, Shin SJ, Yoo BM, Cho SW, Cheong JY. Tenofovir-based rescue therapy in chronic hepatitis B patients with suboptimal responses to adefovir with prior lamivudine resistance. *J Med Virol* 2015 Sep;**87**(9):1532–8. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=25940352.
46. Buster EH, Hansen BE, Lau GK, Piratvisuth T, Zeuzem S, Steyerberg EW, et al. Factors that predict response of patients with hepatitis B e antigen-positive chronic hepatitis B to peginterferon-alfa. *Gastroenterology* 2009 Dec;**137**(6):2002–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=19737568.
47. Wong GL, Chan HL, Yiu KK, Lai JW, Chan VK, Cheung KK, et al. Meta-analysis: the association of hepatitis B virus genotypes and hepatocellular carcinoma. *Aliment Pharmacol Ther* 2013 Mar;**37**(5):517–26. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=23305043.
48. Ono A, Suzuki F, Kawamura Y, Sezaki H, Hosaka T, Akuta N, et al. Long-term continuous entecavir therapy in nucleos(t)ide-naive chronic hepatitis B patients. *J Hepatol* 2012 Sep;**57**(3):508–14. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=22659518.
49. Suzuki F, Tsubota A, Arase Y, Suzuki Y, Akuta N, Hosaka T, et al. Efficacy of lamivudine therapy and factors associated with emergence of resistance in chronic hepatitis B virus infection

in Japan. *Intervirology* 2003;**46**(3):182–9. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12867757.

50. Komiya Y, Katayama K, Yugi H, Mizui M, Matsukura H, Tomoguri T, et al. Minimum infectious dose of hepatitis B virus in chimpanzees and difference in the dynamics of viremia between genotype A and genotype C. *Transfusion* 2008 Feb; **48**(2):286–94. http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=18028278.

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SUMMARY. The hepatitis C virus (HCV) epidemic was forecasted through 2030 for 15 countries in Europe, the Middle East and Asia, and the relative impact of two scenarios was considered: [1] increased treatment efficacy while holding the annual number of treated patients constant and [2] increased treatment efficacy and an increased annual number of treated patients. Increasing levels of diagnosis and treatment, in combination with improved treatment efficacy, were critical for achieving substantial reductions in disease burden. A 90% reduction in total HCV infections within 15 years is feasible in most countries studied, but it required a coordinated effort to introduce harm reduction programmes to reduce new infections, screening to identify those already infected and treatment with high cure rate therapies. This suggests that increased capacity for screening and treatment will be critical in many countries. Birth cohort screening is a helpful tool for maximizing resources. Among European countries, the majority of patients were born between 1940 and 1985. A wider range of birth cohorts was seen in the Middle East and Asia (between 1925 and 1995).

Keywords: diagnosis, disease burden, elimination, epidemiology, hepatitis C, hepatitis C virus, incidence, mortality, prevalence, scenarios, strategy, treatment.

Abbreviations: DAA, direct-acting antiviral agent; G, Genotype; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; IDU, injection drug use; Peg-IFN, Pegylated interferon; RBV, ribavirin; SVR, sustained viral response.

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INTRODUCTION

Although the prevalence of hepatitis C virus (HCV) is decreasing in many countries, there is an expected increase in HCV-related morbidity and mortality as the prevalent population ages and progresses to more advanced disease stages. The previous publications in this supplement demonstrated the expected disease burden if no changes were made to the current treatment paradigm. However, it is reasonable to assume that changes will occur, due to current and future adoption of new, more efficacious therapies. This study was designed to demonstrate the potential impact that various disease control strategies, both conservative and aggressive, might have on the future HCV disease burden in individual countries. The results are not intended to stipulate the adoption of these specific strategies, but rather to illustrate what outcomes might be possible should similar intervention strategies be implemented.

METHODOLOGY

The details of the model used to forecast HCV disease burden were described previously [1–3]. The model interface allowed for changing assumptions of the number of patients treated, the proportion of cases eligible for treatment, the reduction in treatment restrictions, the average sustained viral response (SVR) by genotype, the number of newly diagnosed individuals and the number of new infections at five different points in time. The year in which these changes took effect was also an input field. A variety of new therapies were considered, including: direct-acting antivirals (DAAs) + pegylated interferon (Peg-IFN) + ribavirin (RBV), DAA + RBV, interferon-free all-oral, second-generation DAA combinations and third-generation combinations. All changes took effect immediately, and the co-existence of multiple therapies was handled by modifying the average SVR.

