

performed standard virion titration by immunofluorescent antibody staining. The result showed that the virus titer of J6/JFH/Luc supernatant, collected at day 4 post-RNA transfection, was 5×10^3 fluorescent-focus forming units (ffu) per ml. In contrast, the titer of JFH/Luc supernatant was below the detection limit ($<1 \times 10^2$ ffu/ml). Interestingly, the peaks of the core release and the infectivity were slightly different, i.e., the peak of the core release of J6/JFH1 was on day 6, and that of the infectivity was on day 4 post-electroporation. Collectively, these data revealed that J6/JFH1 had a greater ability to release progeny virions than JFH1, though the levels of intracellular replication were comparable between J6/JFH1 and JFH1.

3.3. Characterization of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we examined whether the J6/JFH1/Luc-derived supernatants had the features of a virus and thus could be used as a surrogate for HCV. The supernatants collected from each culture of reporter RNA-electroporated cells were irradiated with ultra-violet (UV) for 5 min, and the supernatants were then inoculated into naïve Huh7.5 cells. As shown in Fig. 3A, the infectivity of the reporter virus was completely abrogated by UV-irradiation. The results indicated that the luciferase activity transduced by the supernatants was derived from the genome of the reporter virus, not from incorporation of the luciferase protein into the virion. The entry of the HCV virion was mediated by binding between the cellular surface protein CD81 and the HCV envelope protein E2 [15]. Therefore, the naïve Huh7.5 cells were pre-treated with a recombinant monoclonal antibody against CD81. After 1 h pre-treatment, the J6/JFH1/Luc supernatant was inoculated into the cells, and the luciferase activity of cells was analyzed at 48 h post-inoculation (Fig. 3B). Normal mouse IgG showed no effect on the infectivity of the J6/JFH/Luc supernatant. In contrast, the infectivity of the J6/JFH/Luc supernatant was decreased by pre-treatment with anti-CD81 antibody in a dose-dependent manner. The results suggested that the supernatant from luciferase reporter J6/JFH1/Luc-transfected cells contained a virus with characteristics similar to HCV,

and that this reporter virus could be utilized to investigate all the steps of virus replication, including the intra-cellular viral replication, the virus production and the virus entry as a surrogate model of HCV.

3.4. Analysis of a potential role for ESCRT family proteins in HCV virus production

Prior to the recent establishment of the JFH1-based cell-culture system, there was no system for producing the HCV virus, and thus many aspects of the virus production of HCV still remain poorly understood. Generally, the production of the enveloped virus requires a multi-step process that includes the proper transport of viral proteins and organization of viral proteins on the cellular membrane, and these steps are coordinated by a variety of cellular factors [16,17]. From numerous intensive studies, it has been revealed that the process of budding of many enveloped viruses utilizes the ESCRT machinery, which is responsible for the formation of luminal vesicles of endosomal multivesicular bodies (MVB) [16,18–20]. The ESCRT machinery consists of a number of cellular proteins that make up three functional sub-complexes – ESCRT-I, ESCRT-II and ESCRT-III – and other related factors; i.e., Vps4 and AIP/Alix are also participated in the function of ESCRT machinery [20]. A series of analyses about ESCRT networks has revealed the consensus amino acid motifs of viral proteins; the P(T/S)AP motif was observed to interact with Tsg101, and the YPxL motif was seen in the case of AIP/Alix [19]. We searched for these motifs in the J6 and JFH1 genomes, and found one AIP/Alix interacting the YPxL motif in the NS5B region (aa. 2604 to 2607; YPDL). Therefore, the relation between ESCRT and HCV was examined by analyzing the virus production using a luciferase reporter HCV system. First, we constructed the expression plasmids of the ESCRT-I protein Tsg101, and the ESCRT-associated proteins Nedd4L and AIP/Alix. The ESCRT expression plasmids were transfected into the J6/JFH1/Luc or JFH1/Luc RNA-transfected Huh7.5 cells. After 48 h of transfection, the culture supernatants were collected and inoculated into the culture of the naïve Huh7.5 cells. The effects of over-expression of ESCRT proteins on intra-cellular virus

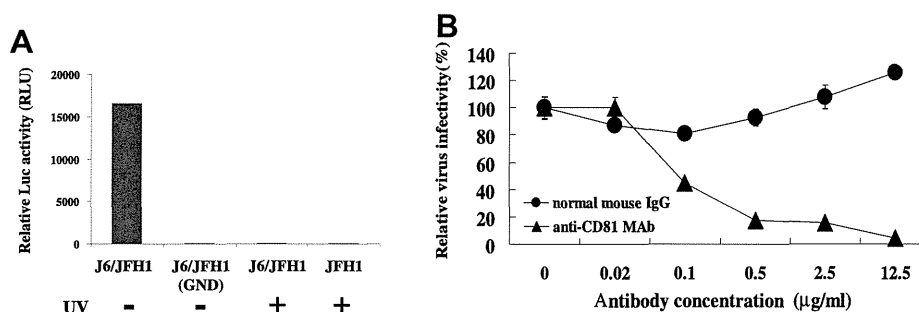


Fig. 3. Anti-CD81 antibody blocks luciferase reporter HCV infection. The reporter viruses containing supernatants were prepared as described in Materials and methods. (A) The JFH1/Luc and J6/JFH1/Luc supernatants were irradiated with UV at 5 min, and then added to naïve Huh7.5 cells. The infectivity was analyzed by luciferase assay. (B) Huh7.5 cells were pre-treated with anti-CD81 monoclonal antibody or control mouse IgG at 1 h before infection. Cells were then infected with J6/JFH1/Luc reporter viruses for 6 h. At 48 h post-infection, the cells were lysed and assayed for luciferase activity. Activities are expressed as the relative activity compared to that of the null antibody-treated sample. The assays were repeated at least three times, and the mean values are presented.

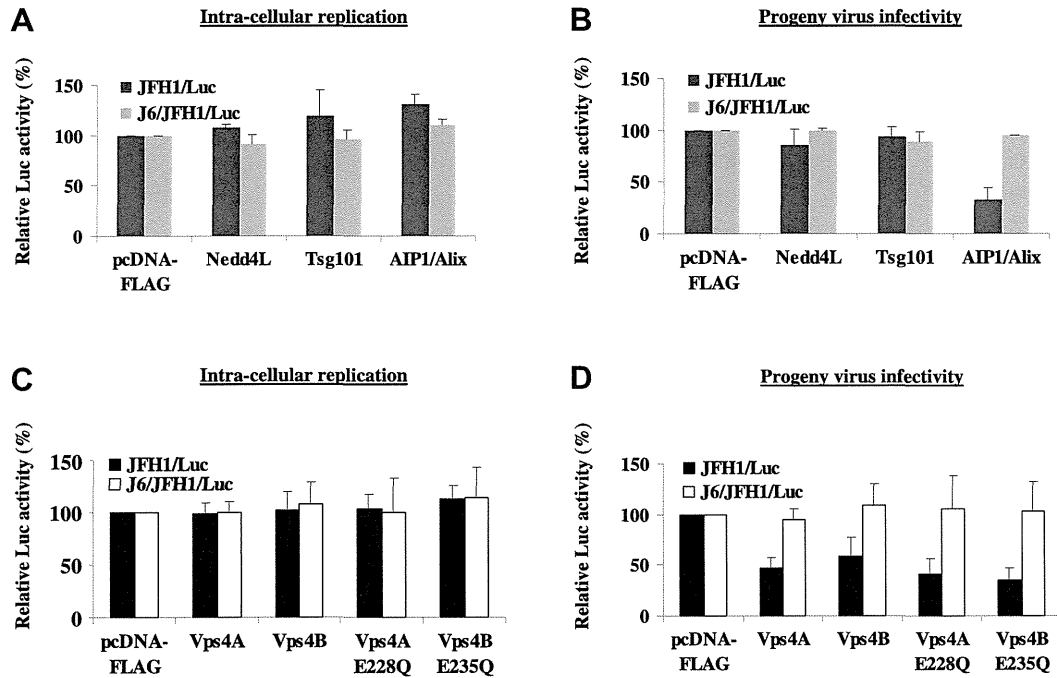


Fig. 4. Effect of ESCRT family protein expression on intra-cellular replication and progeny virus production in Huh7.5 cells. Huh7.5 cells were electroporated with JFH1/Luc and J6/JFH1/Luc RNA, respectively. The RNA-electroporated cells were then transfected with ESCRT protein expression plasmids. At 96 h after transfection, the culture supernatants were collected, and the cells were harvested. (A, C) Cell lysates were assayed for luciferase activity to assess intra-cellular virus replication. (B, D) Collected supernatants were added to naïve Huh7.5 cells and incubated for 48 h, and then the luciferase activity of the cells was analyzed to assess progeny virus infectivity. The data relative to that of luciferase activity in the absence of ESCRT protein (pcDNA-FLAG) is indicated. The assays were repeated at least three times, and the mean and standard error are presented.

replication and virus production were analyzed by monitoring the luciferase activity of reporter RNA-transfected cells (Fig. 4A), and the luciferase activity expressed by supernatant virus (Fig. 4B). As shown in Fig. 4A, the overexpression of Nedd4L, Tsg101, and AIP/Alix had no effect on the intra-cellular replication of either reporter HCV. As shown in Fig. 4B, the virus production from J6/JFH1/Luc also was not affected by these ESCRT protein expressions. In contrast, the expression of AIP/Alix decreased the virus production from JFH1/Luc by 50%. This result implied that the ESCRT machinery might have played some role in the difference in the efficacy of virus production observed between JFH1 and J6/JFH1. AAA-ATPase Vps4, which is present in humans in two isoforms (Vps4A and Vps4B), is a key modulator protein for the final step of ESCRT machinery. To analyze the role of ESCRT in HCV virus production, we constructed expression vectors for Vps4A and 4B, as well as expression vectors for a dominant-negative Vps4A(E228Q) and Vps4B(E235Q) [19]. As shown in Fig. 4C, the intra-cellular replications of JFH1 and J6/JFH1 were not influenced by the wild-type or dominant-negative Vps4 expression. In contrast, the levels of virus production of JFH1/Luc were reduced up to 50% by the expression of both dominant-negative Vps4 mutants (Fig. 4D). Interestingly, neither dominant-negative Vps4 influenced the virus production of J6/JFH1/Luc. These results implied that JFH1 might utilize the ESCRT machinery for release of infectious virus particles.

