Real-time RT-PCR analysis

Total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA was used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using Quanti Tect SYBR Green PCR Master Mix (QIAGEN, Valencia, CA) and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers used were as follows: HCV-JFH1 sense (positions 7090 to 7109; 5′-TCA GAC AGA GCC TGA GTC CA-3′), HCV-JFH1 antisense (positions 7404 to 7423; 5′-AGT TGC TGG AGG GCT TCT GA-3′), beta-actin sense (5′-ACA ATG AAG ATC AAG ATC ATT GCT CCT CCT-3′), and beta-actin antisense (5′-TTT GCG GTG GAC GAT GGA GGG GCC GGA CTC-3′).

Quantification of HCV core antigen in the culture supernatant

The culture supernatants of JFH1-RNA transfected Huh-7.5.1 cells were collected on the days indicated, passed through a 0.45 μm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80 °C. The levels of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Tanabe et al., 2004; Yokota et al., 2003). Briefly, 10 μg of total cell lysate was separated by SDS–PAGE and blotted onto a polyvinylidene fluoride (PVDF) western blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti-IgG antibody and visualized by chemiluminescence using the ECL western blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody 2H9 (provided by Dr. Wakita), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-eIF2-alpha, anti-phospho-eIF2-alpha rabbit polyclonal antibody (Cell Signaling, Danvers, CA), and anti-beta-actin antibody (Sigma).

Immunocytochemistry

HCV-JFH1-transfected or infected Huh-7.5.1 cells were cultured in Lab-Tek® Chamber Slide™ (Nalge Nunc International, Rochester, NY) or on 22-mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV-core and GRP78, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 h at 37 °C and with Alexa Fluor 488 goat anti-mouse IgG antibody or Alexa Fluor 568 donkey anti-goat IgG antibody (Molecular Probes, Eugene, OR) for 1 h at room temperature. To analyze apoptosis of HCV-JFH1 infected cells, double staining for annexin V-FITC binding and for cellular DNA using propidium iodide (PI) was performed using an annexin V-Fluorescein Staining Kit (Wako, Osaka, Japan). Cells were visualized by a fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Plaque assay

Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at a density of $2–4 \times 10^5$ cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed, and the cell monolayers were overlaid with 8 ml of culture medium (DMEM, 2 mmol/l L-glutamine and 10% fetal bovine serum) that contained 0.8% methylcellulose. After 7 to 12 days of incubation under normal culture conditions, formation of cytopathic plaque was visualized by staining the cell monolayers with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (PFU/ml). Similarly, the titers of infectivity were evaluated by performing immunocytochemistry to detect foci of HCV-core-positive cells and calculating the infectious focus-forming units (FFU/ml).

Sequence analyses

The cDNA from the isolated JFH1 plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequence determination. Nucleotide sequences were read from both strands using Big Dye Terminator Cycle Sequencing Ready Reaction kits (Applied Biosystems) and an automated DNA sequencer (ABI PRISM® 310 Genetic Analyzer; Applied Biosystems).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5B region of JFH1, plasmid pJFH1 was digested with *Hin*dIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescriptII SK+ phagemid vector (Stratagene, La Jolla, CA). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-ChangeII Site-Directed Mutagenesis Kit; Stratagene): C9153T and G9295C, respectively. Finally, these *Hin*dIII–*Hin*dIII fragments were subcloned back into the parental plasmid pJFH1. The mutation T7662A-introduced PCR fragment (nt. 7421–7839) was subcloned into the T-Vector (pGEM-T Easy Vector Systems; Promega) and digested with *Rsr*II and *Bsr*GI. Finally, these *Rsr*II–*Bsr*GI fragments were subcloned back into the parental plasmid.

Statistical analyses

Statistical analyses were performed using the Student's *t*-test, and *p*-values of less than 0.05 were considered as statistically significant.

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References

Bartenschlager, R., Lohmann, V., 2000. Replication of hepatitis C virus. J. Gen. Virol. 81 (Pt 7), 1631–1648.

Benali-Furet, N.L., Chami, M., Houel, L., De Giorgi, F., Vernejoul, F., Lagorce, D., Buscail, L., Bartenschlager, R., Ichas, F., Rizzuto, R., Paterlini-Brechot, P., 2005. Hepatitis C virus core triggers apoptosis in liver cells by inducing ER stress and ER calcium depletion. Oncogene 24 (31), 4921–4933.

Blight, K.J., Kolykhalov, A.A., Rice, C.M., 2000. Efficient initiation of HCV RNA replication in cell culture. Science 290 (5498), 1972–1974.

Blight, K.J., McKeating, J.A., Rice, C.M., 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. J. Virol. 76 (24), 13001–13014.

Borisevich, V., Seregin, A., Nistler, R., Mutabazi, D., Yamshchikov, V., 2006. Biological properties of chimeric West Nile viruses. Virology 349 (2), 371–381.

Canbay, A., Friedman, S., Gores, G.J., 2004. Apoptosis: the nexus of liver injury and fibrosis. Hepatology 39 (2), 273–278.

Cerny, A., Chisari, F.V., 1999. Pathogenesis of chronic hepatitis C: immunological features of hepatic injury and viral persistence. Hepatology 30 (3), 595–601.

Choukhi, A., Ung, S., Wychowski, C., Dubuisson, J., 1998. Involvement of endoplasmic reticulum chaperones in the folding of hepatitis C virus glycoproteins. J. Virol. 72 (5), 3851–3858.

Chuma, M., Sakamoto, M., Yamazaki, K., Ohta, T., Ohki, M., Asaka, M., Hirohashi, S., 2003. Expression profiling in multistage hepatocarcinogenesis: identification of HSP70 as a molecular marker of early hepatocellular carcinoma. Hepatology 37 (1), 198–207.

Despres, P., Frenkiel, M.P., Deubel, V., 1993. Differences between cell membrane fusion activities of two dengue type-1 isolates reflect modifications of viral structure. Virology 196 (1), 209–219.

Despres, P., Flamand, M., Ceccaldi, P.E., Deubel, V., 1996. Human isolates of dengue type 1 virus induce apoptosis in mouse neuroblastoma cells. J. Virol. 70 (6), 4090–4096.

Ferri, K.F., Kroemer, G., 2001. Organelle-specific initiation of cell death pathways. Nat. Cell Biol. 3 (11), E255–E263.

Ghavami, S., Hashemi, M., Kadkhoda, K., Alavian, S.M., Bay, G.H., Los, M., 2005. Apoptosis in liver diseases—detection and therapeutic applications. Med. Sci. Monit. 11 (11), RA337–RA345.

Gosert, R., Egger, D., Lohmann, V., Bartenschlager, R., Blum, H.E., Bienz, K., Moradpour, D., 2003. Identification of the hepatitis C virus RNA replication complex in Huh-7 cells harboring subgenomic replicons. J. Virol. 77 (9), 5487–5492.

Harding, H.P., Zhang, Y., Ron, D., 1999. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature 397 (6716), 271–274.

He, B., 2006. Viruses, endoplasmic reticulum stress, and interferon responses. Cell Death Differ. 13 (3), 393–403.

Hinshaw, V.S., Olsen, C.W., Dybdahl-Sissoko, N., Evans, D., 1994. Apoptosis: a mechanism of cell killing by influenza A and B viruses. J. Virol. 68 (6), 3667–3673.

Jordan, R., Wang, L., Graczyk, T.M., Block, T.M., Romano, P.R., 2002. Replication of a cytopathic strain of bovine viral diarrhea virus activates PERK and induces endoplasmic reticulum stress-mediated apoptosis of MDBK cells. J. Virol. 76 (19), 9588–9599.

Kato, T., Furusaka, A., Miyamoto, M., Date, T., Yasui, K., Hiramoto, J., Nagayama, K., Tanaka, T., Wakita, T., 2001. Sequence analysis of hepatitis C virus isolated from a fulminant hepatitis patient. J. Med. Virol. 64 (3), 334–339.

Kato, T., Date, T., Miyamoto, M., Furusaka, A., Tokushige, K., Mizokami, M., Wakita, T., 2003. Efficient replication of the genotype 2a hepatitis C virus subgenomic replicon. Gastroenterology 125 (6), 1808–1817.

Kato, N., Nakamura, T., Dansako, H., Namba, K., Abe, K., Nozaki, A., Naka, K., Ikeda, M., Shimotohno, K., 2005. Genetic variation and dynamics of hepatitis C virus replicons in long-term cell culture. J. Gen. Virol. 86 (Pt 3), 645–656.

Kaufman, R.J., 1999. Stress signaling from the lumen of the endoplasmic reticulum: coordination of gene transcriptional and translational controls. Genes Dev. 13 (10), 1211–1233.

Koutsoudakis, G., Herrmann, E., Kallis, S., Bartenschlager, R., Pietschmann, T., 2007. The level of CD81 cell surface expression is a key determinant for productive entry of hepatitis C virus into host cells. J. Virol. 81 (2), 588–598.

Kummerer, B.M., Meyers, G., 2000. Correlation between point mutations in NS2 and the viability and cytopathogenicity of Bovine viral diarrhea virus strain Oregon analyzed with an infectious cDNA clone. J. Virol. 74 (1), 390–400.

Leifeld, L., Nattermann, J., Fielenbach, M., Schmitz, V., Sauerbruch, T., Spengler, U., 2006. Intrahepatic activation of caspases in human fulminant hepatic failure. Liver Int. 26 (7), 872–879.

