

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 IFN α 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 \pm 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

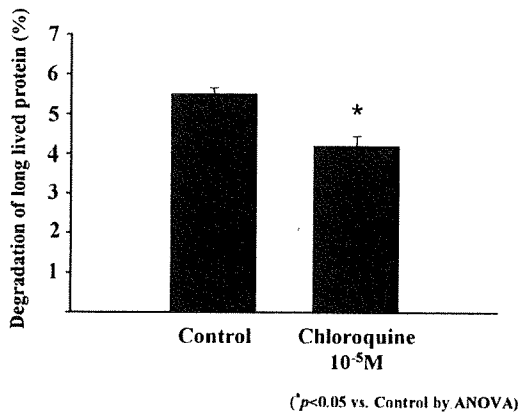
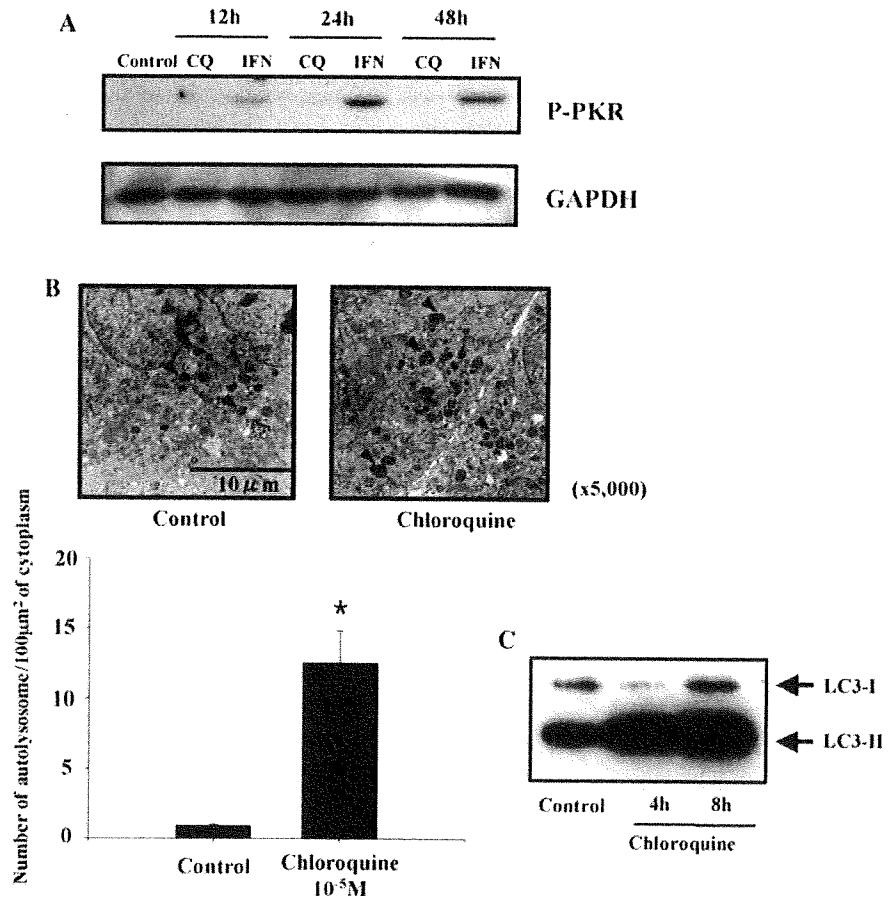


Fig. 6 Turnover of long lived protein. Huh-7 Rep/Feo cells were labeled with [^{14}C]leucin for 4 h, then degradation of long-lived protein in chloroquine treated cells was measured as described in Materials and Methods. The percentage of protein degradation was calculated by dividing the amount of acid-soluble radioactivity in the medium at that time by the amount of acid-precipitable radioactivity present in the cells at time zero. Data are mean \pm 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 IFN α (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 IFN α on HCV replication (Fig. 4a). Although IFN α possesses strong anti-HCV effects, re-propagation of HCV replicon was observed after 3 weeks following 7 days of treatment with IFN α . Interestingly, co-incubation with IFN α 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 IFN α 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.

