transfected Huh7.5 cells were monitored by immunoblotting using the anti-FLAG antibody (Sigma–Aldrich, St. Louis, MO).

2.6. Quantification of HCV core protein

HCV core protein in the cells or cell-culture supernatants was quantified by using a highly sensitive enzyme immunoassay (Ortho HCV antigen ELISA kit; Ortho Clinical Diagnostics). To determine the intra-cellular amounts of core, cell lysates were prepared as described by Schaller et al. [14].

2.7. Blocking of virus attachment and entry with anti-CD81 antibody

Blocking of virus attachment and entry with anti-CD81 antibody was performed essentially as described previously [9]. Huh7.5 cells (6×10^4 cells/well of a 24-well plate) were pre-treated with anti-CD81 antibody (clone JS-81; BD Biosciences) or an isotype-matched control antibody (purified mouse IgG1, isotype control; BD Biosciences) as indicated for 1 h. Cells were then infected with the reporter viruses for 6 h. The viruses were removed, and then the culture medium was replaced with complete DMEM. On day 2 post-infection, the cells were lysed with a passive lysis buffer as mentioned above. The efficiency of infection was monitored by measuring the luciferase activity of the cell lysate.

2.8. Transfection of microRNA inhibitor

Huh7.5 cells were electroporated with luciferase reporter HCV RNA as mentioned above, and then the cells were seeded in a well of a 24-well plate. To analyze the effect of inhibition of microRNA (miR), both a specific miRNA inhibitor (Anti-miR™ miRNA) and a non-targeting negative control (Anti-miR™miRNA Inhibitors—Negative Control) were purchased from Ambion, Inc. 50 pmol of a specific miRNA inhibitor or negative control were transfected into luciferase reporter RNA-electroporated Huh7.5 cells by using a siPORT™ NeoFX™ Transfection Agent (Ambion) according to the manufacturer's instructions. At 48 h post-transfection, the cells were harvested, and viral replication was determined by luciferase assay of the cell lysate.

2.9. Polyions

The polyanions heparin (mol. wt. 3000), dextran sulfate (mol. wt. 50,000) and polyvinyl sulfate (mol. wt. 150,000), and the polycations polybrene (mol. wt. 3000), DEAE-dextran (mol. wt. 100,000), and poly-L-lysine (mol. wt. 500,000) (all purchased from Sigma) were dissolved in PBS.

3. Results

3.1. Construction and characterization of luciferase reporter HCV

To construct a reporter HCV that can permit easy monitoring of both virus production and intra-cellular viral growth kinetics, we constructed the bicistronic HCV constructs by inserting a luciferase reporter gene into the 5′ end of the coding sequence of the JFH1 or J6/JFH1 full-genome plasmids clone as shown in Fig. 1A. In the transcript derived from bicistronic reporter HCV clone, the HCV and EMCV IRESs are responsible for the translation of the luciferase protein and all HCV proteins, respectively. A reporter construct with NS5B GDD to GND mutation, which disrupts viral polymerase function, was also constructed by site-directed mutagenesis, and served as a negative control for viral genome replication. To examine the replication level of reporter HCVs, we prepared the RNAs from each construct by *in vitro* transcription, and then transfected them into Huh7.5 cells by an electroporation technique. The viral replication was quantified up to 10 days post-transfection by using an HCV core-specific ELISA and luciferase reporter assay. As shown in Fig. 1B, the transfection of RNAs of both the JFH1/Luc and J6/JFH1/Luc reporter clones induced intra-cellular HCV core protein expression, which peaked on day 2 post-transfection. Both JFH1/Luc and J6/JFH1/Luc showed similar kinetics, and the high level core protein expression continued until day 10 post-transfection. As expected, the GND mutant exhibited 100-fold lower intra-cellular core protein expression on day 2 post-transfection. The level of core expression by the GND mutant continued to decline thereafter, and fell below the detection limit on day 10 post-transfection. As shown in Fig. 1C, both JFH1/Luc and J6/JFH1/Luc induced similar levels of luciferase activity in Huh7.5 cells at 4 h after electroporation. This result indicated that both RNAs were electroporated with similar efficiency because RNA replication had not started at that time and all the luciferase was translated from the input RNA. At 4 days post-electroporation, the luciferase activities of both JFH1/Luc and J6/JFH1/Luc were 10-fold greater than those measured at 4 h after electroporation. Subsequently, JFH1/Luc and J6/JFH1/Luc showed almost the same kinetics of luciferase activity until 10 days post-transfection. At 3 days post-electroporation, both JFH1/Luc and J6/JFH1/Luc electroporated cells were stained with HCV-positive patient sera, and the rate of intra-cellular replication was then visualized using immunofluorescent microscopy as previously reported [12]. As a result, the HCV-positive rates were 17% and 19% for JFH1/Luc and J6/JFH1/Luc, respectively (Fig. 1D). These results indicated that the luciferase activity of reporter HCV-transfected cells reflected the intra-cellular viral replication, and also suggested that both JFH1 and J6/JFH1 had similar intra-cellular replication ability in Huh7.5 cells.

