

# Critical role of PA28 $\gamma$ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis

Kohji Moriishi\*, Rika Mochizuki\*, Kyoji Moriya<sup>†</sup>, Hironobu Miyamoto\*, Yoshio Mori\*, Takayuki Abe\*, Shigeo Murata<sup>‡</sup>, Keiji Tanaka<sup>‡</sup>, Tatsuo Miyamura<sup>§</sup>, Tetsuro Suzuki<sup>§</sup>, Kazuhiko Koike<sup>†</sup>, and Yoshiharu Matsuura\*<sup>¶1</sup>

\*Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan; <sup>†</sup>Department of Internal Medicine, Graduate School of Medicine, University of Tokyo, Tokyo 113-8655, Japan; <sup>‡</sup>Department of Molecular Oncology, Tokyo Metropolitan Institute of Medical Science, Tokyo 113-8613, Japan; and <sup>§</sup>Department of Virology II, National Institute of Infectious Diseases, Tokyo 162-8640, Japan

Edited by Peter Palese, Mount Sinai School of Medicine, New York, NY, and approved December 1, 2006 (received for review August 23, 2006)

Hepatitis C virus (HCV) is a major cause of chronic liver disease that frequently leads to steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC). HCV core protein is not only a component of viral particles but also a multifunctional protein because liver steatosis and HCC are developed in HCV core gene-transgenic (CoreTg) mice. Proteasome activator PA28 $\gamma$ /REG $\gamma$  regulates host and viral proteins such as nuclear hormone receptors and HCV core protein. Here we show that a knockout of the PA28 $\gamma$  gene induces the accumulation of HCV core protein in the nucleus of hepatocytes of CoreTg mice and disrupts development of both hepatic steatosis and HCC. Furthermore, the genes related to fatty acid biosynthesis and *srebp-1c* promoter activity were up-regulated by HCV core protein in the cell line and the mouse liver in a PA28 $\gamma$ -dependent manner. Heterodimer composed of liver X receptor  $\alpha$  (LXR $\alpha$ ) and retinoid X receptor  $\alpha$  (RXR $\alpha$ ) is known to up-regulate *srebp-1c* promoter activity. Our data also show that HCV core protein enhances the binding of LXR $\alpha$ /RXR $\alpha$  to LXR-response element in the presence but not the absence of PA28 $\gamma$ . These findings suggest that PA28 $\gamma$  plays a crucial role in the development of liver pathology induced by HCV infection.

fatty acid | proteasome | sterol regulatory element-binding protein (SREBP) | RXR $\alpha$  | LXR $\alpha$

Hepatitis C virus (HCV) belongs to the Flaviviridae family, and it possesses a positive, single-stranded RNA genome that encodes a single polyprotein composed of  $\approx$ 3,000 aa. The HCV polyprotein is processed by host and viral proteases, resulting in 10 viral proteins. Viral structural proteins, including the capsid (core) protein and two envelope proteins, are located in the N-terminal one-third of the polyprotein, followed by nonstructural proteins.

HCV infects >170 million individuals worldwide, and then it causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) (1). The prevalence of fatty infiltration in the livers of chronic hepatitis C patients has been reported to average  $\approx$ 50% (2, 3), which is higher than the percentage in patients infected with hepatitis B virus and other liver diseases. However, the precise functions of HCV proteins in the development of fatty liver remain unknown because of the lack of a system sufficient to investigate the pathogenesis of HCV. HCV core protein expression has been shown to induce lipid droplets in cell lines and hepatic steatosis and HCC in transgenic mice (4–6). These reports suggest that HCV core protein plays an important role in the development of various types of liver failure, including steatosis and HCC.

Recent reports suggest that lipid biosynthesis affects HCV replication (7–9). Involvement of a geranylgeranylated host protein, FBL2, in HCV replication through the interaction with NSSA suggests that the cholesterol biosynthesis pathway is also important for HCV replication (9). Increases in saturated and monounsaturated fatty acids enhance HCV RNA replication, whereas increases in polyunsaturated fatty acids suppress it (7). Lipid homeostasis is regulated by a family of steroid regulatory element-binding proteins (SREBPs), which activate the expression of >30 genes involved in

the synthesis and uptake of cholesterol, fatty acids, triglycerides, and phospholipids. Biosynthesis of cholesterol is regulated by SREBP-2, whereas that of fatty acids, triglycerides, and phospholipids is regulated by SREBP-1c (10–14). In chimpanzees, host genes involved in SREBP signaling are induced during the early stages of HCV infection (8). SREBP-1c regulates the transcription of acetyl-CoA carboxylase, fatty acid synthase, and stearyl-CoA desaturase, leading to the production of saturated and monounsaturated fatty acids and triglycerides (15). SREBP-1c is transcriptionally regulated by liver X receptor (LXR)  $\alpha$  and retinoid X receptor (RXR)  $\alpha$ , which belong to a family of nuclear hormone receptors (15, 16). Accumulation of cellular fatty acids by HCV core protein is expected to be modulated by the SREBP-1c pathway because RXR $\alpha$  is activated by HCV core protein (17). However, it remains unknown whether HCV core protein regulates the *srebp-1c* promoter.

We previously reported (18) that HCV core protein specifically binds to the proteasome activator PA28 $\gamma$ /REG $\gamma$  in the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. PA28 $\gamma$  is well conserved from invertebrates to vertebrates, and amino acid sequences of human and murine PA28 $\gamma$ s are identical (19). The homologous proteins, PA28 $\alpha$  and PA28 $\beta$ , form a heteroheptamer in the cytoplasm, and they activate chymotrypsin-like peptidase activity of the 20S proteasome, whereas PA28 $\gamma$  forms a homoheptamer in the nucleus, and it enhances trypsin-like peptidase activity of 20S proteasome (20). Recently, Li and colleagues (21) reported that PA28 $\gamma$  binds to steroid receptor coactivator-3 (SRC-3) and enhances the degradation of SRC-3 in a ubiquitin- and ATP-independent manner. However, the precise physiological functions of PA28 $\gamma$  are largely unknown *in vivo*. In this work, we examine whether PA28 $\gamma$  is required for liver pathology induced by HCV core protein *in vivo*.

## Results

**PA28 $\gamma$ -Knockout HCV Core Gene Transgenic Mice.** To determine the role of PA28 $\gamma$  in HCV core-induced steatosis and the development of HCC *in vivo*, we prepared PA28 $\gamma$ -knockout core gene transgenic mice. The PA28 $\gamma$ -deficient, PA28 $\gamma$ <sup>-/-</sup> mice were born without

Author contributions: K. Moriishi, K.T., T.M., T.S., K.K., and Y. Matsuura designed research; K. Moriishi, R.M., K. Moriya, H.M., Y. Mori, and T.A. performed research; S.M. contributed new reagents/analytic tools; Y. Matsuura analyzed data; and K. Moriishi, K.K., and Y. Matsuura wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS direct submission.

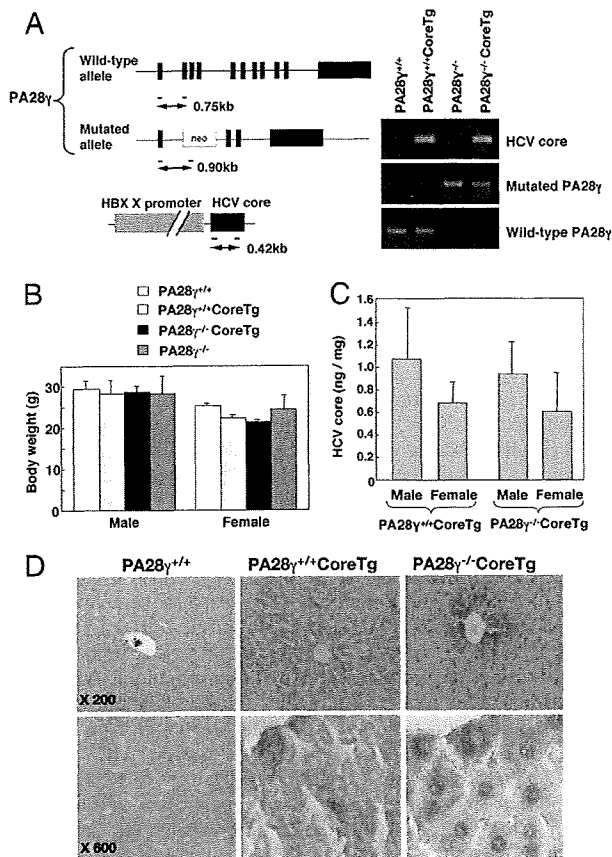
Freely available online through the PNAS open access option.

Abbreviations: CoreTg, HCV core gene-transgenic; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; LXR, liver X receptor; LXRE, liver X receptor-response element; MEF, mouse embryonic fibroblast; ROS, reactive oxygen species; RXR, retinoid X receptor; SRC-3, steroid receptor coactivator-3; SREBP, sterol regulatory element-binding protein.

<sup>¶</sup>To whom correspondence should be addressed. E-mail: matsuura@biken.osaka-u.ac.jp.

This article contains supporting information online at [www.pnas.org/cgi/content/full/0607312104/DC1](http://www.pnas.org/cgi/content/full/0607312104/DC1).

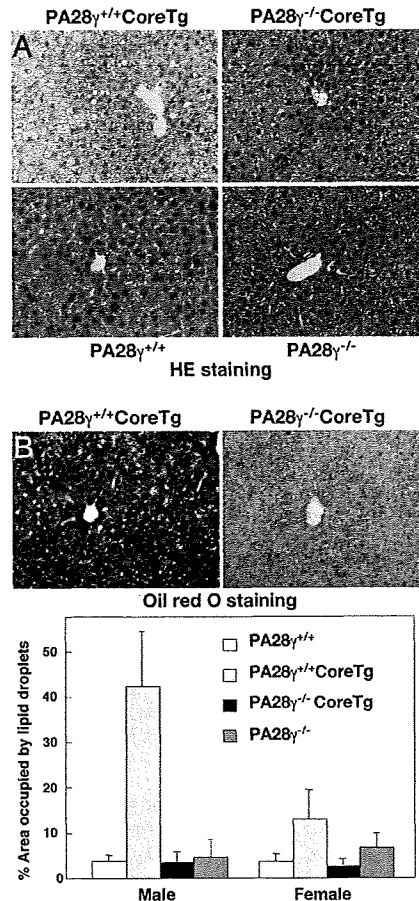
© 2007 by The National Academy of Sciences of the USA



**Fig. 1.** Preparation and characterization of PA28 $\gamma$ -knockout HCV core-transgenic mice. (A) The structures of the wild-type and mutated PA28 $\gamma$  genes and the transgene encoding the HCV core protein under the control of the HBV X promoter were investigated. Positions corresponding to the screening primers and sizes of PCR products are shown. PCR products of the HCV core gene as well as wild-type and mutated PA28 $\gamma$  alleles were amplified from the genomic DNAs of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice. (B) Body weights of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, PA28 $\gamma^{-/-}$ CoreTg, and PA28 $\gamma^{-/-}$  mice at the age of 6 months. (C) HCV core protein levels in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice were determined by ELISA (mean  $\pm$  SD,  $n = 10$ ). (D) Localization of HCV core protein in the liver. Liver sections of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{+/+}$ CoreTg, and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 2 months were stained with anti-HCV core antibody.

appreciable abnormalities in all tissues examined, with the exception of a slight retardation of growth (22). HCV core gene-transgenic (PA28 $\gamma^{+/+}$ CoreTg) mice were bred with PA28 $\gamma^{-/-}$  mice to create PA28 $\gamma^{-/-}$ CoreTg mice. The PA28 $\gamma^{-/-}$ CoreTg offspring were bred with each other, and PA28 $\gamma^{-/-}$ CoreTg mice were selected by PCR using primers specific to the target sequences (Fig. 1A). No significant differences in body weight were observed among the 6-month-old mice, although PA28 $\gamma^{-/-}$  mice exhibited a slight retardation of growth (Fig. 1B). A similar level of PA28 $\gamma$  expression was detected in PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{+/+}$  mice (see Fig. 5B). The expression levels and molecular size of HCV core protein were similar in the livers of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1C; see also Fig. 5B).

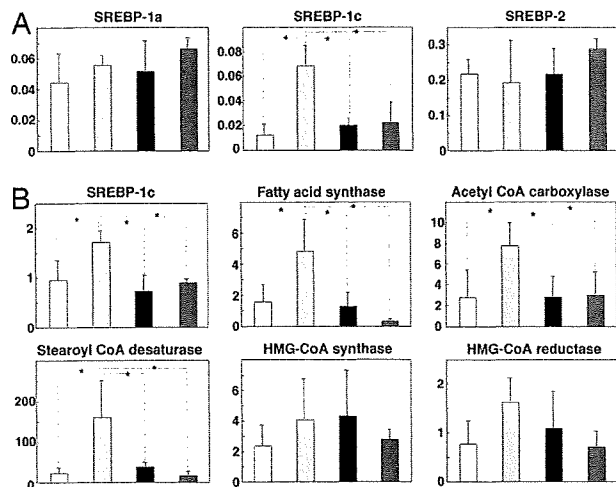
**PA28 $\gamma$  Is Required for Degradation of HCV Core Protein in the Nucleus and Induction of Liver Steatosis.** HCV core protein has been detected at various sites, such as the endoplasmic reticulum, mitochondria, lipid droplets, and nucleus of cultured cell lines, as well as in hepatocytes of PA28 $\gamma^{+/+}$ CoreTg mice and hepatitis C patients



**Fig. 2.** Accumulation of lipid droplets by expression of HCV core protein. (A) Liver sections of the mice at the age of 6 months were stained with hematoxylin/eosin (HE). (B) (Upper) Liver sections of PA28 $\gamma^{+/+}$ CoreTg and PA28 $\gamma^{-/-}$ CoreTg mice at the age of 6 months were stained with oil red O. (Lower) The area occupied by lipid droplets of PA28 $\gamma^{+/+}$  (white), PA28 $\gamma^{+/+}$ CoreTg (gray), PA28 $\gamma^{-/-}$ CoreTg (black), and PA28 $\gamma^{-/-}$  (dark gray) mice was calculated by Image-Pro software (MediaCybernetics, Silver Spring, MD) (mean  $\pm$  SD,  $n = 10$ ).

