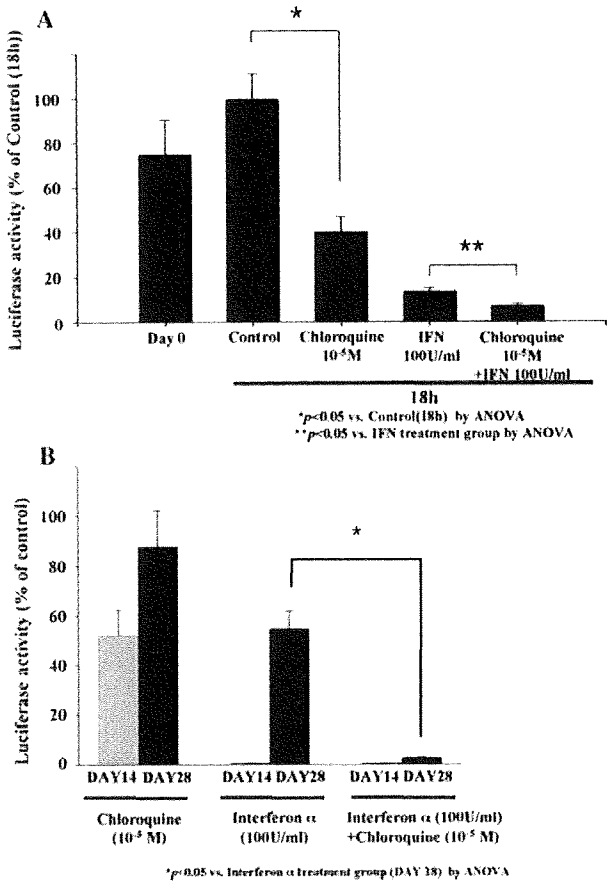

Fig. 4 Combination effect of chloroquine with IFNα on HCV replication. a Huh-7 Rep/Feo cells were treated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 18 h. Values are shown as percentages of the control cells [$*P < 0.05$ vs. control (18 h) by ANOVA, $**P < 0.05$ vs. IFNα treatment group by ANOVA]. b Assessment of re-propagation of HCV replicon after long term treatment of chloroquine and/or IFNα. Huh-7 Rep/Feo was incubated with chloroquine (10^{-5} M) and/or IFNα (100 U/ml) for 7 days, then drugs were removed from the medium and incubation continued for another 21 days. Luciferase assay was performed at the 7th and 21st days from cessation of drugs. Values are shown as percentages of the control cells [$*P < 0.05$ vs. IFNα treatment group (day 28) by ANOVA]

resulted in a significant decrease of HCV replicon to about 40% of control. On the other hand, incubation with IFNα for 18 h inhibited the replication of HCV replicon to the levels about 15% of controls as expected. However, co-incubation with 100U/ml of IFNα and 10^{-5} M of chloroquine further decreased HCV replication significantly (Fig. 4a).

To determine whether long-term chloroquine treatment inhibits post-treatment re-propagation of HCV replicon, we followed up luciferase activity of the cells at the 7th and 21st days after 7 days of treatment with chloroquine and/or IFNα (Fig. 4b). In HCV replicon cells treated by chloroquine, luciferase activities recovered to 53 and 88% on 7 and 21 days after cessation of treatment. In cells that were treated by IFNα, luciferase activity maintained background level for 7 days post-treatment. However, it reappeared in 21 days. In sharp contrast, co-incubation with IFNα and chloroquine for 7 days suppressed HCV replication for the extensive period up to 21 days, even in the absence of these drugs (Fig. 4b).

Anti-HCV effect of chloroquine independent of IFN signaling pathway

IFN-inducible double-stranded RNA-activated protein kinase R (PKR) plays a key antiviral role against hepatitis C virus [26, 27]. To elucidate the mechanisms of the inhibitory effect of chloroquine on HCV replication, phosphorylated PKR (P-PKR) was evaluated by western blotting analysis. P-PKR was detectable in cells treated with IFNα after 24 h; this increase in P-PKR expression peaked at 24 h after IFNα treatment and was reduced at 48 h (Fig. 5a). In contrast, P-PKR was not observed in cells treated with chloroquine at any time point.

Chloroquine blunts autophagic proteolysis in cells transfected with HCV replicon

It is reported that chloroquine disrupts lysosomal function, preventing effective autophagic protein degradation, leading to the accumulation of ineffective autophagosomes [28]. Therefore, we investigated if chloroquine led to the accumulation of autolysosomes as a result of suppression of proteolysis. We performed electron microscopic investigation to evaluate quantities of autophagosomes and autolysosomes. Ultrastructural analysis identified 0.94 ± 0.1 vacuoles/100 μm^2 of autolysosomes in control cells; however, treatment with chloroquine increased the number of autolysosomes dramatically to about 13-fold over control (Fig. 5b). Furthermore, the molecular form of LC3 protein of the cells, which is a component of autophagosomes, was examined by western blot analysis to ensure that chloroquine treatment leads to the accumulation of autophagosomes and autolysosomes. As shown in Fig. 5c, immunopositive protein bands for LC3-I and LC3-II forms were clearly evident in control cells. After chloroquine treatment, LC3-II expression increased at 4 h (Fig. 5c) to about threefold over control without enhancing LC3-I expression, and at 8 h (Fig. 5c) LC3-II expression was further enhanced. Finally, we evaluated turnover of the long-lived protein leucine, which was mainly degraded by autophagy. Huh7/Rep-Feo cells were labeled with [^{14}C]leucine for 24 h, and degradation of [^{14}C]leucine in cells treated with or without chloroquine was measured. Chloroquine treatment decreased degradation of leucin to 76% of control, indicating that chloroquine blunts degradation of proteins via an autophagic

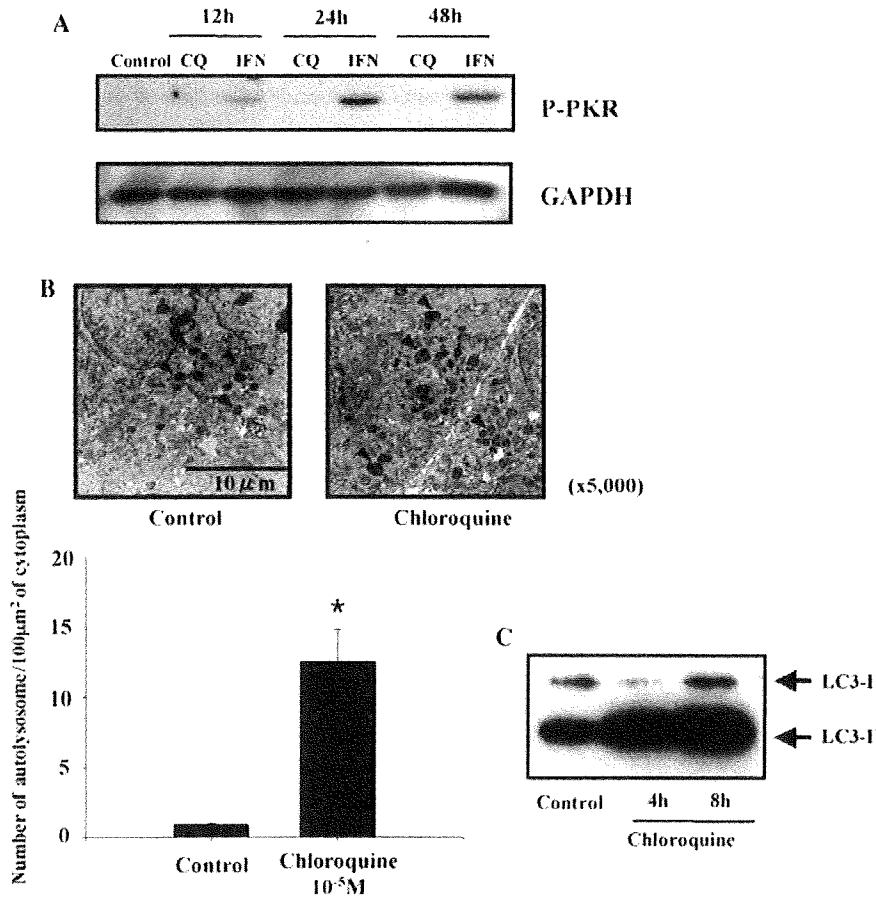

Fig. 5 Chloroquine suppresses autophagic protein degradation, not interferon pathways. **a** Cells were treated with 10^{-5} M of chloroquine (CQ) or 100 U/ml of IFNα for 24–48 h. Phosphorylation of PKR was assessed by western blot analysis. GAPDH was used as loading control. **b** Ultrastructural analysis showing the effect of chloroquine on the number of autolysosomes. Huh-7/Rep-Feo cells were incubated with chloroquine for 18 h. Autolysosomes were identified as the double membrane vesicles (arrow heads) of cytoplasm in Huh-7 Rep/Feo. The number of autolysosomes in 100 μm^2 of cytoplasm was counted by using transmission electron microscopy. Data represent mean ± SEM of individual preparations from pictures (*$P < 0.05$ vs. control by ANOVA). **c** Western blot analysis of LC3 in Huh7 Rep/Feo. The lysate of Huh7 Rep/Feo treated with chloroquine for 4–8 h were immunoblotted with LC3. GAPDH was used as loading control