3.2. Production of cell-free infectious progeny virions in luciferase reporter HCV RNA-transfected cells

Next, we assessed the potential of the reporter HCV to produce infectious progeny virions. Huh7.5 cells were electroporated with the reporter RNAs, and the culture supernatant was collected at various time points. To analyze the release of progeny virions from the reporter RNA-electroporated cells, the amounts of core protein in culture supernatants were

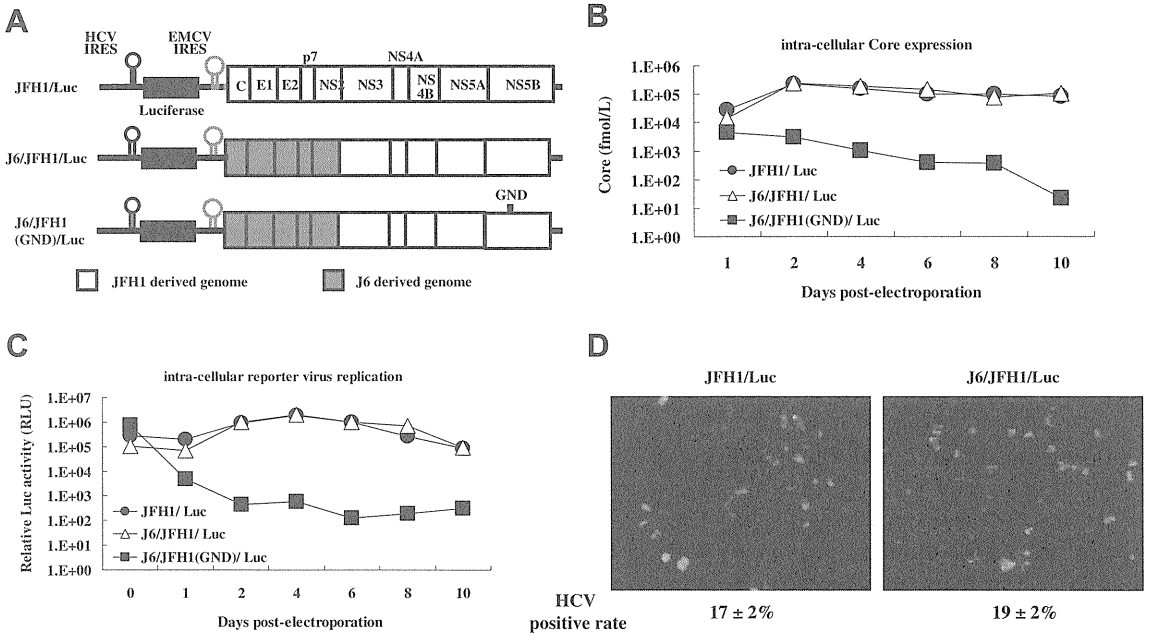

Fig. 1. Schematics of luciferase reporter HCV in this study. (A) Organization of luciferase reporter HCV. The luciferase gene is depicted as a black box. The JFH1-derived open reading frame and J6-derived open reading frame are depicted as a gray box and white box, respectively. As a negative control, a GND mutation was introduced to NS5B RdRp. (B, C) Virus replication kinetics in Huh7.5 cells of luciferase reporter HCV. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and the cells were assayed for core protein ELISA (B) and luciferase activity (C) at intervals as indicated. The assays were repeated at least three times, and the mean values are presented. Huh7.5 cells electroporated with JFH1/Luc or J6/JFH1/Luc RNA were subjected to indirect immunofluorescence analysis at 3 days post-electroporation (D). Cells were incubated with an HCV-infected patient's serum followed by FITC-labeled goat anti-human IgG (green). In parallel, the cells were stained with Hoechst 33342 to visualize the nuclei (blue). The HCV-positive rate was calculated by counting the number of HCV-positive cells among the total cells, and the data represent the means and SE of three independent experiments.

analyzed by ELISA. As shown in Fig. 2A, electroporation of both reporter viral RNAs with Huh7.5 cells released the HCV core protein into the culture supernatants. The levels of core protein released from both reporter HCV RNAs peaked at 6 days post-electroporation. The amount of core protein of the J6/JFH1/Luc supernatants was 2–4 fold greater than that of JFH1/Luc among all the time points tested. In parallel, to analyze the infectivity of progeny virions produced from reporter RNA-electroporated cells, these supernatants were used as inocula for naïve Huh7.5 cells. The cells inoculated with these supernatants were harvested at 48 h post-inoculation, and the luciferase activity of the cell lysate was analyzed (Fig. 2B). These supernatants infected naïve Huh7.5 cells, and transduced luciferase activity in the cells. The infectious virus of both reporter HCVs was initially detected on day 2 and peaked on day 4 post-electroporation. However, the infectivity was decreased after day 6 post-electroporation. Furthermore, the infectivity of J6/JFH1/Luc supernatants was significantly higher than that of JFH1/Luc (approximately 10-fold). To compare the luciferase activity and the virus titer, we

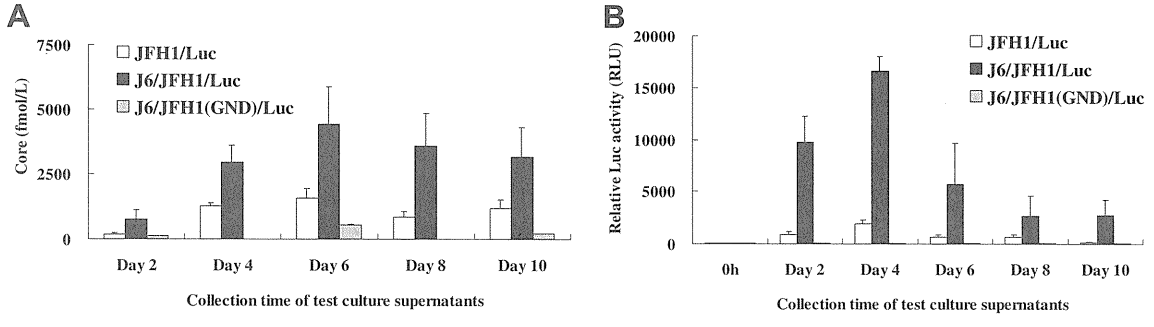

Fig. 2. Progeny virus production from luciferase reporter RNA-transfected Huh7.5 cells. The cells were electroporated with luciferase reporter RNA as described in Materials and methods, and culture supernatants of the cells were collected at the indicated time points. The amount of progeny virus in the supernatant was measured by the HCV core protein ELISA. (A) In parallel, the supernatants were added to naïve Huh7.5 cells. At 48 h post-addition, the cells were lysed, and assayed for luciferase activity to assess the infectivity of progeny virus from reporter HCV RNA. (B) The assays were repeated at least three times, and the mean values are presented.