(6, 23, 24). Although HCV core protein is predominantly detected in the cytoplasm of the liver cells of PA28 $\gamma^{+/+}$ CoreTg mice, as reported in ref. 6, in the present study a clear accumulation of HCV core protein was observed in the liver cell nuclei of PA28 $\gamma^{-/-}$ CoreTg mice (Fig. 1D). These findings clearly indicate that at least some fraction of the HCV core protein is translocated into the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. Mild vacuolation was observed in the cytoplasm of the liver cells of 4-month-old PA28 $\gamma^{+/+}$ CoreTg mice, and it became more severe at 6 months, as reported in ref. 25. Hematoxylin/eosin-stained liver sections of 6-month-old PA28 $\gamma^{+/+}$ CoreTg mice exhibited severe vacuolating lesions (Fig. 2A), which were clearly stained with oil red O (Fig. 2B Upper), whereas no such lesions were detected in the livers of PA28 $\gamma^{-/-}$ CoreTg, PA28 $\gamma^{+/+}$ , or PA28 $\gamma^{-/-}$  mice at the same age. The areas occupied by the lipid droplets in the PA28 $\gamma^{+/+}$ CoreTg mouse livers were  $\approx 10$  and 2–4 times larger than those of male and female of PA28 $\gamma^{+/+}$ , PA28 $\gamma^{-/-}$ , and PA28 $\gamma^{-/-}$ CoreTg mice, respectively (Fig. 2B Lower). These results suggest that PA28 $\gamma$  is required for the induction of liver steatosis by HCV core protein in mice.

**PA28 $\gamma$  Is Required for the Up-Regulation of SREBP-1c Transcription by HCV Core Protein in the Mouse Liver.** To clarify the effects of a knockout of the PA28 $\gamma$  gene in PA28 $\gamma^{+/+}$ CoreTg mice on lipid



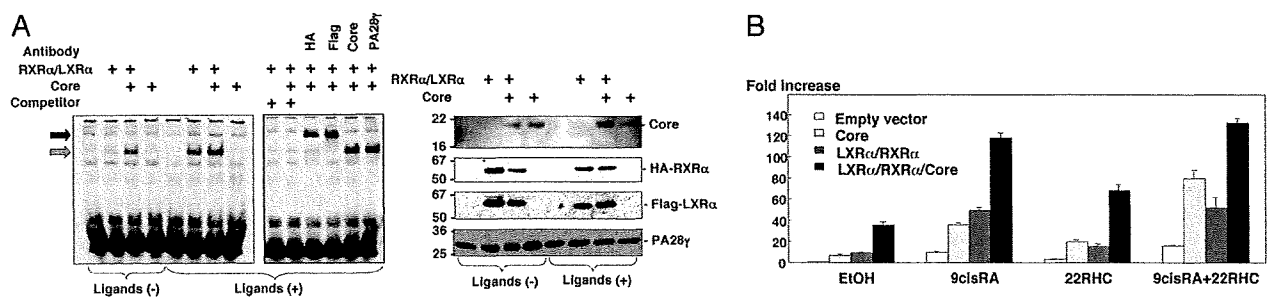
**Fig. 3.** Transcription of genes regulating lipid biosynthesis in the mouse liver. (A) Total RNA was prepared from the livers of 2-month-old mice; and the transcription of genes encoding SREBP-1a, SREBP-1c, and SREBP-2 was determined by real-time PCR. (B) The transcription of genes encoding SREBP-1c, fatty acid synthase, acetyl-CoA carboxylase, stearyl-CoA desaturase, HMG-CoA synthase, and HMG-CoA reductase of 6-month-old mice was measured by real-time PCR. The transcription of the genes was normalized with that of hypoxanthine phosphoribosyltransferase, and the values are expressed as relative activity ( $n = 5$ ; \*,  $P < 0.05$ ; \*\*,  $P < 0.01$ ). The transcription of each gene in  $PA28\gamma^{+/+}$ ,  $PA28\gamma^{+/+}CoreTg$ ,  $PA28\gamma^{-/-}CoreTg$ , and  $PA28\gamma^{-/-}$  mice is indicated by white, gray, black, and dark gray bars, respectively.

metabolism, genes related to the lipid biosyntheses were examined by real-time quantitative PCR. Transcription of SREBP-1c was higher in the livers of  $PA28\gamma^{+/+}CoreTg$  mice than in those of  $PA28\gamma^{+/+}$ ,  $PA28\gamma^{-/-}$ , and  $PA28\gamma^{-/-}CoreTg$  mice at 2 months of age, but no such increases in SREBP-2 and SREBP-1a were observed (Fig. 3A). Although transcription of SREBP-1c and its regulating enzymes, such as acetyl-CoA carboxylase, fatty acid synthase, and stearyl-CoA desaturase, was also enhanced in the livers of 6-month-old  $PA28\gamma^{+/+}CoreTg$  mice compared with the levels in the livers of  $PA28\gamma^{+/+}$ ,  $PA28\gamma^{-/-}$ , and  $PA28\gamma^{-/-}CoreTg$  mice, no statistically significant differences were observed with respect to the transcription levels of cholesterol biosynthesis-related genes that are regulated by SREBP-2 (e.g., HMG-CoA synthase and HMG-CoA reductase) (Fig. 3B). These results suggest the

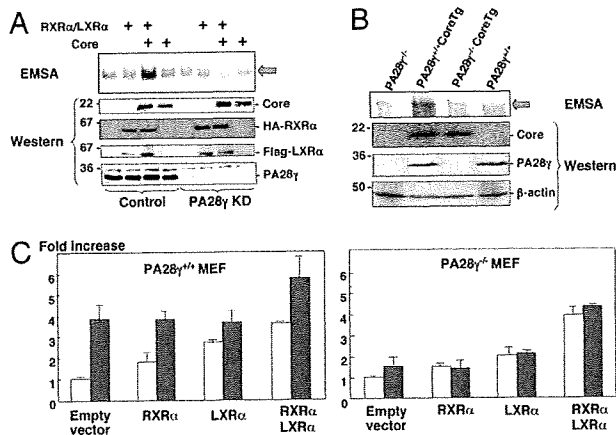
following: (i) the up-regulation of SREBP-1c transcription in the livers of mice requires both HCV core protein and  $PA28\gamma$ ; and (ii) the nuclear accumulation of HCV core protein alone, which occurs because of the lack of degradation along a  $PA28\gamma$ -dependent proteasome pathway, does not activate the *srebp-1c* promoter.

**HCV Core Protein Indirectly Potentiates *srebp-1c* Promoter Activity in an  $LXR\alpha/RXR\alpha$ -Dependent Manner.**  $LXR\alpha$ , which is primarily expressed in the liver, forms a complex with  $RXR\alpha$  and synergistically potentiates *srebp-1c* promoter activity (16). Activation of  $RXR\alpha$  by HCV core protein suggests that cellular fatty acid synthesis is modulated by the SREBP-1c pathway, although HCV core protein was not included in the transcription factor complex in the electrophoresis mobility shift assay (EMSA) (17). To analyze the effect of HCV core protein and  $PA28\gamma$  on the activation of the *srebp-1c* promoter, we first examined the effect of HCV core protein on the binding of the  $LXR\alpha/RXR\alpha$  complex to the LXR-response element (LXRE) located upstream of the SREBP-1c gene (Fig. 4A). Although a weak shift of the labeled LXRE probe was observed by incubation with nuclear extracts prepared from 293T cells expressing FLAG-tagged  $LXR\alpha$  and HA-tagged  $RXR\alpha$ , a clear shift was obtained by the treatment of cells with 9-*cis*-retinoic acid and 22(*R*)-hydroxycholesterol, ligands for  $LXR\alpha$  and  $RXR\alpha$ , respectively. In contrast, coexpression of HCV core protein with  $LXR\alpha$  and  $RXR\alpha$  potentiated the shift of the probe irrespective of the treatment with the ligands. Addition of 500 times the amount of nonlabeled LXRE probe (competitor) diminished the shift of the labeled probe induced by the ligands and/or HCV core protein. Furthermore, coinubation of the nuclear fraction with antibody to FLAG or HA tag but not with antibody to either HCV core or  $PA28\gamma$  caused a supershift of the labeled probe. These results indicate that HCV core protein does not participate in the  $LXR\alpha/RXR\alpha$ -LXRE complex but indirectly enhances the binding of  $LXR\alpha/RXR\alpha$  to the LXRE.

The activity of the *srebp-1c* promoter was enhanced by the expression of HCV core protein in 293T cells, and it was further enhanced by coexpression of  $LXR\alpha/RXR\alpha$  (Fig. 4B). Enhancement of the *srebp-1c* promoter by coexpression of HCV core protein and  $LXR\alpha/RXR\alpha$  was further potentiated by treatment with the ligands for  $LXR\alpha$  and  $RXR\alpha$ . The cells treated with 9-*cis*-retinoic acid exhibited more potent enhancement of the *srebp-1c* promoter than those treated with 22(*R*)-hydroxycholesterol. HCV core protein exhibited more potent enhancement of the *srebp-1c* promoter in cells treated with both ligands than in those treated with either ligand alone. These results suggest that HCV core protein poten-



**Fig. 4.** Activation of the *srebp-1c* promoter by HCV core protein. (A) FLAG- $LXR\alpha$  and HA- $RXR\alpha$  were expressed in 293T cells together with or without HCV core protein. Ligands for  $LXR\alpha$  and  $RXR\alpha$  dissolved in ethanol [Ligands (+)] or ethanol alone [Ligands (-)] were added to the culture supernatant at 24 h posttransfection. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA in the presence or absence of antibody (100 ng) against HA, FLAG, HCV core or  $PA28\gamma$ , or nonlabeled LXRE probe (Competitor). (Left) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase/streptavidin. The mobility shift of the LXRE probe and its supershift are indicated by a gray and black arrow, respectively. (Right) Expression of HCV core, HA- $RXR\alpha$ , FLAG- $LXR\alpha$ , and  $PA28\gamma$  in cells was detected by immunoblotting. (B) Effects of ligands for  $RXR\alpha$ , 9-*cis*-retinoic acid (9*cis*RA), and for  $LXR\alpha$ , 22(*R*)-hydroxycholesterol (22RHC), on the activation of the *srebp-1c* promoter in 293T cells expressing  $RXR\alpha$ ,  $LXR\alpha$ , and/or HCV core protein. Ligands were added into the medium at a concentration of 5  $\mu M$ , and the cells were harvested after 24 h of incubation.



**Fig. 5.** PA28 $\gamma$  is required for HCV core-dependent activation of the *srebp-1c* promoter. (A) Effect of PA28 $\gamma$  knockdown on the LXR $\alpha$ /RXR $\alpha$ -DNA complex. FLAG-LXR $\alpha$  and HA-RXR $\alpha$  were expressed in FLC4 (control) or PA28 $\gamma$ -knockdown (PA28 $\gamma$  KD) cells together with or without HCV core protein. Cells were harvested at 48 h posttransfection, and nuclear extracts were mixed with the reaction buffer for EMSA. (Upper) The resulting mixtures were subjected to PAGE and blotted with horseradish peroxidase-streptavidin. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) Expression of HCV core, HA-RXR $\alpha$ , FLAG-LXR $\alpha$ , and PA28 $\gamma$  in cells was detected by immunoblotting. (B) Effect of PA28 $\gamma$  knockout on the LXR $\alpha$ /RXR $\alpha$ -DNA complex in the mouse liver. (Upper) Nuclear extracts were prepared from the livers of 2-month-old PA28 $\gamma$ <sup>-/-</sup>, PA28 $\gamma$ <sup>+/+</sup>CoreTg, PA28 $\gamma$ <sup>-/-</sup>CoreTg, and PA28 $\gamma$ <sup>+/+</sup> mice and subjected to EMSA. The mobility shift of the LXRE probe is indicated by an arrow. (Lower) The expression of HCV core, PA28 $\gamma$ , and  $\beta$ -actin in the livers of the mice was detected by immunoblotting. (C) Effect of HCV core protein on *srebp-1* promoter activity in PA28 $\gamma$ -knockout fibroblasts. A plasmid encoding firefly luciferase under the control of the *srebp-1c* promoter was transfected into MEFs prepared from PA28 $\gamma$ <sup>+/+</sup> (Left) or PA28 $\gamma$ <sup>-/-</sup> (Right) mice together with a plasmid encoding a *Renilla* luciferase. An empty plasmid or plasmids encoding mouse RXR $\alpha$  or LXR $\alpha$  were also cotransfected into the cells together with (gray bars) or without (white bars) a plasmid encoding HCV core protein. Luciferase activity under the control of the *srebp-1c* promoter was determined, and it is expressed as the fold increase in relative luciferase activity after standardization with the activity of *Renilla* luciferase.

tates *srebp-1c* promoter activity in an LXR $\alpha$ /RXR $\alpha$ -dependent manner.

**HCV Core Protein Activates the *srebp-1c* Promoter in an LXR $\alpha$ /RXR $\alpha$ - and PA28 $\gamma$ -Dependent Manner.** To examine whether PA28 $\gamma$  is required for HCV core-induced enhancement of *srebp-1c* promoter activity in human liver cells, a PA28 $\gamma$ -knockdown human hepatoma cell line (FLC4 KD) was prepared. Enhancement of binding of the LXRE probe to LXR $\alpha$ /RXR $\alpha$  by coexpression of HCV core protein and LXR $\alpha$ /RXR $\alpha$  in FLC4 cells was diminished by knockdown of the PA28 $\gamma$  gene (Fig. 5A). Furthermore, formation of the LXR $\alpha$ /RXR $\alpha$ -LXRE complex was enhanced in the livers of PA28 $\gamma$ <sup>+/+</sup>CoreTg mice but not in those of PA28 $\gamma$ <sup>-/-</sup>, PA28 $\gamma$ <sup>+/+</sup>, or PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (Fig. 5B). The expression of the HCV core protein in the mouse embryonic fibroblasts (MEFs) of PA28 $\gamma$ <sup>+/+</sup> mice induced the activation of the mouse *srebp-1c* promoter through the endogenous expression of LXR $\alpha$  and RXR $\alpha$  (Fig. 5C Left). Further enhancement of the activation of the *srebp-1c* promoter by HCV core protein in PA28 $\gamma$ <sup>+/+</sup> MEFs was achieved by the exogenous expression of both LXR $\alpha$  and RXR $\alpha$ . However, no enhancing effect of HCV core protein on *srebp-1c* promoter activity was observed in PA28 $\gamma$ <sup>-/-</sup> MEFs (Fig. 5C Right). These results support the notion that HCV core protein enhances the activity of the *srebp-1c* promoter in an LXR $\alpha$ /RXR $\alpha$ - and PA28 $\gamma$ -dependent manner.