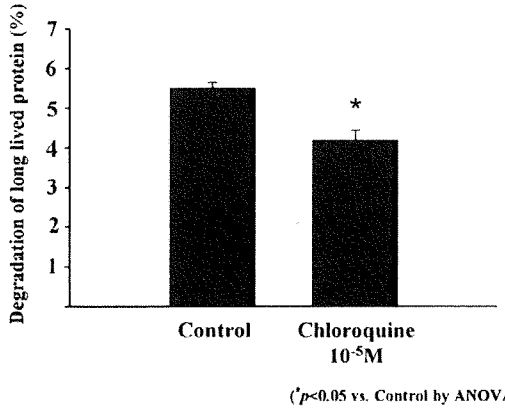

Fig. 6 Turnover of long lived protein. Huh-7 Rep/Feo cells were labeled with [^{14}C]leucin for 4 h, then degradation of long-lived protein in chloroquine treated cells was measured as described in Materials and Methods. The percentage of protein degradation was calculated by dividing the amount of acid-soluble radioactivity in the medium at that time by the amount of acid-precipitable radioactivity present in the cells at time zero. Data are mean ± SEM of value of triplicate in each group (*$P < 0.05$)

pathway (Fig. 6). These results demonstrate that chloroquine-induced the accumulation of autolysosomes was due to disruption of autophagic proteolysis.

Discussion

Previous reports have disclosed that autophagy plays a pivotal role on the replication of several RNA viruses [10–12]. Our present results demonstrate that autophagy is induced by transfection of HCV replicon and is reduced by deletion of replicon due to IFNα (Fig. 1a, b). These results suggest that autophagy is induced in the presence of HCV replication in its host cells. However, the role of autophagy in the pathogenesis of HCV is largely unclear. We found that the inhibition of autophagosome formation and autophagic proteolysis blunt the replication of genotype 1b subgenomic HCV replicon (Fig. 2a, c). Sir et al. [13] reported that inhibition of autophagy also reduced the replication of the JFH1-based full length genotype 2a genome. Therefore, the utilization of autophagy on viral replication is shown by HCV strains across different genotypes.

On the other hand, not only a silencing of autophagic gene but also pharmacological inhibition of autophagic proteolysis possesses anti-HCV effects (Fig. 2a, c). However, treatment with both chloroquine and the mixture of E64d and pepstatin induced the accumulation of

autophagosomes in cytoplasm. Therefore, it is likely that HCV does not utilize the double membrane structure as the localization of the viral replication formation. These results support the hypothesis that protein degradation due to autophagy is important for HCV replication.

Chloroquine is a well-known inhibitor of autophagic protein degradation and is often used as an anti-malarial agent. Moreover, the anti-viral effect of chloroquine on other RNA viruses has been already reported in clinical trials [15, 16]. In our results, chloroquine inhibits the intracellular replication of an HCV replicon in a dose-dependent manner (Fig. 3a). This antiviral effect of chloroquine was clearly not due to cytotoxic effects (Fig. 3b). Moreover, chloroquine possesses a synergistic effect with IFNα on HCV replication (Fig. 4a). Although IFNα possesses strong anti-HCV effects, re-propagation of HCV replicon was observed after 3 weeks following 7 days of treatment with IFNα. Interestingly, co-incubation with IFNα and chloroquine for 7 days prevented re-propagation of HCV replicon (Fig. 4b). Chloroquine is a lysosomal weak base that is known to affect acid vesicles leading to dysfunction of several proteins [29]. It was demonstrated that disruption of lysosomal function impairs maturation of viruses through inhibiting the low-pH dependent proteases in trans-Golgi vesicles in HIV and the SARS coronavirus infection in vitro [15, 29]. However, little is understood about the mechanism of its antiviral effect. In previous reports, various drugs which possess inhibitory effects on the replication of HCV and have a synergistic action with IFNα have been proposed as new therapeutic agents to treat HCV. Some of them have proved to exhibit their anti-HCV effects through augmentation of IFN-induced antiviral gene responses [30, 31]. However, the anti-HCV effect of chloroquine was not associated with activation of one of IFN receptors signaling molecule PKR (Fig. 5a). Our results showed chloroquine induced the accumulation of ineffective autophagosomes in cytoplasm of Huh7/Rep-Feo cells (Fig. 5b) and inhibited the degradation of long-lived protein leucine (Fig. 6). These findings imply that chloroquine effectively impairs the function of autophagy in our experiment. These results indicated that chloroquine is a new anti-HCV agent that targets the autophagic proteolysis.

Previous reports have shown that chloroquine possesses anti-viral effects on various RNA viruses. Its best-studied effects are those against HIV replication, which are being tested in clinical trials [17, 18]. HCV co-infection is common in HIV-positive patients in USA and Europe [32, 33]. Since HIV infection accelerates the progression of HCV-related liver disease, treatment of HCV is generally recommended. However, co-infected patients have a greater risk of antiretroviral therapy-associated hepatotoxicity than patients with HIV only [34]. Moreover, treatment with ribavirin is believed to increase the risk of anemia in patients taking the HIV drug zidovudine [35]. A clinical study designed for HIV patients showed the safety and efficacy of chloroquine used for long terms up to 48 weeks [36]. Therefore, the combination therapy of interferon and chloroquine is, possibly, a hopeful therapy for HCV–HIV co-infected patients. Since chloroquine is known as one of the inexpensive drugs, therefore, chloroquine might provide a new effective, safe and economical therapeutic option for patients with HCV. In conclusion, autophagic proteolysis might be a new therapeutic target on the replication of HCV.

References

1. Yotsuyanagi H, Koike K. Drug resistance in antiviral treatment for infections with hepatitis B and C viruses. J Gastroenterol. 2007;42:329–35.
2. Bruno S, Stroffolini T, Colombo M, Bollani S, Benvegnù L, Mazzella G, et al. Sustained virological response to interferon-alpha is associated with improved outcome in HCV-related cirrhosis: a retrospective study. Hepatology. 2007;45:579–87.
3. Shiffman ML, Cooksley WG, Dusheiko GM, Lee SS, Balart L, Reindollar R, et al. Peginterferon alfa-2a in patients with chronic hepatitis C and cirrhosis. N Engl J Med. 2000;343:1673–80.
4. Sezaki H, Suzuki F, Kawamura Y, Yatsuji H, Hosaka T, Akuta N, et al. Poor response to pegylated interferon and ribavirin in older women infected with hepatitis C virus of genotype 1b in high viral load. Dig Dis Sci. 2009;54:1317–24.
5. Honda T, Katano Y, Urano F, Murayama M, Hayashi K, Ishigami M, et al. Efficacy of ribavirin plus interferon-α in patients aged 60 years with chronic hepatitis C. J Gastroenterol Hepatol. 2007;22:989–95.
6. Wang CW, Klionsky DJ. The molecular mechanism of autophagy. Mol Med. 2003;9:65–76.
7. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000;290:1717–21.
8. Reggiori F, Klionsky DJ. Autophagy in the eukaryotic cell. Eukaryot Cell. 2002;1:11–21.
9. Prentice E, Jerome WG, Yoshimori T, Mizushima N, Denison MR. Coronavirus replication complex formation utilizes components of cellular autophagy. J Biol Chem. 2004;279:10136–41.
10. Randolph VB, Winkler G, Stollar V. Acidotropic amines inhibit proteolytic processing of flavivirus prM protein. Virology. 1990;174:450–8.
11. Posthuma CC, Pedersen KW, Lu Z, Joosten RG, Roos N, Zevenhoven-Dobbe JC, et al. Formation of the arterivirus replication/transcription complex: a key role for nonstructural protein 3 in the remodeling of intracellular membranes. J Virol. 2008;82:4480–91.
12. Suhy DA, Giddings TH Jr, Kirkegaard K. Remodeling the endoplasmic reticulum by poliovirus infection and by individual viral proteins: an autophagy-like origin for virus-induced vesicles. J Virol. 2000;74:8953–65.
13. Sir D, Chen WL, Choi J, Wakita T, Yen TS, Ou JH. Induction of incomplete autophagic response by hepatitis C virus via the unfolded protein response. Hepatology. 2008;48:1054–61.

