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Elimination of Hepatitis C Virus from Hepatocytes by a Selective Activation of Therapeutic Molecules

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Abstract

To eliminate hepatitis C virus (HCV) from infected hepatocytes, we generated two therapeutic molecules specifically activated in cells infected with HCV. A dominant active mutant of interferon (IFN) regulatory factor 7 (IRF7) and a negative regulator of HCV replication, VAP-C (Vesicle-associated membrane protein-associated protein subtype C), were fused with the C-terminal region of IPS-1 (IFNβ promoter stimulator-1), which includes an HCV protease cleavage site that was modified to be localized on the ER membrane, and designated cIRF7 and cVAP-C, respectively. In cells expressing the HCV protease, cIRF7 was cleaved and the processed fragment was migrated into the nucleus, where it activated various IFN promoters, including promoters of IFNα6, IFNβ, and IFN stimulated response element. Activation of the IFN promoters and suppression of viral RNA replication were observed in the HCV replicon cells and in cells infected with the JFH1 strain of HCV (HCVcc) by expression of cIRF7. Suppression of viral RNA replication was observed even in the IFN-resistant replicon cells by the expression of cIRF7. Expression of the cVAP-C also resulted in suppression of HCV replication in both the replicon and HCVcc infected cells. These results suggest that delivery of the therapeutic molecules into the liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

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Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases. A high risk of chronicity is the major concern of HCV infection, since chronic HCV infection often leads to liver cirrhosis and hepatocellular carcinoma [1,2]. Although the proportion of patients achieving a sustained virological response (SVR) has been increased by the recent used of combination therapy with pegylated-interferon-α (PEG-IFNα) and ribavirin (RBV), half of patients still exhibit no response to this therapy, suggesting that the IFN signaling pathway is modulated by HCV infection. In addition, various side effects have been reported in more than 20% of patients treated with this combination therapy [3].

HCV belongs to the family *Flaviviridae* and possesses a single positive-stranded RNA genome that encodes a single polyprotein composed of about 3,000 amino acids. The HCV polyprotein is processed into 10 viral proteins by host and viral proteases. Viral structural proteins, including the capsid protein and two envelope proteins, are located in the N-terminal one third of the polyprotein, followed by nonstructural proteins. The NS2 protease cleaves its own carboxyl terminus and NS3 cleaves the downstream positions to produce NS4A, NS4B, NS5A and NS5B. Although laboratory strains of HCV propagating in cell culture (HCVcc) have been established based on the full-length genome of the genotype 2a JFH1 strain [4], establishment of a robust cell culture system capable of propagating serum-derived HCV from hepatitis C patients has not yet been achieved.

Type I IFN exhibits potent antiviral effects through the regulation of hundreds of IFN-stimulated genes (ISGs) which encode proteins involved in the establishment of antiviral state in cells [5]. IFNs induce transcription of ISGs through activation of the Jak-STAT pathway [6]. Binding of type I IFN to the IFN receptor induces phosphorylation of the receptor-associated tyrosine kinases, Jak1 and Tyk2, and then these kinases activate STAT1 and STAT2. The phosphorylated STATs migrate into the nucleus and activate ISG promoters through binding to the specific responsible elements. HCV infection has been suggested to impair the IFN production through multiple pathways. The IFN-induced Jak-STAT signaling is inhibited in cells and transgenic mice expressing HCV proteins and in the liver biopsy samples of chronic hepatitis C patients [7–9].

Induction of type I IFN upon infection with pathogens is crucial for innate immunity, and it is mediated by the activation of pattern-recognition receptors, including Toll-like receptors (TLRs) and cytosolic receptors, such as RIG-I and MDA5 [10–12]. The induction of type I IFN is primarily controlled at the gene transcriptional level, wherein a family of transcription factors known as IFN regulatory factors (IRFs) play a pivotal role. IRF3

and IRF7 are known to be essential for the RIG-I-, MDA5-, and TLR-mediated type I IFN production pathways. IRF3 is induced primarily by a response to initiate IFNβ production, whereas IRF7 is induced by IFNβ and participates in the late phase for IFNβ induction [13]. All TLRs, except for TLR3, activate the MyD88-dependent pathway, whereas TLR3 and TLR4 activate the TRIF-dependent pathway. HCV NS3/4A protease has been shown to impair the production of IFNβ as well as the subsequent IFN-inducible genes through the inactivation of the adaptor molecules involved in the TLR-dependent and -independent signaling pathways [14–18]. On the other hand, Vilasco *et al.* suggested that impairment of IKKi - which, along with TBK1, is one of the important factors participating in IRF3 phosphorylation and activation - in the HCV replicon cells plays at least a partial role in the restoration of type I IFN signaling pathways [19]. In addition, IRF7 was shown to participate in the positive feedback of type I IFN signaling through the IFN receptor [13]. Therefore, we tried to examine the effect of exogenous expression of IRF7 under the assumption that IRF7 is a potent type I IFN inducer and capable of modulating the viral propagation in hepatocytes infected with HCV.

In this study, we generated two therapeutic molecules consisting of a dominant active mutant of IRF7 or VAP-C, a negative regulator of HCV replication [20], followed by the C-terminal region of IFN promoter stimulator 1 (IPS-1), including the cleavage site of the HCV NS3/4A protease, which was modified so that the cleavage site localized on the ER membrane [21]. The expression of the plasmids encoding these molecules in the HCV replicon and HCVcc-infected cells resulted in a substantial suppression of HCV propagation, suggesting the possibility that these or other similar molecules could be used therapeutically to eliminate HCV from hepatocytes infected with HCV.

Results

IRF7m, a dominant active mutant of IRF7, activates the IFN promoters in cells replicating HCV

Previous studies have shown that an IRF7 mutant, IRF7m, lacking the amino acid residues from 284 to 454, a region that includes the auto-inhibitory domain (from amino acid residue 305 to 467), and an IRF3 mutant, IRF3m, carrying the substitution of Ser^{396} to Asp in the carboxyl terminal region (Fig. 1A), induced a potent activation of type I IFN promoter in non-hepatic cell lines irrespective of viral infection [22–25]. We first examined the effect of the expression of the IRF dominant active mutants on the inhibition of HCV RNA replication through the production of type I IFN. HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding either wild-type or dominant active mutant of IRF3 or IRF7 together with the reporter plasmids encoding a luciferase gene under the control of the promoters of IFNα6, IFNβ and ISRE, respectively. Among these examined constructs, we observed significant activation of the promoters of IFNα6 and ISRE in the replicon and HCVcc-infected cells compared with naïve and mock-infected cells upon expression of IRF7m, while we observed no activation of the IFNα6 promoter in cells expressing IRF3m (Figs. 1 B and 1C). Potent stimulation of the IFNβ promoter was observed in the replicon cells expressing IRF7m but not in cells infected with HCVcc. Next we examined the antiviral activity of the IRF constructs in both replicon (Fig. 1D) and HCVcc-infected cells (Fig. 1E). The expression of the plasmid encoding IRF7m resulted in potent suppression of viral protein and viral RNA syntheses in both cell types. Although expression of IRF3m induced a slight suppression of viral propagation in cells infected with HCVcc, expression of the IRF constructs except for IRF7m did not induce the significant suppression of viral replication and propagation. These results suggest the possibility of elimination of HCV through a specific induction of type I IFN by the expression of IRF7m in HCV-infected cells.

cIRF7, a chimeric construct of IRF7m, specifically activates the IFN promoters in cells replicating HCV

To induce IFNs in cells infected with HCV but not in uninfected cells through a selective activation of IRF7m, we constructed a chimeric IRF7 (cIRF7) consisting of the IRF7m fused with FLAG-tag and the C-terminal amino acid residues from 503 to 540 of IPS-1 modified to be localized on ER (Fig. 2A upper) [21]. HCV NS3/4A protease cleaves the carboxyl site of Cys^{508} in the C-terminal domain of IPS-1. Although cIRF7 is anchored in the ER and exhibits no activation in uninfected cells, cIRF7 would be cleaved by the NS3/4A protease in cells infected with HCV and the released N-terminal fragment would migrate into the nucleus and activate various IFN promoters (Fig. 3). Immunoblot analyses revealed that cIRF7 was cleaved in 293T cells expressing HCV NS3/4A protease of a wild type but not in those expressing the mutant protease NS3/4A(S139A), and a mutant cIRF7(C508A) which has a substitution of Cys^{508} to Ala, exhibited resistance to the cleavage by the HCV protease (Fig. 2A bottom). To assess a specific activation of the IFN promoters after cleavage of the cIRF7 by HCV NS3/4A, 293T cells expressing FLAG-tagged HCV proteases were transfected with the plasmids encoding the luciferase gene under the control of the promoter of IFNα6, IFNβ or ISRE together with the plasmid encoding either cIRF7 or cIRF7(C508A). Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFNα6, IFNβ and ISRE promoters in cells expressing HCV NS3/4A protease but not in those expressing the mutant protease NS3/4A(S139A) (Fig. 2B). Next we examined the activation of the IFN promoters associated with the expression of the plasmid encoding cIRF7 in the replicon and HCVcc-infected cells. Expression of cIRF7 but not of cIRF7(C508A) induced the activation of the IFN promoters in both cell types (Figs. 2C and 2D). On the other hand, these promoters were not activated by the expression of cIRF7 in the replicon cells harboring subgenomic RNA of Japanese encephalitis virus (JEV) and Huh7 cells infected with JEV (Fig. 2E). These results suggest that the cIRF7 expression is a feasible method for specifically activating the IFN promoters in cells infected with HCV.

Specificity of activation of the IFN promoters by the expression of cIRF7

To further examine the specificity of the activation of the IFN promoters by the expression of cIRF7 in cells replicating HCV, a plasmid encoding either cIRF7 or IRF7m was co-transfected with that encoding the luciferase gene under the ISRE promoter into the HCV replicon or HCVcc-infected cells and cultured in the presence or absence of inhibitors for HCV replication. Treatment with an HCV protease inhibitor (BILN2061) or cyclosporine A (CsA) inhibited the activation of the ISRE promoter by the expression of cIRF7 in the HCV replicon and HCVcc-infected cells in a dose-dependent manner, in contrast to the resistance to the treatments in cells expressing the IRF7m (Fig. 4A and Fig. 4B). Recently, it was shown that an NS3/4A protease of GB virus B (GBV-B), which is the virus genetically related most closely to HCV, also impairs the dsRNA-induced IFN production through a cleavage of IPS-1[26]. Therefore, to assess the possibility of activation of cIRF7 by other flaviviral proteases, cleavage of cIRF7

