Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, Ishitobi T, Nonaka M, Chayama K.	Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia.	Metabolism	57(12)	1711-8	2008
Iwamoto K, Kanno K, Hyogo H, Yamagishi S, Takeuchi M, Tazuma S, Chayama K.	Advanced glycation end products enhance the proliferation and activation of hepatic stellate cells.	J Gastroenterol	43(4)	298-304	2008
Jeong S, Kawakami Y, Kitamoto M, Ishihara H, Tsuji K, Aimitsu S, Kawakami H, Uka K, Takaki S, Kodama H, Waki K, Imamura M, Aikata H, Takahashi S, Chayama K.	Prospective study of short-term peginterferon-alpha-2a monotherapy in patients who had a virological response at 2 weeks after initiation of interferon therapy.	J Gastroenterol Hepatol	23(4)	541-5.	2008
Utoh R, Tateno C, Yamasaki C, Hiraga N, Kataoka M, Shimada T, Chayama K, Yoshizato K.	Susceptibility of chimeric mice with livers repopulated by serially subcultured human hepatocytes to hepatitis B virus	Hepatology	47(2)	435-46	2008
Igarashi Y, Tateno C, Tanaka Y, Tachibana A, Utoh R, Kataoka M, Ohdan H, Asahara T, Yoshizato K.	Engraftment of human hepatocytes in the livers of rats bearing bone marrow reconstructed with immunodeficient mouse bone marrow cells. Xenotransplantation	Xenotransplantation	15	235-45	2008;
Lin YC, Goto S, Tateno C, Nakano T, Cheng YF, Jawan B, Kao YH, Hsu LW, Lai CY, Yoshizato K, Chen CL.	Induction of indoleamine 2,3-dioxygenase in livers following hepatectomy prolongs survival of allogeneic hepatocytes after transplantation.	Transplant Proc	40	2706-8	2008
Kimura T, Imamura M, Hiraga N, Hatakeyama T, Miki D, Noguchi C, Mori N, Tsuge M, Takahashi S, Fujimoto Y, Iwao E, Ochi H, Abe H, Maekawa T, Arataki K, Tateno C, Yoshizato K, Wakita T, Okamoto T, Matsuura Y, Chayama K.	Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice	J Gen Virol.	89	2108-13	2008
Sato Y, Yamada H, Iwasaki K, Tateno C, Yokoi T, Yoshizato K, Horii I.	Human hepatocytes can repopulate mouse liver: histopathology of the	Toxicol Pathol	36	581-91	2008

	liver in human hepatocyte-transplanted chimeric mice and toxicologic responses to acetaminophen.				
Park TJ, Jeong BR, Tateno C, Kim HS, Ogawa T, Lim IK, <u>Yoshizato K.</u>	Pleiotrophin inhibits transforming growth factor betal-induced apoptosis in hepatoma cell lines	Mol Carcinog	47	784-96	2008
Motoi, N., Suzuki, K., Hirota, R., Johnson, P., Oofusa, K., Kikuchi, Y., and <u>Yoshizato, K.</u>	Identification and characterization of nucleoplasmin 3 as a histone-binding protein in embryonic stem cells.	Devel. Growth & Differ.	50	307-320	2008
Tatsumi K, Ohashi K, Kataoka M, Tateno C, Shibata M, Naka H, Shima M, Hisanaga M, Kanehiro H, Okano T, <u>Yoshizato K</u>, Nakajima Y, Yoshioka A.	Successful in vivo propagation of factor I X-producing hepatocytes in mice: potential for cell-based therapy in haemophilia B.	Thromb Haemost.	99	883-91	2008
Emoto C, Yamato Y, Sato Y, Ohshita H, Katoh M, Tateno C, Yokoi T, <u>Yoshizato K</u>, Iwasaki K.	Non-invasive method to detect induction of CYP3A4 in chimeric mice with a humanized liver.	Xenobiotica	38	239-48	2008
Katoh M, Tateno C, <u>Yoshizato K</u>, Yokoi T.	Chimeric mice with humanized liver.	Toxicology	246	9-17	2008
Utoh R, Tateno C, Yamasaki C, Hiraga N, Kataoka M, Shimada T, Chayama K, <u>Yoshizato K.</u>	Susceptibility of chimeric mice with livers repopulated by serially subcultured human hepatocytes to hepatitis B virus.	Hepatology	47	435-46	2008
Ura S, Honda M, Yamashita T, Ueda T, Takatori H, Nishino R, Sunakozaka H, Sakai Y, Horimoto K, <u>Kaneko S.</u>	Differential microRNA expression between hepatitis B and hepatitis C leading disease progression to hepatocellular carcinoma.	Hepatology	19		2008
Yamashita T, Ji J, Budhu A, Forgues M, Yang W, Wang HY, Jia H, Ye Q, Qin LX, Wauthier E, Reid LM, Minato H, Honda M,	EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features.	Gastroente rology	136	1012-24	2009

Kaneko S, Tang ZY, Wang XW.					
Sakai Y, Honda M, Fujinaga H, Tatsumi I, Mizukoshi E, Nakamoto Y, Kaneko S.	Common transcriptional signature of tumor-infiltrating mononuclear inflammatory cells and peripheral blood mononuclear cells in hepatocellular carcinoma patients.	Cancer Res	68	10267-79	2008
Minagawa H, Yamashita T, Honda M, Tabuse Y, Kamijo K, Tsugita A, Kaneko S.	Comparative analysis of proteome and transcriptome in human hepatocellular carcinoma using 2D-DIGE and SAGE.	Protein J	27	409-19	2008
Yamashita T, Honda M, Takatori H, Nishino R, Minato H, Takamura H, Ohta T, Kaneko S.	Activation of lipogenic pathway correlates with cell proliferation and poor prognosis in hepatocellular carcinoma.	J Hepatol	50	100-10	2009
Yamashita T, Honda M, Kaneko S.	Application of Serial Analysis of Gene Expression in cancer research.	Curr Pharm Biotechnol	9	375-82	2008
Mizukoshi E, Honda M, Arai K, Yamashita T, Nakamoto Y, Kaneko S.	Expression of multidrug resistance-associated protein 3 and cytotoxic T cell responses in patients with hepatocellular carcinoma.	J Hepatol	49	946-54	2008
Nishino R, Honda M, Yamashita T, Takatori H, Minato H, Zen Y, Sasaki M, Takamura H, Horimoto K, Ohta T, Nakanuma Y, Kaneko S.	Identification of novel candidate tumour marker genes for intrahepatic cholangiocarcinoma.	J Hepatol	49	207-16	2008
Minagawa H, Honda M, Miyazaki K, Tabuse Y, Teramoto R, Yamashita T, Nishino R, Takatori H, Ueda T, Kamijo K, Kaneko S.	Comparative proteomic and transcriptomic profiling of the human hepatocellular carcinoma.	Biochem Biophys Res Commun.	366	186-92	2008
Takamura T, Misu H, Matsuzawa-Nagata N, Sakurai M, Ota T, Shimizu A, Kurita S, Takeshita Y, Ando H, Honda M, Kaneko S	Obesity Upregulates Genes Involved in Oxidative Phosphorylation in Livers of Diabetic Patients.	Obesity			2008
Hussein H. Aly, Kunitada Shimotohno, Hijikata M.	3D cultured immortalized human hepatocytes useful to develop	Biochem. Biophys.	244(9)	330-334	2009

		drugs for blood-borne HCV	Res. Commun.			
土方　誠	HCV と肝発癌	医学のあゆみ	51(1)		826-831	2008
宮成　悠介、臼田　信光、土方　誠、下　遠野　邦忠	C 型肝炎ウイルスの生活環と発がん	化学と生物	46(12)		1089-1097	2008
土方　誠、アリ・ハッサン・フセイン、　下遠野邦忠	3D 細胞培養系を用いた患者　血液由来 HCV 培養	肝・胆・膵	57(5)		679-687	2008
Mitsui M, Nishikawa M, Zang L, Ando M, Hattori K, Takahashi Y Watanabe Y, Takakura Y	Effect of the content of unmethylated CpG dinucleotides in plasmid DNA on sustainability of transgene expression	J Gene Med	In press			2009
Nishikawa M Nakayama A Takahashi Y Fukuhara Y, Takakura Y	Reactivation of Silenced Transgene Expression in Mouse Liver by Rapid, Large-volume Injection of Isotonic Solution	Int. J. Cancer	In press			2009
Takahashi Y, Yamaoka K, Nishikawa M Takakura Y	Quantitative and temporal analysis of gene silencing in tumor cells induced by small interfering RNA or short hairpin RNA expressed from plasmid vectors	J Pharm Sci	98(1)		74-80	2008
Takahashi Y, Nishikawa M, Suehara T, Takiguchi N, Takakura Y	Gene silencing of b-catenin in melanoma cells retards their growth but promotes the formation of pulmonary metastasis in mice	Int J Cancer	123(10)		2315-20	2008
Takahashi Y Kaneda H, Takasuka N Hattori K Nishikawa M Watanabe Y Takakura Y	Enhancement of anti-proliferative activity of interferons by RNAi-mediated silencing of SOCS gene expression in tumor cells	Cancer Sci	99(8)		1650-5	2008
Miyazaki M, Miyazaki K, Itoi M, Katoh Y, Guo Y, Kanno R, Katoh-Fukui Y, Honda H, Amagai T, van Lohuizen M, Kawamoto H and Kanno M.	Pre-T-cell-receptor signaling induced thymocyte proliferation is epigenetically maintained through the Polycomb gene product Bmi-1-mediated cdkn2a repression	Immunity	28		231-45	2008
Sekine-Osajima Y, Sakamoto N, Nakagawa M, Itsui Y, Tasaka M, Nishimura-Sakurai Y, Chen CH, Suda G, Mishima K, Onuki Y, Yamamoto M, Maekawa S, Enomoto N, Kanai T, Tsuchiya	Two flavonoids extracts from Glycyrrhizae radix inhibit in vitro hepatitis C virus replication.	Hepatol Res	39		60-9	2008

