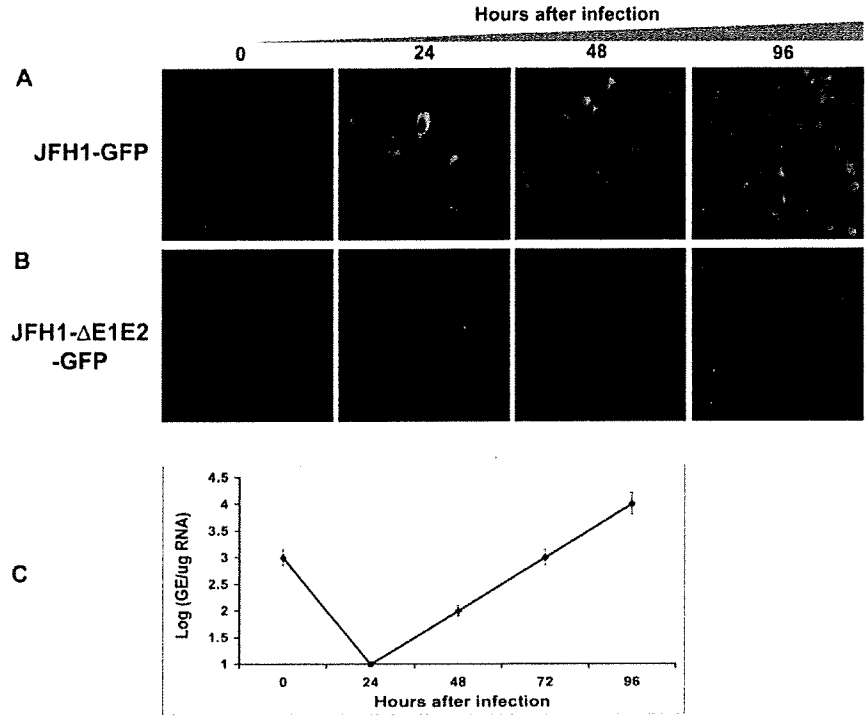

Figure 4 Infectivity of virus particles produced from Huh-7.5 cells transfected with JFH1-GFP chimeric genome and JFH1-ΔE-E2-GFP deleted mutant clone. Huh-7.5 cells were transfected with 20 μg of *in vitro* transcribed HCV RNA. After 72 hours, cells along with supernatants were harvested. Four rounds of freezing and thawing using dry ice lysed the cells. Cell free supernatants were collected by centrifugation at 3500 rpm using a tabletop centrifuge. The titer of HCV in the supernatant was determined by real-time RT-PCR. The TCID50 of infectious supernatant was determined by using 10-serial dilution of the virus stock. (A) Intracellular GFP expression in the infected Huh-7.5 cells at 0, 24, 48, 72 and 96 h using MOI of 10 or TCID50 (i.e 10^5 virus particle/ml). At different time intervals, cells were taken out from the culture, fixed and GFP examined under a fluorescence microscope. Increased expression of GFP in the infected culture was seen. (B) Intracellular GFP expression in Huh-7.5 cells infected using supernatants of E1-E2 deleted mutant construct. No GFP signal was seen in cells infected using culture supernatants of E1-E2 deleted clone. (C) Real-time RT-PCR was used to quantify the HCV RNA level in the infected cells using a primer targeted to the HCV 5'UTR region. HCV RNA titer in the infected cultures was increased with time suggesting that replication of HCV genome in the infected culture.

cell colonies that appeared on the plate (Fig. 5). No colonies developed in the culture transfected with the GND mutant sub-genomic HCV RNA. Individual cell colonies were picked and stable Huh-7 cell lines supporting replication of HCV-GFP sub-genomic RNA were developed. The absence of stable DNA integration in these cell lines was confirmed by direct PCR analysis for neo gene followed by southern blot analysis. High levels of GFP expression due to replication of sub-genomic HCV 2a clone was seen in sensitive and resistant Huh-7 clones (Fig. 6A). The expression of HCV-GFP chimera protein was seen exclusively in the cytoplasm in the majority of Huh-7 cells in the culture. These cell lines have maintained stable GFP expression over more than one year when cultured in a growth medium supplemented with G-418 (500 μg/ml). Two types of stable replicon cell lines were prepared using Huh-7 cells with or without functional Jak-Stat pathway. Stable HCV-GFP replicon cell lines prepared using IFN sensitive (S-Huh-7) cells were named as S3-GFP and S10-GFP replicons. Replicon cell lines, also prepared using IFN resistant Huh-7 cell lines (R-Huh-7), were named as R4-GFP and R8-GFP replicons. The level of GFP expression in the IFN sensitive and resistant replicon Huh-7 cell lines was quantitatively determined by flow analysis. The results of these experiments suggest that more than 80% of replicon cells express GFP (Fig. 6B).

Antiviral activity of IFN-α against full-length HCV 2a is blocked in Huh-7 cell clone (R-Huh 7) with a defective Jak-Stat pathway

The development of JFH1-GFP chimera using the HCV 2a clone allowed us to quantify the antiviral properties of IFN-α in Huh-7 cells. One important predictive factor associated with IFN response is the viral genotype. It has been reported by a number of investigators that the sustained virological response in patients infected with HCV genotype 2 is much higher than in patients

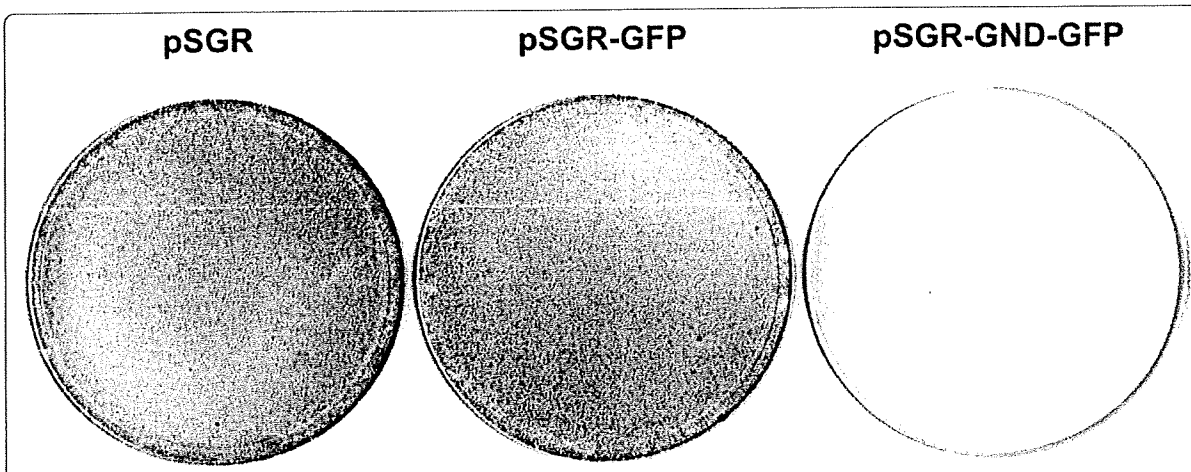

Figure 5 Replication of unmodified sub-genomic HCV 2a RNA clone and GFP labeled sub-genomic HCV 2a chimera in Huh-7 cells. Huh-7 cells were transfected with 10 μg of in vitro transcribed RNA by the electroporation method and then cultured in the medium containing G-418 (500 μg/ml). After 4-weeks, G-418 resistant cell colonies were stained with Giemsa Stain (Sigma Chemical). Both the unmodified (pSGR) and GFP tagged sub-genomic clone (pSGR-GFP) replicated at equal efficiencies based on the development of number of G-418 resistant Huh7 cell colonies. No G-418 resistant colonies were present in Huh-7 cells with GND mutant sub-genomic RNA with GFP (pSGR-GND-GFP).

infected with genotype 1 virus. We conducted experiments to determine whether the replication of an HCV 2a strain could be inhibited in liver cells (R-Huh-7) having a defective Jak-Stat pathway. Both S-Huh-7 and R-Huh-7 cells were transfected individually with full-length JFH1-GFP RNA and then treated with an increasing concentration of IFN-α. We first determined that both S-Huh-7 and R-Huh-7 cells developed in our laboratory supported HCV 2a replication and infection. The ability of IFN-α to inhibit full-length HCV 2a replication in these two different Huh-7 clones was examined in a kinetic study at 24 to 96 hours. Results shown in the upper panel of Fig. 7A suggest that GFP expression can be efficiently inhibited in S-Huh-7 cell clones. There was no reduction in GFP expression in the R-Huh-7 cell clones with a defective Jak-Stat pathway at all time points (lower panel of Fig. 7A). The antiviral effect of IFN-α against HCV 2a in these two cell clones (S-Huh-7 and R-Huh-7) was also quantified by flow cytometric analysis. We found a time dependent effect of IFN-α on HCV 2a replication in S-Huh-7 cells and the number of GFP positive cells was decreased from 4.2% to 0.2% as compared to resistant Huh-7 cell line (Fig. 7B). To verify that the inhibition of GFP is also associated with the reduction of viral RNA in the interferon treated cells, RNA extracts were assayed for HCV RNA by RPA assay using a probe targeted to the 5' UTR region of HCV genome. We found that interferon treatment decreased HCV RNA levels in S-Huh-7 clones and the levels of HCV RNA remained unchanged after interferon treatment in the resistant clone. (Fig. 7C). The ability of IFN-α to stop viral RNA replication in the infected cells was also examined using these two Huh-7 cell clones. IFN-α treatment efficiently inhibited HCV replication as measured by GFP expression in S-Huh-7 cells within 24 hours (Fig 8A). However, antiviral activity of IFN-α against the full-length HCV 2a replication was prevented in R-Huh-7 cells with the defective Jak-Stat pathway (Fig. 8B). The results of these experiments indicate that antiviral activity of IFN-α to inhibit replication of full-length HCV 2a clone was prevented in R-Huh-7 clone with defective Jak-Stat pathway.