3.5. Effect of polyions on the infectivity of the J6/JFH1/Luc reporter virus

Next, we tested the usefulness of the J6/JFH1/luc reporter system for virus entry analysis. The binding of the viral and cellular receptors is coordinated with the ionic conditions, indicating that compounds that affect the ionic charge of the receptor surface might be potent inhibitors of virus infection [21,22]. Polyions with a positive or negative charge are frequently used for virus entry analyses, and exhibit inhibitory activity on virus infection [21,22]. Therefore, we investigated the effect of different polyions on the infectivity of the J6/JFH1/Luc virus in order to clarify the influence of electrostatic interactions in virus binding to cell membranes. As candidate compounds, we used both polymers having a positive charge (polybrene (size of 3000 Da), DEAE-dextran (100,000 Da), and poly-L-lysine (500,000 Da)) and those having a negative charge (heparin (15,000 Da), dextran sulfate (50,000 Da), and polyvinyl sulfate (150,000 Da)). These polymers were added to the Huh7.5 cells at 1 h before inoculation of the J6/JFH1/Luc virus into the cells. After 48 h of inoculation, the cells were harvested and the luciferase activity was analyzed (Fig. 5A and B). As shown in Fig. 5A, two polyanions, heparin and polyvinyl sulfate, decreased the infectivity of J6/JFH1/Luc virus in a dose-dependent manner, whereas one polyanion, dextran sulfate, enhanced the infectivity up to 2-fold. In the case of polycations, the addition of polybrene enhanced virus

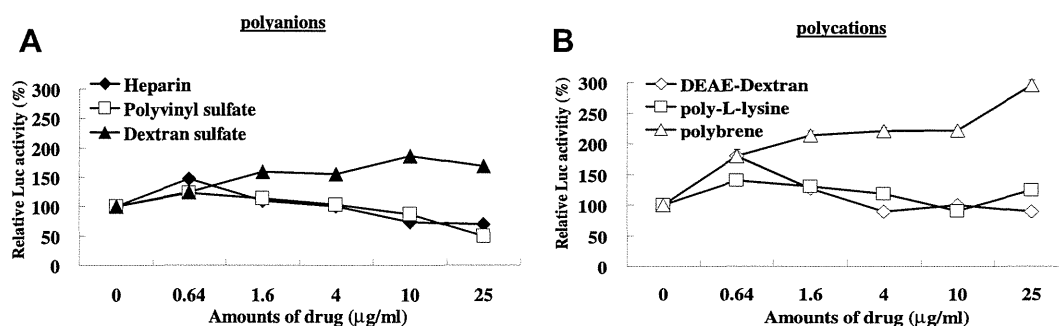


Fig. 5. Effect of multiple polyions on J6/JFH1/Luc virus infection of Huh7.5 cells. Huh7.5 cells were infected with J6/JFH1/Luc virus in the presence of each of the polyions for 6 h, and a luciferase assay was performed 48 h later. The data was expressed as the relative activity compared to the luciferase activity in the absence of polyions. The assays were repeated at least three times, and the mean values are presented.

infection up to 3-fold in a dose-dependent manner, although poly-L-lysine and DEAE-dextran showed no effect on the infectivity of the J6/JFH1/Luc virus (Fig. 5B). The effect shown by compounds belonging to positive and negative polyions suggested that the electric charge is not sufficient by itself to explain the inhibitory or enhancing activity of these drugs on the HCV virus entry. These results indicated that the J6/JFH1/Luc virus was useful to easily monitor HCV virus entry.

3.6. Screening of microRNA inhibition on intra-cellular HCV replication

To confirm the usefulness of the J6/JFH1/Luc reporter system in the analysis targeting intra-cellular replication of HCV, we analyzed the possible involvement of micro RNAs (miRNAs) in HCV infection. miRNAs are evolutionarily conserved, small, non-coding RNA molecules that regulate gene expression at the level of translation [23,24]. Recently, it has been reported that some miRNAs influence the replication of HCV in the cells [25–27]. For example, the expression of miR-122 in the cells might be essential for HCV replication [25]. In addition, the number of miRNAs has been increasing due to numerous strenuous analyses in recent years. Therefore, we compared the full sequences of the viral genome among 4

different HCV strains (H77C, Con1, J6, JFH1) with the sequences of 630 human miRNAs using the miRNA database program (RegRNA: <http://regrna.mbc.nctu.edu.tw/index.php>), and then identified 54 miRNAs that matched with at least one HCV strain. 10 of the 54 miRNAs matched with all four HCV strains. Hence, we focused on analysis of the function of the 10 miRNAs on HCV replication and prepared commercially available miRNA inhibitors (Anti-miR™ miRNA inhibitor, Ambion) that were chemically modified, single-stranded nucleic acids designed to specifically bind to and inhibit endogenous target miRNA molecules. The J6/JFH1/Luc RNA-electroporated cells were transfected with each of the 10 specific miRNA inhibitors and the luciferase activities were analyzed at 48 h post-transfection of the inhibitors. None of the miRNA inhibitors significantly affected the cell viability (data not shown). As shown in Fig. 6A, the inhibition of miR-122 reduced the level of intra-cellular virus replication by up to 50% as previously reported [25]. A similar reduction of viral replication was also observed by treatment with the miR-34b inhibitor. The treatment with an anti-miR negative control that is a random sequence anti-miR molecules that has been extensively tested in human cell lines and validated to not produce identifiable effects on known miRNA functions showed no significant effect on HCV replication. None of the other inhibitors showed any significantly greater effect on the

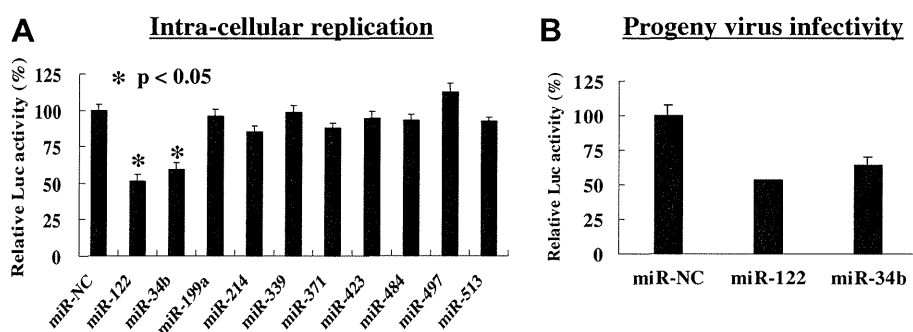


Fig. 6. Effect of miRNA inhibitor on intra-cellular replication of J6/JFH1/Luc RNA in Huh7.5 cells. Target cells were electroporated with J6/JFH1/Luc RNA, and then transfected with a miRNA-specific or a non-target negative control miRNA inhibitor. At 48 h post-transfection, cells were harvested and analyzed for luciferase activity (A). In parallel, the culture supernatants were collected at 48 h post-transfection to assess the effect of miRNA inhibitors on the virus production. The supernatants were added to naïve Huh7.5 cells, and the progeny virus infectivities were then analyzed by luciferase assay. (B) The data relative to the luciferase activity obtained from a non-target negative control miRNA inhibitor are indicated. The assays were repeated at least three times, and the mean and standard error are presented. Statistical significance relative to the negative control miRNA samples as calculated by *t*-test is shown (* $p < 0.05$).

virus replication than the anti-miR negative control. The miR-34b and miR-122 inhibitors decreased the virus production to the levels 64% and 53% of the control, respectively (Fig. 6B). Since the extent of the reduction in virus production was comparable with that of intra-cellular HCV RNA levels (Fig. 6A), it was likely that these miRNA inhibitors affected the intra-cellular viral replication rather than interfering with the particle formation and the release of the virion. These results suggest that the function of miR-34b could affect the replication of HCV, and also suggested that the J6/JFH1/Luc system was useful to analyze the intra-cellular replication of HCV.

4. Discussion

In this report, we generated two bicistronic luciferase reporter HCV clones from JFH1 and J6/JFH1, and established a unifying system that can monitor intra-cellular viral replication, virion production, and virus entry. Using two constructs, we initially compared the potential of intra-cellular viral replication and virus production. After transfection of reporter RNAs, the level of the intra-cellular core protein and the luciferase activity in RNA-transfected cells showed similar kinetics for JFH1/Luc and J6/JFH1/Luc (Fig. 1B and C). In contrast, both the efficacy of core protein production into the culture supernatant and the infectivity of supernatant virus from J6/JFH1/Luc were significantly higher than that of JFH1/Luc (Fig. 2A and B). These results indicated two possibilities that JFH1 and J6/JFH1 utilize different machinery for progeny virus packaging and budding, or that they utilize the same machinery for the virus production but to a different degree. To evaluate the difference in the virus production between JFH1 and J6/JFH1, we analyzed the role of ESCRT machinery in virus production (Fig. 4A–D). Dominant-negative Vps4 expression inhibited JFH1/Luc virus production, but did not influence J6/JFH1/Luc virus production. In the course of preparing this manuscript, Corless et al. reported that HCV requires late components of the ESCRT pathway for release of infectious virus particles [28]. They showed that a dominant-negative Vps4 expression inhibited the production of virus-like particles derived from JFH1 in a dose-dependent manner. The findings reported by Corless et al. and the findings of our present study emphasize that the ESCRT machinery plays an essential role in JFH1 virus production.

To examine the virus entry, we analyzed the effect of anti-CD81 antibody and polyions on reporter virus infectivity (Figs. 3B and 5A and B). The pre-treatment with anti-CD81 antibody decreased the infectivity of the reporter J6/JFH1 virus in a dose-dependent manner. The result suggested that the reporter J6/JFH1 virus, similar to HCVcc, utilized the CD81 as a major entry receptor, and that our reporter virus could be used as a surrogate model of HCV entry analysis. As a result of polyions analysis, one of the polycations (dextran sulfate) and one of the polyanions (polybrene) increased the reporter virus infectivity, and the remainder of the polyions inhibited the virus infectivity. These results indicate the

possibility that not only the electrostatic condition of polyions but also their molecular weight may be a determinant of the receptor binding of HCV. Considering that several membrane molecules have been identified as candidate cellular receptors for HCV entry [15,29,30], the polyions could interact with a different molecule(s) to influence virus production. As for heparin, it was reported that cell surface heparan sulfate proteoglycans play an important role in mediating HCV envelope–target cell interaction [31]. Basu et al. [32] also reported that heparin treatment completely blocked HIV/HCV E1–E2 pseudotype infection. In their analysis, however, the inhibitory effect of heparin against cell culture-grown HCV H77 was somewhat lower than that of HIV/HCV E1–E2 pseudotypes. In our present study, the level of inhibitory effect of heparin on J6/JFH1 reporter virus infection was not so prominent. Collectively, these data suggest a possibility that cell surface heparan sulfate proteoglycans contribute to the infection of both HIV/HCV E1–E2 pseudotype and cell culture-grown HCV with a different degree. Therefore, to develop a polyion-based anti-HCV drug, a more detailed assessment of the interaction between each candidate receptor and polyion is necessary.

Using microRNA inhibitors, the decrease of miR-34b expression suppressed intra-cellular HCV replication (Fig. 6A). miR-34b belongs to the evolutionary conserved microRNA family of miR-34s [33], known for their role in the p53 tumor suppressor network [34]. miR-34s have been shown to be controlled in a tissue-specific manner by p53. Both wild-type and mutant-type p53 protein expressions in serum and cytoplasm of liver tissue were more pronounced in patients with hepatocellular carcinoma associated with HCV infection [35]. Wild-type p53 binds to a transcriptional regulatory element of miR-34s, thereby up-regulating miR-34 expression [34]. However, it is not understood whether the mutant-type p53 increases miR-34b expression. Furthermore, HCV replication in chronic hepatitis is higher than that of hepatocellular carcinoma [36]. Therefore, more detailed research is needed to reveal the significance of miR-34b expression in HCV replication and hepatocellular carcinoma.

As mentioned above, we have generated a recombinant luciferase reporter HCV, and have shown that the reporter HCV could be used for the quantitative analyses of intra-cellular replication, virus entry, and virion production. In general, the intra-cellular HCV replication has been analyzed by the quantitative real-time RT-PCR method that could detect a small amount of viral RNA because of the greatly high sensitivity. However, the real-time RT-PCR method involves multi-step procedures of the RNA extraction, the reverse transcription and the PCR reaction, which require skillfulness to perform. The high sensitivity and the multiple-steps of the real-time RT-PCR system sometimes cause an experimental error(s) when conducted by less-experienced individuals. On the other hand, our HCV luciferase reporter system is simpler and easier to perform compared to the real-time PCR system. The significant advantage of the reporter HCV is that it can analyze a large number of samples at a time in a time- and cost-saving manner. Also, it can be used to evaluate all the

events of viral life cycle. By using it, we have started the screening of anti-HCV substances from the natural resource chemical libraries and found a number of potential candidates for the analysis. Thus, this system can be applicable for robust screening analyses of chemical compounds to discover a potential therapeutic target of HCV.