K, Watanabe M.					
Jin H, Yamashita A, Maekawa S, Yang P, He L, Takayanagi S, Wakita T, Sakamoto N, Enomoto N, Ito M.	Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication in vitro.	Hepatol Res.	38	909-18	2008
Amemiya F, Maekawa S, Itakura Y, Kanayama A, Matsui A, Takano S, Yamaguchi T, Itakura J, Kitamura T, Inoue T, Sakamoto M, Yamauchi K, Okada S, Yamashita A, Sakamoto N, Itoh M, Enomoto N.	Targeting lipid metabolism in the treatment of hepatitis C virus infection.	J Infect Dis..	197	361-70	2008
Sakamoto N, Tanabe Y, Yokota T, Satoh K, Sekine-Osajima Y, Nakagawa M, Itsui Y, Tasaka M, Sakurai Y, Cheng-Hsin C, Yano M, Ohkoshi S, Aoyagi Y, Maekawa S, Enomoto N, Kohara M, Watanabe M.	Inhibition of hepatitis C virus infection and expression in vitro and in vivo by recombinant adenovirus expressing short hairpin RNA.	J Gastroente rol Hepatol.	23	1437-47	2008
Okamoto K., Mori Y., Komoda Y., Okamoto T., Okochi M., Takeda M., Suzuki T., Moriishi K., and Matsuura Y.	Intramembrane processing by signal peptide peptidase regulates the membrane localization of hepatitis C virus core protein and viral propagation.	J. Virol.	82	8349-61	2008
Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, Date T, Matsuura Y, Miyamura T, Wakita T, and Suzuki T.	Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles.	J. Virol.	82	7964-76	2008
Aizaki H, Morikawa K., Fukasawa M, Hara H, Inoue Y, Tani H, Saito K, Hanada K, Matsuura Y, Lai M.M.C., Miyamura T, Wakita T, and Suzuki T.	A critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection.	J. Virol.	82	5715-24	2008
Okamoto T., Omori H., Kaname Y., Abe T., Nishimura Y., Suzuki T., Miyamura T.,	A single amino acid mutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral	J. Virol.	82	3480-89	2008

Yoshimori T., Moriishi K., and Matsuura Y.	replication.				
Taguwa S., Okamoto T., Abe T., Mori Y., Suzuki T., Moriishi K., and Matsuura Y.	Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication.	J. Virol.	82	2631-41	2008

Ⅳ．研究成果の刊行物・別刷

Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice

Takashi Kimura,[1,2] Michio Imamura,[1,2] Nobuhiko Hiraga,[1,2] Tsuyoshi Hatakeyama,[1,2] Daiki Miki,[1,2] Chiemi Noguchi,[1,2] Nami Mori,[1,2] Masataka Tsuge,[1,2] Shoichi Takahashi,[1,2] Yoshifumi Fujimoto,[1,2] Eiji Iwao,[3] Hidenori Ochi,[2,4] Hiromi Abe,[1,2,4] Toshiro Maekawa,[4] Keiko Arataki,[5] Chise Tateno,[2,6] Katsutoshi Yoshizato,[2,6] Takaji Wakita,[7] Toru Okamoto,[8] Yoshiharu Matsuura[8] and Kazuaki Chayama[1,2,4]

Correspondence
Kazuaki Chayama
chayama@hiroshima-u.ac.jp

[1]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

[2]Liver Research Project Center, Hiroshima University, Hiroshima, Japan

[3]Research Division, Mitsubishi Tanabe Pharma Corporation, Osaka, Japan

[4]Laboratory for Liver Disease, SNP Research Center, Institute of Physical and Chemical Research (RIKEN), Yokohama, Japan

[5]Hirosimakinen-Hospital, Internal Medicine, Hiroshima, Japan

[6]Developmental Biology Laboratory, Department of Biological Science, Graduate School of Science, Hiroshima University, Higashihiroshima, Japan

[7]Department of Virology II, National Institute of Infectious Diseases, Shinjuku-ku, Japan

[8]Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka, Japan

The establishment of clonal infection of hepatitis C virus (HCV) in a small-animal model is important for the analysis of HCV virology. A previous study developed models of molecularly cloned genotype 1a and 2a HCV infection using human hepatocyte-transplanted chimeric mice. This study developed a new model of molecularly cloned genotype 1b HCV infection. A full-length genotype 1b HCV genome, HCV-KT9, was cloned from a serum sample from a patient with severe acute hepatitis. The chimeric mice were inoculated intrahepatically with in vitro-transcribed HCV-KT9 RNA. Inoculated mice developed viraemia at 2 weeks post-infection, and this persisted for more than 6 weeks. Passage experiments indicated that the sera of these mice contained infectious HCV. Interestingly, a similar clone, HCV-KT1, in which the poly(U/UC) tract was 29 nt shorter than in HCV-KT9, showed poorer in vivo infectivity and replication ability. An in vitro study showed that no virus was produced in the culture medium from HCV-KT9-transfected cells. In conclusion, this study developed a genetically engineered genotype 1b HCV-infected mouse. This mouse model will be useful for the study of HCV virology, particularly the mechanism underlying the variable resistance of HCV genotypes to interferon therapy.

INTRODUCTION

Hepatitis C virus (HCV), a positive-sense, single-stranded RNA virus, infects and replicates efficiently only in the

hepatocytes of humans and chimpanzees. There are many genotypes of HCV distributed worldwide (Simmonds et al., 1993); among them genotype 1b is the major genotype in Asia, including Japan, and is known to be one of the most resistant genotypes to interferon (IFN) therapy (Fried et al., 2002). Until recently, studies of HCV replication have long been hampered by the lack of a virus culture system. The development of HCV replicon systems has allowed the

study of the mechanisms of replication of HCV (Lohmann et al., 1999). However, these replicons lack structural proteins, do not replicate efficiently without adaptive mutations and do not produce infectious virions. Recently, it was reported that the genotype 2a full-length JFH-1 genome replicated efficiently in Huh7 cells without adaptive mutations and produced virions that were infectious for both naïve cells and chimpanzees, as well as for a human hepatocyte-transplanted chimeric mouse (Wakita et al., 2005; Zhong et al., 2005; Lindenbach et al., 2006). To date, five full-length genotype 1b clones, HCV-N (Beard et al., 1999), Con-1 (Bukh et al., 2002), HCV-J4 (Okamoto et al., 1992), HCV-CG1b (Thomson et al., 2001) and HCV-BK (Takamizawa et al., 1991), have been demonstrated to be infectious by intrahepatic inoculation of transcribed HCV RNA into the liver of chimpanzees. Among these, only the HCV-CG1b genome is reported to produce HCV particles when transfected into Huh7 cells (Heller et al., 2005).

Although the chimpanzee is a useful animal model for the study of HCV infection, there are ethical restrictions on the use of this animal. Instead, Mercer et al. (2001) developed a useful small-animal model for the study of HCV infection using chimeric urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) mice (which are immunodeficient and undergo liver failure) with engrafted human hepatocytes. This HCV-infected mouse model is reported to be useful for evaluating anti-HCV drugs such as IFN-α and anti-NS3 protease (Kneteman et al., 2006). We have previously described methods to improve the replacement levels of human hepatocytes in this mouse model (Tateno et al., 2004) and we have developed a reverse genetics system for hepatitis B virus (Tsuge et al., 2005) and HCV (Hiraga et al., 2007). In the present study, we report the establishment of an infectious genotype 1b HCV clone that infects and replicates efficiently in human hepatocyte chimeric mice.

METHODS

Cloning of infectious genotype 1b HCV isolate. Serum samples were obtained from a 43-year-old physician who developed severe acute hepatitis after needle stick exposure from a patient with chronic hepatitis C. On admission, the serum total bilirubin concentration was 10.0 mg dl^{-1} and the prothrombin time was 40 %. The patient tested positive for HCV antibodies by a third-generation radioimmunoassay (Ortho-Clinical Diagnostics) and for HCV RNA by RT-PCR. Serum HCV RNA was quantified using an Amplicor Monitor HCV test (Roche Diagnostics). The HCV RNA titre was 2.5×10^6 copies ml^{-1} on admission and then decreased gradually. Fig. 1 shows the serial changes in alanine aminotransferase (ALT) as a measure of liver function and HCV RNA levels in this patient. Serum samples obtained in the early phase of infection were used for cloning the full-length genome.