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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 glycycomarin, which were from *Glycyrrhizae*

radix. Dose-effect analyses showed that 50% effective concentrations were $6.2 \pm 1.0 \mu\text{g/mL}$ and $15.5 \pm 0.8 \mu\text{g/mL}$ for isoliquiritigenin and glycycomarin, 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 glycycomarin, 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-gamma¹² 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
<i>Glycyrrhizae radix</i>	Isoliquiritigenin
	Glycycomarin
	Isoliquiritin
	Licuroside
<i>Paeoniae radix</i>	Paeoniflorin
	1,2,3,6-tetra-O-galloyl- β -D-glucose
<i>Rhei Rhizoma</i>	Rhein 8-O- β -glucoside
<i>Rehmanniae radix</i>	Acteoside
	Martynoside
	Isoacteoside
<i>Artemisiae capillari spica</i>	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-Chai-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix*, *Pinelliae tuber*, *Scutellariae radix*, *Ginseng radix*, *Glycyrrhizae 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 C¹⁶ 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 radix*; *Rehmanniae 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 $\mu\text{g}/\text{mL}$ 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 phosphotransferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

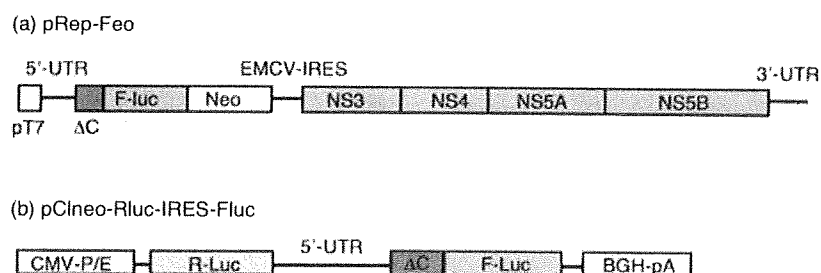


Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo". 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 \pm 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 \pm 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 μ g 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 RNA²⁵ 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 glycycomarin

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 radix*, *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 glycycomarin, 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 $\mu\text{g}/\text{mL}$ for isoliquiritigenin and glycycomarin, 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 glycycomarin, 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 isoliquirit-

