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ORIGINAL ARTICLE

Inhibition of hepatitis C virus replication through adenosine monophosphate-activated protein kinase-dependent and -independent pathways

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ABSTRACT

Persistent infection with hepatitis C virus (HCV) is closely correlated with type 2 diabetes. In this study, replication of HCV at different glucose concentrations was investigated by using J6/JFH1-derived cell-adapted HCV in Huh-7.5 cells and the mechanism of regulation of HCV replication by AMP-activated protein kinase (AMPK) as an energy sensor of the cell analyzed. Reducing the glucose concentration in the cell culture medium from 4.5 to 1.0 g/L resulted in suppression of HCV replication, along with activation of AMPK. Whereas treatment of cells with AMPK activator 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) suppressed HCV replication, compound C, a specific AMPK inhibitor, prevented AICAR's effect, suggesting that AICAR suppresses the replication of HCV by activating AMPK in Huh-7.5 cells. In contrast, compound C induced further suppression of HCV replication when the cells were cultured in low glucose concentrations or with metformin. These results suggest that low glucose concentrations and metformin have anti-HCV effects independently of AMPK activation.

Key words 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR), adenosine monophosphate-activated protein kinase (AMPK), diabetes, metformin.

Hepatitis C virus, which is classified within the family *Flaviviridae*, is a small enveloped virus that possesses a positive-sense single-stranded RNA genome. HCV infection proceeds to a persistent stage at a high rate, leading to cirrhosis and hepatocellular carcinoma. Despite recent advances in the development of antiviral therapies, certain patient populations are difficult to treat (1) due to host factors such as obesity, hyperglycemia and insulin resistance (2–4).

Adenosine monophosphate-activated protein kinase is a major cellular energy sensor that is activated by cellular stresses that increase intracellular AMP (5). ZMP, which mimics AMP, also activates AMPK (6). AMPK is a heterotrimer composed of a catalytic α subunit and regulatory β and γ subunits (7). Phosphorylation of Thr^{172} in its activation loop of α subunit by upstream kinases, namely, LKB1 (8,9) and Ca^{2+}/calmodulin-dependent kinase kinase (10,11) can increase its kinase activity (12). Activated AMPK inhibits the synthesis of fatty acids, cholesterol, proteins and gluconeogenesis in hepatocytes (13–16). Phosphorylation of $Ser^{485/491}$ by protein kinase B is known to inhibit AMPK activity (17).

Hepatitis C virus infection suppresses cellular glucose uptake through down-regulation of cell surface expression

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List of Abbreviations: AICAR, 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside; AMP, adenosine monophosphate; AMPK, AMP-activated protein kinase; compound C, 6-(4-[2-piperidin-1-yl-ethoxy])-phenyl)-3-pyridin-4-yl-pyrrazolo(1,5-a)-pyrimidine; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; LKB1, liver kinase B1; MOI, multiplicity of infection; NS3, non-structural protein 3; PRPP, phosphoribosyl pyrophosphate; ZMP, 5-aminoimidazole-4-carboxamide ribonucleotide; ZTP, 5-aminoimidazole 4-carboxamide ribonucleoside 5-triphosphate.

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of glucose transporters (18). Our preliminary experiments demonstrated that HCV infection alters the expression of 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/inosine monophosphate cyclohydrolase, which catalyzes ZMP in purine nucleotide synthesis. ZMP is known to mimic the activating effects of AMP on AMPK (6). We postulated that glucose usage and/or activation of AMPK might affect the infection and replication of HCV.

In this study, we have investigated HCV replication with different glucose concentrations in the culture medium, with treatment of cells with AMPK activators (AICAR, metformin) or with the AMPK inhibitor compound C in the cell culture medium.

MATERIALS AND METHODS

Cells

The Huh-7.5 cell line used in this study, a highly HCV-susceptible subclone of Huh7 cells, was a kind gift from Dr. C. M. Rice (Center for the Study of Hepatitis C, The Rockefeller University, New York, NY, USA) (19). The cells were propagated in Dulbecco's modified Eagle medium supplemented with 10% heat-inactivated FBS and 0.1 mM nonessential amino acids.

Viruses

The virus stock was prepared as described previously (20,21). The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1, was kindly provided by Dr. C. M. Rice. The HCV RNA genome was transcribed *in vitro* from pFL-J6/JFH1 and transfected to Huh-7.5 cells. The supernatant was harvested as a virus stock. In this study we used an adapted strain of the virus obtained by passaging the HCV genotype 2a, J6/JFH1, infected cells 47 times (20,22). Virus infection was performed at a MOI of three. Culture supernatants of uninfected cells were used as controls (mock preparation). Virus infectivity was measured by indirect immunofluorescence analysis as described previously (20).

Reagents

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside and uridine were purchased from Sigma (St. Louis, MO, USA), compound C from Chemdea (Ridgewood, NJ, USA), metformin from Enzo Life Sciences (Plymouth Meeting, PA, USA) and Hoechst 33258 from Wako (Osaka, Japan).

Immunoblotting

Immunoblotting was essentially as described previously (23). Cells were solubilized in a lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 100 mM NaF, 0.1 mM Na_3VO_4, 10 mM EDTA, 1% Triton-X, and protease inhibitor cocktail [Sigma]). Cell debris was removed by centrifugation and resulted supernatants were diluted 1:2 (v/v) with 3×sampling buffer. Protein quantification was carried out using a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Rockford, IL, USA). Equal amounts of soluble proteins were subjected to SDS-PAGE and transferred onto a polyvinylidene difluoride transfer membrane (Millipore, Billerica, MA, USA). After blocking in 5% milk in TBST (25 mM Tris, pH 8.0, 150 mM NaCl, and 0.1% Tween 20), the blots were reacted with the respective primary antibodies. The primary antibodies used were anti-phospho-AMPKα (Thr172) monoclonal antibody (clone D79.5E, Cell Signaling Technology, Danvers, MA, USA), anti-AMPKα antibody (Cell Signaling Technology), anti-HCV core monoclonal antibody (clone C7–50, Thermo Fisher Scientific), anti-AMPKα antibody (Phospho-$Ser^{485/491}$) (anti-pAMPK [Ser485/491]) (GenScript, Piscataway, NJ, USA), and anti-HCV NS3 monoclonal antibody (Millipore). Horseradish peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) were used as secondary antibodies. The respective protein bands were visualized by enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA) (24,25). Protein loading was normalized by probing with anti-GAPDH monoclonal antibody (clone 6C5, Millipore).

Indirect immunofluorescence

Cells seeded in 96-well plates were infected with HCV at a MOI of 3.0 for 4 hr or left uninfected. The cells were incubated for 30 hr and fixed with cold methanol for 10 min at room temperature. After being washed with PBS twice, the cells were stained with anti-HCV core monoclonal antibody and visualized by using horseradish peroxidase-conjugated goat anti-mouse IgG (Bio-Rad Laboratories, Hercules, CA, USA) and the tyramide signal amplification cyanine 3 system (Perkin Elmer). Stained cell samples were examined by fluorescence microscopy (Olympus IX70 microscope system, Tokyo, Japan).

Statistical analysis

The one-tailed Student *t*-test was applied to evaluate the statistical significance of differences found. A *P* value of <0.05 was considered statistically significant.