RNA extraction, cDNA synthesis, plasmid construction and RNA transcription. Total RNA was extracted from 100 μl serum samples using SepaGene RV-R (Sanko Junyaku) and reverse transcribed with random hexamers and ReverTra Ace reverse transcriptase (Toyobo) according to the manufacturer's instructions. PCR primers were designed based on the sequence of HCV-Con1 (GenBank accession
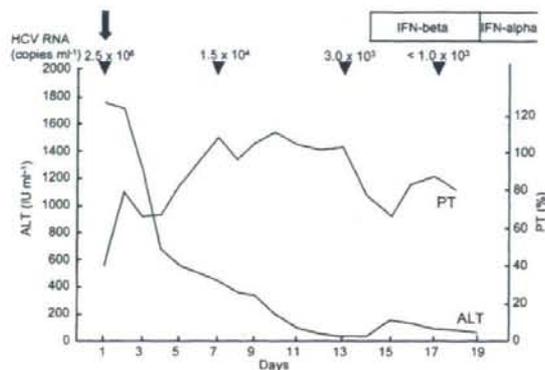
Fig. 1. Clinical course of a patient with severe acute hepatitis C. Alanine aminotransferase (ALT) and prothrombin time (PT) are shown from the day of admission (day 1). The patient was treated daily with 10^6 U IFN-β intravenously for 5 days, followed by 10^6 U IFN-α intramuscularly three times a week for 6 months. HCV RNA was measured on days 1, 7, 13 and 17 (arrowheads). A serum sample was taken on day 1 (arrow) and used to clone the full-length HCV genome.

no. AJ238799; Bukh et al., 2002). Five overlapping cDNA segments (nt 1–2292, 2269–6715, 6696–9094, 7564–9404 and 9361–9605; nucleotide numbers are those of HCV-Con1) were amplified by PCR with TaKaRa LA *Taq* polymerase (Takara Biochemicals) using the above cDNA. Amplified products were separated by agarose gel electrophoresis. Nucleotide sequences were determined using a Big Dye Terminator Mix Cycle Sequencing kit (Applied Biosystems Japan) with an automated DNA sequencer (model 310; PE Biosystems). We corrected the nucleotide sequences of the obtained clones by site-directed mutagenesis and made them identical to the nucleotide sequences obtained by direct sequencing. Naturally occurring restriction enzyme cutting sites were utilized to clone each segment. We utilized the vector pBR322 and created a multiple-cloning site under the control of the T7 promoter by ligating a linker at restriction enzyme cutting sites as they appeared in order from 5' to 3' in the HCV sequences (Fig. 2a). Each segment of HCV was cloned into this vector to generate the full-length clones. The HCV-KT9 clone was established using the 3'-terminal fragment with the longest poly(U/UC) tract length (115 nt), which should have a high replication ability (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). A clone with a shorter poly(U/UC) tract length (86 nt), HCV-KT1, was also generated. A polymerase-deficient mutant with an amino acid substitution in the GDD motif (GDD→GND; HCV-KT9-GND) was generated using a Quick Change Site-Directed Mutagenesis kit (Stratagene). After digesting the plasmid with *Xba*I (New England BioLabs) at the 3' end of the HCV cDNA, HCV RNA was transcribed using T7 RNA polymerase (MEGAscript; Ambion) at 37 °C for 3 h in a 100 μl reaction mixture, according to the manufacturer's instructions. The RNA was analysed using denaturing agarose gel electrophoresis and kept at −80 °C until use.

Construction of a phylogenetic tree. A phylogenetic tree was constructed based on the entire nucleotide sequences of 26 full-length genotype 1b clones plus HCV-KT9. The total number of synonymous and non-synonymous substitutions among the nucleotide sequences was estimated using the method of Gojobori et al. (1982) and a phylogenetic tree was constructed by the neighbour-joining method (Saitou & Nei, 1987).

(a)

Spel · · · Xhol 2281 · · · EcoRI 6699 · Kpni 9077 · Clai 9367 · Xbai 9621

T7P 5′ · Core E1 E2 P7 NS2 NS3 NS 4A NS4B NS5A NS5B 3′

I200M
L290R
F285L
V301M

N395R
D463G
F627T
A444H
N478P
A752V

M81+V
Q1021G
L899I

V1061I
N1369T
S1425G
S1061T
G1409N
P1621Q

Q1703K

T2319H
V2360L
T2939H
T2956A
S2975D

(b)

(c)

HCV-KT9
HCV-N (AF139594) *
HCV-BK (M58335) *
HCV-CG1b (AF333324) *
HCV-S (AY460204)
J33 (D14484)
M1LE (AB080299)
HCV-JS (D85516)
HCVT094 (AB049088)
HCVT142 (AB049091)
D89815 (D89815)
HCV-AD78P1 (AJ132997)
HCVT140 (AB049090)
XB016785 (AB016785)
HCV-O (AB191333)
HCVT109 (AB049089)
HCVT169 (AB049095)
Con1 (AJ238799) *
HCVT221 (AB049101)
HCV-K1-R1 (D50480)
JT (D11355)
HCV-J (D90208)
HCV-J4 (AF054250) *
pCV-J4L6S (AF054247)
pCV-J4L2S (AF054248)
274933RU (AF176573)
pCV-J4L4S (AF054249)

0 1

Fig. 2. (a) Schematic diagram of the organization of the cDNA clone HCV-TK9. The T7 RNA promoter (T7P) is located immediately upstream of the HCV genome. Restriction enzyme sites that were used to create clone HCV-KT9 are labelled according to their nucleotide position within the HCV sequence. Amino acid sequences unique to HCV-KT9 compared with 26 other HCV genotype 1b isolates are indicated at the bottom of the figure, with the position of the repaired amino acid residues noted within the polyprotein. (b) Length of the poly(U/UC) tracts of HCV-KT1, HCV-KT9 and 22 other HCV genotype 1b clones reported previously. Asterisks indicate clones confirmed to be infectious by experiments using chimpanzees. (c) Phylogenetic tree constructed with HCV-KT9 and 26 genotype 1b HCV whole-genome sequences. Bar, number of nucleotide substitutions per site. Asterisks indicate clones confirmed to be infectious in experiments using chimpanzees.

Intrahepatic injection experiments in human hepatocyte chimeric mice. We used methods described previously (Tateno *et al.*, 2004) to generate uPA$^{+/+}$/SCID$^{+/+}$ mice and transplant human hepatocytes. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mouse serum concentrations of human serum albumin (HSA) correlate with the repopulation index and were measured as described previously (Tateno *et al.*, 2004). Intrahepatic injection of RNA, extraction of serum samples and euthanasia were performed under ether anaesthesia. Briefly, 500 μl RNA solution containing 30 μg transcribed HCV RNA was injected into the liver of anaesthetized chimeric mice through a small abdominal incision. RNA extraction from mouse serum samples, quantification of HCV RNA and nested PCR were performed as described previously (Hiraga *et al.*, 2007). All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments and under the approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Cell culture, RNA transfection and measurement of HCV core antigen. The human hepatoma cell line Huh7 was maintained in Dulbecco's modified Eagle's medium (Sigma) containing 10 % fetal calf serum. RNA transfection and measurement of HCV core antigen in the culture medium were performed as described previously (Wakita *et al.*, 2005).

Statistical analysis. The infectious ratio of chimeric mice was compared and the differences assessed using a χ^2 test. Differences in HCV RNA replication ability *in vitro* were analysed statistically by one-way analysis of variance followed by Scheffe's test. A *P* value of less than 0.05 was considered statistically significant.

RESULTS

Characteristics of genotype 1b clones HCV-KT9 and HCV-KT1

The entire genome of HCV cDNA was assembled from five DNA fragments (Fig. 2a). We obtained 24 3′-extremity clones with different poly(U/UC) tract lengths. We selected the clone with the longest (U/UC) tract because a previous study indicated that the length of poly(U/UC) tract correlates with HCV replication in an HCV replicon system (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). The length of the poly(U/UC) tract in the longest 3′ clone was 115 nt. The entire genome length of the HCV-KT9 clone using this longest 3′ clone was 9621 nt. We also generated the clone HCV-KT1 with a shorter (86 nt) poly (U/UC) tract to compare the replication abilities of these clones. The lengths of the poly(U/UC) tracts of 22 clones deposited in GenBank are shown in Fig. 2(b). All infectious clones had a poly(U/UC) tract longer than 80 nt. Fig. 2(c) shows a phylogenetic tree constructed using the nucleotide sequences of the 26 full-length genotype 1b clones published to date. Interestingly, the sequence of HCV-KT9 was closest to that of HCV-CG1b (GenBank accession no. AF333324), which has been reported to be infectious, and formed a cluster with two other infectious clones, HCV-N (Beard *et al.*, 1999) and HCV-BK (Takamizawa *et al.*, 1991). We compared the amino acid

sequences of HCV-KT9 with an alignment of the sequences of the 26 other genotype 1b strains. All HCV full-length clones reported from Japan were included in these 26 strains. Based on these comparisons, we identified 25 aa unique to HCV-KT9 (Fig. 2a). We found that the amino acid sequence of the IFN sensitivity-determining region in the NS5A region, which has been suggested to mediate IFN resistance via interaction with the cellular protein kinase R (Enomoto et al., 1996; Gale et al., 1997), was that of the wild-type.