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References

- [1] T.L. Tellinghuisen, M.J. Evans, T. von Hahn, S. You, C.M. Rice, Studying hepatitis C virus: making the best of a bad virus, *J. Virol.* 81 (2007) 8853–8867.
- [2] N. Kato, M. Hijikata, Y. Ootsuyama, M. Nakagawa, S. Ohkoshi, T. Sugimura, K. Shimotohno, Molecular cloning of the human hepatitis C virus genome from Japanese patients with non-A, non-B hepatitis, *Proc. Natl. Acad. Sci. U.S.A.* 87 (1990) 9524–9528.
- [3] S. Chevaliez, J.M. Pawlotsky, Hepatitis C virus: virology, diagnosis and management of antiviral therapy, *World J. Gastroenterol.* 13 (2007) 2461–2466.
- [4] M. Laguno, C. Cifuentes, J. Murillas, S. Veloso, M. Larrousse, A. Payeras, L. Bonet, F. Vidal, A. Milinkovic, A. Bassa, C. Villalonga, I. Perez, C. Tural, M. Martinez-Rebollar, M. Calvo, J.L. Blanco, E. Martinez, J.M. Sanchez-Tapias, J.M. Gatell, J. Mallolas, Randomized trial comparing pegylated interferon alpha-2b versus pegylated interferon alpha-2a, both plus ribavirin, to treat chronic hepatitis C in human immunodeficiency virus patients, *Hepatology* 49 (2009) 22–31.
- [5] A. El-Shamy, M. Nagano-Fujii, N. Sasase, S. Imoto, S.R. Kim, H. Hotta, Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy, *Hepatology* 48 (2008) 38–47.
- [6] D.P. Webster, P. Klenerman, J. Collier, K.J. Jeffery, Development of novel treatments for hepatitis C, *Lancet Infect. Dis.* 9 (2009) 108–117.
- [7] P.J. Lukavsky, Structure and function of HCV IRES domains, *Virus. Res.* 139 (2009) 166–171.
- [8] V. Lohmann, F. Korner, J. Koch, U. Herian, L. Theilmann, R. Bartenschlager, Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line, *Science* 285 (1999) 110–113.
- [9] T. Wakita, T. Pietschmann, T. Kato, T. Date, M. Miyamoto, Z. Zhao, K. Murthy, A. Habermann, H.G. Krausslich, M. Mizokami, R. Bartenschlager, T.J. Liang, Production of infectious hepatitis C virus in tissue culture from a cloned viral genome, *Nat. Med.* 11 (2005) 791–796.
- [10] B.D. Lindenbach, M.J. Evans, A.J. Syder, B. Wolk, T.L. Tellinghuisen, C.C. Liu, T. Maruyama, R.O. Hynes, D.R. Burton, J.A. McKeating, C. M. Rice, Complete replication of hepatitis C virus in cell culture, *Science* 309 (2005) 623–626.
- [11] T. Kato, T. Date, M. Miyamoto, M. Sugiyama, Y. Tanaka, E. Orito, T. Ohno, K. Sugihara, I. Hasegawa, K. Fujiwara, K. Ito, A. Ozasa, M. Mizokami, T. Wakita, Detection of anti-hepatitis C virus effects of interferon and ribavirin by a sensitive replicon system, *J. Clin. Microbiol.* 43 (2005) 5679–5684.
- [12] L. Deng, T. Adachi, K. Kitayama, Y. Bungyoku, S. Kitazawa, S. Ishido, I. Shoji, H. Hotta, Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway, *J. Virol.* 82 (2008) 10375–10385.
- [13] K. Kamada, T. Igarashi, M.A. Martin, B. Khamsri, K. Hachio, T. Yamashita, M. Fujita, T. Uchiyama, A. Adachi, Generation of HIV-1 derivatives that productively infect macaque monkey lymphoid cells, *Proc. Natl. Acad. Sci. U.S.A.* 103 (2006) 16959–16964.
- [14] T. Schaller, N. Appel, G. Koutsoudakis, S. Kallis, V. Lohmann, T. Pietschmann, R. Bartenschlager, Analysis of hepatitis C virus superinfection exclusion by using novel fluorochrome gene-tagged viral genomes, *J. Virol.* 81 (2007) 4591–4603.
- [15] P. Pileri, Y. Uematsu, S. Campagnoli, G. Galli, F. Falugi, R. Petracca, A. J. Weiner, M. Houghton, D. Rosa, G. Grandi, S. Abrignani, Binding of hepatitis C virus to CD81, *Science* 282 (1998) 938–941.
- [16] A. Calistri, C. Salata, C. Parolin, G. Palu, Role of multivesicular bodies and their components in the egress of enveloped RNA viruses, *Rev. Med. Virol.* 19 (2009) 31–45.
- [17] S. Welsch, B. Muller, H.G. Krausslich, More than one door – budding of enveloped viruses through cellular membranes, *FEBS Lett.* 581 (2007) 2089–2097.
- [18] B. Strack, A. Calistri, S. Craig, E. Popova, H.G. Gottlinger, AIP1/ALIX is a binding partner for HIV-1 p6 and EIAV p9 functioning in virus budding, *Cell* 114 (2003) 689–699.
- [19] P.D. Bieniasz, Late budding domains and host proteins in enveloped virus release, *Virology* 344 (2006) 55–63.
- [20] J.H. Hurley, P.I. Hanson, Membrane budding and scission by the ESCRT machinery: it's all in the neck, *Nat. Rev. Mol. Cell Biol.* 11 (2010) 556–566.
- [21] O. Bagasra, P. Whittle, B. Heins, R.J. Pomerantz, Anti-human immunodeficiency virus type 1 activity of sulfated monosaccharides: comparison with sulfated polysaccharides and other polyions, *J. Infect. Dis.* 164 (1991) 1082–1090.
- [22] J. Haldar, D. An, L. Alvarez de Cienfuegos, J. Chen, A.M. Klivanov, Polymeric coatings that inactivate both influenza virus and pathogenic bacteria, *Proc. Natl. Acad. Sci. U.S.A.* 103 (2006) 17667–17671.
- [23] A. Ventura, T. Jacks, microRNAs and cancer: short RNAs go a long way, *Cell* 136 (2009) 586–591.
- [24] D.P. Bartel, microRNAs: target recognition and regulatory functions, *Cell* 136 (2009) 215–233.
- [25] C.L. Jopling, M. Yi, A.M. Lancaster, S.M. Lemon, P. Sarnow, Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA, *Science* 309 (2005) 1577–1581.
- [26] G. Randall, M. Panis, J.D. Cooper, T.L. Tellinghuisen, K.E. Sukhodolets, S. Pfeffer, M. Landthaler, P. Landgraf, S. Kan, B.D. Lindenbach, M. Chien, D.B. Weir, J.J. Russo, J. Ju, M.J. Brownstein, R. Sheridan, C. Sander, M. Zavolan, T. Tuschl, C.M. Rice, Cellular cofactors affecting hepatitis C virus infection and replication, *Proc. Natl. Acad. Sci. U.S.A.* 104 (2007) 12884–12889.
- [27] Y. Murakami, H.H. Aly, A. Tajima, I. Inoue, K. Shimotohno, Regulation of the hepatitis C virus genome replication by miR-199a, *J. Hepatol.* 50 (2009) 453–460.
- [28] L. Corless, C.M. Crump, S.D. Griffin, M. Harris, Vps4 and the ESCRT-III complex are required for the release of infectious hepatitis C virus particles, *J. Gen. Virol.* 91 (2010) 362–372.
- [29] A. Ploss, M.J. Evans, V.A. Gaysinskaya, M. Panis, H. You, Y.P. de Jong, C.M. Rice, Human occludin is a hepatitis C virus entry factor required for infection of mouse cells, *Nature* 457 (2009) 882–886.
- [30] M.J. Evans, T. von Hahn, D.M. Tscherne, A.J. Syder, M. Panis, B. Wolk, T. Hatzioannou, J.A. McKeating, P.D. Bieniasz, C.M. Rice, Claudin-1 is a hepatitis C virus co-receptor required for a late step in entry, *Nature* 446 (2007) 801–805.

- [31] H. Barth, C. Schafer, M.I. Adah, F. Zhang, R.J. Linhardt, H. Toyoda, A. Kinoshita-Toyoda, T. Toida, T.H. Van Kuppevelt, E. Depla, F. Von Weizsacker, H.E. Blum, T.F. Baumert, Cellular binding of hepatitis C virus envelope glycoprotein E2 requires cell surface heparan sulfate, *J. Biol. Chem.* 278 (2003) 41003–41012.
- [32] A. Basu, T. Kanda, A. Beyene, K. Saito, K. Meyer, R. Ray, Sulfated homologues of heparin inhibit hepatitis C virus entry into mammalian cells, *J. Virol.* 81 (2007) 3933–3941.
- [33] M. Fabbri, C.M. Croce, G.A. Calin, microRNAs, *Cancer J.* 14 (2008) 1–6.
- [34] L. He, X. He, S.W. Lowe, G.J. Hannon, microRNAs join the p53 network – another piece in the tumour-suppression puzzle, *Nat. Rev. Cancer* 7 (2007) 819–822.
- [35] A.M. Attallah, G.E. Shiha, H. Ismail, S.E. Mansy, R. El-Sherbiny, I. El-Dosoky, Expression of p53 protein in liver and sera of patients with liver fibrosis, liver cirrhosis or hepatocellular carcinoma associated with chronic HCV infection, *Clin. Biochem.* 42 (2009) 455–461.
- [36] M. Colombo, Natural history and pathogenesis of hepatitis C virus related hepatocellular carcinoma, *J. Hepatol.* 31 (Suppl. 1) (1999) 25–30.

A Point Mutation at Asn-534 That Disrupts a Conserved N-Glycosylation Motif of the E2 Glycoprotein of Hepatitis C Virus Markedly Enhances the Sensitivity to Antibody Neutralization

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The molecular basis of antibody neutralization against hepatitis C virus (HCV) is poorly understood. The E2 glycoprotein of HCV is critically involved in viral infectivity through specific binding to the principal virus receptor component CD81, and is targeted by anti-HCV neutralizing antibodies. A previous study showed that a mutation at position 534 (N534H) within the sixth N-glycosylation motif of E2 of the J6/JFH1 strain of HCV genotype 2a (HCV-2a) was responsible for more efficient access of E2 to CD81 so that the mutant virus could infect the target cells more efficiently. The purpose of this study was to analyze the sensitivity of the parental J6/JFH1, its cell culture-adapted variant P-47 possessing 10 amino acid mutations and recombinant viruses with the adaptive mutations to neutralization by anti-HCV antibodies in sera of HCV-infected patients. The J6/JFH1 virus was neutralized by antibodies in sera of patients infected with HCV-2a and -1b, with mean 50% neutralization titers being 1:670 and 1:200, respectively ($P < 0.00001$). On the other hand, the P-47 variant showed 50- to 200-times higher sensitivity to antibody neutralization than the parental J6/JFH1 without genotype specificity. The N534H mutation, and another one at position 416 (T416A) near the first N-glycosylation motif to a lesser extent, were shown to be responsible for the enhanced sensitivity to antibody neutralization. The present results suggest that the residues 534, and 416 to a lesser extent, of the E2 glycoprotein are critically involved in the HCV infectivity

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INTRODUCTION

Hepatitis C virus (HCV), a member of the family *Flaviviridae*, the genus *Hepacivirus*, is an enveloped, positive-stranded RNA virus that infects an estimated 170 million people worldwide. The virus evades the

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host immune system to establish chronic infection, which often leads to serious liver diseases, such as cirrhosis and hepatocellular carcinoma. Even with a current standard treatment with pegylated interferon *plus* ribavirin, sustained viral clearance is obtained for only approximately 50% of patients infected with HCV genotype 1b (HCV-1b). Neither antibody-based prophylaxis nor an effective vaccine is currently available.

A better understanding of the interplay between viral and host factors that determine HCV clearance or persistence is needed for the design of effective passive immunotherapy and effective vaccines. A growing body of evidence from studies in humans and chimpanzees suggests that HCV-specific T-cell immunity plays an important role in the viral clearance [Bowen and Walker, 2005]. Also, several studies have indicated a role for humoral immunity in HCV infection [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Netski et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. However, this aspect remains poorly characterized.