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/Ailx [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

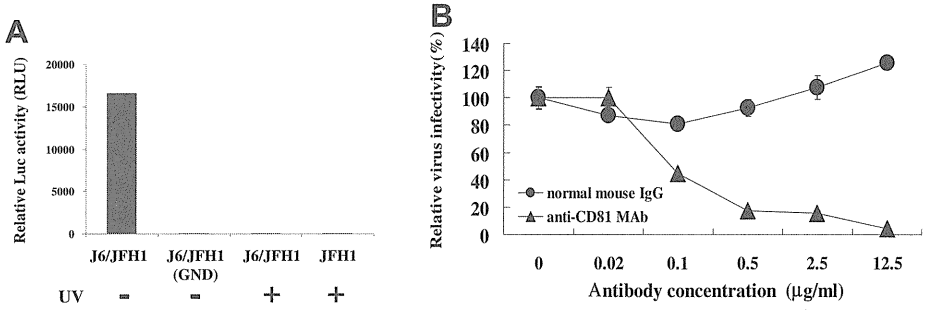

Fig. 3. Anti-CD81 antibody blocks luciferase reporter HCV infection. The reporter viruses containing supernatants were prepared as described in Materials and methods. (A) The JFH1/Luc and J6/JFH1/Luc supernatants were irradiated with UV at 5 min, and then added to naïve Huh7.5 cells. The infectivity 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.

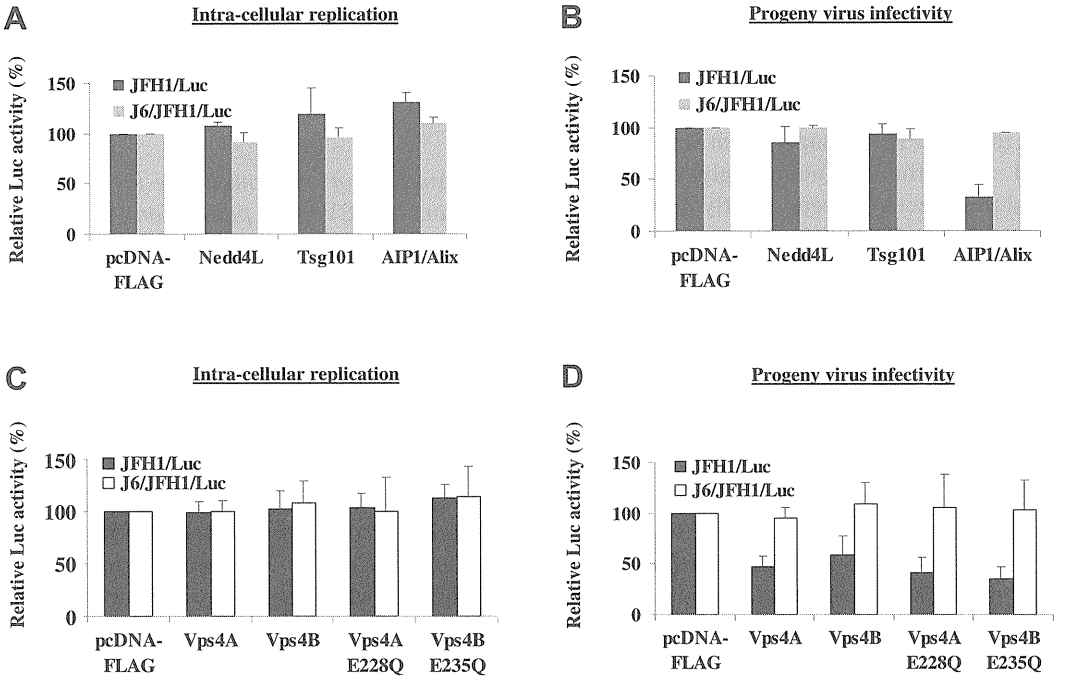

Fig. 4. Effect of ESCRT family protein expression on intra-cellular replication and progeny virus production in Huh7.5 cells. Huh7.5 cells were electroporated with JFH1/Luc and J6/JFH1/Luc RNA, respectively. The RNA-electroporated cells were then transfected with ESCRT protein expression plasmids. At 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

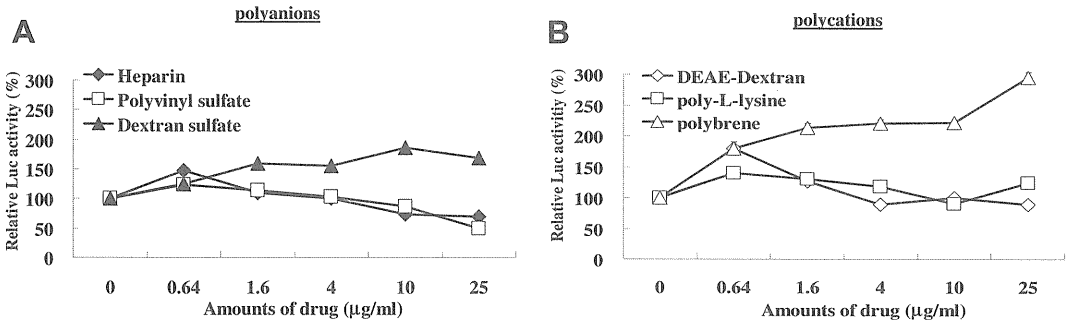

Fig. 5. Effect of multiple polyions on J6/JFH1/Luc virus infection of Huh7.5 cells. Huh7.5 cells were infected with J6/JFH1/Luc virus in the presence of each of the polyions for 6 h, and a luciferase assay was performed 48 h later. The data was expressed as the relative activity compared to the luciferase 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