The future number of treated patients was capped by (i) number diagnosed, (ii) number eligible and (iii) unrestricted cases. The latter related to implicit (defined by physician's practice) and/or explicit (defined by treatment guidelines) restrictions. These restrictions could be modified by changing the upper and lower end of patients' age and their stage of fibrosis (≥F4, ≥F3, ≥F2, ≥F1 or ≥F0). Review of treatment guidelines and interviews with expert panels were used to identify both. While age restrictions were applied to all genotypes, the restrictions by the stage of liver disease were applied to specific genotypes. Patients with decompensated cirrhosis, irrespective of genotype, were considered ineligible for any treatment that involved Peg-IFN. The fibrotic stages eligible for treatment are shown in Figs 1–15. When the number of treated patients was greater than those diagnosed, eligible and unrestricted, the number of newly diagnosed cases was increased or the treatment restrictions were relaxed. The focus of the analysis was to highlight how many cases have to be diagnosed to achieve a strategy rather than to forecast the screening capacity in a country.

According to the literature, approximately 40–60% of HCV patients are eligible for Peg-IFN/RBV treatment [4–6]. The definition of eligibility included lack of contraindications to the drugs (e.g. psychiatric conditions) as well as patients' preference. For all countries, a treatment eligibility of 60% was used for all therapies that included Peg-IFN/RBV. When Peg-IFN could be eliminated, the eligibility was increased. The increase in eligibility did not increase treatment in the future. However, it did increase the pool of diagnosed and eligible patients who could be treated. Any changes in treatment were implemented using a separate input.

In this analysis, three strategies were considered – base, increased efficacy only and increased efficacy and treatment. The base strategy was defined as the case when all assumptions (the number of acute cases, treated patients, percent of patients eligible for treatment, treatment restrictions, the number of newly diagnosed and the average SVR by genotype) remained the same as today. This was assumed to be the most conservative, but feasible, scenario. Even more conservative scenarios are possible (e.g., stop treating HCV patients completely), but those were deemed to be unlikely. The base scenario for each country was described in detail previously [1]. In the second strategy, the impact of increasing the SVR of therapies was considered. The number of treated patients remained the same as in the base strategy. In a few countries, treatment restrictions were relaxed if there were no longer enough patients left to treat in the future. However, all other assumptions remained consistent with the base strategy.

The third scenario included an increase in both SVR and treatment uptake. The assumptions for the number of treated patients in the future were often driven by a desire to achieve a certain goal (i.e. control HCV disease burden) and were developed in discussion with expert panels in each country. To achieve some of these strategies, expanding access to patients with early stages of fibrosis (F0–F2) was considered. In most instances, the number of newly diagnosed cases also had to be increased to keep up with the depletion of the diagnosed eligible patient pool.

Scenario inputs, including SVR, fibrosis stage and medical eligibility are provided, by genotype and year, in Figs 1–15. The numbers of treated and diagnosed patients necessary to achieve the desired scenario outputs are also provided.

In all instances, viremic infections represented current HCV or chronic HCV infections. The term viremic was used throughout this study to highlight the presence of HCV virus. The term incidence was used for new HCV infections and not newly diagnosed. Hepatocellular carcinoma (HCC) referred to the total number of viremic HCV-related HCC cases, rather than new cases. Additionally, all reductions

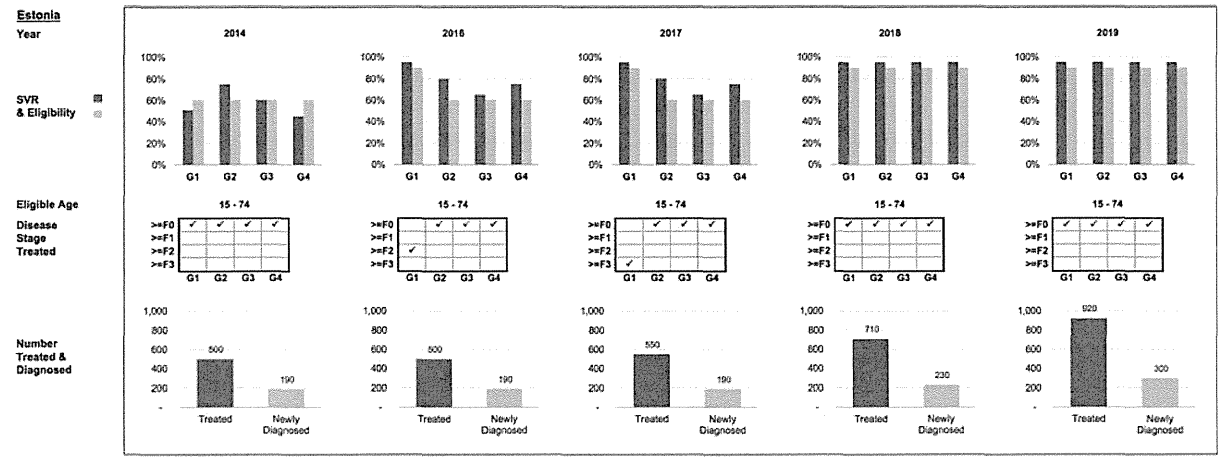

Fig. 1 Estonia model inputs, by year.