The E2 glycoprotein of HCV plays an important role in viral attachment and, therefore, becomes a major target of anti-HCV neutralizing antibodies. Identification of protective epitopes in E2 conserved among different HCV strains is a major challenge in vaccine design [Tarr et al., 2006; Helle et al., 2007; Gal-Tanamy et al., 2008; Keck et al., 2008]. The development of infectious retroviral pseudoparticles (HCVpp) bearing HCV envelope glycoproteins helps us study interactions between E2 epitopes and the virus receptor CD81 or neutralizing antibodies [Bartosch et al., 2003; Logvinoff et al., 2004; Lavillette et al., 2005; Pestka et al., 2007; Dowd et al., 2009]. More significantly, authentic HCV particles produced by the HCV cell culture system (HCVcc) are currently available for this purpose [Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005; Fournier et al., 2007].

Recently, it was demonstrated using HCVcc that a mutation at position 534 from Asn to His (N534H) in the E2 glycoprotein of the HCV J6/JFH1 strain confers an advantage to the mutant viruses at the entry level probably through more efficient access to CD81 [Bungyoku et al., 2009]. The Asn-534 is located in the sixth of 11 *N*-linked glycosylation sites and the N534H mutation is predicted to remove this glycosylation. The present study has shown that the N534H mutation in the E2 glycoprotein of HCV J6/JFH1 markedly enhances the sensitivity of the virus to neutralization by specific neutralizing antibodies in sera of patients infected with HCV.

MATERIALS AND METHODS

Cells and Viruses

Huh-7.5 cells [Blight et al., 2002] and pFL-J6/JFH1 [Lindenbach et al., 2005] were kindly provided by

Dr. C. M. Rice (Rockefeller University, New York, NY, USA). Huh-7.5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Biowest, Nuaille, France), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA), penicillin (100 IU/ml), and streptomycin (100 μ g/ml) (Invitrogen) at 37°C in a CO₂ incubator. Propagation of HCV J6/JFH1, its cell culture-adapted mutant P-47 and recombinant viruses possessing each of the adaptive mutations was described previously [Deng et al., 2008; Bungyoku et al., 2009].

Human Sera and Anti-HCV Neutralization Test

Sera were collected from 89 patients infected chronically with HCV-1b or HCV-2a, who were treated with pegylated interferon α -2b and ribavirin, as described previously [El-Shamy et al., 2007, 2008]. Sera were also collected from 11 patients with acute HCV-1b infection, either severe acute hepatitis or mild self-resolving hepatitis. The study protocol was approved by the Ethic Committees in Kobe University and Yamagata University and informed written consent provided by patients and volunteers. Sera collected from healthy volunteers who were negative for anti-HCV antibodies served as a control. The sera were inactivated at 56°C for 30 min before being used for the virus neutralization test.

An HCV neutralization test was performed as described previously [Sasayama et al., 2010]. In brief, serially diluted serum samples were mixed with the same amount of HCV solution containing 1×10^4 cell-infecting units. After incubation at 37°C for 1 hr, the mixtures were inoculated to Huh-7.5 cells (2×10^5 cells per well in 24-well plates) and incubated in a 5% CO₂ incubator. After 3 hr, the inocula were removed and fresh complete DMEM were added to the cells. At 24 hr postinfection, cells were fixed with ice-cold methanol, blocked with 5% goat serum in phosphate-buffered saline and subjected to immunofluorescence analysis using mouse monoclonal antibody against HCV core antigen (2H9) [Wakita et al., 2005] and Alexa Fluor 488-conjugated goat anti-mouse IgG (H + L) (Molecular Probes, Eugene, OR). The immunostained cells were counterstained with Hoechst 33342 (Molecular Probes) at room temperature for 5 min and observed under a fluorescence microscope (BZ-9000; Keyence, Osaka, Japan). The number of HCV-infected cells in each well was counted by using a software BZ-H1C (Keyence). The serum dilutions that neutralized 50% of the virus infectivity was calculated by curvilinear regression analysis [Abe et al., 2003]. Titers were expressed as 50% neutralization titers (NT₅₀).

Statistical Analysis

Student's *t*-test was used to compare the data between different groups. A *P*-value of <0.05 was considered to be significant.

RESULTS

Anti-HCV Neutralizing Antibodies in Sera of Patients Infected With HCV

Sera were obtained from patients chronically infected with HCV-1b or -2a, and tested for anti-HCV neutralizing activities. Representative results of neutralization curves using the parental J6/JFH1 and the P-47 mutant as challenge viruses are shown in Figure 1. When measured against J6/JFH1, NT₅₀ titers of sera of patients infected with HCV-1b ranged from 1:10 to 1:700, with the mean NT₅₀ titer being 1:197, whereas those of patients infected with HCV-2a ranged from 1:100 to 1:1,500, with the mean value being 1:670 (Table I). The difference in NT₅₀ between patients infected with HCV-1b and -2a was statistically significant ($P < 0.00001$). When measured against P-47, on the other hand, unexpectedly high NT₅₀ titers were obtained ranging from 1:4,000 to 1:182,000, with the mean values being 1:40,500 and 1:32,900 for patients infected with HCV-1b and -2a, respectively. These results suggest the possibility that an adaptive mutation(s) of P-47, most probably present in the envelope glycoproteins, confers higher sensitivity to neutralization by anti-HCV antibodies.

Unlike the case with J6/JFH1, when P-47 was used as a challenge virus, no significant difference in NT₅₀ titers was observed between patients infected with HCV-1b and -2a (Table I). This result suggests the possible presence of a genotype-dominant neutralization epitope(s) on the envelope glycoproteins of J6/JFH1 although anti-HCV neutralizing antibodies in patients' sera are reactive to both HCV-1b and -2a. The broad reactivity of the neutralizing antibodies in patients' sera across different HCV genotypes is consistent with previous observations by other researchers [Logvinoff et al., 2004; Meunier et al., 2005; Fournier et al., 2007; Pestka et al., 2007; Scheel et al., 2008].

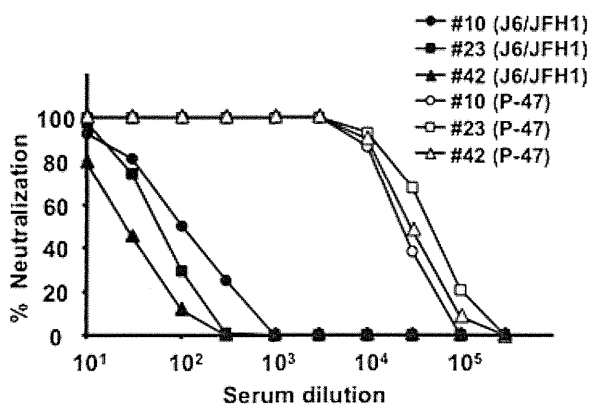


Fig. 1. Neutralization curves (NT₅₀ assay) of sera obtained from HCV-infected patients against HCV J6/JFH1 and its adaptive mutant P-47. J6/JFH1 or P-47 was incubated with serial dilutions of HCV-infected patients (nos. 10, 23, and 42; all infected with HCV-1b) and tested for neutralization activities. The neutralization rates at each dilution were plotted. Filled and open symbols indicate data obtained with J6/JFH1 and P-47, respectively.

Sera obtained from patients with acute hepatitis C contained much lower titers of anti-HCV neutralizing antibodies compared to those in sera from chronic hepatitis patients, with the average NT₅₀ titers against J6/JFH1 and the adaptive mutant P-47 being 1:15 and 1:126, respectively (Table I). Two patients with severe acute hepatitis C with elevated serum alanine aminotransferase levels of >1,000 IU/ml [Saito et al., 2004; unpublished], possessed relatively high NT₅₀ titers against P-47 (1:150 and 1:1,100) compared to the remaining nine patients who experienced mild self-resolving hepatitis (<1:10 to 1:50).

A Single-Point Mutation (N534H or T416A) of the HCV E2 Glycoprotein Increases Sensitivity to Neutralization by Anti-HCV Antibodies

Neutralization of virus infectivity by antibodies usually involves their interaction with viral envelope glycoproteins. It has been reported that the cell culture-adapted mutant P-47 possesses 10 amino acid mutations, including four mutations in E2, compared to the parental J6/JFH1 [Bungyoku et al., 2009]. To examine which mutation(s) in E2 is responsible for the increased sensitivity of P-47 to neutralization by antibodies in patients' sera, recombinant viruses possessing each one of the four mutations in E2 were used (Fig. 2A). The result obtained revealed that a recombinant virus possessing a single-point mutation at position 534 from Asn to His (N534H) and another one possessing four mutations (E2) were as sensitive as P-47 to neutralization by sera of chronic hepatitis patients (Fig. 2B) and the two patients with acute hepatitis C (data not shown). The T416A and T396A mutants were also significantly more sensitive than J6/JFH1, but less sensitive than P-47, N534H, and E2 mutants, to neutralization by antibodies in patients' sera. In this connection, it was recently reported that a JFH1 virus-based T416A mutant showed increased sensitivity to antibody neutralization [Dhillon et al., 2010].

DISCUSSION

The present results revealed that sera of patients infected with HCV-1b possessed cross-genotypic neutralizing antibodies against the J6/JFH1 strain of HCV-2a, albeit with significantly lower titers (ca. one-third) compared to the homotypic neutralization titers observed for patients infected with HCV-2a (Table I). When measured against the adaptive mutant P-47 derived from J6/JFH1, neutralizing antibody titers of the patients sera increased markedly to the level 50- to 200-times higher than that measured against J6/JFH1. Also, the partial genotype-specificity observed with J6/JFH1 was no longer evident when measured against P-47. The marked increase in the sensitivity of P-47 to antibody neutralization was assigned to a mutation at position 534 (N534H), and another one at position 416 (T416A) to a lesser extent, of the E2 glycoprotein (Fig. 2).

TABLE I. NT₅₀ Titers in Sera of HCV-Infected Patients With Chronic or Acute Hepatitis C

CH/AH	Genotype	NT ₅₀ titer ^a measured against	
		J6/JFH1	P-47
CH	HCV-1b (n = 69)	197 ± 164 (1)	40,500 ± 31,800 (206)
CH	HCV-2a (n = 20)	670 ± 652 ^b (3.4)	32,900 ± 26,500 ^c (167)
AH	HCV-1b (n = 11)	15 ± 28 (0.08) (<10–100)	126 ± 326 (0.6) (<10–1,100)

CH, chronic hepatitis; AH, acute hepatitis.

^aMean ± SD. The number in the parenthesis means the ratio when compared to the mean titer that was obtained with sera of HCV-1b-infected CH patients against J6/JFH1.

^bP < 0.00001, compared to the mean titer obtained with sera of HCV-1b-infected patients against J6/JFH1 (Student's *t*-test).

^cP = 0.33, compared to the mean titer obtained with sera of HCV-1b-infected patients against P-47 (Student's *t*-test).

The N534H and T416A mutations are located at the sixth, and in close proximity to the first, respectively, of the conserved 11 *N*-linked glycosylation sites of the HCV E2 glycoprotein [Helle et al., 2007; Bungyoku et al., 2009]. It was recently reported that the positions 416 and 534 are conformationally located in the former and the latter halves of the central domain 1 (DIa and DIb), respectively, of E2 and that the two parts of DI domain interact to form the CD81-binding region [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011]. This region is, therefore, considered as the possible target for neutralizing antibodies that inhibit E2-CD81 interactions [Helle and Dubuisson,

2008; Law et al., 2008; Owsianka et al., 2008; Perotti et al., 2008].

The N534H mutation removes glycans at this position as it disrupts the consensus sequence for *N*-linked glycosylation. The removal of glycans at positions 417, 532, and 645 (the first, sixth, and eleventh glycosylation site, respectively) of the H77 isolate (HCV-1a) was shown to increase the sensitivity of HCVpp to neutralizing antibodies and to enhance the access of CD81 to its binding site on E2 [Falkowska et al., 2007; Helle et al., 2007]. It should be noted, however, that the HCVpp system relies on retroviral pseudoparticles bearing HCV envelope glycoproteins that assemble at the plasma membrane or in multivesicular bodies whereas HCV virions assemble on the endoplasmic reticulum membranes that are closely associated with lipid droplet [Miyazaki et al., 2007; Helle and Dubuisson, 2008]. Therefore, the virus neutralization data obtained with HCVpp should be verified using the HCVcc system in which virion assembly and maturation take place through the authentic process.

