stretch that regulates extranuclear export of HDAg (Lee *et al.*, 2001) (Fig. 5).

DISCUSSION

In the present study, we initially sequenced the full-length HDV genome from 33 patients in Japan with chronic HDV infection and demonstrated a new HDV genotype IIb variant in the Miyako Islands, Okinawa, the Japanese islands nearest to Taiwan. Patients infected with genotype IIb-M showed greater progression to CH or liver cirrhosis than genotype IIb-infected patients with similar clinical backgrounds. HDV genotype IIb-M has specific genetic structures in the RNA editing site and the packaging signal sequence of HDAg, which could potentially influence the efficiency of HDV replication (Casey, 2002; Hsu *et al.*, 2002; Lee *et al.*, 1995, 2001; Wu *et al.*, 1995b; Yang *et al.*, 1995). The observed correlation between HDV genetic structure and clinical characteristics suggests a critical role for variations in the RNA editing site and packaging signal of the HDAg gene in determining the diversity of clinical outcome, even among patients infected with the same genotype of HDV.

We identified the new HDV genotype IIb variant by phylogenetic analysis of the complete genomes of 33 HDV isolates. Among them, 30 isolates, mostly from the Miyako Islands, were classified as genotype IIb (Wu *et al.*, 1998) or its variant, IIb-M. In previous studies including our own, HDV genotypes in the Miyako Islands have been considered as genotype IIb (Sakugawa *et al.*, 1999; Ma *et al.*, 2003; Arakawa *et al.*, 2000). However, the present detailed phylogenetic analysis using the full genome successfully identified a cluster distinct from the prototype IIb cluster. In fact, the nucleotide homologies between genotype IIb and IIb-M and among genotype IIb-M were clearly different, i.e. 88–90 % and 94–97 %, respectively. HDV genotype II is divided into two types in Taiwan (i.e. IIa and IIb), with 77 % nucleotide homology between the complete sequences of genotype IIa and IIb (Wu *et al.*, 1998). Although the criteria for defining identical genotype by homology analysis were not determined, the difference between IIb and IIb-M seems to be less than that between IIa and IIb, as shown by phylogenetic tree analysis. In fact, a IIa variant was recently reported in Siberia (IIa-Yakutia), which in comparison with IIa showed a similar degree of genetic differences (Ivaniushina *et al.*, 2001). Based on these results, we conclude that IIb-M should be considered as a genetically relevant IIb variant.

Genotype II is confined to East Asia (mainly Siberia, Japan and Taiwan), in contrast to the ubiquitous global distribution of genotype I (Gerin *et al.*, 2002). Genotype IIb was first identified in Taiwan (Wu *et al.*, 1998) and we subsequently reported it among patients from the Miyako Islands (Sakugawa *et al.*, 1999). However, the origin of clusters of IIb-M cannot be precisely determined. If in the future the precise evolution rate of the HDV genome can be determined, then the temporal estimation of the spread of HDV using a molecular clock might be possible.

One of the most important findings in the present study is that the clinical pictures differ between genotype IIb and IIb-M. Our previous studies demonstrated that HDV genotype II is predominant in this area and that these patients show heterogeneous clinical pictures ranging from ASC to HCC (Sakugawa *et al.*, 1999; Nakasone *et al.*, 1998); however, the reason for this diversity could not be explained based on the known clinical and virological factors of HBV. In the present study, all of the patients with chronic HDV genotype IIb infection were ASC or CH and none were at the LC or HCC stage. In contrast, 55 % and 45 % of patients with genotype IIb-M were in the CH and LC stages, respectively, and none of them was ASC. These findings indicate that patients with genotype IIb-M are more likely to progress to LC and HCC than those with genotype IIb and that differences in HDV genotype could cause the different clinical pictures observed in this population.

The main cause of the difference in liver disease between patients with IIb and IIb-M seems to be the diversity of HDV itself. Although the severity of liver disease in hepatitis D can be influenced by a variety of host factors including genetic backgrounds as well as HBV status, no apparent differences were found between patients with genotype IIb and IIb-M. In particular, in most patients, serum HBV DNA levels were below 10^5 copies ml^{-1} with negative HBeAg, which were too low to cause HBV-related liver injury (Sakugawa *et al.*, 2001; Lok *et al.*, 2001). Similarly, the HBV genotype, which is also known to cause diversity of liver disease (Kao *et al.*, 2000; Orito *et al.*, 2001), was genotype B in all of the patients from the Miyako Islands. Differences in HDV genotype are known to affect the pathogenesis and diverse clinical pictures of HDV infection (Casey *et al.*, 1993; Wu *et al.*, 1995a; Ivaniushina *et al.*, 2001). Genotype III, exclusively found in the northern part of South America, is associated with fulminant hepatitis (Casey *et al.*, 1993). On the other hand, genotype II in Taiwan is generally associated with a more favourable outcome than genotype I, which causes liver disease with diverse clinical presentation from asymptomatic carrier to rapidly progressive CH (Wu *et al.*, 1995a). A IIa variant recently reported in Yakutia, Siberia, Russia, also causes a severe hepatitis comparable with genotype I in this cohort (Ivaniushina *et al.*, 2001). These findings strongly suggest that the genetic structure of HDV can profoundly influence the pathogenesis of liver injury in HDV infection. However, the genetic structure responsible for such clinical features could not be readily determined because the genetic differences between the different genotypes are too diverse, as seen in Fig. 2. In contrast, despite the different clinical pictures between IIb and IIb-M, the genetic differences are small enough to enable the definition of the genetic features of HDV pathogenesis and replication *in vivo*.

By comparative analysis between the genotype IIb and IIb-M genomes, the highest difference was found in the

hypervariable region (nt 1598–657) and moderately high in HDAg (nt 957–1597), whereas the autocatalytic regions encoding ribozyme activity were well conserved (Wu & Lai, 1989). The hypervariable region was markedly variable even within the same genotype, supporting the notion that this region cannot confer any relevant biological function aside from the formation of the rod structure of HDV RNA required for RNA synthesis by RNA polymerase II (Modahl & Lai, 2000). On the other hand, the requirement for strict secondary or tertiary structure of the autocatalytic domain seems to be so crucial for full activity of the ribozyme needed for the rolling-circle mechanism of HDV replication that divergence of this region could not exist among isolates. Therefore, HDV genetic regions other than the hypervariable region or the autocatalytic domain, i.e. the HDAg coding region, confer the clinical difference between IIb and IIb-M. In the HDAg coding region, we found that the most prominent differences are in the RNA editing site and the packaging signal in the C terminus of the large HDAg (Modahl & Lai, 2000). Although the coiled-coil domain (Wang & Lemon, 1993) also showed modest differences, the leucine zipper motif (Chen *et al.*, 1992) was preserved, and the nuclear localizing signal (Xia *et al.*, 1992) and RNA binding domain (Lin *et al.*, 1990) were identical in IIb and IIb-M, indicating that these regions are not responsible for liver damage.

In genotype IIb-M, there was particular disruption of the base-pairing structure two bases upstream of the editing site, resulting in a characteristic structure in this region distinct from genotype IIb and IIa (Fig. 3). There is a possibility that the unique structure of the RNA editing site of genotype IIb-M may affect the observed difference in pathogenesis between genotype IIb and IIb-M. RNA editing is a pivotal event during the HDV replication cycle (Casey *et al.*, 1992), where initially in HDV infection, small HDAg transactivates HDV RNA synthesis by RNA polymerase II (Wu *et al.*, 1995b). Large HDAg, which has 19 additional amino acids (the packaging signal sequence) at the C terminus of small HDAg, is produced in the late stage of infection by RNA editing of the umber stop codon (UAG) to a tryptophan codon (UGG) in the small HDAg gene by the host adenosine deaminase (Modahl & Lai, 2000). Large HDAg suppresses HDV RNA replication and promotes virion assembly by extranuclear export of the HDAg–RNA complex and binding to HBsAg. The regulatory mechanism of this RNA editing is not fully understood, but the secondary structure of the antigenomic region corresponding to the 3′ end of the small HDAg gene influences the editing efficiency (Casey *et al.*, 1992; Wu *et al.*, 1995b; Casey, 2002). A recent *in vitro* mutational study clearly demonstrated that the base-pairing structure surrounding the RNA editing site profoundly influences RNA editing efficiency (Hsu *et al.*, 2002). In genotype I, the base pairing surrounding this site is particularly strong (four base pairs on each side), whereas a weaker secondary structure is found within genotype II that is associated with milder liver disease. In addition, the distinct structure of genotype III is thought to be involved in fulminant hepatitis (Casey, 2002). Collectively, the specific differences in the base-paired structure of the RNA editing site might explain to some extent the difference in virulence among HDV genotypes. Therefore, although *in vitro* confirmation is necessary, it appears that the loose structure around the RNA editing site found in genotype IIb-M might influence the editing efficiency in comparison with genotype IIb, leading to the observed clinical differences.

In addition to the difference in the RNA editing site, there are four characteristic amino acid differences (codons 198, 200, 201 and 203) in the packaging signal sequence of the large HDAg between genotype IIb and IIb-M (Fig. 5). This region is almost completely conserved among IIb-M isolates. As mentioned above, addition of this packaging signal reverses the property of HDAg (Modahl & Lai, 2000; Chang *et al.*, 1993). The exact molecular mechanism of this phenomenon is not completely understood, but, as shown in Fig. 4, a sequence of 19 amino acids was highly genotype specific. *In vitro* analysis demonstrated that swapping the packaging signal sequence of genotype IIa with that of genotype I HDAg decreases the virus replication of genotype I, while the replication of genotype II was intensified, indicating that this region directly regulates HDV RNA replication (Hsu *et al.*, 2002). Thus, the structural characteristics of this region in IIb-M can profoundly influence virus replication. In particular, two of the four amino acid differences found in IIb-M were located in the proline residues, which are implicated in the assembly process by extranuclear export of the HDAg–RNA complex. In fact, in a recent study with cultured cells, mutation of the proline residue in this region attenuated the extranuclear export of large HDAg (Lee *et al.*, 2001). However, these data did not directly prove that the C-terminal domain structure of HDAg influences the pathogenesis. In the future, *in vitro* mutational studies should be performed to verify the hypothesis that differences in the packaging signal sequence in genotype IIb-M can modulate HDV replication and lead to progressive disease.

In conclusion, we have identified a new genetic subclass of HDV genotype IIb in the Miyako Islands, Okinawa, Japan. This HDV variant is associated with more aggressive liver disease and has specific genetic changes in the C-terminal packaging signal of large HDAg as well as the RNA editing sequence. These findings should prompt further investigation into the relationship between HDV genetic structures and their function and pathogenesis. This study provides valuable information for molecular epidemiology and diagnosis, contributes to a better understanding of HDV biology and offers the potential for new therapies for HDV – a disease for which no effective therapy has yet been established.

ACKNOWLEDGEMENTS

This work was supported in part by a Grant-in-aid (08457164) from the Ministry of Education, Sciences, Culture and Sports of Japan.

REFERENCES

Arakawa, Y., Moriyama, M., Taira, M., Hayashi, N., Tanaka, N., Okubo, H. & Sugitani, M. (2000). Molecular analysis of hepatitis D virus infection in Miyako Island, a small Japanese island. *J Viral Hepat* **7**, 375–381.