Antiviral activity of IFN-α is impaired against HCV 2a sub-genomic clone in Huh-7 cell clone with a defective Jak-Stat pathway

The role of the Jak-Stat pathway in the IFN-α response to HCV 2a was also studied using an IFN sensitive (S3-GFP) and IFN resistant (R4-GFP) stable Huh-7 cell line that replicates sub-genomic RNA. Replication of HCV 2a sub-genomic RNA in the S3-GFP after IFN-α treatment was studied by measuring the intracellular GFP expression directly under a fluorescence microscope. It was found that GFP expression in the stable cell line (S3-GFP) diminished over time (Fig 9A). Where as no reduction of the HCV-GFP signal in R4-GFP replicon was observed even when treated with a similar concentration of IFN-α for an extended period. To quantify the IFN antiviral effect intracellular GFP expression was analyzed by flow analysis. The GFP peak disappeared after IFN treatment only in the S3-GFP replicon cell line (53% to 2%). The percentage of GFP positive cells did not decrease (58% to 55%) when similar experiments were carried out using R4-GFP cells (Fig. 9B). To

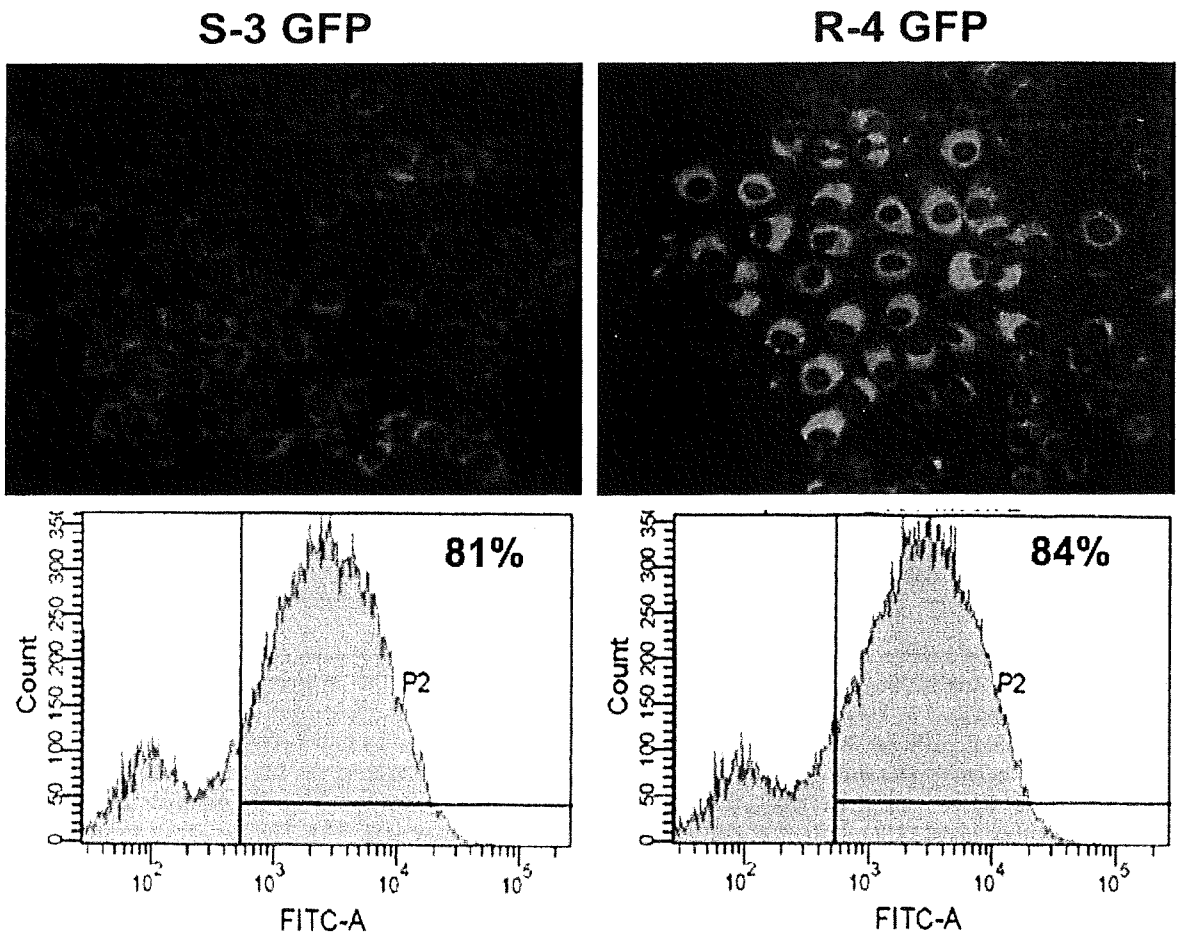

Figure 6 Preparation of stable replicon cell line replicating HCV 2a sub-genomic RNA. Interferon sensitive (S3-GFP) and interferon resistant (R4-GFP) Huh-7 cells were transfected with pSGR-GFP replicon RNA and then selected with G-418 (500 μg/ml). Single G-418 resistant cell clones were picked and stable cell lines were generated. (A) Intracellular GFP expression in S3-GFP and R4-GFP stable replicon cell lines. (B) Quantification of GFP expression in these IFN-sensitive (S3-GFP) and resistant (R4-GFP) cells was performed flow analysis. Approximately 81% of S3-GFP and 84% of R4-GFP cells in the culture showed intracellular GFP expression. High-level expression of HCV-GFP was seen in both cell lines suggesting that both the sensitive and resistant Huh-7 clones support high level HCV replication.

correlate the results of GFP expression, intracellular HCV RNA after IFN-α treatment was also measured by RPA. The results of RPA assays demonstrate that HCV RNA replication is not inhibited by IFN-α treatment in the R4-GFP replicon cell line (Fig. 9C). The level of HCV RNA was also quantified by real-time PCR in these two cell lines after IFN treatment. IFN-α treatment inhibited the HCV RNA level in a dose dependent manner in S3-GFP but the HCV RNA level remained the same in the R4-GFP replicon. There was a significant difference in the level of HCV RNA between the IFN sensitive replicon and resistant replicon after IFN treatment measured by real-time PCR (Fig. 9D). These results suggest that replication of HCV 2a full-length as well as sub-genomic RNA can not be inhibited by IFN-α in R-Huh-7 cells with a defective Jak-Stat pathway.

HCV infection and replication did not alter the state of Jak-Stat pathway in S-Huh-7 and R-Huh-7 cell clones

Experiments were carried out to examine whether infection or replication of HCV in both S-Huh-7 and R-Huh-7 cells could have any impact on the IFN-α induced Jak-Stat signaling. The levels of pStat1 and pStat2 proteins in the lysates of S-Huh-7 and R-Huh-7 cells after 96 hours of HCV infection were examined by western blot analysis. Results shown in Fig. 10A and 10B clearly show that IFN-α treatment induced pStat1 and pStat2 protein in the infected as well uninfected S-Huh-7 only. However, pStat1 or pStat2 protein was not

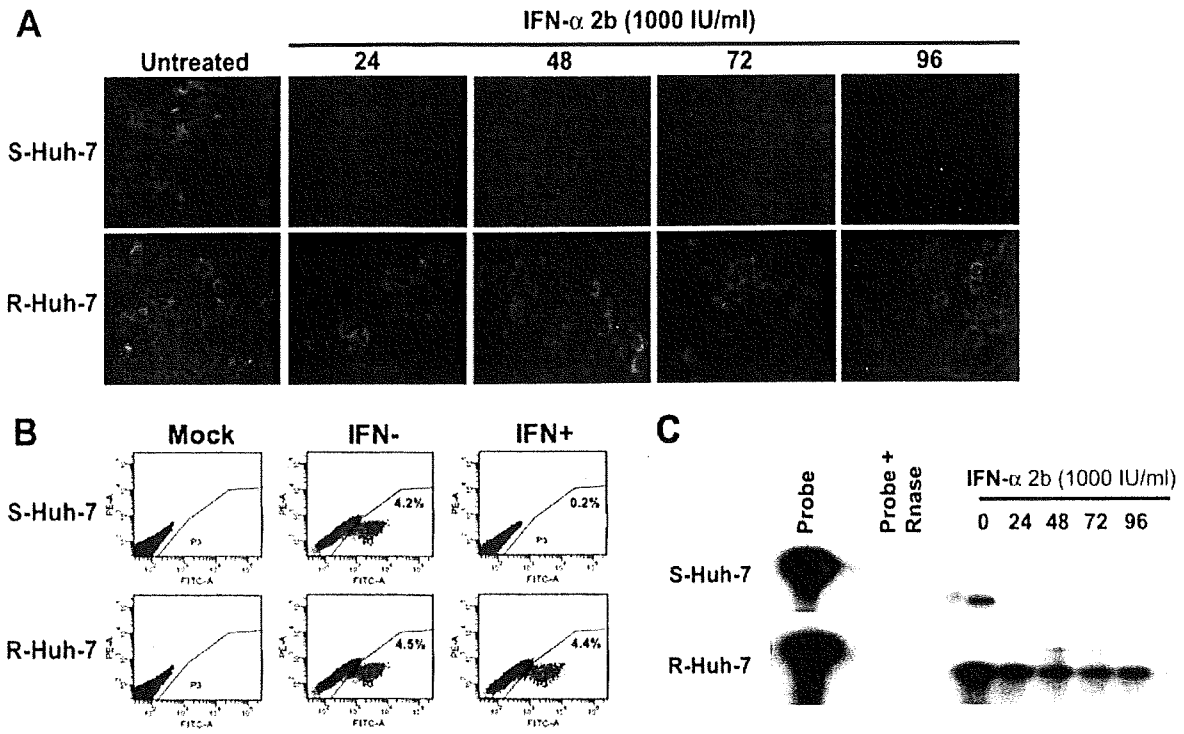

Figure 7 Antiviral effect IFN-α on the replication of JFH1-GFP RNA using S-Huh-7 and R-Huh-7 cells in culture. (A) S-Huh-7 and R-Huh-7 cells were transfected with full-length JFH1-GFP RNA and next day the cultures were treated with IFN-α (1000 IU/ml). Intracellular GFP expression was measured after 24, 48, 72 and 96 hours of post-transfection. Upper panel shows the intracellular GFP expression after IFN-α treatment in S-Huh-7 cells. Bottom panel shows the intracellular GFP expression in R-Huh-7 cells after IFN-α treatment (B) Quantification of antiviral effect of IFN-α (1000 IU/ml) between these two cell lines by flow analysis after 72 hours of post-transfection. In the S-Huh-7 cell line the number of GFP positive cells were decreased from 4.2% to 0.2% after IFN-α treatment. There is no decrease in the number of GFP positive cells after IFN-α treatment in the transfected R-Huh-7 cells. The upper and lower left panel shows the untransfected cells as mock. (C) Intracellular HCV mRNA levels in the IFN treated cells measured by RPA. The experiments were carried out same way as described in the panel A; except that the RPA analysis was performed using the total RNA isolated from the transfected cells at different time points. Upper panel shows the HCV RNA level in the transfected S-Huh-7 cells after IFN-α treatment. Bottom panel shows the HCV RNA levels in the transfected R-Huh-7 cells after IFN-α treatment.

detected in the infected R-Huh-7 cells even after interferon treatment. These results were confirmed by a co-localization of pStat1 protein in the GFP labeled replicon cells after IFN-treatment. We show that pStat1 is induced only in the sensitive replicon (S3-GFP) and localizes to the nucleus. The nuclear translocation of pStat1 is correlated with a decrease in GFP expression after IFN-α at 72 hours in the S-Huh-7 cells only (Fig. 10C). The pStat1 protein was undetectable in R4-GFP cells after IFN-α treatment. To examine if the effect of HCV infection or replication in both S-Huh-7 and R-Huh-7 could alter the overall Jak-Stat signaling, the ISRE-luciferase promoter activity was examined by a transient transfection assay. Interferon induced activity of ISRE-luciferase did not change significantly in R-Huh-7 cells after HCV infection (Fig. 10D).