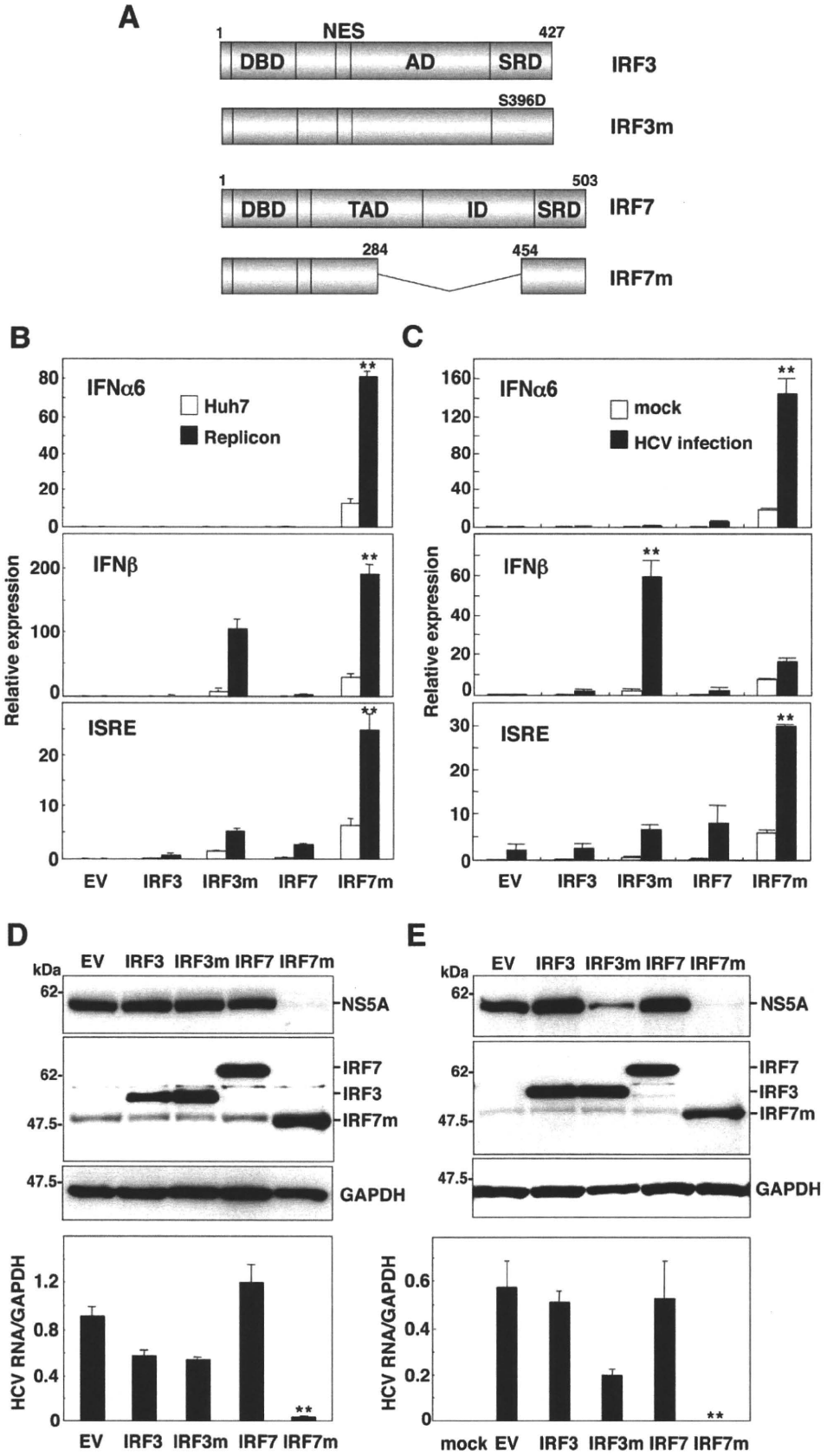

Figure 1. Dominant active mutant of IRF7 activates IFN promoters in cells replicating HCV. (A) Structures of IRF3, IRF7 and the dominant active mutants, IRF3m and IRF7m. The DNA-binding domain, nuclear export sequence, transactivation domain, association domain, inhibitory domain, and signal response domain are indicated as DBD, NES, TAD, AD, ID, and SRD, respectively. Huh7 cells and HCV replicon cells (1×10^5 cells/

well) (B), and Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (C) were transfected with 100 ng of plasmid encoding the luciferase gene under the control of the IFNα6, IFNβ, or ISRE promoter together with an empty vector (EV) or a plasmid encoding each of the IRF constructs. The relative luciferase activity of cell lysates was determined at 24 h post-transfection. HCV replicon cells (3×10^5 cells/well) (D) and Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h (E) were transfected with EV or a plasmid encoding each of the IRF constructs and the expressions of NS5A, IRFs, and GAPDH (upper panel) and synthesis of viral RNA (lower panel) at 72 h post-transfection were determined by immunoblotting and real-time PCR after standardization with GAPDH, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (**$P<0.01$) versus the control cells or mock-infected cells.
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and activation of the IFN promoters were evaluated in 293T cells expressing the viral proteases of HCV, GBV and JEV. Immunoblot analyses revealed that cIRF7 was processed by the viral proteases of HCV and GBV but not by that of JEV and the activation of the IFN promoters was well correlated with the cleavability of the cIRF7 (Fig. 4C). Although the GBV protease exhibited an efficient activation of cIRF7 comparable to HCV protease, processing of cIRF7 and activation of the IFN promoters by the GBV protease was not inhibited by the pretreatment with the HCV protease inhibitor (Figs. 4D and 4E). These results indicate that cIRF7 is capable of activating the IFN promoters through a specific cleavage by the protease in cells infected with HCV.

Nuclear localization of cIRF7 in cells expressing HCV protease

From these results, it was suggested that cIRF7 is cleaved by the HCV protease and the processed fragment migrates into the nucleus and activates IFN promoters (Fig. 3). To confirm the nuclear localization of the cleaved cIRF7, we constructed an EGFP-cIRF7 and determined its subcellular localization in cells expressing the HCV protease and in the HCV replicon cells by confocal microscopy. Nuclear accumulation of the cIRF7 was observed in cells expressing EGFP-cIRF7 together with NS3/4A, but not in those with NS3/4A(S139A) or NS5A and also not in cells co-expressing EGFP-cIRF7(C508A) and NS3/4A (Fig. 5A). Furthermore, expression of EGFP-cIRF7 but not of EGFP-cIRF7(C508A) induced a nuclear accumulation of cIRF7 in the HCV replicon cells, and nuclear localization of the cIRF7 abrogated the expression of viral antigen (NS3), in contrast to the co-localization of EGFP-cIRF7(C508A) and the ER marker PDI, which had no discernible antiviral effect (Fig. 5B). These results suggest that cIRF7 is capable of suppressing HCV replication through an HCV protease-dependent cleavage, migration into the nucleus and activation of the IFN promoters.

Suppression of HCV replication by the expression of cIRF7

To examine the inhibitory effect of the expression of cIRF7 on HCV replication, a plasmid encoding either cIRF7 or cIRF7(C508A) was transfected into the HCV replicon and HCVcc-infected cells, and HCV replication was evaluated by immunoblotting and real-time PCR. The expression of cIRF7 but not of cIRF7(C508A) resulted in cleavage by the HCV protease, and a clear reduction of viral protein and RNA syntheses in both replicon and HCVcc-infected cells (Figs. 6A and 6B). In addition, we examined the effect of cIRF7 on the replication of HCV in the 4βR replicon cells [27,28], which have been shown to exhibit more resistant to the IFNα treatment than Con1 replicon cells (Fig. 6C upper left). Expression of the cIRF7 in the 4βR replicon cells but not in those cured HCV RNA (4βRc cells) induced an activation of the ISRE promoter (Fig. 6C lower left). Expression of cIRF7 but not of cIRF7(C508A) also resulted in processing by the HCV protease and suppression of viral protein and RNA syntheses in the 4βR replicon cells (Fig. 6C right panels).

Suppression of HCV replication by the expression of cVAP-C

Human vesicle-associated membrane protein-associated protein subtype A (VAP-A) and B (VAP-B) are known to be involved in the regulation of membrane trafficking, lipid transport and metabolism, and the unfolded protein response [29]. VAP-A and VAP-B have been shown to be involved in the replication of HCV, and we have shown recently that human VAP-C, a splicing variant of VAP-B, negatively regulates HCV replication by interfering with the interaction of VAP-A and VAP-B with HCV NS5B [20]. We next examined the possibility of using a selective activation of VAP-C to suppress HCV replication in cells infected with HCVcc. We generated expression plasmids encoding a chimeric VAP-C fused with the IPS-1 sequence (cVAP-C), a cVAP-C(C508A) which is made resistant to the HCV protease by a substitution in the cleavage site similar to the substitutions made in cIRF7(C508A), or VAP-C (Fig. 7A). The cVAP-C was cleaved in cells infected with HCVcc, and expression of cVAP-C and VAP-C suppressed expression of NS5A, in contrast to the weak reduction of NS5A in the infected cells expressing cVAP-C(C508A), probably due to a slight cleavage of cVAP-C(C508A) (Fig. 7B, top). Furthermore, the production of viral RNA and infectious particles in the culture supernatants of cells infected with HCVcc was also impaired by the expression of cVAP-C and VAP-C, but not of cVAP-C(C508A) in a dose-dependent manner (Fig. 7B, middle and bottom). Collectively, these results suggest that delivery of the therapeutic molecules into liver of hepatitis C patients, followed by selective activation of the molecules in HCV-infected hepatocytes, is a feasible method for eliminating HCV.

Discussion

An effective prophylactic vaccine against HCV has not been developed yet. Although combination therapy consisting of PEG-IFNα and RBV has been introduced for the treatment of hepatitis C patients, and 50% of individuals infected with genotype 1 achieved a SVR, this treatment is sometimes associated with serious side effects, including depression and anemia [3]. Therefore, new anti-HCV drugs targeted to HCV protease and polymerase and capable of optimizing therapy are currently in the early stages of the development [30,31]. However, it is difficult to achieve a complete removal of viruses by antiviral drugs targeted to the viral enzymes from patients persistently infected with RNA viruses that exhibit a quasispecies nature, such as human immunodeficiency virus (HIV) and HCV. Viral quasispecies are not a simple collection of diverse mutants but a group of interactive variants capable of adapting to new environments [32]. Furthermore, introduction of antiviral drugs may induce an emergence of drug-resistant breakthrough viruses as seen in the case of HIV infection. Therefore, a novel therapeutic approach for hepatitis C patients in addition to the current chemotherapies is required to overcome serious adverse effects and improve the ratio of patients achieving SVR.