Casey, J. L. (2002). RNA editing in hepatitis delta virus genotype III requires a branched double-hairpin RNA structure. *J Virol* **76**, 7385–7397.

Casey, J. L., Bergmann, K. F., Brown, T. L. & Gerin, J. L. (1992). Structural requirements for RNA editing in hepatitis delta virus: evidence for a uridine-to-cytidine editing mechanism. *Proc Natl Acad Sci U S A* **89**, 7149–7153.

Casey, J. L., Brown, T. L., Colan, E. J., Wignall, F. S. & Gerin, J. L. (1993). A genotype of hepatitis D virus that occurs in northern South America. *Proc Natl Acad Sci U S A* **90**, 9016–9020.

Chang, M. F., Sun, C. Y., Chen, C. J. & Chang, S. C. (1993). Functional motifs of delta antigen essential for RNA binding and replication of hepatitis delta virus. *J Virol* **67**, 2529–2536.

Chen, P. J., Chang, F. L., Wang, C. J., Lin, C. J., Sung, S. Y. & Chen, D. S. (1992). Functional study of hepatitis delta virus large antigen in packaging and replication inhibition: role of the amino-terminal leucine zipper. *J Virol* **66**, 2853–2859.

Chomczynski, P. & Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. *Anal Biochem* **162**, 156–159.

Enomoto, N., Kurosaki, M., Tanaka, Y., Marumo, F. & Sato, C. (1994). Fluctuation of hepatitis C virus quasispecies in persistent infection and interferon treatment revealed by single-strand conformation polymorphism analysis. *J Gen Virol* **75**, 1361–1369.

Gerin, J. L., Casey, J. L. & Purcell, R. H. (2002). Hepatitis delta virus. In *Viral Hepatitis*, pp. 169–182. Edited by F. Blaine Hollinger. Philadelphia: Lippincott Williams & Wilkins.

Glenn, J. S., Watson, J. A., Havel, C. M. & White, J. M. (1992). Identification of a prenylation site in delta virus large antigen. *Science* **256**, 1331–1333.

Hsu, S. C., Syu, W. J., Sheen, I. J., Liu, H. T., Jeng, K. S. & Wu, J. C. (2002). Varied assembly and RNA editing efficiencies between genotypes I and II hepatitis D virus and their implications. *Hepatology* **35**, 665–672.

Imazeki, F., Omata, M. & Ohto, M. (1991). Complete nucleotide sequence of hepatitis delta virus RNA in Japan. *Nucleic Acids Res* **19**, 5439.

Ivaniushina, V., Radjef, N., Alexeeva, M., Gault, E., Semenov, S., Salhi, M., Kiselev, O. & Deny, P. (2001). Hepatitis delta virus genotypes I and II cocirculate in an endemic area of Yakutia, Russia. *J Gen Virol* **82**, 2709–2718.

Kamisango, K., Kamogawa, C., Sumi, M., Goto, S., Hirao, A., Gonzales, F., Yasuda, K. & Iino, S. (1999). Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. *J Clin Microbiol* **37**, 310–314.

Kao, J. H., Chen, P. J., Lai, M. Y. & Chen, D. S. (2000). Hepatitis B genotypes correlate with clinical outcomes in patients with chronic hepatitis B. *Gastroenterology* **118**, 554–559.

Lee, C. H., Chang, S. C., Wu, C. H. & Chang, M. F. (2001). A novel chromosome region maintenance 1-independent nuclear export signal of the large form of hepatitis delta antigen that is required for the viral assembly. *J Biol Chem* **276**, 8142–8148.

Lee, C. Z., Chen, P. J. & Chen, D. S. (1995). Large hepatitis delta antigen in packaging and replication inhibition: role of the carboxyl-terminal 19 amino acids and amino-terminal sequences. *J Virol* **69**, 5332–5336.

Lin, J. H., Chang, M. F., Baker, S. C., Govindarajan, S. & Lai, M. M. (1990). Characterization of hepatitis delta antigen: specific binding to hepatitis delta virus RNA. *J Virol* **64**, 4051–4058.

Lok, A. S., Heathcote, E. J. & Hoofnagle, J. H. (2001). Management of hepatitis B: 2000 – summary of a workshop. *Gastroenterology* **120**, 1828–1853.

Ma, S. P., Sakugawa, H., Makino, Y., Tadano, M., Kinjo, F. & Saito, A. (2003). The complete genomic sequence of hepatitis delta virus genotype IIb prevalent in Okinawa, Japan. *J Gen Virol* **84**, 461–464.

Makino, S., Chang, M. F., Shieh, C. K., Kamahora, T., Vannier, D. M., Govindarajan, S. & Lai, M. M. (1987). Molecular cloning and sequencing of a human hepatitis delta (δ) virus RNA. *Nature* **329**, 343–346.

Mizokami, M., Nakano, T., Orito, E., Tanaka, Y., Sakugawa, H., Mukaide, M. & Robertson, B. H. (1999). Hepatitis B virus genotype assignment using restriction fragment length polymorphism patterns. *FEBS Lett* **450**, 66–71.

Modahl, L. E. & Lai, M. M. (2000). Hepatitis delta virus: the molecular basis of laboratory diagnosis. *Crit Rev Clin Lab Sci* **37**, 45–92.

Nakano, T., Shapiro, C. N., Hadler, S. C., Casey, J. L., Mizokami, M., Orito, E. & Robertson, B. H. (2001). Characterization of hepatitis D virus genotype III among Yucpa Indians in Venezuela. *J Gen Virol* **82**, 2183–2189.

Nakasone, H., Sakugawa, H., Shokita, H. & 8 other authors (1998). Prevalence and clinical features of hepatitis delta virus infection in the Miyako Islands, Okinawa, Japan. *J Gastroenterol* **33**, 850–854.

Orito, E., Mizokami, M., Sakugawa, H., Michitaka, K., Ishikawa, K., Ichida, T., Okanoue, T., Yotsuyanagi, H. & Iino, S. (2001). A case–control study for clinical and molecular biological differences between hepatitis B viruses of genotypes B and C. Japan HBV Genotype Research Group. *Hepatology* **33**, 218–223.

Rizzetto, M., Canese, M. G., Gerin, J. L., London, W. T., Sly, D. L. & Purcell, R. H. (1980). Transmission of the hepatitis B virus-associated delta antigen to chimpanzees. *J Infect Dis* **141**, 590–602.

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

Sakugawa, H., Nakasone, H., Nakayoshi, T. & 7 other authors (1999). Hepatitis delta virus genotype IIb predominates in an endemic area, Okinawa, Japan. *J Med Virol* **58**, 366–372.

Sakugawa, H., Nakasone, H., Nakayoshi, T., Kawakami, Y., Yamashiro, T., Maeshiro, T., Kinjo, F., Saito, A. & Zukeran, H. (2001). Hepatitis B virus concentrations in serum determined by sensitive quantitative assays in patients with established chronic hepatitis delta virus infection. *J Med Virol* **65**, 478–484.

Tamura, I., Kurimura, O., Koda, T., Ichimura, H., Katayama, S., Kurimura, T. & Inaba, Y. (1993). Risk of liver cirrhosis and hepatocellular carcinoma in subjects with hepatitis B and delta virus infection: a study from Kure, Japan. *J Gastroenterol Hepatol* **8**, 433–436.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Wang, J. G. & Lemon, S. M. (1993). Hepatitis delta virus antigen forms dimers and multimeric complexes in vivo. *J Virol* **67**, 446–454.

Wang, K. S., Choo, Q. L., Weiner, A. J. & 7 other authors (1986). Structure, sequence and expression of the hepatitis delta (δ) viral genome. *Nature* **323**, 508–514.

Wu, H. N. & Lai, M. M. (1989). Reversible cleavage and ligation of hepatitis delta virus RNA. *Science* **243**, 652–654.

Wu, J. C., Choo, K. B., Chen, C. M., Chen, T. Z., Huo, T. I. & Lee, S. D. (1995a). Genotyping of hepatitis D virus by restriction-fragment length polymorphism and relation to outcome of hepatitis D. *Lancet* **346**, 939–941.

Wu, T. T., Bichko, V. V., Ryu, W. S., Lemon, S. M. & Taylor, J. M. (1995b). Hepatitis delta virus mutant: effect on RNA editing. *J Virol* **69**, 7226–7231.

Wu, J. C., Chiang, T. Y. & Sheen, I. J. (1998). Characterization and phylogenetic analysis of a novel hepatitis D virus strain discovered by restriction fragment length polymorphism analysis. *J Gen Virol* **79**, 1105–1113.

Xia, Y. P., Yeh, C. T., Ou, J. H. & Lai, M. M. (1992). Characterization of nuclear targeting signal of hepatitis delta antigen: nuclear transport as a protein complex. *J Virol* **66**, 914–921.

Yang, A., Papaioannou, C., Hadzyannis, S., Thomas, H. & Monjardino, J. (1995). Base changes at positions 1014 and 578 of delta virus RNA in Greek isolates maintain base pair in rod conformation with efficient RNA editing. *J Med Virol* **47**, 113–119.

Available online at www.sciencedirect.com
SCIENCE @ DIRECT®
ELSEVIER
Hepatology Research 26 (2003) 275–280
Hepatology Research
www.elsevier.com/locate/ihepcom

Prevalence of hepatitis virus types B through E and genotypic distribution of HBV and HCV in Ho Chi Minh City, Vietnam

Huy Thien-Tuan Tran [a,b,c], Hiroshi Ushijima [b], Vo Xuan Quang [c], Nguyen Phuong [c], Tian-Cheng Li [d], Shigeki Hayashi [e], Truong Xuan Lien [f], Tetsutaro Sata [a], Kenji Abe [a,*]

[a] *Department of Pathology, National Institute of Infectious Diseases, Toyama 1-23-1, Shinjuku-ku, Tokyo 162-8640, Japan*
[b] *Department of Developmental Medical Sciences, Graduate School of Medicine, University of Tokyo, Tokyo, Japan*
[c] *Department of Gastroenterology and Hepatology, Cho Ray Hospital, Ho Chi Minh City, Viet Nam*
[d] *Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan*
[e] *National Disaster Medical Center, Tokyo, Japan*
[f] *Department of Biological Analysis, Pasteur Institute Ho Chi Minh City, Ho Chi Minh City, Viet Nam*

Received 30 September 2002; received in revised form 20 February 2003; accepted 10 March 2003

Abstract

A molecular epidemiological survey of various hepatitis viral infections, including hepatitis B virus (HBV), hepatitis C virus (HCV) and hepatitis D virus (HDV), was carried out in Ho Chi Minh City, Vietnam. This study included of 295 patients with liver disease (234 viral related and 61 non-viral related) and 100 healthy individuals. The infection rates of HBV and HCV in 234 liver disease patients with acute hepatitis, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma, were 31.2 and 19.2%, respectively. On the other hand, detection rates of these viruses in healthy populations were 10 and 2%, respectively ($P < 0.005$ and < 0.0001, respectively). None of cases tested was positive for HDV RNA. The most common viral genotypes were type B and C of HBV (43 and 57%) and type 2a of HCV (33.3%). Surprisingly, high prevalence of HBV pre-S2 deletion mutant was found in 22 of 87 (25.3%) patients with chronic liver disease. Moreover, antibody to hepatitis E virus (HEV) immunoglobulin G (IgG) was detected in 78 of 185 (42%) and IgM in 1 of 185 (0.5%) patients. The age prevalence of anti-HEV IgG was reached 61.9% in 21–40-year-olds. These results suggest that these hepatitis viruses, except for HDV, are spreading among liver disease patients in Ho Chi Minh city, Vietnam and HBV was the most important causative agent correlated with liver disease in this area.
© 2003 Elsevier Science B.V. All rights reserved.