Discussion

The JFH1 full-length cDNA clone of HCV 2a strain was isolated from a chronically infected Japanese patient by Wakita and his coworkers [14]. JFH1 derived clones replicate at a greater efficiency than all other HCV strains, making it the system of choice for biochemical studies that address HCV replication mechanisms and virus host interactions. The replication of full-length virus in cell culture is assessed by the detection of viral RNA by using a highly sensitive RT-PCR method. Viral proteins were detected by western blot analysis, ELISA or immunocytochemistry. These methods are highly specific and accurately determine the replication kinetics but are complex and time consuming. To overcome these difficulties, we prepared a chimeric clone of JFH1 by inserting the coding sequence of EGFP-N1 in the NS5A coding sequences. We noticed that a high-level

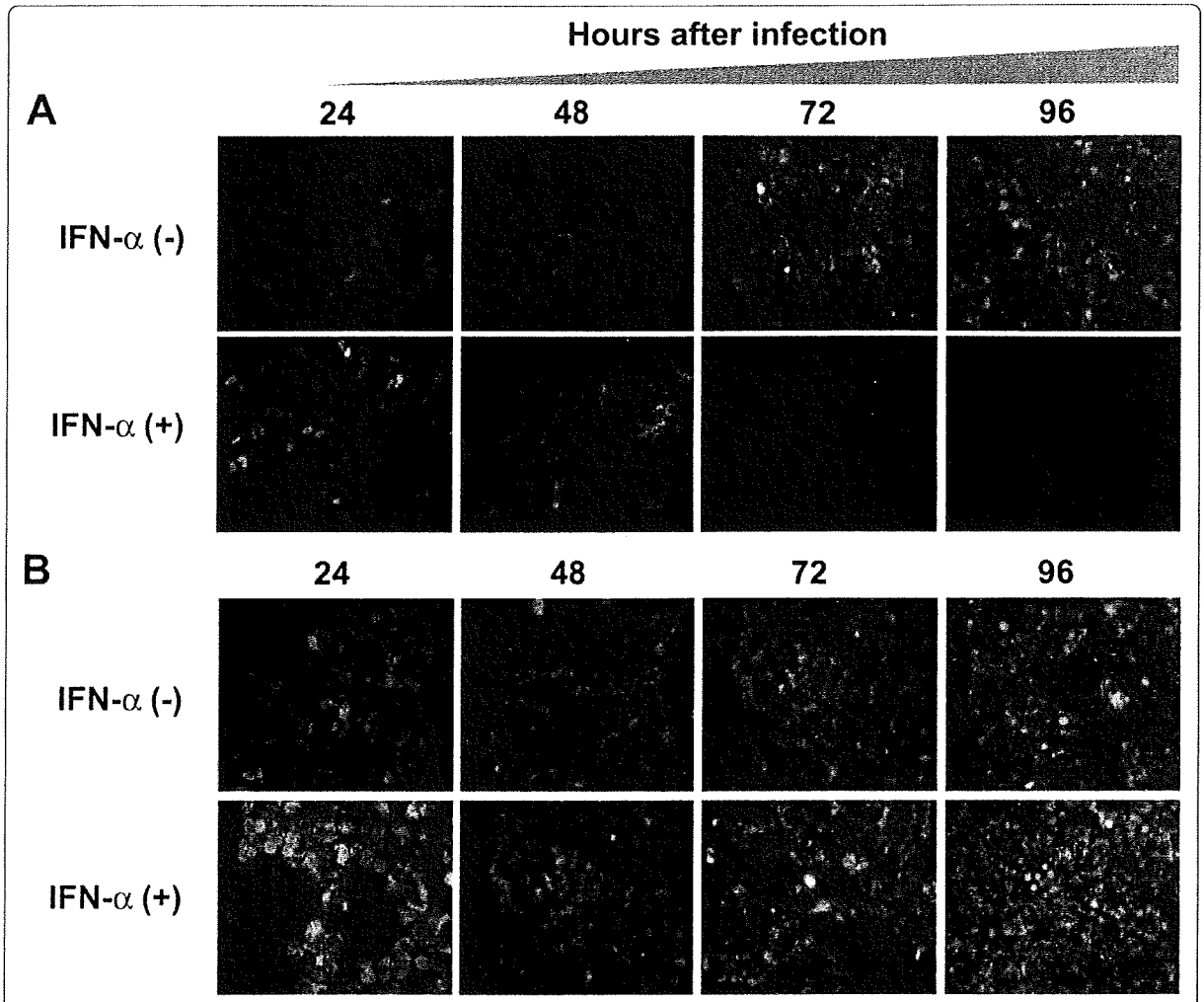

Figure 8 Antiviral effect of IFN-α against HCV replication in the infected Huh-7 cells in culture. S-Huh-7 and R-Huh-7 cells were seeded at density of 1×10^4 cell per well in a chamber slide. The next day cultures were infected with infectious HCV GFP virus at the TCID50 (1×10^5 virus particles/ml) or MOI of 10 using the standard protocol described in the material and method section. After 24 hours cultures were treated with IFN-α (1000 IU/ml). (A) Intracellular GFP expression in the infected S-Huh-7 cells in culture at 24, 48, 72 and 96 hours of post-infection in the presence and absence of IFN-α. The results show that HCV RNA replication in the infected cells can be inhibited over time by treatment with alpha interferon in S-Huh-7 cells. (B) Intracellular GFP expression in the infected R-Huh-7 cells in culture at 24, 48, 72 and 96 hours of post-infection in the presence and absence of IFN-α. The results suggest that HCV RNA replication remain resistant to IFN-α treatment at all time points in infected R-Huh-7 cells with a defective Jak-Stat pathway.

expression of this JFH1-GFP chimera was seen in Huh-7.5 cells 24 h after transfection. The expression of GFP in the transfected cells is an indication of active replication of the HCV genome since no GFP expression was detected in cells transfected with a GND mutant RNA. Replication of JFH1-GFP RNA in the transfected cell is supported by the results of detection of positive and negative strand RNA. We also showed that the transfected cells produced infectious virus particles. The infection can be transferred to naïve Huh-7 cells in a culture. The expression of GFP protein and viral RNA increased over time in the infected culture suggest that the replication of HCV occurred over time after natural infection. We also prepared a JFH1 sub-genomic clone with GFP as a fusion protein. Multiple stable replicon cell lines containing the GFP chimeric clone and neomycin selection marker were prepared in Huh-7 cells. Replication of sub-genomic clone of HCV 2a in the Huh-7 cells was stable. High-level expression and replication of HCV sub-genomic RNA was observed in the cells for over one year, and can be assayed by flow analysis. Stable cell lines replicating HCV sub-genomic

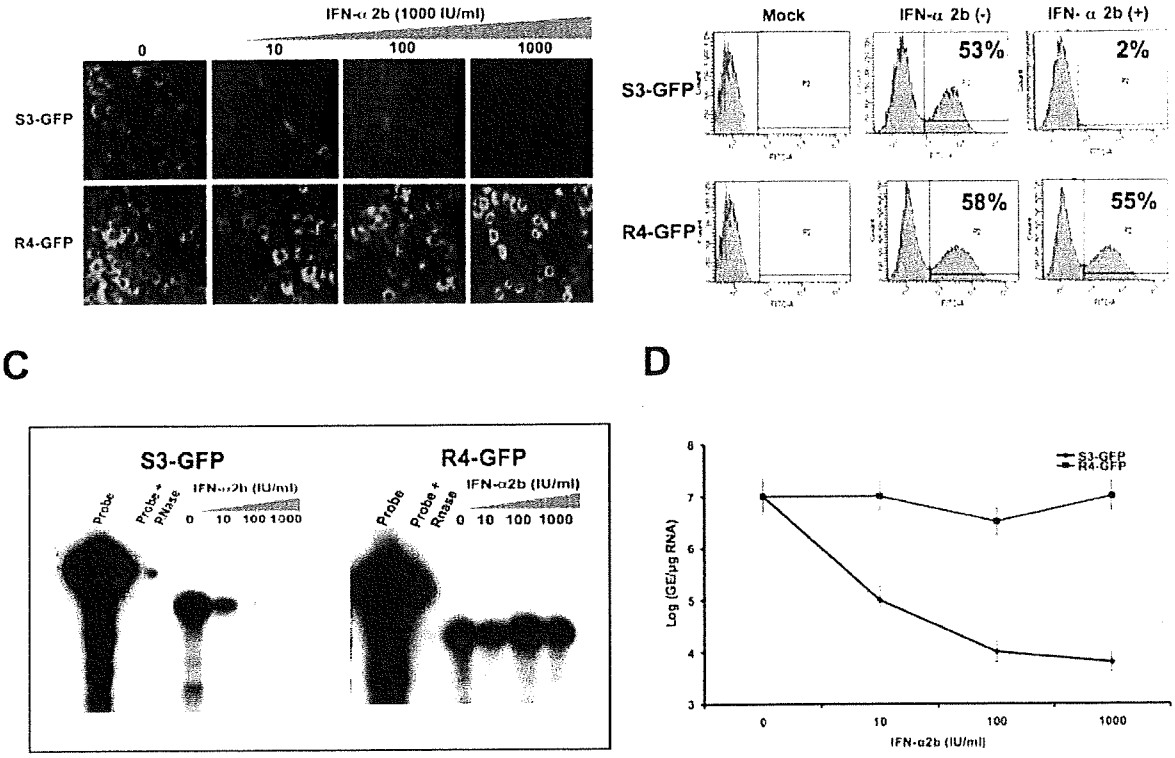

Figure 9 Antiviral effect of IFN-α using stable cell lines replicating GFP labeled sub-genomic RNA of HCV 2a. S3-GFP and R4-GFP were treated with IFN-α from 10 to 1000 IU/ml. (A) Upper panel shows the antiviral effect of IFN-α against HCV 2a sub-genomic RNA replication in S3-GFP cells. There was a dose dependent decrease in the GFP expression in the S3-GFP cells with increasing concentration of IFN-α. Lower panel shows no effect of GFP expression in R4-GFP cells. (B) The GFP fluorescence was quantitatively measured by flow cytometry analysis after IFN-α treatment (1000 IU/ml) for 72 hours using S3-GFP and R4-GFP cells. A significant reduction in the number of GFP positive S3-GFP cells (53% to 2%) was seen after IFN treatment. The number of GFP-positive cells did not decrease when treated with interferon (58% to 55%) using R4-GFP cells. (C) RPA shows the intracellular HCV RNA level in the S3-GFP and R4-GFP after IFN-α treatment. Left panel shows HCV RNA levels in S3-GFP after IFN-α treatment at 72 hours. Right panel shows the HCV RNA level in R4-GFP after IFN-α treatment at 72 hours. There is a gradual reduction of HCV mRNA level in the S3-GFP replicon cells compared to the R4-GFP cells. (D) Shows the quantification of HCV RNA levels in the S3-GFP and R4-GFP after IFN-α treatment at 72 hours by real-time RT-PCR. In both the cells the HCV RNA level was detected up to a level of titer of log 7 GE/μg of total RNA. The HCV RNA levels reduced significantly after IFN-α treatment at 1000 IU/ml in the S3-GFP replicon cells. There was no decrease in the HCV RNA level in R4-GFP cells after IFN-α treatment.

RNA were prepared using IFN-sensitive (S-Huh7) and resistant Huh-7 cells (R-Huh7). We now clearly showed that replication of HCV-GFP chimera cannot be inhibited by IFN-α in Huh-7 cells with defective Jak-Stat pathway.