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RESULTS

Glucose shortage in the culture medium suppresses the replication of hepatitis C virus along with activation of adenosine monophosphate-activated protein kinase

Any virus requires an energy source for replication. We surmised that glucose shortage in the cell culture medium would have a harmful effect on energy metabolism in HCV-infected Huh-7.5 cells. We used Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV throughout this study. First, we examined the effect of alterations in glucose concentration in the cell culture medium on the replication of HCV. Reducing the glucose concentration from 4.5 to 1.0 g/L resulted in a significant decrease in HCV replication, as demonstrated by decreased virus infectivity in culture supernatants (Fig. 1a), and decreased production of HCV core protein (Fig. 1b, third panel). In order to estimate the intracellular energy status, we examined the kinase activity of AMPK by the immunoblotting of phosphorylated Thr^{172} in AMPK. Reducing the glucose concentration from 4.5 to 2.0 g/L resulted in a dramatic increase in phosphorylation of Thr^{172} in AMPK, suggesting that Huh-7.5 cells sensed poor nutrition when cultured with 2.0 g/L of glucose in the medium, irrespective of infection with HCV (Fig. 1b). Phosphorylation of $Ser^{485/491}$ of AMPK was not affected by infection with HCV (Fig. 1c). Phosphorylation of AMPK was not affected by infection with HCV, although almost all of the cells were infected in this experiment (Fig. 1d). These results demonstrate that glucose shortage in the cell culture medium suppresses replication of HCV along with activation of AMPK in Huh-7.5 cells. Glucose shortage activates AMPK regardless of HCV infection.

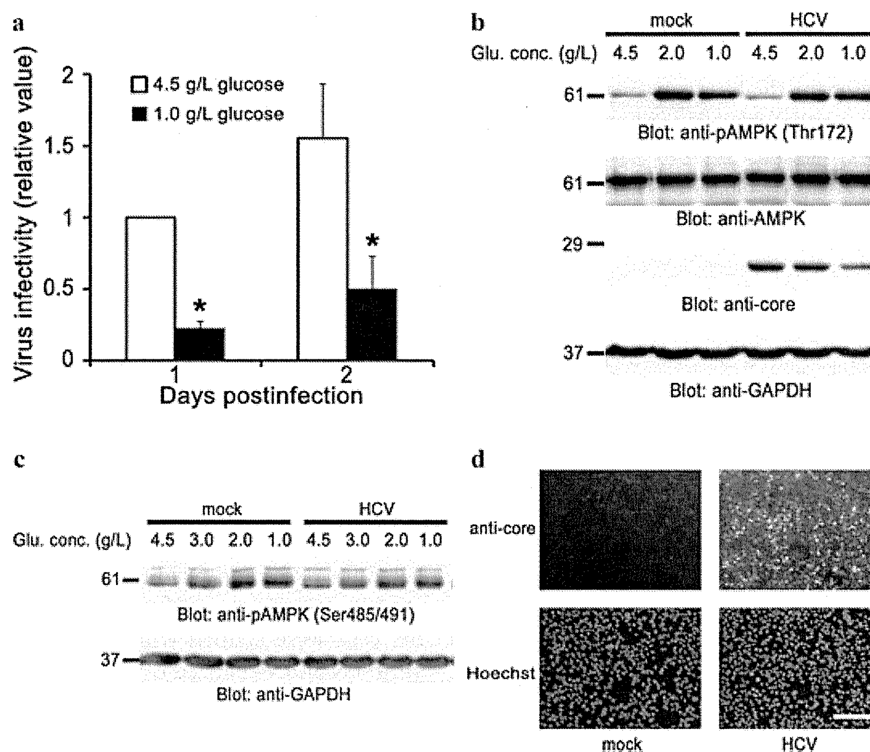

Fig. 1. Glucose shortage suppresses HCV replication and activates AMPK. (a) Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr and then incubated at the indicated concentration of glucose in serum-free DMEM. Infectivity titer in culture supernatants of HCV-infected cells cultured in medium containing 4.5 g/L glucose at day 1 postinfection was arbitrarily expressed as 1.0. Data are expressed as means ± standard deviations (SD) of three independent experiments. *, $P < 0.05$, compared with the control. (b and c) Huh-7.5 cells were mock infected or infected with HCV at a MOI of 3.0 for 4 hr and then incubated at the indicated concentrations of glucose in serum-free DMEM for 30 hr. (b) Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-phospho-AMPKα (Thr172) (anti-pAMPK (Thr172)), anti-AMPK, anti-HCV core, anti-GAPDH antibodies as indicated. (c) Cell lysates were analyzed by immunoblotting with anti-AMPKα antibody (anti-pAMPK (Ser485/491)) and anti-GAPDH monoclonal antibody. (d) Huh-7.5 cells mock infected or infected with HCV at a MOI of 3.0 for 4 hr were subjected to indirect immunofluorescence analysis by anti-HCV core antibody. Nuclei were stained with Hoechst 33258. Scale bar, 200 μm. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments. Conc., concentration; glu., glucose.

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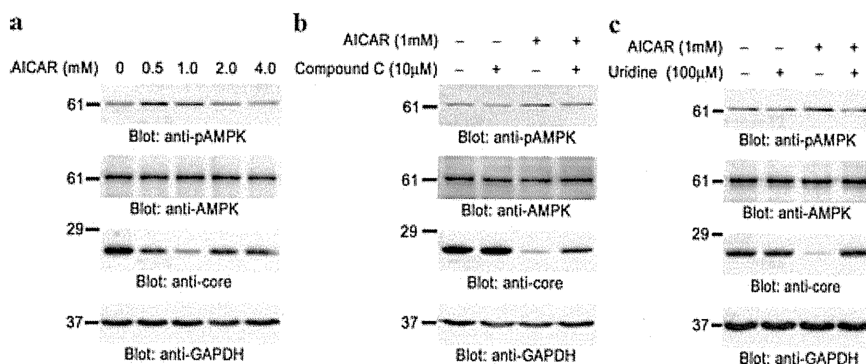

Fig. 2. AICAR suppresses HCV replication by activating AMPK. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. (a) The cells were treated with the indicated concentrations of AICAR for 20 hr. (b) 10 μM compound C was added to the cells 30 min prior to the addition of AICAR and was present in the medium during the entire 20 hr of incubation with AICAR. (c) The cells were treated with 1 mM AICAR for 20 hr with or without supplementation with 100 μM uridine. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as Figure 1. The results are representative of three independent experiments.

5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside suppresses the replication of hepatitis C virus by activating adenosine monophosphate-activated protein kinase in Huh-7.5 cells

Activated AMPK inhibits the synthesis of fatty acids, cholesterol, proteins and gluconeogenesis (13–16). Imbalance of these metabolic pathways in liver cells might affect the replication of HCV. Therefore we next examined whether activated AMPK suppresses the replication of HCV by using an activator (AICAR) and an inhibitor (compound C) of AMPK. Mankauri J. *et al.* have previously reported that treatment of Huh-7 parental cells with AICAR suppresses the replication of JFH-1 (26). In this study, we adopted a more efficient HCV replication system, Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV. Similar to the previous finding, activation of AMPK by AICAR suppressed the expression of HCV core protein in Huh-7.5 cells (Fig. 2a, lanes 1–3). Activation of AMPK by AICAR was observed when the cells were treated with relatively low concentrations (0.5 or 1.0 mM), but not with higher concentrations (2.0 or 4.0 mM). Possible reasons for the latter effect are that higher concentrations of AICAR could suppress the synthesis of purine nucleotides and/or increase the concentration of ZTP, thus inhibiting AMPK (6,27).