**Table 1.** HCC in mice at 16–18 months of age

Mouse and sex	Total no. of mice	No. of mice developing HCC	Incidence, %
PA28 $\gamma$ <sup>+/+</sup> CoreTg			
Male	17	5	29.4
Female	28	3	10.7
PA28 $\gamma$ <sup>+/+</sup>			
Male	16	0	0
Female	4	0	0
PA28 $\gamma$ <sup>-/-</sup>			
Male	23	0	0
Female	13	0	0
PA28 $\gamma$ <sup>-/-</sup> CoreTg			
Male	15	0	0
Female	21	0	0

**PA28 $\gamma$  Plays a Crucial Role in the Development of HCC in PA28 $\gamma$ <sup>+/+</sup>CoreTg Mice.** The incidence of hepatic tumors in male PA28 $\gamma$ <sup>+/+</sup>CoreTg mice older than 16 months was significantly higher than that in age-matched female PA28 $\gamma$ <sup>+/+</sup>CoreTg mice (6). We reconfirmed here that the incidence of HCC in male and female PA28 $\gamma$ <sup>+/+</sup>CoreTg mice at 16–18 months of age was 29.4% (5 of 17 mice) and 10.7% (3 of 28 mice), respectively. To our surprise, however, no HCC developed in PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (males, 15; females, 21), although, as expected, no HCC was observed in PA28 $\gamma$ <sup>-/-</sup> (males, 16; females, 4) and PA28 $\gamma$ <sup>-/-</sup> mice (males, 23; females, 13) (Table 1). These results clearly indicate that PA28 $\gamma$  plays an indispensable role in the development of HCC induced by HCV core protein.

#### Discussion

HCV core protein is detected in the cytoplasm and partially in the nucleus and mitochondria of culture cells and hepatocytes of transgenic mice and hepatitis C patients (6, 23, 24, 26). Degradation of HCV core protein was enhanced by deletion of the C-terminal transmembrane region through a ubiquitin/proteasome-dependent pathway (27). We previously reported (18) that PA28 $\gamma$  binds directly to HCV core protein and then enhances degradation of HCV core protein in the nucleus through a proteasome-dependent pathway because HCV core protein was accumulated in nucleus of human cell line by treatment with proteasome inhibitor MG132. In this work, accumulation of HCV core protein was observed in nucleus of hepatocytes of PA28 $\gamma$ <sup>-/-</sup>CoreTg mice (Fig. 1D). This result directly demonstrates that HCV core protein migrates into the nucleus and is degraded through a PA28 $\gamma$ -dependent pathway. However, HCV core protein accumulated in the nucleus because knockout of PA28 $\gamma$  gene abrogated the ability to cause liver pathology, suggesting that interaction of HCV core protein with PA28 $\gamma$  in the nucleus is prerequisite for the liver pathology induced by HCV core protein. We have previously shown (18) that HCV core protein is degraded through a PA28 $\gamma$ -dependent pathway, and Minami *et al.* (28) reported that PA28 $\gamma$  has a cochaperone activity with Hsp90. Therefore, degradation products of HCV core protein by means of PA28 $\gamma$ -dependent processing or correct folding of HCV core protein through cochaperone activity of PA28 $\gamma$  might be involved in the development of liver pathology. We do not know the reason why knockout of the PA28 $\gamma$  gene does not affect the total amount of HCV core protein in the liver of the transgenic mice. PA28 $\gamma$ -dependent degradation of HCV core protein may be independent of ubiquitination, as shown in SRC-3 (21), whereas knockdown of PA28 $\gamma$  in a human hepatoma cell line enhanced the ubiquitination of HCV core protein [supporting information (SI) Fig. 6], suggesting that lack of PA28 $\gamma$  suppresses a ubiquitin-independent degradation but enhances a ubiquitin-dependent degradation of HCV core protein. Therefore, the total amount of HCV

core protein in the liver of the mice may be unaffected by the knockout of the PA28 $\gamma$  gene.

Our results suggest that the interaction of HCV core protein with PA28 $\gamma$  leads to the activation of the *srebp-1c* promoter along an LXR $\alpha$ /RXR $\alpha$ -dependent pathway and the development of liver steatosis and HCC. HCV core protein was not included in the LXR $\alpha$ /RXR $\alpha$ -LXRE complex (Fig. 3A), suggesting that HCV core protein indirectly activates the *srebp-1c* promoter. Cytoplasmic HCV core protein was shown to interact with Sp110b, which is a transcriptional corepressor of RAR $\alpha$ -dependent transcription, and this interaction leads to the sequestering of Sp110b in the cytoplasm, resulting in the activation of RAR $\alpha$ -dependent transcription (29). The sequestration of an unidentified corepressor of the LXR $\alpha$ /RXR $\alpha$  heterodimer in the cytoplasm by HCV core protein may also contribute to the activation of the *srebp-1c* promoter. Although the precise physiological function of PA28 $\gamma$ -proteasome activity in the nucleus is not known, PA28 $\gamma$  has previously been shown (21) to regulate nuclear hormone receptors by means of the degradation of its coactivator SRC-3 and to participate in the fully Hsp90-dependent protein refolding (28). It appears reasonable to speculate that degradation or refolding of HCV core protein in a PA28 $\gamma$ -dependent pathway might be involved in the modulation of transcriptional regulators of various promoters, including the *srebp-1c* promoter. Saturated or monounsaturated fatty acids have been shown to enhance HCV RNA replication in Huh7 cells containing the full-length HCV replicon (7). The up-regulation of fatty acid biosynthesis by HCV core protein may also contribute to the efficient replication of HCV and to the progression of HCV pathogenesis.

Expression of HCV core protein was reported to enhance production of reactive oxygen species (ROS) (30), which leads to carbonylation of intracellular proteins (31). Enhancement of ROS production may trigger double-stranded DNA breaks and result in the development of HCC (30, 32, 33). HCV core protein could enhance the protein carbonylation in the liver of the transgenic mice in the presence but not in the absence of PA28 $\gamma$  (SI Fig. 7), suggesting that PA28 $\gamma$  is required for ROS production induced by HCV core protein. Development of HCC was observed in PA28 $\gamma^{+/+}$ CoreTg mice but not in PA28 $\gamma^{-/-}$ CoreTg mice (Table 1). Enhancement of ROS production by HCV core protein in the presence of PA28 $\gamma$  might be involved in the development of HCC in PA28 $\gamma^{+/+}$ CoreTg mice.

It is well known that resistant viruses readily emerge during the treatment with antiviral drugs targeting the viral protease or replicase, especially in the case of infection with RNA viruses. Therefore, antivirals targeting the host factors that are indispensable for the propagation of viruses might be an ideal target for the development of antiviral agents because of a lower rate of mutation than that of viral genome, if they have no side effects to patients. Importantly, the amino acid sequence of PA28 $\gamma$  of mice is identical to that of human, and mouse PA28 $\gamma$  is dispensable because PA28 $\gamma$  knockout mice exhibit no abnormal phenotype except for mild growth retardation. Therefore, PA28 $\gamma$  might be a promising target for an antiviral treatment of chronic hepatitis C with negligible side effects.

In summary, we observed that a knockout of the PA28 $\gamma$  gene from PA28 $\gamma^{+/+}$ CoreTg mice induced the accumulation of HCV core protein in the nucleus and disrupted the development of both steatosis and HCC. Activation of the *srebp-1c* promoter was up-regulated by HCV core protein both *in vitro* and *in vivo* through a PA28 $\gamma$ -dependent pathway, suggesting that PA28 $\gamma$  plays a crucial role in the development of liver pathology induced by HCV infection.

## Materials and Methods

Histology and immunohistochemistry, real-time PCR, and detection of proteins modified by ROS are discussed in *SI Materials and Methods*.

**Plasmids and Reagents.** Human PA28 $\gamma$  cDNA was isolated from a human fetal brain library (18). The gene encoding HCV core protein was amplified from HCV strain J1 (genotype 1b) (34) and cloned into pCAG-GS (35). Mouse cDNAs of RXR $\alpha$  and LXR $\alpha$  were amplified by PCR from the total cDNAs of the mouse liver. The RXR $\alpha$  and LXR $\alpha$  genes were introduced into pEF-FLAGGspGBK (36) and pcDNA3.1 (Invitrogen, Carlsbad, CA), respectively. The targeting fragment for human PA28 $\gamma$  knockdown (GGATCCGGTGGATCAGGAAGTGAAGTTCAAGAGACTTCACTTCCTGATCCACCTTTTTTGGAAAAGCTT) was introduced into the BamHI and HindIII sites of pSilencer 4.1 U6 hygro vector (Ambion, Austin, TX). Mouse anti-FLAG (M2) and mouse anti- $\beta$ -actin antibodies were purchased from Sigma (St. Louis, MO). Rabbit polyclonal antibody against synthetic peptides corresponding to amino acids 70–85 of PA28 $\gamma$  was obtained from AFFINITI (Exeter, U.K.). Horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit IgGs were purchased from ICN Pharmaceuticals (Aurora, OH). Rabbit anti-HCV core protein was prepared by immunization with recombinant HCV core protein (amino acids 1–71), as described in ref. 24. Mouse monoclonal antibody to HCV core protein was kindly provided by S. Yagi (37). The plasmid for expression of HA-tagged ubiquitin was described in ref. 27.

**Preparation of PA28 $\gamma$ -Knockout HCV CoreTg Mice.** The generation of C57BL/6 mice carrying the gene encoding HCV core protein genotype 1b line C49 and that of PA28 $\gamma^{-/-}$  mice have been reported previously (22, 25). Both strains were crossbred with each other to create PA28 $\gamma^{-/-}$ CoreTg mice. PA28 $\gamma^{-/-}$ CoreTg mice were identified by PCR targeted at the PA28 $\gamma$  or HCV core gene (22, 25). Using 1  $\mu$ g of genomic DNA obtained from the mouse tail, the PA28 $\gamma$  gene was amplified by PCR with the following primers: sense, PA28-3 (AGGTGGATCAGGAAGTGAAGCTCAA); and antisense, PA28 $\gamma$ -5cr (CACCTCACTGTGATCCGCTCTCTGAAAGAATCAACC). The targeted sequence for the PA28 $\gamma$ -knockout mouse was detected by PCR using the PA28-3 primer and the PAKO-4 primer (TGCAGTTCATTGAGGGCACCGGACAG). The transgene encoding HCV core protein was detected by PCR as described in ref. 25. The expression of PA28 $\gamma$  and HCV core protein in the livers of 6-month-old mice was confirmed by Western blotting with mouse monoclonal antibody to HCV core protein, clone 11-10, and rabbit antibody to PA28 $\gamma$ . Mice were cared for according to the institutional guidelines. The mice were given ordinary feed, CRF-1 (Charles River Laboratories, Yokohama, Japan), and they were maintained under specific pathogen-free conditions.

All animal experiments conformed to the Guidelines for the Care and Use of Laboratory Animals, and they were approved by the Institutional Committee of Laboratory Animal Experimentation (Research Institute for Microbial Diseases, Osaka University).

**Preparation of Mouse Embryonic Fibroblasts.** MEFs were prepared as described in ref. 22. MEFs were cultured at 37°C under an atmosphere of 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% FBS, penicillin, streptomycin, sodium pyruvate, and nonessential amino acids.

**Transfection and Immunoblotting.** Plasmid vectors were transfected into the MEFs and 293T cells by liposome-mediated transfection by using Lipofectamine 2000 (Invitrogen). The amount of HCV core protein in the liver tissues was determined by an ELISA as described in ref. 37. The cell lysates were subjected to SDS/PAGE (12.5% gel), and they were then transferred onto PVDF membranes. Proteins on the membranes were treated with specific antibody and Super Signal Femto (Pierce, Rockford, IL). The results were then visualized by using an LAS3000 imaging system (Fuji Photo Film, Tokyo, Japan). The method of immunoprecipitation test is described in ref. 18.

**Reporter Assay for *srebp-1c* Promoter Activity.** The genomic DNA fragment encoding the *srebp-1c* promoter region (located from residues -410 to +24) was amplified from a mouse genome. The fragment was introduced into the KpnI and HindIII sites of pGL3-Basic (Promega, Madison, WI), and it was designated as pGL3-*srebp-1c*Pro. The plasmids encoding RXR $\alpha$  and LXR $\alpha$  were transfected into MEFs together with pGL3-*srebp-1c*Pro and a control plasmid encoding *Renilla* luciferase (Promega). The total DNA for transfection was normalized by the addition of empty plasmids. Cells were harvested at 24 h posttransfection. The ligand of RXR $\alpha$ , 9-*cis*-retinoic acid (Sigma), and that of LXR $\alpha$ , 22(R)-hydroxycholesterol (Sigma) were added at a final concentration of 5  $\mu$ M each to the culture medium of 293T cells transfected with pGL3-*srebp-1c*Pro together with expression plasmids encoding RXR $\alpha$ , LXR $\alpha$ , and HCV core protein at 24 h posttransfection. Cells were harvested 24 h after treatment. Luciferase activity was measured by using the dual-luciferase reporter assay system (Promega). Firefly luciferase activity was standardized with that of *Renilla* luciferase, and the results are expressed as the fold increase in relative luciferase units.

**Electrophoresis Mobility Shift Assay (EMSA).** EMSA was carried out by using a LightShift Chemiluminescent EMSA kit (Pierce) according to the manufacturer's protocol. Nuclear extract of the cell lines and liver tissue was prepared with an NE-PER nuclear

and cytoplasmic extraction reagent kit (Pierce). Briefly, double-stranded oligonucleotides for EMSA were prepared by annealing both strands of each LXRE of the *srebp-1c* promoter (5'-GGACGCCCGCTAGTAACCCCGGC-3') (16). Both strands were labeled at the 5' ends with biotin. The annealed probe was incubated for 20 min on ice with nuclear extract (3  $\mu$ g of protein) in a reaction buffer containing 10 mM Tris-HCl (pH 7.5), 50 mM KCl, 1 mM DTT, 0.05  $\mu$ g/ $\mu$ l poly(dI-dC), 2.5% glycerol, 0.05% Nonidet P-40, and 0.1 nM labeled probe, with or without 1 mM nonlabeled probe. The resulting mixture was subjected to PAGE (5% gel) at 120 V for 30 min in 0.5 $\times$  TBE. The DNA-protein complex was transferred to a Hybond N+ membrane (Amersham, Piscataway, NJ), incubated with horseradish peroxidase-conjugated streptavidin, and visualized by using an LAS3000 imaging system.

**Statistical Analysis.** The results are expressed as the mean  $\pm$  SD. The significance of differences in the means was determined by Student's *t* test.

We thank H. Murase for secretarial work and D. C. S. Huang for providing the plasmids. This work was supported in part by grants-in-aid from the Ministry of Health, Labor, and Welfare; the Ministry of Education, Culture, Sports, Science, and Technology; the 21st Century Center of Excellence Program; and the Foundation for Biomedical Research and Innovation.