The availability of a full-length GFP clone and stable replicon cell lines have allowed us to examine the antiviral mechanisms of IFN-α against HCV 2a strain in cell culture. There are reports suggesting that the effectiveness of the IFN response depends on the viral genotype. We performed a study to examine differences in the level of IFN response of HCV using the HCV 2a replication system. Previously, we have demonstrated that both the HCV 1a and HCV 1b strain can be efficiently inhibited by IFN-α within 72 h in a concentration dependent manner [18,19]. In this study we provide evidence suggesting that interferon alpha treatment inhibited HCV RNA replication of full-length as well as replication of HCV sub-genomic RNA in a dose dependent manner. These results are also consistent with a previous report suggesting that IFN inhibits replication of HCV 2a and HCV 1b strain in a dose dependent manner [29]. The role of virus and the Jak-Stat pathway of host cell in the IFN response using HCV 2a cell culture system were examined. We showed here that IFN-α treatment induced phosphorylation of Stat1 and Stat2 proteins in the infected S-Huh-7 cells and successfully inhibited HCV RNA replication. However, we could not

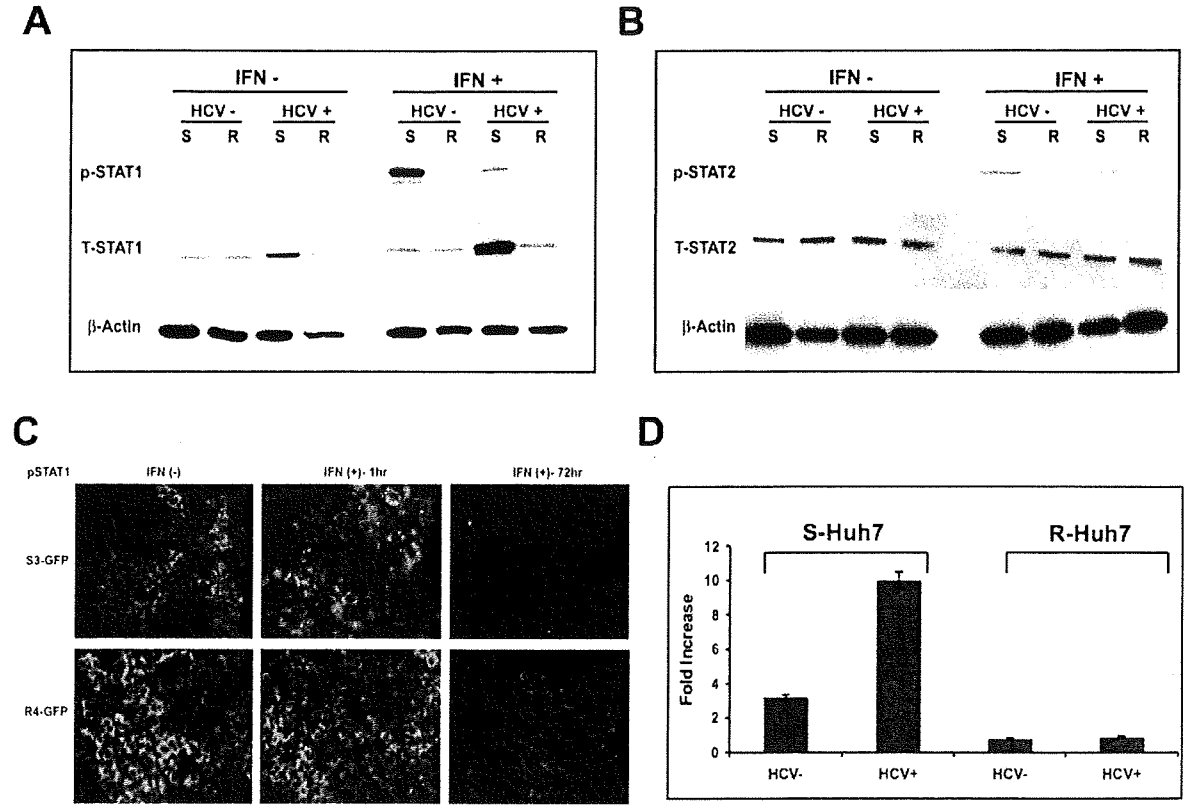

Figure 10 IFN-α induced Jak-Stat signaling in both S-Huh-7 and R-Huh-7 cells after HCV 2a infection. (A) Both S-Huh-7 and R-Huh-7 cells were infected with JFH1-GFP chimera virus for 96 hours. The phosphorylation of Stat1 protein in the infected cultures after 30 minutes of IFN-α treatment (1000 IU/ml) was determined by western blot analysis. Equal amounts of proteins were used in the blot to assay for total Stat1 and beta-actin levels. (B) IFN-α induced pStat2 protein in S-Huh-7 and R-Huh-7 cells with or without HCV 2a infection. The experiment was carried out as described in panel A. Equal amounts of proteins were used in the blot to assay for total Stat2 and beta-actin levels. (C) Demonstrates IFN-α induced nuclear localization of pStat1 in the S3-GFP and R4-GFP stable replicon cells at 24 and 72 hours by immunofluorescence microscopy using anti-mouse pStat1 (1:1000 dilution) and Alexaflour labeled anti-mouse secondary antibody (1:2000 dilution). The pStat1 protein was detected in the nucleus of S3-GFP replicon cells only at 24 and 72 hours after IFN-α treatment. The pStat1 translocation is associated with negative GFP expression after 72 hours of IFN-α treatment only in S3-GFP cells. (D) IFN-α induced ISRE-luciferase activity in S-Huh-7 and R-Huh-7 cells after HCV 2a infection. Both S-Huh-7 and R-Huh-7 cells were were infected with full-length HCV. After 72 hours, infected cells were transfected with μgm of pISRE-luciferase plasmid. After 24 hours, ISRE-luciferase activity in the cell lysates were measured in the presence and absence of IFN-α(1 IU/ml) treatment for 24 hours. The values were calculated as fold increase with respect to untreated cells.

detect phosphorylated Stat1 or Stat2 protein in the HCV infected R-Huh-7 cells after IFN-α treatment. The IFN-α induced Jak-Stat mediated ISRE-luciferase activity of R-Huh-7 cells did not change significantly with or without HCV infection. We showed here that IFN-α is not able to inhibit the replication of full-length as well as sub-genomic HCV 2a virus in R-Huh-7 cell clone suggesting a dominant role of cellular Jak-Stat pathway in the response to the interferon treatment [22].

The overall sustained virological response of patients infected with HCV genotype 2 and 3 is about 80% as compared to only 50% in the case of chronic HCV patients that are infected with HCV genotype 1 strain [3]. The mechanisms that determine the response at the genotype level are not clear. There has been a report suggesting that the IFN treatment response alters two phases of viral replication kinetics [30]. The first phase is the dose dependent reduction of HCV RNA levels in the liver within the first 24 hours after treatment. The second phase of IFN-induced decline of HCV RNA occurs over weeks to months. The first phase viral decay may be due to the direct action of interferon on HCV production and the second phase may be due to death of infected cells. In our analysis, we have found that there is no difference in the efficacy of IFN upon replication of HCV 2a and HCV genotype 1b viruses. It will be important to determine if there are differences in death of hepatocytes when they are infected with HCV

genotype 1 and HCV 2a virus. Our study provides evidence suggesting that cells with defective Jak-Stat pathway of IFN-signaling can prevent the antiviral response after IFN-α treatment. This conclusion supports results from previous studies using HCV cell cultures [22,31] as well as by a recent multicenter study using clinical samples from HALT-C trial suggesting that response to IFN therapy is dependent upon the host genetic polymorphisms of Tyk2 in the liver cells [32]. In summary, results of this investigation support the importance of host cell factors in the mechanisms of IFN-resistance in chronic HCV infection. The development of IFN-sensitive and IFN-resistant GFP tagged HCV 2a replicon cell lines will allow us to further understand the mechanisms of resistance against HCV in tissue culture. In particular, GFP labeled IFN-resistant replicon cells should be very useful to develop alternative antiviral strategies to overcome IFN resistance against HCV.

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Authors' contributions

SH performed most of the biochemical experiments, prepared the chimeric constructs, cell lines, and participated in the design of the study. PKC contributed in the full-length infectivity assay. BP prepared RPA probe for negative strand detection and SND helped in western blot experiments. TPF and GK provided us the temporary laboratory space to recover the cell lines at the time of hurricane Katrina. TW provided the initial JFH1 constructs. RFG helped in editing the manuscript. SD overall supervised, helped to design the study and wrote the manuscript. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no competing interests.

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2′,5′-Oligoadenylate synthetase-like gene highly induced by hepatitis C virus infection in human liver is inhibitory to viral replication *in vitro*

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ABSTRACT

We found the 2′,5′-*oligoadenylate synthetase-like (OASL)* gene to be significantly elevated by high virus loads in human liver infected with hepatitis C virus (HCV). Here, we determined whether OASL inhibited HCV replication using an *in vitro* system. We constructed three expression vectors of OASL to produce isoform a (OASLa), isoform b (OASLb), and the C-terminal ubiquitin-like domain of isoform a (Ub). When Huh7 JFH-1 HCV replicon cells were separately transfected with these three vectors, colony formation of HCV-replicating cells was inhibited by 95%, 94%, and 65%, respectively. Both OASLa and OASLb were also inhibitory for cells as well as the virus because colony formation of OASL-producing cells was reduced to 41% and 8%, respectively. Stable Huh7 clones producing each of the three OASLs were established and assessed for their inhibition of HCV replication using luciferase reporter gene-containing JFH-1 replicon RNA. HCV replication was inhibited by 50–90% in several stable OASL clones. Association analysis in six Ub clones expressing different levels of Ub mRNA showed that the degree of inhibition of HCV replication was significantly associated with the amount of Ub present. In conclusion, OASL possesses two domains with HCV inhibitory activity. The N-terminal OAS-homology domain without OAS activity is inhibitory for cell growth as well as HCV replication, whereas C-terminal Ub is inhibitory only for HCV replication. Therefore, OASLa, a major isoform of this molecule induced in human liver, may mediate anti-HCV activity through two different domains.

Introduction

Hepatitis C virus (HCV) causes serious liver diseases including chronic hepatitis, liver cirrhosis and hepatocellular carcinoma. Host hepatic responses to HCV infection include innate immune reactions in the liver, such as interferon (IFN) α/β production, followed by induction of different interferon-stimulated genes (ISGs) [1–3]. In fact, IFN αβ in combination with ribavirin is currently the most effective therapy for a sizeable fraction of patients with chronic hepatitis C. Although it is known that IFN αβ induces more than 300 cellular genes, it is unclear which ISG(s) function effectively to control HCV in the human liver. Transcriptome analyses have demonstrated that 30–40 ISGs can be identified in association with HCV infection in the chimpanzee and human liver [1–3]. A few of them, such as viperin [3,4], IFN-inducible protein 6 [5] and ISG-20 [4] have been overexpressed in Huh7 replicon cells, in which it was established that they are inhibitory to HCV replication. To isolate active natural anti-HCV factors effective *in vivo*, we previously investigated differential gene expression in infected human liver containing different levels of HCV. We identified a gene up-regulated in the presence of a high HCV load; that is the 2′,5′-oligoadenylate synthetase-like (OASL) gene.