Keywords: HBV DNA; HCV RNA; HBV genotyping; HCV genotyping; HBV pre-S deletion mutant; Anti-HEV prevalence in Ho Chi Minh City; Vietnam

1. Introduction

Viral hepatitis is still one of major public health problems throughout the world. Particularly, hepatitis B virus (HBV) and hepatitis C virus (HCV) are the causative agents responsible for parenteral transmitted diseases. It is known that the prevalence of HBV and HCV infections vary according to geographical areas and that their infections appear to correlate with the severity of chronic liver diseases such as liver cirrhosis and hepatocellular carcinoma. In Vietnam, an adequate level of information on the molecular epidemiology of hepatitis viruses has not been available so far, although some reports have appeared [1,2]. Here we report the molecular-based epidemiological characterization of hepatitis viruses, including types B, C, D and E in Ho Chi Minh City, which is located in the southern region and is the biggest city in Vietnam.

* Corresponding author. Tel.: +81-3-5285-1111x2624; fax: +81-3-5285-1189.
E-mail address: kenjiabe@nih.go.jp (K. Abe).

1386-6346/03/$ - see front matter © 2003 Elsevier Science B.V. All rights reserved.
doi:10.1016/S1386-6346(03)00166-9

2. Materials and methods

2.1. Study population

We tested 395 serum samples, including samples of 295 patients with liver disease from Cho Ray Hospital and 100 healthy persons from Pasteur Institute in Ho Chi Minh City, Vietnam. All subjects are residents in Ho Chi Minh City or its suburbs. Patients with liver disease were classified into two groups, i.e. viral and non-viral related liver disease, according to clinical manifestation, laboratory test, imaging (ultrasound and CT scan), serology and liver histopathology. Healthy group were persons who had had health check-up in medical centers. They had been showed neither clinical symptoms nor abnormalities in laboratory tests. Informed consent for participation in this study was obtained from each individual. These serum samples were collected from 1998 to 2001 and stored at −40 °C or below until use.

2.2. Extraction of nucleic acids and detection of HBV DNA and HCV RNA by multiplex PCR method

Both DNA and RNA were extracted simultaneously from 100 μl of serum by using the SepaGene RV-R kit (Sanko Junyaku Co., Ltd., Tokyo, Japan), precipitated with isopropanol, and washed in ethanol. The resulting pellet was resuspended in 50 μl of RNase-free water. The sequences of PCR primers were as follows. (1) For HBV (X region): 5′-TGCCAACTGGATCCTTCGCGG-GACGTCCTT-3′ (MD24, sense primer, nucleotide [nt] 1392–1421) and 5′-GTTCACGGTGGTCTCCATG-3′ (MD26, antisense primer, nt 1625–1607) for the outer primer pairs (233 bases), and 5′-GTCCCCTTCTTCATCTGCCGT-3′(HBx1, sense primer, nt 1487–1507) and 5′-ACGTGCAGAGGT-GAAGCGAAG-3′ (HBx2, antisense primer, nt 1604–1584) for the inner primer pairs (118 bases). (2) For HCV (5′-untranslated region): 5′-GCGACACTCCAC-CATAGAT-3′ (19, sense primer, nt 2–20 and 5′-GCTCATGGTGCACGGTCTA-3′ (20, antisense primer, nt 312–330) for the outer primer pairs (329 bases), and 5′-CTGTGAGGAACTACTGTCT-3′ (21, sense primer, nt 28–46) and 5′-ACTCGCAAGCACCC-TATCA-3′ (22, antisense primer, nt 277–295) for the inner primer pairs (268 bases). The nucleotide positions were deduced from HBVadr4 [3] for HBV and HC-J1 [4] for HCV. To obtain simultaneous detection of hepatitis B and C viral genomes, we used multiplex PCR method as described previously [5]. It was performed in a one-step that combines cDNA synthesis and PCR in a single tube. That is, for HCV, the first PCR was combined with the reverse transcriptase (RT) step in the same tube containing 50 μl of a reaction buffer prepared as follows: 10 units of RNase inhibitor (Promega, Madison, WI, USA), 100 units of Moloney murine leukemia virus RT (Promega), 40 ng of each outer primer for HBV and HCV, 300 μM of each of the four deoxynucleotides, 2 unit of AmpliTaq Gold DNA polymerase (Perkin–Elmer, Norwalk, CT, USA), and 1 × reaction buffer containing 1.5 mM $MgCl_2$. To obtain an automatic hot-start reaction, we used AmpliTaq Gold DNA polymerase instead of regular thermostable DNA polymerase. The thermocycler was programmed first to incubate the samples for 50 min at 37 °C for the initial RT step and then preheat at 95 °C for 10 min to activate Ampli*Taq* Gold followed by 40 cycles consisting of 94 °C for 30 s, 50 °C for 45 s, and 72 °C for 1 min using a Perkin–Elmer 2400 or 9700 Thermal Cycler (Perkin–Elmer). For the second reaction, 2 μl (1/25 volume) of the first PCR product were added to a tube containing the second set of each inner primer, deoxynucleotides, AmpliTaq Gold DNA polymerase, and PCR buffer as in the first reaction, but without reverse transcriptase and omitting the initial 50 min incubation at 37 °C. Amplification was performed for 40 cycles with the following parameters; preheat at 95 °C for 10 min, 20 cycles of amplification at 94 °C for 30 s, annealing at 53 °C for 45 s and extension at 72 °C for 1 min, followed by an additional 20 cycles of 94 °C for 30 s, 55 °C for 45 s and 72 °C for 1 min. The PCR products were run on 3% agarose gel, stained with ethidium bromide, and evaluated under UV light. The sizes of the PCR products were estimated according to the migration pattern of a 50-bp DNA ladder (Pharmacia Biotech, Piscataway, NJ, USA). To avoid the risk of false-positive results, PCR assays were done with strict precautions against cross-contaminations. Furthermore, all PCR assays were performed in duplicate to confirm reproducibility.

2.3. Detection of hepatitis D virus (HDV) RNA by PCR

HDV RNA was screened by nested RT-PCR method using primer combinations reported by Fukai et al. [6]. We used HBV DNA-positive samples to be determined HDV RNA.

2.4. Genotyping of HBV and HCV by PCR assay

Genotyping of HBV and HCV was determined by PCR method using type-specific primers as reported previously [7,8].

2.5. Nucleotide sequencing of HBV pre-S2 gene

Using HBV DNA-positive samples, we amplified the HBV pre-S2 gene by semi-nested PCR using the primers as follows; P1: 5′-TCACCATATTCTTGGGAA-CAAGA-3′ (sense, nt 2817–2839) and S1-2: 5′-CGAACCACTGAACAAATGGC-3′ (antisense, nt

704–685) for the outer primer pairs, and P1 and S2-2: 5′-GGCACTAGTAAACTGAGCCA-3′ (antisense, nt 687–668) for the inner primer pairs. PCR products were separated by 2% agarose gel electrophoresis and purified using the QIAquick gel extraction kit (Qiagen Inc., Chatsworth, CA, USA). Recovered PCR products were subjected to direct sequencing from both directions using the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin–Elmer). Sequences of amplified cDNA were determined using a sequencer (ABI model 377 and 310; Applied Biosystems, Foster City, CA, USA).

2.6. *Quantitation of HBV DNA by real-time PCR*

Quantitation of HBV DNA level was measured by real-time PCR reported by Chen et al. [9]. The real-time PCR was done using an ABI PRISM 7900HT Sequence Detector (Applied Biosystems).

2.7. *Assay for antibody to HEV by enzyme-linked immunosorbent assay (ELISA)*

Immunoglobulin G (IgG) and IgM antibodies to HEV were measured by ELISA. The ELISA to detect anti-HEV using virus-like particles expressed by a recombinant baculovirus was performed as reported previously [10].

2.8. *Statistical analysis*

All statistical calculations were made by the SPSS 11.0 statistical software package (SPSS Inc., Chicago, IL, USA). Fisher's exact test was used for the comparison of categorical variables, and the Student's *t*-test was used for the comparison of HBV DNA level between pre-S2 mutant and wild type. Differences with a P value <0.05 were considered significant.

3. Results

3.1. *Prevalence of HBV, HCV and HDV*

HBV DNA and HCV RNA were detected in ten (10%) and two (2%), respectively, of a population of 100 healthy individuals (Table 1). In contrast, these viruses were detected in 73 (31.2%) and 45 (19.2%), respectively ($P<0.005$ and <0.00001, respectively), of 234 liver disease patients with acute hepatitis, chronic hepatitis, liver cirrhosis or hepatocellular carcinoma. In particular, patients with hepatocellular carcinoma were infected with HBV (34.8%), followed by HCV (15.7%). Among 61 other subjects with non-viral liver disease, such as fatty liver, liver abscess, parasitic infection, liver cyst and cancer metastasis to the liver, these viruses were detected in seven (11.5%) and three (4.9%), respectively. The prevalence of HBV and HCV in viral liver disease patients was significantly higher than in non-viral liver disease group ($P<0.05$ and <0.01, respectively). No positive case for HDV RNA was found among 90 HBV-infected individuals examined.

Table 1
Rate of HBV and HCV infections in Ho Chi Minh City, Vietnam

Category	*n*	HBV DNA	HCV DNA	Co-infection[b]
Acute hepatitis	6	1 (16.7)[a]	1 (16.7)	0
Chronic hepatitis	16	4 (25.0)	4 (25.0)	2 (12.5)
Liver cirrhosis	123	37 (30.1)	26 (21.1)	10 (8.1)
Hepatocellular carcinoma	89	31 (34.8)	14 (15.7)	7 (6.7)
Subtotal	234	73 (31.2)[c,e]	45 (19.2)[d,f]	18 (7.7)
Fatty liver	4	0	0	0
Liver abscess	37	5 (13.5)	3 (8.1%)	0
Fascioliasis	4	0	0	0
Liver cyst	5	0	0	0
Liver metastases	7	1 (14.3)	0	0
Miscelanous	4	1 (25.0)	0	0
Subtotal	61	7 (11.5)	3 (4.9)	0
Healthy individual	100	10 (10.0)[e]	2 (2.0)[f]	0
Total	395	90 (22.8)	50 (12.7)	18 (4.6%)

[a] Number in parentheses indicate the percentage of each result according to each diagnosis.
[b] HBV+HVC.
[c] $P=0.0018$.
[d] $P=0.0057$.
[e] $P=0.003$.
[f] $P<0.00001$ (Fisher's exact test).