Intrahepatic injection of HCV-KT1 and HCV-KT9 RNAs into human hepatocyte chimeric mice

In the next experiments, 30 μg in vitro-transcribed RNA of HCV-KT1, HCV-KT9 or HCV-KT9-GND was injected into the livers of chimeric mice. Eight of 10 (80 %) HCV-KT9-injected mice developed measurable viraemia at 2 weeks post-inoculation (Table 1 and Fig. 3), with the HCV RNA titre reaching 1.1×10^6 to 8.8×10^6 copies ml^{-1} at 6 weeks post-inoculation (Fig. 3). To check for the presence of infectious HCV in the serum of HCV-KT9-infected mice, each of five naïve mice was injected with 10 μl serum sample (containing 3.5×10^5 copies of HCV) obtained from an HCV-KT9-infected mouse 6 weeks after inoculation. All five naïve mice became positive for HCV RNA, as confirmed by nested PCR, at 2 weeks post-inoculation and two mice developed persistent viraemia (Fig. 4). These results indicated that the serum of HCV-KT9-injected mice contained infectious HCV. In contrast to HCV-KT9, none of the three mice injected with HCV-KT9-GND RNA developed viraemia (Table 1). These results indicated that HCV-KT9 replicates efficiently in mice livers and produces infectious virus continuously. On the other hand, only one out of seven HCV-KT1-injected mice (14 %) developed measurable viraemia (Table 1 and Fig. 3). The level of viraemia was low in this HCV-KT1-infected mouse, HCV RNA was negative by nested PCR at 2 weeks after inoculation and the titre was only 2.2×10^4 copies ml^{-1} at 4 weeks post-inoculation (Fig. 3). These results confirmed the importance of the poly(U/UC) tract length in experimentally induced viraemia.

The nucleotide and amino acid sequences of the viral genome isolated from an HCV-KT9-injected mouse (Fig. 3)

Table 1. Correlation between length of the poly(U/UC) tract and HCV infection

Clone	Length of poly(U/UC) tract	Number of mice			Infection ratio
		Infected	Not infected	Total	
HCV-KT1	86	1	6	7	14 %
HCV-KT9	115	8	2	10	80 %*
HCV-KT9-GND	115	0	3	3	0 %

*$P=0.015$, compared with HCV-KT1.
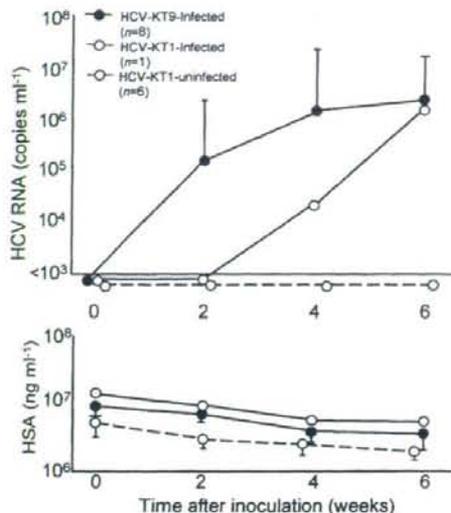
Fig. 3. Changes in HCV RNA levels and HSA concentrations in the sera of mice infected with clonal HCV. Mice were inoculated intrahepatically with 30 μg in vitro-transcribed HCV RNA. Eight of the ten HCV-KT9-infected mice (80 %), one of the seven HCV-KT1-infected mice (14 %) and none of the three HCV-KT9-GND-infected mice became positive for HCV RNA. The results for six HCV-KT1-uninfected mice are also shown. Mice serum samples were obtained every 2 weeks post-infection for analysis of HCV RNA titres. Data are shown as mean ± SD.

at 6 weeks after RNA injection were identical to the injected HCV-KT9 (data not shown). We tried to reclone the poly(U/UC) tract in the HCV-KT1-infected mouse, but it was impossible to reamplify the HCV cDNA using the remaining small amount of serum.

Analysis of virus production from HCV-KT9-transfected cells

Next, we evaluated the ability of the HCV-KT9 clone to replicate in transfected Huh7 cells. In these experiments, we used JFH-1 RNA, which is known to replicate efficiently in cell cultures, as control (Wakita et al., 2005). Core protein was secreted efficiently from JFH-1 RNA-transfected Huh7 cells. In contrast, we did not observe any measurable levels of core protein in the supernatant of HCV-KT9-transfected cells (Fig. 5), suggesting a minimal replication ability of HCV-KT9 to produce and release virus into the supernatant.

DISCUSSION

In this study, we described the establishment of a genotype 1b clone, HCV-KT9, that replicated efficiently following injection of the transcribed RNA into chimeric mouse liver.
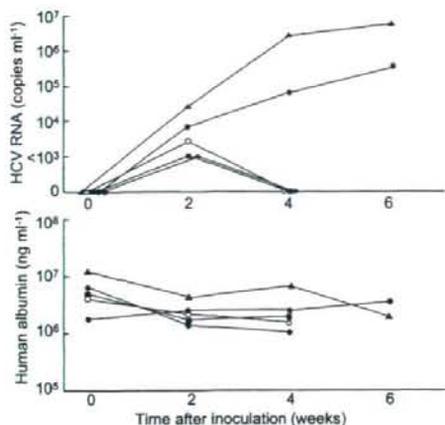
Fig. 4. Passage experiments of HCV in naïve chimeric mice. Five naïve chimeric mice were inoculated intravenously with 10 µl serum sample (containing $3.5×10^5$ copies HCV) obtained from an HCV-KT9-infected mouse at week 6 post-inoculation. Serum samples were obtained at the indicated time intervals for the measurement of HCV RNA levels and HSA concentrations. Data represent the changes in five individual mice.

The key factor that determines the infectivity of HCV clones has not yet been established. We previously established a clone from HCV that replicated in a chimeric mouse after injection of serum from a chronically HCV-infected patient. However, we did not observe viraemia after intrahepatic injection of the transcribed RNA from this clone (unpublished results). In contrast, injection of HCV-KT9 RNA in the present study resulted in viraemia in eight out of ten mice (80 %). The fact that the nucleotide
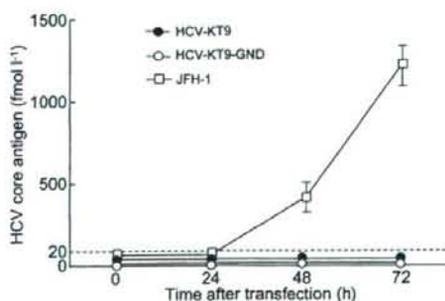
Fig. 5. Time-course studies of HCV core protein secretion into the culture medium of HCV RNA-transfected cells. Huh7 cells were transfected with 10 µg HCV-KT9, HCV-KT9-GND or JFH-1 RNA. HCV core antigen in the culture medium was measured at 24, 48 and 72 h after transfection. Data are shown as mean ± SD of HCV core protein levels obtained from three independent transfection experiments.

and amino acid sequences of the virus recovered from the infected mice were identical to those of the HCV-KT9 clone indicated that no adaptive mutation was necessary for this clone to replicate in the chimeric mouse.

Interestingly, the clone was obtained from a patient with severe acute hepatitis. This is similar to JFH-1, an HCV clone with a strong replication ability in cultured cell lines, chimpanzees and chimeric mice, which was cloned from serum samples of a patient who developed acute fulminant hepatitis with a high virus titre (Wakita *et al.*, 2005). A virus that replicates in the early stage of infection may have strong replication ability, which may be lost in the chronic phase of infection.

A key amino acid substitution may be present in one (or some) of the amino acids unique to this clone (Fig. 2a). We also showed that clone HCV-KT1, which differs from HCV-KT9 only in the length of the poly(U/UC) tract, had a poorer replication ability in mice (Table 1 and Fig. 3). However, there is a possibility that a shorter poly(U/UC) tract only slows down the rate of infection, as the HCV RNA titre in the HCV-KT1-infected mouse at 6 weeks after inoculation was similar to that in HCV-KT9-infected mice (Fig. 3). It has been reported that the length and composition of the poly(U/UC) tract is important for the replication of HCV replicons (Friebe & Bartenschlager, 2002; Yi & Lemon, 2003; You & Rice, 2008). However, no replication advantage of a poly(U/UC) tract longer than 86 bp was revealed in this study. This may be due to differences *in vitro* and *in vivo*, where the innate immune response against the virus may be more robust than in cell culture.

As shown in the present study, reverse genetics of HCV has become available for studies of HCV replication. The important factors for virus replication suggested above can be analysed further using this system.