OASL is a member of the OAS family and is an IFN-induced cellular protein [6]. mRNAs of both OAS and OASL are induced by HCV infection in patients livers, as shown by our study and that of others [3,7]. OAS is known to synthesize the activator of cellular RNase L, 2′,5′-oligoadenylate, which inhibits viral replication through general degradation of RNA [8]. Indeed, OAS1 is known to inhibit HCV replication in replicon cells [5]. However, whether OASL possesses anti-viral activity has remained unclear for a long time because OASL lacks OAS enzyme activity due to an amino acid substitution at the N-terminal OAS-homology domain [6]. There are two possible hypotheses regarding the viral infection-related activity of OASL. The first attributes anti-viral activity to the C-terminal region, which has two ubiquitin-like domains (Ub) unique to

Abbreviations: HCV, hepatitis C virus; IFN, interferon; ISGs, interferon-stimulated genes; OAS, 2′,5′-oligoadenylate synthetase; OASL, 2′,5′-oligoadenylate synthetase-like; OASLa, OASL isoform a; OASLb, OASL isoform b; Ub, two C-terminal ubiquitin-like domains of OASLa; JFH-1, HCV isolate (genotype 2a) from a patient with type-C fulminant hepatitis

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OASL. The second is that OASL has a dominant-negative effect on OAS activity, i.e. it acts as an antagonist of the anti-viral activity of OAS. In the present study, we investigated whether OASL was inhibitory or stimulatory for HCV replication in an *in vitro* system. In addition, we assessed domain activity by separating OASL into two parts, the OAS-homology domain and Ub.

Materials and methods

Liver specimens and cells. HCV-positive liver samples were obtained from non-tumorous liver tissues of patients with hepatocellular carcinoma. All 44 cases had solitary tumor less than 5 cm in diameter, and no metastasis to lymph nodes and other organs. Histopathology of all non-tumorous liver tissues was consistent with a diagnosis of chronic hepatitis without liver cirrhosis. HCV-negative liver samples were obtained from non-tumorous liver tissues of patients with hepatic metastasis of colon cancer. Informed, written consent was obtained from each patient. Our study protocol was previously approved by the ethics committee of our school and was in accordance with the Helsinki Declaration of 1975. Huh7 and JFH-1 HCV subgenomic replicon-containing Huh7 cells were described previously [9]. HCV replicon cells were cultured in complete medium containing 1 mg/ml G418 (Invitrogen, Carlsbad, CA).

Quantification of mRNA. Total RNA was extracted from liver tissues or cultured cells with TRIzol reagent (Invitrogen), treated with DNase I (Takara Bio, Shiga, Japan), after which cDNA was synthesized using PrimeScript reverse transcriptase (Takara Bio). PCR was performed in 20 μl of reaction mixture containing SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 10 ng of cDNA template, and 0.3 μM of each primer by Rotor-Gene 3000 (Corbett Life Science, Mortlake, Australia) at 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 1 min. The standard for quantification was the plasmid DNA containing individual genes or a sample of human liver cDNA that showed the highest expression of each gene. Gene expression was normalized against the quantity of 18S rRNA used as an endogenous control gene, and the relative value was expressed as an arbitrary unit. Primer sequences were designed by primer 3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and are shown in Supplementary Table 1.

Transfection of OASL expression plasmids. Three cDNA fragments of OASL were amplified from human liver cDNA using the primers shown in Supplementary Table 1. The cDNA was inserted into the pEF6/V5-His vector containing a blasticidin-resistance gene (Invitrogen). The pEF6/V5-His vector was used as a negative control. 1×10^5 HCV replicon cells or Huh7 cells per well were incubated in 6-well plates at 37 °C overnight, followed by transfection with 5 μg of the plasmid DNA using Lipofectamine LTX together with Plus reagent (Invitrogen). To establish stable clones of OASL-positive Huh7 cells, the cells were harvested again 24 h after transfection and cultured in 10-cm dishes with complete medium containing 10 μg/ml of blasticidin (Invitrogen). After three weeks, colonies were isolated and cultured individually as single clones.

Immunofluorescence staining. Cells 24 h after transfection were harvested and cultured on cover slips. After fixation with cold acetone for 3 min, the cells were blocked with 1% bovine serum albumin in phosphate-buffered saline, incubated with the first antibody (either 5 μg/mL of rabbit anti-OASL antibody (Abgent, San Diego, CA), 5 μg/mL of mouse monoclonal anti-V5 antibody (Invitrogen), or 400-fold diluted HCV-positive human serum), and then stained with 4 μg/ml of the second antibody conjugated to AlexaFluor 647 or AlexaFluor 488 (Invitrogen). The stained cells mounted on glass slides with ProLong Gold antifade reagent + DAPI (Invitrogen), were visualized with BIOREVO BZ-9000 (KEYENCE, Osaka, Japan) and iCys Research Imaging Cytometer (CompuCyte, Westwood, MA). The fluorescence intensity of HCV proteins was determined with the iCys Cytometer enumerating approximately 600 cells positive for OASL per cover slip. As a negative control, pEF6/V5-His plasmid transfectants were used to determine the fluorescence intensity of HCV proteins by randomly selecting the cells corresponding to the same area as the OASL-positive cells. The transfection experiment was done twice and the immunofluorescence staining on cover slips was done in duplicate per experiment.

Western blotting. Cell lysate was prepared as described previously [10]. Twenty micrograms of protein were electrophoresed on an SDS–10% polyacrylamide gel, and then transferred to a nitrocellulose membrane. The target protein was detected by ECL Advance Western Blotting Detection kit (GE Healthcare, Buckinghamshire, UK). The first antibodies were as follows: 0.2 μg/ml of rabbit anti-OASL antibody (Abgent), 0.2 μg/ml of mouse monoclonal anti-V5 antibody (Invitrogen), 0.2 μg/ml of mouse monoclonal anti-β-actin antibody (Abcam, Cambridge, UK), or 5000-fold diluted HCV-positive human serum. The chemiluminescence was detected with Light-Capture AE-6972 (ATTO, Tokyo, Japan) and quantified by CS Analyzer version 2.07 software (ATTO).

Colony formation assay for HCV replication. 1×10^5 HCV replicon cells transfected with plasmid DNA 24 h previously were transferred to 6-cm dishes containing 10 μg/ml of blasticidin with and without 1 mg/ml of G418. After 3 weeks of culture, cells were stained with 0.1% crystal violet/10% formalin solution. The stained dishes were photographed and the area of the colonies was measured with Colony version 1.1 software (Fujifilm, Tokyo, Japan).

Reporter assay for HCV replication. Reporter replicon RNA was transcribed *in vitro* from the plasmid pSGR-JFH1/Luc and transfected into Huh7 clones producing OASLa, OASLb, and Ub individually as described previously [11]. Luciferase activity was measured by Luciferase Assay Systems (Promega, Madison, WI) as described previously, using Infinite F200 (TECAN, Männedorf, Switzerland).

Statistical analysis. The Student *t* test, Mann–Whitney *U* test and Pearson's correlation analysis were used to analyze the data. *P* values less than 0.05 were considered statistically significant.

Results

OASL mRNA in human liver and HCV replicon cells

OASL and OAS1 mRNAs were quantified in the liver tissues of three groups carrying high, low and no HCV loads, respectively (Fig. 1). After measuring the copy number of HCV RNA in 44 samples of non-tumorous liver tissues, 17 and 14 samples were selected and assigned as representatives of groups with high and low viral loads, respectively. The HCV load was markedly different (>100-fold) between the two positive groups (Fig. 1), although underlying factors such as clinicopathological features were similar (Supplementary Table 2). Although there was significant induction of OASL mRNA in the liver following HCV infection, the amount was much greater in livers with high HCV load than in those with low viral load (Fig. 1). On the other hand, levels of mRNA for OAS1, which is a major ISG with anti-viral activity, were not significantly different between the high and low HCV groups (Fig. 1). To investigate the anti-viral activity of OASL using HCV replicon cells, we quantified HCV RNA, OASL and OAS1 mRNAs in the HCV replicon cells compared to the parent Huh7 cells (Fig. 1). HCV RNA was found to be $\geqslant$10-fold higher in HCV replicon cells than in the high HCV group of human livers, but the amount of OASL mRNA was quite low, similar to the low HCV group (Fig. 1). No OASL mRNA was detected in Huh7 cells. Thus, OASL mRNA was highly induced in human liver with a high HCV load but not in HCV replicon cells, whereas the reciprocal pattern was seen with OAS1 mRNA.

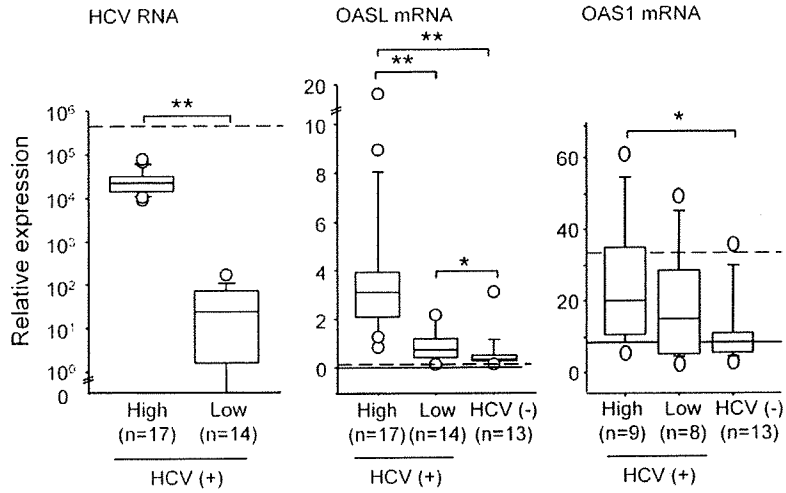

Fig. 1. Quantity of HCV RNA, OASL, and OAS1 mRNAs in patient liver tissues. The mRNA levels were quantified by real-time PCR and compared among the three groups: high, chronic hepatitis liver with high HCV viral load; Low, chronic hepatitis liver with low HCV viral load; HCV (−), HCV-negative non-tumorous liver from patients with hepatic metastasis of colon cancer. The number of patients examined is shown in parentheses; the number for OAS1 is a part of that group. Solid and dotted horizontal lines in each graph indicate the expression levels in Huh7 and HCV replicon cells, respectively. $^{*}P < 0.05$; $^{**}P < 0.01$.

Transient expression of OASLa in HCV replicon cells

There are two isoforms of OASL, a (OASLa) and b (OASLb) (Supplementary Fig. 1A). OASLa was the major isoform in human liver (Supplementary Fig. 1B). We asked whether OASLa protein was produced transiently in HCV replicon cells; the effect on HCV proteins was also assessed in OASLa-positive cells. We found that OASLa was present in 43% of HCV replicon cells after transfection and that it was localized in the cytoplasm (Fig. 2A). There was a 30% reduction in HCV proteins in OASLa-positive cells 72 h after transfection compared with OASLa-negative cells, as assessed by relative fluorescence (Fig. 2A). Western blot analysis confirmed that HCV proteins were reduced (in this assay by approximately 40%) 72 h post-transfection (Fig. 2B). These data suggest that OASLa is inhibitory to HCV.