3.2. *Genotype distributions of HBV and HCV*

Genotype C of HBV (57%) was the most prevalent, followed by type B (43%) in 90 patients tested. For HCV, among 21 samples, genotype 2a (33.3%) was the most common, followed by genotype 1a (23.8%), 1b (19%), 6a (14.3%) and 4 (4.8%). 4.8% of HCV cases examined were unclassifiable in these populations. There was no significant difference in the genotypic distribution of HBV and HCV between viral related and non-viral-related liver disease.

3.3. *HBV pre-S2 deletion mutant*

We analyzed the nucleotide sequences of the HBV pre-S2 region obtained from 87 Vietnamese. The results revealed that 22 of 87 (25.3%) HBV isolates had a deletion mutant in this region. The number of the nucleotide deleted ranged from 3 to 93 bases, but was not associated with a frame shift of the amino acid sequences (Fig. 1). All HBV isolates with such mutation were identified from chronic liver disease patients.

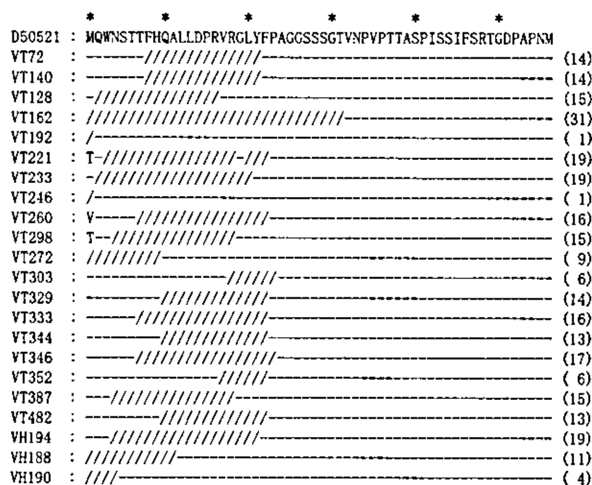

Fig. 1. Vietnamese HBV isolates with pre-S2 deletion mutant. Number in parenthesis indicates number of amino acid deleted. D50521 is wild type from database. /, Deletion.

Moreover, serum HBV DNA level ($4.29 \pm 0.66 \log_{10}$ copies/ml) in pre-S2 deletion mutant was significant lower than that of wild type ($5.42 \pm 1.23 \log_{10}$ copies/ml) by real-time PCR analysis ($P < 0.01$).

3.4. Prevalence of anti-HEV antibody

The prevalence of anti-HEV antibodies was 42% (78/185) for the IgG class and 0.5% (1/185) for the IgM class. The age-specific prevalence already reached 61.9% (13/21) in 21–40-year-olds, 50% (24/48) in 41–60-year-olds and 52.4% (11/21) in over 61-year-olds, respectively (Table 2).

4. Discussion

Hepatitis virus infection is now a major problem both in developed and developing countries. In particular, hepatitis viruses are responsible for one of the most widespread infectious diseases in Asian countries. In Vietnam, it is known that HBV is spreading and its main cause of liver diseases [1,2]. But, most of the studies are mainly based on serological assays such as virus-related antigen/antibody. An adequate level of information on the molecular characteristic of various types of hepatitis viruses including genotypic distribution of HBV and HCV in Vietnam has not been available so far. In the present study, we focused molecular-based epidemiology of these viral infections in Ho Chi Minh City, Vietnam, although we could not use a sufficient serum samples, because there was strict rules to take out of patient's samples from Vietnam. We omitted the serological data such as antigen/antibody related to HBV and HCV due to insufficient quantity of each serum samples in this study. Our results presented here have shown that Ho Chi Minh City was a high endemic area of HBV infection.

Table 2
Age-specific prevalence of anti-HEV among 90 subjecs in Ho Chi Minh City, Vietnam

Age (years)	n	Anti-HEV antibody[a]	
		IgG	IgM
21–40	21	13 (61.9%)	0
41–60	48	24 (50%)	0
>61	21	11 (52.4%)	0
Total	90	48 (53.3%)	0

[a] Determined by ELISA.

In general, the route of viral infection in tropical areas is not clear. The routes and risk factors involved were not identified in the present study. Currently, we are conducting an investigation to clarify the transmission route of these viruses in Vietnam. Genotyping of HBV and HCV are important tools to clarify the route and pathogenesis of these viruses [11,12]. In particular, examination of sequence diversity among different isolates of the virus is important because variants may differ in their patterns of serologic reactivity, pathogenicity, virulence and responses to therapy. On the other hand, HBV and HCV have genetic variations, which correspond to the geographic distribution [12,13]. In this study, we found that the major genotype distributed among Vietnamese people is type C, followed by type B for HBV; and type 2a, followed by type 1a for HCV. It is known that types B and C of HBV are mainly distributed in Asian counties [14]. In HCV genotyping, 4.8% of HCV RNA-positive cases were untypeable in these populations. These results suggest that HCV variants were not classifiable into known genotypes prevail in the south region of Vietnam. In fact, Tokita et al. [15,16] reported that 41% of HCV isolates from Vietnamese patients could not classified into known HCV genotypes and they were grouped into novel genotypes. In this study, we tried to identify the untypable HCV by sequencing analysis, however, it was failure. Our study presented here showed that there was no significant difference in the genotypic distribution of HBV and HCV between viral and non-viral liver disease although it remains to be further investigated. To clarify this point, investigation of the relationship between genotypes of HBV/HCV and clinical outcome is now underway by collaboration with several different countries. Further studies to evaluate the viral genotypes and clinical significance in HBV-infected patients will be needed in order to draw valid conclusions.

Surprisingly, we found a high prevalence (25%) of HBV pre-S mutant. The mutation was occurred in the 5′ terminus half of this region and truncated partially with various size. It is known that this region have several

important functions of HBV such as neutralizing antibody site, binding site for human serum albumin and epitopes for HLA-A_3-restricted CTL and B-cell neutralization [17]. The emergence of pre-S mutants may affect viral replication and evade the immune surveillance. In fact, pre-S2 delete mutant have been suggested to be immune escape variants of HBV by in vitro study [18]. In addition, Fan et al. reported that the pre-S2 deletion mutant appeared to prevail at low or nonreplicative phases of HBV [19]. Our study also found that HBV DNA level in serum was lower in pre-S2 deletion mutant than in wild type. More investigations are needed to clarify the biologic significance of the spread of the HBV with pre-S1/S2 mutant in this area. To address this question, we are now conducting a global investigation of pre-S2 mutant sero-epidemiology in 14 different geographic regions, including developed and developing countries, and plan to report separately on this further study. We also investigated the seroprevalence of HDV in this country, however, our results showed that Vietnam is not an endemic area of HDV infection.

HEV, previously referred to as enterically transmitted non-A, non-B hepatitis, is a major cause of epidemic hepatitis and of acute, sporadic hepatitis in developing countries [20]. Many outbreaks of HEV-induced hepatitis have been reported in India, Southeast and Central Asia, Africa, and Mexico [21]. Our results indicated that there was a high prevalence of anti-HEV in the age group of over 21 years in Vietnam. This suggests that the enterically transmitted infectious disease is prevailing in this country.

In conclusion, hepatitis virus infections were the main cause of liver diseases in Ho Chi Minh City, Vietnam. In particular, nearly 35% of the hepatocellular carcinoma patients in Ho Chi Minh City, were found to be infected with HBV, followed in frequency by HCV. Establishment of prevention, serological diagnosis and treatment of hepatitis viruses constitute an important subject in this area.

Acknowledgements

We thank Hideo Naito, Xin Ding, Yoko Iwaki, Makoto Hirano, Naokazu Takeda, Yutaka Takebe, at National Institute of Infectious Diseases (Japan), Akira Muraoka at International Medical Center of Japan (Japan), Banh Vu Dien at Cho Ray Hospital (Vietnam), Vu Thuy Yen at Pasteur Institute Ho Chi Minh City (Vietnam), Tran Van Be at Blood Transfusion Hematology Center (Vietnam) and staffs of Department of Gastroenterology and Hepatology, Cho Ray Hospital (Vietnam), for their kindly cooperation during this study. This study was supported in part by grants-in-aid for Science Research of the Ministry of Education, Culture, Sports, Science and Technology of Japan and the Ministry of Health, Labour and Welfare of Japan and the International Medical Cooperation Research Grant in Japan.

References

[1] Song P, Duc DD, Hien B, et al. Markers of hepatitis C and B virus infections among blood donors in Ho Chi Minh City and Hanoi, Vietnam. Clin Diagn Lab Immunol 1994;1:413–8.

[2] Kakumu S, Sato K, Morishita T, et al. Prevalence of hepatitis B, hepatitis C, and GB virus C/hepatitis G virus infections in liver disease patients and inhabitants in Ho Chi Minh, Vietnam. J Med Virol 1998;54:243–8.

[3] Fujiyama A, Miyanohara A, Nozaki C, Yoneyama T, Ohtomo N, Matsubara K. Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. Nucleic Acids Res 1983;11:4601–10.

[4] Okamoto H, Okada S, Sugiyama Y, et al. The 5′-terminal sequence of the hepatitis C virus genome. Jpn J Exp Med 1990;60:167–77.

[5] Konomi N, Yamaguchi M, Naito H, et al. Simultaneous detection of hepatitis B, C and G viral genomes by multiplex PCR method. Jpn J Infect Dis 2000;53:70–2.

[6] Fukai K, Yokosuka O, Fujiwara K, et al. Etiologic considerations of fulminant non-A non-B viral hepatitis in Japan: analyses by nucleic acid amplification method. J Infect Dis 1998;178:325–33.

[7] Naito H, Hayashi S, Abe K. Rapid and specific genotyping system for hepatitis B virus corresponding to six major genotypes by PCR using type-specific primers. J Clin Microbiol 2001;39:362–4.

[8] Ohno O, Mizokami M, Wu RR, et al. New hepatitis C virus (HCV) genotyping system that allows for identification of HCV genotypes 1a, 1b, 2a, 2b, 3a, 3b, 4, 5a and 6a. J Clin Microbiol 1997;35:201–7.

[9] Chen RW, Piiparinen H, Seppanen M, Koskela P, Sarna S, Lappalainen M. Real-time PCR for detection and quantitation of hepatitis B virus DNA. J Med Virol 2001;65:250–6.

[10] Li TC, Yamakawa Y, Suzuki K, et al. Expression and self-assembly of empty virus-like particles of hepatitis E virus. J Virol 1997;71:7207–13.

[11] Kidd-Ljunggren K, Miyakawa Y, Kidd AH. Genetic variability in hepatitis B viruses. J Gen Virol 2002;83:1267–80.

[12] Zein NN. Clinical significance of hepatitis C virus genotypes. Clin Microbiol Rev 2000;13:223–35.

[13] Magnius LO, Norder H. Subtypesgenotypes and molecular epidemiology of the hepatitis B virus as reflected by sequence variability of the S-gene. Intervirology 1995;38:24–34.

[14] Chu CJ, Lok AS. Clinical significance of hepatitis B virus genotypes. Hepatology 2002;35:1274–6.

[15] Tokita H, Okamoto H, Iizuka H, et al. The entire nucleotide sequences of three hepatitis C virus isolates in genetic groups 7–9 and comparison with those in the other eight genetic groups. J Gen Virol 1998;79(Pt. 8):1847–57.