In this study, we have generated two therapeutic molecules, cIRF7 and cVAP-C, which are selectively activated in cells

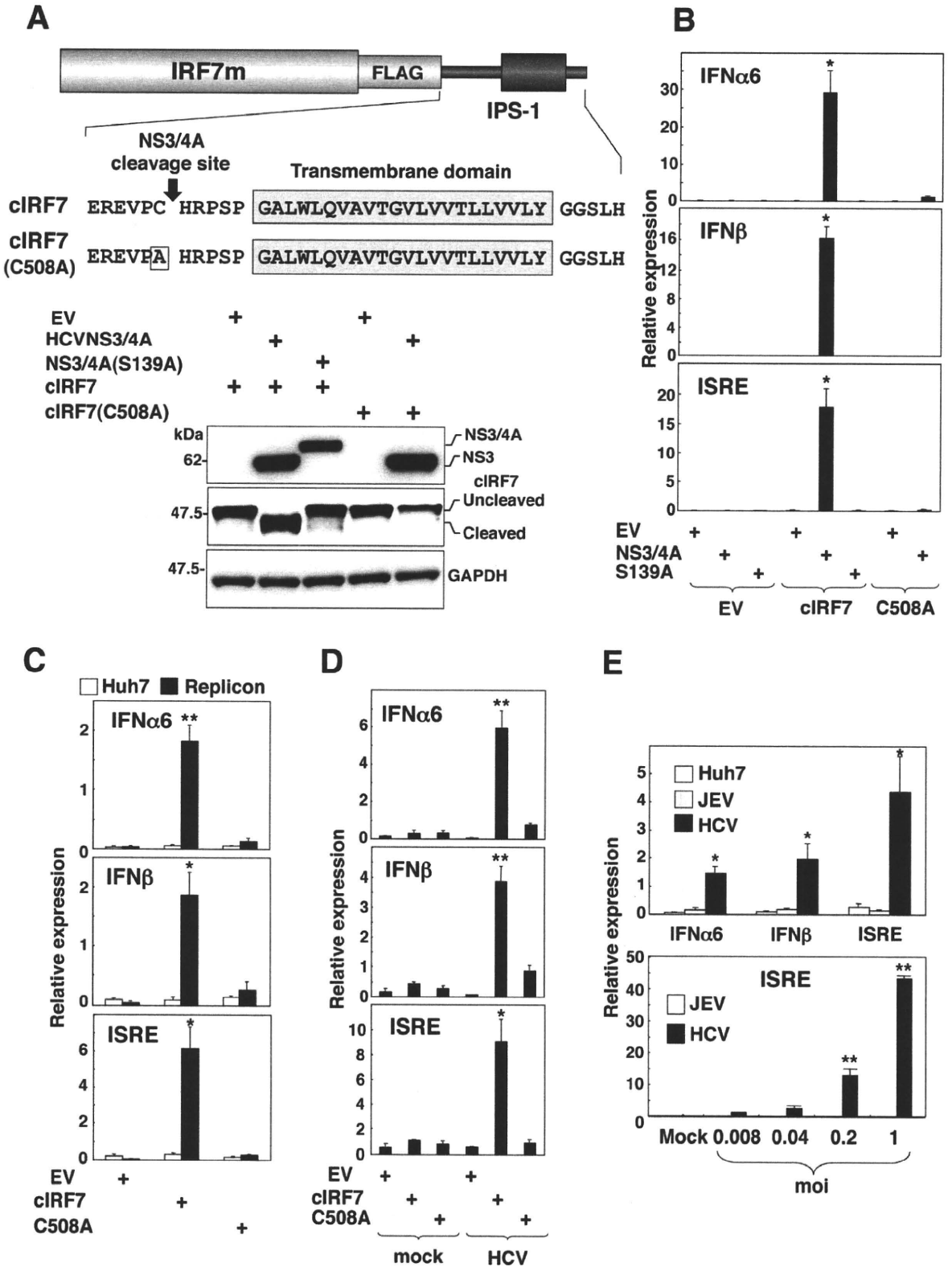

Figure 2. Construction of cIRF7 capable of activating the IFN promoters in cells replicating HCV. (A top) Schematic representation of the cIRF7 constructs. cIRF7 consists of IRF7m, FLAG-tag, and IPS-1 (503 to 540 amino acid residues) sequences containing a cleavage site by HCV NS3/4A protease, a transmembrane domain and a cytoplasmic region modified to localize on the ER. cIRF7(C508A) has a substitution of Cys508 to Ala which renders it resistant to the cleavage by the HCV protease. (A bottom) Immunoblot analyses of 293T cells transfected with a plasmid encoding either cIRF7 or cIRF7(C508A) together with either an empty vector (EV) or a plasmid encoding either FLAG-tagged HCVNS3/4A or FLAG-tagged HCVNS3/4A (S139A). (B) 293T cells (2×10^5 cells/well) were transfected with a plasmid of EV, FLAG-tagged HCVNS3/4A or FLAG-tagged HCVNS3/4A(S139A) in combination with a plasmid of EV, cIRF7 or cIRF7 (C508A) together with 100 ng of the reporter plasmid encoding the luciferase gene under the

control of the IFNα6, IFNβ or ISRE promoter, and luciferase activity was determined at 24 h post-transfection. (C) HCV replicon cells (1.5×10^5 cells/well) and (D) Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with 100 ng of each of the reporter plasmids together with plasmid of EV, cIRF7 or cIRF7(C508A) and luciferase activity was determined at 24 h post-transfection. (E) Huh7 cells, HCV subgenomic replicon cells, and JEV subgenomic replicon cells (1×10^5 cells/well) (top) and Huh7OK1 cells (7.5×10^4 cells/well) infected with JEV and HCV (bottom) at an moi of 0.008, 0.04, 0.2, and 1 and incubated for 24 h and 72 h, respectively, were transfected with 100 ng of each of the reporter plasmids together with cIRF7 and the luciferase activity was determined at 24 h post-transfection. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$) versus the control cells or mock-infected cells.
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replicating HCV. To tightly regulate activation of the molecules in HCV- infected cells, we employed the C-terminal amino acid sequence of human IPS-1, which has been identified as an adaptor molecule involved in the RIG-like receptor (RLR) signaling pathways. It has been demonstrated that HCV NS3/4A protease efficiently cleaves the upstream position of the transmembrane region of IPS-1 on the mitochondrial outer membrane and disrupts the IFN signaling pathway [15–18]. Furthermore, to avoid induction of mitochondrial dysfunction and cell death due to the expression of the therapeutic molecules on the mitochondria, we replaced three arginine residues among the C-terminal five residues of IPS-1 with non-charged amino acid glycine residues (RRRLH to GGGLH) so that these three residues would be localized on the ER membrane [21]. HCV is suggested to replicate on the ER membrane, and therefore subcellular localization and distance of the cleavage site of the substrates from the membrane could be crucial for an efficient processing. The tightly regulated activation of the therapeutic molecules in cells replicating HCV observed in this study might be largely attributable to the ER localization of the therapeutic molecules.

Irrespective of IFN sensitivity, the expression of cIRF7 in the HCV replicon cells induced the activation of type I IFN promoter and inhibited the viral RNA replication, suggesting the possibility that cIRF7 could be used for the treatment of hepatitis C patients who are infected with HCV resistant to IFNα therapy. The expression of IRF3m in cells infected with HCVcc induced a higher antiviral response than that in the Con1 replicon cells in spite of the comparable transcription of IFNβ mRNA between the two cell types Fig. 1), suggesting that differences among HCV genotypes might be caused to the difference to the sensitivity of IFNβ. To assess the real efficacy of cIRF7 for suppression of HCV replication, we must await the establishment of robust cell culture systems capable of propagating various genotypes of HCV derived from the sera of hepatitis C patients.

It has been shown previously that HCV interferes with the induction of type I IFN through the cleavage of IPS-1 by NS3/4A protease [15–18], the interaction of NS5A with MyD88, a major adaptor molecule of TLRs [33], and the intervention of the IFNα-activated Jak-STAT signaling pathway by HCV proteins [7–9]. After cleavage by the HCV protease, the processed cIRF7 migrates into the nucleus and activates various IFN promoters, and it may participate in regulation of the expression of hundreds of ISGs, suggesting that cIRF7 is capable of inducing an antiviral response through the Jak-STAT-independent pathway. Although it has been reported previously that the basal expression of IRF7 and the IRF7-induced activation of the IFNα promoter are impaired in the HCV replicon cells [34], in this study we have shown that cIRF7 is activated in cells infected with HCVcc and capable of inducing type I IFN. Collectively, these results suggest that cIRF7 is capable of eliminating HCV that persistently infects

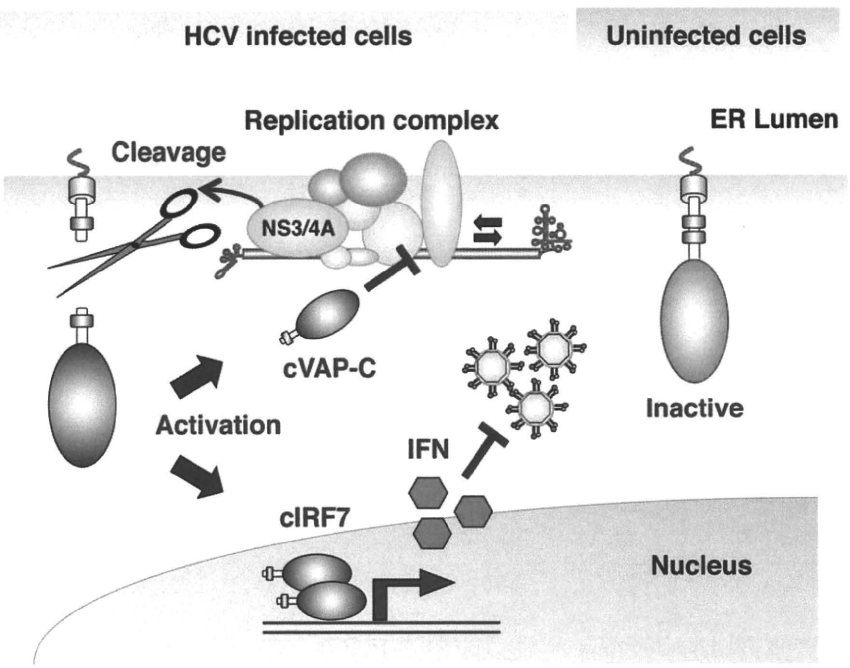

Figure 3. Scheme of activation of the therapeutic molecule in cells infected with HCV. The chimeric molecules are cleaved by HCV NS3/4A protease and the released fragments inhibit propagation of HCV through induction of IFN after translocation into the nucleus (cIRF7) or disruption of the replication complex (cVAP-C), whereas the molecule is stably anchored in the ER within uninfected cells.
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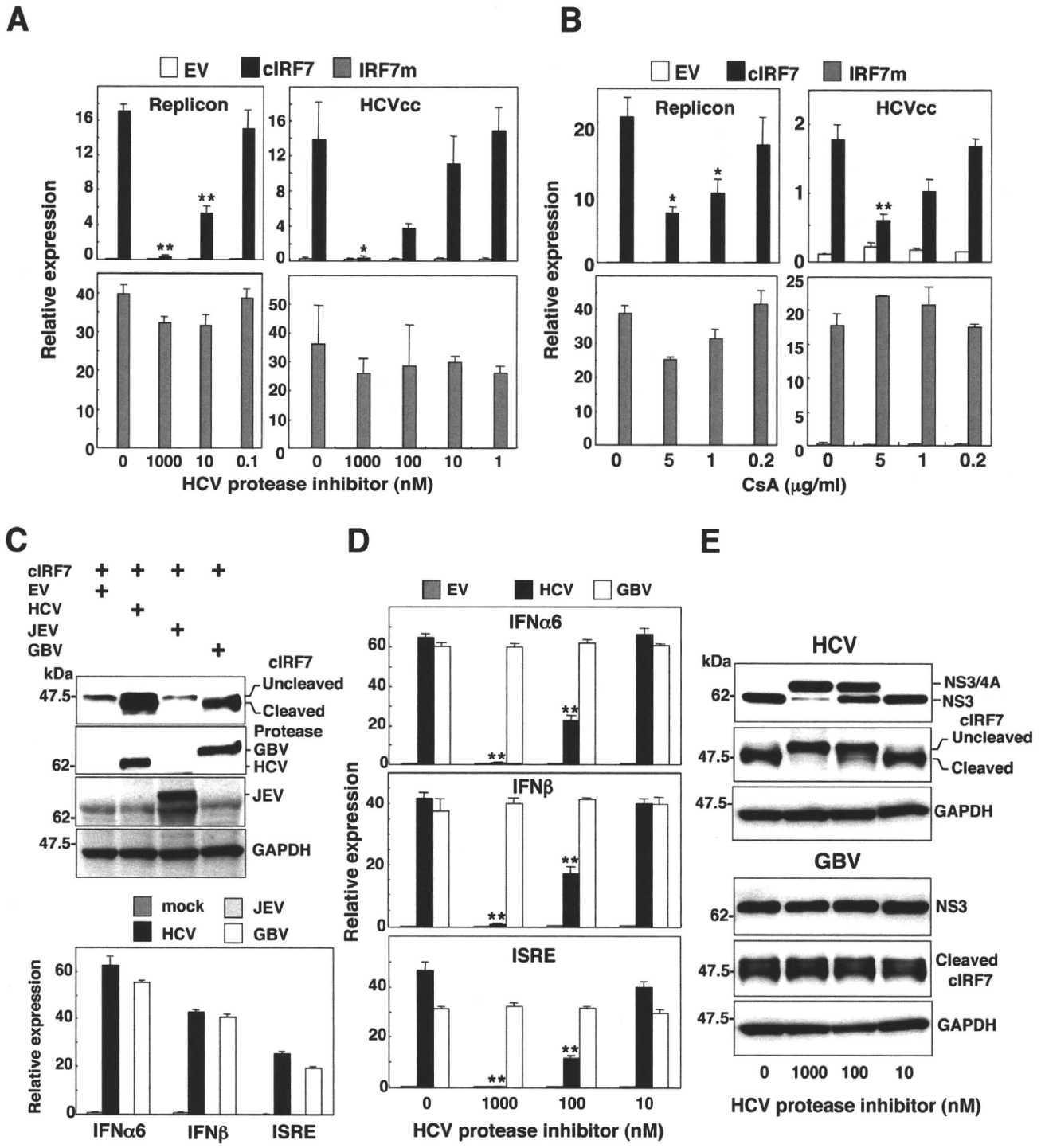