Lesburg, C.A., Cable, M.B., Ferrari, E., Hong, Z., Mannarino, A.F., Weber, P.C., 1999. Crystal structure of the RNA-dependent RNA polymerase from hepatitis C virus reveals a fully encircled active site. Nat. Struct. Biol. 6 (10), 937–943.

Liberman, E., Fong, Y.L., Selby, M.J., Choo, Q.L., Cousens, L., Houghton, M., Yen, T.S., 1999. Activation of the grp78 and grp94 promoters by hepatitis C virus E2 envelope protein. J. Virol. 73 (5), 3718–3722.

Lindenbach, B.D., Evans, M.J., Syder, A.J., Wolk, B., Tellinghuisen, T.L., Liu, C.C., Maruyama, T., Hynes, R.O., Burton, D.R., McKeating, J.A., Rice, C.M., 2005. Complete replication of hepatitis C virus in cell culture. Science 309 (5734), 623–626.

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 (5424), 110–113.

Maekawa, S., Enomoto, N., Sakamoto, N., Kurosaki, M., Ueda, E., Kohashi, T., Watanabe, H., Chen, C.H., Yamashiro, T., Tanabe, Y., Kanazawa, N., Nakagawa, M., Sato, C., Watanabe, M., 2004. Introduction of NS5A mutations enables subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. J. Viral Hepatitis 11 (5), 394–403.

Mendez, E., Ruggli, N., Collett, M.S., Rice, C.M., 1998. Infectious bovine viral diarrhea virus (strain NADL) RNA from stable cDNA clones: a cellular insert determines NS3 production and viral cytopathogenicity. J. Virol. 72 (6), 4737–4745.

Meyers, G., Thiel, H.J., 1996. Molecular characterization of pestiviruses. Adv. Virus Res. 47, 53–118.

Mita, A., Hashikura, Y., Tagawa, Y., Nakayama, J., Kawakubo, M., Miyagawa, S., 2005. Expression of Fas ligand by hepatic macrophages in patients with fulminant hepatic failure. Am. J. Gastroenterol. 100 (11), 2551–2559.

Mori, K., 2000. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101 (5), 451–454.

Morikawa, K., Zhao, Z., Date, T., Miyamoto, M., Murayama, A., Akazawa, D., Tanabe, J., Sone, S., Wakita, T., 2007. The roles of CD81 and glycosaminoglycans in the adsorption and uptake of infectious HCV particles. J. Med. Virol. 79 (6), 714–723.

Mottola, G., Cardinali, G., Ceccacci, A., Trozzi, C., Bartholomew, L., Torrisi, M.R., Pedrazzini, E., Bonatti, S., Migliaccio, G., 2002. Hepatitis C virus nonstructural proteins are localized in a modified endoplasmic reticulum of cells expressing viral subgenomic replicons. Virology 293 (1), 31–43.

Munro, S., Pelham, H.R., 1986. An Hsp70-like protein in the ER: identity with the 78 kD glucose-regulated protein and immunoglobulin heavy chain binding protein. Cell 46 (2), 291–300.

Nakagawa, M., Sakamoto, N., Tanabe, Y., Koyama, T., Itsui, Y., Takeda, Y., Chen, C.H., Kakinuma, S., Oooka, S., Maekawa, S., Enomoto, N., Watanabe, M., 2005. Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. Gastroenterology 129 (3), 1031–1041.

Pahl, H.L., 1999. Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol. Rev. 79 (3), 683–701.

Patel, T., Gores, G.J., 1995. Apoptosis and hepatobiliary disease. Hepatology 21 (6), 1725–1741.

Pavio, N., Romano, P.R., Graczyk, T.M., Feinstone, S.M., Taylor, D.R., 2003. Protein synthesis and endoplasmic reticulum stress can be modulated by the hepatitis C virus envelope protein E2 through the eukaryotic initiation factor 2alpha kinase PERK. J. Virol. 77 (6), 3578–3585.

Quaresma, J.A., Barros, V.L., Pagliari, C., Fernandes, E.R., Guedes, F., Takakura, C.F., Andrade Jr., H.F., Vasconcelos, P.F., Duarte, M.I., 2006. Revisiting the liver in human yellow fever: virus-induced apoptosis in hepatocytes associated with TGF-beta, TNF-alpha and NK cells activity. Virology 345 (1), 22–30.

Rodrigues, C.M., Brites, D., Serejo, F., Costa, A., Ramalho, F., De Moura, M.C., 2000. Apoptotic cell death does not parallel other indicators of liver damage in chronic hepatitis C patients. J. Viral Hepatitis 7 (3), 175–183.

Rust, C., Gores, G.J., 2000. Apoptosis and liver disease. Am. J. Med. 108 (7), 567–574.

Ryo, K., Kamogawa, Y., Ikeda, I., Yamauchi, K., Yonehara, S., Nagata, S., Hayashi, N., 2000. Significance of Fas antigen-mediated apoptosis in human fulminant hepatic failure. Am. J. Gastroenterol. 95 (8), 2047–2055.

Sato, H., Takimoto, T., Tanaka, S., Ogura, H., Shiraishi, K., Tanaka, J., 1989. Cytopathic effects induced by Epstein–Barr virus replication in epithelial nasopharyngeal carcinoma hybrid cells. J. Virol. 63 (8), 3555–3559.

Shinoura, N., Yoshida, Y., Tsunoda, R., Ohashi, M., Zhang, W., Asai, A., Kirino, T., Hamada, H., 1999. Highly augmented cytopathic effect of a fiber-mutant E1B-defective adenovirus for gene therapy of gliomas. Cancer Res. 59 (14), 3411–3416.

Shuda, M., Kondoh, N., Imazeki, N., Tanaka, K., Okada, T., Mori, K., Hada, A., Arai, M., Wakatsuki, T., Matsubara, O., Yamamoto, N., Yamamoto, M., 2003. Activation of the ATF6, XBP1 and grp78 genes in human hepatocellular carcinoma: a possible involvement of the ER stress pathway in hepatocarcinogenesis. J. Hepatol. 38 (5), 605–614.

Su, H.L., Liao, C.L., Lin, Y.L., 2002. Japanese encephalitis virus infection initiates endoplasmic reticulum stress and an unfolded protein response. J. Virol. 76 (9), 4162–4171.

Sumpter Jr., R., Loo, Y.M., Foy, E., Li, K., Yoneyama, M., Fujita, T., Lemon, S.M., Gale Jr., M., 2005. Regulating intracellular antiviral defense and permissiveness to hepatitis C virus RNA replication through a cellular RNA helicase, RIG-I. J. Virol. 79 (5), 2689–2699.

Takashima, M., Kuramitsu, Y., Yokoyama, Y., Iizuka, N., Toda, T., Sakaida, I., Okita, K., Oka, M., Nakamura, K., 2003. Proteomic profiling of heat shock protein 70 family members as biomarkers for hepatitis C virus-related hepatocellular carcinoma. Proteomics 3 (12), 2487–2493.

Tanabe, Y., Sakamoto, N., Enomoto, N., Kurosaki, M., Ueda, E., Maekawa, S., Yamashiro, T., Nakagawa, M., Chen, C.H., Kanazawa, N., Kakinuma, S., Watanabe, M., 2004. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J. Infect. Dis. 189 (7), 1129–1139.

Tardif, K.D., Mori, K., Siddiqui, A., 2002. Hepatitis C virus subgenomic replicons induce endoplasmic reticulum stress activating an intracellular signaling pathway. J. Virol. 76 (15), 7453–7459.

Tardif, K.D., Mori, K., Kaufman, R.J., Siddiqui, A., 2004. Hepatitis C virus suppresses the IRE1-XBP1 pathway of the unfolded protein response. J. Biol. Chem. 279 (17), 17158–17164.

Thompson, C.B., 1995. Apoptosis in the pathogenesis and treatment of disease. Science 267 (5203), 1456–1462.

Vaughn, D.W., Hoke Jr., C.H., 1992. The epidemiology of Japanese encephalitis: prospects for prevention. Epidemiol. Rev. 14, 197–221.

Wakita, T., Pietschmann, T., Kato, T., Date, T., Miyamoto, M., Zhao, Z., Murthy, K., Habermann, A., Krausslich, H.G., Mizokami, M., Bartenschlager, R., Liang, T.J., 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat. Med. 11 (7), 791–796.

Waxman, L., Whitney, M., Pollok, B.A., Kuo, L.C., Darke, P.L., 2001. Host cell factor requirement for hepatitis C virus enzyme maturation. Proc. Natl. Acad. Sci. U. S. A. 98 (24), 13931–13935.

Yanagiya, A., Jia, Q., Ohka, S., Horie, H., Nomoto, A., 2005. Blockade of the poliovirus-induced cytopathic effect in neural cells by monoclonal antibody against poliovirus or the human poliovirus receptor. J. Virol. 79 (3), 1523–1532.

Yi, M., Ma, Y., Yates, J., Lemon, S.M., 2007. Compensatory mutations in E1, p7, NS2, and NS3 enhance yields of cell culture-infectious intergenotypic chimeric hepatitis C virus. J. Virol. 81 (2), 629–638.

Yokota, T., Sakamoto, N., Enomoto, N., Tanabe, Y., Miyagishi, M., Maekawa, S., Yi, L., Kurosaki, M., Taira, K., Watanabe, M., Mizusawa, H., 2003. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 4 (6), 602–608.