[16] Tokita H, Okamoto H, Tsuda F, et al. Hepatitis C virus variants from Vietnam are classifiable into the seventh, eighth and ninth major genetic groups. Proc Natl Acad Sci USA 1994;91:11022–6.

[17] Neurath AR, Kent SB, Parker K, et al. Antibodies to a synthetic peptide from the preS 120–145 region of the hepatitis B virus envelope are virus neutralizing. Vaccine 1986;4:35–7.

[18] Tai PC, Suk FM, Gerlich WH, Neurath AR, Shih C. Hypermodification and immune escape of an internally deleted middle-envelope (M) protein of frequent and predominant hepatitis B virus variants. Virology 2002;292:44–58.

[19] Fan YF, Lu CC, Chen WC, et al. Prevalence and significance of hepatitis B virus (HBV) pre-S mutants in serum and liver at

different replicative stages of chronic HBV infection. Hepatology 1995;33:277–86.
[20] Pawlotsky JM, Belec L, Cresenguet G, Deforges L, Bouvier M, Duval J, Dhumeaux D. High prevalence of hepatitis B, C and E in young sexually active adults from the Central African Republic. J Med Virol 1995;46:269–73.
[21] Bradley DW, Balayan MS. Virus of enterically transmitted non-A non-B hepatitis. Lancet 1988;1:819.

Short Communication

Intervirology

Intervirology 2002;45:101–104

Received: December 17, 2001
Accepted after revision: January 21, 2002

Genotyping of Hepatitis E Virus from Vietnam

Minako Hijikata[a] Shigeki Hayashi[b] Ngoc Thi Trinh[e] Le Dang Ha[e]
Hiroshi Ohara[c] Yohko K. Shimizu[a] Naoto Keicho[a] Hiroshi Yoshikura[d]

[a]Department of Respiratory Diseases, Research Institute, [b]Department of Gastroenterology, [c]Bureau of International Cooperation, International Medical Center of Japan, [d]National Institute of Infectious Diseases, Tokyo, Japan; [e]The Institute for Clinical Research in Tropical Medicine, Bach Mai Hospital, Hanoi, Vietnam

Key Words
Hepatitis E virus · Genotype · Vietnam

Abstract
To identify the genotype of Vietnamese isolates of human hepatitis E virus (HEV), phylogenetic analysis was performed for the open reading frame (ORF) 1 and ORF2 nucleotide sequences of the viral genome. HEV was detected by RT-PCR in 9 out of 141 sera collected from patients with a diagnosis of acute sporadic hepatitis in Hanoi, Vietnam. All of them had sequences related most closely to genotype 4. In addition, the Vietnamese isolate had a single nucleotide insertion in the ORF 3 region, a characteristic reported for genotype 4 with the possible change of initiation of ORF 3 and ORF 2.

Copyright © 2002 S. Karger AG, Basel

Hepatitis E virus (HEV) is a causative agent for epidemic and sporadic hepatitis in developing countries. The prototype sequence of HEV was derived from a Burmese isolate [1]. Up to now, divergent HEV sequences have been reported from many countries, and they are divided into four major genotypes [2]. Nucleotide sequences from most Asian isolates have high identity with the Burmese prototype and are called genotype 1. A complete nucleotide sequence from epidemic hepatitis E in Mexico has less than 77% identity with the Asian prototype, and it constitutes a discrete group, i.e. genotype 2 [3]. The third genotype was discovered in the United States from swine HEV [4] and from sporadic cases of hepatitis E in humans [5]. Genotype 4 was found in Chinese patients with hepatitis E [6], and recently its complete nucleotide sequence was reported [7].

In the present study, we analyzed nucleotide sequences from HEV isolates in Vietnam, which have been poorly studied. We found that Vietnamese isolates, together with Chinese isolates, belong to genotype 4.

Sera from 141 patients with a clinical diagnosis of acute sporadic hepatitis were collected in a hospital of Hanoi, Vietnam, in 2001. For the detection of HEV RNA, nucleic acids were extracted from 50 μl of serum with SMITEST ExR&D (Genome Science Laboratories) and reverse transcribed at 42°C for 40 min using random hexamers with SuperScriptII RNase H⁻ Reverse Transcriptase (Invitrogen). Then, cDNA was subjected to a nested PCR using oligonucleotide primers that detect the open reading frame (ORF) 2 region with *Taq* Gold polymerase (Applied Biosystems). The external primers were HEVORF2-1s (5′-GCC GAC AGA ATT GAT TTC GTC GGC-3′, nt 6295–6318 of the Burmese isolate; GenBank accession No. M73218) and HEVORF2-1as (5′-GTC CTG CTC ATG TTG GTT GTC ATA-3′, nt 6496–6473).

KARGER
Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

© 2002 S. Karger AG, Basel
0300–5526/02/0452–0101$18.50/0

Accessible online at:
www.karger.com/journals/int

Minako Hijikata, MD
Department of Respiratory Diseases
Research Institute, International Medical Center of Japan
1-21-1 Toyama, Shinjuku-ku, Tokyo 162-8655 (Japan)
Tel. +81 3 3202 7181 (ext. 2875), Fax +81 3 3207 1038, E-Mail minakoh@ri.imcj.go.jp

The internal primers were HEVORF2-2s (5′-CCG TCG TCT CAG CCA ATG GCG AGC-3′, nt 6345–6368) and HEVORF2-2as (5′-CTC ATG TTG GTT GTC ATA ATC CTG-3′, nt 6490–6467). Thermal cycling conditions were 95°C for 9 min plus 35 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 30 s plus 72°C for 7 min. PCR products were electrophoresed in 3% agarose gels.

Samples found positive for HEV RNA were further subjected to RT-nested PCR to amplify other regions of the HEV genome. Sequences ORF 1 were amplified as described above with external primers HE5-1 (5′-TCG ATG CCA TGG AGG CCC-3′ [8] and HEVORF1-1as (5′-GGC CAT TGC CTC CGC AAC ATC-3′, nt 562–542 of the genotype 4 isolate; GenBank accession No. AJ272108) and internal primers HE5-2 (5′-GCC YTK GCG AAT GCT GTG G-3′ [8]) and HEVORF1-2as (5′-ACC ATC AAA GCA GTA AGT CCG-3′, nt 472–452). For amplification of the ORF 3 region, cDNA was synthesized with external antisense primer HEVORF3-1as (5′-AAC AAC ACG ATA CTG GGC ATA-3′, nt 5745–5725 of AJ272108), and PCR was carried out using TaKaRa LA *Taq* with GC Buffer I (Takara) with primers HEVORF3-1s (external sense, 5′-CCC GGA GAG GGC GGA GCA GCT-3′, nt 4918–4938), HEVORF3-1as (external antisense, sequence shown above), HEVORF3-2s (internal sense, 5′-CCA GGT TTG TGT GGA TGT TGT-3′, nt 4984–5004) and HEVORF3-2as (internal antisense, 5′-ATT AGA TGC TTC AGT AGC CAT-3′ nt 5724–5704). Thermal cycling conditions were 95°C for 2 min plus 35 cycles of 95°C for 15 s, 55°C for 15 s and 72°C for 1 min and 30 s plus 72°C for 7 min.

PCR products were purified with Wizard PCR Preps DNA Purification System (Promega), and direct sequencing was performed in an ABI model 310 DNA sequencer (Applied Biosystems) using a BigDye terminator cycle sequencing FS ready reaction kit (Applied Biosystems) with internal PCR primers. Additional primers used for sequencing of the ORF 3 region were HEV1s (5′-TGT CGG GTG GAA TGA ATA ACA-3′, nt 5135–5155), HEV2s (5′-CCT ATG TTG CCC GCG CCA CCG-3′, nt 5230–5250), HEV3as (5′-GCT GGG CCT GGT CAC GCC AAG-3′, nt 5458–5438) and HEV4as (5′-AGC TGT GGT AGG TCT ACG ACG-3′, nt 5496–5476). Genetic analyses were performed using GenetyxMac Ver. 9.1 (Software Development).

Nine sera were positive for HEV RNA with the ORF 2 primers. Their sequences of 98 nucleotides were identical to each other with 81.4–100% identity. They had 76.5–81.4% identity with genotype 1 (accession No. M73218), 77.6–83.7% identity with genotype 2 (M74506), 72.4–76.5% identity with genotype 3 (AF060668) and 86.6–89.6% identity with genotype 4 (AJ272108).

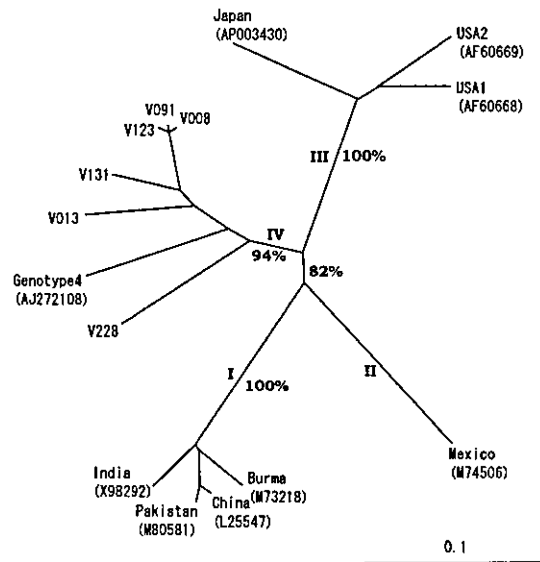

Fig. 1. Phylogenetic tree constructed by the neighbor-joining method based on a 329-nucleotide sequence in ORF 1. The scale representing nucleotide substitutions per position is shown. The internal node numbers indicate the bootstrap values as a percentage of trees obtained from 1,000 replicates. Database accession numbers are in parentheses.

Other regions of the HEV genome were amplified with primers that were based on the genotype 4 sequence. Six of the 9 samples were positive with the ORF 1 primer sets. In the other 3 samples, the ORF 1 sequence was not detected, presumably due to their low HEV titers (data not shown). Phylogenetic analysis performed on 329 nucleotides in the ORF 1 region revealed that the Vietnamese sequences had 75.3–77.1% identity with genotype 1 (accession No. M73218), 77.4–78.7% identity with genotype 2 (M74506), 75.6–77.7% identity with genotype 3 (AF060668) and 84.5–87.5% identity with genotype 4 (AJ272108). Identity among the Vietnamese isolates was 82.6–98.8%. IN a phylogenetic tree (fig. 1), the branches of the Vietnamese isolates extended off the branch of genotype 4. Their bootstrap values were 94%.