By using the HCVcc system, it was shown that a variant virus possessing the N534K mutation spread faster than the parental JFH1 virus [Delgrange et al., 2007], with the result suggesting the possibility that removal of glycans on residue 534 resulted in more efficient access of E2 to CD81. It is also possible that removal of glycans on this residue might allow more efficient access of neutralizing antibodies to the CD81-binding region of E2, resulting in increased sensitivity to antibody neutralization. In fact, Helle et al. [2010] recently reported that removal of glycans at five (the first, second, fourth, sixth, and eleventh) *N*-linked glycosylation sites in E2 markedly increased the sensitivity of JFH1 virus to antibody neutralization, suggesting that the glycans interfere with the access of neutralizing antibodies to a determinant crucial for virus infectivity. It was also reported that mutations at positions 415 (N415D) and 416 (T416A) near the first glycosylation site of JFH1 virus increased the sensitivity to neutralizing antibodies in patients' sera [Dhillon et al., 2010]. Also, a mutation at position 451 (G451R), which is located in the domain 2 (DII) but still in close proximity to DI [Helle et al., 2010; Krey et al., 2010; Albecka et al., 2011],

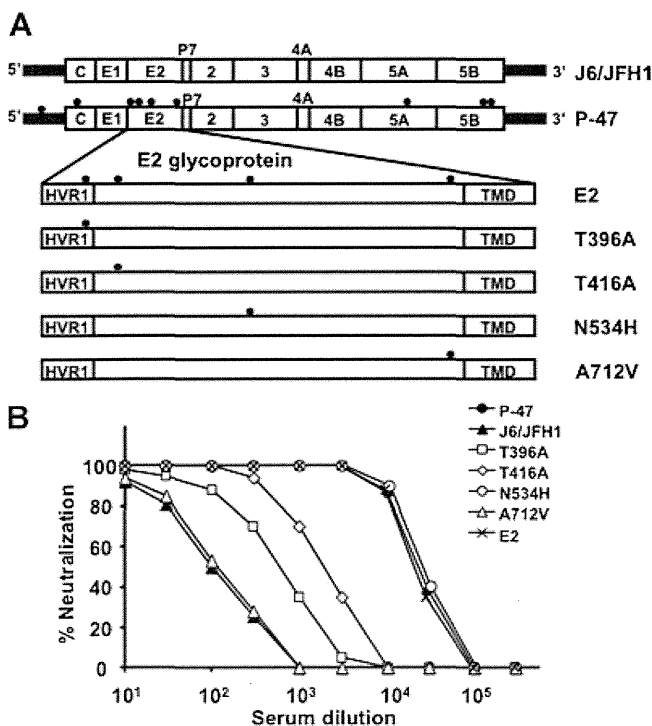


Fig. 2. Effects of amino acid mutations at positions 396, 416, 534, and 712 of the HCV E2 glycoprotein on neutralization by anti-HCV antibodies in patients' sera. A: A schematic diagram of the mutations seen in the adaptive mutant P-47 and recombinant viruses carrying each (T396A, T416A, N534H, and A712V) and all (E2) of the four mutations in E2. Filled circles indicate the positions of the mutations. B: A representative result of virus neutralization by anti-HCV antibodies in an HCV-infected patient (no. 10; HCV-1b).

increased the sensitivity of JFH1 virus to antibody neutralization [Grove et al., 2008].

In conclusion, the present study using J6/JFH1 virus, another HCVcc strain, has demonstrated that the N534H mutation within the sixth *N*-glycosylation site of the E2 glycoprotein, and the T416A mutation near the first *N*-glycosylation site to a lesser extent, markedly enhances sensitivity to neutralization by antibodies in sera of HCV-infected patients. These results suggest that glycans on Asn-534 of the HCV E2 glycoprotein plays an important role in protecting the virus from humoral immune mechanisms of the host.

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REFERENCES

- Abe M, Kuzuhara S, Kino Y. 2003. Establishment of an analyzing method for a Japanese encephalitis virus neutralization test in vero cells. *Vaccine* 21:1989–1994.
- Albecka A, Monserret R, Krey T, Tarr AW, Diesis E, Ball JK, Descamps V, Duverlie G, Rey F, Penin F, Dubuisson J. 2011. Identification of new functional regions in hepatitis C virus envelope glycoprotein E2. *J Virol JVI accepts*, 85:1777–1792.
- Bartosch B, Bukh J, Meunier JC, Granier C, Engle RE, Blackwelder WC, Emerson SU, Cosset FL, Purcell RH. 2003. In vitro assay for neutralizing antibody to hepatitis C virus: Evidence for broadly conserved neutralization epitopes. *Proc Natl Acad Sci USA* 100:14199–14204.
- Blight KJ, McKeating JA, Rice CM. 2002. Highly permissive cell lines for subgenomic and genomic hepatitis C virus RNA replication. *J Virol* 76:13001–13014.
- Bowen DG, Walker CM. 2005. Adaptive immune responses in acute and chronic hepatitis C virus infection. *Nature* 436:946–952.
- Bungyoku Y, Shoji I, Makine T, Adachi T, Hayashida K, Nagano-Fujii M, Ide YH, Deng L, Hotta H. 2009. Efficient production of infectious hepatitis C virus with adaptive mutations in cultured hepatoma cells. *J Gen Virol* 90:1681–1691.
- Delgrange D, Pillez A, Castelain S, Cocquerel L, Rouillé Y, Dubuisson J, Wakita T, Duverlie G, Wychowski C. 2007. Robust production of infectious viral particles in Huh-7 cells by introducing mutations in hepatitis C virus structural proteins. *J Gen Virol* 88:2495–2503.
- Deng L, Adachi T, Kitayama K, Bungyoku Y, Kitazawa S, Ishido S, Shoji I, Hotta H. 2008. Hepatitis C virus infection induces apoptosis through a Bax-triggered, mitochondrion-mediated, caspase 3-dependent pathway. *J Virol* 82:10375–10385.
- Dhillon S, Witteveldt J, Gatherer D, Owsianka AM, Zeisel MB, Zahid MN, Rychłowska M, Fong SK, Baumert TF, Angus AG, Patel AH. 2010. Mutations within a conserved region of the hepatitis C virus glycoprotein that influence virus-receptor interaction and sensitivity to neutralizing antibodies. *J Virol* 84:5494–5507.
- Dowd KA, Netski DM, Wang XH, Cox AL, Ray SC. 2009. Selection pressure from neutralizing antibodies drives sequence evolution during acute infection with hepatitis C virus. *Gastroenterology* 136:2377–2386.
- El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2007. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pre-treatment sera. *Microbiol Immunol* 51:471–482.
- El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. 2008. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. *Hepatology* 48:38–47.
- Falkowska E, Kajumo F, Garcia E, Reinus J, Dragic T. 2007. Hepatitis C virus envelope glycoprotein E2 glycans modulate entry, CD81 binding, and neutralization. *J Virol* 81:8072–8079.
- Fournier C, Duverlie G, François C, Schnuriger A, Dedeurwaerder S, Brochet E, Capron D, Wychowski C, Thibault V, Castelain S. 2007. A focus reduction neutralization assay for hepatitis C virus neutralizing antibodies. *Virol J* 4:35.
- Gal-Tanamy M, Keck ZY, Yi M, McKeating JA, Patel AH, Fong SK, Lemon SM. 2008. In vitro selection of a neutralization-resistant hepatitis C virus escape mutant. *Proc Natl Acad Sci USA* 105:19450–19455.
- Grove J, Nielsen S, Zhong J, Bassendine MF, Drummer HE, Balfe P, McKeating JA. 2008. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. *J Virol* 82:12020–12029.
- Helle F, Dubuisson J. 2008. Hepatitis C virus entry into host cells. *Cell Mol Life Sci* 65:100–112.
- Helle F, Goffard A, Morel V, Duverlie G, McKeating J, Keck ZY, Fong S, Penin F, Dubuisson J, Voisset C. 2007. The neutralizing activity of anti-hepatitis C virus antibodies is modulated by specific glycans on the E2 envelope protein. *J Virol* 81:8101–8111.
- Helle F, Vieyres G, Elkrief L, Popescu CI, Wychowski C, Descamps V, Castelain S, Roingeard P, Duverlie G, Dubuisson J. 2010. Role of *N*-linked glycans in the functions of hepatitis C virus envelope proteins incorporated into infectious virions. *J Virol* 84:11905–11915.
- Keck ZY, Olson O, Gal-Tanamy M, Xia J, Patel AH, Dreux M, Cosset FL, Lemon SM, Fong SK. 2008. A point mutation leading to hepatitis C virus escape from neutralization by a monoclonal antibody to a conserved conformational epitope. *J Virol* 82:6067–6072.
- Krey T, d'Alayer J, Kikuti CM, Saulnier A, Damier-Piolle L, Petitpas I, Johansson DX, Tawar RG, Baron B, Robert B, England P, Persson MA, Martin A, Rey FA. 2010. The disulfide bonds in glycoprotein E2 of hepatitis C virus reveal the tertiary organization of the molecule. *PLoS Pathog* 6:e1000762.
- Lavillette D, Morice Y, Germanidis G, Donot P, Soulier A, Pagkalos E, Sakellariou G, Intrator L, Bartosch B, Pawlotsky JM, Cosset FL. 2005. Human serum facilitates hepatitis C virus infection, and neutralizing responses inversely correlate with viral replication kinetics at the acute phase of hepatitis C virus infection. *J Virol* 79:6023–6034.
- Law M, Maruyama T, Lewis J, Giang E, Tarr AW, Stamataki Z, Gastaminza P, Chisari FV, Jones IM, Fox RI, Ball JK, McKeating JA, Kneteman NM, Burton DR. 2008. Broadly neutralizing antibodies protect against hepatitis C virus quasispecies challenge. *Nat Med* 14:25–27.
- Lindenbach BD, Evans MJ, Syder AJ, Wölk B, Tellinghuisen TL, Liu CC, Maruyama T, Hynes RO, Burton DR, McKeating JA, Rice CM. 2005. Complete replication of hepatitis C virus in cell culture. *Science* 309:623–626.
- Logvinoff C, Major ME, Oldach D, Heyward S, Talal A, Balfe P, Feinstone SM, Alter H, Rice CM, McKeating JA. 2004. Neutralizing antibody response during acute and chronic hepatitis C virus infection. *Proc Natl Acad Sci USA* 101:10149–10154.
- Meunier JC, Engle RE, Faulk K, Zhao M, Bartosch B, Alter H, Emerson SU, Cosset FL, Purcell RH, Bukh J. 2005. Evidence for cross-genotype neutralization of hepatitis C virus pseudo-particles and enhancement of infectivity by apolipoprotein C1. *Proc Natl Acad Sci USA* 102:4560–4565.
- Miyazawa Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K. 2007. The lipid droplet is an important organelle for hepatitis C virus production. *Nat Cell Biol* 9:1089–1097.
- Netski DM, Mosbrugger T, Depla E, Maertens G, Ray SC, Hamilton RG, Roundtree S, Thomas DL, McKeating J, Cox A. 2005. Humoral immune response in acute hepatitis C virus infection. *Clin Infect Dis* 41:667–675.
- Owsianka AM, Tarr AW, Keck ZY, Li TK, Witteveldt J, Adair R, Fong SK, Ball JK, Patel AH. 2008. Broadly neutralizing human monoclonal antibodies to the hepatitis C virus E2 glycoprotein. *J Gen Virol* 89:653–659.
- Perotti M, Mancini N, Diotti RA, Tarr AW, Ball JK, Owsianka A, Adair R, Patel AH, Clementi M, Burioni R. 2008. Identification of a broadly cross-reacting and neutralizing human monoclonal