14. Poole B, Ohkuma S. Effect of weak bases on the intralysosomal pH in mouse peritoneal macrophages. J Cell Biol. 1981;90: 665–9.
15. Vincent MJ, Bergeron E, Benjannet S, Erickson BR, Rollin PE, Ksiazek TG, et al. Chloroquine is a potent inhibitor of SARS coronavirus infection and spread. Virol J. 2005;2:69.
16. Savarino A, Gennero L, Chen HC, Serrano D, Malavasi F, Boelaert JR, et al. Anti-HIV effects of chloroquine: mechanisms of inhibition and spectrum of activity. AIDS. 2001;15:2221–9.
17. Savarino A, Boelaert JR, Cassone A, Majori G, Cauda R. Effects of chloroquine on viral infections: an old drug against today's diseases? Lancet Infect Dis. 2003;3:722–7.
18. Sperber K, Chiang G, Chen H, Ross W, Chusid E, Gonchar M, et al. Comparison of hydroxychloroquine with zidovudine in asymptomatic patients infected with human immunodeficiency virus type 1. Clin Ther. 1997;19:913–23.
19. Paton NI, Aboulhab J. Hydroxychloroquine, hydroxyurea and didanosine as initial therapy for HIV-infected patients with low viral load: safety, efficacy and resistance profile after 144 weeks. HIV Med. 2005;6:13–20.
20. Tanabe Y, Sakamoto N, Enomoto N, Kurosaki M, Ueada E, Maekawa S, et al. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. J Infect Dis. 2004;189:1129–39.
21. Yokota T, Sakamoto N, Enomoto N, Tanabe Y, Miyagishi M, Maekawa S, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep. 2003;4:602–8.
22. Seglen PO, Gordon PB. 3-Methyladenine: specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. Proc Natl Acad Sci USA. 1982;79:1889–92.
23. Ueno T, Ishidoh K, Mineki R, Tanida I, Murayama K, Kadowaki M, et al. Autolysosomal membrane-associated betaine homocysteine methyltransferase. Limited degradation fragment of a sequestered cytosolic enzyme monitoring autophagy. J Biol Chem. 1999;274:15222–9.
24. Klionsky DJ, Emr SD. Autophagy as a regulated pathway of cellular degradation. Science. 2000;290:1717–21.
25. Gronostajski RM, Pardee AB. Protein degradation in 3T3 cells and tumorigenic transformed 3T3 cells. J Cell Physiol. 1984;119:127–32.
26. Clemens MJ. PKR–a protein kinase regulated by double-stranded RNA. Int J Biochem Cell Biol. 1997;29:945–9.
27. Gale MJ Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology. 1997;230:217–27.
28. Glaumann H, Ahlberg J. Comparison of different autophagic vacuoles with regard to ultrastracture, enzymatic composition, and degradation capacity—formation of crinosomes. Exp Mol Pathol. 1987;47:346–62.
29. Thorens B, Vassalli P. Chloroquine and ammonium chloride prevent terminal glycosylation of immunoglobulins in plasma cells without affecting secretion. Nature. 1986;321:618–20.
30. Dev A, Patel K, McHutchison JG. New therapies for chronic hepatitis C virus infection. Curr Gastroenterol Rep. 2004;6:77–86.
31. Lin K, Kwong AD, Lin C. Combination of a hepatitis C virus NS3-NS4A protease inhibitor and alpha interferon synergistically inhibits viral RNA replication and facilitates viral RNA clearance in replicon cells. Antimicrob Agents Chemother. 2004;48:4784–92.
32. Staples CT Jr, Rimland D, Dudas D. Hepatitis C in the HIV (human immunodeficiency virus) Atlanta V.A. (Veterans Affairs Medical Center) Cohort Study (HAVACS): the effect of coinfection on survival. Clin Infect Dis. 1999;29:150–4.
33. Denis F, Adjide CC, Rogez S, Delpeyroux C, Rogez JP, Weinbreck P. Seroprevalence of HBV, HCV and HDV hepatitis markers in 500 patients infected with the human immunodeficiency virus. Pathol Biol (Paris). 1997;45:701–8.
34. Sulkowski MS, Benhamou Y. Therapeutic issues in HIV/HCV-coinfected patients. J Viral Hepat. 2007;14:371–86.
35. Alvarez D, Dieterich DT, Brau N, Moorehead L, Ball L, Sulkowski MS. Zidovudine use but not weight-based ribavirin dosing impacts anaemia during HCV treatment in HIV-infected persons. J Viral Hepat. 2006;13:683–9.
36. Paton NI, Aboulhab J, Karim F. Hydroxychloroquine, hydroxycarbamide, and didanosine as economic treatment for HIV-1. Lancet. 2002;359:1667–8.

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Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae radix*. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneo-delS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

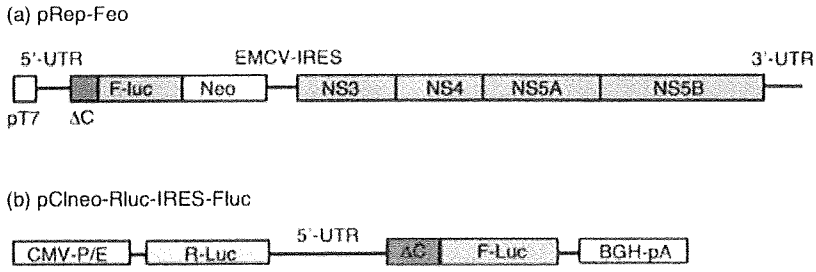

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3' UTR, 3' untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency. The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[9] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

Statistical analyses were performed using Student's *t*-test. *P*-values of less than 0.05 were considered statistically significant.

RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma*, and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix*, suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