To examine whether the inhibitory effect of AICAR on HCV replication is mediated by activation of AMPK, we tested an AMPK inhibitor (compound C) in this experiment. We found that pretreatment of cells with 10 μM compound C attenuates AICAR-mediated suppression of HCV core protein expression (Fig. 2b, lane 4). This suggests that AICAR-mediated suppression of HCV replication is mediated by activation of AMPK. Addition of compound C to the cell culture medium without AICAR did not affect the expression of HCV core protein, suggesting that this inhibitor does not affect the replication of HCV under nutritious condition in which AMPK is inactive (Fig. 2b, lane 2).

In the presence of AICAR, the amounts of uridine triphosphate and cytidine triphosphate are decreased in the cultured cells as a result of PRPP depletion (27). PRPP is an important precursor for pyrimidine nucleotide synthesis. PRPP-derived pyrophosphate can increase ZTP/ZMP which are then no longer able to activate AMPK (6,28). To complement the pyrimidine shortage in Huh-7.5 cells treated with AICAR, the cells were co-incubated with 100 μM uridine in the presence of 1 mM AICAR. This resulted in the complete prevention of AICAR-mediated activation of AMPK and the resulting suppression of HCV (Fig. 2c, lane 4). Taken together, these data demonstrate that AICAR suppresses the replication of HCV by activating AMPK in Huh-7.5 cells.

Glucose shortage and metformin have an anti-hepatitis C virus effect independently of adenosine monophosphate-activated protein kinase activation

It is important to note that glucose shortage activates AMPK because of cellular energy limitations, whereas AICAR can activate AMPK regardless of cellular energy status. Therefore we tried another AMPK activator, metformin, which activates AMPK by impairing complex 1 of the mitochondrial respiratory chain (29,30). In addition, in mice metformin has a LKB1/AMPK-independent

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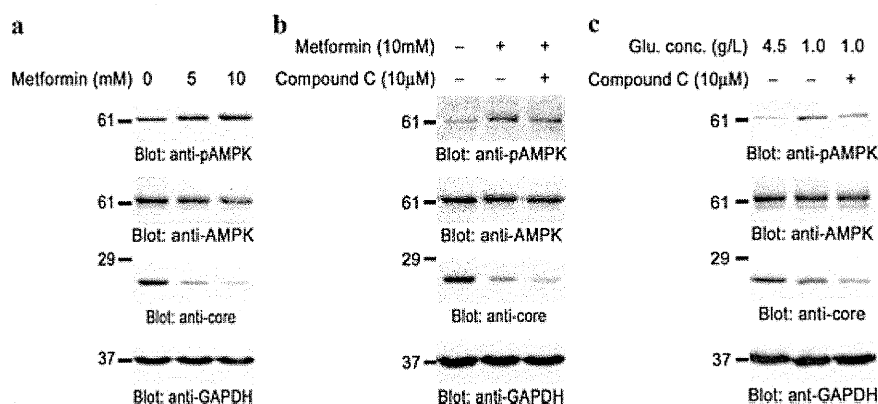

Fig. 3. Compound C, an AMPK inhibitor, stimulates the anti-HCV effects of glucose shortage or metformin. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. (a) The cells were treated with the indicated concentration of metformin for 20 hr. (b) 10 μM compound C was added to the cells 30 min prior to the addition of metformin and present in the medium during the entire 20 hr of incubation with metformin. (c) The cells were incubated with or without 10 μM compound C at the indicated glucose concentrations in serum-free DMEM for 15 hr. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as Figure 1. The results are representative of three independent experiments. Conc., concentration; glu., glucose.

inhibitory role on gluconeogenesis by decreasing the hepatic energy state (31). Treatment of cells with metformin activated AMPK and suppressed replication of HCV in a concentration-dependent manner in Huh-7.5 cells (Fig. 3a). However, co-incubation of cells with compound C, an inhibitor of AMPK, did not prevent metformin-mediated suppression of HCV replication (Fig. 3b, lane 3). Relatively speaking, compound C enhances the suppression of HCV replication induced by metformin. Likewise, compound C promoted suppression of HCV replication when the cells were cultured under conditions of glucose shortage (Fig. 3c, lane 3). These results demonstrate that glucose shortage and metformin inhibit HCV replication independently of AMPK activation.

The effects of adenosine monophosphate-activated protein kinase activators/inhibitor on hepatitis C virus non-structural protein 3

Finally, we tested the effects of AMPK activators/inhibitor on the expression of other HCV protein besides core protein (Fig. 4). As shown, treatment of cells with AICAR, metformin or glucose shortage suppressed the expression of NS3 protein. In addition, compound C attenuated the anti-HCV effect of AICAR, whereas it enhanced the anti-HCV effect of metformin or glucose shortage. These results support the conclusion that AMPK activators/inhibitor affect the replication of HCV, as demonstrated by the expression of HCV core protein (Figs. 1–3).

DISCUSSION

Previous reports have suggested that HCV infection directly causes insulin resistance, resulting in the progression of diabetes (32,33). Moreover it has been reported that insulin resistance is a negative predictor of the response to antiviral therapy in chronic hepatitis C patients treated with peginterferon plus ribavirin (4). However, the association between virus proliferation and hyperglycemia due to insulin resistance remains elusive. In this study, we have demonstrated that HCV proliferation is promoted in Huh-7.5 cells cultured at 4.5 g/L glucose, the equivalent of the blood glucose concentrations of diabetes patients (Fig. 1). This result suggests that intensive control of glucose concentrations would aid antiviral therapy in hepatitis C patients with diabetes.

We have demonstrated that activation of AMPK suppresses HCV replication (Fig. 2). This result suggests that AMPK as a potential target for the treatment of chronic hepatitis C. Therapeutic interest has recently been increased by the findings that hepatic AMPK is activated by adiponectin (34) and by thiazolidinedione-type antidiabetic drugs (35). Pharmacological activation of AMPK may provide a new strategy for both the management of chronic hepatitis C itself and metabolic hepatic disorders linked to HCV infection.

Adenosine monophosphate-activated protein kinase, a major energy sensor, is activated by energy depletion. AMPK is directly activated by AICAR, being metabolized to ZMP in the cell, regardless of the cellular energy status (6). We have demonstrated that AICAR-mediated suppression of HCV proliferation is AMPK-dependent

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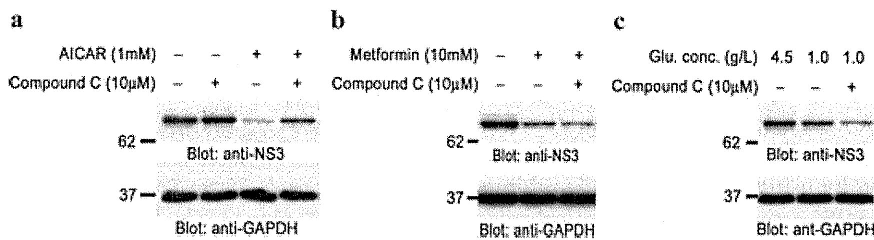

Fig. 4. AMPK activators/inhibitors' effect on the expression of HCV NS3 protein. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. The cells were treated with the indicated reagents. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-HCV NS3 and anti-GAPDH antibodies. The results are representative of three independent experiments.