Yu, C.Y., Hsu, Y.W., Liao, C.L., Lin, Y.L., 2006. Flavivirus infection activates the XBP1 pathway of the unfolded protein response to cope with endoplasmic reticulum stress. J. Virol. 80 (23), 11868–118680.

Zheng, Y., Gao, B., Ye, L., Kong, L., Jing, W., Yang, X., Wu, Z., Ye, L., 2005. Hepatitis C virus non-structural protein NS4B can modulate an unfolded protein response. J. Microbiol. 43 (6), 529–536.

Zhong, J., Gastaminza, P., Cheng, G., Kapadia, S., Kato, T., Burton, D.R., Wieland, S.F., Uprichard, S.L., Wakita, T., Chisari, F.V., 2005. Robust hepatitis C virus infection in vitro. Proc. Natl. Acad. Sci. U. S. A. 102 (26), 9294–9299.

Zhong, J., Gastaminza, P., Chung, J., Stamataki, Z., Isogawa, M., Cheng, G., McKeating, J.A., Chisari, F.V., 2006. Persistent hepatitis C virus infection in vitro: coevolution of virus and host. J. Virol. 80 (22), 11082–11093.

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特集II 高齢者C型慢性肝炎に対する治療のあり方

ISDRからみた高齢者のC型慢性肝炎に対する治療法*

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Key Words：interferon, ISDR, amino acid mutations in HCV core region

はじめに

C型慢性肝炎に対するインターフェロン(interferon：IFN)治療は，PEG-IFNとRibavirin併用療法により格段に進歩した．しかし，いまだ難治例が存在し，1型高ウイルス量症例ではウイルス排除率(Sustained virologic response：SVR)率は50%に満たない[1)2)]．とくに高齢者では，治療完遂しても治療効果が劣るとともに，副作用の出現率が高いことから十分な治療が行えないことも，大きな要因と考えられている．一方，治療効果を規定する因子としては，年齢・性別などの宿主因子のみならず，ウイルス側の因子も重要視されている．とくに1b型C型肝炎ウイルス(Hepatitis C virus：HCV)のIFN感受性領域(Interferon sensitivity determining region：ISDR)は，治療効果を規定する因子として重要であることを，われわれは報告してきた[3)4)]．そこで，本稿ではISDRからみた高齢者に対するIFN療法について述べる．

1b高ウイルス量症例に対するPEG-IFN/Ribavirin併用療法の治療成績

当科および関連施設で構成する共同研究Y-PERS(Yamanashi-PEG-Interferon α2b- Ribavirin Study)で集積されたPEG-IFN/Ribavirin併用療法を施行した465症例を検討した．初回治療の場合は高ウイルス量症例に限られるが，1b型に対する48週治療，2a型および2b型の24週治療のSVR率はそれぞれ45%(63/140)，77%(17/22)，68%(13/19)であった(図1)．とくに，難治の1b型かつ高ウイルス量症例に関して，60歳未満/以上，男性/女性に分けて検討すると，60歳未満では男女差がないものの，60歳以上では，男性44%に対し女性22%のSVR率で，高齢女性のSVR率がきわめて悪いことが明らかになった(図2)．この理由としては，高齢者，とくに女性では，治療中止・減量率が高いことが考えられた．そこで，治療完遂率と完遂者におけるSVRの関連について検討した．この結果は，60歳以上の女性では治療完遂率は61%と，他と比べて若干劣るものの，有意な差はみられなかった．しかし，SVR率は36%であり，60歳以上の高齢女性は，たとえ治療完遂してもSVR率が悪いことが判明した．したがって，高齢者の治療成績が劣ることは，単に副作用による治療中止・減量によるものではなく，他の原因があると考えられた．これまで，この理由として，HCVの持続感染期間の差による線維化進展の可能性(すなわち高齢者では線維化進展例が多い)や，女性特有のホルモン環境の相違，

* Mutations in ISDR on efficacy of interferon therapy for elderly chronic hepatitis C patients.

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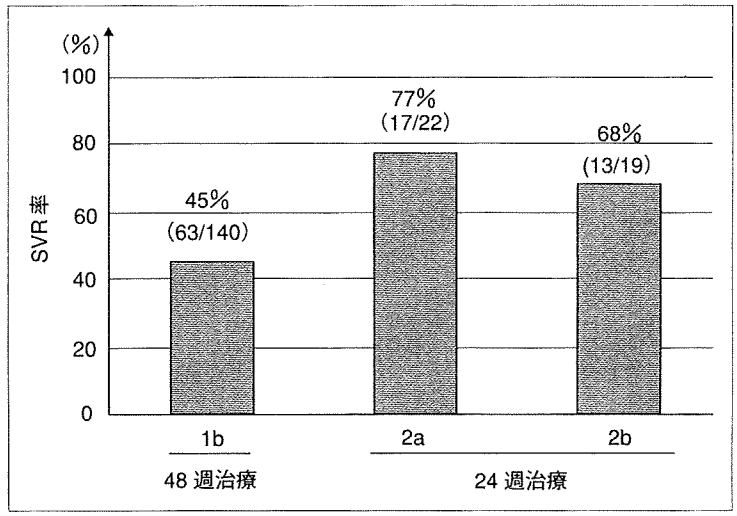

図1 PEG-IFN/Ribavirin併用療法におけるgenotypeとウイルス排除率(SVR)率

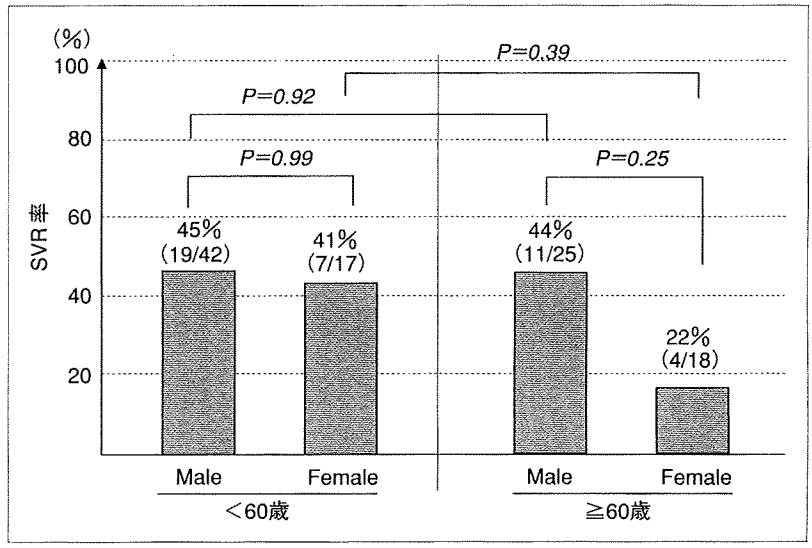

図2 PEG-IFN/Ribavirin併用療法における年齢・性別別のウイルス排除率(SVR)率
(n=102)

宿主の免疫反応によるHCV陰性化時期の遅延などが論じられてきたが，いまだ，明らかな結論は得られていない．

ISDRからみたPEG-IFN/Ribavirin併用療法の治療成績

1b型のHCVにおいては，IFN単独療法の治療成績を規定する因子として，ISDRの関与が明らかになっている．これはHCVのNS5A領域内の40アミノ酸領域で，この領域にアミノ酸変異のまったくない野生型(Wild type)では IFN単独療法のSVR率はきわめて低く，4個以上の変異がある変異型(Mutant type)では，きわめて高いSVR率を示し，1～3個の変異がある中間型(Intermediate type)では，この中間のSVR率を示す．PEG-IFN/Ribavirin併用療法においても，ISDR別にSVR率を検討すると，野生型では37%(29/78)，中間型では44%(19/43)であるのに対し，変異型では80%(12/15)であり，変異型は中間型や野生型に比べ有意にSVR率が高かった(図3)[5]．この理由は，

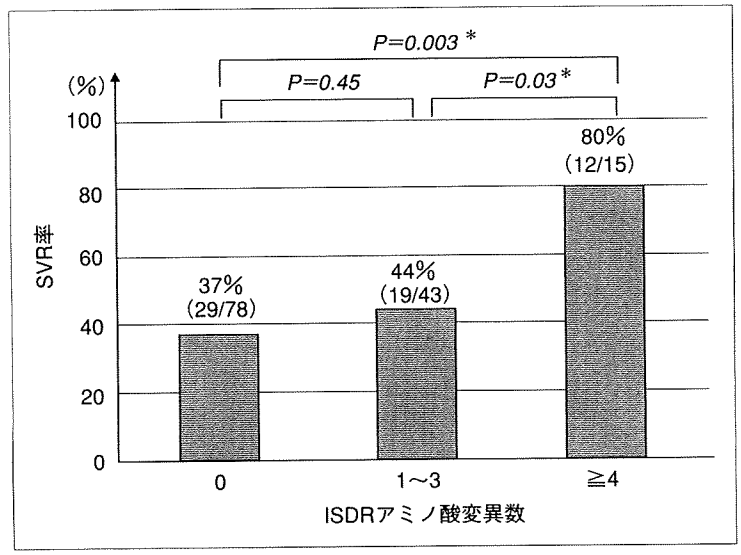

図3　PEG-IFN/Ribavirin併用療法におけるIFN感受性領域ウイルス排除率(SVR)率(1b症例 n=140)