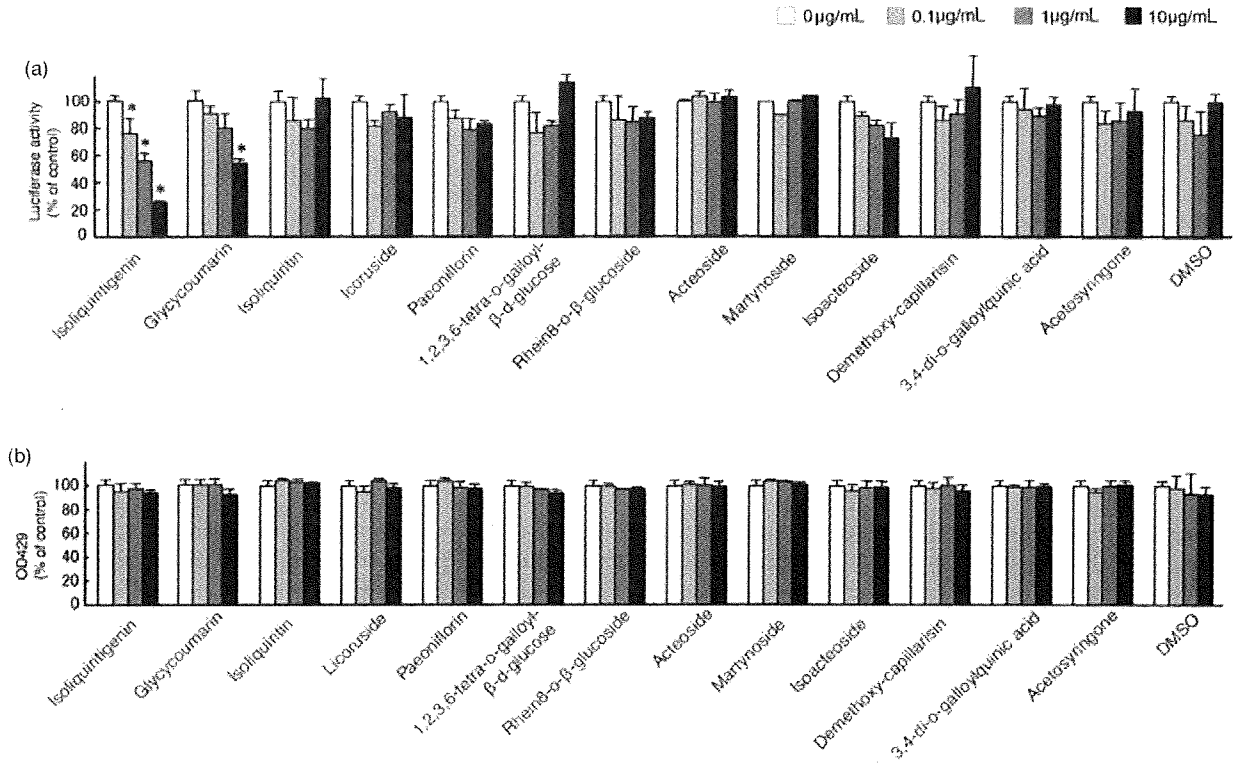

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

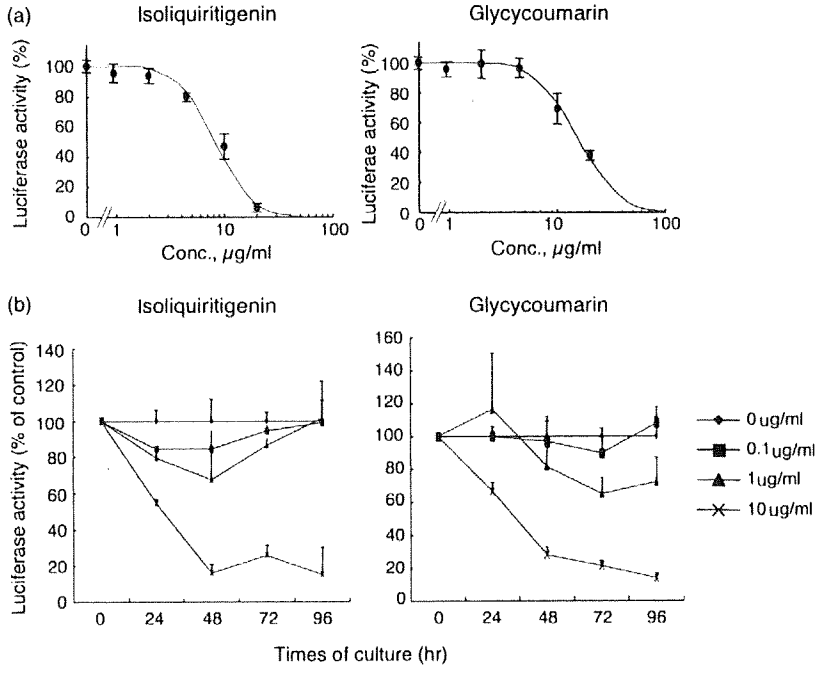

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

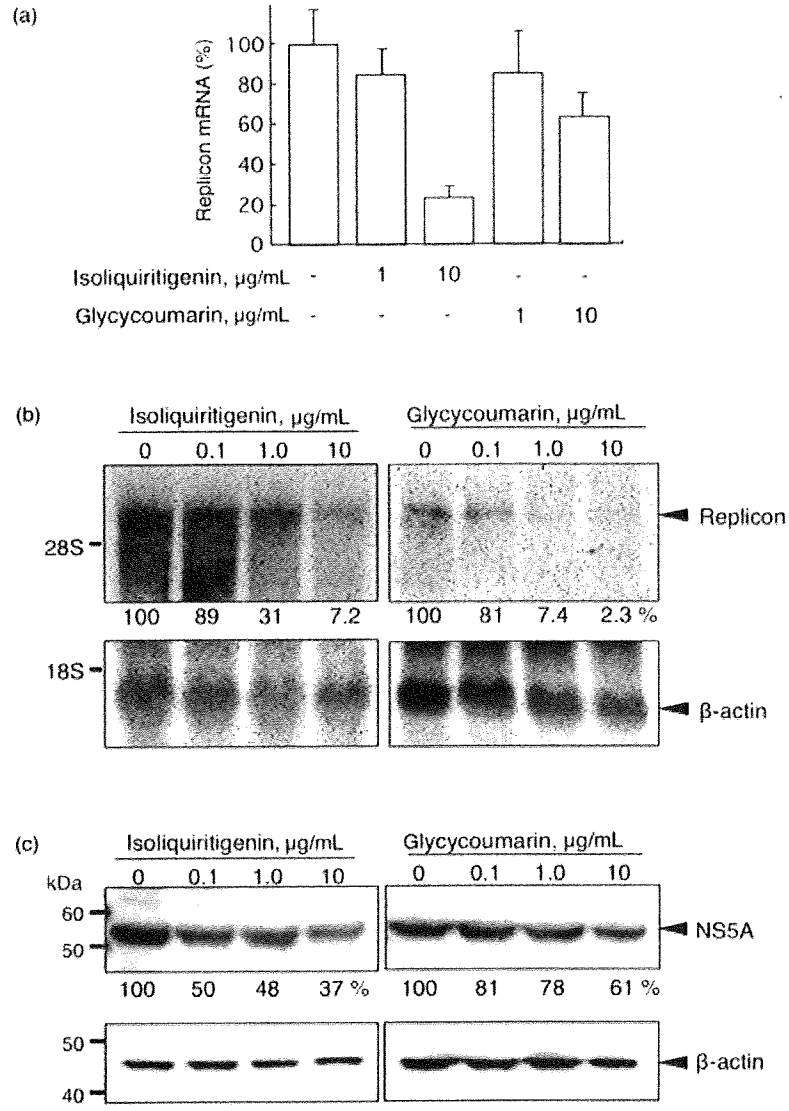

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycycoumarin than the untreated culture. The effect of glycycoumarin was partly reversed on day six probably due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycycoumarin (Fig. 7b).

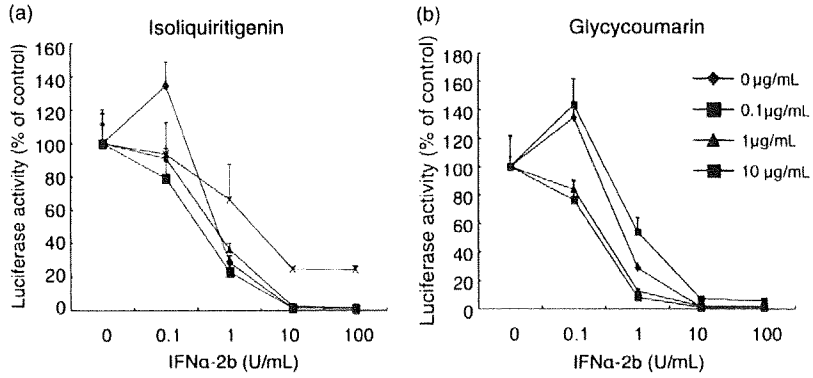

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

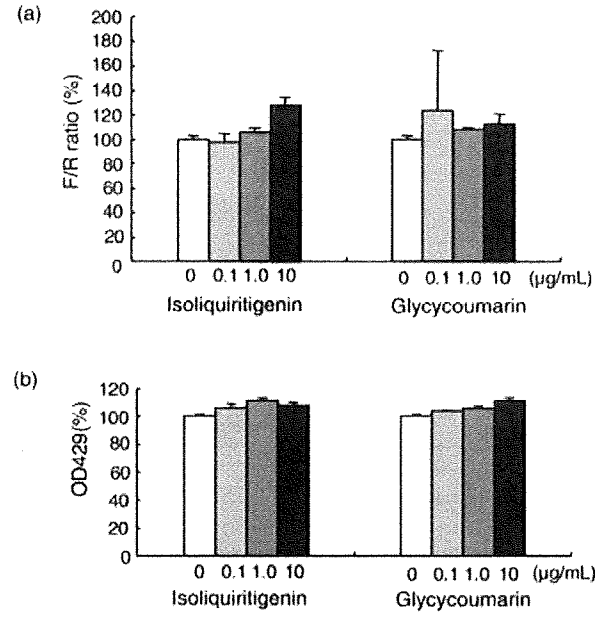

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.

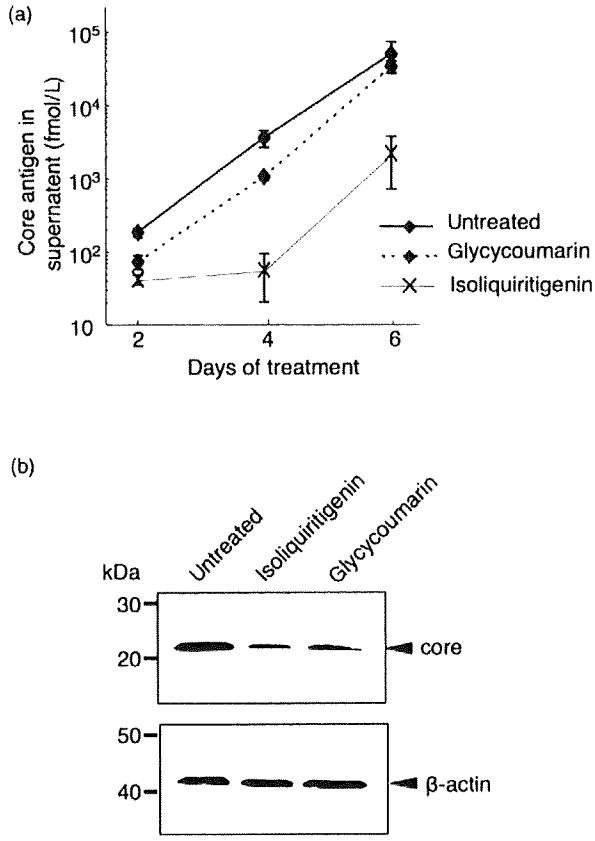

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes, Haemophilus infuenzae* and *Moraxella catarrhalis*,[36] and methicillin-resistant *Staphylococcus aureus*,[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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REFERENCES

1 Alter MJ. Epidemiology of hepatitis C. *Hepatology* 1997; 26: 62S–65S.
2 Tong MJ, el-Farra NS, Reikes AR, Co RL. Clinical outcomes after transfusion-associated hepatitis C. *N Engl J Med* 1995; 332: 1463–6.
3 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; 347: 975–82.