We also examined the response of HCV-KT9-infected mice to IFN treatment. Three HCV-KT9-infected mice were treated with daily intramuscular injections of 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks. This regimen resulted in a reduction in HCV RNA levels of only 1.0 log copies ml^{-1} (data not shown). These results are consistent with our previous study, which showed a similar low-level reduction in HCV RNA in mice infected with a genotype 1a clone, and differ from our previous results in mice infected with HCV genotype 2a, which became negative for HCV RNA following daily treatment with 1000 IU IFN-α (g body weight)$^{-1}$ for 2 weeks (Hiraga *et al.*, 2007). These results are in agreement with our clinical experience that genotype 1 is more resistant to IFN therapy than genotype 2. As shown in the present study and previously (Hiraga *et al.*, 2007), reverse genetics of HCV with three genotypes, 1a, 1b and 2a, is now available. By recombination of these clones or the establishment of mutants with nucleotide and amino acid sequences similar to each other, it may be possible to clarify the mechanism underlying the variability in susceptibility of HCV genotypes to IFN.

In this study, HCV-KT9 showed no virus production ability *in vitro*. Recently, Kato *et al.* (2007) reported that the genotype 1b HCV clone CG1b replicated in Huh7.5.1 cells and produced infectious HCV. It will be of interest to create chimeric viruses of HCV-KT9 and HCV-CG1b, and to determine the mutations that are important for virus production *in vitro*.

In summary, we established an infection model of a genotype 1b HCV clone using human hepatocyte chimeric mice. This model will be useful for studies of HCV replication, particularly the mechanism underlying the variable resistance of HCV genotypes to IFN therapy.

ACKNOWLEDGEMENTS

REFERENCES

Beard, M. R., Abell, G., Honda, M., Carroll, A., Gartland, M., Clarke, B., Suzuki, K., Lanford, R., Sangar, D. V. & Lemon, S. M. (1999). An infectious molecular clone of a Japanese genotype 1b hepatitis C virus. *Hepatology* 30, 316–324.

Bukh, J., Pietschmann, T., Lohmann, V., Krieger, N., Faulk, K., Engle, R. E., Govindarajan, S., Shapiro, M., St Claire, M. & other authors (2002). Mutations that permit efficient replication of hepatitis C virus RNA in Huh-7 cells prevent productive replication in chimpanzees. *Proc Natl Acad Sci U S A* 99, 14416–14421.

Enomoto, N., Sakuma, I., Asahina, Y., Kurosaki, M., Murakami, T., Yamamoto, C., Ogura, Y., Izumi, N., Marumo, F. & other authors (1996). Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. *N Engl J Med* 334, 77–81.

Friebe, P. & Bartenschlager, R. (2002). Genetic analysis of sequences in the 3′ nontranslated region of hepatitis C virus that are important for RNA replication. *J Virol* 76, 5326–5338.

Fried, M. W., Shiffman, M. L., Reddy, K. R., Smith, C., Marinos, G., Goncales, F. L., Jr, Haussinger, D., Diago, M., Carosi, G. & other authors (2002). Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 347, 975–982.

Gale, M. J., Jr, Korth, M. J., Tang, N. M., Tan, S. L., Hopkins, D. A., Dever, T. E., Polyak, S. J., Gretch, D. R. & Katze, M. G. (1997). Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. *Virology* 230, 217–227.

Gojobori, T., Ishii, K. & Nei, M. (1982). Estimation of average number of nucleotide substitutions when the rate of substitution varies with nucleotide. *J Mol Evol* 18, 414–423.

Heller, T., Saito, S., Auerbach, J., Williams, T., Moreen, T. R., Jazwinski, A., Cruz, B., Jeurkar, N., Sapp, R. & other authors (2005). An in vitro model of hepatitis C virion production. *Proc Natl Acad Sci U S A* 102, 2579–2583.

Hiraga, N., Imamura, M., Tsuge, M., Noguchi, C., Takahashi, S., Iwao, E., Fujimoto, Y., Abe, H., Maekawa, T. & other authors (2007). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. *FEBS Lett* 581, 1983–1987.

Kato, T., Matsumura, T., Heller, T., Saito, S., Sapp, R. K., Murthy, K., Wakita, T. & Liang, T. J. (2007). Production of infectious hepatitis C virus of various genotypes in cell cultures. *J Virol* 81, 4405–4411.

Kneteman, N. M., Weiner, A. J., O'Connell, J., Collett, M., Gao, T., Aukerman, L., Kovelsky, R., Ni, Z. J., Zhu, Q. & other authors (2006). Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. *Hepatology* 43, 1346–1353.

Lindenbach, B. D., Meuleman, P., Ploss, A., Vanwolleghem, T., Syder, A. J., McKeating, J. A., Lanford, R. E., Feinstone, S. M., Major, M. E. & other authors (2006). Cell culture-grown hepatitis C virus is infectious in vivo and can be recultured in vitro. *Proc Natl Acad Sci U S A* 103, 3805–3809.

Lohmann, V., Korner, F., Koch, J., Herian, U., Theilmann, L. & Bartenschlager, R. (1999). Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 285, 110–113.

Mercer, D. F., Schiller, D. E., Elliott, J. F., Douglas, D. N., Hao, C., Rinfret, A., Addison, W. R., Fischer, K. P., Churchill, T. A. & other authors (2001). Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* 7, 927–933.

Okamoto, H., Kojima, M., Okada, S., Yoshizawa, H., Iizuka, H., Tanaka, T., Muchmore, E. E., Peterson, D. A., Ito, Y. & other authors (1992). Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. *Virology* 190, 894–899.

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

Simmonds, P., Holmes, E. C., Cha, T. A., Chan, S. W., McOmish, F., Irvine, B., Beall, E., Yap, P. L., Kolberg, J. & other authors (1993). Classification of hepatitis C virus into six major genotypes and a series of subtypes by phylogenetic analysis of the NS-5 region. *J Gen Virol* 74, 2391–2399.

Takamizawa, A., Mori, C., Fuke, I., Manabe, S., Murakami, S., Fujita, J., Onishi, E., Andoh, T., Yoshida, I. & other authors (1991). Structure and organization of the hepatitis C virus genome isolated from human carriers. *J Virol* 65, 1105–1113.

Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K. & other authors (2004). Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* 165, 901–912.

Thomson, M., Nascimbeni, M., Gonzales, S., Murthy, K. K., Rehermann, B. & Liang, T. J. (2001). Emergence of a distinct pattern of viral mutations in chimpanzees infected with a homogeneous inoculum of hepatitis C virus. *Gastroenterology* 121, 1226–1233.

Tsuge, M., Hiraga, N., Takaishi, H., Noguchi, C., Oga, H., Imamura, M., Takahashi, S., Iwao, E., Fujimoto, Y. & other authors (2005). Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis B virus. *Hepatology* 42, 1046–1054.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H. G. & other authors (2005). Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11, 791–796.

Yi, M. & Lemon, S. M. (2003). 3′ Nontranslated RNA signals required for replication of hepatitis C virus RNA. *J Virol* 77, 3557–3568.

You, S. & Rice, C. M. (2008). 3′ RNA elements in hepatitis C virus replication: kissing partners and long poly(U). *J Virol* 82, 184–195.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D. R., Wieland, S. F., Uprichard, S. L., Wakita, T. & other authors (2005). Robust hepatitis C virus infection *in vitro*. *Proc Natl Acad Sci U S A* 102, 9294–9299.

Randomized Trial of High-Dose Interferon-α-2b Combined With Ribavirin in Patients With Chronic Hepatitis C: Correlation Between Amino Acid Substitutions in the Core/NS5A Region and Virological Response to Interferon Therapy

Nami Mori, Michio Imamura, Yoshiiku Kawakami, Hiromi Saneto, Tomokazu Kawaoka, Shintaro Takaki, Hiroshi Aikata, Shoichi Takahashi, Kazuaki Chayama,* and Hiroshima Liver Study Group

Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Minami-ku, Hiroshima, Japan

The aim of this study was to compare the efficacy of high-dose interferon (IFN)-α-2b with standard dose of IFN-α-2b in combination with ribavirin (RBV) for patients with chronic hepatitis C virus (HCV) infection, and to investigate the predictive factors associated with virological response. Two hundred Japanese patients with high HCV viral load (>100 KIU/ml) were randomized to 6 or 10 mega units (MU) of 24-week IFN-α-2b with RBV. Predictive factors were investigated; including pretreatment amino acid (aa) sequences of the core region and the IFN-sensitive determining region (ISDR). The sustained virological response rate was not different in the two groups (24% vs. 30%) but the incidence of depression was significantly higher in the 10 MU group than 6 MU group (7% vs. 0%, $P = 0.02$). Younger age (<60) and HCV genotype (2a/b) were significant predictors of sustained virological response. In patients infected with genotype 1b, substitutions of core aa 70 and/or 91 were predictive for non-virological response ($P < 0.001$), and substitutions in the ISDR was observed frequently in virological responders. Early viral kinetics study showed that serum HCV core antigen decreased more slowly in both patients with aa 70 and/or 91 substitutions in the core and with absence of substitutions in the ISDR. In conclusion, the use of a higher dose of IFN-α-2b in combination with RBV did not improve virological response but resulted in higher incidence of depression. Amino acid substitutions in the core and ISDR are predictive of virological response to the therapy in patients with genotype 1b and high viral load. *J. Med. Virol. 81:640–649, 2009.*
© 2009 Wiley-Liss, Inc.

