Differences in the Serum 4β-hydroxycholesterol Levels of Patients with Chronic Hepatitis C Virus (HCV) Infection: A Possible Impact on the Efficacy and Safety of Interferon (IFN)-free Treatment

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Abstract:
Objective Since the majority of direct-acting antivirals (DAAs) that are used in the treatment of hepatitis C virus (HCV) infection are mainly metabolized by CYP3A4, it is hypothesized that inter-individual differences in CYP3A4 activity may be associated with the bioavailability of these agents.

Methods The level of serum 4β-hydroxycholesterol (4βHC), a surrogate marker of CYP3A4 activity, was determined by LC-MS/MS in samples obtained from patients with HCV infection (CHCs) as well as healthy control subjects (CTLs). Serum samples obtained from patients treated with either asunaprevir/daclatasvir (ASV/DCV) or ombitasvir/paritaprevir/ritonavir (OTV/PTV/r) were used for additional assays.

Results The serum 4βHC level in CHCs was significantly higher than that in CTLs, and a gender difference was seen among CHCs. In patients treated with OTV/PTV/r, the serum 4βHC level was observed to gradually decrease during the treatment period. In the cohort treated with ASV/DCV, 4 of 83 patients showed virological treatment failure. In pretreatment testing, an Invader assay detected a low prevalence of resistance-associated variants in these four patients. The average serum concentration of DCV/ASV in the treatment-failed group tended to be lower than that in the sustained virological response (SVR) group. The pretreatment serum 4βHC level in patients with treatment failure was significantly higher than that in patients with an SVR but in whom the prevalence of resistance-associated variants was low in the pretreatment setting.

Conclusion The evaluation of CYP3A4 activity by measuring 4βHC before treatment may provide additional information that can potentially be used to select cost- and efficacy-optimized treatment of HCV.

Key words: HCV, direct acting antivirals, cytochrome P450 (CYP) 3A4, oxysterol, 4β-hydroxycholesterol

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Introduction

Recent advances in anti-viral therapy for hepatitis C virus (HCV) infection have led to drastic changes in the use of drugs as well as the selection of patients who are eligible for treatment. The so-called interferon (IFN)-free regimens, which consist of two or three oral direct-acting antivirals (DAA) combinations but not IFN, have been approved and have now replaced IFN-based regimens as the mainstay of anti-HCV therapy worldwide. For example, the combination use of daclatasvir (DCV, a NS5A replication complex inhibitor) and asunaprevir (ASV, a NS3/4A protease inhibitor) was approved in Japan in 2014 as an all-oral treatment for HCV genotype 1b infection. Clinical trials in Japan have shown that 24-week treatment yielded an sustained virologi-
cal response (SVR) rate of 85% in both non-responders to previous combined peglated (PEG)-IFN plus ribavirin therapies and patients who were either intolerant of the combination therapies or ineligible for treatment with combination therapies (1, 2). Although this combination can safely and very effectively achieve viral eradication, several issues have emerged. The most important concern is that the relatively lower genetic barrier of these DAAs (in comparison to other DAA combinations) can cause virologic failure, which is usually accompanied by the appearance of resistance-associated variants (RAVs) of HCV. Relationships between pre-existing RAVs by direct sequencing and clinical antiviral responses to ASV and DCV have been reported (3); thus, a pretreatment test for RAVs is recommended by the Japanese clinical guidelines (4). On the other hand, an article by Kumada et al. showed the tendency for the plasma trough concentrations of DCV and ASV to be lower in patients with virologic failure (2). Although the detailed pharmacokinetics and pharmacodynamics of ASV/DCV have not been fully elucidated, these drugs are known as substrates of cytochrome P450 (CYP) 3A4. In addition, ASV has been reported to function as a CYP3A4-inducer. The pharmacodynamics of the majority of other combinations of DAAs are also reported to be strongly correlated with CYP3A4 activity.

On the other hand, another DAA combination, ombitasvir/paritaprevir/ritonavir (OTV/PTV/r), which is designed to maintain the concentration of paritaprevir and to make a once daily dose possible (through the combination use of ritonavir, an HIV-protease inhibitor), also possesses a strong inhibitory action against CYP3A. Because of this drug design, the potential risk of drug-drug interactions between this DAA and some commonly co-administered medications (i.e., calcium channel blockers) has been reported. In DAA treatment for HCV, a careful evaluation of potential drug-drug interactions is essential to prevent adverse events or the unnecessary risk of treatment failure.

Taken together, understanding the inter-individual difference of basal CYP3A4 activity, which possibly affects the plasma concentration of DAAs and potential drug-drug interactions with concomitant medications, among patients under DAA treatment has clinical relevance when customizing potent therapeutic strategies.

It has been discovered that the endogenous oxysterol 4β-hydroxycholesterol (4βHC) is formed from cholesterol by CYP3A4/5 (5). Patients treated with drugs that are known to induce CYP3A4, for example, carbamazepine, phenytoin, and phenobarbital, had significantly increased plasma levels of 4βHC in comparison to control subjects, but this was not observed in patients treated with valproic acid (5). It was suggested that the high levels of 4βHC in carbamazepine-treated patients occurred due to increased synthesis rather than a decreased rate of elimination (6). In addition, the long half-life of 4βHC results in small intra-individual variations in the plasma concentration. Taken together, 4βHC was suggested to be a potential endogenous marker of the CYP3A4/5 enzyme activity (6-9).