4 Hadziyannis SJ, Sette H Jr, Morgan TR *et al.* Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. *Ann Intern Med* 2004; **140**: 346–55.
5 Hayashi N, Takehara T. Antiviral therapy for chronic hepatitis C: past, present, and future. *J Gastroenterol* 2006; **41**: 17–27.
6 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon-alpha. *J Infect Dis* 2004; **189**: 1129–39.
7 Watashi K, Hijikata M, Hosaka M, Yamaji M, Shimotohno K. Cyclosporin A suppresses replication of hepatitis C virus genome in cultured hepatocytes. *Hepatology* 2003; **38**: 1282–8.
8 Nakagawa M, Sakamoto N, Enomoto N *et al.* Specific inhibition of hepatitis C virus replication by cyclosporin A. *Biochem Biophys Res Commun* 2004; **313**: 42–7.
9 Nakagawa M, Sakamoto N, Tanabe Y *et al.* Suppression of hepatitis C virus replication by cyclosporin a is mediated by blockade of cyclophilins. *Gastroenterology* 2005; **129**: 1031–41.
10 Yokota T, Sakamoto N, Enomoto N *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; **4**: 602–8.
11 Kapadia SB, Brideau-Andersen A, Chisari FV. Interference of hepatitis C virus RNA replication by short interfering RNAs. *Proc Natl Acad Sci USA* 2003; **100**: 2014–18.
12 Frese M, Schwarzle V, Barth K *et al.* Interferon-gamma inhibits replication of subgenomic and genomic hepatitis C virus RNAs. *Hepatology* 2002; **35**: 694–703.
13 Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. *Hepatology* 2006; **44**: 117–25.
14 Kim SS, Peng LF, Lin W *et al.* A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. *Gastroenterology* 2007; **132**: 311–20.
15 Kanda T, Yokosuka O, Imazeki F *et al.* Inhibition of subgenomic hepatitis C virus RNA in Huh-7 cells: ribavirin induces mutagenesis in HCV RNA. *J Viral Hepat* 2004; **11**: 479–87.
16 Yamashiki M, Nishimura A, Suzuki H, Sakaguchi S, Kosaka Y. Effects of the Japanese herbal medicine "Sho-saiko-to" (TJ-9) on in vitro interleukin-10 production by peripheral blood mononuclear cells of patients with chronic hepatitis C. *Hepatology* 1997; **25**: 1390–7.
17 Oka H, Yamamoto S, Kuroki T *et al.* Prospective study of chemoprevention of hepatocellular carcinoma with Sho-saiko-to (TJ-9). *Cancer* 1995; **76**: 743–9.
18 Arase Y, Ikeda K, Murashima N *et al.* The long term efficacy of glycyrrhizin in chronic hepatitis C patients. *Cancer* 1997; **79**: 1494–500.
19 van Rossum TG, Vulto AG, Hop WC, Schalm SW. Glycyrrhizin-induced reduction of ALT in European patients with chronic hepatitis C. *Am J Gastroenterol* 2001; **96**: 2432–7.
20 Guo JT, Bichko VV, Seeger C. Effect of alpha interferon on the hepatitis C virus replicon. *J Virol* 2001; **75**: 8516–23.
21 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon- alpha. *J Infect Dis* 2004; **189**: 1129–39.
22 Itsui Y, Sakamoto N, Kurosaki M *et al.* Expressional screening of interferon-stimulated genes for antiviral activity against hepatitis C virus replication. *J Viral Hepat* 2006; **13**: 690–700.
23 Sakamoto N, Sato C, Haritani H *et al.* Detection of hepatitis C viral RNA in sporadic acute non-A, non-B hepatitis by polymerase chain reaction. Its usefulness for the early diagnosis of seronegative infection. *J Hepatol* 1993; **17**: 28–33.
24 Yamashiro T, Sakamoto N, Kurosaki M *et al.* Negative regulation of intracellular hepatitis C virus replication by interferon regulatory factor 3. *J Gastroenterol* 2006; **41**: 750–7.
25 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–6.
26 Zhong J, Gastaminza P, Cheng G *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294–9.
27 Sekine-Osajima Y, Sakamoto N, Nakagawa M *et al.* Development of plaque assays for hepatitis C virus and isolation of mutants with enhanced cytopathogenicity and replication capacity. *Virology* 2008; **371**: 71–85.
28 Arima H, Ashida H, Danno G. Rutin-enhanced antibacterial activities of flavonoids against Bacillus cereus and Salmonella enteritidis. *Biosci Biotechnol Biochem* 2002; **66**: 1009–14.
29 Musonda CA, Chipman JK. Quercetin inhibits hydrogen peroxide (H2O2)-induced NF-kappaB DNA binding activity and DNA damage in HepG2 cells. *Carcinogenesis* 1998; **19**: 1583–9.
30 Haraguchi H, Ishikawa H, Mizutani K, Tamura Y, Kinoshita T. Antioxidative and superoxide scavenging activities of retrochalcones in Glycyrrhiza inflata. *Bioorg Med Chem* 1998; **6**: 339–47.
31 Tawata M, Aida K, Noguchi T *et al.* Anti-platelet action of isoliquiritigenin, an aldose reductase inhibitor in licorice. *Eur J Pharmacol* 1992; **212**: 87–92.
32 Aida K, Tawata M, Shindo H *et al.* Isoliquiritigenin: a new aldose reductase inhibitor from glycyrrhizae radix. *Planta Med* 1990; **56**: 254–8.
33 Tamir S, Eizenberg M, Somjen D, Izrael S, Vaya J. Estrogen-like activity of glabrene and other constituents isolated from licorice root. *J Steroid Biochem Mol Biol* 2001; **78**: 291–8.

34 Kim DC, Choi SY, Kim SH *et al.* Isoliquiritigenin selectively inhibits H(2) histamine receptor signaling. *Mol Pharmacol* 2006; 70: 493–500.
35 Finney RS, Somers GF. The antiinflammatory activity of glycyrrhetinic acid and derivatives. *J Pharm Pharmacol* 1958; 10: 613–20.
36 Tanaka Y, Kikuzaki H, Fukuda S, Nakatani N. Antibacterial compounds of licorice against upper airway respiratory tract pathogens. *J Nutr Sci Vitaminol (Tokyo)* 2001; 47: 270–3.
37 Hatano T, Shintani Y, Aga Y, Shiota S, Tsuchiya T, Yoshida T. Phenolic constituents of licorice. VIII. Structures of glicophenone and glicoisoflavanone, and effects of licorice phenolics on methicillin-resistant Staphylococcus aureus. *Chem Pharm Bull (Tokyo)* 2000; 48: 1286–92.

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REVIEW

Viral factors influencing the response to the combination therapy of peginterferon plus ribavirin in chronic hepatitis C

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Abstract Hepatitis C virus (HCV) is a single-stranded RNA virus known for its high genetic variability owing to the lack of a proofreading mechanism of its RNA dependent RNA polymerase. Until now, numerous studies have been undertaken to clarify the correlation between pretreatment HCV genetic variability and the therapeutic response. Even with the recent combination therapy of peginterferon plus ribavirin for chronic hepatitis C, viral response is variable, and only half of treated patients could clear the virus [sustained viral response (SVR)]. In this review, the contribution of viral genetic variability affecting the treatment outcome is discussed according to each HCV genomic region.

Keywords Hepatitis C virus · Peginterferon plus ribavirin therapy · Viral predictive factor

Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases worldwide; 180 million people, or some 3% of the world's population, are infected with HCV. Seventy percent of acute infections become persistent, and 50–75% of patients with chronic HCV infection progress to hepatocellular carcinoma. Though interferon-based therapy for HCV has been greatly advanced, half of patients still cannot eradicate the virus [sustained virological response (SVR)] even with the most recent combination therapy of peginterferon plus ribavirin [1].

HCV has a 9.5 kb single positive stranded RNA genome, and contains a single open reading frame flanked by 5′ and 3′ untranslated regions (UTR). HCV is classified as hepacivirus, a family of flaviridae. HCV is known for its high mutation rate owing to the lack of proofreading activity of its RNA dependent RNA polymerase (1.4×10^{-3} to 1.9×10^{-3} substitutions/nucleotide/year [2, 3]). In accord with this mechanism, HCV presents a high degree of genetic variability, and the resultant molecular polymorphisms of HCV are suspected as one of the major causes determining the treatment responses.

HCV genotypes

By phylogenetic analysis, HCV is classified into six major genotypes, and then further classified into subtypes in each genotype determined by their genetic distances [4]. Among all the viral factors investigated, viral genotypes are the most important, and a well-established predictive factor determining the treatment outcome. Geographically, genotypes 1–3 are associated with worldwide epidemic, while genotypes 4–6 are endemic. In comparison among major genotypes 1–3, a high SVR rate (~84%) was observed in patients with genotype 2 or 3, while a low SVR rate(~42%) was observed in genotype 1 [5–7]. Comparing between genotypes 2 and 3, genotype 2 could have more favorable outcomes [8, 9]. The study of genotype 4 was mainly from Egypt, and the SVR rate was reported to be intermediate (55–69%) [10, 11]. In genotypes 5 and 6, the SVR rate has been considered to be intermediate between the SVR of genotype 1 and genotypes 2–3, but studies

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focusing on the response of genotype 5 and 6 are limited because of their minor distribution [12].

Genomic regions and the treatment response

5′UTR

The 5′UTR of the HCV genome is 341 nucleotides long, and is the most conserved region throughout the HCV genome among different HCV genotypes. The 5′UTR together with the first 30 nucleotides of the core region acts as an internal ribosome entry site (IRES) regulating the cap-independent translation of HCV RNA to polyprotein. Secondary and tertiary conformation of IRES has the critical role in the initiation of polyprotein translation, and an IRES contains four highly structured domains (Domain I–IV). Since the structures play a pivotal role in HCV replication, changes in the conformation of an IRES, as well as changes in primary nucleotide sequence, result in a decrease of efficiency of protein translation. Therefore, it was suspected that IRES heterogeneity might correlate with the response to interferon-based therapy clinically. Several studies for interferon-based therapy, including peginterferon plus ribavirin, have been undertaken to date, however, its clinical value as a predictive factor for therapy is still in question, since most of these studies showed conflicting results of the relationship between 5′UTR variability and treatment response [13–18].