- Wasley A, Alter MJ (2000) *Semin Liver Dis* 20:1-16.
- Bach N, Thung SN, Schaffner F (1992) *Hepatology* 15:572-577.
- Lefkowitz JH, Schiff ER, Davis GL, Perrillo RP, Lindsay K, Bodenheimer HC, Jr., Balart LA, Ortego TJ, Payne J, Dienstag JL, et al. (1993) *Gastroenterology* 104:595-603.
- Barba G, Harper F, Harada T, Kohara M, Goulinet S, Matsuura Y, Eder G, Schaff Z, Chapman MJ, Miyamura T, Brechot C (1997) *Proc Natl Acad Sci USA* 94:1200-1205.
- Hope RG, McLauchlan J (2000) *J Gen Virol* 81:1913-1925.
- Moriya K, Fujie H, Shintani Y, Yotsuyanagi H, Tsutsumi T, Ishibashi K, Matsuura Y, Kimura S, Miyamura T, Koike K (1998) *Nat Med* 4:1065-1067.
- Kapadia SB, Chisari FV (2005) *Proc Natl Acad Sci USA* 102:2561-2566.
- Su AI, Pezacki JP, Wodicka L, Brideau AD, Supekova L, Thimme R, Wieland S, Bukh J, Purcell RH, Schultz PG, Chisari FV (2002) *Proc Natl Acad Sci USA* 99:15669-15674.
- Wang C, Gale M, Jr, Keller BC, Huang H, Brown MS, Goldstein JL, Ye J (2005) *Mol Cell* 18:425-434.
- Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) *J Clin Invest* 101:2331-2339.
- Pai JT, Guryev O, Brown MS, Goldstein JL (1998) *J Biol Chem* 273:26138-26148.
- Shimano H, Horton JD, Hammer RE, Shimomura I, Brown MS, Goldstein JL (1996) *J Clin Invest* 98:1575-1584.
- Shimano H, Horton JD, Shimomura I, Hammer RE, Brown MS, Goldstein JL (1997) *J Clin Invest* 99:846-854.
- Shimano H, Shimomura I, Hammer RE, Herz J, Goldstein JL, Brown MS, Horton JD (1997) *J Clin Invest* 100:2115-2124.
- Repa JJ, Liang G, Ou J, Bashmakov Y, Lobaccaro JM, Shimomura I, Shan B, Brown MS, Goldstein JL, Mangelsdorf DJ (2000) *Genes Dev* 14:2819-2830.
- Yoshikawa T, Shimano H, Amemiya-Kudo M, Yahagi N, Hasty AH, Matsuzaka T, Okazaki H, Tamura Y, Iizuka Y, Ohashi K, et al. (2001) *Mol Cell Biol* 21:2991-3000.
- Tsutsumi T, Suzuki T, Shimoike T, Suzuki R, Moriya K, Shintani Y, Fujie H, Matsuura Y, Koike K, Miyamura T (2002) *Hepatology* 35:937-946.
- Moriishi K, Okabayashi T, Nakai K, Moriya K, Koike K, Murata S, Chiba T, Tanaka K, Suzuki R, Suzuki T, et al. (2003) *J Virol* 77:10237-10249.
- Masson P, Andersson O, Petersen UM, Young P (2001) *J Biol Chem* 276:1383-1390.
- Li J, Rechsteiner M (2001) *Biochimie* 83:373-383.
- Li X, Lonard D, Jung SY, Malovannaya A, Feng Q, Qin J, Tsai SY, Tsai M, O'Malley BW (2006) *Cell* 124:381-392.
- Murata S, Kawahara H, Tohma S, Yamamoto K, Kasahara M, Nabeshima Y, Tanaka K, Chiba T (1999) *J Biol Chem* 274:38211-38215.
- Falcon V, Acosta-Rivero N, China G, Gavilondo J, de la Rosa MC, Menendez I, Duenas-Carrera S, Vina A, Garcia W, Gra B, et al. (2003) *Biochem Biophys Res Commun* 305:1085-1090.
- Suzuki R, Sakamoto S, Tsutsumi T, Rikimaru A, Tanaka K, Shimoike T, Moriishi K, Iwasaki T, Mizumoto K, Matsuura Y, et al. (2005) *J Virol* 79:1271-1281.
- Moriya K, Yotsuyanagi H, Shintani Y, Fujie H, Ishibashi K, Matsuura Y, Miyamura T, Koike K (1997) *J Gen Virol* 78:1527-1531.
- Yasui K, Wakita T, Tsukiyama-Kohara K, Funahashi SI, Ichikawa M, Kajita T, Moradpour D, Wands JR, Kohara M (1998) *J Virol* 72:6048-6055.
- Suzuki R, Tamura K, Li J, Ishii K, Matsuura Y, Miyamura T, Suzuki T (2001) *Virology* 280:301-309.
- Minami Y, Kawasaki H, Minami M, Tanahashi N, Tanaka K, Yahara I (2000) *J Biol Chem* 275:9055-9061.
- Watashi K, Hijikata M, Tagawa A, Doi T, Marusawa H, Shimotohno K (2003) *Mol Cell Biol* 23:7498-7509.
- Machida K, Cheng KT, Lai CK, Jeng KS, Sung VM, Lai MM (2006) *J Virol* 80:7199-7207.
- Nystrom T (2005) *EMBO J* 24:1311-1317.
- Bromberg JF, Wrzeszczynska MH, Devgan G, Zhao Y, Pestell RG, Albanese C, Darnell JE, Jr (1999) *Cell* 98:295-303.
- Carballo M, Conde M, El Bekay R, Martin-Nieto J, Camacho MJ, Monteseirin J, Conde J, Bedoya FJ, Sobrino F (1999) *J Biol Chem* 274:17580-17586.
- Aizaki H, Aoki Y, Harada T, Ishii K, Suzuki T, Nagamori S, Toda G, Matsuura Y, Miyamura T (1998) *Hepatology* 27:621-627.
- Niwa H, Yamamura K, Miyazaki J (1991) *Gene* 108:193-199.
- Huang DC, Cory S, Strasser A (1997) *Oncogene* 14:405-414.
- Aoyagi K, Ohue C, Iida K, Kimura T, Tanaka E, Kiyosawa K, Yagi S (1999) *J Clin Microbiol* 37:1802-1808.

## Review

# Drug resistance in antiviral treatment for infections with hepatitis B and C viruses

HIROSHI YOTSUYANAGI and KAZUHIKO KOIKE

Department of Infectious Diseases, Internal Medicine, Graduate School of Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-8655, Japan

Treatments for infections with hepatitis B and C viruses have recently developed markedly, and range from non-specific interferon-based treatments to specific antiviral treatments, such as those that inhibit hepatitis virus-coded protein production or activity. These developments have contributed to the achievement of excellent enhancement of the antiviral effect. On the other hand, the development of specific antiviral therapies has created unprecedented problems. Antiviral drug-resistant strains of viruses have emerged, leading to a poor prognosis for infected patients. Clarification of the mechanisms underlying the emergence of such resistance to drugs will be useful for the treatment of such patients. In this review, we outline pathological conditions associated with hepatitis B and C viruses and their treatments, and discuss the current situation and mechanisms underlying the emergence of antiviral drug-resistant strains.

**Key words:** Interferon, lamivudine, entecavir, adefovir, ribavirin

### Introduction

Infections with hepatitis B and C viruses (HBV and HCV, respectively) are a problem worldwide. Many patients are infected with HBV and HCV, and these infections are not only a major medical burden but also a socioeconomic burden because of their possible progression from chronic hepatitis to cirrhosis and hepatocellular carcinoma (HCC) if left untreated.<sup>1,2</sup> The treatments against these hepatitis viruses have been mainly nonspecific, based on the use of interferon (IFN). Specific antiviral drugs developed recently that inhibit

hepatitis viral replication, for example, by inhibition of reverse transcriptase in HBV, have shown remarkable efficacy.<sup>3,4</sup> However, these treatments have also turned out to be a double-edged sword because they have led to the emergence of strains resistant to these drugs. The emergence of antiviral drug-resistant strains of these viruses may hinder the development of treatments against them.

### HBV infection

HBV belongs to the family *Hepadnaviridae*. HBV is a DNA virus that has an approximately 3.2-kb circular incomplete double-stranded DNA genome. When it replicates, HBV forms DNA by using its own reverse transcriptase with RNA as a replicative intermediate. Worldwide, the distribution of HBV carriers and the incidence of HCC are closely correlated. The number of HBV carriers in Japan is estimated to be approximately one million, many of whom became infected via perinatal mother-to-child transmission. Hepatitis B e antigen (HBeAg) is a protein produced by the wild-type HBV strain as it replicates that appears in the blood. Generally, the presence of HBeAg indicates an abundance of the virus, and the detection of anti-HBe antibody often indicates a marked decrease in the HBV load.

HBV carriers are HBeAg-positive asymptomatic carriers (AsC) in the early stages of HBV infection. Their blood HBV level is very high in these stages. They can develop symptoms of hepatitis at any time of their life, but generally do between adolescence and their early 30s. At that time, the HBV load decreases markedly, and symptoms of hepatitis improve in 2 to 3 years in 80%–90% of HBV patients. Most of these patients then become negative for HBeAg and positive for the anti-HBe antibody. The annual incidence of seroconversion from being HBeAg-positive to anti-HBe antibody-

Received: February 21, 2007 / Accepted: February 25, 2007  
Reprint requests to: H. Yotsuyanagi

positive has been reported to be approximately 5%.<sup>5,6</sup> However, symptoms of hepatitis persist in about 10%–20% of these patients even after seroconversion; such patients often develop cirrhosis and HCC.<sup>7</sup>

#### *Treatment of HBV infection*

There are several choices for treatment of chronic hepatitis B, but they all have limited efficacy. HBV elimination [i.e., serum HB surface antigen (HBsAg)-negative status] is difficult in HBV carriers. Seroconversion from HBeAg-positive to anti-HBe antibody-positive is generally the goal of HBV infection treatment. It should also be understood that many HBV AsCs do not need to or often must not undergo treatment with any anti-HBV agent.

In approximately 80% of patients with chronic hepatitis B, seroconversion occurs spontaneously. Consequently, chronic hepatitis B does not progress to cirrhosis in most chronic hepatitis B patients. The 15%–20% of HBV carriers who do not show seroconversion ultimately develop cirrhosis and/or HCC and should undergo antiviral treatment for chronic hepatitis B. IFN and reverse transcriptase inhibitors, including lamivudine, adefovir dipivoxil, and entecavir, have been approved for the treatment of chronic hepatitis B. Among these, lamivudine was the first used for chronic hepatitis B patients.

Lamivudine recipients are more likely than placebo recipients to show a histological response (52% vs. 23%,  $P < 0.001$ ), the disappearance of HBeAg in serum (32% vs. 11%,  $P = 0.003$ ), a sustained decrease in serum HBV DNA level to undetectable levels (44% vs. 16%,  $P < 0.001$ ), and a sustained normalization of the serum alanine aminotransferase (ALT) level (41% vs. 7%,  $P < 0.001$ ) after 52 weeks of treatment.<sup>8</sup>

Entecavir has an antiviral effect comparable to that of lamivudine. In one study of HBeAg-positive patients,<sup>9</sup> histological improvement after 48 weeks occurred in 226 of 314 patients in an entecavir-treated group (72%) and in 195 of 314 patients in a lamivudine-treated group (62%,  $P = 0.009$ ). More patients in the entecavir-treated group than in the lamivudine-treated group had undetectable serum HBV DNA levels according to a polymerase chain reaction (PCR) assay (67% vs. 36%,  $P < 0.001$ ), and normalization of ALT levels (68% vs. 60%,  $P = 0.02$ ). However, the seroconversion rate was not very high in either group.

#### *Emergence of antiviral resistance in HBV infection*

The treatment efficacy against HBV infection has improved, as mentioned above. The primary reason for chronic hepatitis B becoming resistant to treatment is the emergence of drug-resistant strains.

Mechanisms underlying development of resistance to IFN-based treatments

IFNs are administered in a wide range of doses according to various protocols for chronic hepatitis B. Such variation in protocols must be taken into account in determining the therapeutic efficacy of such drugs. Viral factors potentially involved in drug resistance include the HBV genotype and the presence of HBeAg, a precore gene mutation, or a core promoter mutation. HBeAg negativity, HBV genotypes C and D, precore gene 1896 mutation, and 1762/1764 mutations in the core promoter have been reported to be responsible for a poor response to IFN treatment. Strictly, these factors do not indicate “drug resistance” to IFN. The acquisition of the precore gene 1896 mutation also decreases the efficacy of IFN treatment.<sup>10</sup>

Mutation of the HBV polymerase domain

The HBV polymerase (*Pol*) gene is shown in Fig. 1. The Pol/RT region of this gene encodes HBV reverse transcriptase (RT). In HBV gene replication, reverse transcription from pregenomic RNA to viral gene DNA occurs, and reverse transcriptase is used in this process. The Pol/RT region is further divided into five domains from A to E. The Pol/RT active center is reported to be in domain C, domains B and E have been reported to be important for RNA template binding, and domains A and D for binding to nucleic acids.

Nucleic acid analog formulations are incorporated into the viral genome involved in reverse transcription instead of nucleic acids to inhibit viral replication competitively. However, if a Pol/RT gene mutation emerges, the nucleic acid analog formulation administered may be unable to inhibit viral replication because of interference with its binding to RT. Acquisition of this mutation of the Pol/RT gene thus results in the emergence of a drug-resistant viral strain. This mutation is shown in Fig. 1.

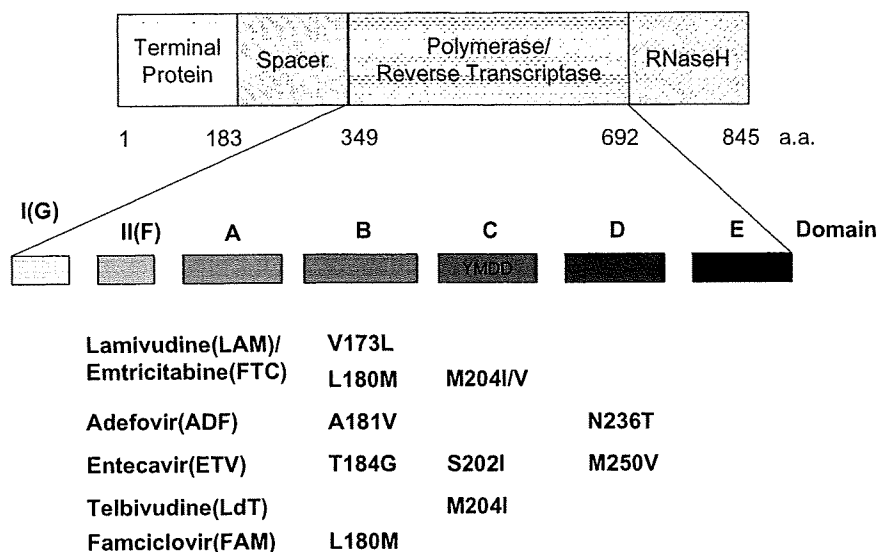
Resistance to lamivudine

Among nucleic acid analog formulations, lamivudine (LAM) has been used longest for the treatment of chronic hepatitis B, and its use continued until resistance to this drug emerged. Many factors underlying this resistance have been identified.