The ORF 3 region could be amplified in one sample, and the 609-nucleotide sequence was compared to the reported sequences. It had 80.6% identity with genotype 1

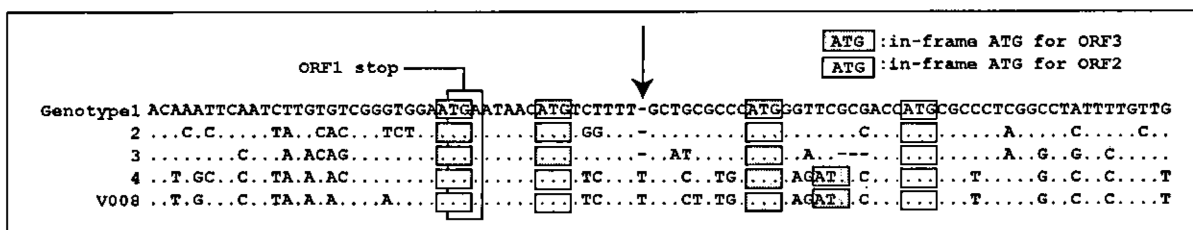

Fig. 2. Comparison of the sequences for the initiation of translation of ORF 2 and ORF 3. A dot indicates a residue identical to that in the top sequence, and a dash indicates a deletion. The arrow indicates the inserted T residue of the genotype 4 isolate (accession No. AJ272108) and Vietnamese isolate (V008, AB075971). Potential initiation codons for ORF 2 (open boxes) and ORF 3 (shaded boxes) are shown. Compared sequences are genotype 1 (M73218), genotype 2 (M74506) and genotype 3 (AF060668).

(accession No. M73218), 80.0% identity with genotype 2 (M74506), 83.4% identity with genotype 3 (AF060668) and 90.0% identity with genotype 4 (AJ272108). A single nucleotide insertion in the ORF 3 region observed in genotype 4 by Wang et al. [7] existed in this isolate as well, resulting in the same possible change in the initiation of ORF 3 and ORF 2 (fig. 2). Deletion of 6 nucleotides reported by Wang et al. [7] resulting in the deletion of two amino acids of the ELISA-reactive peptide in ORF 2 was not observed in this isolate.

Phylogenetic analyses were also performed on 98-nucleotide sequences in the ORF 2 region and on 609-nucleotide sequences of the ORF 3 region. The obtained phylogenetic trees were similar to that for 329-nuelcotide sequences of the ORF 1 region.

All of the HEV isolates from Hanoi in this study had sequences closely related to genotype 4. Since the ORF 2 primers used in our study for the detection of HEV RNA were located in the conserved sequences among all genotypes, they could have amplified other genotypes than genotype 4 if they had been present. Therefore, it was not likely that the remaining 132 cases were negative for HEV RNA due to primer mismatch. Genotype 4 was initially found in Chinese patients from Beijing, Liaoning province and Henian province [6]. These regions are in northern China and not near to the China-Vietnam border. Some of the Taiwanese isolates reported by Hsieh et al. [9] have also been indicated to belong to genotype 4 [2]. The previous data together with the present data may suggest that genotype 4 is distributed widely from northern China to Vietnam. Therefore, it is predicted that genotype 4 is prevalent in the southern provinces of China, though there have been no reports of HEV from this region up to now.

A most interesting finding in this study was the single nucleotide insertion in the ORF 3 region. Wang et al. [7] confirmed this insertion in two isolates from China (HEV-T1 and T11). In the present study, the Vietnamese isolate had the same insertion; therefore, it should be characteristic of the genotype 4 isolate. If ORF 2 and ORF 3 polypeptides are translated from the first available initiation codons, ORF 2 of genotype 4 has an additional 14 codons and ORF 3 of genotype 4 lacks 9 codons compared to other genotypes. However, there are other in-frame initiation codons for ORFs 2 and 3 that are shared by all genotypes (fig. 2). If these common initiation codons were used, the translational strategy may be identical among all genotypes.

The nucleotide sequences reported in this paper will appear in the DDBJ/GenBank/EMBL databases under the accession numbers AB075956–AB075971.

Acknowledgement

This work was supported by a Grant for International Health Cooperation Research (12C-4) from the Ministry of Health, Labor and Welfare of Japan.

References

1 Tam AW, Smith MM, Guerra ME, Huang CC, Bradley DW, Fry KE, Reyes GR: Hepatitis E virus (HEV): Molecular cloning and sequencing of the full-length viral genome. Virology 1991;185:120–131.

2 Schlauder GG, Mushahwar IK: Genetic heterogeneity of hepatitis E virus. J Med Virol 2001; 65:282–292.

3 Huang CC, Nguyen D, Fernandez J, Yun KY, Fry KE, Bradley DW, Tam AW, Reyes GR: Molecular cloning and sequencing of the Mexico isolate of hepatitis E virus (HEV). Virology 1992;191:550–558.

4 Meng XJ, Purcell RH, Halbur PG, Lehman JR, Webb DM, Tsareva TS, Haynes JS, Thacker BJ, Emerson SU: A novel virus in swine is closely related to the human hepatitis E virus. Proc Natl Acad Sci USA 1997;94:9860–9865.

5 Schlauder GG, Dawson GJ, Erker JC, Kwo PY, Knigge MF, Smalley DL, Rosenblatt JE, Desai SM, Mushahwar IK: The sequence and phylogenetic analysis of a novel hepatitis E virus isolated from a patient with acute hepatitis reported in the United States. J Gen Virol 1998;79:447–456.

6 Wang Y, Ling R, Erker JC, Zhang H, Li H, Desai S, Mushahwar IK, Harrison TJ: A divergent genotype of hepatitis E virus in Chinese patients with acute hepatitis. J Gen Virol 1999; 80:169–177.

7 Wang Y, Zhang H, Ling R, Li H, Harrison TJ: The complete sequence of hepatitis E virus genotype 4 reveals an alternative strategy for translation of open reading frames 2 and 3. J Gen Virol 2000;81:1675–1686.

8 Takahashi K, Iwata K, Watanabe N, Hatahara T, Ohta Y, Baba K, Mishiro S: Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. Virology 2001;287:9–12.

9 Hsieh SY, Yang PY, Ho YP, Chu CM, Liaw YF: Identification of a novel strain of hepatitis E virus responsible for sporadic acute hepatitis in Taiwan. J Med Virol 1998;55:300–304.

Journal of Gastroenterology and Hepatology (2002) 17, 1198–1204

INJECTION THERAPY FOR CHRONIC VIRAL HEPATITIS

Efficacy of Stronger Neo-Minophagen C compared between two doses administered three times a week on patients with chronic viral hepatitis

KAZUHIKO MIYAKE,[*] TOSHIRO TANGO,[†] YASUHIKO OTA,[‡] KEIJI MITAMURA,[§] MAKOTO YOSHIBA,[¶] MAKOTO KAKO,[**,¶¶¶¶] SHIGEKI HAYASHI,[††] YUSEI IKEDA,[‡‡] NORIMASA HAYASHIDA,[§§] SHOGO IWABUCHI,[¶¶,*****] YUZURU SATO,[***] TOSHIAKI TOMI,[†††] NAOYA FUNAKI,[‡‡‡] NAOAKI HASHIMOTO,[§§§] TOMOYUKI UMEDA,[¶¶¶] JUN MIYAZAKI,[****] KATSUAKI TANAKA,[††††] YASUO ENDO[‡‡‡‡] AND HIROSHI SUZUKI[§§§§]

[*]*Teikyo University School of Medicine, Tokyo,* [†]*National Institute of Public Health, Tokyo,* [‡]*Toshiba Hospital, Tokyo,* [§]*Showa University School of Medicine, Tokyo,* [¶]*Showa University Fujigaoka Hospital, Kanagawa,* [**]*Teikyo University Mizonokuchi Hospital, Kanagawa,* [††]*International Medical Center of Japan, Tokyo,* [‡‡]*Tokyo Kosei-Nenkin Hospital, Tokyo,* [§§]*Kawakita General Hospital, Kanagawa,* [¶¶]*Kawasaki Abdominal Hospital, Kanagawa,* [***]*Kanto Medical Center of NTT East Corporation, Tokyo,* [†††]*Jiseikai Hospital, Tokyo,* [‡‡‡]*Tokyo Senbai Hospital, Tokyo,* [§§§]*Tokyo Postal Services Agency Hospital, Tokyo,* [¶¶¶]*Tokyo Metropolitan Ebara Hospital, Tokyo,* [****]*Tokyo Hitachi Hospital, Tokyo,* [††††]*Yokohama City University Medical Center, Kanagawa,* [‡‡‡‡]*Sanraku Hospital, Tokyo and* [§§§§]*Yamanashi Medical University, Yamanashi, Japan*

Abstract

Background: A daily injection of glycyrrhizin (Stronger Neo-Minophagen C (SNMC) containing 40 mg glycyrrhizin in a 20 mL ampoule) lowers alanine aminotransferase (ALT) levels in patients with chronic viral hepatitis.

Methods: The therapeutic effects of intermittent administration of SNMC three times a week for 12 weeks were evaluated and compared between two doses (40 and 100 mL) in a randomized clinical trial.

Results: Overall, the therapeutic response was better in the 53 patients allocated 100 mL than the 59 who were allocated to have 40 mL SNMC ($P=0.0243$). At the completion of SNMC treatment, ALT levels decreased more extensively in the patients on 100 mL than those on 40 mL SNMC (–29 *vs* –50% in comparison with the baseline value, $P=0.0002$). Minor side-effects occurred in both the patients on 100 mL (20%) and those on 40 mL (12%), but they did not require any therapies.

Conclusions: Intermittent SNMC would be efficient in suppressing ALT levels in patients with chronic viral hepatitis in a dose-dependent manner. Taken along with infrequent and very mild side-effects, long-term intermittent SNMC would benefit patients with chronic hepatitis by maintaining their quality of life with easier compliance.

© 2002 Blackwell Publishing Asia Pty Ltd

Key words: chronic hepatitis, glycyrrhizin, hepatitis B virus, hepatitis C virus, liver cirrhosis.

Correspondence: Professor K Miyake, Department of Internal Medicine, Teikyo Univeristy School of Medicine, 2-11-1 Kaga, Itabashi-ku, Tokyo 173-8605, Japan. Email: kmiyake@med.teikyo-u.ac.jp

[¶¶¶¶]Present address: Social Insurance Tonan General Hospital, Tokyo, Japan.

[*****]Present address: Ohfuna Chuo Hospital, Kanagawa, Japan.

Accepted for publication 10 June 2002.

INTRODUCTION

Stronger Neo-Minophagen C (SNMC) is an intravenous drug containing glycyrrhizin as a principal ingredient along with glycine and L-cysteine. In Japan, SNMC has been used for the treatment of allergic diseases since 1948. Later, in 1958, SNMC was also used for the treatment of chronic liver disease. Suzuki *et al.* conducted a randomized, double-blinded multicenter clinical trial with SNMC on patients with chronic liver disease.[1] They found that SNMC can significantly decrease serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT) and γ-glutamyltranspeptidase (γ-GTP). Based on their evidence, SNMC has been approved for use in the improvement of liver function abnormalities in chronic liver disease since 1979 in Japan. Because of very infrequent side-effects, SNMC has gained popularity in the treatment of chronic liver disease since then.

A regular therapeutic regimen of SNMC consists of 40–60 mL administered daily. There are patients, however, who do not respond to it and fail to lower AST and ALT levels satisfactorily. Iino *et al.* gave patients with chronic liver disease a daily dose of 40 mL SNMC, and they further treated the patients without having a sufficient response to either 40 or 100 mL SNMC daily.[2] They found significant differences between the two SNMC doses in terms of its ability to lower serum transaminase levels.