Colony formation of OASL-positive HCV replicon cells

Next, to assess their inhibitory effects on HCV replication, three forms of OASL—OASLa, OASLb, and two ubiquitin-like domains of OASLa at the C terminus (Ub)—were produced in HCV replicon cells (Supplementary Fig. 1). The colony formation of these replicon cells was then assessed in the presence of G418 and blasticidin. Both OASLb and Ub localized in the cytoplasm, similar to OASLa (Fig. 3A). Colony formation in the presence of blasticidin alone was reduced to 41% (54.2/131) and 8% (10.2/131) in OASLa- and OASLb-transfectants, respectively, but slightly enhanced to 139% (182/131) in Ub-transfectants (Fig. 3B). Of these colonies, those in which HCV was replicating were further reduced to 5% (2.69/54.2), 6% (0.565/10.2), and 35% (62.9/182), respectively, compared with 82% (107/131) in negative control transfectants (Fig. 3C). Inhibitory activity of OASLa, OASLb, and Ub relative to the negative control was calculated as 94%, 93%, and 57%, respectively. However, it was noted that OASLa, and especially OASLb, acted as strong inhibitors of cell growth, whereas Ub slightly enhanced it. The efficiency of transfection with OASLa and OASLb was similar to that of control lacZ (pEF6/V5-His/*lacZ* plasmid) (Supplementary Fig. 2), indicating that the reduction in blasticidin-resistant colonies of transfectants with OASLa and OASLb was not due to the low efficiency of transfection.

HCV replication in OASL-positive cell clones

We established 19, 17, and 17 Huh7 clones producing OASLa, OASLb, and Ub, respectively. Representative clones were examined for HCV replication by luciferase reporter assay. Inhibition of HCV replication by more than 50% was observed in OASLa-46, OASLa-40, OASLb-11, Ub-9, and Ub-55 (Fig. 4A). The expression of OASL mRNAs and proteins was confirmed by quantitative PCR and Western blotting, respectively (Fig. 4B and C). The growth curves of established cell clones were similar to control clones (Supplementary Fig. 3). Especially, HCV replication was inversely correlated with the Ub mRNA level in the Ub clones ($P < 0.0001$, $r = -0.997$ by Pearson's correlation analysis) (Fig. 4D).

Discussion

We demonstrated in this study that OASL is inhibitory, not stimulatory, to HCV replication in HCV replicon cells; its anti-HCV activity is complex in that there are two separate functional domains, both with individual anti-HCV activity via different mechanisms. Thus, the OAS-homology domain without OAS enzyme activity, naturally occurring in OASLb, was inhibitory to cell growth and additionally to HCV replication. The Ub, C-terminal domain of OASLa, in contrast, was slightly stimulatory for cell growth but inhibitory to HCV replication. Thus, OASLa has the total sum activity of the two domains, that is, modest cell growth inhibition and strong anti-HCV activity.

Established cell clones producing OASLs were helpful for directly estimating the anti-HCV activity of OASLs because the clones had overcome the inhibitory effect on cell growth and all showed similar growth activity. The Ub clones clearly showed inhibitory activity against HCV replication in a dose-dependent manner. As OASLa and OASLb clones were difficult to establish, the number of clones examined was limited but a few clones did allow demonstration of this inhibitory activity. Further work, such as knocking down OASLs in these clones, would be necessary to prove the inhibitory activity of OASLs on HCV replication. Several clones such as Oa-46, Oa-40, Ob-11, Ub-9, and Ub-55 will be helpful for studying the anti-viral mechanism of OASLs.

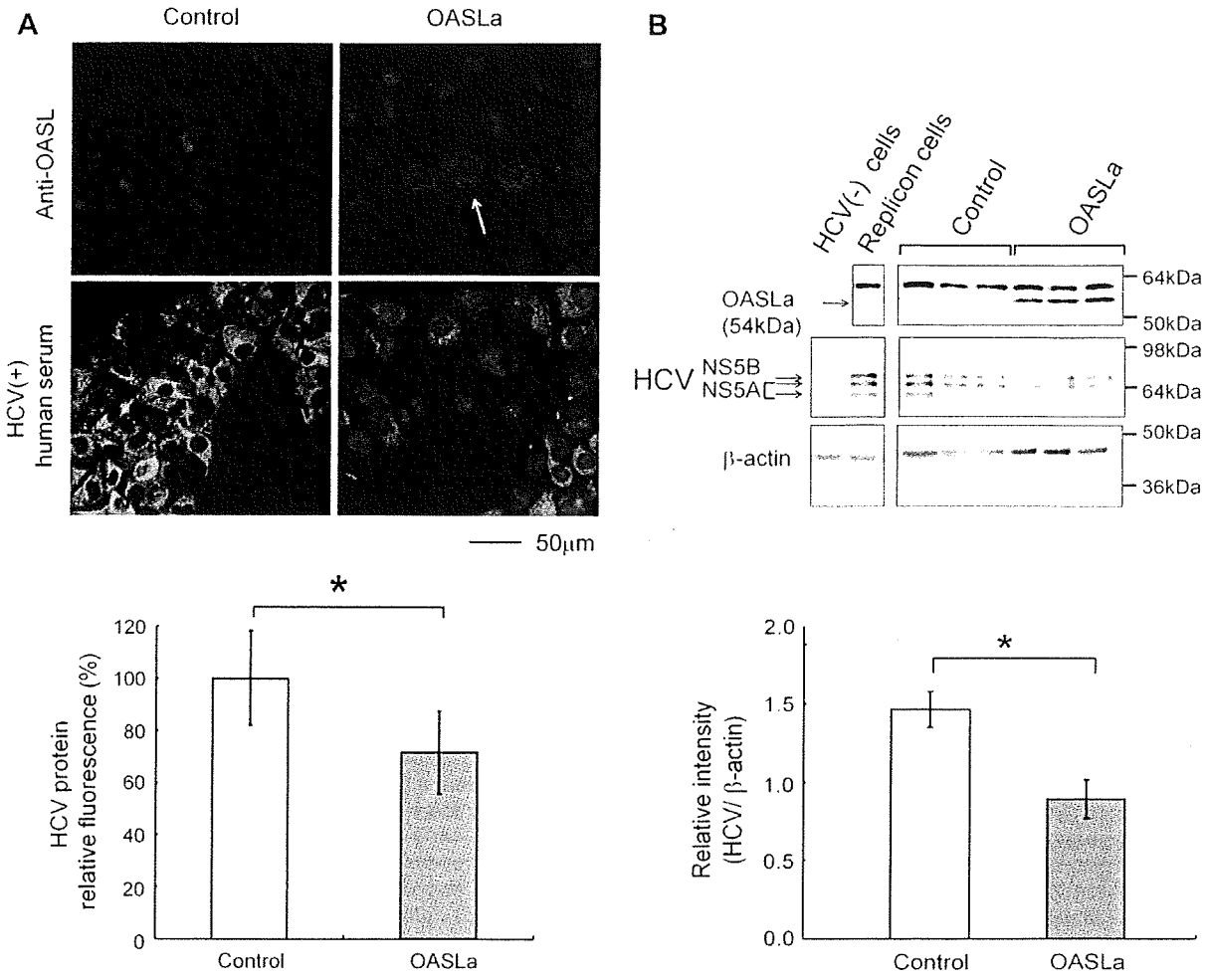

Fig. 2. Transient expression of OASLa in HCV replicon cells. (A) Immunofluorescence staining of OASLa and HCV proteins. HCV replicon cells 72 h after transfection with the expression plasmid were stained for OASLa (red) and HCV proteins (green) by immunofluorescence and for nuclei (blue) with DAPI. Control was transfected with the pEF6/V5-His plasmid. An arrow shows a representative cell positive for OASLa and negative for HCV. HCV-positive and OASLa-negative cells were also observed. The relative fluorescence was expressed by using the HCV fluorescence intensity of control cells 72 h after transfection as 100% (lower panel). Bars indicate the standard error of duplicate assays. (B) Western blot analyses of OASLa, HCV proteins and β-actin 72 h after transfection. Triplicate cultures of the transfectants were individually subjected to Western blotting. A signal of 54 kDa was specific for transfected OASLa, and the upper signal of 61 kDa is from an endogenous protein cross-reactive to rabbit anti-OASL antibody but not OAS (the anti-OASL antibody used in this study was specific for the C-terminal OASLa). Three signals were specific for HCV proteins, and were absent in Huh7 cells. Relative intensity of HCV proteins to β-actin was calculated by signals of HCV proteins and β-actin and expressed as a mean ± SD (lower panel). $^{*}P < 0.05$.

Recently, the biological function of OASL following infection with other viruses has been investigated *in vitro*. Thus, ectopic expression of OASL was found to be inhibitory to encephalomyocarditis virus infection but not to herpes simplex virus 1 in Vero cells. The anti-viral activity against encephalomyocarditis virus was lost in a truncated form of OASL lacking the C-terminal Ub [12]. Hence, anti-viral activity attributed to the C-terminal Ub is similar to our findings here. Furthermore, we demonstrated direct anti-viral activity of an isolated Ub, a truncated OASL lacking the N-terminal OAS-homology domain. In contrast, in the encephalomyocarditis virus study, truncated OASL equivalent to OASLb in our study, was found to lack anti-viral activity, as well as effects on cell growth. This apparent discrepancy may be due to the different cells used, different viruses and/or selectivity of the infected cells examined.