KEY WORDS: HCV; interferon; ribavirin; core region; ISDR

INTRODUCTION

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [Kiyosawa et al., 1990; Niederau et al., 1998]. Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection. The most effective therapy available at present is the combination therapy of pegylated (PEG)-IFN and ribavirin (RBV) [Manns et al., 2001; Fried et al., 2002; Hoofnagle et al., 2003]. Among HCV genotypes, genotype 1b is the most resistant genotype to IFN therapy [Fried et al., 2002]. The limitation of use of the combination therapy for HCV infection with genotype 1b is due to the low response rate during therapy and high relapse rate after the therapy [McHutchison et al., 1998]. Several studies have evaluated the potential benefits of a larger dose of IFN with varying results [Lindsay et al., 1996; Fried et al., 2000; Ferenci et al., 2001; Hadziyannis et al., 2001; Di Marco et al., 2002; Brouwer et al., 2004]. Although treatment has been

switched to the combination of PEG-IFN and RBV in recent years, it is important to know if a larger dose of IFN is beneficial to patients with chronic hepatitis C.

Many molecular mechanisms through which HCV evades host innate immunity have been reported to date. HCV core, E2 and NS5A proteins have been reported to inhibit the IFN signaling system [Gale et al., 1997; Taylor et al., 1999; Blindenbacher et al., 2003; Bode et al., 2003; Foy et al., 2003; Lin et al., 2006; Ciccaglione et al., 2007]. Variations of amino acid (aa) sequences in the E2 and the NS5A region have been reported to correlate with the effect of IFN therapy [Enomoto et al., 1996; Chayama et al., 1997, 2000; Polyak et al., 1998, 2000; Hashimoto et al., 1999; Puig-Basagoiti et al., 2001; Pascu et al., 2004; Gaudy et al., 2005; Brillet et al., 2007; Torres-Puente et al., 2008]. Recently, Akuta et al. [2005, 2006, 2007a, b] reported that substitution of aa 70 and/or 91 in the core region is an independent and significant predictor of non-virological response.

The aim of the present study was to evaluate the therapeutic efficacy and safety of a large dose of IFN-α-2b combined with RBV. For this purpose, a randomized trial was conducted to compare the therapeutic effects of high-dose (10 MU) versus standard dose (6 MU) of IFN-α-2b combined with RBV in patients with high HCV viral titers. The second endpoint of this study was to analyze the predictive factors associated with virological response including aa substitutions in the core region and the NS5A region.

PATIENTS AND METHODS

Patient Selection

Two hundred adult patients enrolled into the study. The inclusion criteria were positivity for antibody to HCV, HCV RNA levels higher than 100 KIU/ml, and the diagnosis of chronic hepatitis C was confirmed by liver biopsy. The liver biopsy specimens were evaluated as described by Desmet et al. [1994], and classified into F0 to F3. None of the patients included in this study had liver cirrhosis (F4). Other exclusion criteria included leukocytopenia (leukocyte <4,000/mm^3) and anemia (hemoglobin concentration <10 g/dl). Patients with human immunodeficiency or hepatitis B super infection, previous organ transplantation, other causes of liver disease, poorly controlled diabetes, de-compensated renal disease, pre-existing psychiatric disease, seizure disorders, cardiovascular disease, hemophilia or auto-immune type diseases were also excluded.

Study Design

The double-blind, multi-center randomized clinical trial was conducted in 23 centers in Hiroshima city (The Hiroshima Liver Study Group). The study was approved by the Ethics Committee of Hiroshima University. Written informed consent was obtained form all participants. Eligible patients were assigned randomly into either of the two groups without further stratification using sequentially numbered cards in sealed envelopes.

Patients were randomized to treatment with combination of IFN-α-2b (Intron A, Shering Plough, Kenilworth, NJ) at a dose of 6 MU (Group A) or 10 MU (Group B) plus RBV (Rebetol, Shering Plough). IFN-α-2b was administered intramuscularly daily over the initial 2 weeks and three times weekly in the remaining 22 weeks. The dose of RBV was adjusted according to body weight (600 mg/day for ≤60 kg, 800 mg/day for >60 kg). Adverse events were monitored clinically by careful interview and hematological examination throughout the study. The dosage of RBV was reduced in patients who experienced a decrease in hemoglobin concentration to <10 g/dl.

Blood samples were taken 2 and 4 weeks after the beginning of therapy and every 4 weeks thereafter. Biochemical and hematological tests were performed in each center, including alanine amino transferase (ALT). Part of the serum samples were kept frozen at −80°C until further analysis. Viral genotypes were determined by phylogenetic analysis after reverse transcription (RT)-polymerase chain reaction (PCR) and direct sequencing.

Assessment of Efficacy

Serum HCV RNA was detected by nested PCR assay (Cobas Amplicor HCV test v 2.0, Roche Diagnostics, Tokyo, Japan; limit of detection, 50 IU/ml) at weeks 2, 4 and every 4 weeks during treatment and 24 weeks after the cessation of therapy. Positive samples were analyzed further by quantitative assay (Cobas Amplicor HCV monitor v 2.0, Roche Diagnostics; limit of detection, 500 IU/ml).

The primary endpoint of this study was sustained virological response, defined as undetectable serum HCV RNA by qualitative PCR test and normalization of ALT 24 weeks after the treatment. Non-virological response was applied to those patients with positive qualitative HCV RNA PCR tests in all examinations. Virological response was used to define the remaining patients who became PCR negative at least once during the treatment.

Nucleotide Sequencing of the Core and NS5A Gene

The core aa 61–110 and NS5A aa 2209–2248 (IFN-sensitive determining region [ISDR] [Enomoto et al., 1996]) sequences were determined by direct sequencing using stored serum samples obtained just before therapy. HCV RNA was extracted from serum samples and reverse transcribed with random primers and MMLV reverse transcriptase (Takara Bio Inc., Shiga, Japan). DNA fragments were amplified by PCR using the following primers. (a) Nucleotide sequences of the core region: The first-round PCR was performed with primers CC11 (forward, 5′-GCC ATA GTG GTC TGC GGA AC-3′) and e14 (reverse, 5′-GGA GCA GTC CTT CGT GAC ATG-3′), and the second-round PCR with primers CC9 (forward, 5′-GCT AGC CGA GTA GTG TT-3′) and e14 (reverse) as described by Akuta et al. [2005, 2006, 2007a, b]. After denaturation at 95°C for 5 min, 35

cycles of amplification were set as follows; denaturation for 30 sec at 94°C, annealing of primers for 1.5 min at 57°C, and extension for 1 min at 72°C, followed by final extension at 72°C for 7 min. The second PCR was carried out with the same amplification conditions used in the first PCR, except that the second PCR primers were used instead of the first PCR primers. (b) Nucleotide sequences of ISDR in NS5A: PCR was performed with IM11 (forward, 5'-TTC CAC TAC GTG ACG GGC AT-3') and 5OA2KI (reverse, 5'-CCC GTC CAT GTG TAG GAC AT-3'). After denaturation at 98°C for 30 sec, 35 cycles of amplification were set as follows; denaturation for 10 sec at 98°C, annealing of primers for 30 sec at 66°C, and extension for 15 sec at 72°C, followed by final extension at 72°C for 5 min. The amplified PCR products were separated in a 2% agarose gel and purified by GENE-CLEAN II kit (Q-Bio Gene, Carlsbad, CA). Nucleotide sequences were determined using Big Dye Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer, Tokyo, Japan). Nucleotide and aa sequences were compared with the nucleotide sequences of genotype 1b HCV-J (Gene Bank accession number; D90208) [Kato et al., 1990].

Quantitation of HCV Core Antigen

HCV core antigen levels were measured using stored serum samples just before and 4 weeks after the start of the therapy as described previously [Aoyagi et al., 1999].

Statistical Analysis

The baseline characteristics of the patients in the two groups were compared and the differences were assessed by Chi-square test with Yate's correction and Mann–Whitney U-test. To assess the sustained virological response rates, an intention-to-treat (ITT) analysis and a per-protocol (PP) analysis were conducted. The response rates and substitutions in the core region and the ISDR were compared by Fisher's exact test. All P values reported are two-sided and those less than 0.05 were considered significant. To determine the predictors of sustained virological and non-virological responses, univariate and multivariate logistic regression analyses were carried out. Potential predictive factors included the following variables: age, sex, alcohol consumption, past history of IFN monotherapy, body mass index, ALT, hemoglobin, platelets, HCV RNA level, genotype, liver histology, total RBV dose (adjusted for body weight [mg/kg]) and total dose of IFN-α-2b. The odds ratio and 95% confidence intervals (95% CI) were also calculated. Variables with statistical significance ($P < 0.05$) or marginal significance ($P < 0.10$) on univariate analysis were entered into multiple logistic regression analysis to identify significant independent factors. Statistical analyses were performed using the SPSS software (SPSS, Inc., Chicago, IL).