* Significant difference

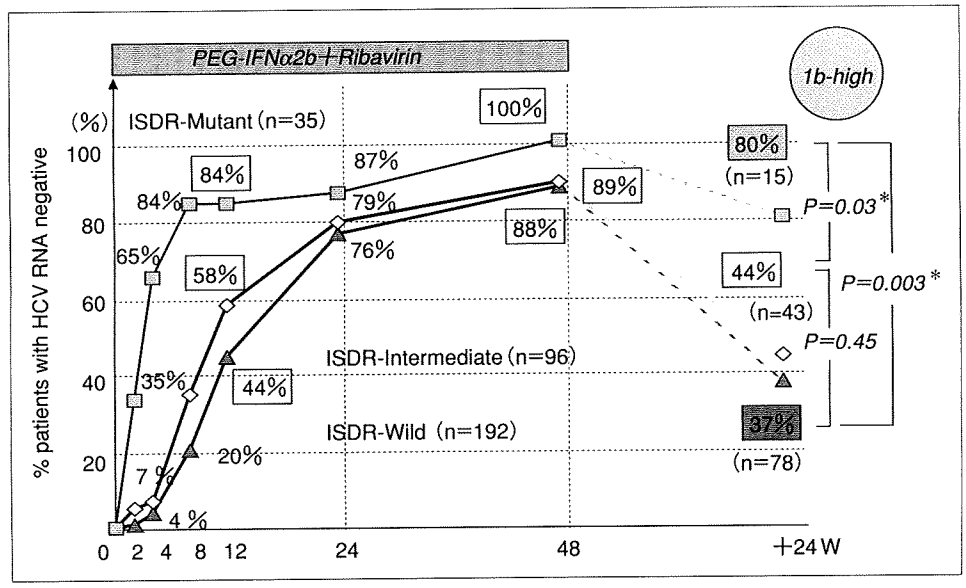

図4　PEG-IFN/Ribavirin併用療法におけるIFN感受性領域(ISDR)と経時的ウイルス陰性化率(1b症例 n=323)

* Significant difference

ISDRが治療早期のウイルス量の減少と関係しているためと考えられている．すなわち，ISDR変異型では野生型・中間型に比較して投与開始2週間ないしは4週間のウイルス量の減少率が高く，12週以内のウイルス消失(Early virologic response：EVR)に影響していた(図4)．EVRは治療効果(SVR)予測する重要な因子とされ[6]，1b型高ウイルス量症例ではEVRが得られた症例では48週間の治療により高率にSVRとなる．したがって，HCVのIFN反応性はウイルスダイナミクスにより判定することが可能であるが，これは，ISDRとも強く関連しているということである．さらに

表1 PEG-IFN/Ribavirin併用療法(1b)のウイルス排除率(SVR)に寄与する因子(多変量解析)(n=140)

	odds ratio (95% CI)	*P* value
年齢(<60歳/≧60歳)	0.453(0.191-1.073)	0.0719
肝線維化(F1/F2~4)	0.356(0.152-0.833)	0.0173
HCVコア蛋白量	0.454(0.196-1.055)	0.0663
ISDR(変異数<2/≧2)	5.125(1.590-16.520)	0.0062

多変量ロジステック回帰分析

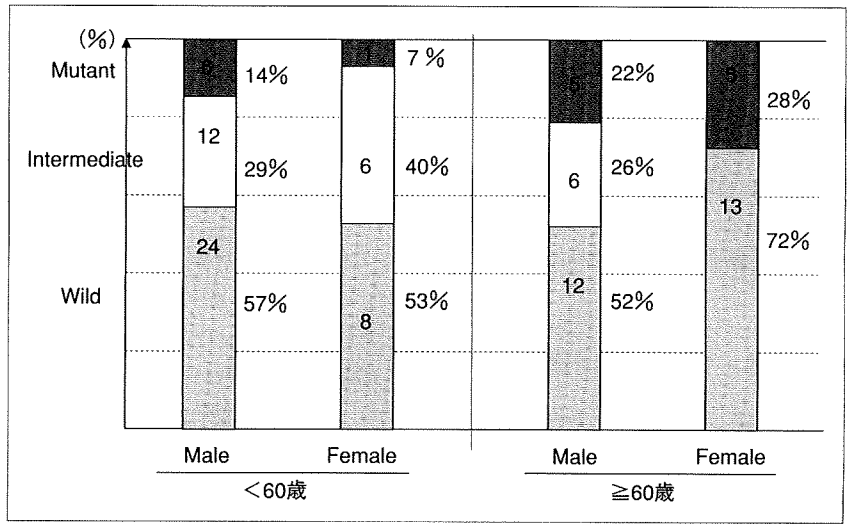

図5 年齢・性別とIFN感受性領域(ISDR)の分布(1b-high)(n=101)

SVRに寄与する因子について宿主側の因子を含めて，多変量ロジステック回帰分析を行なうと，抽出された因子は年齢，肝線維化(F2以上とそれ以外)とISDR変異数(2未満と2以上のみ)のみであり，この中でもISDRがもっとも強い因子であった(表1)．また，ISDRは治療開始前のHCV RNA量と相関しているが，多変量解析ではRNA量はISDRに勝る因子ではなく，投与前のウイルス量を1,000 KIU以上と未満とに分けて検討すると，同等のウイルス量群の中でも，変異型の効果が高いことが判明し，上記を裏づけているものと思われた[7]．

しかし，ISDR変異数が同じであっても治療反応性が異なる症例が存在し，ISDR変異数0ないしは1の難治症例でも，SVR例と非SVR例が存在する．そこで，われわれは，ISDR変異数0ないしは1の症例で，他のウイルス学的条件と臨床的条件がそろった症例で，IFN反応性が良好な症例とそうでない症例について，HCV全ゲノムの相違を検討した．その結果，両者に相違がみられたのは，コア領域とNS2領域のアミノ酸であり，統計学に有意な相違がみられたのはコア領域の70番目のアミノ酸のみであった．さらにこの領域に注目してretrospectiveに，IFN治療効果とこのアミノ酸変異との関連を検討すると，ISDRが0ないしは1の難治が予測される症例であっても，このアミノ酸がHCVのプロトタイプのHCV-Jにみられるアルギニン(R)であれば高率にSVRが期待できるものの，グルタミン(Q)に変異しているとIFN治療反応性がきわめて悪く，主治医の判断で治療を中断した症例や，治療完遂してもSVRにならない症例が多数を占めていた．同様の報告はAkutaらにより，コア領域の70番目と91番目のアミノ酸に変異がみられるとインターフェロン治療効果が劣り，とくに50歳以上の女性ではその傾向が顕著であることが報告されている[8]．

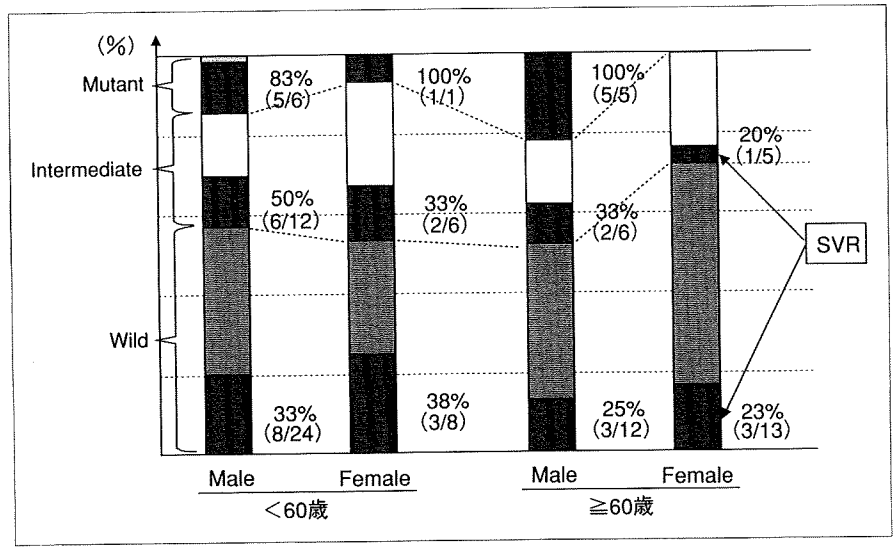

図6　年齢・性別とIFN感受性領域(ISDR)の分布とウイルス排除率(SVR)の関係(n=101)

ISDRと年齢・性別との関連

上記のようにISDRは治療効果と密接に関連しているが，これと年齢・性別との関連についても検討した．本検討では，ISDRの分布は野生型が多くを占め，中間型や変異型は少なかったが，60歳以上の女性には変異型の比率が低いことが判明した(図5)．各年代，男性/女性におけるISDR別の治療成績には差がほとんどみられないことから，ISDRの分布が各年代の治療性成績に関連し，60歳以上の女性のSVR率が低いことの一つの理由にISDRの分布の差が関与していることが推測された(図6)．しかし，この分布の理由については，変異型のHCVが，過去の治療や自然経過で駆逐されており，現在のPEG-IFN/Ribavirin併用療法の治療対象でないことが推測された．そこで，約10年ほど前のIFN単独療法時のISDRについても検討した．しかし，10年ほど前の50歳以上の女性でもISDRのmutant typeは少なく，もともとある年代以上の，女性にはこのtypeが少ない可能性が考えられた．すなわちこの集団の感染経路が異なりISDRの分布が異なる可能性や，女性ではmutant typeは持続感染しにくく，ある年代以降で自然治癒している可能性などが考えられるが，一定の結論が得られるような証拠は存在しない．しかし，かつてのIFN単独療法の時代でも，wild typeやintermediate typeの比率が高い高齢女性の治療成績はきわめて悪く，ISDRが関与している可能性も否定できなかった．しかし，この傾向は他の地域でも普遍的にみられるのかどうかなど，今後の検討課題も存在する．