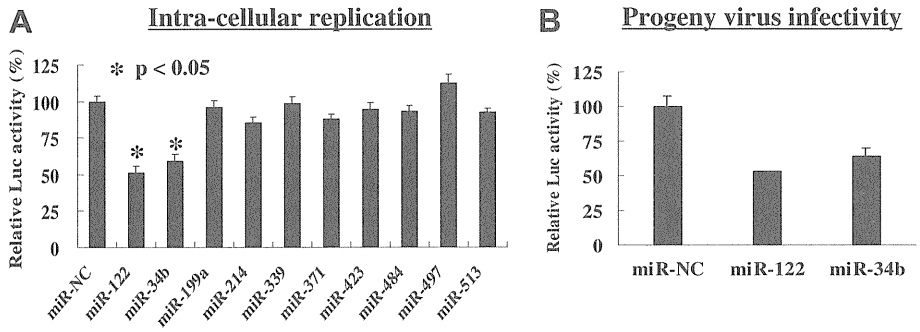

Fig. 6. Effect of miRNA inhibitor on intra-cellular replication of J6/JFH1/Luc RNA in Huh7.5 cells. Target cells were electroporated with J6/JFH1/Luc RNA, and then transfected with a miRNA-specific or a non-target negative control miRNA inhibitor. At 48 h post-transfection, cells were harvested and analyzed 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/JFH 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.

Acknowledgments

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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. Hatcho, 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. Klibanov, 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. Hatziioannou, 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.

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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:*** Pretreatment 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≥6), 18 (75%) patients achieved sustained virological response (SVR), whereas only 11 (25%) of 44 patients infected with HCV having IRRDR≤5 did. IRRDR≥6 was significantly associated with SVR ($p < 0.0001$). On the other hand, ISDR≥2 was significantly 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 (Gln^{70}) was significantly associated with null-response ($p < 0.05$). Multivariate analysis identified IRRDR≥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≥6 is a useful marker for prediction of SVR.

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 (Arg^{70}/Leu^{91}), 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 Arg^{70}/Leu^{91} was defined as wild-core while the other patterns as non-wild-core. The possible correlation of either Arg^{70} alone or Leu^{91} 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 as according to the method of Okamoto et al. [10]. Patients were treated with PEG-IFNα-2b (Pegintron®; Schering-Plough, Kenilworth, N.J., USA) (1.5 μg/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 μl 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' demographical, 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≥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≥6 and those of IRRDR≤5.

Next, the possible correlation between IRRDR polymorphism and the ultimate treatment responses was examined. Among 24 patients infected with HCV isolates of IRRDR≥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≤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≥6 and IRRDR≤5 were significantly different. Furthermore, patients infected with HCV isolates with Ala at position 2360 (Ala^{2360}) in IRRDR had a more significant likelihood of SVR than those infected with HCV isolates with non-Ala^{2360}, 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 Ala^{2360} 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≥4), the initial criterion of IFN responsiveness proposed by Enomoto et al. [6] was tested. Since the prevalence of ISDR≥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≥2) and those of ISDR≤1, a small but significant difference in the proportions of SVR and relapse (ETR-relapse *plus* breakthrough) was observed between ISDR≥2 and ISDR≤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≥6	IRRDR≤5	p value
Age, mean ± SD	58.71 ± 8.44	59.61 ± 10.30	0.71
Sex, male/female	17/7	18/26	0.02
Body weight, kg	59.87 ± 9.56	58.20 ± 11.92	0.56
Platelets, $\times 10^4/mm^3$	17.22 ± 5.5	14.96 ± 4.71	0.16
Hemoglobin, g/dl	14.25 ± 1.48	13.55 ± 1.77	0.11
γ-GTP, IU/l	49.50 ± 44.29	55.60 ± 65.60	0.69
GPT, IU/l	47.54 ± 33.09	49.33 ± 34.78	0.84
HCV-RNA, KIU/ml	2,070.21 ± 1,720.27	2,038.57 ± 1,963.05	0.95
HCV core antigen, fmol/l	6,750.87 ± 6,859.82	9,320.52 ± 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 *plus* breakthrough)	p value		
							SVR vs. non-SVR	SVR vs. null-response	SVR vs. relapse (ETR-relapse *plus* breakthrough)
NS5A	IRRDR≥6	24	18 (75)[c]	6 (25)	3 (12.5)	3 (12.5)	<0.0001	0.005	0.0006
	IRRDR≤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≥2	18	10 (56)	8 (44)	6 (33)	2 (11)	0.27	1.0	0.048
	ISDR≤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; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70; Met^{91} = 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 (Arg^{70}/Leu^{91}), 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