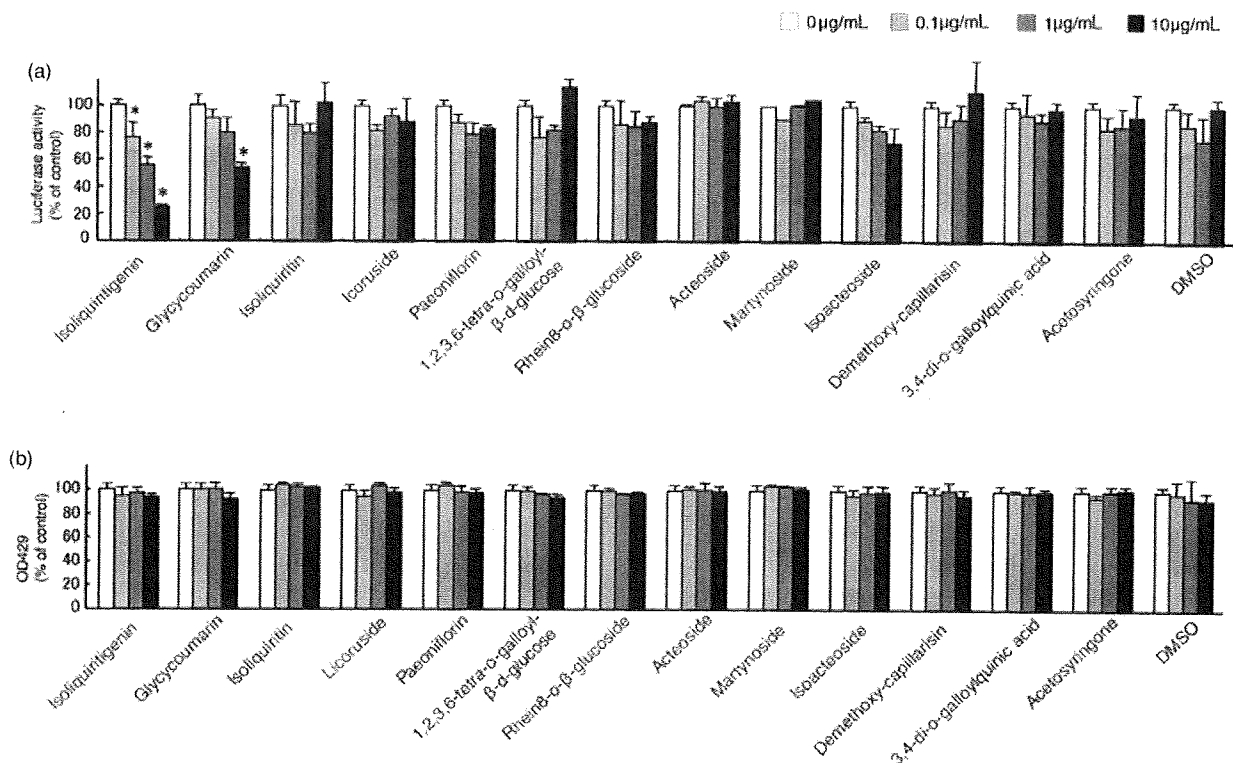


Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 $\mu\text{g}/\text{mL}$. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean \pm 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 \pm SD.

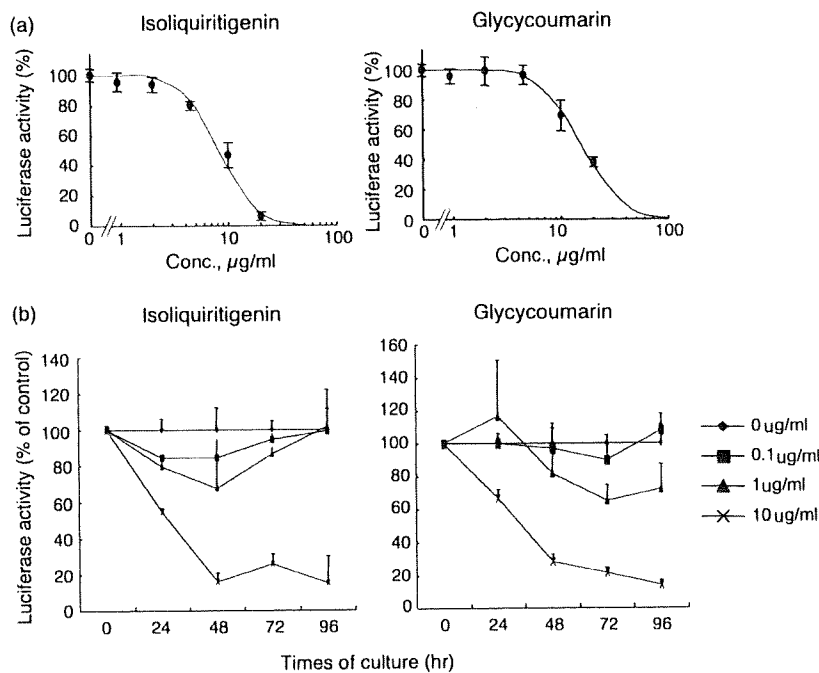


Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritigenin and glycy coumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycy coumarin. Error bars indicate mean \pm SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo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

itigenin and glycy coumarin (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 glycy coumarin (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 glycy coumarin

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 IFN α -2b and isoliquiritigenin or glycy coumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycy coumarin of 0, 0.1, 1, 10 μ g/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 glycy coumarin 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

glycy coumarin. 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 glycy coumarin 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 pCneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycy coumarin 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 glycy coumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycy coumarin 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