ISDRからみた高齢者のC型慢性肝炎に対する治療法

これまでの検討で，ISDRはPEG-IFNを含むIFN単独治療ないしはPEG-IFN/Ribavirin併用療法において，治療効果を規定する重要な因子であることが明らかとなった．高齢女性にはISDR mutant typeが少ないことから，治療効果が低いことの一つの説明であることが推測された．しかし，この傾向が，他の地域や集団で普遍的なものであるかどうかなどは，今後の検討に委ねられるものと考えられる．しかしながら，ISDRは治療効果予測因子として非常に重要であり，とくに，副作用の出現率が高い高齢者では，治療法や治療期間を選択するうえで重要な情報となりえる可能性がある．われわれは，年齢・性別などの宿主要因とウイルス型，ウイルス量などのウイルス要因にISDRやコア領域変異を参考に治療方針を個々に検討している．すなわち，1bかつ高ウイルス量で高齢者のきわめて難治が予測される症例であってもISDRがmutant typeであれば積

極的に治療を行うが，wild typeかつコア70番のアミノ酸がQであれば，仮に治療開始してもNull responseであれば，副作用を鑑み早期に治療を中断ないしは，肝癌抑止のための治療に切り替えるなどの検討を行っている．今後は，さらなる症例の蓄積と解析により，ISDRの解析が高齢者を含めた個々の症例に応じたテーラーメード治療を可能にするものと考えられる．

文　献

1) Manns MP, McHutchison JG, Gordon SC, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C : a randomised trial. Lancet 2001 ; 358 : 958-65.
2) Fried NW, Schffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002 ; 347 : 975-82.
3) Enomoto N, Sakuma I, Asahina Y, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest 1995 ; 96 : 224-30.
4) Enomoto N, Sukuma I, Asahina Y, 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.
5) 坂本　穣，榎本信幸．ウイルス性慢性肝炎：診断と治療の進歩．日本内科学会誌 2008 ; 97 : 57-63.
6) Davis GL, Wong JB, McHutchison JG, et al. Early virologic response to treatment with peginterferon alfa-2b plus ribavirin in patients with chronic hepatitis C. Hepatology 2003 ; 38 : 645-52.
7) 坂本　穣，榎本信幸．ISDRと初期抗ウイルス効果からみた治療効果．In：坪内博仁・監，岡上　武，小俣政男，林　紀夫，ほか・編．コンセンサス肝疾患2007．B型肝炎・C型肝炎．東京：日本メディカルセンター；2007．p. 96-101.
8) Akuta N, Suzuki F, Sezaki H, et al. Predictors of Viral Kinetics to Peginterferon Plus Ribavirin Combination Therapy in Japanese Patients Infected With Hepatitis C Virus Genotype 1b. J Med Virol 2007 ; 79 : 1686-95.

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Interferon sensitivity determining region : ISDR

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索引用語：C型慢性肝炎，インターフェロン，ISDR，個別化医療

1 はじめに

C型慢性肝炎に対する従来のインターフェロン単独療法の，ウイルス排除(SVR: sustained virological response)率は30％程度に過ぎなかったが，ペグインターフェロンとリバビリン併用療法が行われるようになり，約70％でウイルス排除が可能となった．しかし，難治とされるgenotype 1bかつ高ウイルス量の慢性肝炎では，併用療法を48週間行っても，約半数でのみウイルス排除可能であるにすぎない．このインターフェロン治療反応性の違いについては，さまざまな検討がなされてきたが，ウイルス側の要因として，C型肝炎ウイルス(HCV)の遺伝子型(genotype)をはじめとする，さまざまな遺伝子変異の存在が明らかになってきた．特に，HCVの非翻訳領域(Nonstructural region: NS) 5Aに存在するインターフェロン感受性領域(Interferon sensitivity determining region: ISDR)のアミノ酸変異は，治療効果の予測因子として臨床応用可能であることがみいだされている．

2 C型肝炎ウイルス（HCV）の遺伝子構造とインターフェロン感受性領域（Interferon sensitivity determining region: ISDR)

C型肝炎ウイルス(Hepatitis C virus: HCV)は＋1本鎖のRNAウイルスであり，ゲノムの両端に非翻訳領域が存在し，中央部には約3,010個のアミノ酸からなる1本のポリ蛋白前駆体をコードするopen reading frameが存在する．この領域にはHCVの構造蛋白(コア，エンベロープ蛋白)とウイルス増殖に必要な種々の酵素をコードする非構造領域(Nonstructural region: NS)が存在する．このうち，NS5A領域のC末端よりの40アミノ酸(NS5A a.a. 2209-2248)領域は，かつて，1b型のHCVに対するインターフェロン単独療法の著効症例と無効症例の全塩基配列の比較検討から，治療効果に関連した遺伝子領域とし

Minoru SAKAMOTO et al : Interferon sensitivity determining region: ISDR
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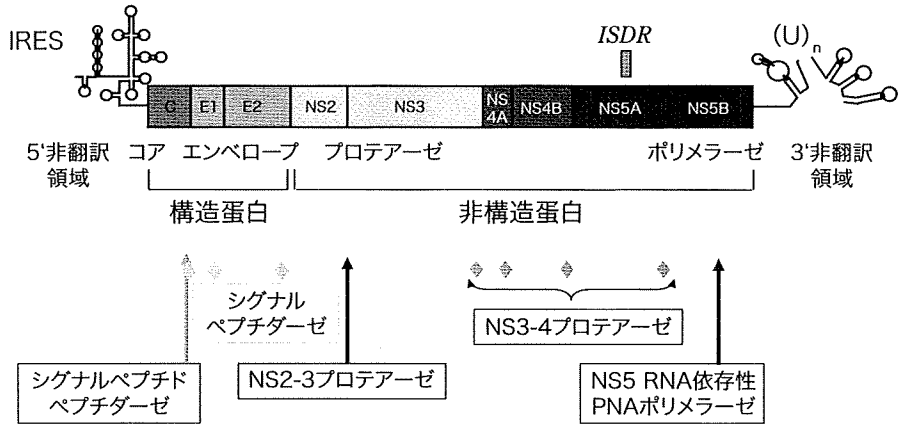

図1　C型肝炎ウイルスの遺伝子構造

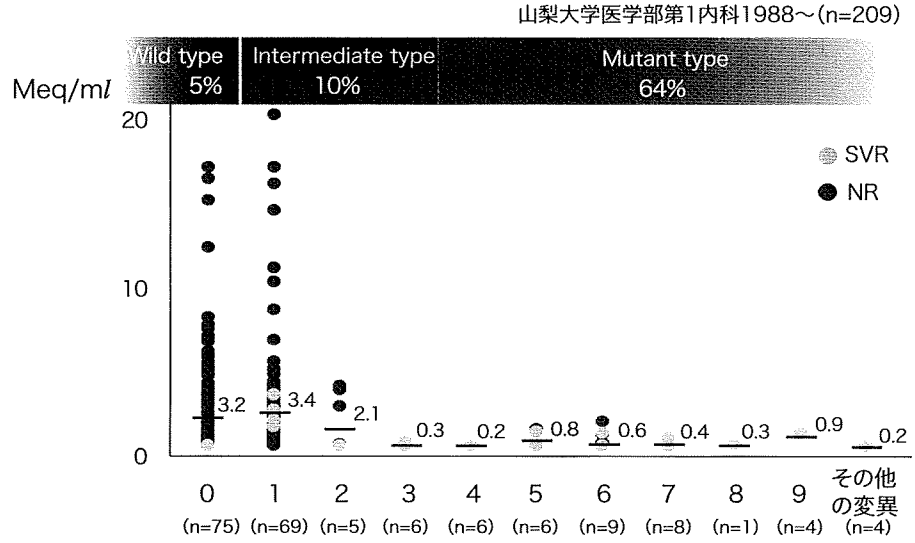

図2　IFN単独療法(6カ月)のISDR変異数と治療効果とウイルス量の関係

てみいだされ，インターフェロン感受性領域(Interferon sensitivity determining region: ISDR)と命名された(図1)[1,2]．すなわち，1b型のHCVの標準株であるHCV-Jと比較して，変異のない野生型(wild type)ではインターフェロン単独療法ではSVRとなる可能性は極めて低いのに対し，4個以上の変異がある変異型(mutant type)では高いSVR率を示し，1～3個の変異がある中間型(intermediate type)ではこの中間のSVR率を示す．この領域はPKR Binding domainのN末端側に位置し，ウイルス増殖と密接に関連している部位と考えられ，レプリコンを用いた細胞培養モデルでも適応変異(adaptive mutations)の集積部位である[3]．さらにISDRを含むNS5A領域から翻訳される蛋白は，種々のtyrosine kinas活性を調節していることも推測されており，この領域のアミノ酸変異が