Figure 4. Specificity of activation of the IFN promoters by the expression of cIRF7. (A) HCV replicon cells (1.5×10^5 cells/well) or Huh7OK1 cells (7.5×10^4 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were treated with various concentrations of HCV protease inhibitor (A) or cyclosporine A (CsA) (B), transfected with an empty vector (EV) (white bars) or plasmids encoding cIRF7 (black bars) or IRF7m (gray bars) together with 100 ng of a reporter plasmid encoding the luciferase gene under the control of the ISRE promoter, and luciferase activity was determined at 24 h post-transfection. (C top) A plasmid encoding cIRF7 was co-transfected with a plasmid encoding either FLAG-tagged HCVNS3/4A, FLAG-tagged GBVNS3/4A, or HA-tagged JEVNS2b/3 into 293T cells, and the expressions of cIRF7, viral proteases and GAPDH were determined by immunoblotting. (C bottom) 293T cells (2×10^5 cells/well) transfected with a plasmid encoding either EV (dark gray bars), FLAG-tagged HCVNS3/4A (black bars), FLAG-tagged GBVNS3/4A (white bars), or HA-tagged JEVNS2b/3 (gray bars) together with 100 ng of the plasmid encoding the luciferase gene under the control of the promoter of either IFNα6, IFNβ or ISRE, and luciferase activity was determined at 24 h post-transfection. (D) 293T cells (2×10^5 cells/well) were transfected with 100 ng of the reporter plasmids together with plasmids encoding EV (gray bars), FLAG-tagged HCVNS3/4A (black bars) or FLAG-tagged GBVNS3/4A (white bars) in the presence or absence of the HCV protease inhibitor, and luciferase activity was determined at 24 h post-transfection. (E) cIRF7 was co-expressed with FLAG-tagged HCVNS3/4A or FLAG-tagged GBVNS3/4A in 293T cells in the presence or absence of the HCV protease inhibitor, and the expressions of cIRF7, viral proteases and GAPDH were determined by immunoblotting. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$) versus the control cells or mock-infected cells.
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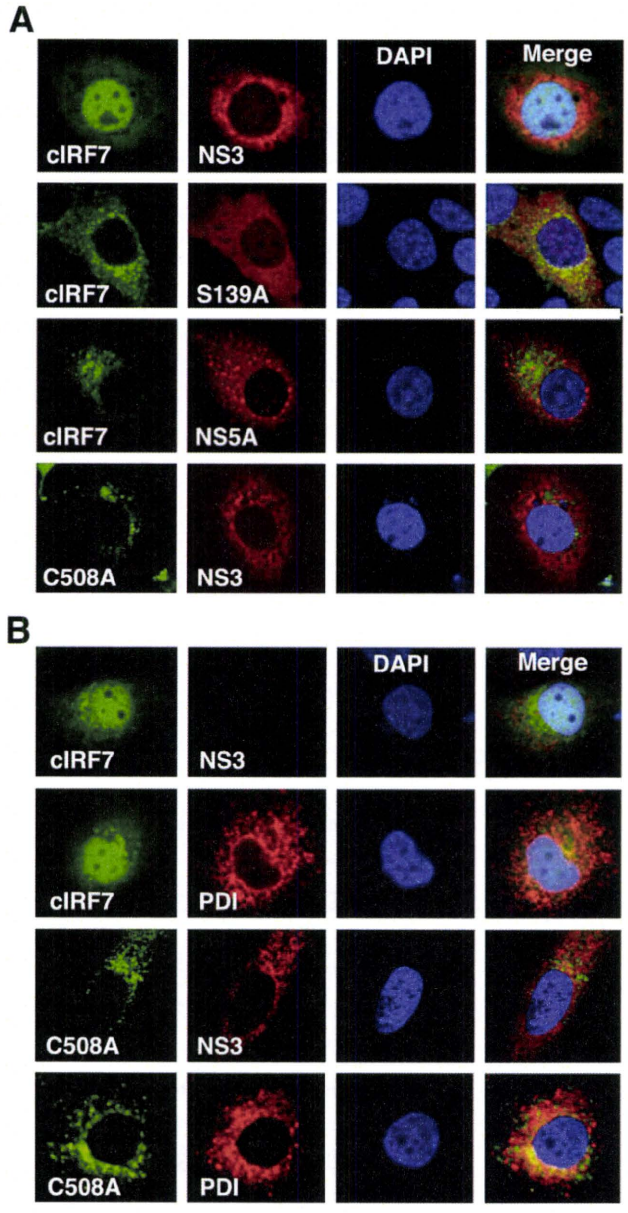

Figure 5. Activation of cIRF7 in cells expressing HCV protease. (A) Huh7OK1 cells (5×10^4 cells/well) were co-transfected with plasmids encoding either EGFP-cIRF7 or EGFP-cIRF7(C508A) and plasmids encoding either HCVNS3/4A, HCVNS3/4A(S139A) or NS5A, harvested at 24 h post-transfection, fixed with 4% paraformaldehyde in PBS, and permeabilized with 0.25% saponin. HCV NS3 and NS5A were stained with the appropriate antibodies, followed by staining with AF594-conjugated second antibodies. (B) HCV replicon cells (5×10^4 cells/well) were transfected with plasmids encoding either EGFP-cIRF7 or EGFP-cIRF7(C508A), and endogenous expression of HCV NS3 and an ER marker, PDI, was detected in cells treated and stained with the appropriate antibodies as described above. Subcellular localization of cIRF7s, HCV proteins and PDI was determined by confocal microscopy after staining of nuclei by DAPI. The data shown in this figure are representative of three independent experiments.
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human hepatocytes through an induction of sufficient amounts of type I IFN.

It is well known that patients achieving a rapid viral clearance by the treatment with PEG-IFNα showed a significant up-regulation of ISG, whereas a high level expression of ISG is observed in nonresponsive patients before IFN therapy, probably due to a rapid induction of negative regulators for the IFN signaling pathway, such as the suppressor of cytokine signaling proteins [35,36]. These results suggest that chronic hepatitis C patients with a pre-activated IFN signaling pathway respond poorly to IFN therapy. In this study we also demonstrated that activation of various IFN promoters by the expression of the dominant active mutants of IRFs was more accentuated in cells replicating HCV rather than naïve cells, probably due to an undetectable expression of ISG in cells replicating HCV RNA as described previously [37]. However, the precise mechanisms underlying the enhancement of IFN activity by the expression of a dominant active mutant of IRFs in cells replicating HCV remain unknown. Fillipowicz *et al.* suggested the possibility of recovery of the sensitivity to IFN therapy by the restoration of the endogenous IFN system to a "naïve" state through a blockage of the IFN response in nonresponders before treatment [36]. However, modulation of ISG expression before IFN therapy may induce a flare of HCV propagation in the liver of chronic hepatitis C patients. Therefore, it might be interesting to examine whether an effectiveness of cIRF7 are sustained in a state of occurring a negative regulator for IFN signaling pathway and preactivated IFN signaling pathway in cells replicating HCV.

VAP-A and VAP-B are suggested to be involved in the construction of the HCV replication complex consisting of viral proteins and host cellular lipid components, and that VAP-C interrupts the VAP-A and VAP-B functions and negatively regulates the HCV propagation and not expressed in human hepatocytes probably involves in the determination of tissue tropism of HCV [20]. Although further studies will be needed to elucidate the effectiveness of the molecules *in vivo* experiment using drug delivery systems including viral and non-viral vectors in more detail, therapeutic molecules consisting of host factors involved in IFN induction such as IRF7 and in the suppression of HCV replication such as VAP-C fused with the IPS-1 sequences specifically cleaved by the HCV protease might be a promising approach capable of eliminating HCV without induction of severe cellular toxicity.

Materials and Methods

Cells and viruses

Vero and 293T cell lines were purchased from American Type Culture Collection (Manassas, VA). Huh7 cell line was kindly provided by Ralf Bartenschlager. Huh7OK1 cell line was previously established from interferone-treated Huh7 cells including HCV replicon and exhibited high susceptibility to HCVcc propagation [38]. These cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum (FCS). Huh-9–13 cells harboring an HCV subgenomic RNA replicon of genotype 1b [39] were cultured in DMEM supplemented with 10% FCS, 1 mg/ml G418 and nonessential amino acids. The infectious RNA of the JFH1 strain was introduced into Huh7OK1 cells and the infectious titers were expressed as focus-forming units (FFU) [4]. Huh7 cells harboring a JEV subgenomic RNA replicon (Nakayama strain) were cultured in DMEM supplemented with 10% FCS and 1 μg/ml puromycin. Preparation of the HCV subgenomic replicon cells 4βR exhibiting an IFN-resistant phenotype and their cured cells 4βRc were described previously [27,28]. All cells were cultured at 37°C in a humidified atmosphere with 5% CO_2.