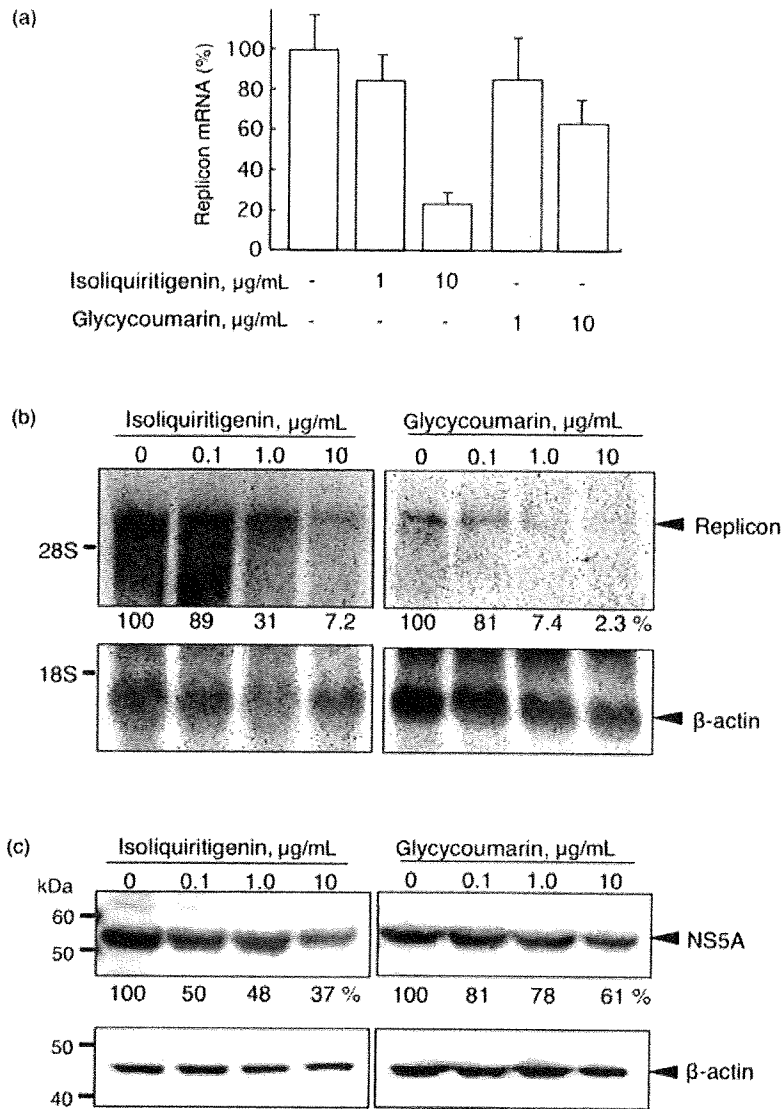


Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycy coumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycy coumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot 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 glycy coumarin than the untreated culture. The effect of glycy coumarin 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 glycy coumarin (Fig. 7b).

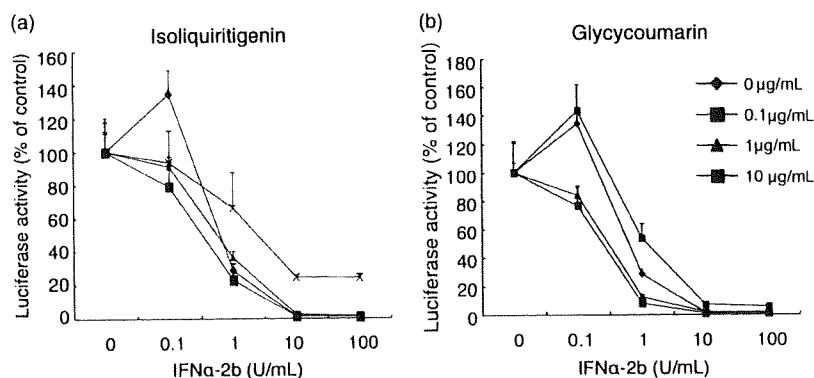


Figure 5 Effects of (a) isoliquiritigenin and (b) glycycomarin used in combination with interferon(IFN)- α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN- α -2b and isoliquiritigenin or glycycomarin at concentrations indicated. The internal luciferase activities were measured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