The question, then, is how does OASL inhibit HCV replication? OASL has two independent anti-HCV functional domains, as shown in this study. The inhibitory effect against cell growth of the N-terminal OAS-homology domain, OASLb, is probably related to the anti-HCV activity. Scholle et al. showed that HCV RNA replication was strongly decreased in poorly proliferating cells and highly dependent on cell growth [13]. A similar functional link between cell cycle regulation and HCV RNA replication might occur in OASLa- or OASLb-induced HCV replicon cells. Another functional domain is the C-terminal ubiquitin-like (UBL) domain, Ub, of OASLa. The UBL-containing proteins are classified into two groups, ubiquitin-like modifiers (ULM) and ubiquitin-like domain proteins (UDP). ULM are small molecules of 20 kDa or less, conjugated to proteins and functioning in a "ubiquitin-like" manner [14,15]. UDP is not conjugated to proteins, but is an adaptor that escorts ubiquitylated substrates to the 26S proteasome. Thus, the UBL domain is a proteasome-binding domain. Both ULM and UDP function in essential protein degradation pathways [15]. OASL is considered a UDP, with a function possibly related to the degradation of target proteins. UBQLN1, a member of the UDP family, regulates HCV RNA replication [16]. UBQLN1 has, in addition to a UBL domain, an ubiquitin-associated (UBA) domain which binds to the ubiquitin of ubiquitylated substrates. UBQLN1 binds to ubiquitylated NS5B through the

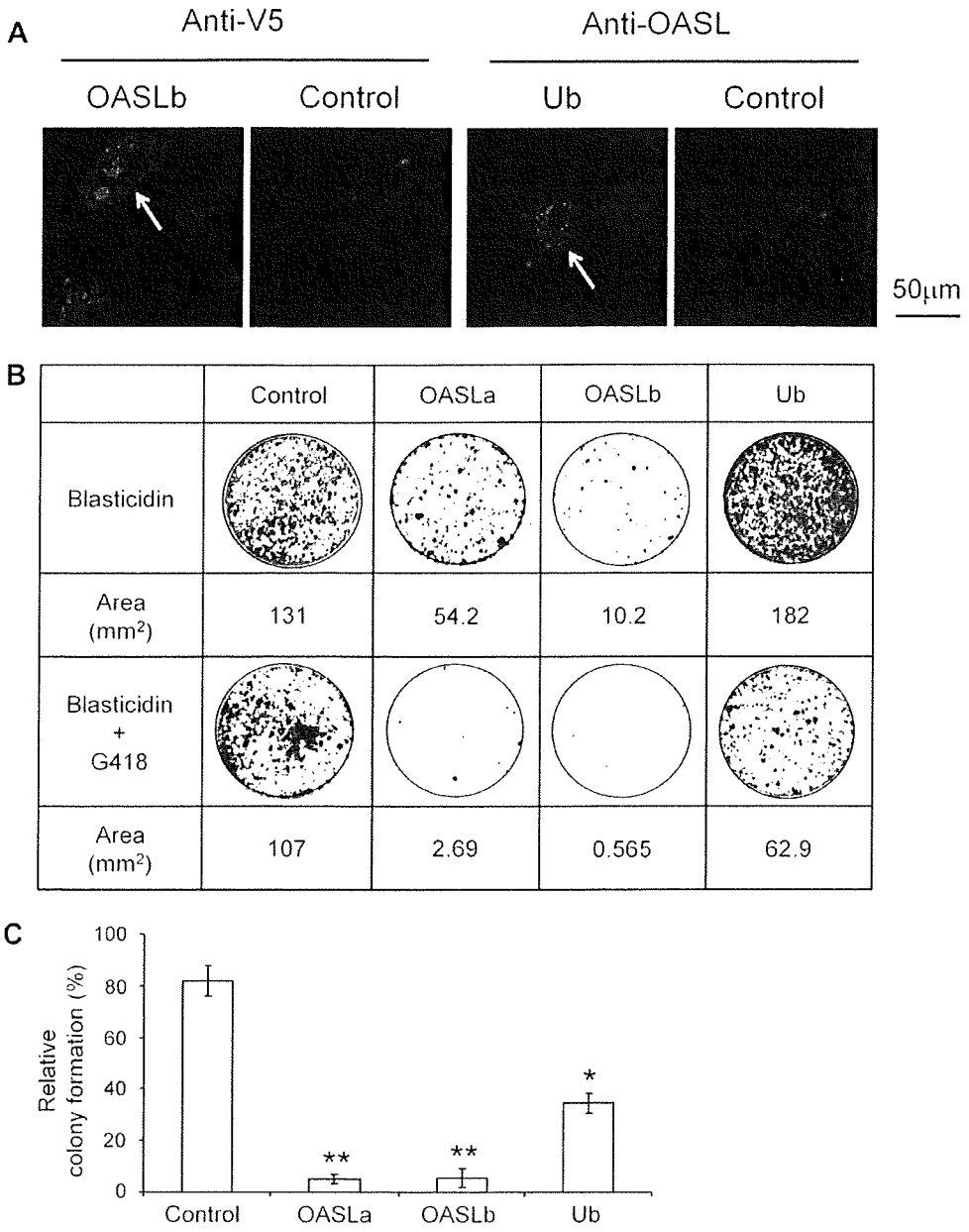

Fig. 3. Immunofluorescence staining of OASLb and Ub (A) and colony formation assay for HCV replication in OASL-producing cells (B, C). (A) HCV replicon cells 72 h after transfection with the plasmid of OASLb or Ub were stained for OASLb (red, arrow) or Ub (red, arrow), and nuclei (blue). OASLb with V5 epitope at the C terminus and Ub were detected by mouse monoclonal anti-V5 antibody and rabbit anti-OASL antibody, respectively. Control was transfected with the pEF6/V5-His plasmid. (B) Colony formation of HCV replicon cells transfected with vectors containing OASLa, OASLb and Ub. After culture with blasticidin in the presence or absence of G418 for 3 weeks in triplicate, the colony area was measured in an individual dish and shown as an average. (C) Relative efficiency of colony formation of HCV-replicating cells. The relative colony formation is expressed as% ratio of colony areas of blasticidin/G418 culture to blasiticidin culture obtained from (B). Bars indicate standard deviation of triplicates. $^{*}P < 0.05$; $^{**}P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this paper.)

UBA domain, recruits it to the proteasome through the UBL domain and promotes the degradation of NS5B through a ubiquitin-dependent pathway [16]. Although a substrate-binding domain like UBA is not found in OASLa, a possible mechanism of anti-HCV function may be that OASLa binds to a certain modified protein from HCV or the host cell, and shuttles it to the proteasome through the UBL domain, Ub, resulting in degradation of that protein. In such a case, a substrate recognition site is probably located in the OAS-homology domain. On the other hand, another mechanism responsible for the anti-HCV effect of ectopic Ub in Ub-clones may be that Ub, a small molecule of 19 kDa, potentially functions as a ULM to modify a target protein, tagging it for proteasome degradation. Thus, knowledge of what interacts with OASLa, OASLb or Ub in the HCV replicon cells and results in down-regulating HCV replication is crucial for understanding the anti-HCV mechanism.

In summary, OASL possesses two domains with HCV inhibitory activity. The OAS-homology domain without OAS activity is inhibitory for cell growth as well as HCV replication, whereas C-terminal Ub is inhibitory only for HCV replication. Therefore, OASLa, a major isoform induced in human liver, may mediate anti-HCV activity

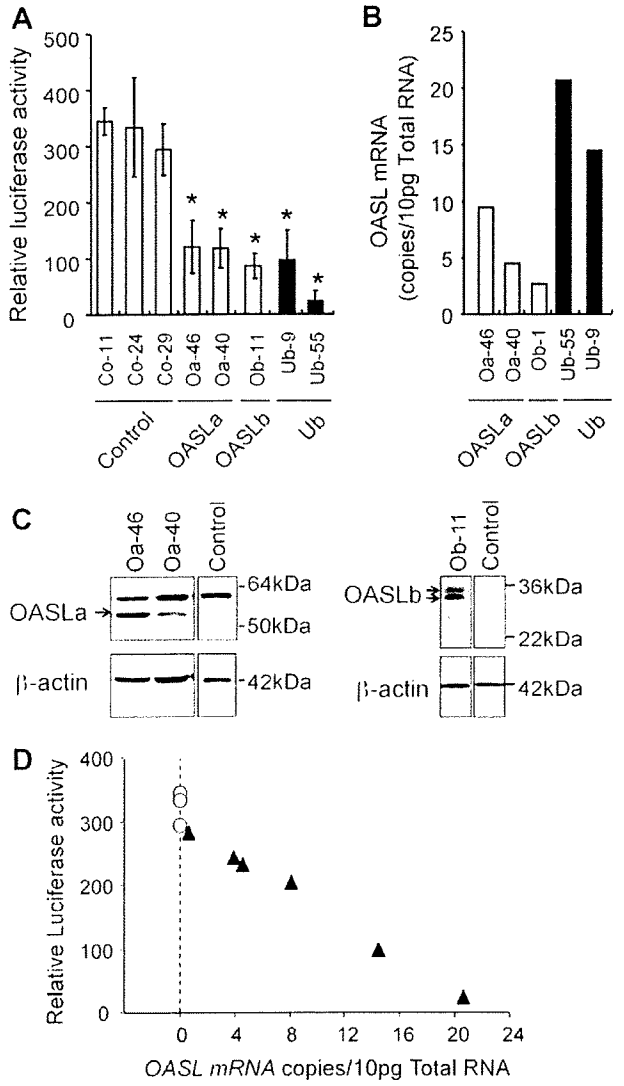

Fig. 4. Luciferase reporter assay of HCV replication (A, D) in Huh7 clones with stable expression of OASLa, OASLb, and Ub (B, C). (A) Relative luciferase activity of different stable clones. Luciferase activity was measured 4 h and 48 h after transfection with pSGR-JFH1/Luc in triplicate, and the relative luciferase activity was expressed as the ratio between the two values. Stable clones transfected with pEF6/V5-His vector were used as controls (Co). *$P < 0.05$. (B) Quantity of OASL mRNA in established cell clones. (C) Western blot analyses of OASLa and OASLb in established cell clones. OASLa, 54 kDa; OASLb, 33 kDa, 35 kDa. (D) Correlation of HCV RNA replication and OASL mRNA quantity in Ub stable clones. The relationship between the relative luciferase activity and OASL mRNA quantity of stable clones was analyzed by Pearson's correlation analysis. Open circle, pEF6; closed triangle, Ub ($P < 0.0001$, $r = -0.997$).

through two different domains. Regarding practical aspects of anti-HCV therapy, enhancement of IFN signaling is known to be useful. However, considering the tolerance to IFN signaling in chronic hepatitis C patients in whom it is pre-activated by HCV infection [17], it is clear that ISGs functional against HCV must be selected for induction by the therapy. OASL is a candidate for clinical application, and a truncated OASL, Ub, may be rather more useful as an anti-HCV molecule lacking toxicity. Further study on viral counteraction against OASL is also required to clarify the reason why a high level of OASL is not sufficient to reduce the HCV load in patients' livers.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.01.034.

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Identification of hepatitis C virus genotype 2a replicon variants with reduced susceptibility to ribavirin

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ABSTRACT

Ribavirin (RBV), a nucleoside analogue, is used in the treatment of hepatitis C virus (HCV) infection in combination with interferons. However, potential mechanisms of RBV resistance during HCV replication remain poorly understood. Serial passage of cells harboring HCV genotype 2a replicon in the presence of RBV resulted in the reduced susceptibility of the replicon to RBV. Transfection of fresh cells with RNA from RBV-resistant replicon cells demonstrated that the RBV resistance observed is largely replicon-derived. Four major amino acid substitutions: T1134S in NS3, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B region, were identified. Site-directed mutagenesis of these mutations into the replicon indicated that Y2471H plays a role in the reduced susceptibility to RBV and leads to decrease in replication fitness. The results, in addition to analysis of sequence database, suggest that HCV variants with reduced susceptibility to RBV identified are preferential to genotype 2a.

1. Introduction

Hepatitis C virus (HCV) is a leading cause of chronic liver diseases, such as chronic hepatitis, cirrhosis and hepatocellular carcinoma, affecting approximately 170 million people worldwide (WHO, 2000). HCV belongs to the genus Hepacivirus of the family Flaviviridae, and its genome is a single-stranded, positive-sense RNA of 9.6 kb. HCV displays marked genetic heterogeneity and is currently classified into 6 major genotypes and more than 50 subtypes. HCV genotypes have regional distribution and, of those, genotypes 1 and 2 are detected worldwide (Simmonds et al., 2000). Current standard therapy for chronic hepatitis C consists of the combination of pegylated interferon alpha (IFN-α) in combination with ribavirin (RBV). However, approximately 50% of treated patients infected with genotype 1 do not respond or show only a partial or transient response and treatment is limited by the adverse effects of both agents (Manns et al., 2001; Fried et al., 2002).