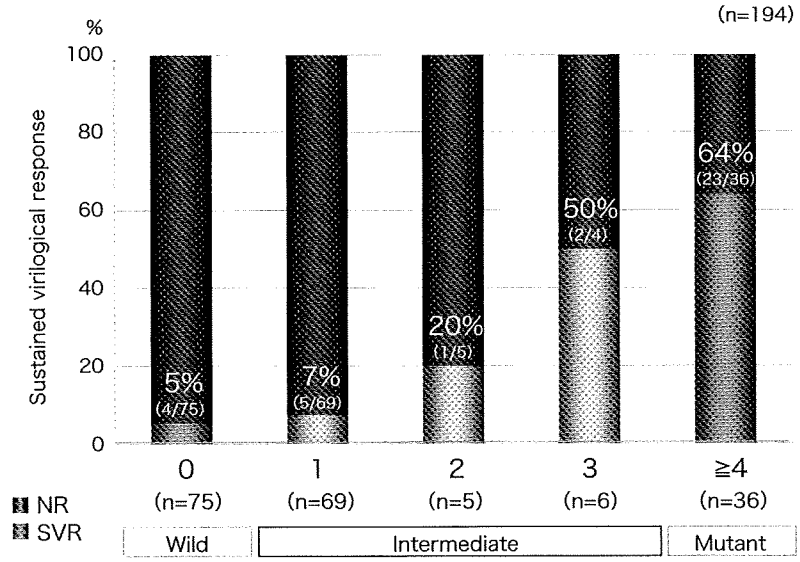

図3　IFN単独療法(6カ月)のISDR別SVR率

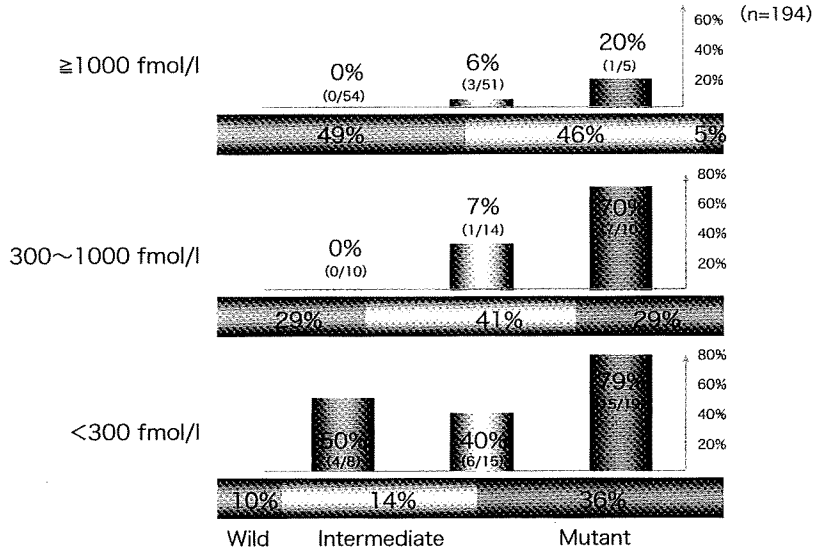

図4　IFN単独療法(6カ月)のHCV量とISDR別SVR率

HCVの増殖やインターフェロン感受性と関連することは，ウイルス学的にも容易に推測される．また，臨床的には，野生型ではウイルス量が多く，変異型ではウイルス量は低く，ISDRの変異数が増すほどウイルス量は減少することが明らかになっている(図2)．

3　インターフェロン単独療法とISDR変異

もともと，ISDRはインターフェロン単独療法の時代にみいだされたもので，1b型のC型慢性肝炎に対するインターフェロン単独6カ月間の治療成績をISDRのアミノ酸変異数

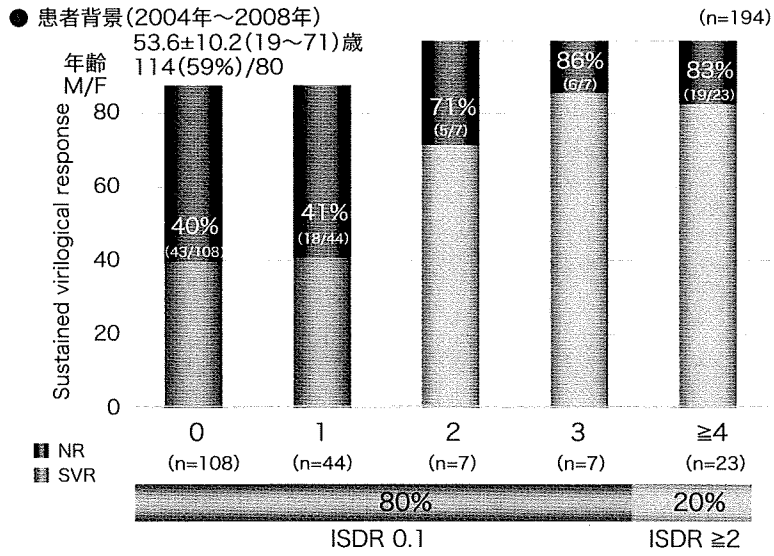

図5　PEG + Riba治療(12カ月)のISDR別SVR率(高ウイルス症例)

表1　PEG + Riba治療(1b)のSVRに寄与する因子(12カ月治療)(n=194)

		odds比	95% CI	p
年齢	＜60/≧60	0.255	0.057-0.886	0.0329
F因子	0-1/2-4	0.139	0.034-0.563	0.0061
白血球数	＜4600/≧4600	3.427	0.582-13.787	0.0828
ISDR変異数	0-1/2-	164.571	8.458-＞999.9	0.0008
コアAA70	Q/R	13.840	2.464-77.739	0.0028

多変量ロジステック回帰分析

別に検討すると，Wild type, Intermediate type, Mutant typeのSVR率はそれぞれ5%，10%，64%であり，ISDRにおけるアミノ酸変異数の増加に伴い，著効率は上昇するもののISDR変異数4個以上が重要であることが明らかにされている(図3)．インターフェロン単独療法では，治療効果はウイルス量と相関し，HCVRNA量が高いほど治療効果が劣るが，ISDR変異とHCVRNA量は相関することから，ISDR変異は単にウイルス量を反映したに過ぎないとの指摘もある．しかし，多変量解析では，ISDRはウイルス量とは関係なく，SVRに寄与する独立した因子として認められるし，ウイルス量が同等であれば，ISDR変異数が多いほど治療効果は高い(図4)．一方，genotype 2aないしは2bにおいては，総じて治療効果が高いため，ISDRは1b型ほど有意な治療効果規定因子とはなりえないが，ISDRのアミノ酸変異数が多いほど治療効果が高い傾向がある．また，最近では，インターフェロン単独療法はペグインターフェロン(PEG-IFN α 2a: ペガシス)を用いることが多いが，この現象はPEG-IFN α 2a (12カ月治療)でも認められる．

表2　PEG/Riba治療(1b-ISDR 0·1)のSVRに寄与する因子(n=157)

		odds比	95% CI	p
年齢	＜60/≧60	0.122	0.023-0.641	0.0129
F因子	0-1/2-4	0.066	0.011-0.407	0.0035
T.Chol	＜160/≧160	10.757	1.630-70.990	0.0136
コアAA70	Q/R	49.457	4.499-543.583	0.0014

多変量ロジステック回帰分析

4 ペグインターフェロン＋リバビリン併用療法とISDR変異

現在，1b型のHCVに対する標準治療は，ペグインターフェロン＋リバビリン併用療法であり，難治である1b型かつ高ウイルス量症例の治療成績を大きく向上させた．この治療法においても，ISDR変異はSVRを規定する独立した因子であり，宿主因子である肝の線維化とならび，最も重要な因子であった[4]．しかし，インターフェロン単独療法でSVRが得られるためにはISDRのアミノ酸変異は4個以上必要であったが，ペグインターフェロン＋リバビリン併用療法においては，2個以上の変異があれば80％以上の高い確率でSVRを期待できることが明らかになった(表1)．これは，インターフェロン単独療法に比較して，PEG-IFN＋リバビリン療法の抗ウイルス効果が格段に高いことに由来していると考えられるが，臨床的な血中リバビリン濃度では，HCVに対する単独の抗ウイルス効果はわずかに過ぎない．しかし，レプリコンシステムを用いた*in vitro*の検討では，インターフェロンにリバビリンを少量添加すると，濃度依存的にインターフェロンのHCV増殖抑制効果を高めることが確認され，リバビリンはインターフェロンの効果を相乗的に高めることが報告されている．さらに，リバビリンは，ウイルスのmutagenとして作用して，NS5A領域の遺伝子変異を増加させる現象がみいだされており，リバビリンの作用機序のひとつとして注目されている[5]．しかし，ISDRのアミノ酸変異数が0ないし1個であっても，SVRとなる症例は存在する．そこで，ISDRのアミノ酸変異数が0ないし1個の症例に限り，治療開始後4週間でウイルス量が法が2 log 以上低下したsteep responderと1 log未満しか低下しなかったflat responderにつき，HCV全ゲノムの相違を検討した．その結果，両者に相違がみられたのは，コア領域とNS2領域のアミノ酸であり，統計学に有意な相違がみられたのはコア領域の70番目のアミノ酸のみであった．さらにこの領域に注目してretrospectiveに，IFN治療効果とこのアミノ酸変異との関連を検討すると，ISDRが0ないしは1の難治が予測される症例であっても，このアミノ酸が，HCVのプロトタイプのHCV-Jにみられるアルギニン(R)であれば高率にSVRが期待できるものの，グルタミン(Q)に変異していると，IFN治療反応性が極めて悪く，主治医の判断で治療中断した症例や，治療完遂してもSVRにならない症例が多数を占め，ISDR変異数0ないし1個の症例ではコア70番のアミノ酸変異が，SVRを予測する最も重要な因子であった(表2)．1b型症例全体でもコア70番のアミノ酸変異は治療効果予測因子のひとつであるが，ISDR変異により，症例を細分化した