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycomarin, 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 anticarcinogenic 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 properties³³ and selective inhibition of H2 receptor-mediated signaling.³⁴

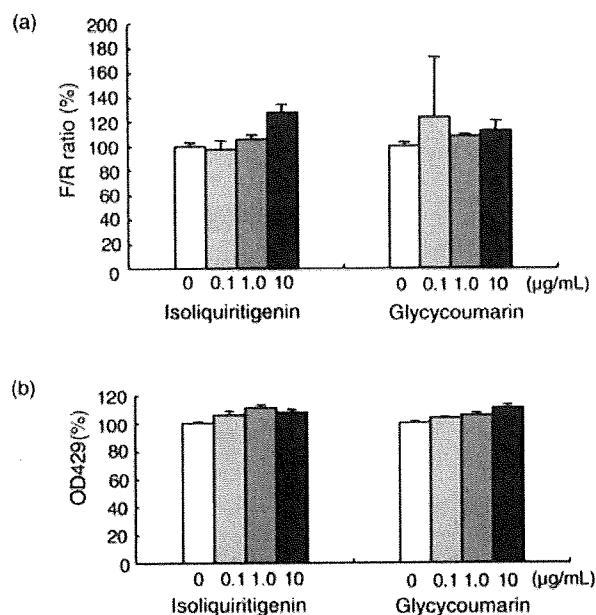


Figure 6 Isoliquiritigenin and glycycomarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably transfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycomarin 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 \pm SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycomarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean \pm SD.

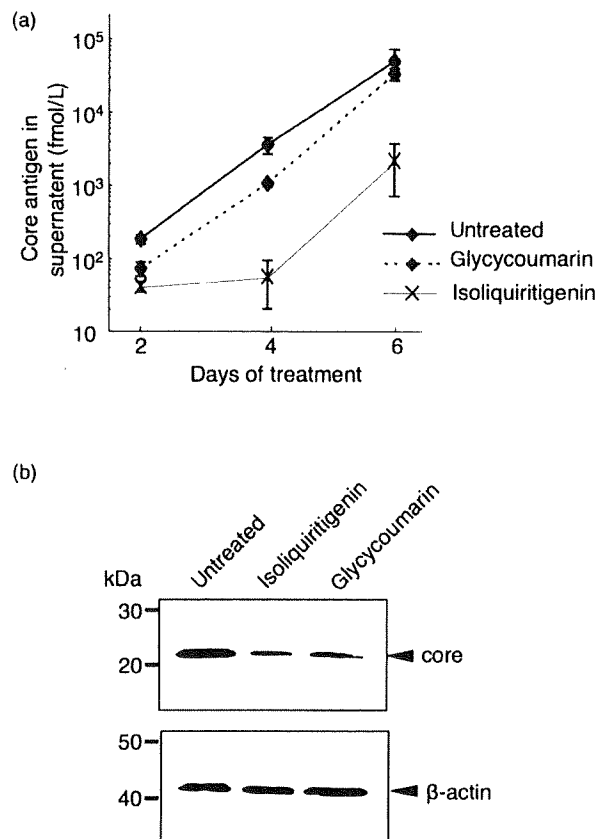


Figure 7 Suppression of HCV-JFH1 virus expression by *isoliquiritigenin* and *glycycomarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean \pm 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 *Glycyrrhizae 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 glycycomarin. Glycycomarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes*, *Haemophilus influenzae* 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 glycycomarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glycyrrhizae 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 glycycomarin, 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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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 pre-treatment 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-2 α phosphorylation homology domain (PePHD). The PePHD motif is similar to the phosphorylation sites of PKR and eIF2 α . 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 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 help 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.

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