- antibody directed against the hepatitis C virus E2 protein. *J Virol* 82:1047–1052.
- Pestka JM, Zeisel MB, Bläser E, Schürmann P, Bartosch B, Cosset FL, Patel AH, Meisel H, Baumert J, Viazov S, Rispeter K, Blum HE, Roggendorf M, Baumert TF. 2007. Rapid induction of virus-neutralizing antibodies and viral clearance in a single-source outbreak of hepatitis C. *Proc Natl Acad Sci USA* 104:6025–6030.
- Saito T, Watanabe H, Shao L, Okumoto K, Hattori E, Sanjo M, Misawa K, Suzuki A, Takeda T, Sugahara K, Ito JI, Saito K, Togashi H, Kawata S. 2004. Transmission of hepatitis C virus quasispecies between human adults. *Hepatology* 39:57–62.
- Sasayama M, Deng L, Kim SR, Ide Y, Shoji I, Hotta H. 2010. Analysis of neutralizing antibodies against hepatitis C virus in patients who were treated with pegylated-interferon plus ribavirin. *Kobe J Med Sci* 56:E60–E66.
- Scheel TK, Gottwein JM, Jensen TB, Prentoe JC, Hoegh AM, Alter HJ, Eugen-Olsen J, Bukh J. 2008. Development of JFH1-based cell culture systems for hepatitis C virus genotype 4a and evidence for cross-genotype neutralization. *Proc Natl Acad Sci USA* 105:997–1002.
- Tarr AW, Owsianka AM, Timms JM, McClure CP, Brown RJ, Hickling TP, Pietschmann T, Bartenschlager R, Patel AH, Ball JK. 2006. Characterization of the hepatitis C virus E2 epitope defined by the broadly neutralizing monoclonal antibody AP33. *Hepatology* 43:592–601.
- Wakita T, Pietschmann T, Kato T, Date T, Miyamoto M, Zhao Z, Murthy K, Habermann A, Kräusslich HG, Mizokami M, Bartenschlager R, Liang TJ. 2005. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 11:791–796.
- Zhong J, Gastaminza P, Cheng G, Kapadia S, Kato T, Burton DR, Wieland SF, Uprichard SL, Wakita T, Chisari FV. 2005. Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 102:9294–9299.

Polymorphisms of Hepatitis C Virus Non-Structural Protein 5A and Core Protein and Clinical Outcome of Pegylated-Interferon/Ribavirin Combination Therapy

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Key Words

Hepatitis C virus · Non-structural protein 5A · Interferon/ribavirin resistance-determining region · Interferon sensitivity-determining region · Core protein · Sustained virological response · Prediction

Abstract

Objective: Hepatitis C virus (HCV genome) polymorphisms are thought to influence the outcome of pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy. This study aimed to examine non-structural protein 5A (NS5A) polymorphisms, e.g. IFN/RBV resistance-determining region (IRRDR) and IFN sensitivity-determining region (ISDR), and core protein polymorphism as predictive therapeutic markers. **Methods:** Pre-treatment sequences of NS5A and core regions were analyzed in 68 HCV-1b-infected patients treated with PEG-IFN/RBV. **Results:** Of 24 patients infected with HCV having an IRRDR with 6 or more mutations (IRRDR \geq 6), 18 (75%) patients achieved sustained virological response (SVR), whereas only 11 (25%) of 44 patients infected with HCV having IRRDR \leq 5 did. IRRDR \geq 6 was significantly associated with SVR ($p < 0.0001$). On the other hand, ISDR \geq 2 was significant-

ly associated with relapse (either before [breakthrough] or after end-of-treatment response [ETR-relapse]) ($p < 0.05$) and a point mutation of the core protein from Arg to Gln at position 70 (Gln⁷⁰) was significantly associated with null-response ($p < 0.05$). Multivariate analysis identified IRRDR \geq 6 as the only viral genetic factor that independently predicted SVR. **Conclusion:** NS5A (IRRDR and ISDR) and core protein polymorphisms are associated with the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR \geq 6 is a useful marker for prediction of SVR.

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Introduction

Hepatitis C virus (HCV) is the major cause of chronic liver diseases worldwide [1]. As a consequence of the long-term persistence of chronic hepatitis C, the number of patients with hepatocellular carcinoma is expected to increase further over the next 20 years [2]. To reduce the impact of this worldwide health problem, efficient treatment is required. Currently, a combination therapy of pegylated-interferon- α and ribavirin (PEG-IFN/RBV) is a

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standard treatment for chronic hepatitis C [3]. However, this therapy is sometimes difficult to tolerate and results in a sustained virological response (SVR) in only ~50% of patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b [3]. Given the considerable side effects, the possibility of discontinuation and the high cost of this treatment, prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients in more accurately assessing the likelihood of an SVR and thus in making more informed treatment decisions [4].

Since the HCV genotype is one of the major factors affecting the IFN-based therapy response, IFN resistance is, at least partly, genetically encoded by HCV itself [5]. In this context, non-structural protein 5A (NS5A) has been widely discussed for its correlation with IFN responsiveness. Enomoto et al. [6] proposed that sequence variations within a region in NS5A spanning from amino acids (aa) 2,209 to 2,248, called the IFN sensitivity-determining region (ISDR), is correlated with IFN responsiveness. Recently, we identified a new region near the C-terminus of NS5A spanning from aa 2,334 to 2,379, which we referred to as the IFN/RBV resistance-determining region (IRRDR) [7]. The degree of sequence variation within IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions within the core protein in Japanese patients infected with HCV-1b has also been proposed [8, 9]. In multivariate analysis, the criterion of double-wild core, presence of Arg at position 70 and Leu at position 91 (Arg⁷⁰/Leu⁹¹), was identified as an independent SVR predictor.

This study aimed to examine NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism as predictive markers for HCV treatment outcome. The core protein with Arg⁷⁰/Leu⁹¹ was defined as wild-core while the other patterns as non-wild-core. The possible correlation of either Arg⁷⁰ alone or Leu⁹¹ alone with the clinical outcome of PEG-IFN/RBV therapy was also examined.

Patients and Methods

Patients

A total of 68 patients seen at Kobe Asahi Hospital in Kobe, Japan, who were chronically infected with HCV-1b, with diagnoses based on anti-HCV antibody detection and HCV-RNA detection, were enrolled in the study. HCV subtype was determined according to the method of Okamoto et al. [10]. Patients were treated with PEG-IFN α -2b (Pegintron[®]; Schering-Plough, Kenilworth,

N.J., USA) (1.5 μ g/kg b.w., once weekly, s.c.) and RBV (Rebetol[®]; Schering-Plough) (600–800 mg daily, per os), according to a standard treatment protocol for Japanese patients established by a hepatitis study group of the Ministry of Health, Labor and Welfare, Japan. All patients received >80% of scheduled dosage of PEG-IFN and RBV. Serum samples were collected from the patients at intervals of 4 weeks before, during and after the treatment, and tested for HCV RNA and core antigen titers as reported previously [11].

The study protocol was approved beforehand by the Ethic Committee in Kobe Asahi Hospital, and written informed consent was obtained from each patient prior to the treatment.

Sequence Analysis of HCV NS5A and Core

HCV RNA was extracted from 140 μ l of serum using a commercially available kit (QIAmp viral RNA kit; Qiagen, Tokyo, Japan). Amplification of full-length NS5A and core regions of the HCV genome were performed as described elsewhere [7, 11, 12]. The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing. The aa sequences were deduced and aligned using Genetyx Win software version 7.0 (Genetyx Corp., Tokyo, Japan).

Statistical Analysis

Statistical differences in the patients' baseline parameters according to the degree of IRRDR polymorphism were determined by Student's t test for numerical variables and Fisher's exact probability test for categorical variables. Likewise, statistical differences in treatment responses according to NS5A and core polymorphisms were determined by Fisher's exact probability test. Kaplan-Meier HCV survival curve analysis was performed based on serum HCV-RNA positivity data during the treatment period (48 weeks) according to NS5A and core polymorphisms. The data obtained were evaluated by the log-rank test. Uni- and multivariate logistic analyses were performed to identify variables that independently predicted the treatment outcome. Variables with a p value of <0.1 in univariate analysis were included in a multivariate logistic regression analysis. The odds ratios and 95% confidence intervals (95% CI) were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS Inc., Chicago, Ill., USA). Unless otherwise stated, a p value <0.05 was considered as statistically significant.

Nucleotide Sequence Accession Numbers

The sequence data reported in this paper have been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases under the accession numbers AB285035 through AB285081, AB354116 through AB354118, and AB518774 through AB518861.

Results

Patients' Responses to PEG-IFN/RBV Combination Therapy

Among 68 patients enrolled in this study, HCV-RNA negativity was achieved by 8 (12%) patients at week 4 (rapid virological response [RVR]), 36 (53%) patients at week 12 (early virological response [EVR]), 47 (69%) patients at

Table 1. Proportions of various virological responses of patients treated with PEG-IFN/RBV

Virological response	Proportion, patients	
	n/total	%
RVR	8/68	12
EVR	36/68	53
ETR	47/68	69
SVR	29/68	43
Non-SVR	39/68	57
Null-response	17/68	25
ETR-relapse	18/68	26
Breakthrough	4/68	6

PEG-IFN/RBV = Pegylated-interferon/ribavirin; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response; SVR = sustained virological response.

week 48 (end-of-treatment response [ETR]) and 29 (43%) patients at week 72 (SVR) (table 1). A total of 39 patients (57%) failed to achieve SVR and they were referred to as non-SVR. Non-SVR can be further divided into three categories: (i) null-response, which is defined by continued presence of serum HCV RNA during the entire period of the treatment and follow-up; (ii) breakthrough, defined as transient disappearance of HCV RNA followed by its reappearance before the end of the 48-week treatment, and (iii) ETR-relapse, defined by reappearance of HCV RNA after ETR has been achieved. Seventeen (25%) patients were null-response while 18 (26%) and 4 (6%) patients were ETR-relapse and breakthrough, respectively (table 1).

Correlation between NS5A Polymorphism and Treatment Responses

Using a receiver operating characteristic curve analysis, 6 mutations in IRRDR were previously estimated as an optimal cutoff number of mutations for SVR prediction [7]. Initially the correlation between the patients' demographic, hematological, biochemical and virological baseline parameters and the degree of IRRDR polymorphism was examined. This analysis revealed that patient's sex was the only factor that significantly correlated to the degree of IRRDR polymorphism since 49% (17/35) of males were infected with HCV isolates having IRRDRs with 6 mutations or more (IRRDR \geq 6) compared to 21% (7/33) of females ($p = 0.02$) (table 2). HCV-RNA titers or HCV core antigen titers did not differ significantly between patients infected with HCV isolates of IRRDR \geq 6 and those of IRRDR \leq 5.

Next, the possible correlation between IRRDR polymorphism and the ultimate treatment responses was examined. Among 24 patients infected with HCV isolates of IRRDR \geq 6, 18 (75%), 6 (25%), 3 (12.5%) and 3 (12.5%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3). By contrast, among 44 patients infected with HCV isolates of IRRDR \leq 5, 11 (25%), 33 (75%), 14 (32%) and 19 (43%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. The proportions of different treatment responses among HCV isolates with IRRDR \geq 6 and IRRDR \leq 5 were significantly different. Furthermore, patients infected with HCV isolates with Ala at position 2360 (Ala²³⁶⁰) in IRRDR had a more significant likelihood of SVR than those infected with HCV isolates with non-Ala²³⁶⁰, who tended to be non-SVR, in particular null-response (table 3; fig. 1).

As the IRRDR polymorphism was closely correlated with the ultimate treatment responses, it was also significantly correlated with the on-treatment responses, in particular EVR and ETR (table 4). However, there was no significant correlation between the IRRDR polymorphism and RVR. Also, the presence of Ala²³⁶⁰ was correlated significantly with ETR.