表3　C型慢性肝炎に対するテーラーメイド治療

1)遺伝子型
　2型：　インターフェロン単独
　　高ウイルス量ではペグインターフェロン+リバビリン24週
　1型：　ISDRとコア領域変異測定による方針決定
2) 1型におけるISDRとコアアミノ酸70番変異
　ISDR変異数4個以上：　インターフェロン単独
　ISDR変異数2個以上：　ペグインターフェロン+リバビリン48週
　ISDR変異数0・1個の場合：　下記のペグインターフェロン+リバビリンの成績をふまえ，検討する

<table>
<tr><th>ISDR変位数
コア70番アミノ酸</th><th>0</th><th>1</th><th>2個以上</th></tr>
<tr><td>R</td><td>72週投与投与により
70%</td><td>86%</td><td rowspan="2">81%</td></tr>
<tr><td>NonR（Q）</td><td colspan="2">72週投与(8週以内の陰性化例)により
40%</td></tr>
</table>

うえで，難治が予測されるISDR変異数0ないし1の症例においては，コアアミノ酸変異を検討することが重要であると考えられる．

5　ISDRからみたC型慢性肝炎に対する治療方針

HCVのゲノム解析により，インターフェロン治療の効果予測は可能となりつつある．すなわち遺伝子型が2aないしは2b型であれば，遺伝子変異に関わらず，インターフェロン単独療法でもある程度効果期待できるが，ウイルス量が多い場合は，ペグインターフェロン+リバビリン併用療法が望ましい．さらに，1b型でもISDR変異数が4個以上あれば，単独療法でも治癒可能であるし，2個以上あればペグインターフェロン+リバビリン療法48週間の治療でSVRが期待できる．またISDR変異数が0ないし1であってもコア70番のアミノ酸がRであればSVRが期待できるが，Qであれば治療には慎重である必要がある．しかし，ISDR変異数0・1でコア70番がQであっても，治療早期(8週以内)にウイルス陰性化が得られれば，72週間投与によりSVRとなる可能性が残されており，さらに，宿主因子である性別，年齢，肝線維化・脂肪化，初回・再治療，前治療の効果を考慮することによって，さらに詳細な治療効果予測が可能になると思われ，近い将来の，いわゆる個別化医療にHCVのゲノム解析の臨床応用が重要な意味を持っているものと考えられる(表3)．

文　献

1) Enomoto N, Sukuma I, Asahina Y 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 334 : 77–81, 1996
2) Enomoto N, Sakuma I, Asahina Y et al : Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino

acid substitutions in the NS5A region. J Clin Invest 96 : 224–230, 1995
3) Maekawa S, Ennomoto N, Sakamoto N et al : Intriduction of NS5A mutations enable subgenomic HCV replicon derived from chimpanzee-infectious HC-J4 isolate to replicate efficiently in Huh-7 cells. J Virol hepatitis 11 : 394–403, 2004
4) 坂本穣，榎本信幸：ウイルス性慢性肝炎：診断と治療の進歩.日本内科学会誌 97：57–63，2008
5) Tanabe Y, Sakamoto N, Enomoto N et al : Synergistic inhibition of intracellular hepatitis C virus replication by vombination of ribavirin and interferon-alpha. J Infect Dis 189 : 1129–1139, 2004

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Mutations in the interferon sensitivity determining region and virological response to combination therapy with pegylated-interferon alpha 2b plus ribavirin in patients with chronic hepatitis C-1b infection

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Abstract
Background Pegylated-interferon-alpha 2b (PEG-IFN) plus ribavirin (RBV) therapy is currently the de-facto standard treatment for hepatitis C virus (HCV) infection. The aims of this study were to analyze the clinical and virological factors associated with a higher rate of response in patients with HCV genotype 1b infection treated with combination therapy.
Methods We analyzed, retrospectively, 239 patients with chronic hepatitis C-1b infection who received 48 weeks of combination therapy. We assessed clinical and laboratory parameters, including age, gender, pretreatment hemoglobin, platelet counts, HCV RNA titer, liver histology, the number of interferon sensitivity determining region (ISDR) mutations and substitutions of the core amino acids 70 and 91. Drug adherence was monitored in each patient. We carried out univariate and multivariate statistical analyses of these parameters and clinical responses.
Results On an intention-to-treat (ITT) analysis, 98 of the 239 patients (41%) had sustained virological responses (SVRs). Patients with more than two mutations in the ISDR had significantly higher SVR rates ($P < 0.01$). Univariate analyses showed that stage of fibrosis, hemoglobin, platelet counts, ISDR mutations, serum HCV RNA level, and adherence to PEG-IFN plus RBV were significantly correlated with SVR rates. Multivariate analysis in subjects with good drug adherence extracted the number of ISDR mutations (two or more: odds ratio [OR] 5.181).
Conclusions The number of mutations in the ISDR sequence of HCV-1b (≥ 2) is the most effective parameter predicting a favorable clinical outcome of 48-week PEG-IFN plus RBV therapy in patients with HCV genotype 1b infection.

Keywords Hepatitis C virus (HCV) · Chronic hepatitis C · PEG-IFN plus RBV therapy · Combination therapy · Interferon sensitivity determining region (ISDR)

Abbreviations

HCV	Hepatitis C virus
IFN	Interferon
PEG	Polyethylene glycol
PEG-IFN	Pegylated-interferon-alpha 2b
RBV	Ribavirin
ISDR	Interferon sensitivity determining region
BMI	Body mass index

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ALT	Alanine transaminase
dM	Double mutant
ITT analysis	Intention-to-treat analysis
PP analysis	Per protocol analysis
SVR	Sustained virological response
ETR	End of treatment response
PKR	Double stranded RNA-dependent protein kinase
TLR	Toll-like receptor
MyD88	Myeloid differentiation primary response gene 88

Introduction

Hepatitis C virus (HCV) is one of the major pathogens causing chronic hepatitis [1, 2] and eradication of the virus by the host occurs infrequently during the natural course of infection once it becomes chronic. Interferon (IFN) has been used widely as the most effective antiviral agent for chronic hepatitis C. Although ribavirin (RBV), a synthetic guanosine analog, alone does not decrease the serum HCV RNA level [3–5], it has been shown that combination therapy with IFN-alpha (given 3 times weekly) and daily RBV gives a higher sustained response rate than IFN monotherapy [6–8]. Pegylation is the process by which an inert molecule of polyethylene glycol (PEG) is covalently attached to a protein, and the addition of PEG to IFN produces a biologically active molecule with a longer half-life and more favorable pharmacokinetics than the natural molecule. These characteristics allow more convenient, once-weekly dosing [9]. Pegylated (PEG)-IFN plus RBV is significantly more effective than IFN plus RBV or PEG-IFN alone for the treatment of chronic hepatitis C, with sustained virological response rates of $\sim$50% in patients infected with HCV genotype 1b [10].

We reported previously a close correlation between the number of mutations in the nonstructural 5A (NS5A) region of the HCV genome encoding amino acids (aa) at positions 2209–2248 [the IFN sensitivity determining region (ISDR)] and IFN efficacy in patients with HCV genotype 1b infection [11–13]. The aims of this study were to analyze clinical and virological factors associated with a higher rate of response by patients with HCV genotype 1b infection who were treated with combination therapy with pegylated-IFN-alpha 2b (PEG-IFN) plus RBV, and to clarify the relationship between ISDR mutations and virological response to the combination therapy.

Methods

Patients and methods

We analyzed, retrospectively, 239 patients with chronic HCV-1b infection who received combination therapy with PEG-IFN plus RBV between December 2004 and April 2008 at Tokyo Medical and Dental University Hospital (Tokyo, Japan) and associated hospitals participating in the Ochanomizu-Liver Conference Study Group. All patients had histologically or clinically proven chronic active hepatitis and were positive for anti-HCV antibodies and serum HCV RNA by reverse transcription polymerase chain reaction (RT-PCR). Patients with a positive test for serum hepatitis B surface antigen, coinfection with other HCV genotypes, coinfection with human immunodeficiency virus, other causes of hepatocellular injury (such as alcoholism, autoimmune hepatitis, primary biliary cirrhosis, or a history of treatment with hepatotoxic drugs), and a need for hemodialysis were excluded.

The following factors were analyzed to determine whether they were related to the efficacy of combination therapy: age; gender; body mass index (BMI); previous IFN therapy; grade of inflammation and stage of fibrosis on liver biopsy; pretreatment biochemical parameters, such as hemoglobin, alanine transaminase (ALT) level, platelet count, low density lipoprotein (LDL) cholesterol, serum HCV RNA level (Log IU/ml); and the amino acid sequence of the IFN sensitivity determining region (aa 2209–2248, ISDR). Liver biopsy specimens were evaluated according to the grade of inflammation and the stage of fibrosis; this was done blindly by an independent interpreter who was not aware of the clinical data. Activity of inflammation was graded on a scale of 0–3: A0 shows no activity, A1 shows mild activity, A2 shows moderate activity, and A3 shows severe activity. Fibrosis was staged on a scale of 0–4: F0 shows no fibrosis, F1 shows moderate fibrosis, F2 shows moderate fibrosis with few septa, F3 shows severe fibrosis with numerous septa without cirrhosis, and F4 shows cirrhosis.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki and was approved by the ethics committee of our hospital, and informed written consent was obtained from each patient.