(Fig. 2). In terms of phosphorylation of AMPK, the most effective concentration of AICAR was 0.5 mM, whereas the most effective concentration of AICAR for suppression of HCV replication was clearly 1.0 mM. One of the possible explanations of this discrepancy is that the former immunoblot shows the state of AMPK phosphorylation at the endpoint of the experiment, whereas the latter immunoblot reflects the accumulation of the expression of core protein during the whole period of the experiment. Compound C, a specific AMPK inhibitor that competes with ATP (36), could inhibit the effect of AICAR on HCV proliferation (Fig. 2b). Compound C did not completely reverse the suppressive effect of AICAR treatment on HCV replication. In general, inhibitors do not completely suppress the effect of reagents or enzymes; however it is still possible that the suppression of HCV replication by AICAR cannot be explained purely by activation of AMPK. Uridine as a source of pyrimidine could also prevent the effect of AICAR (Fig. 2c). Moreover, we focused on another mechanism of AICAR-mediated inhibition of HCV replication. Activated AMPK causes inhibitions of fatty acids and cholesterol synthesis. Recent reports have shown a crucial involvement of fatty acids, cholesterol and lipid droplets in infectious virion production (37–40). Therefore, we predicted that AICAR-mediated inhibition of HCV replication might be due to lipid depletion. To investigate this possibility, we added mevalonolactone and/or oleic acid in the presence of AICAR to the cell culture medium, and then examined the replication of HCV. However, the addition of lipids had almost no effect on AICAR-mediated suppression of HCV (Fig. S1). This suggests that HCV replication does not require additional lipids when the cells are treated with AICAR, which shifts cellular metabolism from energy expenditure to energy production by activating AMPK.

Cell confluency is known to activate AMPK, and LKB1 is a major kinase that activates AMPK. Replication of the HCV replicon is known to be inhibited in confluent Huh-7 cells (41). Replication of HCV replicon in HeLa cells, a known LKB1-deficient cell line, is not affected by their confluence (42). These data suggest that confluence-mediated suppression of HCV replication requires the LKB1-AMPK pathway. Our experiments demonstrated that confluence of cells can activate AMPK and suppress replication of HCV in Huh-7.5 cells (Fig. S2). It is still not clear whether this anti-HCV effect is due to relative undernutrition resulting from increased cell numbers or the activation of AMPK by the confluence itself.

Culturing cells under a shortage of glucose or with metformin can activate cellular AMPK and suppress replication of HCV in the cells (Fig. 1 and 3). Under such low energy conditions, compound C, a specific AMPK inhibitor, can induce further suppression of HCV replication. The explanation of this phenomenon is as follows: AMPK is activated in order to restore energy status. In the presence of glucose depletion or energy limitations by metformin, compound C-induced AMPK inhibition may lead to failure to maintain ATP concentrations. Various compensatory mechanisms may maintain intracellular ATP concentrations. In other words, under energy limitations the breakdown of the fuel gauge, AMPK, may proceed to imbalance in metabolism leading to poor replication of HCV. Recent reports have shown that metformin therapy is associated with a reduced hepatocarcinogenesis risk in type 2 diabetes patients (43) and an improvement of sustained virological response in chronic hepatitis C patients (44). The present study provides evidence for the possibility that not only metformin monotherapy, but also AMPK inhibitor and metformin combination therapy, may be helpful in the treatment of chronic hepatitis C.

In a previous study using JFH-1 strain of HCV and Huh-7 cells, it was reported that HCV-infection causes $Ser^{485/491}$ phosphorylation of AMPK and inhibits the kinase activity of AMPK. Inhibition of AMPK facilitates HCV replication (26). In the present study using Huh-7.5 cells and a J6/JFH1-derived, cell-adapted strain of HCV, inhibition of AMPK by HCV replication was not observed. Moreover, phosphorylation of $Ser^{485/491}$ was not affected by HCV-infection (Fig. 1c). Since this experimental system using Huh-7.5 and the cell-adapted HCV strain produces

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infectious HCV particles efficiently, inhibition of AMPK by HCV replication may play a minor role in efficient HCV replication. In addition, a previous study having shown that AMPK activators suppress HCV replication, we further investigated the mechanisms of AMPK involvement in HCV replication by using a specific AMPK inhibitor. AICAR-induced AMPK activation plays a critical role in the suppression of HCV (Fig. 2), meanwhile AMPK inhibitor rather potentiates the anti-HCV effects of metformin or glucose shortage (Fig. 3). These data suggest that AMPK activation does not simply lead to an anti-HCV effect.

In conclusion, we have shown the replication of HCV by AMPK-dependent and -independent mechanisms in Huh-7.5 cells. HCV does not replicate efficiently under the low energy conditions that activate AMPK. Hence, correction of hyperglycemia in hepatitis C patients should have a beneficial effect on anti-HCV therapy and the clinical course of hepatitis C. We suggest that AMPK is a therapeutic target for the treatment of chronic hepatitis C patients.

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DISCLOSURE

The authors who have taken part in this study declare that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article:

Fig. S1. Exogenous lipid has no effect on AICAR-mediated suppression of HCV. Huh-7.5 cells were infected with HCV at a MOI of 3.0 for 4 hr. The cells were

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treated with 1 mM AICAR for 20 hr with or without supplementation with100 μM mevalonolactone (Sigma) and 100 μM oleic acid (Sigma) (Lipid). Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as shown in Figure 1b. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

Fig. S2. Cell confluence activates AMPK and suppresses HCV replication. 10^5 (sub-confluent) or 4×10^5 Huh-7.5 cells (confluent) were seeded in 24-well plates overnight. 4×10^5 Huh-7.5 cells resulted in a 100% confluent monolayer of cells in the culture plates. The cells were infected with HCV at a MOI of 3.0 for 4 hr and incubated for 20 hr. Cell lysates were separated by SDS-PAGE and analyzed by immunoblotting as shown in Figure 1b. Molecular size markers are indicated at the left in kilodaltons. The results are representative of three independent experiments.

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ORIGINAL ARTICLE

Sequence heterogeneity of NS5A and core proteins of hepatitis C virus and virological responses to pegylated-interferon/ribavirin combination therapy

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ABSTRACT

Both host and viral factors have been implicated in influencing the response to pegylated-interferon/ribavirin (PEG-IFN/RBV) therapy for hepatitis C virus (HCV) infection. Among the viral factors, sequence heterogeneity within NS5A and core regions has been proposed. This study aimed to clarify the relationship between virological responses to PEG-IFN/RBV therapy and sequence heterogeneity within NS5A, including the IFN/RBV resistance-determining region (IRRDR), the interferon sensitivity-determining region (ISDR) and the core region. Pretreatment sequences of NS5A and the core regions were analyzed in 57 HCV-1b-infected patients who were to be treated with PEG-IFN/RBV. Of 40 patients infected with HCV having an IRRDR with four or more mutations (IRRDR $\geq$ 4), 28 (70%) patients achieved a sustained virological response (SVR). On the other hand, only 4 (24%) of 17 patients infected with HCV having an IRRDR with three or fewer mutations (IRRDR $\leq$ 3) achieved a SVR ($P = 0.001$). Similarly, 22 (71%) of 31 patients infected with HCV and having an ISDR with one or more mutations (ISDR $\geq$ 1) achieved a SVR while 10 (38%) of 26 patients infected with HCV and having an ISDR without any mutations (ISDR = 0) achieved a SVR ($P = 0.014$). As for the core region, there was significant correlation between a single mutation at position 70 (Gln^{70}) and non-SVR ($P = 0.02$). Notably, Gln^{70} was more prominently associated with the null response ($P = 0.0007$). In conclusion, sequence heterogeneity within the IRRDR and ISDR, and a single point mutation at position 70 of the core region of HCV-1b are likely to be correlated with virological responses to PEG-IFN/RBV therapy.

Key words Core, interferon/ribavirin resistance-determining region, interferon sensitivity-determining region, pegylated-interferon/ribavirin.