RESULTS

Patient Demographics

Patient enrollment started in January 2002, and the trial ended in March 2005. The disposition of patients throughout the trial is shown in Figure 1. A total of 200 patients were randomized to treatment, and 198 patients met the eligibility criteria and underwent
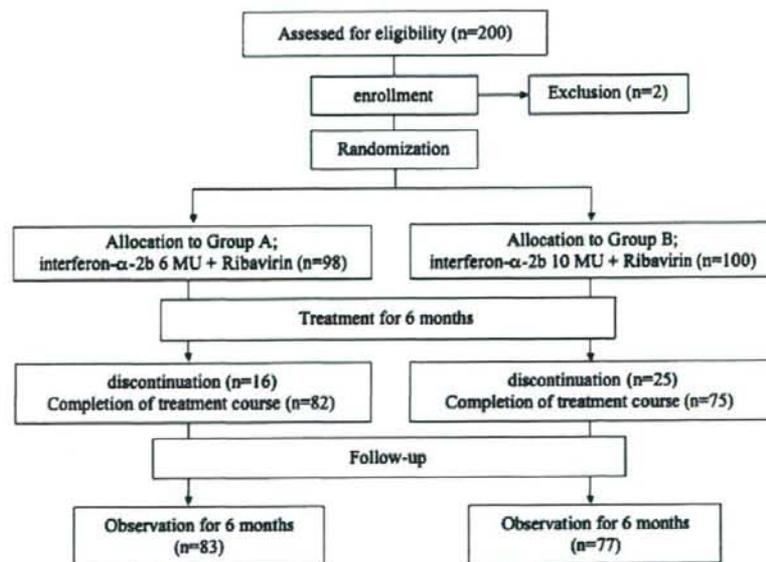

Fig. 1. Flow chart of number of patients throughout the trial. A total of 200 patients were included in this study. One hundred ninety-eight patients met the eligibility criteria and they underwent randomization, 98 patients in Group A and 100 patients in Group B.

TABLE I. Baseline Characteristics of the Patients

Characteristic	Group A (n = 98)	Group B (n = 100)	P
Age (years)[a]	55 ± 10.3	55 ± 11.0	0.43
Male sex (%)	63	75	0.07
Alcohol consumption (%)[b]	23	20	0.61
Past history of IFN monotherapy (%)	33	35	0.72
Body-mass index (kg/m^2)[a]	23.3 ± 2.9	24.2 ± 3.6	0.05
ALT (IU/L)[a]	79.2 ± 45.3	109.4 ± 111.2	0.31
Hemoglobin (g/dl)[a]	14.2 ± 1.4	14.5 ± 1.2	0.02
Platelets ($\times 10^4$/mm^3)[a]	14.8 ± 4.8	16.5 ± 5.0	<0.05
HCV RNA (KIU/ml) (%)			
100–850	49	47	
≥850	51	53	0.80
Genotype (%)			
1b	82	72	
2a/2b	17	28	0.32
3a/3b	1	0	
Liver histology[a,c]	2.0 ± 0.84	1.8 ± 0.82	0.05

ALT, alanine aminotransferase.
[a]Values are mean ± SD.
[b]Percentage of patients who consumed alcohol at >30 g/day.
[c]Liver fibrosis was scored 0 (F0), no fibrosis; 1 (F1), periportal expansion; 2 (F2), portoportal septa; 3 (F3), portocentral linkage or bridging fibrosis.

randomization. Ninety-eight patients were assigned to Group A and 100 patients to Group B. Patients were observed for 24 weeks after the treatment. Sixteen patients of Group A and 25 patients of Group B discontinued the treatment because of adverse events. Table I lists the baseline characteristics of the patients. Hemoglobin concentrations and platelet counts were higher in group B patients. The other parameters were similar between the two groups.

Overall Sustained Virological Response

The effect of therapy in the two groups is summarized in Table II. The sustained virological response rate was lower significantly in patients of group B with genotype 2a/b relative to those of group A (ITT analysis). This reflects the fact that a larger number of patients dropped out form the protocol because of the adverse effects (1 [6%] of 16 in group A and 10 [43%] of 23 in group B, $P = 0.02$). All patients who stopped treatment did not achieve sustained virological response. Patients with genotype 1b had a lower sustained virological response rate than those with genotype 2a/b (33/124 [27%] vs. 26/39 [67%], $P < 0.01$).

Dose Reduction or Discontinuation and Adverse Events

Table III summarizes the laboratory abnormalities and the dose reduction and discontinuation of IFN-α-2b and RBV due to adverse events. The overall discontinuation rate was 16% for group A and 25% for group B (not significant). The most frequent adverse event associated with dose reduction was anemia. A larger number of patients of group B developed depression ($P = 0.02$).

Predictive Factors Associated With Sustained Virological Response

Univariate analysis identified three parameters that correlated with sustained virological response: age (<60 years, $P = 0.007$); genotype (2a/b, $P < 0.001$); and platelet count (>15 × 10^4/mm^3, $P = 0.01$). Multivariate analysis including the above variables identified two parameters that independently predicted sustained virological response: age ($P = 0.02$) and genotype ($P < 0.001$) (Table IV).

TABLE II. Rates of Sustained Virological Response According to Adherence

Genotype	Group A	Group B	P
1b	n = 68	n = 56	
ITT	16/68 (24%)	17/56 (30%)	0.39
PP	16/53 (30%)	17/41 (41%)	0.25
2a/b	n = 16	n = 23	
ITT	15/16 (94%)	11/23 (48%)	0.005
PP	15/15 (100%)	11/13 (85%)	0.21

ITT, intention to treatment analysis; PP, per protocol analysis; IFN, interferon; RBV, ribavirin.

TABLE III. Dose Reduction or Discontinuation and Adverse Events

	Group A (n = 98) % (n)	Group B (n = 100) % (n)	P
Discontinuation	16 (16)	25 (25)	0.13
Dose reduction or discontinuation of			
IFN	20 (20)	41 (41)	0.002
RBV	36 (35)	50 (50)	0.04
IFN and/or RBV	37 (36)	55 (55)	0.01
Depression	0 (0)	7 (7)	0.02

IFN, interferon; RBV, ribavirin.

TABLE IV. Factors Associated With Sustained Virological
Response to Combination Therapy of Interferon Plus
Ribavirin by Multivariate Analysis

Factor	Category	Odds ratio (95% CI)	P
Age (years)	0: ≥60	1	
	1: <60	2.420 (1.173–5.002)	0.020
Genotype	0: 1b	1	
	1: 2a/b	5.301 (2.401–11.702)	<0.001

Only variables that achieved statistical significance ($P < 0.05$) on multivariate logistic regression analysis are shown.

Analysis of aa Sequences in the Core Gene in Genotype 1b Patients

The relationship between aa substitutions in the core region and the viral response to therapy was investigated in patients with genotype 1b using 93 available serum samples. Figure 2 shows the sequences of aa 61–110 of the HCV core region in 93 patients just before commencement of treatment. Table V summarizes the relationship between the response to IFN therapy and
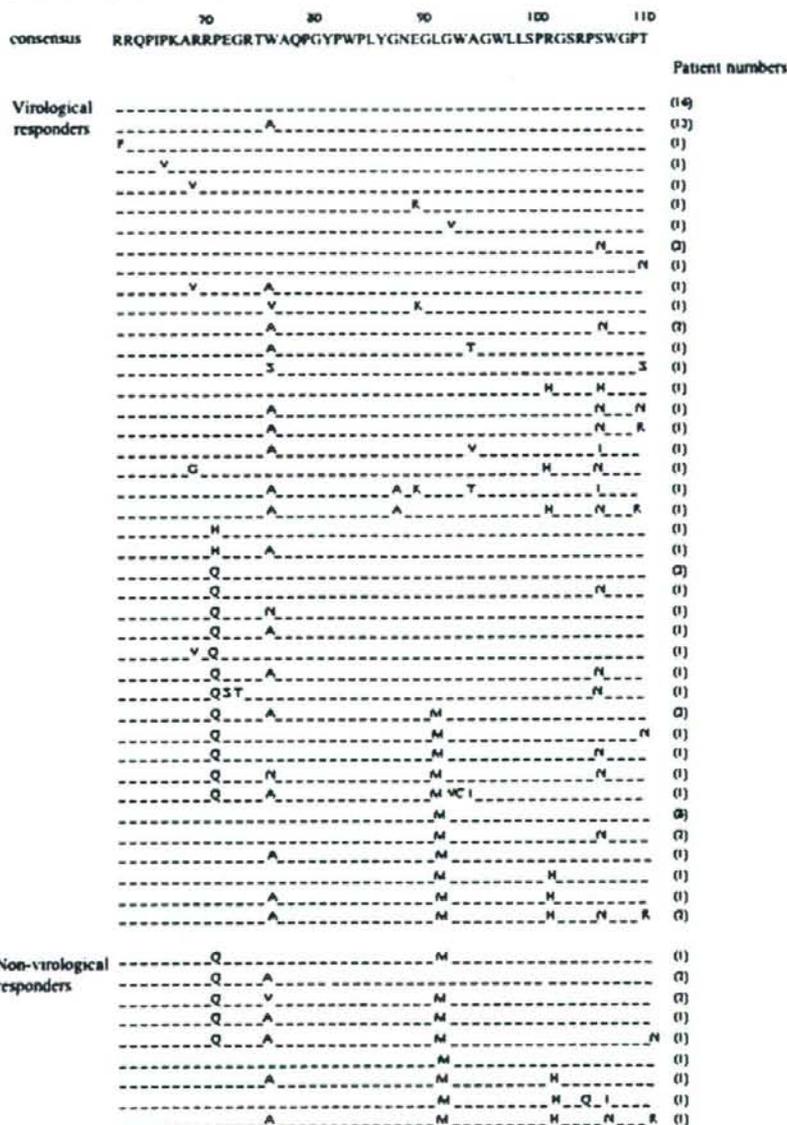
Fig. 2. Sequences of amino acids 61–110 in the core region at commencement of combination therapy in 93 patients infected with hepatitis C virus genotype 1b. Dashes indicate amino acids identical to the consensus sequence of genotype 1b, and substituted amino acids are shown by standard single-letter codes.