LAM resistance is caused by a mutation in which the 204th methionine (M) in the polymerase domain is replaced by valine (V) or isoleucine (I) (rtM204V/I). Because the site containing the 204th methionine is referred to as the YMDD motif, this mutation is also called the YVDD mutation or YIDD mutation. Another mutation associated with rtM204V/I is caused by replacement of the 180th leucine (L) by M (rtL180M).

rtM204V/I was detected in 10% of HBeAg-positive patients with chronic hepatitis B 24 weeks after the start





**Fig. 1.** Structure of the hepatitis B virus polymerase/reverse transcriptase gene and amino acid substitutions associated with resistance to nucleoside analogs. *a.a.*, amino acids

of LAM administration, in 24% after 52 weeks, and in 65% after 5 years.<sup>4</sup> In Japan, in an extensive study on the clinical course of both HBeAg-positive and HBeAg-negative chronic hepatitis B patients, the rate of emergence of LAM-resistant strains was 50%, 5 years after the start of LAM administration.<sup>11</sup> Not all patients having rtM204V/I develop hepatitis. Although the incidence of amino acid mutation of polymerase is high in patients with hepatitis, no specific mutation has been observed.<sup>12</sup>

The case of a patient who was LAM-resistant despite the absence of mutation in the YMDD motif was reported recently. The 181st alanine (A) in the Pol domain was replaced by threonine (T) (rtA181T) in this patient. An experiment using chimeric mice bearing human hepatocytes demonstrated that this mutation causes LAM resistance.<sup>13</sup>

#### Resistance to adefovir dipivoxil

The use of adefovir dipivoxil (ADV) in combination with LAM has been approved in Japan for patients showing LAM resistance. The emergence of ADV-resistant viral strains was first studied with regard to the administration of ADV alone. ADV resistance occurs in approximately 6% of patients 3 years after the start of ADV administration.<sup>14</sup> The replacement of the 236th asparagine (N) in the Pol domain by T (rtN236T) and that of the 181st A in the Pol domain by V (rtA181V) were observed in ADV-resistant strains.

Following the administration of ADV to patients exhibiting LAM resistance, strains with rtA181V/T or rtN236T emerged in 18% of the patients 1 year after the start of ADV administration.<sup>15</sup> rtA181T was also observed in LAM-resistant strains in the absence of mutation in the YMDD motif. It was reported recently that

some patients exhibiting LAM resistance are non-responders to ADV from the start of ADV administration. These resistant strains had the rtI233V mutation.<sup>16</sup>

#### Resistance to entecavir

Administration of entecavir (ETV) for more than 52 weeks to patients with LAM-resistant chronic hepatitis B leads to the emergence of resistant viral strains in 1.4% of patients,<sup>17</sup> although no results have been reported for long-term administration of ETV. The amino acid sequence of the RT domain in resistant viral strains was analyzed in two patients, and strains with rtM250V and rtI169T were found in one patient and strains with rtT184G and rtS202I in the other.<sup>21</sup> In Japan, strains with rtS202G and rtL269I have been detected.<sup>22</sup> It is notable that all of the patients who acquired ETV resistance were resistant to LAM. Thus, initial treatment with ETV alone may be less likely to lead to ETV-resistant viral strain emergence, but this hypothesis should be confirmed in future studies (Table 1).

#### HCV infection

HCV is a positive-strand RNA virus belonging to the family *Flaviviridae*. Approximately 170 million HCV carriers or patients are estimated to be persistently infected with HCV worldwide, and approximately 1.8 million in Japan. HCV is transmitted to humans by direct contact with infected or contaminated blood. The routes of infection include transfusion of contaminated or HCV-tainted blood or blood products, which have now been eliminated in Japan, sharing needles among drug abusers, acupuncture, and tattoos. Acupuncture and

**Table 1.** Clinical trial of entecavir for the treatment of chronic hepatitis B

No.	Authors	HBeAg	Lam resistance at baseline	ETV resistance at baseline	ETV resistance at end point (1 year)	Biochemical rebound (1 year)	Reference No.
1	Chang et al.	Positive	No		0/339	6/339 (2%)	9
2	Sherman et al.	Positive	Yes	11/186 (6%)	7/134 (5%)		17
3	Colonno et al.	Positive	No		0/354	6/354 (2%)	18
		Negative			1/325 (0.3%)	8/325 (2%)	
4	Lai et al.	Negative	No		0/211	5/211 (2%)	19
5	Chang et al.	Positive	Yes	6/181 (3%)	2/181 (1%)		20
		Negative					

HBeAg, hepatitis B e antigen; LAM, lamivudine; ETV, entecavir

depilation are invasive treatments and should be considered to involve risk for infection with HCV unless disposable medical instruments are used.<sup>23</sup>

HCV infection develops into persistent infection at a very high rate, becoming persistent in 70%–80% of patients with acute HCV infection. Generally, patients develop acute hepatitis 2 to 3 months after their initial infection with HCV. However, many patients are unaware of the onset of hepatitis because of the mild subjective symptoms and mild jaundice, if any. Although 20%–30% of patients developing acute hepatitis recover from the disease spontaneously, acute hepatitis develops into chronic hepatitis (by definition, hepatitis persisting for more than 6 months) in the remaining 70%–80% of patients. Then, chronic hepatitis enters an inactive phase that lasts 10–15 years. Serum ALT level, which indicates the destruction of hepatocytes, is within normal limits during the inactive phase, but viral growth continues.

Chronic hepatitis enters an active phase after 10–15 years in many patients, although there are marked individual differences. Serum ALT level increases to about two to three times the normal level when chronic hepatitis enters the active phase. Once chronic hepatitis C enters the active phase, it will not improve spontaneously. If the disease is left untreated, the risk of progressing from chronic hepatitis to cirrhosis increases. Hepatitis C characteristically progresses gradually but steadily.<sup>24</sup> The risk of developing HCC is high among patients with cirrhosis. The risk of HCC development in cirrhosis patients is 5%–7%.<sup>25</sup> Patients infected with HCV should be diagnosed during the inactive phase of chronic hepatitis C and start treatment for HCV elimination (antiviral treatment) as soon as the hepatitis enters the active phase.

#### Treatment of HCV infection

HCV infection is treated mainly with IFN-based drugs. The treatment efficacy is evaluated 6 months after the end of IFN-based drug administration. If HCV-RNA is

not detected by the sensitive RT-PCR test, the patient is considered to show a sustained virological response (SVR), indicating that HCV has been virtually eliminated.

At present, polyethylene glycol-interferon (Peg-IFN) treatment in combination with ribavirin plays a key role in the treatment of HCV infection. Peg-IFN, an IFN molecule covalently bonded to Peg, is a sustained-release formulation. It needs to be injected only once weekly from the start of treatment, whereas conventional IFN preparations require administration three times weekly. Administration of Peg-IFN alone is more effective than that of a conventional IFN-based drug alone, but the administration of Peg-IFN in combination with ribavirin is even more effective.<sup>26–28</sup> An SVR rate of approximately 50% can be expected even in cases of chronic hepatitis infected with a high viral load of HCV of genotype 1, and an SVR rate of approximately 60% can be generally expected. Peg-IFN is usually administered for 48 consecutive weeks. It is important to continue the treatment for 48 weeks, although the dose may be reduced if adverse drug reactions appear. In addition, extending the administration period to a total of 72 weeks recently proved effective in patients who became HCV-negative after 12 weeks of treatment.<sup>29</sup>

There is a long history of treatment with IFN alone: treatment of non-A, non-B hepatitis with IFN alone dates back to around 1985, before the discovery of HCV. A nationwide survey conducted by the Study Group of the Ministry of Health, Labour and Welfare of Japan in 1995 showed that the SVR rate for treatment with IFN alone for 6 months (administration of 6 to 10 million units) was approximately 30% in all patients. However, in patients with the genotype 1 HCV, which is the major genotype worldwide and in about 70% of Japanese HCV patients, particularly those with high viral loads (determined as an HCV-RNA load of 100 KIU/ml or more), SVR was obtained in only about 2%–7%. The efficacy of treatment with IFN alone is thus low. Hence, Peg-IFN in combination with ribavirin

**Table 2.** Relationship between the ISDR and the response to interferon treatment for chronic hepatitis patients with genotype 1 hepatitis C virus infection

No.	Authors	Interferon	Ribavirin	Relationship between ISDR and viral load	Relationship between ISDR and response	Ethnicity	Ref. no.
1	Enomoto et al.	$\alpha$	No	Yes	Yes	Japanese	30
2	Kurosaki et al.	$\beta$	No	Yes	Yes	Japanese	31
3	Chayama et al.	$\alpha$	No	ND	Yes	Japanese	32
4	Zeuzem et al.	$\alpha$	No	No	No	German	33
5	Squadrito et al.	$\alpha$	No	No	No	French	34
6	Hofgartner et al.	$\alpha$	No	ND	No	American	35
7	Khorsi et al.	$\alpha$	No	ND	No	French	36
8	Saiz et al.	$\alpha$	No	ND	Yes	Spanish	37
9	Frangeul et al.	$\alpha$	No	ND	No	French	38
10	Odeberg et al.	$\alpha$	No	ND	No	Sweden	39
11	Chung et al.	$\alpha$	No	ND	No	American	40
12	Ibarrola et al.	$\alpha$	Yes	ND	No	Spanish	41
13	Sarrazin et al.	$\alpha$	Yes	ND	Yes	German	42
14	McKechmie et al.	$\alpha$	No	ND	No	English	43
15	Yoshioka et al.	$\alpha$	No	ND	Yes	Japanese	44
16	Stratidaki et al.	$\alpha$	No	ND	No	American	45
17	Murphy et al.	$\alpha$	Yes	ND	No	American	46
18	Cappiello et al.	PEG $\alpha$	Yes	ND	No	Italian	47
19	Aslan et al.	$\alpha$	No	ND	No	Turkish	48
20	Murayama et al.	$\alpha$	Yes	ND	Yes	Japanese	49

ISDR, interferon sensitivity determining region; ND, not described; PEG, pegylated

is the first choice for patients with intractable disease, as mentioned above.

#### *Emergence of antiviral resistance in HCV infection*

In the treatment of chronic hepatitis C with IFN alone or IFN (or Peg-IFN) in combination with ribavirin, HCV-RNA does not disappear in some patients, particularly in those with genotype 1 HCV. Approximately 10% of genotype 1 HCV patients with high viral loads never become HCV-RNA-negative during the period of treatment with IFN (or Peg-IFN) in combination with ribavirin.

Not only host factors but also viral factors have been identified as causes for the nonelimination of HCV. The HCV genotype is a typical viral factor, and patients infected with genotype 1 or 4 are more resistant to treatment than those with genotype 2 or 3.

Another reported factor is the interferon sensitivity-determining region (ISDR) in NS5A, a region consisting of 40 amino acids, first reported by Enomoto et al.<sup>30</sup> ISDR is contained in the binding site of interferon  $\alpha$ -inducible RNA-dependent protein kinase (PKR). Mutation in ISDR may cause dysfunction in the binding between the NS5A protein and PKR, leading to a decrease in viral protein translation. In Japan, a close correlation between IFN treatment efficacy and mutation in ISDR in genotype 1b HCV patients was found.<sup>31,32</sup> In Europe and the United States, however, the correlation between amino acid mutation in ISDR and IFN

treatment efficacy is not clear even in patients infected with HCV genotype 1 (Table 2).

In addition, mutation of the PKR/eIF2 $\alpha$  phosphorylation homology domain of the E2 domain has been reported to correlate with IFN-based drug efficacy, but this needs further clarification.

#### *Ribavirin-resistant viral strain*

Ribavirin shows low anti-HCV activity in some patients even when it is administered alone, and chronic hepatitis C has been treated with ribavirin alone. The structure of a ribavirin-resistant viral strain that has emerged has been studied. Mutation of the 415th amino acid (F415Y) in the RNA-dependent RNA polymerase (RdRp) domain of NS5B was detected in strains infecting patients treated with ribavirin alone who became ribavirin-resistant.<sup>50</sup> This mutation was considered to be related to IFN treatment efficacy in patients with genotype 1a HCV. In a study using a replicon, mutations of the 404th and 442nd amino acids (G404S and E442G) were detected.<sup>51</sup>

#### **Conclusions**

An overview of the mechanisms underlying the emergence of drug-resistant HBV and HCV strains has been given above. The emergence of drug-resistant strains of HBV in particular has posed problems. This resistance has resulted from the development of a wide range of

drugs for HBV, ranging from nonspecific IFN-based drugs to viral protein-specific RT inhibitors. Although no serious problem has arisen to date as regards HCV, specific anti-HCV drugs such as protease inhibitors and RNA polymerase inhibitors are beginning to be developed, so the emergence of drug-resistant viral strains is expected to be a major problem. Indeed, the emergence of a strain resistant to VX950, an HCV protease inhibitor with high antiviral activity, following a short period of administration of this drug has already been reported.<sup>52</sup> HBV and HCV do not seem to be very easy to eliminate.

*Acknowledgments.* This work was supported by a Grant-in-Aid for Scientific Research on Priority Area from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by Health Sciences Research Grants from the Ministry of Health, Labour and Welfare.