Plasmids and reagents

The cDNA fragments encoding IRF3 and IRF7 were amplified by PCR from a total RNA from THP-1 cells and cloned into

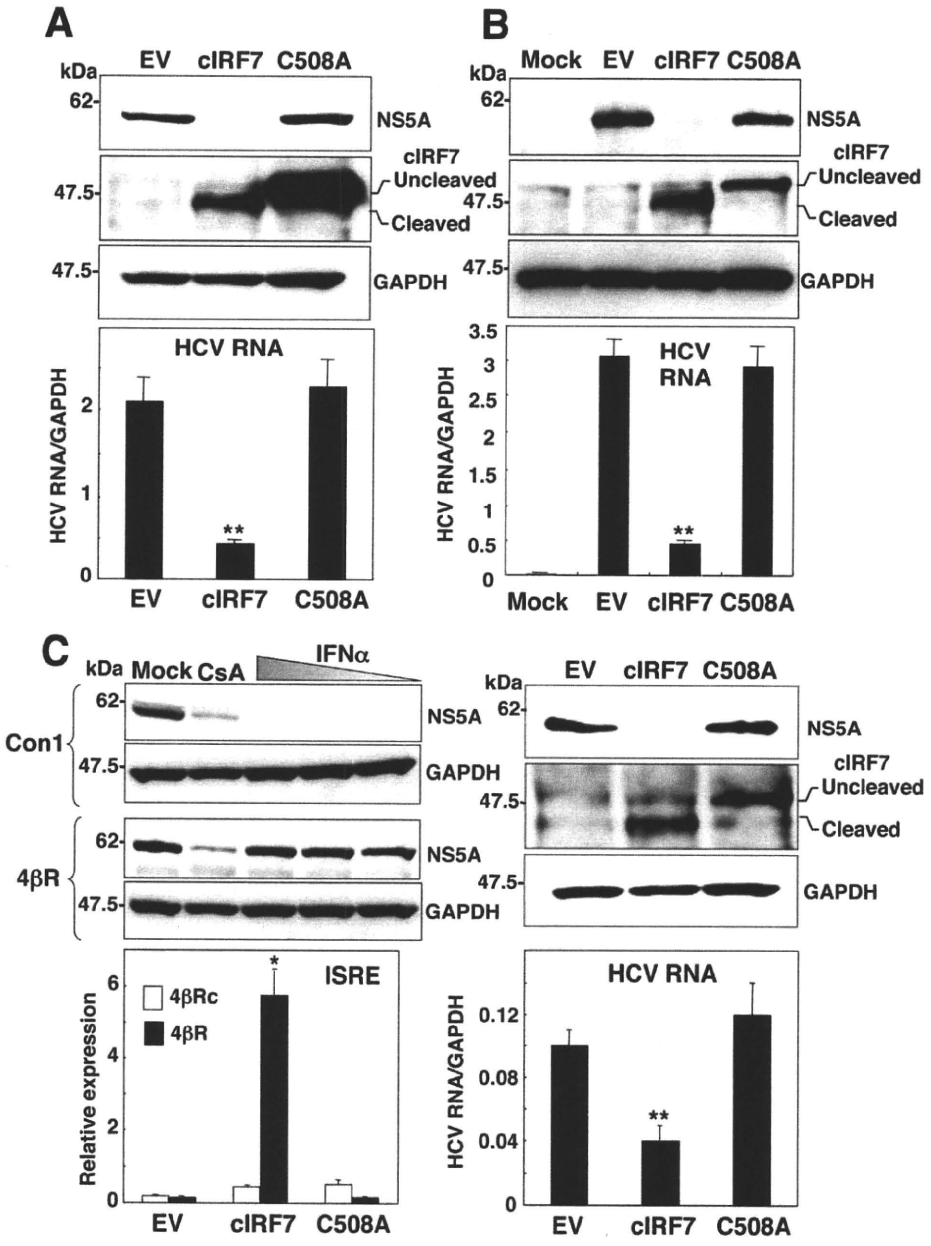

Figure 6. Suppression of HCV replication by the expression of cIRF7. (A) HCV replicon cells (3×10^5 cells/well) and (B) Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with a plasmid encoding either empty vector (EV), cIRF7 or cIRF7(C508A), and the expression of NS5A, cIRF7s and GAPDH (upper panels) and synthesis of viral RNA (lower panels) were determined at 72 h post-transfection by immunoblotting and real-time PCR, respectively. (C upper left) HCV Con1 replicon cells and 4βR replicon cells exhibiting an IFN-resistant phenotype (1.5×10^5 cells/well) were treated with the CsA (5 μg/ml) or 10^4, 10^3, and 10^2 units/ml of recombinant human IFNα and the expressions of NS5A and GAPDH were determined by immunoblotting. The 4βR replicon cells (3×10^5 cells/well) were transfected with EV or plasmid encoding either cIRF7 or cIRF7(C508A), and the expressions of NS5A, cIRF7s and GAPDH (C upper right) and synthesis of viral RNA (C lower right) were determined at 72 h post-transfection by immunoblotting and real-time PCR, respectively. The 4βR cells and their cured cells (4βRc) with the HCV genome eliminated (1×10^5 cells/well) were transfected with EV or plasmid encoding either cIRF7 or cIRF7(C508A) together with 100 ng of plasmid encoding the luciferase gene under the control of the ISRE promoter, and luciferase activity was determined at 24 h post-transfection (C lower left). The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$) versus the control cells or mock-infected cells.
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pcDNA3.1-C-myc-His (Invitrogen, Carlsbad, CA). The mutants carrying a deletion in the auto-inhibitory domain (from amino acid residue 284 to 454) of IRF7 and the substitution of Ser^{396} with phosphomimetic Asp located in the carboxyl terminus of IRF3 were generated by the method of splicing by overlap extension and cloning into pcDNA3.1myc-His and designated as IRF7m and IRF3m, respectively. N-terminally FLAG-tagged wild-type NS3/4A protease and its mutant substituted with Ser^{139} to replaced with Ala (S139A) were prepared as described previously [33]. The cDNA fragment encoding a JEV protease was amplified from a total RNA of Vero cells infected with JEV (AT31 strain) and cloned into pcDNA3.1Flag/HA [40], The cDNA fragment

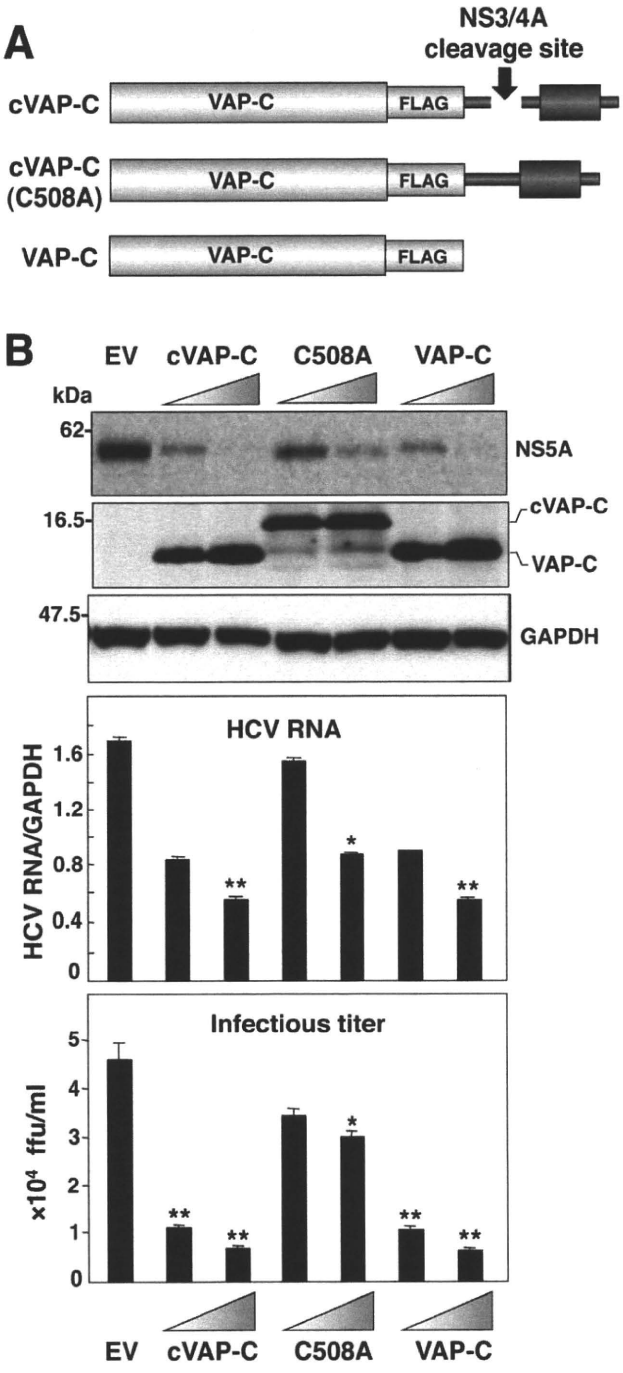

Figure 7. Suppression of HCV replication by the expression of cVAP-C. (A) Schematic representation of cVAP-C, cVAP-C(C508A) and VAP-C. Like cIRF7, cVAP-C is composed of the sequences of VAP-C, FLAG-tag, and the C-terminus domain of IPS-1. (B) Huh7OK1 cells (1.5×10^5 cells/well) infected with HCVcc at an moi of 1 and incubated for 72 h were transfected with EV, or plasmid encoding either cIRF7 or cIRF7(C508A), and the expressions of NS5A, VAP-Cs and GAPDH (top panel), synthesis of viral RNA (middle panel) and infectious titers in the culture supernatants were determined at 72 h post-transfection by immunoblotting, real-time PCR, and focus forming assay, respectively. The data shown in this figure are representative of three independent experiments. The error bars represent the standard deviations. Asterisks indicate significant differences (*$P<0.05$, **$P<0.01$) versus the control cells or mock-infected cells.
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encoding a GBV-B protease was amplified from pGBB (kindly provided by Dr. H. Akari) [41] by PCR and cloned into pcDNA3.1Flag/HA. The chimeric IRF7 (cIRF7) composed of the IRF7m fused with FLAG-tag and the C-terminus of human IPS-1 (from amino acid residues 503 to 540 amino acid residues) containing a cleavage site of HCV NS3/4A, transmembrane domain and the ER retention signal [21] (Fig. 2A) was cloned into pcDNA3.1-c-myc-His. A cIRF7 mutant, C508A, was generated to be resistant to HCV NS3/4A protease by substitution of Cys^{508} of cIRF7 to Ala. The reporter constructs of IFNα6, IFNβ, and ISRE were kindly provided by Drs. T. Kawai and S. Akira. All PCR products were confirmed by sequencing by an ABI PRISM 310 genetic analyzer (Applied Biosystems, Tokyo, Japan). The HCV NS3/4A protease inhibitor, BILN2061 was purchased from Acme Bioscience (Belmont, CA). Human recombinant IFNα and cyclosporine A (CsA) were purchased from PBL Biomedical Laboratories (New Brunswick, NJ) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

Reporter assay

Huh7 cells, HCV replicon cells, and Huh7OK1 cells infected with HCVcc were seeded onto 12-well plates at the concentration of 1.5×10^5 cells/well and transfected with 100 ng of each of the plasmids encoding the luciferase gene under the control of the IFNα6, IFNβ and ISRE promoter together with the various constructs by using FuGene™6 (Roche Molecular Biochemicals, Mannheim, Germany). Luciferase activity was determined by the Dual-luciferase reporter assay system (Promega Inc., Madison, WI) and the *Renilla* luciferase reporter gene was simultaneously transfected as an internal control.