Core

The core protein is considered to form the viral nucleocapsid of HCV, and its mature form consists of a secondary structure made of a large folded multimer of ~24 monomers. It is 21 kDa in size, and is separated into two domains, an N-terminal two-thirds hydrophilic domain (D1, residues 1–117) and a C-terminal one-third hydrophobic domain (D2, residues 118–170), respectively. The D1 domain contains many positively charged amino acids, and is implicated to bind RNA. The D2 domain is required for proper folding of domain D1. The mature core protein shares high homology among HCV genotypes. The core protein has been reported to interact with a variety of cellular proteins and to influence numerous host cell functions, such as its proapoptotic or antiapoptotic actions [19], immunomodulatory roles [20], or oxidative stress [21]. Recently, much attention has been paid to its relationships with liver steatosis, insulin resistance and hepatocellular carcinoma [22, 23]. HCV core proteins of genotype 3a and 1b were reported to interfere with the insulin signaling pathway in different ways depending on genotype-specific mechanisms [24].

As its contribution to the clinical treatment response, Akuta et al. first reported that substitutions of the amino acid 70 and 91 in the core protein were significantly related to the final outcome in the 48 weeks of interferon plus ribavirin combination therapy in 50 Japanese patients infected with genotype 1b HCV [25]. In successive studies, they reported that substitutions in those core regions were related to the final outcome, viral kinetics, early viral response, and extended 72 weeks of therapy [26–29]. They also reported substitution of core protein was associated with elevated alpha-fetoprotein, and hepatocarcinogenesis [29, 30]. Correlation of substitutions in the core protein in the treatment of interferon-based therapy was also reported in several other studies [31–33].

E2

E2 is a type I transmembrane protein 70 kDa in size which assembles with E1 protein forming a heterodimer to become the mature viral envelope. It is a glycoprotein possessing several potential conserved glycosylation sites. Because the protein is essential for the virus's entry into hepatocytes, E2 interacts with potential HCV receptors, CD81, SR-BI [34] and occludin [35]. In E2, hypervariable region 1 (HVR1) was identified in the first 27 amino acids of the E2 ectodomain. HVR1 is known for its significant genomic variability and is suspected to be the target of antibodies. Its significant genetic variability could be induced by antibody selection.

PePHD

A region between amino acid residues of E2 659–670 is well-conserved, and is known as the phosphorylation site of PKR/eIF-2α phosphorylation homology domain (PePHD). The PePHD motif is similar to the phosphorylation sites of PKR and eIF2alpha. The PePHD has been shown to interact with PKR, one of the important antiviral proteins of the host cell, and inhibit antiviral action of PKR in vitro, suggesting a possible mechanism of HCV for countering the antiviral effects of interferon [36–38]. According to those observations, mutations in this PePHD were suspected to influence the clinical response to interferon-based therapy. However, the results of those studies are conflicting, and its clinical importance as the predictive value for treatment outcome has been controversial. Though some studies supported its significance [39–42], other recent studies could not find evident correlations [43–49].

NS5A

NS5A is phosphorylated on multiple serine and threonine residues, and forms two distinct molecules of basal

phosphorylated form (p56) and hyperphosphorylated form (p58), being 56 and 58 kDa in size, respectively. The protein has three distinct domains (domains I, II, and III) being separated by low complexity sequences (LCS I and II). The study of the X-ray crystal structure analysis of domain I suggested that the NS5A is a dimer, and it forms a large putative RNA binding groove. Recent genetic study has shown many residues in domain II are essential for RNA replication, while domain III is less conserved and might be dispensable. Though the true function of NS5A is still under investigation, the protein is considered as a component of the HCV replication complex, where it modulates HCV replication through interaction with other viral proteins. Among all HCV proteins, NS5A has been most extensively explored for its relationship to interferon-based therapy.

ISDR and PKR-BD

The interferon sensitivity determining region (ISDR), located in the C-terminal half of NS5A, was originally identified as the 40 amino acid region (aa2209–2248) significantly related to the treatment outcome in the monotherapy of interferon-alpha in Japanese patients infected with genotype-1b HCV [50, 51]. The "mutant-type," having 4 or more mutations in the region, was associated with a high SVR rate (16/16: 100%), while the SVR rate was low in the "intermediate-type" [1–3 mutations: SVR rate 5/38 (13%)], or the "wild-type" [no mutation: SVR rate 30/30 (0%)]. Following studies from Japan were also concordant with the initial study [52–54]. However, controversy occurred as to the predictive value of ISDR since studies from Europe and North America did not necessarily report evident correlations between ISDR and treatment outcomes [55–60]. However, a recent meta-analysis study clearly confirmed its value, even in the Western countries [61]. Different results observed in North America and Europe might have been been caused partly by smaller rates of mutant-type patients in Western countries, and by the different treatment regimen in Japan compared to Western countries [62–66]. Though ISDR was found in the era of interferon monotherapy, its predictive value in the treatment outcome of the recent peginterferon plus ribavirin regimen has continued to be reported in most large cohort studies [26, 33, 67–69]. In searching for the biological ISDR function, Gale et al. reported that NS5A represses PKR through a direct interaction with the PKR binding domain (PKR-BD, aa2209–2274) and that the PKR-BD contains the ISDR [70]. Thus, they insisted that inactivation of PKR may be one mechanism by which HCV avoids the antiviral effects of interferon.

V3 domain and IRRDR

The V3 domain located in the C-terminal region of NS5A (aa2356–2379) was originally identified as a genomic region of genotype-1b HCV showing a marked heterogeneity between Japanese and American isolates [71]. A correlation of its mutations and the response to interferon-based therapy was first reported by Duverlie et al., and they reported that sequences of the V3 domain were highly conserved in resistant strains, but were highly variable in sensitive strains [72]. Most following studies also reported concordant results [46, 47, 68, 73, 74]. El-Shamy et al. reported a high degree of sequence variations in the V3 and the flanking pre-V3 regions (aa2334–2355) of NS5A, and they designated the region as the interferon/ribavirin resistance-determining region (IRRDR) (aa2334–2379). They reported that substitution number in the IRRDR was closely correlated with early virological response (EVR) by week 16 in 47 HCV-1b-infected patients treated with peginterferon plus ribavirin [75]. In their follow up study for the same group of patients, sequence variation in the IRRDR was also significantly related to the final outcome. The positive predictive values of IRRDR of 6 or more for SVR was 89% (16/18), whereas negative predictive values of IRRDR of 5 or less for non-SVR was 81% (22/27) [76].

Other region in NS5A

Pfeiffer et al. reported that two responsible mutations resided in the C-terminal region of NS5A: G404S and E442G were considered as mechanisms accounting for ribavirin resistance during HCV RNA replication, using HCV replicon-containing cell lines in the presence of increasing concentrations of ribavirin [77]. However, the clinical importance of such mutations and their relevance to ribavirin-related therapy is not evident.

NS5B

NS5B is 68 kDa proteins in size, and known as an RNA-dependent RNA polymerase. The enzyme synthesizes HCV–RNA using HCV–RNA as a template. NS5B is considered as one component of the HCV–RNA replication complex, and its activity as an RNA polymerase is modulated by NS3 and NS5A. Since this enzymatic activity is critical for HCV replication, the correlation between its mutations and treatment response has been explored, to date, in several studies.

Though the viral inhibitory mechanism of ribavirin in the treatment of HCV is unknown, its action as a mutagen is especially focused on the NS5B protein. During

ribavirin monotherapy, Young et al. reported that a specific mutation of NS5B amino acid 415 Phe-to-Tyr (F415Y) had emerged in five out of five patients infected with genotype-1a HCV [78]. To clarify the biological relevance of this mutation in ribavirin monotherapy, they introduced NS5B F415Y mutations into subgenomic HCV replicons, and reported that they observed different drug sensitivities in HCV replicons according to this NS5B polymorphism in a ribavirin dose-dependent manner. However, subsequent studies done in Japan and the UK could not find an evident relationship between specific selection of NS5B 415 mutations and the treatment of combination therapy of peginterferon and ribavirin. Sugihara et al. reported that they did not find specific mutations in NS5B 415 in the both serum obtained before and after therapy in 18 patients infected with genotype-1b HCV [79], and Ward et al. could not find evidence of a relationship of these mutations in the therapy of peginterferon and ribavirin in 15 patients infected with genotype-1a [80]. Hamano et al. explored genetic changes of genotype-1b HCV during the treatment of interferon-alpha and ribavirin, and reported that mutations at positions 300–358 of NS5B, including polymerase motif B–E, occurred more frequently in SVR patients or in end-of-treatment response patients when compared to null-response patients [81]. Mutation rate of NS5B in patients undergoing treatment with ribavirin monotherapy was also explored in patients treated with peginterferon/ribavirin therapy, since error catastrophe from an increase in mutation rate could be a possible mechanism of ribavirin in HCV infection [82]. Lutchman et al. reported that ribavirin was only associated with an early transient increase in the HCV mutation rate, but lethal mutagenesis and error catastrophe was unlikely to be the sole mechanism of ribavirin [83].