## References

- Saito I, Miyamura T, Ohbayashi A, Harada H, Katayama T, Kikuchi S, et al. Hepatitis C virus infection is associated with the development of hepatocellular carcinoma. *Proc Natl Acad Sci U S A* 1990;87:6547-9.
- Williams R. Global challenges in liver disease. *Hepatology* 2006; 44:521-6.
- Okanoue T, Minami M. Update of research and management of hepatitis B. *J Gastroenterol* 2006;41:107-18.
- Lok AS, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, et al. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003;125:1714-22.
- Liaw YF, Chu CM, Lin DY, Sheen IS, Yang CY, Huang MJ. Age-specific prevalence and significance of hepatitis B e antigen and antibody in chronic hepatitis B virus infection in Taiwan: a comparison among asymptomatic carriers, chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. *J Med Virol* 1984;13: 385-91.
- Fattovich G, Rugge M, Brollo L, Pontisso P, Noventa F, Guido M, et al. Clinical, virologic and histologic outcome following seroconversion from HBeAg to anti-HBe in chronic hepatitis type B. *Hepatology* 1986;6:167-72.
- Brunetto MR, Giarin MM, Oliveri F, Chiaberge E, Baldi M, Alfano A, et al. Wild-type and e antigen-minus hepatitis B viruses and course of chronic hepatitis. *Proc Natl Acad Sci U S A* 1991;88:4186-90.
- Dienstag JL, Schiff ER, Wright TL, Perrillo RP, Hann HW, Goodman Z, et al. Lamivudine as initial treatment for chronic hepatitis B in the United States. *N Engl J Med* 1999;341: 1256-63.
- Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, et al. BEHoLD AI463022 Study Group. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006;354:1001-10.
- Brunetto MR, Giarin M, Saracco G, Oliveri F, Calvo P, Capra G, et al. Hepatitis B virus unable to secrete e antigen and response to interferon in chronic hepatitis B. *Gastroenterology* 1993;105: 845-50.
- Akuta N, Suzuki F, Suzuki Y, Sezaki H, Hosaka T, Someya T, et al. Favorable efficacy of long-term lamivudine therapy in patients with chronic hepatitis B: an 8-year follow-up study. *J Med Virol* 2005;75:491-8.
- Suzuki F, Akuta N, Suzuki Y, Sezaki H, Arase Y, Hosaka T, et al. Clinical and virological features of non-breakthrough and severe exacerbation due to lamivudine-resistant hepatitis B virus mutants. *J Med Virol* 2006;78:341-52.
- 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;50:3867-74.
- Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, et al. Adefovir Dipivoxil 438 Study Group. Long-term therapy with adefovir dipivoxil for HBeAg-negative chronic hepatitis B. *N Engl J Med* 2005;352:2673-81.
- 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;43:1385-91.
- Schildgen O, Sirma H, Funk A, Olotu C, Wend UC, Hartmann H, et al. Variant of hepatitis B virus with primary resistance to adefovir. *N Engl J Med* 2006;354:1807-12.
- Sherman M, Yurdaydin C, Sollano J, Silva M, Liaw YF, Cianciara J, et al. AI463026 BEHoLD Study Group. Entecavir for treatment of lamivudine-refractory, HBeAg-positive chronic hepatitis B. *Gastroenterology* 2006;130:2039-49.
- Colonna RJ, Rose R, Baldick CJ, Levine S, Pokornowski K, Yu CF, et al. Entecavir resistance is rare in nucleoside naive patients with hepatitis B. *Hepatology* 2006;44:1656-65.
- Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z, et al. BEHoLD AI463027 Study Group. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006;354:1011-20.
- Chang TT, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, et al. BEHoLD Study Group. A dose-ranging study of the efficacy and tolerability of entecavir in lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005;129:1198-209.
- Tenney DJ, Levine SM, Rose RE, Walsh AW, Weinheimer SP, Discotto L, et al. Clinical emergence of entecavir-resistant hepatitis B virus requires additional substitutions in virus already resistant to Lamivudine. *Antimicrob Agents Chemother* 2004;48: 3498-507.
- Suzuki F, Suzuki Y, Akuta N, Hosaka T, Sezaki H, Someya T, et al. A new entecavir-resistant hepatitis B virus detected from a patient who was treated by entecavir for lamivudine-resistant virus (in Japanese). *Acta Hepatol Jpn* 2005;46:523.
- Kiyosawa K, Tanaka E, Sodeyama T, Yoshizawa K, Yabu K, Furuta K, et al. Transmission of hepatitis C in an isolated area in Japan: community-acquired infection. The South Kiso Hepatitis Study Group. *Gastroenterology* 1994;106:1596-602.
- Koike K. Hepatitis viruses update. *Intern Med* 2001;40:173-5.
- Ikeda K, Saitoh S, Suzuki Y, Kobayashi M, Tsubota A, Koida I, et al. Disease progression and hepatocellular carcinogenesis in patients with chronic viral hepatitis: a prospective observation of 2215 patients. *J Hepatol* 1998;28:930-8.
- Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006;41:17-27.
- Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncalves FL Jr, et al. Peginterferon alpha-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002;347:975-82.
- Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alpha-2b plus ribavirin compared with interferon alpha-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomized trial. *Lancet* 2001;358: 958-65.
- Berg T, von Wagner M, Nasser S, Sarrazin C, Heintges T, Gerlach T, et al. Extended treatment duration for hepatitis C virus type 1: comparing 48 versus 72 weeks of peginterferon-alpha-2a plus ribavirin. *Gastroenterology* 2006;130:1086-97.

30. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 1996;334:77-81.
31. Kurosaki M, Enomoto N, Murakami T, Sakuma I, Asahina Y, Yamamoto C, et al. Analysis of genotypes and amino acid residues 2209 to 2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. *Hepatology* 1997; 25:750-3.
32. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, et al. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. *Hepatology* 1997; 25:745-9.
33. Zeuzem S, Lee JH, Roth WK. Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alpha. *Hepatology* 1997;25:740-4.
34. Squadrito G, Leone F, Sartori M, Nalpas B, Berthelot P, Ramondo G, et al. Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alpha. *Gastroenterology* 1997;113:567-72.
35. Hofgartner WT, Polyak SJ, Sullivan DG, Carithers RL Jr, Gretch DR. Mutations in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. *J Med Virol* 1997;53:118-26.
36. Khorsini H, Castelain S, Wyseur A, Izopet J, Canva V, Rombout A, et al. Mutations of hepatitis C virus 1b NS5A 2209-2248 amino acid sequence do not predict the response to recombinant interferon-alpha therapy in French patients. *J Hepatol* 1997;27: 72-7.
37. Saiz JC, Lopez-Labrador FX, Ampurdanes S, Dopazo J, Fornis X, Sanchez-Tapias JM, et al. The prognostic relevance of the non-structural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. *J Infect Dis* 1998;177:839-47.
38. Frangeul L, Cresta P, Perrin M, Lunel F, Opolon P, Agut H, et al. Mutations in NS5A region of hepatitis C virus genome correlate with presence of NS5A antibodies and response to interferon therapy for most common European hepatitis C virus genotypes. *Hepatology* 1998;28:1674-9.
39. Odeberg J, Yun Z, Sonnerborg A, Weiland O, Lundeberg J. Variation in the hepatitis C virus NS5a region in relation to hypervariable region 1 heterogeneity during interferon treatment. *J Med Virol* 1998;56:33-8.
40. Chung RT, Monto A, Dienstag JL, Kaplan LM. Mutations in the NS5A region do not predict interferon-responsiveness in American patients infected with genotype 1b hepatitis C virus. *J Med Virol* 1999;58:353-8.
41. Ibarrola N, Moreno-Monteagudo JA, Saiz M, Garcia-Monzon C, Sobrino F, Garcia-Buey L, et al. Response to retreatment with interferon-alpha plus ribavirin in chronic hepatitis C patients is independent of the NS5A gene nucleotide sequence. *Am J Gastroenterol* 1999;94:2487-95.
42. Sarrazin C, Berg T, Lee JH, Teuber G, Dietrich CF, Roth WK, et al. Improved correlation between multiple mutations within the NS5A region and virological response in European patients chronically infected with hepatitis C virus type 1b undergoing combination therapy. *J Hepatol* 1999;30:1004-13.
43. McKechnie VM, Mills PR, McCrudden EA. The NS5a gene of hepatitis C virus in patients treated with interferon-alpha. *J Med Virol* 2000;60:364-78.
44. Yoshioka K, Kobayashi M, Orito E, Watanabe K, Yano M, Sameshima Y, et al. Biochemical response to interferon therapy correlates with interferon sensitivity-determining region in hepatitis C virus genotype 1b infection. *J Viral Hepat* 2001;8:421-9.
45. Stratidakis I, Skoulika E, Kelefiotis D, Matrella E, Alexandrakis G, Economou A, et al. NS5A mutations predict biochemical but not virological response to interferon-alpha treatment of sporadic hepatitis C virus infection in European patients. *J Viral Hepat* 2001;8:243-8.
46. Murphy MD, Rosen HR, Marousek GI, Chou S. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. *Dig Dis Sci* 2002;47:1195-205.
47. Cappiello G, Abbate I, Lo Iacono O, Longo R, Solimone M, Ferraro D, et al. ISDR pattern and evolution in patients with chronic hepatitis C treated with standard or PEG-IFN plus ribavirin. *Antivir Ther* 2003;8:105-10.
48. Aslan N, Bozdayi AM, Cetinkaya H, Sarioglu M, Turkay C, Bozkaya H, et al. The mutations in ISDR of NS5A gene are not associated with response to interferon treatment in Turkish patients with chronic hepatitis C virus genotype 1b infection. *Turk J Gastroenterol* 2004;15:21-6.
49. Murayama M, Katano Y, Nakano I, Ishigami M, Hayashi K, Honda T, et al. A mutation in the interferon sensitivity-determining region is associated with responsiveness to interferon-ribavirin combination therapy in chronic hepatitis patients infected with a Japan-specific subtype of hepatitis C virus genotype 1B. *J Med Virol* 2007;79:35-40.
50. Young KC, Lindsay KL, Lee KJ, Liu WC, He JW, Milstien SL, et al. Identification of a ribavirin-resistant NS5B mutation of hepatitis C virus during ribavirin monotherapy. *Hepatology* 2003; 38:869-78.
51. Pfeiffer JK, Kirkegaard K. Ribavirin resistance in hepatitis C virus replicon-containing cell lines conferred by changes in the cell line or mutations in the replicon RNA. *J Virol* 2005;79: 2134-55.
52. Reesink HW, Zeuzem S, Weegink CJ, Forestier N, van Vliet A, van de Wetering de Rooij J, et al. Rapid Decline of Viral RNA in Hepatitis C Patients Treated With VX-950: A Phase Ib, Placebo-Controlled, Randomized Study. *Gastroenterology* 2006; 131:997-1002.



CASE REPORT

## Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report

Yuka Suzuki, Hiroshi Yotsuyanagi, Chiaki Okuse, Yoshihiko Nagase, Hideaki Takahashi, Kyoji Moriya, Michihiro Suzuki, Kazuhiko Koike, Shiro Iino, Fumio Itoh

Yuka Suzuki, Chiaki Okuse, Yoshihiko Nagase, Hideaki Takahashi, Michihiro Suzuki, Fumio Itoh, Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, 2-16-1 Sugao, Miyamae-ku, Kawasaki 216-8511, Japan  
Hiroshi Yotsuyanagi, Kyoji Moriya, Kazuhiko Koike, Division of Infectious Diseases, Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan  
Shiro Iino, Center for Liver Diseases, Seizankai Kiyokawa Hospital, 2-31-12 Asagayaminami, Suginami, Tokyo 166-0004, Japan

Correspondence to: Hiroshi Yotsuyanagi, MD, PhD, Division of Infectious Diseases, Department of Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. hyotsu-ky@umin.ac.jp  
Telephone: +81-3-5800-8720 Fax: +81-3-5800-8796  
Received: 2006-07-05 Accepted: 2006-08-29

### Abstract

We present a case of fatal liver failure caused by the activation of lamivudine-resistant hepatitis B virus (HBV) nine months after lamivudine treatment. A 57-year old man visited our hospital for the treatment of decompensated chronic hepatitis B. Lamivudine was started in December 2001. Subsequently, serum HBV was negative for HBV DNA with seroconversion from HBeAg to anti-HBe and improvement of liver function. However, HBV DNA and HBeAg were again detected in September 2002. He was complicated by breakthrough hepatitis and admitted to our hospital in November for severely impaired liver function. Vidarabine treatment was started and serum HBV DNA and alanine aminotransferase (ALT) decreased transiently. However, after the start of  $\alpha$ -interferon treatment, HBV DNA level increased and liver function deteriorated. He died 1 mo after admission. An analysis of amino acid sequences in the polymerase region revealed that rtM204I/V with rtL80I/V occurred at the time of viral breakthrough. After the start of antiviral treatment, rtL180M was detected in addition to rtM204I/V and rtL80I/V, and became predominant in the terminal stage of the disease. HBV clone with a high replication capacity may be produced by antiviral treatment leading to the worsening of liver function. Antiviral therapy for patients with breakthrough hepatitis in advanced liver disease should be carefully performed.

© 2007 The WJG Press. All rights reserved.

**Key words:** Hepatitis B virus; Lamivudine; Polymerase; Interferon; Tyrosine-methionine-aspartate-aspartate

Suzuki Y, Yotsuyanagi H, Okuse C, Nagase Y, Takahashi H, Moriya K, Suzuki M, Koike K, Iino S, Itoh F. Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report. *World J Gastroenterol* 2007; 13(6):

<http://www.wjgnet.com/1007-9327/13/.asp>

### INTRODUCTION

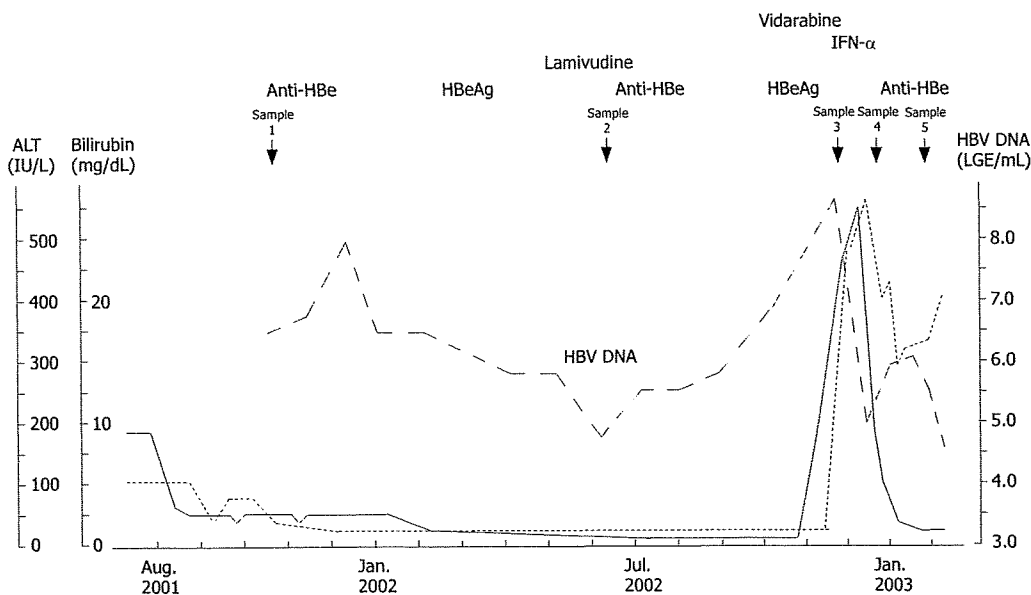
Lamivudine is a nucleoside analogue that interrupts the reverse transcription of hepatitis B viral (HBV) pregenomic RNA. Lamivudine is effective for controlling chronic hepatitis B and currently recommended as the first line of treatment for chronic active hepatitis B<sup>[1,2]</sup>. Even for patients with decompensated liver cirrhosis, lamivudine improves liver function and extends transplantation free intervals<sup>[3-10]</sup>. Since more than 10% of patients with chronic HBV infection are estimated to develop liver cirrhosis and may eventually suffer from decompensated liver cirrhosis or hepatocellular carcinoma, the role of lamivudine in the treatment of advanced liver disease caused by chronic HBV infection is large<sup>[11-14]</sup>.