Nucleotide sequencing of the *NS5A* gene

The serum samples were frozen at −80°C until use. Extraction of RNA from serum and RT-PCR were performed as described previously [14]. The PCR and sequencing primers were synthesized with a DNA synthesizer (model 391; Applied Biosystems Japan, Chiba, Japan).

To determine the nucleotide sequence of the *NS5A 2209–2248* region, we amplified nucleotides (nt) 7296–7320 of HCV complementary DNA by using the outer pair of primers [5′ outer primer, 5′-TGG ATG GAG TGC GGT TGC ACA GGT A-3′ (nt 6703–6727 of HC-J4); 3′ outer primer, 5′-TCT TTC TCC GTG GAG GTG GTA TTG C-3′ (nt 7296–7320)]. We transferred 1 μl of the first PCR product to the second PCR reaction along with the nested 5′ and 3′ primers [5′ inner primer, 5′-TGT AAA ACG ACG GCC AGT CAG GTA CGC TCC GGC GTG CA-3′ (nt 6722–6741), with the M13 forward primer sequence underlined; and 3′ inner primer, 5′-CAG GAA ACA GCT ATG ACC GGG GCC TTG GTA GGT GGC AA-3′ (nt 7275–7294), with the M13 reverse primer sequence underlined]. An M13 forward primer and an M13 reverse primer were attached to the 5′ terminal of the 5′ and 3′ inner primers, respectively, to facilitate direct sequencing with an automated DNA sequencer (model 373S; Applied Biosystems Japan).

Both strands of the PCR products were sequenced with the PRISM dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions. The sequencing primer was the M13 forward primer for the sense strand and the M13 reverse primer for the antisense strand. Deduced aa sequences of *NS5A* 2209–2248 were compared with the *NS5A* 2209–2248 sequences of HCV-J [15], which are prototypic sequences of HCV-1b. The results of the sequencing analysis were confirmed as consistent for each sample by repeating the experiment twice with different PCR products, to rule out the possibility of selection and amplification of minor NS5A quasi species variants in the low-titer specimens.

Nucleotide sequencing of the core gene

Substitutions of amino acids 70 and 91 in HCV-core region were determined according to core sequences obtained as described previously [16, 17]. The pattern of glutamine/histidine (mutant) at aa 70 and methionine (mutant) at aa 91 was evaluated as the double-mutant (dM) type, while the other patterns were non-double-mutant (non dM) type. Two patterns of mutants and competitive were labeled as non-wild. Wild at aa 70 and wild at aa 91 were evaluated as double-wild-type (dW), while the other patterns were considered non-double-wild-type (non dW).

Study design and treatment regimens

Patients were treated with combination therapy with PEG-IFN (Peg-Intron; Schering-Plough Nordic Biotech, Stockholm, Sweden) 1.2–1.5 μg/kg subcutaneously and RBV (Rebetol; Schering-Plough Nordic Biotech) (body weight [b.w.] < 60 kg, 600 mg po daily; b.w. 60–80 kg, 800 mg po daily; b.w. > 80 kg, 1000 mg po daily; in two divided doses). The duration of the combination therapy was set at a standard 48 weeks. Treatment reduction was permitted, to escape side effects, but extended treatment of 72 weeks is not included in this analysis. Achieved rates of PEG-IFN and RBV administration were calculated as the percentage of the actual total dose administered of a standard total dose of 48 weeks according to body weight before therapy. During treatment, patients were assessed as outpatients at weeks 2, 4, 6, and 8, and then every 4 weeks for the duration of treatment and at every 4 weeks after the end of therapy. Biochemical and hematological testing was done by a central laboratory. Serum HCV RNA was measured before treatment, during treatment at 4-weekly intervals, and after therapy at 4-weekly intervals for 24 weeks, by a quantitative PCR assay with a sensitivity of 100 copies/ml (National Genetics Institute, Los Angeles, CA, USA).

Outcomes

The primary end point was a sustained biochemical and virological response. Sustained virological response (SVR) was defined as serum HCV RNA undetectable at 24 weeks after the end of treatment. Secondary end points were end-of-treatment virological responses (HCV RNA undetectable in serum). In addition, tolerability (adverse events) and drug adherence were recorded and factors potentially associated with virological response were explored.

Statistical analysis

SPSS software package (SPSS 12J for Windows; SPSS, Chicago, IL, USA) was used for statistical analysis, which was carried out using the χ^2 or Fisher's exact probability test. Distributions of continuous variables were analyzed by the Mann–Whitney *U*-test. Independent factors possibly affecting response to combination therapy were examined by stepwise multiple logistic-regression analysis. All *P* values were two-tailed and those lessthan 0.05 were considered statistically significant.

Results

Clinical characteristics and response to therapy

The clinical characteristics of the 239 patients are summarized in Table 1. On an intention-to-treat (ITT) analysis, serum HCV RNA levels were undetectable by the end of treatment in 172 of the 239 patients (72%) who were treated with PEG-IFN plus RBV, and among them, 98 of the 239 patients (41%) had an SVR (Table 2). The SVR rate decreased with drug discontinuation and dose

Table 1 Baseline characteristics of participating patients infected with HCV genotype 1b

Total number	239
Age (years)[a]	57 (21–78)
Gender (male/female)	142/97
Body mass index (kg/m^2)[a]	23.3 (15.3–31.0)
Previous interferon therapy (no/yes)	167/72
Histology at biopsy	
Grade of inflammation	
A0/1/2/3	3/65/102/10
Stage of fibrosis	
F0/1/2/3/4	4/73/57/37/9
Hemoglobin (g/dl)[b]	14.3 ± 1.3
ALT (IU/L)[b]	86 ± 67
Platelet count ($\times 10^3/\mu l$)[b]	160 ± 58
LDL cholesterol (mg/dl)[b]	74 ± 19
Serum HCV-RNA level (Log(IU/ml))[b, c]	6.1 ± 0.6
Type of mutations in the core (dM/non dM)	30/166
Type of mutations in the core (dW/non dW)	65/131
Type of ISDR sequence (0/1/2/3/4 or more)	126/45/11/5/18

HCV hepatitis C virus, *LDL* low density lipoprotein, *ALT* alanine transaminase, *ISDR* interferon sensitivity determining region in NS5A $_{2209-2248}$, *dM* double mutant: dual substitutions at amino acids 70 and 91, *non dM* non-double mutant: wild type or substitution at either amino acid 70 or 91, *dW* double wild: wild type at amino acids 70 and 91, *non dW* non-double wild: dual or substitution at either amino acid 70 or 91

[a] Median (range) values are shown

[b] Data are mean ± SD

[c] Data are shown as Log(IU/ml)

Table 2 Sustained response rates to treatment according to drug adherence

Characteristic	Number/total number (%)
Overall	
End of treatment	172/239 (72)
End of follow up	98/239 (41)
PEG-interferon-α2b adherence	
End of treatment	
>80%	131/154 (85)
60–80%	19/27 (70)
<60%	22/58 (38)
End of follow up	
>80%	86/154 (56)
60–80%	7/27 (26)
<60%	5/58 (9)
Ribavirin adherence	
End of treatment	
>80%	113/134 (84)
60–80%	37/46 (80)
<60%	22/59 (37)
End of follow up	
>80%	74/133 (56)
60–80%	18/47 (38)
<60%	6/59 (10)

PEG pegylated

reduction. The SVR rates of patients who received a total cumulative treatment dose of PEG-IFN of more than 80% were almost twice as high as the rates of patients who received less than 80% (56%, 26%, and 9% with >80%, 60%–80% and <60% of the PEG-IFN dose, $P < 0.001$). The SVR rates did not decrease with RBV reduction, as long as the cumulative treatment dose of RBV was more than 60%, but when the RBV reduction fell below 60%, the SVR rates were significantly lower (56%, 38%, and 10% with >80%, 60%–80%, and <60% of the RBV dose, $P < 0.001$).

Factors associated with sustained virological response

Seven parameters that influenced the SVR rate were identified by univariate analysis, including stage of fibrosis at liver biopsy, hemoglobin, platelet count, serum HCV RNA level, the type of ISDR sequence, and adherence to PEG-IFN plus RBV (Table 3). On the other hand, the SVR rate was not related to gender ($P = 0.07$), age or BMI. The amino acid substitution pattern was not significant in the overall analysis, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve SVR. As shown in Table 4, gender differences existed in the mutations in ISDR and core regions based on therapeutic responses. Because there were rather fewer female than male patients, the type of ISDR sequence did not significantly influence the SVR in females. We also analyzed types of mutations in the core, and the amino acid substitution pattern was not significant in the male patients, but female patients with dual substitutions at amino acids 70 and 91 had a low tendency to achieve an SVR, as mentioned above. We also compared results between treatment-naïve patients and those who had failed previous IFN therapy (Table 5). As there were some differences in stage of fibrosis, platelet count, grade of inflammation, and gender in univariate analysis, treatment was comparably effective in both groups.

Finally we performed multivariate analysis in subjects with good drug adherence (Table 6), which identified only one parameter that influenced the SVR rate independently by variable selection: the number of mutations in the ISDR sequence (two or more: odds ratio [OR] = 5.181, $P < 0.05$). This regression model was always obtained regardless of the variable selection method used, including conditional parameter estimation, Wald statistic, and