Hepatitis C virus is a major cause of chronic liver diseases worldwide. Approximately 180 million people, ~3% of the world's population, are infected with HCV. Seventy percent of acute infections become persistent, and 50–75%

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List of Abbreviations: aa, amino acids; Arg^{70}, arginine at position 70 of core protein; AUC, area under the curve; CI, confidence intervals; ETR, end-of-treatment response; EVR, early virological response; Gln^{70}, glutamine at position 70 of core protein; γ-GTP, γ-glutamyl transpeptidase; HCV, hepatitis C virus; IFN, interferon; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; Leu^{91}, leucine at position 91 of core protein; Met^{91}, methionine at position 91 of core protein; NS5A, nonstructural protein 5A; PEG-IFN/RBV, pegylated-interferon/ribavirin; PKR, double-stranded RNA-activated protein kinase; RBV, ribavirin; ROC, receiver operating characteristic; RVR, rapid virological response; SVR, sustained virological response.

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of patients with chronic HCV infection progress to hepatocellular carcinoma (1–5). Therefore, HCV infection is a major global health problem. Although more than two decades have passed since the discovery of HCV, therapeutic options remain limited. Current standard treatment of chronic HCV infection consists of PEG-IFN and RBV, which leads to a SVR in approximately half of treated patients, especially those infected with the most resistant genotypes, HCV-1a and HCV-1b (6, 7). Given the considerable side effects and high cost of this treatment, which result in discontinuation of treatment by some patients, reliable prediction of treatment outcome is needed. An expanded range of predictors may assist clinicians and patients to more accurately assess the likelihood of an SVR and thus to make more reliably informed treatment decisions (8).

Because the SVR rate to PEG-IFN/RBV therapy depends on viral genotypes, it is generally considered that HCV genetics affect the treatment response (9). In this context, NS5A has been widely discussed because of its known correlation with IFN responsiveness. Initially, in the era of IFN monotherapy, it was proposed that sequence variations within a region in NS5A spanning from aa 2209 to 2248, called the ISDR, were correlated with IFN responsiveness (10). Subsequently, in the era of combination therapy with PEG-IFN/RBV, we identified a new region near the C-terminus of NS5A spanning from aa 2334 to 2379, which we referred to as the IRRDR (11). The degree of sequence variations within the IRRDR was significantly associated with the clinical outcome of PEG-IFN/RBV combination therapy. On the other hand, prediction of SVR by aa substitutions at positions 70 and 91 of the core protein in Japanese patients infected with HCV-1b has also been proposed (12–14). More recently, we investigated the impact of NS5A polymorphisms, including those in IRRDR and ISDR, and core polymorphism on virological responses to PEG-IFN/RBV therapy among HCV-1b-infected patients in Hyogo Prefecture, Japan. The criterion of six or more mutations in the IRRDR (IRRDR $\geq$ 6) was identified as the most powerful viral genetic factor that independently predicted SVR (15). In another study curried out on a patient cohort in Yamagata Prefecture, Japan, we proposed that polymorphism in the secondary structure of the N-terminal region of NS3 of HCV-1b influences virological responses to PEG-IFN/RBV therapy, and that virus grouping based on NS3 polymorphism can also be used to predict the outcome of the therapy (16). In the present study, we further analyzed the Yamagata cohort for a possible relationship between heterogeneity of NS5A and the core regions of the HCV genome and virological responses to PEG-IFN/RBV therapy.

MATERIALS AND METHODS

Patients

Fifty-seven patients who were chronically infected with HCV-1b, their diagnoses being based on detection of anti-HCV antibody and HCV RNA, and who had been seen at Yamagata University Hospital in Yamagata, Japan, were enrolled in the study. Their HCV subtypes were determined according to the method of Okamoto *et al.* (17). Patients were treated with PEG-IFNα-2b (Pegintron; Schering-Plough, Kenilworth, NJ, USA) (1.5 μg per kilogram of body weight, once weekly, subcutaneously) and RBV (Rebetol; Schering-Plough) (600~800 mg daily, orally), 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 the scheduled doses of PEG-IFN and RBV. Serum samples were collected from the patients before treatment and at intervals of 4 weeks during the whole observation period (72 weeks), and tested for HCV RNA titers as reported previously (18).

The study protocol was approved beforehand by the Ethics Committee at Yamagata University Hospital, and written informed consent for study participation was obtained from each patient prior to treatment. Also, the study protocol conforms to the provisions of the Declaration of Helsinki.

Sequence analysis of hepatitis C virus NS5A and the core regions of the hepatitis C virus genome

Hepatitis C virus 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 the core regions of the HCV genome were performed as described elsewhere (11, 18, 19). The sequences of the amplified fragments of NS5A and core regions were determined by direct sequencing without subcloning. The aa sequences were deduced and aligned using GENETYX Win software version 7.0 (Genetyx, Tokyo, Japan).

Statistical analysis

To evaluate the optimal threshold of the IRRDR and ISDR mutations for SVR prediction, we constructed an ROC curve and calculated the AUC, sensitivity and specificity (11). Statistical differences in treatment responses according to NS5A and core sequence heterogeneity were determined by the χ^2 test. Likewise, statistical differences in the patients' baseline variables according to the degree of IRRDR polymorphism were determined by Student's *t*

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test for numerical variables and the χ^2 probability test for categorical variables. Univariate and multivariate logistic analyses were performed to identify variables that were independently correlated with the treatment outcome. Variables with a P value of <0.1 in univariate analysis were further included in a multivariate logistic regression analysis. The odds ratios and 95% CI were also calculated. All statistical analyses were performed using SPSS version 16 software (SPSS, Chicago, IL, USA). Unless otherwise stated, a P value of <0.05 was considered 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 AB601987 through AB602043.

RESULTS

Patients' responses to pegylated-interferon/ribavirin combination therapy

Among the 57 patients enrolled in this study, 8 (14%), 36 (63%), 42 (74%) and 32 (56%) patients were negative for HCV-RNA at week 4 (RVR), week 12 (EVR), week 48 (ETR) and week 72 (SVR), respectively (Table 1). SVR was achieved by all (100%) of RVR, 30 (83%) of 36 EVR, and 32 (76%) of 42 ETR patients. Non-SVR patients represented 44% (25/57) of total cases. Twenty-six percent (15/57) of the patients had continuous viremia during the whole observation period (72 weeks), referred to as a null response; whereas 18% (10/57) had transient disappearance of serum HCV RNA at a certain time point followed by a rebound in viremia either before, or after the end of, the treatment course, referred to as a relapse.

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

Virological response	Proportion
RVR	14% (8/57)†
EVR	63% (36/57)
ETR	74% (42/57)
SVR	56% (32/57)
Non-SVR	44% (25/57)
Null response	26% (15/57)
Relapse	18% (10/57)

†, number of patients in the relevant category /total number of patients.