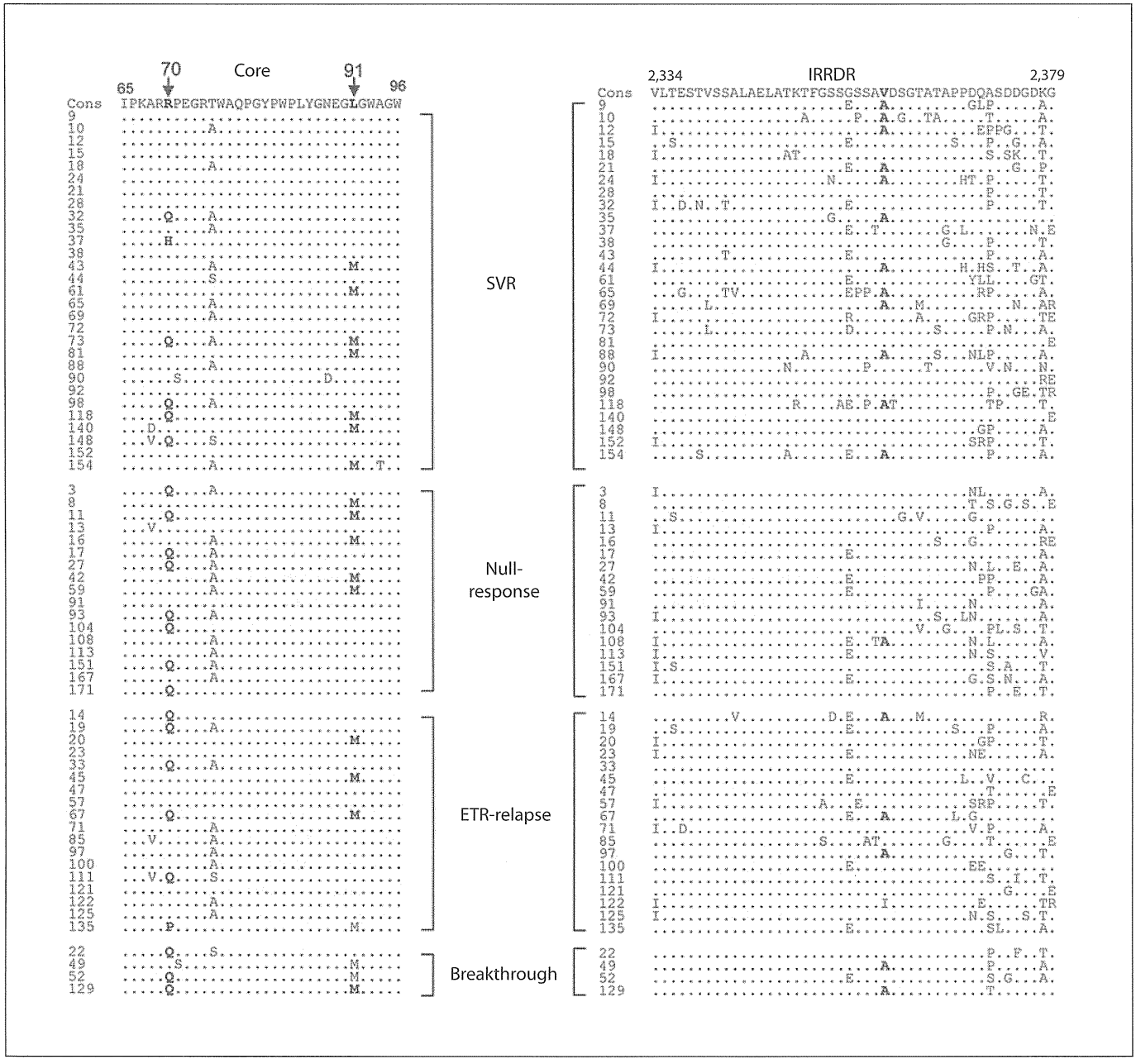

Fig. 1. Sequence alignment of the core protein (aa 65–96) and IRRDR of NS5A obtained from pretreated sera in patients infected with HCV-1b. The consensus (Cons) sequence is shown at the top. Amino acids at positions 70 and 91 of the core protein, and position 2360 of NS5A are shown in boldface.

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 position 70 (Gln^{70} vs. non-Gln^{70}) was significantly correlated with treatment outcome (SVR vs. null-response; $p = 0.04$).

As for the on-treatment responses, wild-core (Arg^{70}/Leu^{91}) was significantly correlated with EVR and ETR, whereas Gln^{70} 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≥6	24	5 (21)[c]	19 (79)	17 (71)	7 (29)	21 (87)	3 (13)	0.12	0.04	0.026
	IRRDR≤5	44	3 (7)	41 (93)	19 (43)	25 (57)	26 (59)	18 (41)			
	Ala^{2360}	18	4 (22)	14 (78)	13 (72)	5 (28)	16 (89)	2 (11)	0.19	0.1	0.04
	Non-Ala^{2360}	50	4 (8)	46 (92)	23 (46)	27 (54)	31 (62)	19 (38)			
	ISDR≥2	18	6 (33)	12 (67)	9 (50)	9 (50)	11 (61)	7 (39)	0.003	0.79	0.39
	ISDR≤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)			
	Gln^{70}	21	2 (10)	19 (90)	6 (29)	15 (71)	10 (48)	11 (52)	1.0	0.009	0.02
	Non-Gln^{70}	47	6 (13)	41 (87)	30 (64)	17 (36)	37 (79)	10 (21)			
	Met^{91}	19	2 (11)	17 (89)	8 (42)	11 (58)	11 (58)	8 (42)	1.0	0.29	0.25
	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; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70; Met^{91} = 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≥6	IRRDR≤5	
Ala^{2360}	50 (12/24)	14 (6/44)	0.003
Non-Ala^{2360}	50 (12/24)	86 (38/44)	
ISDR≥2	42 (10/24)	18 (8/44)	0.047
ISDR≤1	58 (14/24)	82 (36/44)	
Wild-core (Arg^{70}/Leu^{91})	67 (16/24)	39 (17/44)	0.04
Non-wild-core	33 (8/24)	61 (27/44)	
Gln^{70}	21 (5/24)	36 (16/44)	0.27
Non-Gln^{70}	79 (19/24)	64 (28/44)	

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; ISDR = interferon sensitivity-determining region; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = glutamine at position 70.