Immunoblotting

HCV replicon cells and Huh7OK1 cells infected with HCVcc were transfected with the plasmids encoding each of the wild-type and the dominant active mutants of IRFs and harvested at 72 h post-transfection. Cells were washed three times with ice-cold phosphate-buffered saline (PBS), suspended in lysis buffer containing 20 mM Tris-HCl (pH 7.4), 135 mM NaCl, 1% Triton X-100, 10% glycerol and protease inhibitor cocktail tablets (Roche Molecular Biochemicals) and centrifuged at 14,000×g for 15 min at 4°C after incubation for 30 min at 4°C. Cell lysates were subjected to sodium dodecyl sulfate-12.5% polyacrylamide gel electrophoresis (SDS-PAGE) after boiling in sample buffer and transferred to polyvinylidene difluoride membranes (Millipore, Tokyo, Japan). The membranes were blocked with PBS containing 0.05% Tween 20 and 5% skim milk at room temperature for 1 h, incubated with mouse monoclonal anti-FLAG M2 (Sigma), anti-hemagglutinin (HA) 16B12 (HA.11; BabCO, Richmond, CA), anti-NS5A mouse monoclonal antibody (Austral Biologicals, San Ramon, CA), anti-GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-hexahistidine monoclonal antibody (Santa Cruz) at room temperature for 1 h, and then with horseradish peroxidase-conjugated anti-mouse IgG or anti-rabbit IgG antibody at room temperature for 1 h. The immune complexes were visualized with Super Signal West Femto substrate (Pierce, Rockford, IL) and detected by an LAS-3000 image analyzer system (Fujifilm, Tokyo, Japan).

Quantitative reverse-transcription polymerase chain reaction (qRT-PCR)

A total RNA was prepared from HCV replicon cells and Huh7OK1 cells infected with HCVcc transfected with the plasmids encoding each of the IRF constructs using an RNeasy

mini kit (QIAGEN, Valencia, CA) and first-strand cDNA was synthesized by using ReverTra Ace (TOYOBO, Osaka, Japan) and oligo $(dT)_{20}$ primer. The expression of each cDNA was estimated by Platinum SYBR Green qPCR SuperMix UDG (Invitrogen) according to the manufacturer's protocol. Fluorescent signals were analyzed by an ABI PRISM 7000 (Applied Biosystems). The HCV and GAPDH genes were amplified using the primer pairs of 5′-GAGTGTCGTGCAGCCTCCA-3′ and 5′-CACTCGCAAGCACCCTATCA-3′, and 5′-ACCACAGTCCATGCCATCAC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, respectively. The expression of each of mRNA was normalized with that of GAPDH.

Subcellular localization of cIRF7 in HCV- replicating cells

Cells transfected with the plasmids were harvested at 24 h post transfection, washed twice with PBS, fixed with PBS containing 4% paraformaldehyde, and permeabilized by incubation with PBS containing 0.25% saponin for 10 min. Cells were incubated for 1 h at 4°C with 1μg/ml of anti-NS3 (251) mouse monoclonal antibody (Santa Cruz), anti-NS5A mouse monoclonal antibody (Austral Biologicals), or mouse monoclonal antibody to protein disulfide isomerase (PDI) (Affinity Bioreagents, Golden, CO) in PBS containing 10% FCS (PBSF), and then incubated at room temperature for 1 h with 0.5 μg/ml of Alexa Flour 594-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) after three time washes with PBSF. Cell nuclei were stained with 4′, 6-diamidino-2-phenylindole (DAPI). After an extensive wash with PBSF, the samples were examined with a Fluoview FV1000 laser scanning confocal microscope (OLYMPUS, Tokyo, Japan).

Statistical analysis. Results were expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t* test.

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Author Contributions

Conceived and designed the experiments: TA Y. Matsuura. Performed the experiments: XW TA HK ST Y. Mori HT KM. Analyzed the data: NK TS MT. Contributed reagents/materials/analysis tools: NK MT. Wrote the paper: TA Y. Matsuura.

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Analysis of 5′ Nontranslated Region of Hepatitis A Viral RNA Genotype I from South Korea: Comparison with Disease Severities

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Abstract

The aim of the study was to analyze genotype I hepatitis A virus (HAV) 5′ nontranslated region (NTR) sequences from a recent outbreak in South Korea and compare them with reported sequences from Japan. We collected a total of 54 acute hepatitis A patients' sera from HAV genotype I [27 severe disease (prothrombin time INR≥1.50) and 27 mild hepatitis (prothrombin time INR <1.00)], performed nested RT-PCR of 5′ NTR of HAV directly sequenced from PCR products (~300 bp), and compared them with each other. We could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)], all of which were subgenotype IA. Sequence analysis revealed that sequences of severe disease had 93.6%–99.0% homology and of self-limited disease 94.3%–98.6% homology, compared to subgenotype IA HAV GBM wild-type IA sequence. In this study, confirmation of the 5′NTR sequence differences between severe disease and mild disease was not carried out. Comparison with Japanese HAV A10 revealed ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively, although the nucleotide sequences in this study showed high homology (93.6%–100%). In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to Japanese sequences previously reported from Japan, and this region would be considered one of the antiviral targets. Further studies will be needed.

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9 These authors contributed equally to this work.

Introduction

Although hepatitis A vaccination is highly effective, providing herd protection and decreasing mortality and morbidity related to the hepatitis A virus (HAV) [1–3], HAV is still a common cause of hepatitis reportedly leading to occasional lethal acute liver failure in many countries of the world [4–7]. Recently, a rise in the frequency of hepatitis A outbreaks was observed in South Korea, which lies adjacent to Japan, while the number of adult hepatitis A cases in Japan has been progressively decreasing during the last several years. There is a concern regarding a possible HAV epidemic in Japan in the near future, as universal vaccination against hepatitis A is not performed in this country.

HAV is a member of the genus *Hepatovirus* in the *Picornaviridae* family, and has a positive-sense single-stranded RNA genome approximately 7.5 kb in length [8]. The genome codes a large open reading frame (ORF), which is flanked by 5′ nontranslated region (5′NTR) and 3′NTR. The downstream part of 5′NTR represents the internal ribosomal entry site (IRES), which mediates cap-independent translation initiation and is important for HAV replication [9,10]. 5′NTR of HAV is also known as one of the most highly conserved in the HAV genome sequences, making this region one of the likely candidates for antiviral targets [9,11]. It was reported that nucleotide variations in the central portion of 5′NTR of HAV may influence the severity of hepatitis A [12].

Human HAV strains can be grouped into four genotypes (I, II, III and IV) and unique simian strains belong to three additional genotypes (IV, V and VI). Between each of these genotypes, the nucleotide sequence varies by 15–20% of the base positions in the P1 region [13]. Genotype I is the most abundant type worldwide, and genotype IA in particular has been reported from North America, Korea, China, Japan and Thailand [14].

The aim of this study is to characterize the recent HAV genotype I 5′NTR sequences in Korea, to compare them with those reported from Japan and to clarify this region as a target candidate for anti-HAV drugs.

Materials and Methods

Patients

Fifty-four patients infected with HAV subgenotypes IA and IB were included in this study. Serum samples were collected at four

hospitals located in the Seongnam city area, near Seoul, South Korea. Our study was approved by the Seoul National University Bundang Hospital Institutional Review Board (IRB), and we obtained written informed consent from every patient enrolled during Sep 2008 to Aug 2008. We collected serum or plasma samples immediately after hospital admission, and they were stored at −70°C. The 54 patients comprised 27 with severe disease, defined as prolonged prothrombin time [international normalized ratio (INR) > or = 1.5] and 27 with mild disease: self-limited acute hepatitis in this study (Table S1A & S1B).

Primers for PCR and Direct Sequencing

For amplification of HAV sequences and bidirectional direct sequencing of the amplified segments, we prepared several primers for PCR and sequencing as previously described [12]. These primers were prepared with the sequence reported by Cohen et al [8].

Detection of Hepatitis A Virus RNA in Serum

RNA was extracted from sera using the acid guanidinium-phenol-chloroform method. Reverse transcription was performed with HAV genome specific antisense primer (5′-AGTACCTCA-GAGGCAAACAC-3′) as previously described [12].

In the first round PCR, 1 µl of 20 µl of the cDNA solution was used. The first round PCR was performed with 50 µl of reaction mixture containing 25 pmol of outer antisense primer (5′-AGTACCTCAGAGGCAAACAC-3′) and sense primer (5′-TCTTGGAAGTCCATGGTGAG-3′), 200 µM of each dNTP, 50 mM KCl, 10 mM Tris HCL (pH 8.3), 1.5 mM $MgCl_2$, 0.001% gelatin, and 2.5 units of Ex Taq polymerase (Takara Bio Inc., Ohtsu, Shiga, Japan). Amplification conditions consisted of 35 cycles of 95°C for one minute, 50°C for one minute, and 72°C for one minute, and 1 µl of the first round product was used for the second round of PCR with the same PCR mixture, except 1.0 µM of inner sense primer (5′-GGGACTTGATACCT-CACCGC-3′) and antisense primer (5′-CCACATAAGGCCC-CAAAGAA-3′) were used. Amplification conditions for the second round were the same as those for the first round. The second-round PCR products (6 µl) were analyzed by 8% polyacrylamide gel electrophoresis, stained with SyBr green (Takara), and visualized by UV transillumination. In all experiments, the negative samples showed negative results for HAV RNA. HAV genotypes were determined by previously described methods based on the VP1-P2A region [14].

Direct Sequencing of HAV cDNA Fragments

To prepare the sequence template (nucleotides 75-638 of 5′NTR of HAV), PCR products were treated with ExoSAP-ITR (Affymetrix, Inc., Santa Clara, CA), and then sequenced using a BigDye(R) Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Tokyo, Japan). Sequences were analyzed using Applied Biosystems 3730xl (Life Technologies).

Nucleotide Sequence Accession Numbers

The nucleotide sequence data reported in this article will appear in GenBank nucleotide sequence databases with accession numbers AB571027 to AB571045.

Phylogenetic Analysis

To examine the heterogeneity of the viral sequences obtained, a phylogenetic tree was constructed using the neighbor joining methods. To confirm the reliability of the phylogenetic tree, bootstrap resampling tests were performed 10,000 times. These analyses were conducted with the Genetyx-WIN program, version 10 (Software Development, Tokyo, Japan).

Statistical analysis

Differences in proportions among the groups were compared by Fisher's exact probability test, Student's t test and Welch's t test.

Results

Clinical Features of Patients with Acute Hepatitis A Genotype 1 in Korea

Characteristics of these patients at admission are summarized in Table S1. There were no differences in age and gender ratio between the severe and mild disease groups. Mean age of the severe and mild disease groups was 32.1±6.1 and 32.6±5.8 years, respectively. Male gender was dominant in both groups (male/female: 19/8 and 18/9 in the severe and mild disease groups, respectively). Almost all patients of both groups were subgenotype IA, with only two and one being subgenotype IB in the severe and mild disease groups, respectively.

Sequence Analysis of Korean Isolates

Although the VP1/2A region could be detected in the same serum or stool samples of the same patients, we could detect HAV 5′NTR sequences in 19 of the 54 (35.1%) cases [12 of 27 severe cases (44.4%) and 7 of 27 self-limited cases (25.9%)] by reverse-transcription-nested PCR. All these sequences were subgenotype IA. Then we performed further sequence analysis in these 19 patients by the methods of Fujiwara et al [12]. Japanese studies showed that fewer nucleotide variations were found between nucleotides 200 and 500 of 5′NTR in cases of fulminant hepatitis and severe acute hepatitis than in cases of self-limited acute hepatitis [12]. We thusly performed sequence analysis of the region between nucleotides 200 and 500.