Regarding the analysis of ISDR polymorphism and its correlation to the treatment responses, first, the criterion of ISDR with 4 mutations or more (ISDR \geq 4), the initial criterion of IFN responsiveness proposed by Enomoto et al. [6] was tested. Since the prevalence of ISDR \geq 4 was only 9% (6/68) of all isolates analyzed, this criterion did not significantly correlate with the treatment responses (data not shown). Next, the correlation between the treatment responses and ISDR mutations at a cutoff point of 2 mutations, a newly proposed ISDR criterion of PEG-IFN/RBV responsiveness [13, 14] was tested. Although there was no significant difference in the proportions of SVR and non-SVR between HCV isolates with ISDR of 2 mutations or more (ISDR \geq 2) and those of ISDR \leq 1, a small but significant difference in the proportions of SVR and relapse (ETR-relapse *plus* breakthrough) was observed between ISDR \geq 2 and ISDR \leq 1 (table 3). Interestingly, ISDR polymorphism was the only virological factor examined in this study that showed a significant correlation with RVR (table 4). However, this correlation disappeared when further time points of treatment course, such as EVR and ETR, were considered.

Table 2. Correlation between IRRDR polymorphism and patients' demographic characteristics

Factor	IRRDR \geq 6	IRRDR \leq 5	p value
Age, mean \pm SD	58.71 \pm 8.44	59.61 \pm 10.30	0.71
Sex, male/female	17/7	18/26	0.02
Body weight, kg	59.87 \pm 9.56	58.20 \pm 11.92	0.56
Platelets, $\times 10^4/mm^3$	17.22 \pm 5.5	14.96 \pm 4.71	0.16
Hemoglobin, g/dl	14.25 \pm 1.48	13.55 \pm 1.77	0.11
γ -GTP, IU/l	49.50 \pm 44.29	55.60 \pm 65.60	0.69
GPT, IU/l	47.54 \pm 33.09	49.33 \pm 34.78	0.84
HCV-RNA, KIU/ml	2,070.21 \pm 1,720.27	2,038.57 \pm 1,963.05	0.95
HCV core antigen, fmol/l	6,750.87 \pm 6,859.82	9,320.52 \pm 10,636.48	0.30

IRRDR = Interferon/ribavirin resistance-determining region; γ -GTP = γ -guanosine triphosphate; GPT = glutamic pyruvate transaminase.

Table 3. Correlation between NS5A and core protein polymorphisms and ultimate virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total ^a	SVR ^b	Non-SVR	Null-response	Relapse (ETR-relapse <i>plus</i> breakthrough)	p value		
							SVR vs. non-SVR	SVR vs. null-response	SVR vs. relapse (ETR-relapse <i>plus</i> breakthrough)
NS5A	IRRDR \geq 6	24	18 (75) ^c	6 (25)	3 (12.5)	3 (12.5)	<0.0001	0.005	0.0006
	IRRDR \leq 5	44	11 (25)	33 (75)	14 (32)	19 (43)			
	Ala ²³⁶⁰	18	12 (67)	6 (33)	1 (5)	5 (28)	0.026	0.016	0.2
	Non-Ala ²³⁶⁰	50	17 (34)	33 (66)	16 (32)	17 (34)			
	ISDR \geq 2	18	10 (56)	8 (44)	6 (33)	2 (11)	0.27	1.0	0.048
	ISDR \leq 1	50	19 (38)	31 (62)	11 (22)	20 (40)			
Core	Wild-core (Arg ⁷⁰ /Leu ⁹¹)	33	18 (55)	15 (45)	5 (15)	10 (30)	0.1	0.07	0.27
	Non-wild-core	35	11 (31)	24 (69)	12 (34)	12 (34)			
	Gln ⁷⁰	21	5 (24)	16 (76)	8 (38)	8 (38)	0.06	0.04	0.19
	Non-Gln ⁷⁰	47	24 (51)	23 (49)	9 (19)	14 (30)			
	Met ⁹¹	19	7 (37)	12 (63)	5 (26)	7 (37)	0.59	0.74	0.75
	Non-Met ⁹¹	49	22 (45)	27 (55)	12 (24)	15 (31)			

SVR = Sustained virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70; Met⁹¹ = methionine at position 91.

^a Total number of isolates with a given factor.

^b Number of SVR, non-SVR, null-response or relapse (ETR-relapse *plus* breakthrough) cases with a given factor.

^c Values in parentheses are percentages.

Correlation between Core Polymorphism and Treatment Responses

Recently, it was reported that polymorphism at positions 70 and/or 91 of the core protein of HCV-1b correlates with and predicts the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy

[8, 9]. We aimed to test the consistency of this observation among our patient cohort. The result revealed that among 33 patients infected with HCV isolates of wild-core (Arg⁷⁰/Leu⁹¹), 18 (55%), 15 (45%), 5 (15%) and 10 (30%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively (table 3; fig. 1). On

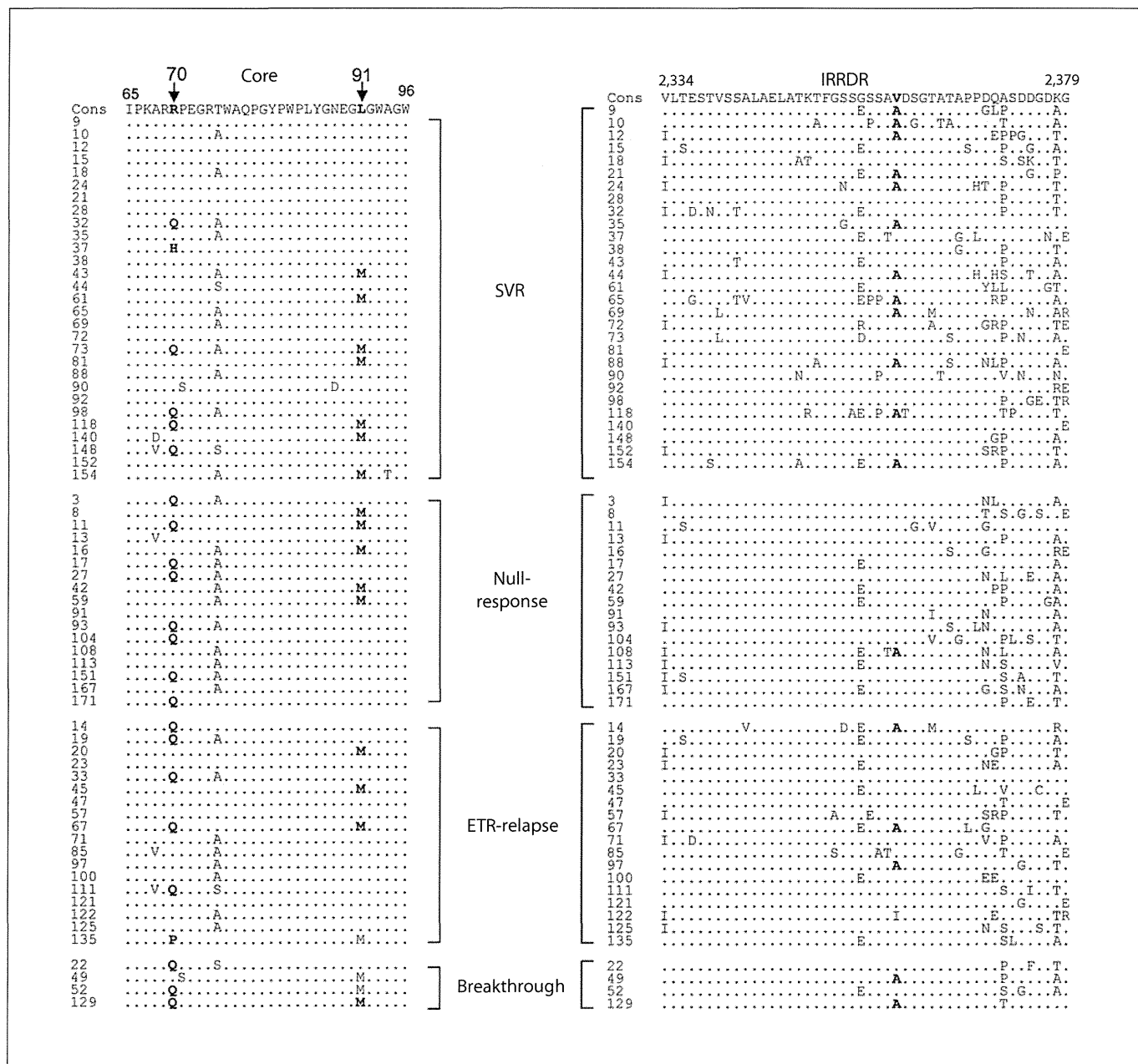


Fig. 1. Sequence alignment of the core protein (aa 65–96) and IRRDR of NS5A obtained from pretreated sera in patients infected with HCV-1b. The consensus (Cons) sequence is shown at the top. Amino acids at positions 70 and 91 of the core protein, and position 2360 of NS5A are shown in boldface.

the other hand, of 35 patients infected with HCV isolates of non-wild-core, 11 (31%), 24 (69%), 12 (34%) and 12 (34%) patients were SVR, non-SVR, null-response and relapse (ETR-relapse *plus* breakthrough), respectively. Thus, there was no significant correlation between wild-core and SVR or non-SVR ($p = 0.1$). However, a single mutation at posi-

tion 70 (Gln⁷⁰ vs. non-Gln⁷⁰) was significantly correlated with treatment outcome (SVR vs. null-response; $p = 0.04$).

As for the on-treatment responses, wild-core (Arg⁷⁰/Leu⁹¹) was significantly correlated with EVR and ETR, whereas Gln⁷⁰ was correlated with non-EVR and non-ETR (table 4).

Table 4. Correlation between NS5A and core protein polymorphisms and on-treatment virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total ^a	RVR ^b	Non-RVR	EVR	Non-EVR	ETR	Non-ETR	p value		
									RVR vs. non-RVR	EVR vs. non-EVR	ETR vs. non-ETR
NS5A	IRRDR \geq 6	24	5 (21) ^c	19 (79)	17 (71)	7 (29)	21 (87)	3 (13)	0.12	0.04	0.026
	IRRDR \leq 5	44	3 (7)	41 (93)	19 (43)	25 (57)	26 (59)	18 (41)	0.19	0.1	0.04
	Ala ²³⁶⁰	18	4 (22)	14 (78)	13 (72)	5 (28)	16 (89)	2 (11)			
	Non-Ala ²³⁶⁰	50	4 (8)	46 (92)	23 (46)	27 (54)	31 (62)	19 (38)	0.003	0.79	0.39
	ISDR \geq 2	18	6 (33)	12 (67)	9 (50)	9 (50)	11 (61)	7 (39)			
ISDR \leq 1	50	2 (4)	48 (96)	27 (54)	23 (46)	36 (72)	14 (28)				
Core	Wild-core (Arg ⁷⁰ /Leu ⁹¹)	33	5 (15)	28 (85)	23 (70)	10 (30)	28 (85)	5 (15)	0.47	0.009	0.009
	Non-wild-core	35	3 (9)	32 (91)	13 (37)	22 (63)	19 (54)	16 (46)	1.0	0.009	0.02
	Gln ⁷⁰	21	2 (10)	19 (90)	6 (29)	15 (71)	10 (48)	11 (52)			
	Non-Gln ⁷⁰	47	6 (13)	41 (87)	30 (64)	17 (36)	37 (79)	10 (21)	1.0	0.29	0.25
	Met ⁹¹	19	2 (11)	17 (89)	8 (42)	11 (58)	11 (58)	8 (42)			
	Non-Met ⁹¹	49	6 (12)	43 (88)	28 (57)	21 (43)	36 (73)	13 (27)			

RVR = Rapid virological response; EVR = early virological response; ETR = end-of-treatment response; IRRDR = interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91;

Gln⁷⁰ = glutamine at position 70; Met⁹¹ = methionine at position 91.

^a Total number of isolates with a given factor. ^b Number of RVR, non-RVR, EVR, non-EVR, ETR or non-ETR cases with a given factor. ^c Values in parentheses are percentages.