Correlation between interferon/ribavirin resistance-determining region polymorphism and treatment responses

The degree of sequence variation within the IRRDR has been proposed as a useful predictor of HCV treatment outcome (11, 15, 20, 21). We performed ROC curve analysis to estimate the optimal cutoff number of IRRDR mutations that differentiated between a SVR and non-SVR in the present patient cohort. Based on the results obtained, we estimated four mutations as the optimal number of IRRDR mutations since this provided the highest sensitivity (88%) and good specificity (52%) with an

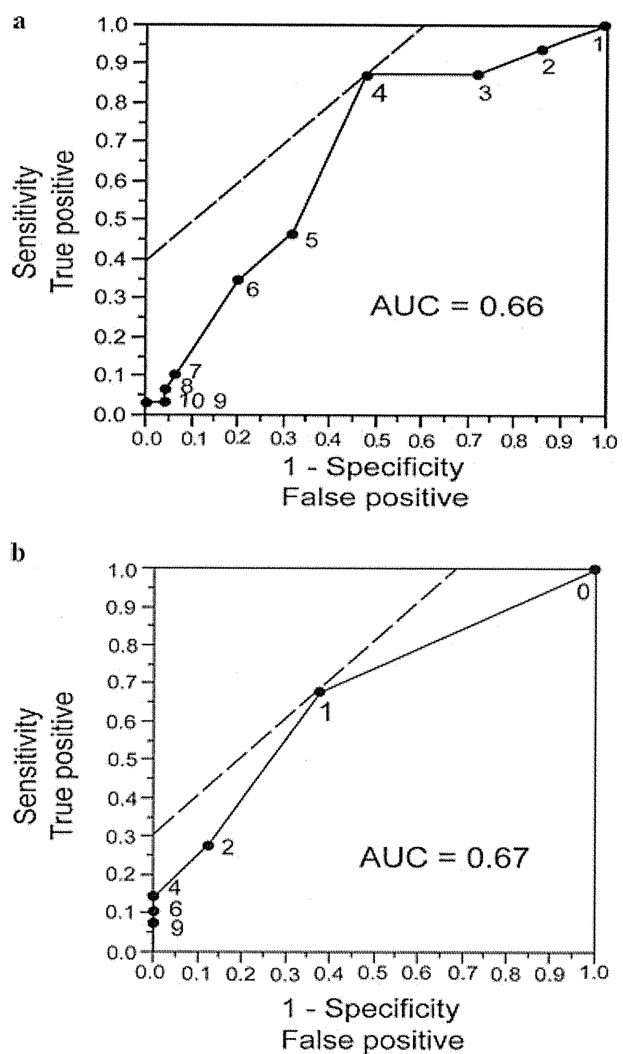

Fig. 1. ROC curve analysis of (a) IRRDR and (b) ISDR sequence heterogeneity for SVR prediction. The curves depicted by solid lines shows the AUC. Solid circles with numerals plotted on the curve represent different numbers of IRRDR and ISDR mutations analyzed. The dashed lines touch the optimal number of IRRDR and ISDR mutations for SVR prediction.

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Table 2. Correlation between NS5A and core protein polymorphisms and virological responses of patients treated with PEG-IFN/RBV

Protein	Factor	Total †	SVR ‡	Non-SVR	Null response	Relapse	*P* value: SVR vs non-SVR	*P* value: SVR vs null response	*P* value: SVR vs relapse
NS5A	IRRDR ≥ 4	40	28 (70%)	12 (30%)	7 (17.5%)	5 (12.5%)	**0.001**	**0.003**	**0.01**
	IRRDR ≤ 3	17	4 (24%)	13 (76%)	8 (47%)	5 (29%)			
	ISDR ≥ 1	31	22 (71%)	9 (29%)	5 (16%)	4 (13%)	**0.014**	**0.02**	0.1
	ISDR = 0	26	10 (38%)	16 (62%)	10 (38%)	6 (24%)			
Core	Wild-core (Arg^{70}/Leu^{91})	24	15 (63%)	9 (37%)	5 (21%)	4 (16%)	0.4	0.4	0.7
	Non-wild-core	33	17 (52%)	16 (48%)	10 (30%)	6 (18%)			
	Gln^{70}	14	4 (29%)	10 (71%)	9 (64%)	1 (7%)	**0.02**	**0.0007**	0.8
	Non- Gln^{70}	43	28 (65%)	15 (35%)	6 (14%)	9 (21%)			
	Met^{91}	22	10 (46%)	12 (54%)	6 (27%)	6 (27%)	0.2	0.6	0.1
	Non- Met^{91}	35	22 (63%)	13 (37%)	9 (26%)	4 (11%)			

†, total number of isolates with a given factor; ‡, number of SVR, non-SVR, null-response or relapse cases with a given factor. *P* values indicating statistically significant difference are written in bold.

AUC of 0.66 (Fig. 1a). In this study, therefore, we used the criteria of four or more mutations in the IRRDR (IRRDR ≥ 4) and IRRDR ≤ 3. In this connection, it should be stated that the criteria of IRRDR ≥ 6 and IRRDR ≤ 5 which were used on different patient cohorts in Hyogo Prefecture (11, 15) were not selected by the ROC curve analysis in this study because of their low sensitivity (34%), although they had higher specificity (80%) than that of IRRDR ≥ 4 (52%). This difference was probably due to the low prevalence of HCV isolates with IRRDR ≥ 6 (28%) in the present patient cohort.

We found that 70%, 30%, 17.5% and 12.5% of patients infected with HCV isolates with IRRDR ≥ 4 were SVR, non-SVR, null response and relapse cases, respectively (Table 2 and Fig. 2). By contrast, 24%, 76%, 47% and 29% of patients infected with HCV isolates with IRRDR ≤ 3 were SVR, non-SVR, null response and relapse cases, respectively. Thus, the proportions of SVR, non-SVR, null response and relapse cases were significantly different among HCV isolates with IRRDR ≥ 4 and IRRDR ≤ 3.

Interestingly, while IRRDR polymorphism was correlated with the final treatment outcome, it was also closely correlated with all the responses during treatment, represented by RVR, EVR and ETR (Table 3).

Next, we investigated the correlations between the patients' demographic, hematological, biochemical and virological baseline variables and the degree of IRRDR polymorphism. This analysis revealed that patient age was the only factor that was significantly correlated with the degree of IRRDR polymorphism, patients who were infected with HCV isolates of IRRDR ≥ 4 being significantly younger on average than patients infected with HCV isolates with IRRDR ≤ 3 ($P = 0.035$) (Table 4).

Correlation between interferon sensitivity-determining region polymorphism and treatment responses

Based on ROC curve analysis, we estimated one mutation in the ISDR as an optimal cut-off number of mutations for SVR prediction since it had the highest sensitivity (69%) combined with the highest specificity (64%) and yielded an AUC of 0.67 (Fig. 1b). Seventy-one percent, 29%, 16% and 13% of patients infected with HCV isolates with one or more mutations in the ISDR (ISDR ≥ 1) were SVR, non-SVR, null response and relapse cases, respectively (Table 2 and Fig. 2). By contrast, 38%, 62%, 38% and 24% of patients infected with HCV isolates with no mutation in the ISDR (ISDR = 0) were SVR, non-SVR, null response and relapse cases, respectively. Thus, the proportions of SVR, non-SVR and null response cases were significantly different among HCV isolates with ISDR ≥ 1 and ISDR = 0.

ISDR polymorphism and the on-treatment responses had significant correlation only with EVR, since 77% of patients infected with HCV isolates with ISDR ≥ 1 were EVR whereas 54% of patients infected with HCV isolates with ISDR = 0 were non-EVR ($P = 0.01$, Table 3).