Sequences between nucleotides 200 and 500 were then compared with the wild-type HAV GBM/WT RNA (X75215) [15]. The nucleotide sequence identities of 5′NTR from severe and mild cases ranged from 93.6% to 99.0% and from 94.3% to 98.6%, respectively, compared with wild-type HAV GBM sequence. The distribution of nucleotide variations is shown in Table S2A & S2B. Sequences from cases of severe and mild diseases were mostly similar. Although there was no statistical significance, ^{214}C, ^{220}T and ^{464}T were found in one case each of the mild disease group (Table S2B). On the other hand, ^{227}deletion of nucleotide and ^{382}A, respectively, were found in two and one cases of the severe disease group (Table S2A). The number of nucleotide substitutions is shown in Figure 1A & 1B. The average number of substitutions between nucleotides 200 and 500 was 10.8 (6.8) [mean (SD)] per case in severe disease and 6.8 (4.5) in mild disease. Differences between severe and mild cases were not statistically significant. We could not construct a phylogenetic tree using these sequences (data not shown).

Comparison to Japanese HAV Sequences Reported from 1984 to 1999

5′NTR of HAV possesses a secondary structure including stems and loops, functions as an IRES, and plays an important role in translation and replication of this virus [9,16]. There are six domains in IRES, which is located between nucleotides 151 and 734. Portions of domains III and IV are present between nucleotides 200 and 500. Domain III is located between nucleotides 99 and 323, and domain IV is located between nucleotides 324 and 586. The region between nucleotides 203 and

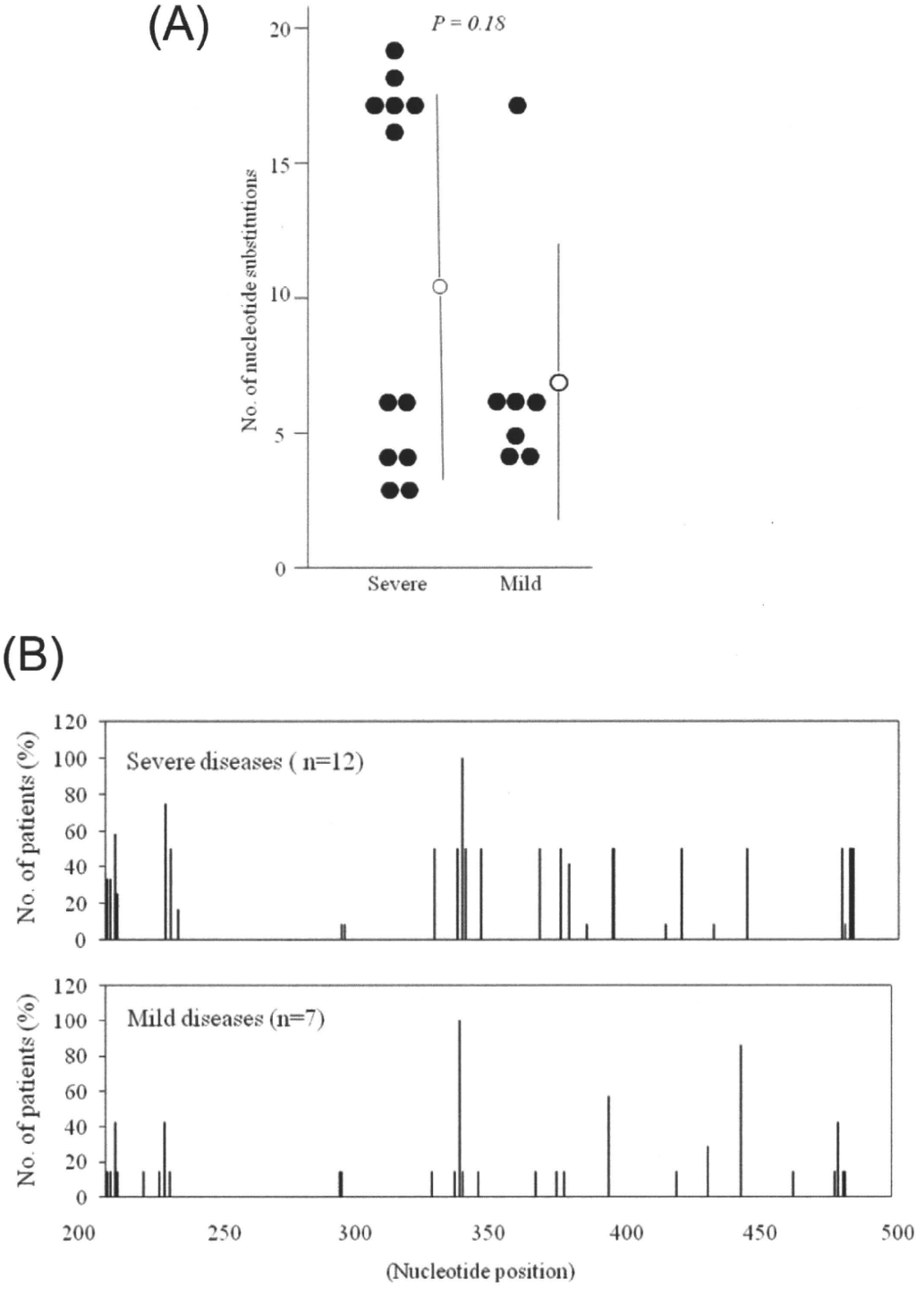

Figure 1. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV GBM. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500. Nucleotide sequences were compared with HAV GBM/WT RNA (X75215) [15]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
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250 is particularly pyrimidine-rich. To examine the homology with the HAV sequences from Japan reported by Fujiwara et al. [12], we compared the sequences from nucleotides 200 to 500 with A10 (AB045328) from Japan [12]. The nucleotide sequence identities of 5′NTR from severe and mild disease groups ranged from 94.3% to 99.6% and from 93.6% to 100%, respectively, compared with the HAV A10 sequence [12] (Table S3A & S3B). In the Korean group, we found ^{222}C to G or T substitution in 8/12 cases of severe disease and ^{222}C to G or T and ^{392}G to A substitutions in 5/7 and 4/7 cases of mild disease, respectively.

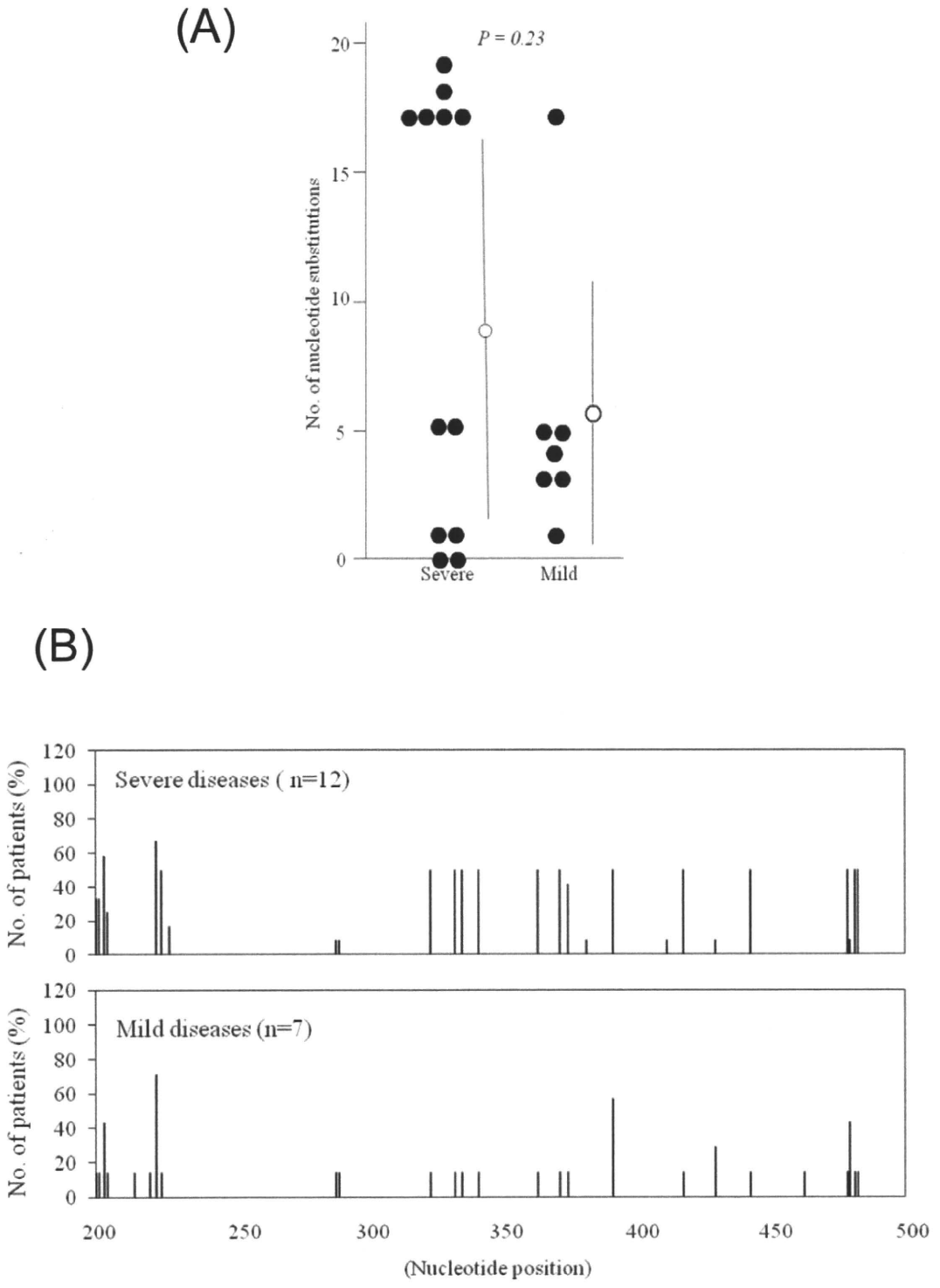

Figure 2. Disease severity and nucleotide substitutions in HAV IRES when compared with HAV A10. (**A**) Number of nucleotide substitutions between nucleotides 200 and 500 Nucleotide sequences were compared with A10 (AB045328) from Japan [12]. Bars represent mean (SD). Severe, severe disease; Mild, mild disease. (**B**) Distribution of nucleotide substitutions between nucleotides 200 and 500 of the 5′ non-translated region. Bars indicate the percentage of cases with substitutions at each nucleotide position.
doi:10.1371/journal.pone.0015139.g002

The number of nucleotide substitutions is shown in Figure 2A & 2B, with the average number between nucleotides 200 and 500 being 9.7 (8.2) [mean (SD)] per case in severe disease and 5.4 (5.2) in mild disease. Again, differences between severe and mild cases were not statistically significant.

Discussion

The number of adult hepatitis A cases has been progressively increasing during the last several years in Korea [6,14]. In Japan, on the other hand, the number of patients with sporadic type A

hepatitis has recently been on the decrease. In the 9 years from 1999 inclusive, 763, 381, 491, 502, 303, 139, 170, 320 and 157 hepatitis A cases were reported to the Infectious Disease Surveillance Center, National Institute of Infectious Diseases, Tokyo, Japan (www.nih.go.jp). Japan lies adjacent to Korea, separated by the Sea of Japan. The two countries have some cultural similarities. In Japan, there is no universal vaccination program against hepatitis A and hepatitis B. These circumstances have raised concerns about a possible HAV epidemic in Japan. We then analyzed HAV genome sequences from Korea and compared them with the reported sequences from Japan over the past several years.