Conclusions

Viral genetic variability of HCV and its potential correlation to the interferon-based treatment response is briefly discussed here. Understanding the biological features of drug-resistant HCV, may helpl us to predict the treatment response in each patient in advance. Furthermore, though trials of HCV specific protease inhibitors are on-going, and are just about to be incorporated into the new standard therapy, understanding those biological features of HCV would further clarify and focus which patients will most benefit from being treated with the new treatment regimens. This viral genetic approach could be crucial even in the era of HCV protease inhibitors for achieving global eradication of HCV.

References

1. Zeuzem S. Interferon-based therapy for chronic hepatitis C: current and future perspectives. Nat Clin Pract Gastroenterol Hepatol. 2008;5(11):610–22.
2. Ogata N, Alter HJ, Miller RH, Purcell RH. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc Natl Acad Sci USA. 1991;88(8):3392–6.
3. Okamoto H, Kojima M, Okada S, Yoshizawa H, Iizuka H, Tanaka T, et al. Genetic drift of hepatitis C virus during an 8.2-year infection in a chimpanzee: variability and stability. Virology. 1992;190(2):894–9.
4. Simmonds P, Bukh J, Combet C, Deleage G, Enomoto N, Feinstone S, et al. Consensus proposals for a unified system of nomenclature of hepatitis C virus genotypes. Hepatology. 2005;42(4):962–73.
5. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med. 2002;347(13):975–82.
6. Hadziyannis SJ, Sette H Jr, Morgan TR, Balan V, Diago M, Marcellin P, et al. Peginterferon-alpha2a and ribavirin combination therapy in chronic hepatitis C: a randomized study of treatment duration and ribavirin dose. Ann Intern Med. 2004;140(5):346–55.
7. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet. 2001;358(9286):958–65.
8. Dalgard O, Mangia A. Short-term therapy for patients with hepatitis C virus genotype 2 or 3 infection. Drugs. 2006;66(14):1807–15.
9. von Wagner M, Huber M, Berg T, Hinrichsen H, Rasenack J, Heintges T, et al. Peginterferon-alpha-2a (40 kDa) and ribavirin for 16 or 24 weeks in patients with genotype 2 or 3 chronic hepatitis C. Gastroenterology. 2005;129(2):522–7.
10. El-Zayadi AR, Attia M, Barakat EM, Badran HM, Hamdy H, El-Tawil A, et al. Response of hepatitis C genotype-4 naive patients to 24 weeks of Peg-interferon-alpha2b/ribavirin or induction-dose interferon-alpha2b/ribavirin/amantadine: a non-randomized controlled study. Am J Gastroenterol. 2005;100(11):2447–52.
11. Kamal SM, El Tawil AA, Nakano T, He Q, Rasenack J, Hakam SA, et al. Peginterferon {alpha}-2b and ribavirin therapy in chronic hepatitis C genotype 4: impact of treatment duration and viral kinetics on sustained virological response. Gut. 2005;54(6):858–66.
12. Nguyen MH, Keeffe EB. Prevalence and treatment of hepatitis C virus genotypes 4, 5, and 6. Clin Gastroenterol Hepatol. 2005;3(10 Suppl 2):S97–101.
13. Araujo FM, Sonoda IV, Rodrigues NB, Teixeira R, Redondo RA, Oliveira GC. Genetic variability in the 5' UTR and NS5A regions of hepatitis C virus RNA isolated from non-responding and responding patients with chronic HCV genotype 1 infection. Mem Inst Oswaldo Cruz. 2008;103(6):611–4.
14. El Awady MK, Azzazy HM, Fahmy AM, Shawky SM, Badreldin NG, Yossef SS, et al. Positional effect of mutations in 5'UTR of hepatitis C virus 4a on patients' response to therapy. World J Gastroenterol. 2009;15(12):1480–6.
15. Katano Y, Hayashi K, Ishigami M, Itoh A, Hirooka Y, Nakano I, et al. Association with 5'-untranslated region and response to interferon in chronic hepatitis C. Hepatogastroenterology. 2007;54(75):854–7.
16. Soler M, Pellerin M, Malnou CE, Dhumeaux D, Kean KM, Pawlotsky JM. Quasi species heterogeneity and constraints on the

evolution of the 5′ noncoding region of hepatitis C virus (HCV): relationship with HCV resistance to interferon-alpha therapy. Virology. 2002;298(1):160–73.
17. Thelu MA, Drouet E, Hilleret MN, Zarski JP. Lack of clinical significance of variability in the internal ribosome entry site of hepatitis C virus. J Med Virol. 2004;72(3):396–405.
18. Yamamoto C, Enomoto N, Kurosaki M, Yu SH, Tazawa J, Izumi N, et al. Nucleotide sequence variations in the internal ribosome entry site of hepatitis C virus-1b: no association with efficacy of interferon therapy or serum HCV-RNA levels. Hepatology. 1997;26(6):1616–20.
19. Kountouras J, Zavos C, Chatzopoulos D. Apoptosis in hepatitis C. J Viral Hepat. 2003;10(5):335–42.
20. Hahn YS. Subversion of immune responses by hepatitis C virus: immunomodulatory strategies beyond evasion? Curr Opin Immunol. 2003;15(4):443–9.
21. Wang T, Weinman SA. Causes and consequences of mitochondrial reactive oxygen species generation in hepatitis C. J Gastroenterol Hepatol. 2006;21(Suppl 3):S34–7.
22. Mori Y, Moriishi K, Matsuura Y. Hepatitis C virus core protein: its coordinate roles with PA28gamma in metabolic abnormality and carcinogenicity in the liver. Int J Biochem Cell Biol. 2008;40(8):1437–42.
23. Serfaty L, Capeau J. Hepatitis C, insulin resistance and diabetes: clinical and pathogenic data. Liver Int. 2009;29(Suppl 2):13–25.
24. Pazienza V, Clement S, Pugnale P, Conzelman S, Foti M, Mangia A, et al. The hepatitis C virus core protein of genotypes 3a and 1b downregulates insulin receptor substrate 1 through genotype-specific mechanisms. Hepatology. 2007;45(5):1164–71.
25. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon–ribavirin combination therapy. Intervirology. 2005;48(6):372–80.
26. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol. 2009;81(3):452–8.
27. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictors of viral kinetics to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b. J Med Virol. 2007;79(11):1686–95.
28. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol. 2007;46(3):403–10.
29. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Amino acid substitutions in the hepatitis C virus core region are the important predictor of hepatocarcinogenesis. Hepatology. 2007;46(5):1357–64.
30. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Substitution of amino acid 70 in the hepatitis C virus core region of genotype 1b is an important predictor of elevated alpha-fetoprotein in patients without hepatocellular carcinoma. J Med Virol. 2008;80(8):1354–62.
31. Donlin MJ, Cannon NA, Yao E, Li J, Wahed A, Taylor MW, et al. Pretreatment sequence diversity differences in the full-length hepatitis C virus open reading frame correlate with early response to therapy. J Virol. 2007;81(15):8211–24.
32. Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka T, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: Correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol. 2009;81(4):640–9.
33. Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, et al. Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J Gastroenterol. 2009;11 [Epub ahead of print].
34. Grove J, Nielsen S, Zhong J, Bassendine MF, Drummer HE, Balfe P, et al. Identification of a residue in hepatitis C virus E2 glycoprotein that determines scavenger receptor BI and CD81 receptor dependency and sensitivity to neutralizing antibodies. J Virol. 2008;82(24):12020–9.
35. Benedicto I, Molina-Jimenez F, Barreiro O, Maldonado-Rodriguez A, Prieto J, Moreno-Otero R, et al. Hepatitis C virus envelope components alter localization of hepatocyte tight junction-associated proteins and promote occludin retention in the endoplasmic reticulum. Hepatology. 2008;48(4):1044–53.
36. Pavio N, Taylor DR, Lai MM. Detection of a novel unglycosylated form of hepatitis C virus E2 envelope protein that is located in the cytosol and interacts with PKR. J Virol. 2002;76(3):1265–72.
37. Taylor DR, Shi ST, Romano PR, Barber GN, Lai MM. Inhibition of the interferon-inducible protein kinase PKR by HCV E2 protein. Science. 1999;285(5424):107–10.
38. Taylor DR, Tian B, Romano PR, Hinnebusch AG, Lai MM, Mathews MB. Hepatitis C virus envelope protein E2 does not inhibit PKR by simple competition with autophosphorylation sites in the RNA-binding domain. J Virol. 2001;75(3):1265–73.
39. Gupta R, Subramani M, Khaja MN, Madhavi C, Roy S, Habibullah CM, et al. Analysis of mutations within the 5′ untranslated region, interferon sensitivity region, and PePHD region as a function of response to interferon therapy in hepatitis C virus-infected patients in India. J Clin Microbiol. 2006;44(3):709–15.
40. Saito T, Ito T, Ishiko H, Yonaha M, Morikawa K, Miyokawa A, et al. Sequence analysis of PePHD within HCV E2 region and correlation with resistance of interferon therapy in Japanese patients infected with HCV genotypes 2a and 2b. Am J Gastroenterol. 2003;98(6):1377–83.
41. Sarrazin C, Bruckner M, Herrmann E, Ruster B, Bruch K, Roth WK, et al. Quasi species heterogeneity of the carboxy-terminal part of the E2 gene including the PePHD and sensitivity of hepatitis C virus 1b isolates to antiviral therapy. Virology. 2001;289(1):150–63.
42. Sarrazin C, Kornetzky I, Ruster B, Lee JH, Kronenberger B, Bruch K, et al. Mutations within the E2 and NS5A protein in patients infected with hepatitis C virus type 3a and correlation with treatment response. Hepatology. 2000;31(6):1360–70.
43. Gaudy C, Lambele M, Moreau A, Veillon P, Lunel F, Goudeau A. Mutations within the hepatitis C virus genotype 1b E2-PePHD domain do not correlate with treatment outcome. J Clin Microbiol. 2005;43(2):750–4.
44. Hung CH, Lee CM, Lu SN, Lee JF, Wang JH, Tung HD, et al. Mutations in the NS5A and E2-PePHD region of hepatitis C virus type 1b and correlation with the response to combination therapy with interferon and ribavirin. J Viral Hepat. 2003;10(2):87–94.
45. Munoz de Rueda P, Casado J, Paton R, Quintero D, Palacios A, Gila A, et al. Mutations in E2-PePHD, NS5A-PKRBD, NS5A-ISDR, and NS5A-V3 of hepatitis C virus genotype 1 and their relationships to pegylated interferon-ribavirin treatment responses. J Virol. 2008;82(13):6644–53.
46. Murphy MD, Rosen HR, Marousek GI, Chou S. Analysis of sequence configurations of the ISDR, PKR-binding domain, and V3 region as predictors of response to induction interferon-alpha and ribavirin therapy in chronic hepatitis C infection. Dig Dis Sci. 2002;47(6):1195–205.