Although no antiviral effects have been attributed to SNMC in the treatment of chronic viral hepatitis,[3,4] SNMC can significantly decrease serum levels of AST and ALT, accompanied by amelioration of necro-inflammatory processes in the liver.[5] Furthermore, long-term SNMC is reported to suppress the development of hepatocellular carcinoma (HCC) in patients with chronic hepatitis C.[6]

Although a daily injection of SNMC achieves indisputable therapeutic efficacy in lowering serum AST and ALT levels in patients with chronic hepatitis, it imposes a substantial burden on the patients who need to visit hospitals every day. Intermittent injections of SNMC, should it prove efficient in normalizing AST and ALT levels or keeping them suppressed, can lessen the burden of patients, thereby enabling long-term treatment for maintaining histological improvements. Although intermittent SNMC administration has been in practise already, evaluation of it has not been made for the determination of an optimal dose. A multicenter clinical trial was conducted for comparing two doses (40 and 100 mL) of intermittent SNMC, three times in a week (t.i.w.), for achieving biochemical responses in patients with chronic hepatitis.

METHODS

Patients

The present study was performed from March 1996 to March 1999 in 17 clinical centers in Japan. The participating patients were diagnosed as having chronic hepatitis (CH) or compensated liver cirrhosis (LC) based on clinical, ultrasonographical and histological criteria, and they possessed ALT levels greater than 1.5-fold the upper limit of normal (ULN) during the 4 weeks before the start of SNMC treatment.

They received care from the following institutions: Department of Internal Medicine, Kawakita General Hospital (attended by Dr Norimasa Hayashida), Department of Surgery, Kawasaki Abdominal Hospital (Dr Shogo Iwabuchi), Department of Gastroenterology, Kanto Rosai Hospital (Dr Yuzuru Sato), Second Department of Gastroenterology, International Medical Center of Japan (Dr Shigeki Hayashi), Department of Internal Medicine, Sanraku Hospital (Dr Yuji Yoshikawa), Department of Internal Medicine, Jiseikai Hospital (Dr Toshiaki Tomi), Second Department of Medicine, Showa University (Dr Keiji Mitamura), Showa University School of Medicine Fujigaoka Hospital (Dr Makoto Yoshiba), Department of Medicine, Teikyo University School of Medicine (Dr Kazuhiko Miyake), Fourth Department of Internal Medicine, Teikyo University School of Medicine (Dr Makoto Kako), Department of Medicine, Tokyo Kose-Nenkin Hospital (Dr Arinari Ikeda), Department of Medicine, Tokyo Senbai Hospital (Dr Naoya Funaki), Department of Medicine, Tokyo Postal Services Agency Hospital (Dr Naoaki Hashimoto), Department of Medicine, Tokyo Metropolitan Ebara Hospital (Dr Tomoyuki Umeda), Tokyo Hitachi Hospital (Dr Jun Miyazaki), Department of Medicine, Toshiba Hospital (Dr Yasuhiko Ohta) and Department of Medicine, Yokohama City University Medical Center (Dr Katsuaki Tanaka).

The study design and execution was controlled by Dr Toshiro Tango of the National Institute of Public Health.

Administration of Stronger Neo-Minophagen C

One ampoule of SNMC, with a volume of 20 mL, contains 40 mg glycyrrhizin, 400 mg glycine and 20 mg L-cysteine. Patients were randomly assigned, by registration and by facsimile numbers, into group A (40 mL SNMC t.i.w. for 12 weeks) and group B (100 mL SNMC t.i.w. for 12 weeks). Stronger Neo-Minophagen C was given to patients via an intravenous injection or infusion with 100 mL saline.

Laboratory tests

Tests were performed in accordance with the original schedule. Liver function tests (AST, ALT, γ-GTP, total bilirubin, serum protein, albumin and total cholesterol), peripheral blood cell counts (erythrocytes, hemoglobin, hematocrit and platelets), electrolytes (Na, K and Cl) were determined 4 and 2 weeks before and on the starting day of the treatment, respectively, as well as 4, 8 and 12 weeks while the patients received SNMC. Hepatitis B surface antigen (HBsAg) and antibody to hepatitis C virus (anti-HCV) were determined before the start of SNMC treatment.

Number of patients required for evaluation

Based on previous experiences, an efficacy of 30–40% was assumed for the patients with ALT > 1.5-fold ULN (in respective institutions) who received 40 mL SNMC, and was 50–65% for those receiving 100 mL SNMC. A statistics power of 80% in a two-tailed statistical analysis with significant *P*-levels set at 0.05 was calculated to be achieved by evaluation on 100 patients in each of group A (40 mL SNMC) and group B (100 mL SNMC), making a total of 200 patients.

Primary end-point

The primary end-point was the improvement in ALT levels in serum at the completion of the 12-week SNMC treatment, and was assessed by the following criteria: (i) marked improvement with a decrease of ALT less than 1.2-fold ULN in the institution; (ii) moderate improvement with that between 1.2- and 1.5-fold ULN; (iii) slight improvement with a decrease in ALT by 25% in comparison with baseline values and failing to clear (i) or (ii) criteria; (iv) non-response with changes less than 25% (plus or minus) in comparison with baseline values and failing to clear (i) or (ii) criteria; and (v) aggravation with an increase in ALT by 25% or more in comparison with baseline values and failing to clear (i) or (ii) criteria.

Statistical analyses

Data were expressed as the median with (25%, 75%) points. Differences were considered statistically significant for two-tailed *P*-values <0.05. Baseline characteristics between groups A and B were compared by using the Fisher's exact test. A comparison of the primary end-point between the two groups was based on the Wilcoxon's rank sum test. Secondary end-point on changes in ALT and AST values was made by using the Fisher's exact test and incidence rates of side-effects by using the Wilcoxon's rank sum test.

RESULTS

Compliance to the clinical trial

The study design aimed to include 200 patients, 100 in group A and 100 in group B. During the period of clinical trial from March 1996 to March 1999, however, 68 patients in each group were preserved, making a total of 136 patients. The exclusion of 11 patients who failed to clear the entry criteria and three for whom the evaluation was not feasible left 62 patients in group A and 60 patients in group B for the evaluation, making a total of 122 patients. Of these patients, SNMC was discontinued on three patients in group A and four patients in group B, and an additional three patients in group B were not to be evaluated for end-point responses. Thus, the final evaluation was possible in 59 patients in group A and 53 patients in group B, making a total of 112 patients. They included 67 (55%) males and 55 females with the median age of 61 years (range: 30–81 years). Their liver diseases were CH in 100 (82%) and LC in 22 patients. There were 10 (8%) carriers of hepatitis B virus (HBV) and 109 (89%) carriers of hepatitis C virus (HCV); two (2%) were coinfected with HBV and HCV.

Table 1 compares the baseline characteristics between patients in groups A and B. There were no appreciable differences in any demographic, clinical, pathological or virological parameters in comparison between the patients in groups A and B.

Table 1 Demographic, clinicopathological and virological characteristics of the patients who received 40 mL (Group A) or 100 mL (Group B) SNMC

	Group A (40 mL) (*n* = 62)	Group B (100 mL) (*n* = 60)	Differences (*P*-value (two-tailed))
Male	33 (53%)	34 (57%)	0.7197†
Age	61 (52, 68)*	61 (51, 59)	0.7499†
Histology: CH/LC	53/9	47/13	0.3517†
Viral markers			
HBV (+)/HCV (–)	5 (6%)	5 (8%)	1.0000†
HBV (–)/HCV (+)	55 (87%)	54 (90%)	
HBV (+)/HCV (+)	1 (2%)	1 (2%)	
HBV (–)/HCV (–)	1 (2%)	0	
Pretreatment with IFN	25 (40%)	28 (47%)	0.5840‡
ALT (IU/L)	124 (79, 190)*	128 (98, 202)	0.2495‡
AST (IU/L)	90 (60, 128)	124 (87, 163)	0.0061‡
γ-GTP (IU/L)	54 (25, 102)*	62 (43, 122)	0.0987‡
Albumin (g/dL)	4.1 (3.9, 4.4)*	4.2 (3.9, 4.4)	0.8950‡

*The 25% and 75% quartile values are shown in parentheses. †Fisher's exact test. ‡Wilcoxon's rank sum test. ALT, alanine animotransferase; AST, aspartate aminotransferase; CH, chronic hepatitis; γ-GTP, gamma glutamyltranspeptidase; HBV, hepatitis B virus; HCV, hepatitis C virus; IFN, interferon; LC, liver cirrhosis; SNMC, Stronger Neo-Minophagen C.

Efficacy in groups A and B treated with Stronger Neo-Minophagen C

Response rates scaled in five categories for the improvement of ALT are compared between groups A and B in Table 2. There were significant differences in total scores between the two groups ($P=0.0243$). The rates of a marked response (16 *vs* 18%) and a moderate response (19 *vs* 32%) were not appreciably different between groups A and B. A better response in group B patients therefore was caused mainly by fewer patients with a non-response or aggravation in it. Changes in ALT values between baseline and end-of-treatment were greater for patients in group B than in group A (−29 *vs* −50% in comparison with the baseline value, $P=0.0002$; Table 3).

A decrease in AST levels, also, was greater in patients in group B than in group A (−42 *vs* −22%, $P=0.0003$). By contrast, there were no significant differences in the changes of total bilirubin, γ-GTP, total protein, albumin and total cholesterol between the patients in group A and B at the completion of the 12-week trial (data not shown).

A secondary assessment was made by using the time course of ALT (Fig. 1). The median ALT values decreased in both groups A and B, and the decrease was maintained throughout the days on SNMC. The median ALT values was significantly lower for group B than A at the end-of-treatment ($P<0.001$).

Side-effects

Side-effects were observed in 16 patients, including six in group A and 10 in group B (Table 4). There was a total of 20 among the 122 patients (16%), and included five (4%) cases of hypokalemia and three (3%) cases of hypertension. Although hypokalemia and/or hypertension tended to occur more frequently in patients in group B than in group A, the difference fell short of being significant.

Table 2 Comparison of primary end-point achieved by the patients in groups A and B after 12 weeks on two different dosages of Stronger Neo-Minophagen C

	Group A (40 mL) ($n=59$)	Group B (100 mL) ($n=53$)	Differences (P-value (2-tailed))
Improvement			
Marked (I)	9 (16)	9 (17)	
Moderate (II)	2 (3)	7 (13)	
Slight (III)	24 (41)	25 (47)	0.0243*
Non-response (IV)	19 (32)	9 (17)	
Aggravated (V)	3 (5)	0	
Indefinite	2 (3)	3 (6)	
Response rate‡			
Marked	9/57 (16)	9/50 (18)	0.8002†
Moderate	11/57 (19)	16/50 (32)	0.1807†

Data in parentheses are percentages. *Wilcoxon's rank sum test. †Fisher's exact test. ‡The patients with marked improvement were considered to have achieved a marked response, while those with marked improvement plus those with moderate improvement were considered to have accomplished moderate response.