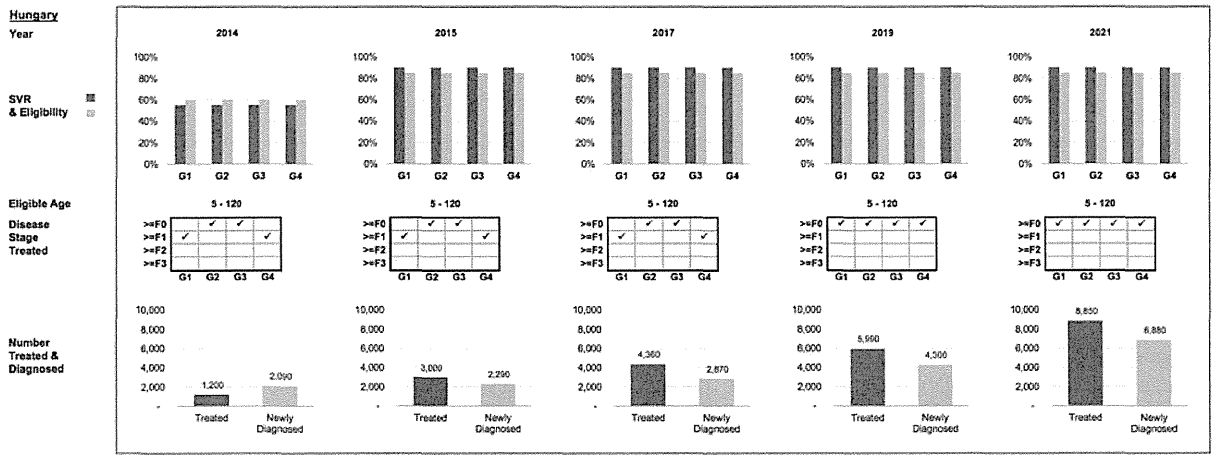

Fig. 2 Hungary model inputs, by year.

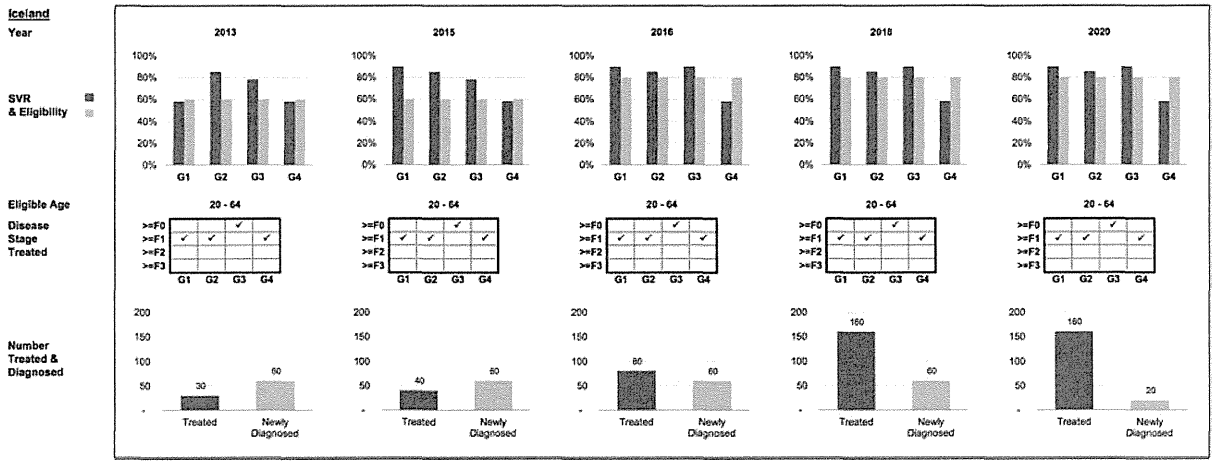

Fig. 3 Iceland model inputs, by year.