HCV replication is associated with a high rate of mutation that gives rise to a mixed and changing population of mutants, known as quasispecies (Martell et al., 1992; Domingo, 1996). The characteristic of HCV may have important implications concerning viral persistence, pathogenicity and resistance to antiviral agents (Domingo, 1996; Forns et al., 1999; Farci and Purcell, 2000). Most previous studies on the possible relationship between HCV quasispecies and response to chemotherapy have been carried out in HCV genotype 1 patients. In addition, several studies have successfully demonstrated that the HCV subgenomic replicon is derived from genotype 1, which typically contains HCV nonstructural genes placed downstream of the neomycin phosphotransferase gene, in selecting variants resistant to antiviral inhibitors. Two studies have demonstrated the identification of HCV genotype 1 mutants responsible for decreased sensitivity to RBV (Young et al., 2003; Pfeiffer and Kirkegaard, 2005). However, little is known about the generation of genotype 2 isolates resistant to antivirals including RBV, or the molecular mechanisms that confer resistance.

In this study, we report the generation and characterization of HCV genotype 2a replicon variants with reduced susceptibility to RBV. The impacts of major amino acid substitutions observed on RBV susceptibility and viral replication capacity were also examined.

2. Materials and methods

2.1. Compounds

RBV and IFN-α were purchased from MP Biomedicals (Eschwege, Germany) and Dainippon Sumitomo Pharma (Osaka, Japan), respectively.

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Table 1
Primers used for PCR and nucleotide sequencing.

Region	Primer name	Nucleotide sequence	Position[a]	Polarity
NS3–4A–4B region	PCR primers			
	JF1S	GAAAAACACGATGATACCATG	1756–1776	Sense
	JF1AS	AACCCAGTCCCACACGTC	4650–4633	Antisense
	Sequencing primers			
	JF5S	CACTTTCAGTGACAACAGCA	2322–2341	Sense
	JF6S	CGCCACCGACGCCCTCATGA	3003–3022	Sense
	JF4AS	CTGGTCGACAACGGACTGGT	4109–4090	Antisense
NS5A–NS5B region	PCR primers			
	JF2S	TGCTCCGGATCCTGGCTC	4612–4629	Sense
	JF2AS	TACCTAGTGTGTGCCGCTCTA	7786–7806	Antisense
	Sequencing primers			
	JF3S	TGAGGTCCATGCTAACAGA	5209–5228	Sense
	JF4S	TCGAGGGGGAGCCTGGAGAT	5870–5889	Sense
	JF3AS	GAGTGTCTAACTGTTTCCCAG	7220–7200	Antisense

[a] Reference strain: Gene Bank accession no. AB114136.

2.2. Cell culture

The human hepatoma cell line Huh-7 was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with MEM non-essential amino acids (Invitrogen) 100 units/ml penicillin, 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 incubator. HCV replicon cells JFH-1/4-1 (Miyamoto et al., 2006), which are Huh-7-derived cells carrying a subgenomic replicon of JFH-1 (Kato et al., 2003) were maintained in the Huh-7 medium as above, supplemented with 1 mg/ml G418 (Nacalai Tesque, Kyoto, Japan).

2.3. Quantification of HCV RNA

Total RNA was isolated from harvested cells using Trizol (Invitrogen). Copy numbers of the viral RNA were determined by real-time RT-PCR involving single-tube reactions and performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, CA, USA), as described previously (Aizaki et al., 2003; Takeuchi et al., 1999).

2.4. Cell viability assay

Cells were seeded at density of 5×10^4 cells/well in 24-well plates and RBV at various concentrations was added on the next day. Cultures were further incubated for 3 days at 37 °C under a humidified 5% CO_2 atmosphere. Cytotoxicity assay was performed by Cell Titer-GLO™ Luminescent Cell Viability Assay (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luciferase activities were quantified with LUMAT LB 9501 (Berthold Technologies, Bad Wilbad, Germany).

2.5. Isolation and nucleotide sequencing of HCV nonstructural regions from replicon-containing cells

Total cellular RNA was isolated from replicon cells with or without RBV treatment as described above. cDNA synthesis was carried out by using Super ScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) with primer JF1AS for NS34AB region and JF2AS for NS5AB region. Two cDNA fragments, corresponding to NS3–NS4B and NS5A–NS5B regions, were amplified by PCR using Takara EX Taq DNA polymerase (Takara BIO, Kyoto, Japan) and specific primers (Table 1; Date et al., 2004). PCR products were subcloned into pGEM-T vector (Promega) and inserts were sequenced using QIA prep^R Spin Mini Prep kit (QIAGEN, Tokyo, Japan). Nucleotide sequences were analyzed with the 3100 Avant Genetic Analyzer (PE Applied Biosystems).

2.6. Plasmid constructions

pSGR-JFH1/luc, a subgenomic replicon construct with luciferase reporter derived from HCV genotype 2a JFH-1 isolate was reported previously (Miyamoto et al., 2006). Mutant replicons carrying T1134S, P1969S, V2405A, and Y2471H were created by PCR-based site-directed mutagenesis and cDNA fragments containing the above mutations were inserted into the corresponding sites of pSGR-JFH/luc. All plasmids were confirmed by sequencing the entire PCR-generated inserts. Each mutant is referred to by the original amino acid (one letter code) followed by the residue positions within the complete open reading frame of full-length JFH-1 and the substituted amino acid (one letter code).

2.7. RNA synthesis and transient replication assay

The transient replication assay method was described previously (Kato et al., 2005). Briefly, purified plasmids of pSGR-JFH1/Luc, -JFH1/Luc-T1134S, -JFH/Luc-P1969S, -JFH/Luc-V2405A and -JFH/Luc-Y2471H were linearized with XbaI and were treated with proteinase K and SDS, followed by phenol–chloroform extraction. RNA was synthesized with Ampliscribe™ T7 Transcription Kits (Epicentre BIO Technologies, Madison, WI, USA). Each transcribed RNA (5 μg) was electroporated into 2.5×10^6 of Huh7 cells pulsed at 290 mV, 975uFD with Gene pulser II apparatus (Bio-Rad Laboratories, Hercules, CA, USA). Transfected cells were resuspended in growth medium without selection antibiotics and were plated in 24-well plates at 6×10^4 cells per well. Cells were harvested at different time points post-transfection and were lysed in Passive Lysis Buffer (Promega). Luciferase activity in cells was determined using the Luciferase Assay System (Promega).

3. Results

3.1. Selection of replicon variants derived from genotype 2a with reduced susceptibility to RBV

It has been reported that RBV inhibits HCV RNA replication in Huh-7 cells bearing the viral subgenomic replicon RNAs with the EC_{50} (50% effective concentration) values of 15–225 μM (Zhou et al., 2003; Tanaka et al., 2004; Kato et al., 2005; aus dem Siepen et al., 2007). To select for RBV-associated replicon variants, cells bearing a genotype 2a HCV replicon were serially passed in the presence of 200 μM RBV as well as 1 mg/ml G418. After 20-week treatment, variant cells were then tested for RBV resistance. HCV RNA levels were determined after a 72-h incubation with various concentrations of RBV in the absence of G418, and about 5-fold-reduced susceptibility to RBV was observed in the variant replicon

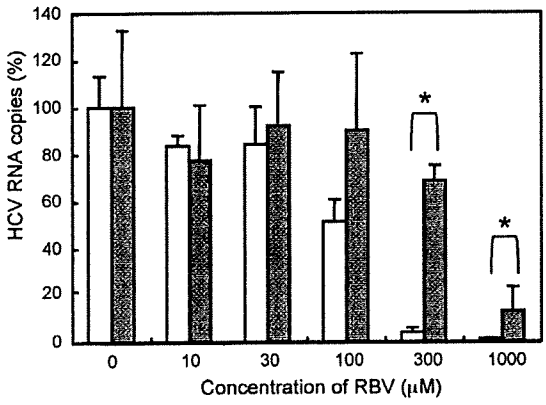

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV: gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. $^*p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

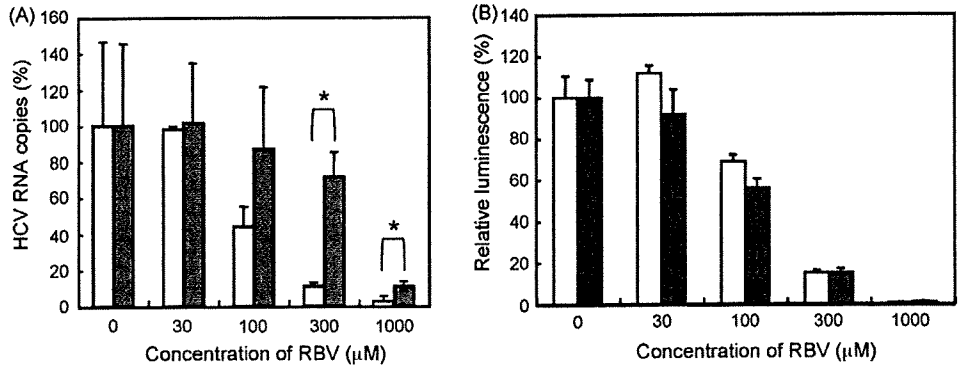

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. $^*p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

[a] Values are means ± standard deviations.
[b] $p < 0.05$ relative to No-treatment by the unpaired t-test.
[c] $p < 0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

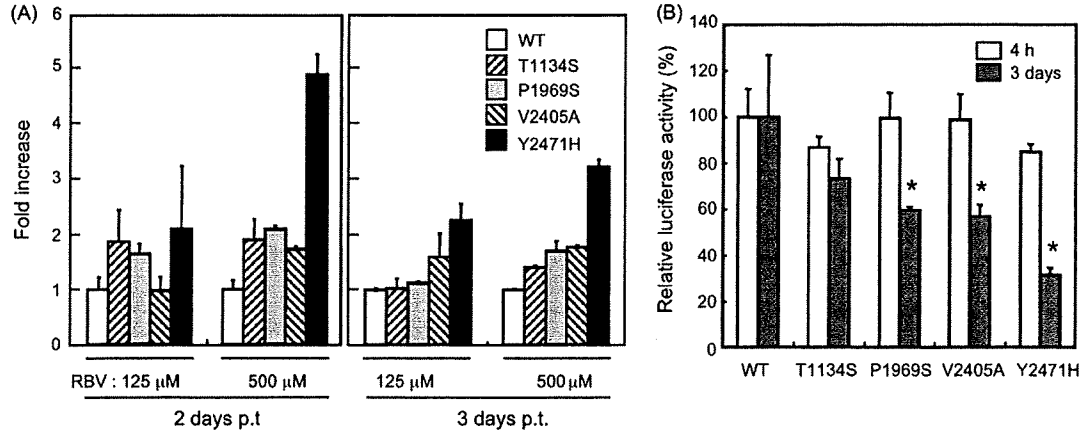

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p < 0.05$ against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

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