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 are useful negative markers for the treatment outcome of Japanese patients treated with PEG-IFN/RBV combination therapy (12–14). We have investigated the impact of various sequences patterns of both positions on treatment responses. We found that 63%, 37%, 21% and 16%

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Group		Isolate	IRRDR (2334–2379)	IRRDR	ISDR	CR/70
		Cons	VLTESTVSSALAELATKTFGSSGSSAVDSGTATAPPDQASDDGDKG			
SVR		80	...................S..DP..T....E.....WSP.H..T.	10	2	R
SVR		77	I..................................LNLL...DNA.	8	1	R
SVR		44	..........................A...I.......PLAS..T.	7	1	R
SVR		22	..S.............R.........A...........LF.E..T.	7	1	H
SVR		105	..........................A...........LFES..T.	6	6	Q
SVR		41	........T..............LP.A..............ND...	6	4	Q
SVR		26	.........V............E..TA...........P.....A.	6	2	R
SVR		54	...................................LNHL..E..T.	6	2	R
SVR		23	......................E...A...........P...DVA.	6	1	R
SVR		107	......................E.......VM.T..A.......R.	6	1	H
SVR		103	.................S....E.Q.A.......L.........N.	6	0	R
SVR		71	...................S..............G...P..N..T.	5	1	R
SVR		86	....................G.E.............G.S.....A.	5	1	R
SVR		2	......................E..T........G........N.E	5	0	R
SVR		88	I..................................LT.P.....A.	5	0	R
SVR		24	..S.......................N.......V..E........	4	9	H
SVR		108	......................EP.TI...................	4	9	R
SVR		27	......................E...A...........P.....A.	4	2	R
SVR		46	.........V................A..............S..A.	4	1	R
SVR		47	..........................I.....S..........G.E	4	1	R
SVR		50	.........................G............VP.....E	4	1	R
SVR		91	..........................A.........G.T.....N.	4	1	R
SVR		102	....................................T.S..E..T.	4	1	R
SVR		11	......................................T...D.RA	4	0	R
SVR		33	...............V...................L..T......E	4	0	R
SVR		59	........T......A..........A...........T.......	4	0	R
SVR		98	..............................I.......PL....T.	4	0	R
SVR		87	.....N....................A..............Q..T.	4	0	Q
SVR		7	......................................T...D...	2	1	R
SVR		38	...................................L..T.......	2	0	Q
SVR		109	......................E...............V.......	2	0	R
SVR		94	...............A..............................	1	1	R
Non-SVR	Relapse	34	..S...................E..V..........GD...N...E	7	0	H
Non-SVR	Relapse	48	...............V....G..........S......L..S..P.	6	1	R
Non-SVR	Relapse	61	...............V......E...........S...P....GA.	6	0	R
Non-SVR	Relapse	15	.........................T............PF.S..T.	5	0	R
Non-SVR	Relapse	32	......................................PF.S..T.	5	1	R
Non-SVR	Relapse	96	......................E...............P.....A.	3	2	R
Non-SVR	Relapse	37	......................................P..S..A.	3	1	R
Non-SVR	Relapse	62	.................N..G.....A...................	3	0	R
Non-SVR	Relapse	97	..................................L.........ER	3	0	Q
Non-SVR	Relapse	1	..........................A...............D...	2	0	R
Non-SVR	Null response	73	......................EP..T..DA......RP..N..T.	9	1	Q
Non-SVR	Null response	82	...............S........LVA....G.............E	6	0	R
Non-SVR	Null response	81	......................E.............G.S...R.T.	5	2	Q
Non-SVR	Null response	29	...............S......E............L.....N...E	5	0	Q
Non-SVR	Null response	101	...................S...............L....G.D..E	5	0	Q
Non-SVR	Null response	25	......................E.............NL......A.	4	2	R
Non-SVR	Null response	52	I.................................L...T.....A.	4	0	R
Non-SVR	Null response	43	......................................P..S..T.	3	1	Q
Non-SVR	Null response	63	....................G.....A..................E	3	0	R
Non-SVR	Null response	28	....GE.	2	0	Q
Non-SVR	Null response	65	...................................L........T.	2	0	R
Non-SVR	Null response	100	......................E...............T.......	2	0	R
Non-SVR	Null response	55	......................E......................E	2	1	Q
Non-SVR	Null response	51	..........................A...................	1	0	Q
Non-SVR	Null response	95	....D...	1	0	Q

Fig. 2. Sequence alignment of IRRDR of NS5A of HCV-1b obtained from pretreatment sera. The consensus sequence is shown at the top (Cons). Dots indicate residues identical to those of the consensus sequence. Number of IRRDR and ISDR mutations, as well as the sequence pattern at aa 70 of the core protein, are shown on the right. The number of ISDR mutations was determined by comparing with the consensus sequence reported by Enomoto *et al.* (10).

of patients infected with HCV isolates with wild-core ($\mathrm{Arg}^{70}/\mathrm{Leu}^{91}$) were SVR, non-SVR, null response and relapse cases, respectively, compared to 52%, 48%, 30% and 18% of patients infected with HCV isolates with non-wild-core (Table 2). Thus, there was no significant correlation between wild-core and SVR or non-SVR ($P = 0.4$). However, the presence of a single point mutation at position 70 (Gln^{70} vs non- Gln^{70}) was significantly associated with

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

Protein	Factor	Total †	RVR‡	Non-RVR	EVR	Non-EVR	ETR	Non-ETR	*P* value		
									RVR vs non-RVR	EVR vs non-EVR	ETR versus non-ETR
NS5A	IRRDR ≥ 4	40	8 (20%)	32 (80%)	30 (75%)	10 (25%)	33 (83%)	7 (17%)	**0.047**	**0.005**	**0.02**
	IRRDR ≤ 3	17	0 (0.0%)	17 (100%)	6 (35%)	11 (65%)	9 (53%)	8 (47%)			
	ISDR ≥ 1	31	5 (16%)	26 (84%)	24 (77%)	7 (23%)	26 (84%)	5 (16%)	0.62	**0.01**	0.057
	ISDR = 0	26	3 (12%)	23 (88%)	12 (46%)	14 (54%)	16 (62%)	10 (38%)			
Core	Wild-core (Arg^{70}/Leu^{91})	24	4 (17%)	20 (83%)	17 (71%)	7 (29%)	19 (79%)	5 (21%)	0.63	0.31	0.42
	Non-wild-core	33	4 (12%)	29 (88%)	19 (58%)	14 (42%)	23 (70%)	10 (30%)			
	Gln^{70}	14	2 (14%)	12 (86%)	4 (29%)	10 (71%)	5 (36%)	9 (64%)	0.97	**0.002**	**0.0002**
	Non- Gln^{70}	43	6 (14%)	37 (86%)	32 (74%)	11 (26%)	37 (86%)	6 (14%)			
	Met^{91}	22	1 (5%)	21 (95%)	12 (54%)	10 (46%)	16 (73%)	6 (27%)	0.1	0.29	0.9
	Non- Met^{91}	35	7 (20%)	28 (80%)	24 (69%)	11 (31%)	26 (74%)	9 (26%)			

†, total number of isolates with a given factor; ‡, number of RVR, non-RVR, EVR, non-EVR, ETR or non-ETR cases with a given factor. *P* values indicating statistically significant difference are written in bold.

Table 4. Correlation between IRRDR polymorphism and patients' demographic characteristics

Factor	IRRDR ≥ 4	IRRDR ≤ 3	*P* value
Age	54.1 ± 9.5†	59.2 ± 6.9	**0.035**
Sex (male/female)	23/17	11/6	0.61
Body weight (Kg)	62.4 ± 18.8	64.2 ± 12.1	0.68
Platelets (× $10^4/mm^3$)	17.0 ± 5.0	17.7 ± 5.0	0.66
Hemoglobin (g/dl)	14.3 ± 1.2	14.7 ± 1.1	0.21
Neutrophil count	2303 ± 822	2432 ± 658	0.57
γ-GTP (IU/L)	52.6 ± 42.3	80.7 ± 70.5	0.15
Glutamate pyruvate transaminase (IU/L)	80.2 ± 60.7	101.6 ± 79.7	0.33
HCV-RNA (KIU/mL)	1719 ± 1298	2273 ± 1571	0.21

†, mean ± S.D. *P* values indicating statistically significant difference are written in bold.

either a non-SVR or null-response (Table 2 and Fig. 2). Gln^{70} was also the only factor of core protein that was strongly associated with non-EVR and non-ETR responses (Table 3).