Table 3 Changes in ALT and AST values before and at the end-of-treatment with SNMC

	Group A (40 mL)	Group B (100 mL)	Differences (P-value (2-tailed))
ALT (IU/L)			
Before	124 (79, 190)* ($n=57$)	128 (98, 202) ($n=53$)	
After (12 weeks)	80 (47, 134) ($n=58$)	63 (48, 104) ($n=50$)	
Changes (%)†	−29 (−47, −7) ($n=57$)	−50 (−63, −36) ($n=50$)	0.0002‡
AST (IU/L)			
Before	90 (60, 128) ($n=57$)	124 (87, 163) ($n=53$)	
After (12 weeks)	73 (43, 100) ($n=58$)	65 (49, 85) ($n=50$)	
Changes (%)‡	−22 (−41, −2) ($n=57$)	−42 (−58, −28) ($n=50$)	0.0003‡

ALT, alanine aminotransferase; AST, aspartate aminotransferase; SNMC, Stronger Neo-Minophagen C. The 25 and 75% quartile values are shown in parentheses. †Changes were calculated by the formula: ((Pretreatment value)—(End-of-treatment value))/pretreatment value × 100. ‡Wilcoxon's rank sum test.

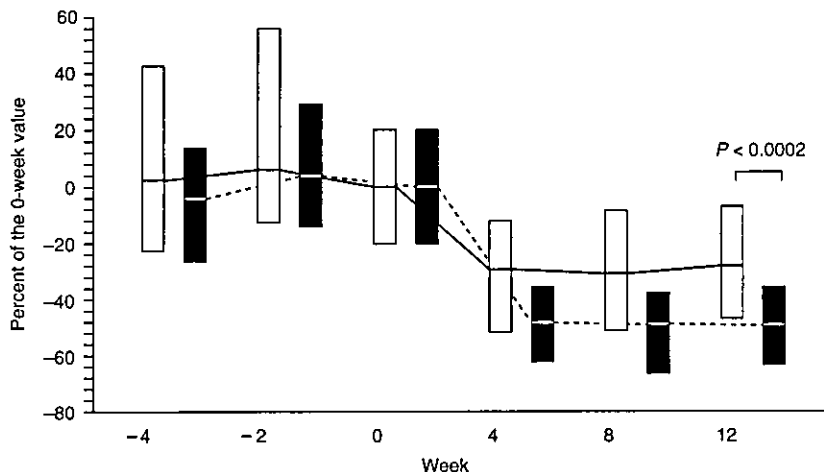

Figure 1 Changes in the alanine aminotransferase (ALT) values in patients in (□) group A (40 mL SNMC, n = 59) and (■) B (100 mL, n = 53). The median as well as (bottom of bar) 25% and (top of bar) 75% quartile values are shown. The ALT values are expressed as a percentage deviation from the value at the start of SNMC treatment (0 week). (—) Group A; (---) group B. SNMC, Stronger Neo-Minophagen C. P = 0.0002.

Table 4 Side-effects in patients who received 40 mL (Group A) or 100 mL (Group B) Stronger Neo-Minophagen C

	Groups		
	A (40 mL) (n = 62)	B (100 mL) (n = 60)	Total (n = 122)
Hypokalemia	1	4	5
Hypertension	1	2	3
Malaise	1	1	2
Numbness	1	1	2
Loss of power	0	1	1
Edema in lower extremities	0	1	1
Anorexia (with or without bodyweight loss)	1	1	1
Nausea	1	0	1
Discomfort in the lower abdomen	1	0	1
Proteinuria	0	1	1
Insomnia due to right back pain	1	0	1
Total	8 (13%)	12 (20%)	20 (10%)

DISCUSSION

More than 30 000 people in Japan die from HCC annually, and deaths caused by HCC are still increasing. Of the patients with HCC, 90% are infected with hepatitis viruses and more than 80% are infected with HCV. Although interferon (IFN) has potent antiviral effects and can terminate HCV infection, a sustained virological response is achieved in only 30% of Japanese patients who receive it. Hepatitis C is reactivated in 50% of the patients who complete IFN and, therefore, there is a pressing need for treatment of the patients who do not respond to it.

Stronger Neo-Minophagen C is an intravenous drug with glycyrrhizin as the active principle. It can lower serum levels of AST and ALT,[1,2,7] which is ascribable to the amelioration of necroinflammation in the liver.[5] Arase *et al.* has reported decreasing the incidence of HCC in the patients with chronic hepatitis C who had received SNMC for 15 years, and who kept low ALT levels in serum.[6]

A major constraint with long-term SNMC is the delivery via intravenous injection. For the purpose of lessening the burden of patients for maintaining the quality of life (QOL), an intermittent injection with SNMC, t.i.w., would be more practical for both patients and attending doctors. Hence, a comparison was made on the therapeutic effect of intermittent SNMC at two doses, 40 and 100 mL t.i.w. in a randomized clinical trial.

Initial plans were to assign 100 patients each to receive 40 or 100 mL SNMC with a total of 200 patients; only 122 (61%) patients participated in the clinical trial, however. The loss was partly caused by a strong wish of some patients to receive daily, rather than intermittent SNMC. Of the patients who entered the clinical trial and those who received two doses of SNMC t.i.w., 8% were infected with HBV and 88% with HCV; the distribution of the two hepatitis viruses reflected that in patients with chronic liver disease in Japan. There were no differences in the demographic, clinical, pathological and virological characteristics between the patients who received 40 and 100 mL SNMC t.i.w., nor were there any differences in the previous treatment with IFN which they had undergone.

The serum levels of ALT decreased in 11 of the 57 (19%) patients who received 40 mL SNMC t.i.w. and in 16 of the 50 (32%) patients who received 100 mL SNMC t.i.w. The response rate was less than 30–40% and 50–65%, respectively, in patients who had been estimated to have their ALT levels decreased by receiving the two doses of SNMC. The expectation, however, was based on the efficacy in patients who receive daily

SNMC at doses of 40 and 100 mL; there were no previous data for the response to intermittent SNMC in the literature. Although the difference in the response to SNMC between 40 and 100 mL t.i.w. did not reach statistical significance, patients treated with 100 mL SNMC achieved a response 1.5-fold higher than those patients who received 40 mL SNMC t.i.w. Hence, there would have been a possibility for a statistical significance in higher patient responses with 100 mL than with 40 mL SNMC administered t.i.w. if more patients had been included in the present study as initially planned.

In the overall assessment on the efficacy in lowering ALT levels after a 12-week intermittent SNMC treatment, 100 mL t.i.w. was found to be better than 40 mL t.i.w. ($P=0.0243$). The differences were caused by having fewer patients on 100 mL SNMC t.i.w. who kept their ALT levels unchanged (nine patients) than those on 40 mL SNMC t.i.w. (19 patients). Likewise, three patients on a 40-mL regimen had their ALT levels increased while none on a 100-mL regimen did so.

In serial follow ups of serum ALT levels, patients on 40 and 100 mL SNMC t.i.w. had them decreased at 4 weeks (the first determination) and thereafter while they were on SNMC. Taken together, intermittent SNMC was found to be effective in lowering serum ALT levels and keeping them decreased, and there was a dose-dependence on intermittent SNMC as has been reported with daily SNMC.[2]

During the period of our clinical trial, two studies have been reported in which different therapeutic regimens of SNMC, in terms of dose and frequency of administration, were compared. In a double-blind study, van Rossum *et al.*[4] compared the dose-effect with 40, 80 and 120 mL SNMC t.i.w. for 4 weeks, and found no significant differences in response to them. However, a significant decrease in ALT levels was achieved by any of the three doses of SNMC in comparison with placebo. Normalization of ALT levels (10 *vs* 0%) and a decrease to less than 1.5-fold ULN (37 *vs* 8%) were accomplished more frequently in the patients who received SNMC than in those who were allocated for placebo. They further treated the patients with daily 100 mL SNMC for an additional 4 weeks, and achieved the normalization of ALT in 20% of them, and a decrease to less than 1.5-fold ULN in 47%.

Iino *et al.* found a dose-effect of daily SNMC.[2] They first treated patients with 40 mL SNMC for 2 weeks. The decrease in ALT levels less than 1.5-fold ULN was accomplished in 43.8% of the patients. The patients who failed to achieve such a decrease were randomized to receive either daily 40 or 100 mL SNMC for an additional 4 weeks. The decrease to less than 1.5-fold ULN was more frequent in the patients receiving 100 than 40 mL SNMC daily (47.4 *v* 23.1%, $P=0.033$).

Understandably, a daily injection with SNMC is superior to intermittent SNMC in decreasing ALT levels of patients with chronic hepatitis. Nevertheless, it is certain that intermittent SNMC injections can decrease ALT levels in a substantial population of patients with chronic viral hepatitis. Taking into account the results obtained in the present study, the most appropriate SNMC regimen needs to determined so that it can be acceptable to patients with high compliance and without much compromise to their QOL. A tentative schedule would be to give patients daily 40 mL SNMC for 2 weeks, and those who achieve a decrease in ALT levels to less than 1.5-fold ULN would be maintained on it. They may as well be placed on an intermittent schedule, 40 mL t.i.w., with close monitoring of their ALT levels. For the patient who fail to achieve this decrease, a daily 100 mL SNMC dose may need to be introduced. When these patients respond by lowering ALT to less than 1.5-fold ULN, 100 mL SNMC t.i.w. may be given. The dose can be reduced further to 40 mL t.i.w. in those patients who have their ALT levels lowered to less than 1.5-fold ULN. Another attempt for determining the optimal therapeutic schedule is in progress in a randomized clinical trial in which 100 mL SNMC is given daily for 3 weeks and then switched to the same dose given once, trice and six t.i.w.

Pseud-aldosteronism is reported to develop in few patients who have received long-term SNMC treatment. In the present clinical trial, hypokalemia developed in 2% of the patients who received 40 mL SNMC t.i.w. and in 5% of those who were placed on 100 mL SNMC t.i.w. They all fared well without any medications. No other serious side-effects occurred in any of the patients who received 40 or 100 mL SNMC t.i.w. for 12 weeks.

In conclusion, a long-term administration of SNMC with a deliberate dose and a therapeutic schedule is efficient in the treatment of patients with chronic hepatitis for suppressing necroinflammatory processes in the liver. Furthermore, it is expected to prevent the development of LC and eventual the evolution into HCC, which represents the most dire health-care problem not only in Japan but also elsewhere in the world where the HCV infection prevails. Because of less burden on patients for better compliance and the importance of maintaining their QOL, intermittent SNMC would be beneficial in the treatment of chronic hepatitis.

REFERENCES

1 Suzuki H, Ota Y, Takino T *et al.* Therapeutic effects of Stronger Neo-Minophagen C (SNMC) in chronic hepatitis: Double blind trial. *Igaku-No-Ayumi* 1977; **102**: 562–78 (in Japanese).

2 Iino S, Tango T, Matsushima T *et al.* Therapeutic effects of Stronger Neo-Minophagen C at different doses on chronic hepatitis and liver cirrhosis. *Hepatol. Res.* 2001; **19**: 31–40.

3 Ito A, Hayashi N, Katayama K *et al.* Effect of glycyrrhizin on viral replication and quasispecies in patients with type C chronic hepatitis. *Int. Hepatol. Commun.* 1997; **6**: 233–8.

4 van Rossum TG, Vulto AG, Hop WC, Brouwer JT, Niesters HG, Schalm SW. Intravenous glycyrrhizin for the treatment of chronic hepatitis C: a double-blind, randomized, placebo-controlled phase I/II trial. *J. Gastroenterol. Hepatol.* 1999; **14**: 1093–9.

5 Hino K, Miyakawa H, Takahashi K *et al.* The effects on hepatohistological patterns of massive administration of