47. Vuillermoz I, Khattab E, Sablon E, Ottevaere I, Durantel D, Vieux C, et al. Genetic variability of hepatitis C virus in chronically infected patients with viral breakthrough during interferon–ribavirin therapy. J Med Virol. 2004;74(1):41–53.
48. Yang SS, Lai MY, Chen DS, Chen GH, Kao JH. Mutations in the NS5A and E2-PePHD regions of hepatitis C virus genotype 1b and response to combination therapy of interferon plus ribavirin. Liver Int. 2003;23(6):426–33.
49. Yoon J, Lee JI, Baik SK, Lee KH, Sohn JH, Lee HW, et al. Predictive factors for interferon and ribavirin combination therapy in patients with chronic hepatitis C. World J Gastroenterol. 2007;13(46):6236–42.
50. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Comparison of full-length sequences of interferon-sensitive and resistant hepatitis C virus 1b. Sensitivity to interferon is conferred by amino acid substitutions in the NS5A region. J Clin Invest. 1995;96(1):224–30.
51. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med. 1996;334(2):77–81.
52. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, et al. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. Hepatology. 1997;25(3):745–9.
53. Komatsu H, Fujisawa T, Inui A, Miyagawa Y, Onoue M. Mutations in the nonstructural protein 5A gene and response to interferon therapy in young patients with chronic hepatitis C virus 1b infection. J Med Virol. 1997;53(4):361–5.
54. Kurosaki M, Enomoto N, Murakami T, Sakuma I, Asahina Y, Yamamoto C, et al. Analysis of genotypes and amino acid residues 2209–2248 of the NS5A region of hepatitis C virus in relation to the response to interferon-beta therapy. Hepatology. 1997;25(3):750–3.
55. Chung RT, Monto A, Dienstag JL, Kaplan LM. Mutations in the NS5A region do not predict interferon-responsiveness in American patients infected with genotype 1b hepatitis C virus. J Med Virol. 1999;58(4):353–8.
56. Hofgartner WT, Polyak SJ, Sullivan DG, Carithers RL Jr, Gretch DR. Mutations in the NS5A gene of hepatitis C virus in North American patients infected with HCV genotype 1a or 1b. J Med Virol. 1997;53(2):118–26.
57. Khorsi H, Castelain S, Wyseur A, Izopet J, Canva V, Rombout A, et al. Mutations of hepatitis C virus 1b NS5A 2209–2248 amino acid sequence do not predict the response to recombinant interferon-alfa therapy in French patients. J Hepatol. 1997;27(1):72–7.
58. Odeberg J, Yun Z, Sonnerborg A, Weiland O, Lundeberg J. Variation in the hepatitis C virus NS5a region in relation to hypervariable region 1 heterogeneity during interferon treatment. J Med Virol. 1998;56(1):33–8.
59. Squadrito G, Leone F, Sartori M, Nalpas B, Berthelot P, Raimondo G, et al. Mutations in the nonstructural 5A region of hepatitis C virus and response of chronic hepatitis C to interferon alfa. Gastroenterology. 1997;113(2):567–72.
60. Zeuzem S, Lee JH, Roth WK. Mutations in the nonstructural 5A gene of European hepatitis C virus isolates and response to interferon alpha. Hepatology. 1997;25(3):740–4.
61. Pascu M, Martus P, Hohne M, Wiedenmann B, Hopf U, Schreier E, et al. Sustained virological response in hepatitis C virus type 1b infected patients is predicted by the number of mutations within the NS5A-ISDR: a meta-analysis focused on geographical differences. Gut. 2004;53(9):1345–51.
62. Pawlotsky JM, Germanidis G, Neumann AU, Pellerin M, Frainais PO, Dhumeaux D. Interferon resistance of hepatitis C virus genotype 1b: relationship to nonstructural 5A gene quasi species mutations. J Virol. 1998;72(4):2795–805.
63. Saiz JC, Lopez-Labrador FX, Ampurdanes S, Dopazo J, Forns X, Sanchez-Tapias JM, et al. The prognostic relevance of the nonstructural 5A gene interferon sensitivity determining region is different in infections with genotype 1b and 3a isolates of hepatitis C virus. J Infect Dis. 1998;177(4):839–47.
64. Sarrazin C, Berg T, Lee JH, Ruster B, Kronenberger B, Roth WK, et al. Mutations in the protein kinase-binding domain of the NS5A protein in patients infected with hepatitis C virus type 1a are associated with treatment response. J Infect Dis. 2000; 181(2):432–41.
65. Sarrazin C, Berg T, Lee JH, Teuber G, Dietrich CF, Roth WK, et al. Improved correlation between multiple mutations within the NS5A region and virological response in European patients chronically infected with hepatitis C virus type 1b undergoing combination therapy. J Hepatol. 1999;30(6):1004–13.
66. Witherell GW, Beineke P. Statistical analysis of combined substitutions in nonstructural 5A region of hepatitis C virus and interferon response. J Med Virol. 2001;63(1):8–16.
67. Shirakawa H, Matsumoto A, Joshita S, Komatsu M, Tanaka N, Umemura T, et al. Pretreatment prediction of virological response to peginterferon plus ribavirin therapy in chronic hepatitis C patients using viral and host factors. Hepatology. 2008; 48(6):1753–60.
68. Veillon P, Payan C, Le Guillou-Guillemette H, Gaudy C, Lunel F. Quasi species evolution in NS5A region of hepatitis C virus genotype 1b during interferon or combined interferon–ribavirin therapy. World J Gastroenterol. 2007;13(8):1195–203.
69. Yen YH, Hung CH, Hu TH, Chen CH, Wu CM, Wang JH, et al. Mutations in the interferon sensitivity-determining region (nonstructural 5A amino acid 2209–2248) in patients with hepatitis C-1b infection and correlating response to combined therapy of pegylated interferon and ribavirin. Aliment Pharmacol Ther. 2008;27(1):72–9.
70. Gale MJ Jr, Korth MJ, Tang NM, Tan SL, Hopkins DA, Dever TE, et al. Evidence that hepatitis C virus resistance to interferon is mediated through repression of the PKR protein kinase by the nonstructural 5A protein. Virology. 1997;230(2):217–27.
71. Inchauspe G, Zebedee S, Lee DH, Sugitani M, Nasoff M, Prince AM. Genomic structure of the human prototype strain H of hepatitis C virus: comparison with American and Japanese isolates. Proc Natl Acad Sci USA. 1991;88(22):10292–6.
72. Duverlie G, Khorsi H, Castelain S, Jaillon O, Izopet J, Lunel F, et al. Sequence analysis of the NS5A protein of European hepatitis C virus 1b isolates and relation to interferon sensitivity. J Gen Virol. 1998;79(Pt 6):1373–81.
73. Puig-Basagoiti F, Forns X, Furcic I, Ampurdanes S, Gimenez-Barcons M, Franco S, et al. Dynamics of hepatitis C virus NS5A quasi species during interferon and ribavirin therapy in responder and non-responder patients with genotype 1b chronic hepatitis C. J Gen Virol. 2005;86(Pt 4):1067–75.
74. Sarrazin C, Herrmann E, Bruch K, Zeuzem S. Hepatitis C virus nonstructural 5A protein and interferon resistance: a new model for testing the reliability of mutational analyses. J Virol. 2002;76(21):11079–90.
75. El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, et al. Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera. Microbiol Immunol. 2007;51(4):471–82.
76. El-Shamy A, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, Hotta H. Sequence variation in hepatitis C virus nonstructural protein 5A predicts clinical outcome of pegylated interferon/ribavirin combination therapy. Hepatology. 2008;48(1):38–47.