TABLE V. Amino Acid Substitutions in the Core Region in Non-Virologic Responders and Virological Responders in 93 Patients With HCV Genotype 1b

Presence of substitution site	Non-virological response (n = 11) % (n)	Virological response (n = 82) % (n)	P
aa 70	64 (7)	23 (19)	0.01
aa 75	73 (8)	45 (37)	0.11
aa 91	82 (9)	30 (25)	0.001
aa 106	27 (3)	31 (26)	1.0
aa 110	18 (2)	12 (10)	62
aa 70 and 91	45 (5)	10 (8)	0.006
aa 70 and/or 91	100 (11)	44 (36)	<0.001

aa, amino acid.

substitutions of aa. Among aa substitutions, only substitutions of aa 70 and 91 were associated with non-virological response. All non-virological responders had aa substitutions at 70 or 91, or both substitutions. In contrast, only 36 of 82 (44%) virological responders had these substitutions ($P < 0.001$, Table V). In contrast to non-virological response, these substitutions were not predictive for sustained virological response ($P = 0.11–0.82$).

Next, the effect of substitutions of aa 70 and 91 in the core region on early viral kinetics was analyzed by dividing patients into four groups according to the pattern of aa substitutions. As shown in Figure 3, the most rapid decrease in core antigen was noted in patients where both aa 70 and 91 were wild-type (double-wild). In contrast, the poorest reduction was

noted in patients with both of aa 70 and 90 substitutions (double-mutant). Patients with either of the two aa substitutions (mutant/wild or wild/mutant) showed decrease in between the above two groups. HCV core antigen decreased below the detectable limit (20 fmol/L) at week 4 in 37 of 40 (93%) patients who had neither aa 70 nor aa 90 substitutions. In contrast, it decreased below the detectable limit in only 5 of 12 patients (42%) who had both aa 70 and 91 substitutions ($P = 0.031$).

Analysis of Nucleotide Sequence of the NS5A Gene

The aa sequences of ISDR in the NS5A gene were determined in 40 patients where PCR for this region was positive. Seventeen of 40 patients had no aa
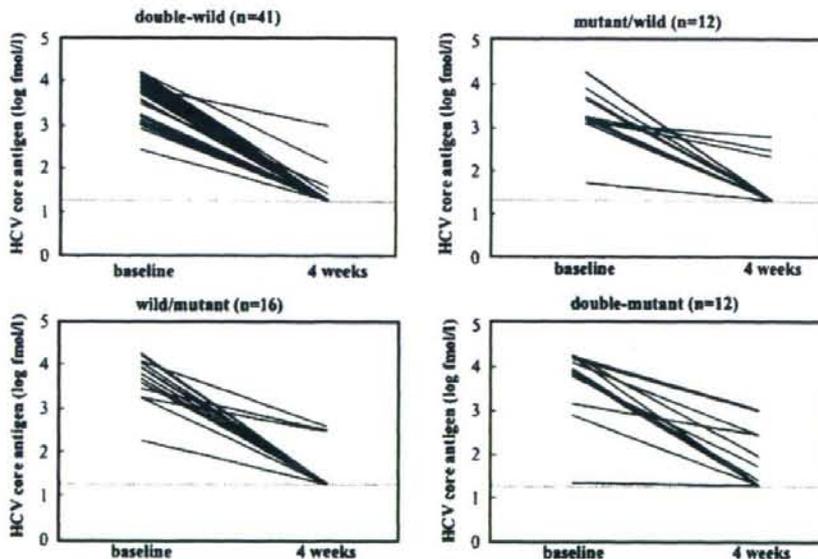

Fig. 3. Reduction of amount of HCV core antigen based on the presence of substitutions at amino acid 70 or 91. Eighty-one patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. The response was divided into four patterns based on the presence of substitution(s) at aa 70 and/or 91. Double-wild; no substitution, neither at aa 70 nor aa 91, mutant/wild; substitution only at aa 70, wild/mutant; substitution only at aa 91, double-mutant; substitutions at both aa 70 and 91. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.

TABLE VI. Amino Acid Substitutions in the IFN-Sensitive Determining Region (ISDR) in Non-Virologic Responders and Virological Responders in 40 Patients With HCV Genotype 1b

ISDR[a]	Non-virological response (n = 8) % (n)	Virological response (n = 32) % (n)	P
Wild-type (n = 17)	36 (6)	64 (11)	
Mutant-type (n = 23)	9 (2)	91 (21)	0.012

aa, amino acid.
[a]Absence of amino acid substitutions was evaluated as wild-type, and presence of one or more amino acid substitutions as mutant-type.

substitutions in ISDR (wild-type), while the remaining 23 patients had one or more substitutions (mutant-type). The relationship between aa substitutions of ISDR and effects of treatment was analyzed. The existence of aa substitution in the ISDR was not predictive for sustained virological response ($P = 0.137$), however, such substitution was observed frequently in virological responders compared to non-virological responders (66% vs. 25%, $P = 0.012$) (Table VI). The use of a different categorization based on the number of substitutions in the ISDR (0/1 vs. $\geq$2) yielded similar results, that is, not predictive for sustained viral response but predictive for virological responders (data not shown).

HCV core antigen decreased more rapidly in patients with ISDR mutant-type compared to those with wild-type (Fig. 4). HCV core antigen decreased below the detectable limit at week 4 in only 6 of 17 (35%) patients with wild-type. In contrast, it decreased below the detectable limit in 19 of 23 (83%) in patients with ISDR mutant-type ($P = 0.006$).

Predictive Factors Associated With Sustained Virological Response and Non-Virological Response in Patients With Genotype 1b

Finally, the predictive factors associated with sustained virological response and non-virological response were analyzed in patients with genotype 1b, including aa substitutions in the core region and ISDR. Univariate analysis showed two parameters correlated with sustained virological response: age (<60 years, $P = 0.004$) and presence of aa substitutions in the core (aa 70 and/or 91, $P = 0.04$). However, multivariate analysis, including the above variables, identified no parameters that influenced sustained virological response independently (age, $P = 0.89$; core, $P = 0.07$). Univariate analysis showed two parameters correlated with non-virological response: age (<65 years, $P = 0.02$) and aa substitutions in the core (double-mutant, $P = 0.01$). Multivariate analysis including the above variables identified aa substitutions in the core as an independent factor that influenced non-virological response (age, $P = 0.40$; core, $P = 0.03$) (Table VII).

DISCUSSION

Treatment of patients with chronic HCV infection had improved by the advent of PEG-IFN and RBV combination therapy. However, a substantial number of patients do not respond to the combination therapy [Taliani et al., 2006]. Several studies described attempts to improve the sustained virological response rate in such patients. Recent trials showed that a longer treatment period results in a higher sustained virological response rate [Berg et al., 2006; Sánchez-Tapias et al., 2006]. However, there are no conclusive studies that compared a larger dose of IFN with standard dose. Although the treatment had shifted in recent years to PEG-IFN and
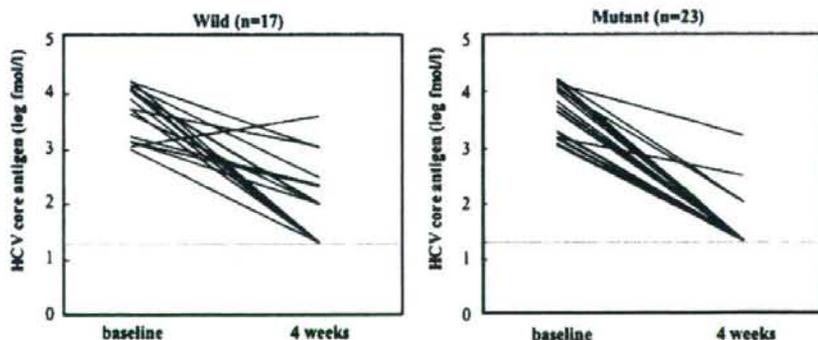

Fig. 4. Reduction of amount of HCV core antigen based on the presence of substitutions in the ISDR. Sixty-five patients infected with hepatitis C virus were treated with combination therapy. Serum HCV core antigen was measured before treatment (baseline) and at week 4. Patients were divided into two groups based on the presence of amino acid substitution(s) in the ISDR. Wild-type; absence of substitutions, mutant-type; presence of one or more substitutions. The fixed-quantity bottom value of HCV core antigen was 20 fmol/L calculated 1.3 in log, indicated by the dotted lines.