Table 5. Correlation between NS5A and core protein polymorphisms

Factor	% (number of subjects/number of subtotal) ^a		p value
	IRRDR \geq 6	IRRDR \leq 5	
Ala ²³⁶⁰	50 (12/24)	14 (6/44)	0.003
Non-Ala ²³⁶⁰	50 (12/24)	86 (38/44)	0.047
ISDR \geq 2	42 (10/24)	18 (8/44)	
ISDR \leq 1	58 (14/24)	82 (36/44)	0.04
Wild-core (Arg ⁷⁰ /Leu ⁹¹)	67 (16/24)	39 (17/44)	
Non-wild-core	33 (8/24)	61 (27/44)	0.27
Gln ⁷⁰	21 (5/24)	36 (16/44)	
Non-Gln ⁷⁰	79 (19/24)	64 (28/44)	

IRRDR = Interferon/ribavirin resistance-determining region; Ala²³⁶⁰ = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = arginine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70.

^a Number of isolates with a certain factor/total number of HCV isolates with IRRDR \geq 6 or IRRDR \leq 5.

Correlation between NS5A and Core Polymorphisms

We then examined the possible correlation among the polymorphic factors in NS5A and core proteins. A significant correlation was observed between IRRDR \leq 5 and non-Ala²³⁶⁰ as the majority (86%) of HCV isolates with IRRDR \leq 5 had non-Ala²³⁶⁰ (p = 0.003) (table 5). Also, a significant correlation was obtained between IRRDR \leq 5 and ISDR \leq 1 since 82% of IRRDR \leq 5 were ISDR \leq 1 (p = 0.047). When IRRDR and core polymorphisms were compared, IRRDR \geq 6 was significantly correlated with wild-core (Arg⁷⁰/Leu⁹¹) (p = 0.04). On the other hand, there was no significant correlation between IRRDR \geq 6 and non-Gln⁷⁰, or IRRDR \leq 5 and Gln⁷⁰, although the majority (79%) of IRRDR \geq 6 were non-Gln⁷⁰.

Influence of NS5A and Core Polymorphisms on HCV Clearance Kinetics during PEG-IFN/RBV Combination Therapy

To investigate the influence of NS5A and core polymorphisms on HCV-RNA kinetics during the entire course of PEG-IFN/RBV combination therapy, Kaplan-Meier HCV survival curve analysis was carried out based on HCV-RNA positivity according to NS5A and core

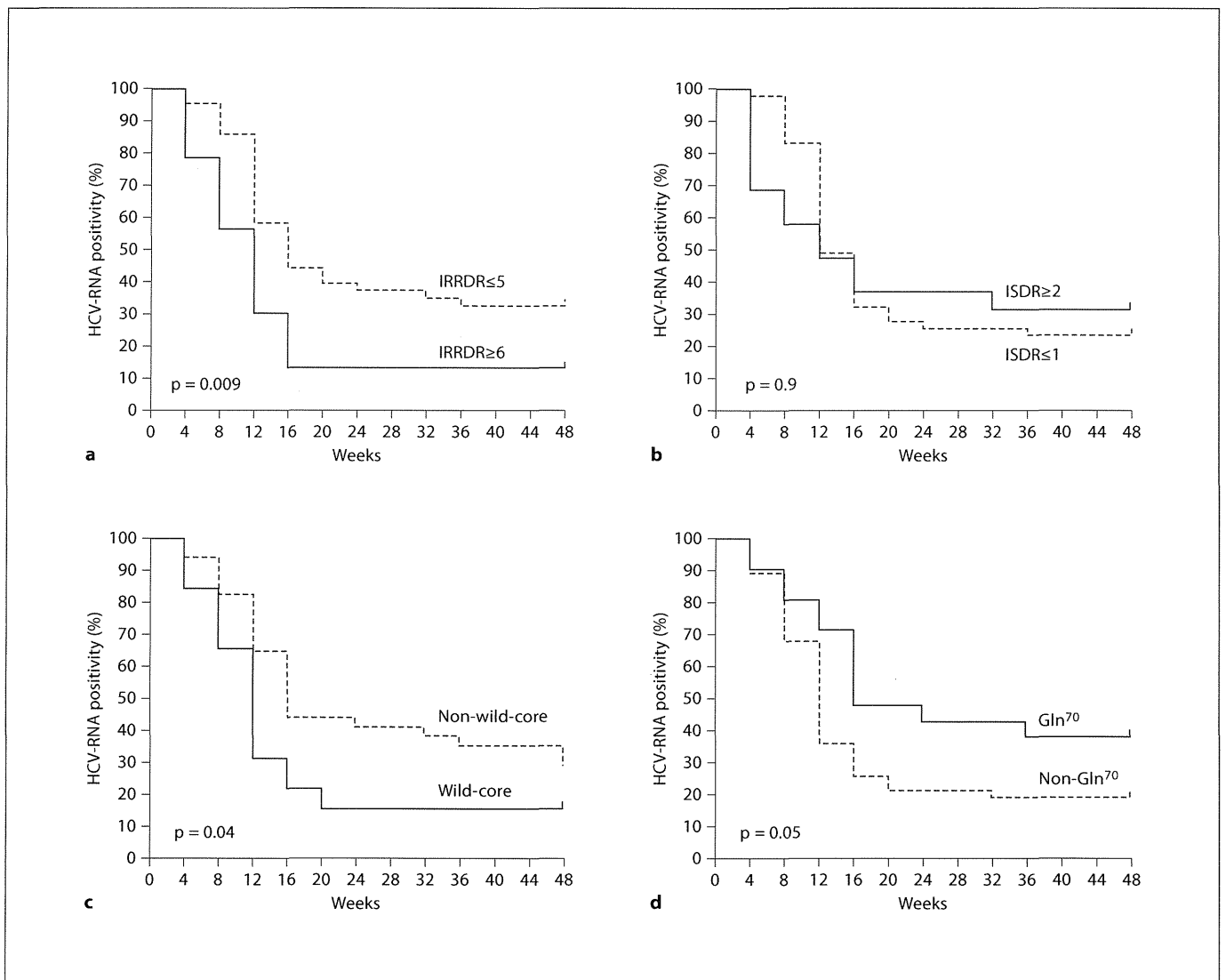


Fig. 2. Kaplan-Meier HCV survival curve analysis based on HCV-RNA positivity during the whole treatment course according to NS5A (**a, b**) and the core protein (**c, d**) polymorphisms. The difference between the analyzed groups was measured by the log-rank test.

polymorphisms. The result showed that HCV isolates of $IRRDR \geq 6$ were cleared from patients' sera more rapidly than those with $IRRDR \leq 5$ (fig. 2a). On the other hand, HCV-RNA clearance kinetics did not differ significantly between HCV isolates of $ISDR \geq 2$ and those of $ISDR \leq 1$ (fig. 2b). As for the core polymorphism, HCV isolates of non-wild-core or Gln^{70} persisted in patients' sera for longer periods of time than those of wild-core (Arg^{70}/Leu^{91}) or non- Gln^{70} (fig. 2c, d).

Next, HCV clearance kinetics during the very early stages of the treatment course, e.g., 24 h, 1, 2 and 4 weeks

after initiation of PEG-IFN/RBV therapy was examined. For this purpose, a possible correlation between the degree of $IRRDR$, $ISDR$ and core polymorphisms and the proportion of patients who achieved significant reduction (1 log after 24 h, 1 log after 1 week, 1.5 log after 2 weeks, and 2 log after 4 weeks) of core antigen titers was analyzed. Interestingly, $IRRDR \geq 6$ was significantly associated with reduction and/or disappearance of serum HCV core antigen titers at 24 h, 1, 2 and 4 weeks after initiation of the treatment (table 6). Again, there was no significant correlation between $ISDR$ sequence variation

Table 6. Correlation between the proportions of patients with rapid reduction of HCV core antigen titers and degree of NS5A and core protein polymorphisms

Protein	Criteria	Number of patients with significant reduction of HCV core antigen titers/number of total							
		24 h ^a (≥1 log) ^b	p value	1 week (≥1 log)	p value	2 weeks (≥1.5 log)	p value	4 weeks (≥2 log)	p value
NS5A	IRRDR≥6	20/23	0.0006	18/23	0.004	17/23	0.018	19/23	0.008
	IRRDR≤5	17/40		16/40		16/40		19/40	
	ISDR≥2	10/19	1.0	11/19	0.59	10/19	1.0	11/19	1.0
	ISDR≤1	24/44		21/44		22/44		27/44	
Core	Wild core (Arg ⁷⁰ /Leu ⁹¹)	23/31	0.01	22/31	0.02	20/31	0.13	24/31	0.005
	Non-wild-core	13/32		13/32		14/32		13/32	
	Gln ⁷⁰	6/19	0.03	5/19	0.01	6/19	0.06	6/19	0.004
	Non-Gln ⁷⁰	28/44		27/44		26/44		32/44	

Note: Patients Nos. 108, 111, 129, 135 and 152 were excluded from this analysis because their core antigen titers at certain time points were missing.

IRRDR = Interferon/ribavirin resistance-determining region; ISDR = interferon sensitivity-determining region; Arg⁷⁰ = argi-

nine at position 70; Leu⁹¹ = leucine at position 91; Gln⁷⁰ = glutamine at position 70.

^a Period after initiation of IFN/RBV therapy.

^b Criteria of significant reduction of HCV core antigen titers.

(ISDR≥2 and ISDR≤1) and reduction of HCV core antigen titers during the very early stages of PEG-IFN/RBV therapy. On the other hand, non-wild-core or Gln⁷⁰ were significantly associated with slow reduction and/or persistence of HCV core antigen in the patients' sera (table 6).

Identification of Independent Predictive Factors for SVR by Uni- and Multivariate Logistic Regression Analyses

Finally, in order to identify significant independent predictive factors of PEG-IFN/RBV treatment outcome, first, all available data of baseline patients' parameters, on-treatment responses and NS5A and core polymorphisms were entered in a univariate logistic analysis. This analysis yielded 11 factors that were correlated or nearly correlated with the treatment outcome; IRRDR mutations categorized as IRRDR≥6 and IRRDR≤5, Ala²³⁶⁰ and non-Ala²³⁶⁰, core protein polymorphism categorized as wild-core (Arg⁷⁰/Leu⁹¹) and non-wild-core, Gln⁷⁰ and non-Gln⁷⁰, RVR and non-RVR, EVR and non-EVR, ETR and non-ETR, HCV core antigen titers, age, platelets count and hemoglobin levels (table 7). Subsequently, these 11 factors were entered in multivariate logistic regression analysis. This analysis yielded IRRDR mutations (p = 0.005), EVR (p = 0.0001) and age (p = 0.02) as independent predictive factors of PEG-IFN/RBV treatment outcome (table 7).

Discussion

Both host and viral genetic polymorphisms influence the outcome of PEG-IFN/RBV therapy for HCV-infected patients [15]. It has recently been reported that host genetic polymorphisms near or within the IL28B gene on chromosome 19 show a significant impact on the treatment outcome for patients infected with HCV genotype 1 (HCV-1a and -1b) [16–18]. Also, HCV genetic polymorphisms have been known to contribute to differences in the treatment outcome, as demonstrated by the observations that SVR rates for patients infected with HCV genotypes 2 and 3 are higher than those for patients infected with HCV genotype 1 [15]. Moreover, viral genetic polymorphisms, especially in the NS5A (ISDR and IRRDR) and the core regions, among HCV isolates of a given genotype have been linked to the difference in SVR rates [6–9, 19, 20]. In the present study, we compared the impact of IRRDR, ISDR and core polymorphisms of HCV-1b isolates on the clinical outcome of PEG-IFN/RBV therapy. Our results suggest that the degree of IRRDR mutations is more dominant than that of ISDR mutations and core polymorphism for predicting the anti-HCV treatment outcome.

IRRDR corresponds to a region near the C-terminus of NS5A. The obtained result that the IRRDR polymorphism influences the clinical outcome of IFN-based anti-HCV therapy can be linked to a recent experimental observation by Tsai et al. [21]. They reported that an HCV