[a] Number of isolates with a certain factor/total number of HCV isolates with IRRDR≥6 or IRRDR≤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≤5 and non-Ala^{2360} as the majority (86%) of HCV isolates with IRRDR≤5 had non-Ala^{2360} (p = 0.003) (table 5). Also, a significant correlation was obtained between IRRDR≤5 and ISDR≤1 since 82% of IRRDR≤5 were ISDR≤1 (p = 0.047). When IRRDR and core polymorphisms were compared, IRRDR≥6 was significantly correlated with wild-core (Arg^{70}/Leu^{91}) (p = 0.04). On the other hand, there was no significant correlation between IRRDR≥6 and non-Gln^{70}, or IRRDR≤5 and Gln^{70}, although the majority (79%) of IRRDR≥6 were non-Gln^{70}.

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

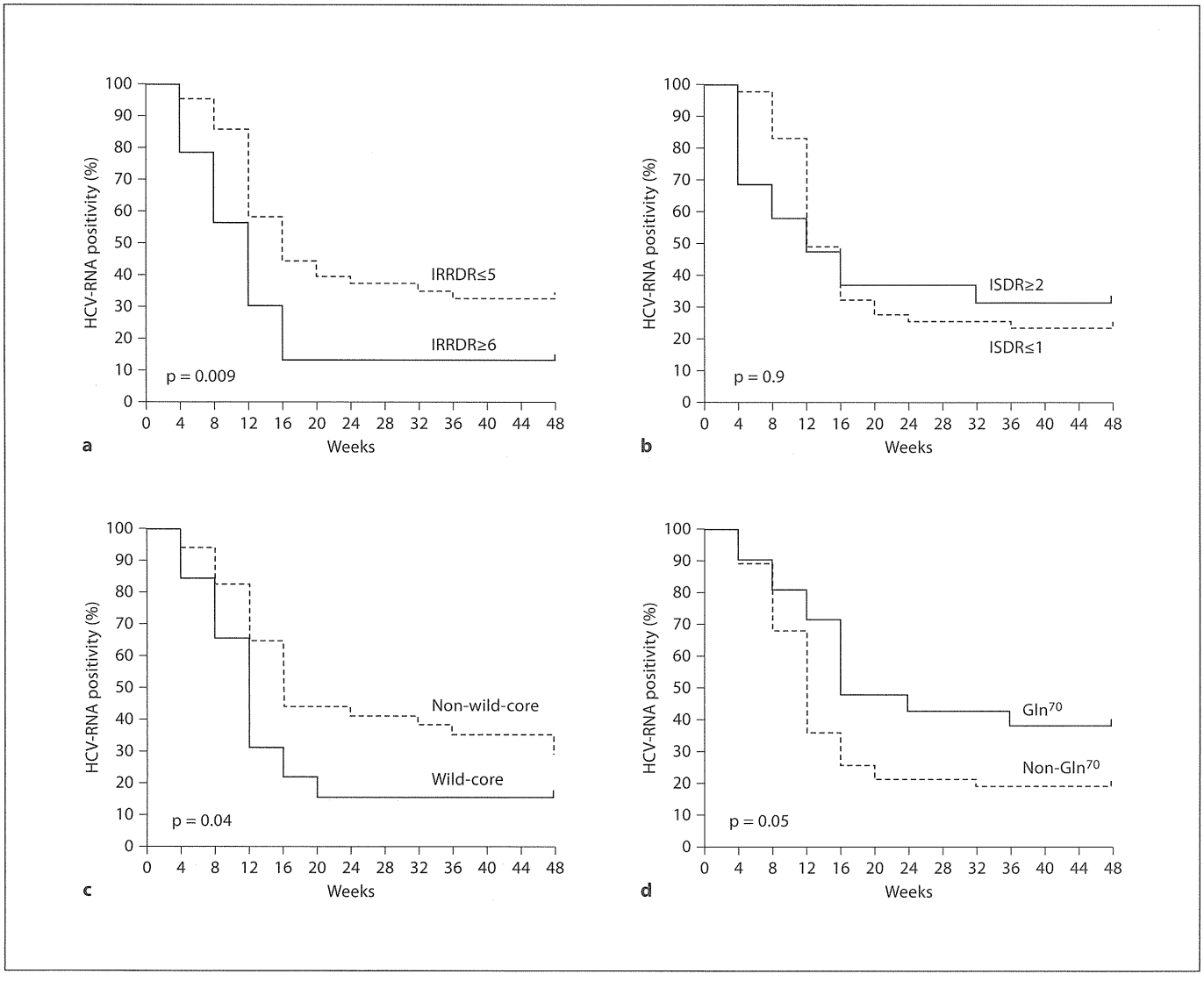

Fig. 2. Kaplan-Meier HCV survival curve analysis based on HCV-RNA positivity during the whole treatment course according to NS5A (**a**, **b**) and the core protein (**c**, **d**) polymorphisms. The difference between the analyzed groups was measured by the log-rank test.

polymorphisms. The result showed that HCV isolates of IRRDR≥6 were cleared from patients' sera more rapidly than those with IRRDR≤5 (fig. 2a). On the other hand, HCV-RNA clearance kinetics did not differ significantly between HCV isolates of ISDR≥2 and those of ISDR≤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≥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; Arg^{70} = arginine at position 70; Leu^{91} = leucine at position 91; Gln^{70} = 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 Gln^{70} 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, Ala^{2360} and non-Ala^{2360}, core protein polymorphism categorized as wild-core (Arg^{70}/Leu^{91}) and non-wild-core, Gln^{70} and non-Gln^{70}, 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

Table 7. Uni- and multivariate logistic regression analyses to identify independent predictive factors for success of PEG-IFN/RBV combination therapy

Univariate		Multivariate	
variable	p value	odds ratio (95% CI)	p value
IRRDR mutations (IRRDR≥6 vs. IRRDR≤5)	<0.0001	14.33 (2.24–91.65)	0.005
Ala^{2360}	0.01	1.75 (0.19–15.36)	0.62
Core polymorphism (wild-core vs. non-wild-core)	0.06	0.41 (0.05–3.28)	0.34
Gln^{70}	0.04		
RVR	<0.0001		
EVR	<0.0001	41.83 (6.12–285.68)	0.0001
ETR	<0.0001		
HCV core antigen, fmol/l	0.05		
Age	0.01	0.91 (0.84–0.99)	0.02
Platelets, $\times 10^4/mm^3$	0.07		
Hemoglobin, g/dl	0.006		