Identification of independent viral factors that are significantly correlated with virological responses to pegylated-interferon/ribavirin therapy

In order to identify which independent viral factors are significantly correlated with final and on-treatment responses to PEG-IFN/RBV therapy, data including all available baseline patient variables, NS5A and core polymorphic factors, and previously published data on polymorphism in an N-terminus of NS3 of the same patient cohort (16) were analyzed by univariate and multivariate logistic regression analyses (Table 5). In regard to the final treatment responses, IRRDR ≥ 4 and group A of the N-terminus of NS3 were identified as independent viral factors that are significantly associated with a SVR, whereas IRRDR ≤ 3 and Gln^{70} of core were identified as independent factors associated with a null response. Regarding on-treatment responses, IRRDR ≥ 4 and non-Gln^{70} were identified as independent factors associated with an EVR and ETR.

DISCUSSION

Pegylated-interferon/ribavirin combination therapy has been used to treat chronic HCV infection, the treatment outcome being thought to be affected by both host and viral factors. Recently, IL28B, which encodes IFNλ3, was identified as the major host factor that determines the treatment outcome (22–24). As for the viral factor(s), we and other research groups have reported that

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Table 5. Univariate and multivariate logistic regression analyses to identify independent factors significantly associated with virological responses to PEG-IFN/RBV therapy

	Univariate		Multivariate	
Response	Variable	*P* value	Odds ratio (95% CI)	*P* value
SVR	**IRRDR ≥ 4**	0.003	5.2 (1.3–20.1)	**0.02**
	ISDR ≥ 1	0.013		
	Non-Gln^{70}	0.016		
	NS3 / A group	0.013	4.2 (1.0–18.3)	**0.05**
	Viral load	0.04		
Null response	**IRRDR ≤ 3**	0.001	0.2 (0.04–0.7)	**0.02**
	ISDR = 0	0.06		
	Gln^{70}	0.0001	7.8 (1.7–35.5)	**0.008**
	Viral load	0.08		
	NS3 / non-A group	0.03		
	Hemoglobin	0.02		
	γ-GTP	0.08		
Relapse	**Age**	0.02	1.2 (1.0–1.3)	**0.03**
	Sex	0.004		
	Hemoglobin	0.03		
RVR	IRRDR ≥ 4	0.05		
	Hemoglobin	0.02	0.4 (0.2–0.9)	**0.03**
EVR	**IRRDR ≥ 4**	0.001	7.0 (1.6–29.8)	**0.009**
	ISDR≥1	0.01		
	Non-Gln^{70}	0.002	0.2 (0.04–0.8)	**0.02**
	NS3 / A group	0.07		
	Viral load	0.015		
	Hemoglobin	0.07		
	γ-GTP	0.09		
ETR	**IRRDR≥4**	0.001	6.2 (1.4–27.7)	**0.02**
	ISDR≥1	0.06		
	Non-Gln^{70}	0.0001	0.1 (0.03–0.6)	**0.008**
	NS3 / A group	0.03		
	Viral load	0.08		
	Hemoglobin	0.02		
	γ-GTP	0.08		

Variables that were shown by multivariate analysis to be significantly correlated with a certain treatment response are written in bold.

heterogeneity of NS5A and the core proteins of HCV-1b are correlated with treatment outcome (11–15). Furthermore, we recently reported that polymorphism in an N-terminus of NS3 is significantly correlated with virological responses to PEG-IFN/RBV therapy (16). In the present study, we have further expanded the previous study by analyzing possible correlations between heterogeneity of NS5A and the core regions of the HCV-1b genome and virological responses to PEG-IFN/RBV therapy. The present study showed that final and on-treatment responses of patients in the same cohort were also significantly influenced by IRRDR ≥ 4, ISDR ≥ 1 of NS5A, and Gln^{70} of the core protein.

We previously reported IRRDR ≥ 6 as an independent viral factor significantly associated with SVR in different patient cohorts in Hyogo Prefecture (11, 15). Also, ISDR ≥ 2 was identified as the optimal threshold for SVR prediction (20, 25–27). However, in the present study IRRDR ≥ 6 or ISDR ≥ 2 did not correlate significantly with a SVR, although there was a trend toward SVR in these criteria (11 of 16 isolates with IRRDR ≥ 6 and 8 of 11 isolates with ISDR ≥ 2 were obtained from SVR patients). This difference might be attributable to the low prevalence of IRRDR ≥ 6 (16/57) and ISDR ≥ 2 (13/57) in the present patient cohort. Accordingly, in this study the IRRDR and ISDR sequences of the HCV isolates were less variable than were those of other studies. It thus appears that the prevalence of HCV isolates of IRRDR ≥ 6 and ISDR ≥ 2 varies from one geographical region to another. This implies the possibility that certain characteristics of HCV isolates, including IFN sensitivity, may also vary from one geographical region to another. Analysis in a large-scale multicenter study is needed to clarify this possibility.

The NS5A- interferon sensitivity-determining region was first identified to be significantly correlated with the probability of a SVR during the era of IFN monotherapy (10). In the more recent era of combination therapy with PEG-IFN/RBV, the NS5A-IRRDR has been identified to be closely associated with a SVR (11). The ISDR interacts with PKR and regulates replication of HCV *in vitro* (28). Mutations in the ISDR affect the interaction with PKR and may inhibit viral replication. In the case of the IRRDR, the molecular mechanism underlying the possible involvement of this region in IFN responsiveness of the virus is still unknown. The significant difference among IRRDR sequence patterns may suggest genetic flexibility of this region. Thus, changes in the IRRDR might be capable of modulating intracellular antiviral activity, or maybe the genetic flexibility of this region is accompanied by compensatory changes elsewhere in the viral genome and these compensatory changes affect overall viral fitness and responses to IFN therapy (29–31)

When we investigated the impact of various sequences patterns at positions 70 and 91 of the core protein, we observed that single point mutation at position 70 (Gln^{70} vs non-Gln^{70}) was the only factor that significantly influenced treatment responses. This result is consistent with recent reports, including a recent multi-center study in Japan that identified Gln^{70} as a predictive factor for poor responses to PEG-IFN/RBV treatment (14, 13, 30). The core region of HCV interacts with several host factors and modulates expression of numerous genes, including down-regulating IFN-induced antiviral genes, thus inhibiting the antiviral action of IFN (32, 33). Therefore, it would also be interesting to investigate the impact of

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polymorphism, both at position 70 and of NS5A, on HCV pathogenesis and IFN sensitivity.

Multivariate logistic regression analysis of all available data, including those of NS5A and core polymorphisms in this study and the data on NS3 polymorphism in the same patient cohort published elsewhere (16), identified IRRDR ≥ 4 and group A of NS3 as independent viral factors that are significantly associated with a SVR, and IRRDR ≤ 3, and Gln^{70} of the core protein as independent factors significantly associated with a null response (Table 5). No combinations of these criteria produced a more significant correlation with virological responses to PEG-IFN/RBV therapy (data not shown).

In conclusion, the present results demonstrate that sequence heterogeneity of NS5A, especially in IRRDR and ISDR, and a single-point mutation at position 70 of the core protein of HCV-1b are significantly correlated with virological responses to PEG-IFN/RBV therapy. Also, the results emphasize the possible functional importance of NS5A and core protein in regulating viral responsiveness to PEG-IFN/RBV.

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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

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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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