In the present study, as most of the HAV strains belonged to subgenotype IA in Korea [14], we chose only genotype I patients for analysis. Among 54 HAV IgM positive sera, 35.1% (n = 19) were positive for HAV RNA by nested RT-PCR for 5′NTR. All these strains belonged to subgenotype IA. We tried to perform phylogenetic tree analysis, but these 19 strains formed a single cluster to which almost all Japanese sequences reported by Fujiwara et al [12] belonged (data not shown). Fujiwara et al [12] found an association between the severity of hepatitis A and nucleotide variations in 5′NTR of Japanese HAV RNA. In the present study, we did not confirm 5′NTR sequence differences between severe disease and mild disease.

The age of HAV sequence-analyzed patients in the present study was 30.5±5.9 and 31.4±5.0 years, respectively, in severe and mild diseases. The gender of HAV sequence-analyzed patients was male-dominant (male/female: 8/4 and 6/1 in the severe disease and mild disease groups, respectively). In the study by Fujiwara et al [12], the patients were also male-dominant, but their age with fulminant hepatitis and severe acute hepatitis (43.1±14.4 year, $P = 0.010$ and 41.6±12.6, $P = 0.010$, respectively) was significantly higher than the age of severe-disease patients. On the other hand, the age of their patients with self-limited acute hepatitis was similar to that of our mild-disease patients. We defined patients with prothrombin time INR ≥1.50 as severe hepatitis in this study, whereas Fujiwara et al [12] defined patients with prothrombin time of less than 40% as severe hepatitis with (fulminant hepatitis) or without encephalopathy (severe acute hepatitis).

In Japan, similar to the situation in Korea [6], young adults seem not to have protective antibody against HAV, and so it appears that hepatitis A cases can be expected to increase in the near future.

A previous study showed that the 5′ border of IRES is located between nucleotides 151 and 257, while the 3′ border extends to the 3′ end of 5′NTR, between nucleotide 695 and the first initiation codon at 735 [17]. ^{222}C to G or T substitution was located on the loop structure at domain IIIa of HAV IRES. A previous Japanese study showed that nucleotide 225 substitutions occurred in 80% of the sequences around nucleotide position 222 [12]. ^{392}G to A substitution located at domain IV of HAV IRES was observed in 64.2% (9/14) of the Korean HAV sequences. Fujiwara et al [12] also reported that substitutions at nucleotide 391 were seen in 32% of Japanese HAV patients. It is possible that these substitutions were non-specific mutations.

In conclusion, HAV 5′NTR subgenotype IA from Korea had relatively high homology to the Japanese sequences previously reported, and this region may represent a viable antiviral target. In Japan, as in Korea, the introduction of childhood vaccination and catch-up vaccination for adolescents and young adults should be considered.

Supporting Information

Table S1 Patient Characteristics. (A) Severe disease, (B) Mild disease.
(DOC)

Table S2 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for HAV GBM/WT RNA (X75215) [15] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

Table S3 Comparison of the nucleotide sequences of the HAV 5′ non-translated region with GBM. (A) Severe disease, (B) Mild disease. The consensus sequence for A10 (AB045328) from Japan [12] is shown on the top. Dots indicate conserved nucleotides; differences are shown by the appropriate single letter nucleotide. -, deletion mutant.
(DOC)

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We dedicate this work in the memory of the late Dr. Koji Yano, Clinical Research Center, NHO Nagasaki Medical Center, Omura, Nagasaki, Japan.

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Author Contributions

Conceived and designed the experiments: TK SHJ FI OY. Performed the experiments: TK SHJ. Analyzed the data: TK SHJ KF. Contributed reagents/materials/analysis tools: TK SHJ FI KF OY. Wrote the paper: TK SHJ KF. Collected the samples: SHJ.

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Quantification of hepatitis C virus in patients treated with peginterferon-alfa 2a plus ribavirin treatment by COBAS TaqMan HCV test

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SUMMARY. Extremely low levels of serum hepatitis C virus (HCV) RNA can be detected by COBAS TaqMan HCV test. To investigate whether the COBAS TaqMan HCV test is useful for measuring rapid virological response (RVR) and early virological response (EVR) to predict sustained virological response (SVR), we compared the virological response to PEG-IFN-alfa 2a plus RBV in 76 patients infected with HCV genotype 1 when undetectable HCV RNA by the COBAS TaqMan HCV test was used, with those when below 1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test was used, which corresponded to the use of traditional methods. Among the 76 patients, 28 (36.8%) had SVR, 13 (17.1%) relapsed, 19 (25.0%) did not respond, and 16 (21.0%) discontinued the treatment due to side effects. The positive predictive values for SVR based on undetectable HCV RNA by COBAS TaqMan HCV test at 24 weeks after the end of treatment [10/10 (100%) at week 4, 21/23 (91.3%) at week 8 and 26/33 (78.7%) at week 12] were superior to those based on <1.7 log IU/mL HCV RNA [17/19 (89.4%) at week 4, 27/38 (71.0%) at week 8, and 27/43 (62.7%) at week 12]. The negative predictive values for SVR based on <1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test [46/57 (80.7%) at week 4, 37/38 (97.3%) at week 8, and 32/33 (96.9%) at week 12] were superior to those based on undetectable HCV RNA [48/66 (72.7%) at week 4, 46/53 (86.7%) at week 8, and 41/43 (95.3%) at week 12]. The utilization of both undetectable RNA and <1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test is useful and could predict SVR and non-SVR patients with greater accuracy.

Keywords: antiviral treatment, chronic hepatitis C, TaqMan PCR, virological response.

INTRODUCTION

Hepatitis C virus (HCV) infection is one of the major causes of chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (HCC) [1]. The increasing number of referrals for liver transplantation reflects the impact of chronic HCV infection as a cause of end-stage liver disease [2]. The current approved therapies for chronic hepatitis C are standard interferon (IFN) and the combination of PEG-IFN-alfa 2a or 2b with or without ribavirin (RBV) therapy. This therapy leads to ~50% sustained virological response (SVR), but non-SVRs persist especially in patients infected with HCV genotype 1 and high viral load [3,4].

The quantitation of serum levels of HCV RNA in chronic hepatitis C has been regarded as providing one of the most important indicators for the outcome of IFN-based therapy because SVR can be expected in patients with a low virus load [5,6]. A rapid virological response (RVR), defined as undetectable HCV RNA at week 4 of treatment, predicts a high likelihood of achieving SVR [7]. Early virological response (EVR), in which HCV RNA disappears [complete EVR (cEVR)], or shows 2-log-reduction at 12 weeks [partial

Abbreviations: EVR, early virological response; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; RVR, rapid virological response; SVR, sustained virological response.

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EVR (pEVR)], is the most accurate predictor of not achieving SVR [7–9]. However, to determine whether the patient's treatment duration could be shortened, RVR is more important than EVR for predicting SVR, and patients with RVR have a good chance of achieving SVR and thus may not need newer antiviral therapy [4].

The COBAS TaqMan HCV test (TaqMan HCV; Roche Molecular Systems, Inc., Branchburg, NJ, USA) is a real-time nucleic acid amplification assay for the qualitative and quantitative detection of HCV RNA in human serum or plasma [10]. Sensitive, accurate detection and quantification of HCV RNA is essential for the diagnosis and management of chronic HCV infection. In the present study, we evaluated HCV RNA in patients with HCV genotype 1 undergoing treatment by COBAS TaqMan HCV test. We compared the proportion of undetectable HCV RNA with that below 1.7 log IU/mL HCV RNA by COBAS TaqMan HCV test, the latter corresponding to that assessed as undetectable by traditional methods such as COBAS AMPLICOR HCV Monitor test, v.2.0, to investigate the differences of detection sensitivity between COBAS TaqMan HCV test and older tests.

MATERIALS AND METHODS

Patients

Patients were recruited from Chiba University Hospital and 28 hospitals in Chiba, Ibaraki, and Saitama Prefectures between March 2008 and March 2010. Patients were eligible if they met the following inclusion criteria: (i) infected with HCV genotype 1 alone, (ii) age ≥20 years, (iii) diagnosed as chronic hepatitis C, (iv) negative for HBs antigen, (v) negative for human immunodeficiency viral test, (vi) no high titres of auto-antibodies, (vii) no severe renal disease, (viii) no severe heart disease, (ix) no mental disorders, (x) no current intravenous drug abuse, and (xi) no pregnancy.

Study design

Seventy-six consecutive patients were enrolled in this study. Informed consent was obtained from all patients prior to enrolment. The Ethics Committee of Chiba University School of Medicine approved the study protocol. In this study, 180 μg of PEG-IFN-alfa 2a per week plus 400–1 200 mg RBV/day were usually given in the treatment of patients for as long as 48 weeks. Clinical and laboratory assessments were performed at least every 4 weeks during treatment and the 12-week follow-up period. Adverse reactions were noted by oral inquiry (patient interview), physical examinations and laboratory tests.

Measurement of HCV RNA in serum

HCV RNA was measured by COBAS TaqMan HCV test (Roche Diagnostics, Tokyo, Japan), with levels ranging from 1.2 to 7.8 log IU/mL. Comparing this with traditional methods such as COBAS AMPLICOR HCV Monitor Test v. 2.0 (range: 0.5–850 kIU/mL, lower limit of detection: 1.7 log IU/mL), when amplified signals were detected at <1.7 log IU/mL, we judged HCV RNA as <1.7 log IU/mL because HCV RNA levels <1.7 log IU/mL are considered as undetectable by COBAS AMPLICOR HCV Monitor Test v. 2.0 [5,11].

Measurement of serum alanine aminotransferase levels, other liver function tests, and haematologic tests

Serum alanine aminotransferase (ALT) measurement and other liver function tests were carried out by standard methods every 4 weeks before, during the treatment, and for at least 12 weeks after the end of treatment.

Definition of treatment response

SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Patients with undetectable HCV RNA within the initial 4 weeks of treatment were considered to have had RVR. Patients who had undetectable HCV RNA within the initial 12 weeks of treatment were considered to have had complete EVR (cEVR) (described as EVR in this article).

Statistical analysis

Data are expressed as mean ± standard deviation (SD). Differences were evaluated by Student's *t*-test, chi-square test, or Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The characteristics of the patients at baseline, including age, gender, ALT, γ-GTP, LDL-C, AFP, HCV RNA levels, and history of previous interferon treatment are given in Table 1. Of 76 patients enrolled, 46 were treatment-naïve and 30 had a history of IFN therapy with or without RBV (Table 1). In the 30 patients previously treated, 3 received PEG-IFN monotherapy, 4 standard IFN monotherapy, 2 standard IFN plus RBV, 16 PEG-IFN-alfa 2b plus RBV, 1 PEG-IFN-alfa 2a plus RBV, and 4 with details unknown.

Virological response

Among the 76 patients, 28 (36.8%) had SVR, 13 (17.1%) relapsed, 19 (25.0%) did not respond, and 16 (21.0%) discontinued treatment due to side effects. In the 46 treatment-naïve patients, 21 (45.6%) had SVR, 10 (21.7%) relapsed, 3 (6.5%) did not respond, and 12 (26.0%) discontinued