IRRDR = Interferon/ribavirin resistance-determining region; Ala^{2360} = alanine at position 2360; Gln^{70} = glutamine at position 70; RVR = rapid virological response; EVR = early virological response; ETR = end-of-treatment response.

subgenomic RNA replicon containing NS5A of HCV-1b exerted more profound inhibitory effects on IFN activity than the original HCV-2a replicon, and that domain swapping between NS5A sequences of HCV-1b and -2a in the V3 and/or a C-terminus region including IRRDR resulted in a transfer of their anti-IFN activity. Since the C-terminal region of NS5A is among the most variable sequences across the different genotypes and subtypes of HCV [22], the difference in IFN responsiveness among different strains of a given HCV subtype could also be attributable, at least partly, to the genetic polymorphism within this region. The molecular mechanism underling the possible involvement of IRRDR in IFN responsiveness of the virus is still unknown. The significant difference in IRRDR sequence pattern may suggest genetic flexibility of this region and, indeed, the C-terminal portion of NS5A was shown to tolerate sequence insertions and deletions [23, 24]. This means that the C-terminal portion of NS5A is not essential for virus replication in cultured cells. It does not exclude the possibility, however, that the same region plays an important role in modulating the interaction with various host systems, including IFN responsiveness. It is also possible that the genetic flexibility of this region, especially IRRDR, is accompanied by compensatory changes elsewhere in the viral genome and that these compensatory changes affect overall viral fitness and responses to IFN therapy [25].

While we observed significant correlation between the overall number of mutations in IRRDR and PEG-IFN/RBV responsiveness, we also found a particular aa mutation, Ala^{2360}, that was significantly associated with SVR (tables 3, 7; fig. 1). It is possible that Ala or Val at this position confers a certain advantage for interaction between NS5A and the other viral or host proteins, which might affect IFN-induced antiviral responses. This issue needs to be elucidated in further studies.

The ISDR polymorphism was the only virological factor examined that showed a significant correlation with RVR (table 4), with the result being consistent with a recent report by other investigators [26]. This significant correlation, however, disappeared as the treatment went on. In contrast, the IRRDR polymorphism did not correlate significantly with RVR, however, it was the dominant viral genetic factor that was correlated with SVR (tables 3, 7). Interestingly, the combination of IRRDR and ISDR polymorphisms (IRRDR≥6 *plus* ISDR≥2) was significantly correlated with RVR and SVR (p = 0.0001 and 0.01, respectively; data not shown). This suggests a possible integrated influence of IRRDR and ISDR polymorphisms, or NS5A as a whole, on the treatment outcome. Further study is needed to clarify the issue.

The core protein polymorphisms (wild-core vs. non-wild-core, and Gln^{70} and non-Gln^{70}) were significantly correlated with the on-treatment HCV clearance kinetics

(fig. 2c, d; tables 4, 6). However, this significant correlation became blurred thereafter and eventually no significant correlation was observed between wild-core (Arg^{70}/Leu^{91}) and the final treatment outcomes (table 3). On the other hand, Gln^{70} was significantly associated with null-response, and almost significantly with non-SVR. This result is consistent, at least partly, with previous reports, including a recent multicenter study in Japan, that identified Gln^{70} as a predictive factor for poor responses to PEG-IFN/RBV treatment [8, 9, 14].

Recently, it was reported that the C-terminal region of NS5A plays a critical role in regulating the early phase of HCV particle formation [27, 28]. Moreover, sequence alteration within this region affected the degree of interaction between NS5A and core protein, which in turn affected the efficiency of progeny virus production [29]. In the present study, we observed a significant correlation between the degree of IRRDR mutations (IRRDR≥6) and the core polymorphism (table 5). Therefore, it would be interesting to investigate the degree of interaction between NS5A with IRRDR of high or low degrees of sequence variation and the wild-type (Arg^{70}/Leu^{91}) or non-wild-type of core protein, and also the impact of these interactions on progeny virus production and IFN sensitivity of the virus.

The present study identified the IRRDR polymorphism as the only viral genetic factor that independently predicted PEG-IFN/RBV treatment outcome (table 7). On the other hand, HCV is likely to utilize an alternative mechanism(s) by which to escape IFN actions through its various structural and non-structural proteins [30]. Also, a different lineage(s) of HCV-1b strains that relies more on the alternative mechanism than on IRRDR may prevail in other regions of the world. It is possible, therefore, that the impact of the IRRDR polymorphism differs with different cohorts. Analysis in a large-scale multicenter study is needed to clarify this issue.

In conclusion, NS5A (IRRDR and ISDR) and core protein polymorphisms are useful viral markers for predicting the outcome of PEG-IFN/RBV therapy for chronic hepatitis C. In particular, IRRDR≥6 is a useful marker for prediction of SVR.