The major problems concerning lamivudine treatment are the viral and biochemical breakthroughs caused by drug resistance. Amino acid mutation in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif can occur six months after treatment and often increases alanine aminotransferase (ALT) level. Although the increase is usually mild, a marked increase in ALT level leading to fatal hepatic failure has been reported<sup>[15-17]</sup>. Factors other than the YMDD motif mutation that are associated with the worsening of liver function remain to be clarified.

Here, we report a case of fatal hepatic failure caused by lamivudine-resistant HBV. A serial analysis of viral amino acid sequences indicated that the acquisition of mutations outside the YMDD motif might be related to the deterioration of the patient's condition.

### CASE REPORT

A 57-year old man visited our hospital in September 2001 for the treatment of decompensated chronic hepatitis



**Figure 1** Clinical course of our patient. HBV DNA level was quantified by transcription-mediated amplification assay. The levels of HBV DNA started to increase 8 mo after treatment with reappearance of HBeAg. Breakthrough hepatitis developed 12 mo after treatment. The timing of serum sample analysis for mutations is shown by the arrowhead.

B. In 1978, He was found to be positive for serum HBs antigen (HBsAg). In July 2001, he was admitted to a nearby hospital for ascites where he was diagnosed as having decompensated cirrhosis with exacerbated chronic hepatitis B. The symptomatic control of his ascites improved his general condition. For further treatment, he was referred to our hospital.

On his first visit, he showed no symptoms or signs of worsening hepatic failure or encephalopathy. No ascites or leg edema was observed. His bulbar conjunctiva was slightly jaundiced. Dilated vasculature was observed in his neck and chest. His ALT, total bilirubin and albumin were 50 IU/L, 3.1 mg/dL and 3.7 g/dL, and his prothrombin time was 76%. He was diagnosed as having liver cirrhosis with a Child-Pugh score of 8. He was negative for HBe antigen (HBeAg) and his HBV DNA level measured by transcription-mediated amplification and hybridization protection assay<sup>[18]</sup> was 106.5 genome copies/mL.

In November 2001, he was found to be positive for HBeAg and showed an increase in HBV DNA level. Because he had a history of decompensated chronic hepatitis B, lamivudine treatment (100 mg/d) was started in December. Figure 1 shows the clinical course. The high serum levels of bilirubin and ALT decreased and normalized within 6 mo after lamivudine treatment was started. The patient became negative for HBV DNA and HBeAg.

However, in September 2002, he was found to be positive for HBeAg again and showed an increase in HBV DNA level. In November 2002, he observed jaundice of his bulbar conjunctiva and was admitted to our hospital. Although he was alert, his bulbar conjunctiva and skin were jaundiced. His ALT, total bilirubin, were 474 IU/L, 11.4 mg/dL and 4.3 g/dL. His HBV DNA level was 108.6 genome copies/mL. He was diagnosed as having breakthrough hepatitis caused by lamivudine-resistant mutants of HBV. HBV with an amino acid substitution in the YMDD motif in the domain C of polymerase region was detected.

Because interferon is not indicated in patients with decompensated cirrhosis, vidarabine, which is effective for the control of active HBV infection<sup>[19-21]</sup>, was administered together with lamivudine under informed consent. Liver function improved transiently with a decrease in HBV DNA within 2 wk. As prolonged vidarabine administration may induce several complications<sup>[22]</sup>, vidarabine was switched to interferon- $\alpha$ . After the start of interferon- $\alpha$  treatment, HBV DNA level increased and liver function worsened. He died of hepatic failure and rupture of esophageal varices 1 mo after his admission.

The histopathology of the patient's liver after necropsy showed cirrhosis with zonal necrosis. Hepatocyte regeneration was scarce (Figure 2).

To elucidate the viral factors affecting early viral breakthrough and fatal outcome, amino acid sequences of the upstream polymerase region (aa 1-250) of HBV DNA in serum were examined at 5 points as shown in Figure 1. The methods were as follows.

First, DNA was extracted from 100  $\mu$ L of a serum sample using the QIAamp DNA blood mini kit (Qiagen Inc., Valencia, CA). Three fragments spanning the upper polymerase region of HBV DNA were amplified by nested PCR with the primers shown in Table 1. The first stage of amplification was carried out using a thermal cycler for 40 cycles (94°C for 1 min, 55°C for 1 min, 72°C for 1 min) in 100  $\mu$ L of reaction mixture containing 200 mmol/L dNTPs, 1.0 mmol/L each of the primers and 1  $\times$  PCR buffer [50 mmol/L KCl, 10 mmol/L Tris-HCl (pH 8.3), 1.5 mmol/L MgCl<sub>2</sub> and 0.001% (w/v) gelatin] and 2 units of Ampli-Taq polymerase gold (Perkin Elmer Cetus Corp., CT). Two microliters of the PCR products was subjected to the second stage of amplification under the same conditions as the first stage.

Second, PCR products were purified using Wizard PCR preps DNA purification resin (Promega, WI) and cloned into a plasmid vector using the TA cloning kit (PCR cloning kit Qiagen, CA). Four clones were selected from each plate, from which recombinant plasmid DNA was

**Table 1** Primers used for amplification and sequencing of polymerase region of HBV

Region 1		
Outer sense	nt 2222-2241	CTTACTTTTGAAGAGAAAAC
Outer antisense	nt 2490-2509	GGACAGTAGAAGAATAAAG
Inner sense	nt 2222-2241	CITACTTTTGAAGAGAAAAC
Inner antisense	nt 2478-2497	GAATAAAGCCCAGTAAAGTT
Region 2		
Outer sense	nt 2413-2434	CGGTCCGAGAAGATCTCAATC
Outer antisense	nt 2816-2835	GTCCCAAGAATATGGTGAC
Inner sense	nt 2434-2452	CTCGGGAATCTCAATGTTAG
Inner antisense	nt 2816-2835	GTCCCAAGAATATGGTGAC
Region 3		
Outer sense	nt 2490-2509	CITTATTCITCTACTGTACC
Outer antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG
Inner sense	nt 2637-2656	ATGCCTGCTAGGTTTTATCC
Inner antisense	nt 3121-3143	CGATTGGTGGAGGCAGGAGGAGG

purified using a commercially available kit (Plasmid midi kit, Qiagen, Valencia, CA). Nucleotide sequences were determined bidirectionally using the dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, CA) and the PCR primers. Sequencing was performed using an automated DNA sequencer (ABI 377; PE Applied Biosystems).

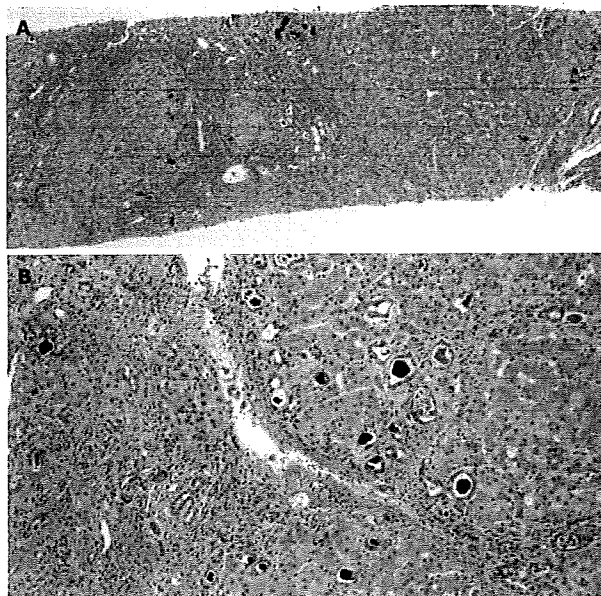
The determined amino acid sequences in the polymerase region are shown in Figure 3. No amino acid sequence changes were found at the start of lamivudine treatment. At the time of viral breakthrough, rtM204I with rtL80I became dominant. After the start of interferon treatment, rtM204I was replaced by rtM204V and rtL80I by rtL80V. At the final stage of the disease, mutation rtL180M appeared besides rtM204V and rtL80V.

## DISCUSSION

Lamivudine monotherapy is effective in suppressing HBV replication and ameliorating liver disease in chronic hepatitis B patients regardless of HBeAg positivity. A one-year study of HBeAg-positive chronic hepatitis B patients showed that 16% of these patients become seroconverted to anti-HBe and 72% of these patients showed normalization of their ALT levels<sup>[23]</sup>. Furthermore, treatment with lamivudine is associated with histologic improvement not only in terms of necroinflammatory score but also in terms of fibrosis score after long-term treatment<sup>[24]</sup>.

One advantage of lamivudine is that it can be used safely in patients with decompensated cirrhosis<sup>[3-10]</sup>. In contrast to IFN- $\alpha$ , lamivudine is well tolerated without any significant side effects even in patients with decompensated cirrhosis. Furthermore, lamivudine can improve liver function and survival prognosis.

However, the emergence of a drug-resistant mutant is a big problem in lamivudine treatment. A large-scale Asian study showed that lamivudine resistant HBV infection occurred in 23 % of patients in year one and 65 % of patients in year five. Hepatitis flares, which occurred more commonly in patients with lamivudine resistant mutations, occurred in 10% of patients in year one, and in 18% to 21% of patients in years two to five. Among patients with



**Figure 2** Histopathological findings of liver specimens. Irregularly-shaped parenchymal cells with massive necrosis (A) and scarce hepatocyte regeneration (B) surrounded by extensive fibrosis (A: HE  $\times$  20; B: HE  $\times$  80).

lamivudine resistant HBV infection, occurrence of hepatic decompensation increased significantly in patients with lamivudine resistant HBV infection for more than 4 years (from 0% to 6%)<sup>[25]</sup>. In this large-scale Asian study, liver-disease-related death occurred in two patients.

The prognosis of patients with lamivudine-resistant HBV infection, particularly those with advanced liver disease, may be determined by the timing and severity of breakthrough hepatitis. However, the viral factors that may influence the severity of this hepatitis remain to be clarified. A recent study indicated that patients with a normal ALT level even after the emergence of a YMDD motif mutant are characterized by HBeAg negativity during pretreatment, HBeAg loss during therapy, a longer duration from the commencement of therapy until the emergence of YMDD mutant, and lack of mixed-type YMDD mutants<sup>[26]</sup>. In contrast, patients with severely exacerbated hepatitis after the emergence of a YMDD mutant tend to have more substitutions in the reverse transcriptase (rt) region within the polymerase gene at the time of hepatitis exacerbation than those without hepatitis exacerbation<sup>[26]</sup>.

Our patient acquired amino acid mutations in the polymerase region one after the other. Amino acid changes in rtM204/I appeared at the time of viral breakthrough. After the initial treatment with vidarabine, rtM204/V substituted for rtM204/I in one of the four clones. During the interferon treatment, rtM204/V became predominant.

Another mutation observed in our patient was rtL80I/V. Ogata *et al*<sup>[27]</sup> showed that rtL180M is accompanied with rtM204I in some patients with resistance to lamivudine. Because the mutation at aa position 80 was found at the same time as that at aa position 204 in our patient, it is not clear whether the mutation at aa position 80 affects the clinical course.