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References

1 Bialek SR, Terrault NA: The changing epidemiology and natural history of hepatitis C virus infection. Clin Liver Dis 2006;10:697–715.
2 Williams R: Global challenges in liver disease. Hepatology 2006;44:521–526.
3 Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, Haussinger D, Diago M, Carosi G, Dhumeaux D, Craxi A, Lin A, Hoffman J, Yu J: Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975–982.
4 Backus LI, Boothroyd DB, Phillips BR, Mole LA: Predictors of response of US veterans to treatment for the hepatitis C virus. Hepatology 2007;46:37–47.
5 Welker MW, Hofmann WP, Welsch C, von Wagner M, Herrmann E, Lengauer T, Zeuzem S, Sarrazin C: Correlation of amino acid variations within nonstructural 4B protein with initial viral kinetics during interferon-α-based therapy in HCV-1b-infected patients. J Viral Hepat 2007;14:338–349.
6 Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, Ogura Y, Izumi N, Marumo F, Sato C: 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.
7 El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H: Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology 2008;48:38–47.
8 Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403–410.
9 Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, Kobayashi M, Saitoh S, Watahiki S, Sato J, Matsuda M, Kobayashi M, Arase Y, Ikeda K, Kumada H: Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372–380.
10 Okamoto H, Sugiyama Y, Okada S, Kurai K, Akahane Y, Sugai Y, Tanaka T, Sato K, Tsuda F, Miyakawa Y, et al: Typing hepatitis C virus by polymerase chain reaction with type-specific primers: application to clinical surveys and tracing infectious sources. J Gen Virol 1992;73:673–679.
11 El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H: Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol 2007;51:471–482.

12 Ogata S, Nagano-Fujii M, Ku Y, Yoon S, Hotta H: Comparative sequence analysis of the core protein and its frameshift product, the F protein, of hepatitis C virus subtype 1b strains obtained from patients with and without hepatocellular carcinoma. J Clin Microbiol 2002;40:3625–3630.
13 Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, Ichijo T, Yoshizawa K, Kiyosawa K, Tanaka E: Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology 2008;48:1753–1760.
14 Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, Tanaka E, Onji M, Toyota J, Chayama K, Yoshioka K, Izumi N, Akuta N, Kumada H: Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J Gastroenterol 2009;44:952–963.
15 Kau A, Vermehren J, Sarrazin C: Treatment predictors of a sustained virologic response in hepatitis B and C. J Hepatol 2008;49:634–651.
16 Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, Heinzen EL, Qiu P, Bertelsen AH, Muir AJ, Sulkowski M, McHutchison JG, Goldstein DB: Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399–401.
17 Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, Bassendine M, Spengler U, Dore GJ, Powell E, Riordan S, Sheridan D, Smedile A, Fragomeli V, Muller T, Bahlo M, Stewart GJ, Booth DR, George J: IL28B is associated with response to chronic hepatitis C interferon-α and ribavirin therapy. Nat Genet 2009;41:1100–1104.
18 Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, Nakagawa M, Korenaga M, Hino K, Hige S, Ito Y, Mita E, Tanaka E, Mochida S, Murawaki Y, Honda M, Sakai A, Hiasa Y, Nishiguchi S, Koike A, Sakaida I, Imamura M, Ito K, Yano K, Masaki N, Sugauchi F, Izumi N, Tokunaga K, Mizokami M: Genome-wide association of IL28B with response to pegylated interferon-α and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105–1109.
19 Murakami T, Enomoto N, Kurosaki M, Izumi N, Marumo F, Sato C: Mutations in nonstructural protein 5A gene and response to interferon in hepatitis C virus genotype 2 infection. Hepatology 1999;30:1045–1053.
20 Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, Hosaka T, Kobayashi M, Kobayashi M, Saitoh S, Arase Y, Ikeda K, Kumada H: Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 2a high viral load and virological response to interferon-ribavirin combination therapy. Intervirology 2009;52:301–309.
21 Tsai YH, Kuang WF, Lu TY, Kao JH, Lai MY, Liu CJ, Chen PJ, Hwang LH: The non-structural 5A protein of hepatitis C virus exhibits genotypic differences in interferon antagonism. J Hepatol 2008;49:899–907.
22 Macdonald A, Harris M: Hepatitis C virus NS5A: tales of a promiscuous protein. J Gen Virol 2004;85:2485–2502.
23 Moradpour D, Evans MJ, Gosert R, Yuan Z, Blum HE, Goff SP, Lindenbach BD, Rice CM: Insertion of green fluorescent protein into nonstructural protein 5A allows direct visualization of functional hepatitis C virus replication complexes. J Virol 2004;78:7400–7409.
24 Appel N, Pietschmann T, Bartenschlager R: Mutational analysis of hepatitis C virus nonstructural protein 5A: potential role of differential phosphorylation in RNA replication and identification of a genetically flexible domain. J Virol 2005;79:3187–3194.
25 Yuan HJ, Jain M, Snow KK, Gale Jr M, Lee WM: Evolution of hepatitis C virus NS5A region in breakthrough patients during pegylated interferon and ribavirin therapy. J Viral Hepat 2009;17:208–216.
26 Enomoto N, Maekawa S: HCV genetic elements determining the early response to peginterferon and ribavirin therapy. Intervirology 2010;53:66–69.
27 Hughes M, Griffin S, Harris M: Domain III of NS5A contributes to both RNA replication and assembly of hepatitis C virus particles. J Gen Virol 2009;90:1329–1334.
28 Appel N, Zayas M, Miller S, Krijnse-Locker J, Schaller T, Friebe P, Kallis S, Engel U, Bartenschlager R: Essential role of domain III of nonstructural protein 5A for hepatitis C virus infectious particle assembly. PLoS Pathog 2008;4:e1000035.
29 Masaki T, Suzuki R, Murakami K, Aizaki H, Ishii K, Murayama A, Date T, Matsuura Y, Miyamura T, Wakita T, Suzuki T: Interaction of hepatitis C virus nonstructural protein 5A with core protein is critical for the production of infectious virus particles. J Virol 2008;82:7964–7976.
30 Gale M Jr, Foy EM: Evasion of intracellular host defence by hepatitis C virus. Nature 2005;436:939–945.

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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 and antibody neutralization. ***J. Med. Virol. 84:229–234, 2012.*** © 2011 Wiley Periodicals, Inc.

KEY WORDS: humoral immune mechanism; evasion; glycan

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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