At the final stage of the disease with deterioration of





- Lamivudine treatment is beneficial in patients with severely decompensated cirrhosis and actively replicating hepatitis B infection awaiting liver transplantation: a comparative study using a matched, untreated cohort. *Hepatology* 2001; **34**: 411-416
- 9 Hann HW, Fontana RJ, Wright T, Everson G, Baker A, Schiff ER, Riely C, Anschuetz G, Gardner SD, Brown N, Griffiths D. A United States compassionate use study of lamivudine treatment in nontransplantation candidates with decompensated hepatitis B virus-related cirrhosis. *Liver Transpl* 2003; **9**: 49-56
  - 10 Manolakopoulos S, Karatapanis S, Elefsiniotis J, Mathou N, Vlachogiannakos J, Iliadou E, Kougioumtzan A, Economou M, Triantos C, Tzourmakliotis D, Avgerinos A. Clinical course of lamivudine monotherapy in patients with decompensated cirrhosis due to HBeAg negative chronic HBV infection. *Am J Gastroenterol* 2004; **99**: 57-63
  - 11 Fattovich G, Broilo L, Giustina G, Noventa F, Pontisso P, Alberti A, Realdi G, Ruol A. Natural history and prognostic factors for chronic hepatitis type B. *Gut* 1991; **32**: 294-298
  - 12 Liaw YF, Tai DI, Chu CM, Chen TJ. The development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Hepatology* 1988; **8**: 493-496
  - 13 Liaw YF, Lin DY, Chen TJ, Chu CM. Natural course after the development of cirrhosis in patients with chronic type B hepatitis: a prospective study. *Liver* 1989; **9**: 235-241
  - 14 Fattovich G, Giustina G, Schalm SW, Hadziyannis S, Sanchez-Tapias J, Almasio P, Christensen E, Krogsgaard K, Degos F, Carneiro de Moura M. Occurrence of hepatocellular carcinoma and decompensation in western European patients with cirrhosis type B. The EUROHEP Study Group on Hepatitis B Virus and Cirrhosis. *Hepatology* 1995; **21**: 77-82
  - 15 Kim JW, Lee HS, Woo GH, Yoon JH, Jang JJ, Chi JG, Kim CY. Fatal submassive hepatic necrosis associated with tyrosine-methionine-aspartate-aspartate-motif mutation of hepatitis B virus after long-term lamivudine therapy. *Clin Infect Dis* 2001; **33**: 403-405
  - 16 Wang JH, Lu SN, Lee CM, Lee JF, Chou YP. Fatal hepatic failure after emergence of the hepatitis B virus mutant during lamivudine therapy in a patient with liver cirrhosis. *Scand J Gastroenterol* 2002; **37**: 366-369
  - 17 Kagawa T, Watanabe N, Kanouda H, Takayama I, Shiba T, Kanai T, Kawazoe K, Takashimizu S, Kumaki N, Shimamura K, Matsuzaki S, Mine T. Fatal liver failure due to reactivation of lamivudine-resistant HBV mutant. *World J Gastroenterol* 2004; **10**: 1686-1687
  - 18 Kamisango K, Kamogawa C, Sumi M, Goto S, Hirao A, Gonzales F, Yasuda K, Iino S. Quantitative detection of hepatitis B virus by transcription-mediated amplification and hybridization protection assay. *J Clin Microbiol* 1999; **37**: 310-314
  - 19 Hoofnagle JH, Minuk GY, Dusheiko GM, Schafer DF, Johnson R, Straus S, Jones EA, Gerin JL, Ishak K. Adenine arabinoside 5'-monophosphate treatment of chronic type B hepatitis. *Hepatology* 1982; **2**: 784-788
  - 20 Weller IV, Lok AS, Mindel A, Karayiannis P, Galpin S, Monjardino J, Sherlock S, Thomas HC. Randomised controlled trial of adenine arabinoside 5'-monophosphate (ARA-AMP) in chronic hepatitis B virus infection. *Gut* 1985; **26**: 745-751
  - 21 Marcellin P, Ouzan D, Degos F, Brechot C, Metman EH, Degott C, Chevalier M, Berthelot P, Trepo C, Benhamou JP. Randomized controlled trial of adenine arabinoside 5'-monophosphate in chronic active hepatitis B: comparison of the efficacy in heterosexual and homosexual patients. *Hepatology* 1989; **10**: 328-331
  - 22 Hoofnagle JH, Hanson RG, Minuk GY, Pappas SC, Schafer DF, Dusheiko GM, Straus SE, Popper H, Jones EA. Randomized controlled trial of adenine arabinoside monophosphate for chronic type B hepatitis. *Gastroenterology* 1984; **86**: 150-157
  - 23 Lai CL, Chien RN, Leung NW, Chang TT, Guan R, Tai DI, Ng KY, Wu PC, Dent JC, Barber J, Stephenson SL, Gray DF. A one-year trial of lamivudine for chronic hepatitis B. Asia Hepatitis Lamivudine Study Group. *N Engl J Med* 1998; **339**: 61-68
  - 24 Dienstag JL, Goldin RD, Heathcote EJ, Hann HW, Woessner M, Stephenson SL, Gardner S, Gray DF, Schiff ER. Histological outcome during long-term lamivudine therapy. *Gastroenterology* 2003; **124**: 105-117
  - 25 Lok AS, Lai CL, Leung N, Yao GB, Cui ZY, Schiff ER, Dienstag JL, Heathcote EJ, Little NR, Griffiths DA, Gardner SD, Castiglia M. Long-term safety of lamivudine treatment in patients with chronic hepatitis B. *Gastroenterology* 2003; **125**: 1714-1722
  - 26 Suzuki F, Akuta N, Suzuki Y, Sezaki H, Arase Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Ikeda K, Kobayashi M, Matsuda M, Satoh J, Watahiki S, Kumada H. Clinical and virological features of non-breakthrough and severe exacerbation due to lamivudine-resistant hepatitis B virus mutants. *J Med Virol* 2006; **78**: 341-352
  - 27 Ogata N, Fujii K, Takigawa S, Nomoto M, Ichida T, Asakura H. Novel patterns of amino acid mutations in the hepatitis B virus polymerase in association with resistance to lamivudine therapy in Japanese patients with chronic hepatitis B. *J Med Virol* 1999; **59**: 270-276
  - 28 Natsuizaka M, Hige S, Ono Y, Ogawa K, Nakanishi M, Chuma M, Yoshida S, Asaka M. Long-term follow-up of chronic hepatitis B after the emergence of mutations in the hepatitis B virus polymerase region. *J Viral Hepat* 2005; **12**: 154-159
  - 29 Janssen HL, Brouwer JT, Nevens F, Sanchez-Tapias JM, Craxi A, Hadziyannis S. Fatal hepatic decompensation associated with interferon alfa. European concerted action on viral hepatitis (Eurohep). *BMJ* 1993; **306**: 107-108
  - 30 Perrillo R, Schiff E, Yoshida E, Statler A, Hirsch K, Wright T, Gutfreund K, Lamy P, Murray A. Adefovir dipivoxil for the treatment of lamivudine-resistant hepatitis B mutants. *Hepatology* 2000; **32**: 129-134
  - 31 Hadziyannis SJ, Tassopoulos NC, Heathcote EJ, Chang TT, Kitis G, Rizzetto M, Marcellin P, Lim SG, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-negative chronic hepatitis B. *N Engl J Med* 2003; **348**: 800-807
  - 32 Marcellin P, Chang TT, Lim SG, Tong MJ, Sievert W, Shiffman ML, Jeffers L, Goodman Z, Wulfsohn MS, Xiong S, Fry J, Brosgart CL. Adefovir dipivoxil for the treatment of hepatitis B e antigen-positive chronic hepatitis B. *N Engl J Med* 2003; **348**: 808-816
  - 33 Chang TT, Gish RG, Hadziyannis SJ, Cianciara J, Rizzetto M, Schiff ER, Pastore G, Bacon BR, Poynard T, Joshi S, Kleszczewski KS, Thiry A, Rose RE, Colonna RJ, Hindes RG. A dose-ranging study of the efficacy and tolerability of entecavir in Lamivudine-refractory chronic hepatitis B patients. *Gastroenterology* 2005; **129**: 1198-1209
  - 34 Chang TT, Gish RG, de Man R, Gadano A, Sollano J, Chao YC, Lok AS, Han KH, Goodman Z, Zhu J, Cross A, DeHertogh D, Wilber R, Colonna R, Apelian D. A comparison of entecavir and lamivudine for HBeAg-positive chronic hepatitis B. *N Engl J Med* 2006; **354**: 1001-1010
  - 35 Lai CL, Shouval D, Lok AS, Chang TT, Cheinquer H, Goodman Z, DeHertogh D, Wilber R, Zink RC, Cross A, Colonna R, Fernandes L. Entecavir versus lamivudine for patients with HBeAg-negative chronic hepatitis B. *N Engl J Med* 2006; **354**: 1011-1020
  - 36 van Bommel F, Wunsche T, Mauss S, Reinke P, Bergk A, Schurmann D, Wiedenmann B, Berg T. Comparison of adefovir and tenofovir in the treatment of lamivudine-resistant hepatitis B virus infection. *Hepatology* 2004; **40**: 1421-1425
  - 37 van der Eijk AA, Hansen BE, Niesters HG, Janssen HL, van de Ende M, Schalm SW, de Man RA. Viral dynamics during tenofovir therapy in patients infected with lamivudine-resistant hepatitis B virus mutants. *J Viral Hepat* 2005; **12**: 364-372

S- Editor Wang GP L- Editor Wang XL E- Editor Lu W

## Original Article

## Amino acid substitutions in the S region of hepatitis B virus in sera from patients with acute hepatitis

Junko Aono,<sup>1,3</sup> Hiroshi Yotsuyanagi,<sup>1</sup> Hideyuki Miyoshi,<sup>2</sup> Takeya Tsutsumi,<sup>1</sup> Hajime Fujie,<sup>2</sup> Yoshizumi Shintani,<sup>1</sup> Kyoji Moriya,<sup>1</sup> Chiaki Okuse,<sup>4</sup> Michihiro Suzuki,<sup>4</sup> Kiyomi Yasuda,<sup>5</sup> Shiro Iino<sup>5</sup> and Kazuhiko Koike<sup>1</sup>

Departments of <sup>1</sup>Infectious Diseases and <sup>2</sup>Gastroenterology, Internal Medicine, University of Tokyo, <sup>3</sup>Center for Liver Diseases, Seizankai Kiyokawa Hospital, Tokyo, <sup>4</sup>Faculty of Pharmaceutical Sciences, Teikyo Heisei University, Chiba, <sup>5</sup>Division of Gastroenterology and Hepatology, Department of Internal Medicine, St. Marianna University School of Medicine, Kawasaki, Japan

**Background:** An increase in the number of acute hepatitis patients with hepatitis B virus (HBV) of non-indigenous genotypes may reduce the efficacy of vaccination against HBV.

**Methods:** We have determined the amino acid (aa) sequences in the major hydrophilic region (MHR) in the S region of HBV in patients with acute hepatitis B and compared those with the ones from HBV strains used for the production of HBV vaccines commercially available in Japan.

**Results:** Of 48 patients studied, 11 were infected with genotype A, 11 with genotype B and 26 with genotype C HBV. The aa sequences of the nine genotype A isolates were the same as the aa sequence of J02205 which is used for the production of one of the commercially available recombinant vaccines. The aa sequences of the 11 genotype B isolates differed from the aa sequence of J02205 in two or three amino acids. Of the

26 genotype C isolates, 22 had the same aa sequence as X01587 which is used for the production of another recombinant vaccine. The remaining genotype C isolates had aa substitutions at aa131, which have a potential to alter the hydrophathy and the three-dimensional structure of the MHR. The differences among the three current HBV vaccines in aa sequences in the MHR theoretically alter the hydrophathy and three-dimensional structure.

**Conclusion:** Our results suggest that the transmission of HBV isolates with different genotypes or with aa substitutions in the MHR might reduce the efficacy of currently available HBV vaccines in the protection of HBV infections.

**Key words:** genotype, hepatitis B virus, major hydrophilic region, vaccine

## INTRODUCTION

ABOUT 300 MILLION people in the world are chronically infected with hepatitis B virus (HBV). Chronic infection may eventually lead to liver cirrhosis or hepatocellular carcinoma.<sup>1-4</sup> To prevent the transmission of this virus, vaccination has been introduced in many countries. Indeed, universal vaccination has not only reduced the number of infected individuals, but also the number of deaths related to HBV.<sup>5,6</sup>

In Japan, in 1985, a national project was started to vaccinate children born to HBV-infected mothers. The chances of vertical transmission from HBV-carrying mothers have decreased. Recently, the prevalence of HBV in Japan has decreased to approximately 0.6%.<sup>7</sup>

Because the number of individuals infected with HBV has decreased, the number of patients with acute hepatitis B, mainly caused by horizontal transmission from HBV carriers, should also have decreased. However, in Japan, the number of patients with acute hepatitis B has recently increased (Yatsushashi H. *et al.*, 2004, unpubl. data).

The increase in the number of patients with acute hepatitis B may, in part, be the result of patients carrying novel HBV genotypes imported from abroad. For

Correspondence: Dr Hiroshi Yotsuyanagi, Department of Infectious Diseases, Internal Medicine, University of Tokyo, 7-3-1 Hongo, Bunkyo, Tokyo 113-8655, Japan. Email: hyotsu-ty@umim.ac.jp  
Received 20 September 2006, revision 31 January 2007; accepted 8 March 2007

example, in recent years, genotype A HBV has often been detected in patients with acute hepatitis B.<sup>8,9</sup>

Genotype A HBV is transmitted from individuals who live in or have immigrated from other countries to Japan. Its infection is characterized by a high viral load and a long hepatitis B surface antigen (HBsAg) positivity period. The transition of acute hepatitis B with genotype A HBV infection to the chronic state has been reported recently.<sup>8,10</sup> Decreasing the transmission rate of genotype A HBV is therefore important for the control of the disease. Introducing universal vaccination for adolescents or adults is a measure to be considered.

The effectiveness of universal vaccination depends on the reactivity of vaccines against HBV. HBsAg binds antibody to hepatitis B surface antigen (anti-HBs) produced against HBV vaccines mainly via the 'a' determinant region (aa124–aa149). This region contains common antigenic epitopes of all subtypes (adw, adr, ayw, ayr) of HBsAg and lies in the major hydrophilic region (MHR) between aa99 and aa169. Amino acid (aa) substitutions in the MHR, particularly in the 'a' determinant region, can alter B cell epitopes of HBsAg, leading to immunological escape from the host immunity induced by either vaccination or previous infection.<sup>11</sup> Therefore, if HBV prevalent in Japan has aa substitutions in the MHR, the effect of universal vaccination may be reduced.

In Japan, three types of HBV vaccine (Bimmugen, The Chemo-Sero-Therapeutic Research Institute, Kumamoto, Japan; Heptavax, Merck & Co., Whitehouse Station, NJ, USA; and Meinyu, Meiji Dairies, Tokyo, Japan) are now available. Efficacy and immunogenicity of vaccines are not always comparable or identical.<sup>12,13</sup> Whether giving a single vaccine effectively prevents the transmission of all genotypes of HBV is an important but still unsolved problem. Elucidating the aa substitutions in the MHR may give a clue to this problem.

The purpose of the present study is to determine the difference of the aa sequences in the MHR of HBV among isolates from patients with acute hepatitis and also the difference of the aa sequences among viral strains used for the production of anti-HBV vaccines, and to find ways to use currently available vaccines as effective prophylaxes.

## METHODS

### Patients

FROM 1992 TO 2001, serum samples were collected from 48 patients diagnosed with acute hepatitis B in our institutions. Only patients whose serum samples

were stored at the onset of hepatitis were included in this study. All the 48 patients ran a self-limited clinical course. No patients subsequently developed fulminant hepatic failure or chronic sequelae.

The criteria for the diagnosis of acute hepatitis B were the following: (i) an acute onset of liver injury without a history of liver dysfunction and positivity for HBsAg in serum; and (ii) immunoglobulin M (IgM) antibody to HBV core antigen (anti-HBc) at a titer of more than 2.5 of cut-off index. Coinfection with a hepatitis A virus or a hepatitis C virus was excluded by serological tests. None of the patients had previously received any vaccination against HBV.

Serum samples from the 48 patients with acute hepatitis B were examined virologically, and the results were examined for correlations with clinical characteristics. Informed consent was obtained from each patient. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the ethics committees of our institutions.

### Determination of HBV-DNA

Hepatitis B virus DNA level was determined using transcription-mediated amplification (TMA) and a hybridization protection assay (Chugai Diagnostics Science, Tokyo, Japan) using the protocol of Kamisango *et al.*<sup>14</sup> The range of detection using TMA was from 3.7 log genome equivalents (LGE)/mL (i.e.  $10^{3.7}$  copies/mL corresponding to 5000 copies/mL) to 8.7 LGE/mL ( $10^{8.7}$  copies/mL). In seven of 34 studied serum samples, the level of HBV-DNA was lower than 3.7 LGE/mL and these were categorized as 3.7 LGE/mL.

### Genotyping HBV

Hepatitis B virus genotypes were determined using commercial enzyme immunoassay kits (Smitest HBV Genotyping kit; Genome Science, Fukushima, Japan). In brief, DNA extracted from serum was amplified by polymerase chain reaction (PCR) using three sense primers (i.e. s1: 5'-ACCAACCCTCTGGGATTCTTTCC-3', s2: 5'-ACCAATCCTCTGGGATTCTCCC-3', and s3: 5'-AGCAATCCTCTAGGATTCCITCC-3' [nt 2902–2924]) and an antisense primer (i.e. as1: 5'-GAGCCTGAGGGCTCCACCC-3' [nt 3091–3073]) biotinylated at the 5' end; their sequences were deduced from conserved sequences in the pre-S1 region of HBV. The biotin-labeled and amplified HBV-DNA was denatured in an alkaline solution, and tested for hybridization to probes specific for one of the seven HBV genotypes (A–G) immobilized on wells of a 96-well