In the present study, we focused on the pre-treatment level of serum 4βHC among CHC patients, and demonstrated its inter-individual variation in patients with different backgrounds. Furthermore, we determined the pre-treatment and on-treatment levels of serum 4βHC among patients treated with ASV/DCV and OTV/PTV/r in order to discuss the association between the serum 4βHC level and treatment outcomes as well as potential drug-drug interactions.

### Materials and Methods

#### Subjects

In this case-control study, we evaluated patients who were diagnosed with chronic hepatitis C (CHC) at the National Hospital Organization Kyushu Medical Center (IFN-Cohort). In all patients, the current and past daily alcohol consumption was <20 g per week. The exclusion criteria (other than excessive alcohol consumption) were as follows: evidence of pregnancy, treatment with corticosteroids, and hormone replacement therapy. Subjects using lipid-lowering medications or food enriched with functional plant sterols or stanols were excluded from the study. Subjects with positive test results for the following disorders were also excluded: drug-induced liver injury, autoimmune hepatitis, primary biliary cholangitis, alpha-1-antitrypsin deficiency, hemochromatosis, Wilson’s disease, and biliary obstruction. Venous blood samples were collected in the morning (following 12-hour overnight fasting) at baseline and 3 months after the initiation of combination treatment with PEG-IFN and ribavirin. Serum samples were stored until use at -20°C.

Fasting serum samples of 113 age- and sex-matched healthy volunteers without obesity, hyperlipidemia, diabetes, or liver dysfunction [obtained for another study group (courtesy of Professor T. Teramoto, Teikyo University), with written informed consent from the healthy volunteers] were used as the control group. The control serum samples were stored and handled as mentioned previously.

Serum samples obtained from a total of 87 CHC patients treated with ASV/DCV (Bristol Myers Scrub, Tokyo, Japan) at Tokyo Medical University Ibaraki Medical Center were analyzed as a DAA cohort. In this cohort, all patients were infected with viral genotype 1b and had no previous history of treatment with other combinations of DAA. The patients’ venous blood samples were collected and stored as mentioned previously. Patient adherence to the drug regimen was monitored using self-checking sheets, which were completed by patients and pharmacists; this was followed up by a direct interview of the patients at every visit to the hospital outpatient clinic. In the same manner, serum samples were collected from a total 10 Japanese patients infected with genotype 1b HCV, who were treated with OTV/PTV/r (Abbvie Godo, Tokyo, Japan). The efficacy endpoint in these DAA treatments was SVR12, defined as HCV RNA < 2.1 log copy/mL at 12 weeks after the end of therapy. The
plasma HCV RNA was measured using the Roche COBAS TaqMan HCV Test (Roche Diagnostics, Tokyo, Japan).

Written informed consent was obtained from all of the patients prior to the collection of blood samples, which was conducted with the approval of the Institutional Review Board of Kyushu Medical Center and Tokyo Medical University Ibaraki Medical Center. This study protocol was in compliance with the Good Clinical Practice Guidelines and the 1975 Declaration of Helsinki.

Quantification of the serum lipid biomarkers

Serum oxysterols, including 24S-hydroxycholesterol (24SHC), 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC), 7α-hydroxycholesterol (7αHC), 4βHC, 22R-hydroxycholesterol (22RHC), and 24S,25-epoxycholesterol, were simultaneously quantified by LC-MS/MS, as described in our previous papers (10-12). Briefly, coprostanol and deuterated oxysterols were added to 10 μL of serum as internal standards, and alkaline hydrolysis was performed in 1 N ethanolic KOH with butylated hydroxytoluene at 37°C for 1 hour. Sterols were extracted with n-hexane, derivatized to picolinyl esters, and injected into the LC-ESI-MS/MS system, which consisted of a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) equipped with an HESI-II probe and a Prominence ultra-fast liquid chromatography (UFLC) system (Shimadzu, Kyoto, Japan).

All samples were thawed for an immediate analysis and were not used subsequently. For purification, all samples were maintained in nitrogen to avoid autoxidation during the assays.

Determination of the serum asunaprevir/daclatasvir concentration

The serum concentrations of ASV and DCV were assayed by CMIC Pharma Science (Tokyo, Japan) using validated LC-MS/MS methods during the period of known analyte stability. The serum samples were frozen at -80°C within 4 hours of collection and were thawed at the time of measurement.

Determination of resistance associated variants (RAVs)

The sequences at AA93 as well as L31 in the NS5A region were determined by direct sequencing (LSI Medience Laboratories, Tokyo, Japan) and/or by an Invader assay (BMI Tokyo, Japan). In the clinical samples, the polymerase chain reaction (PCR)-Invader assays detected drug RAVs that were detected by direct sequencing. Furthermore, they illustrated the presence of RAVs that could not be detected by direct sequencing (13).

Statistical analysis

A one-way analysis of variance (ANOVA) or two-tailed Student's t test were used to analyze the results. The GraphPad Prism software program was used to perform the statistical analyses (GraphPad Software, San Diego, USA). The factors associated with the outcomes of ASV/DCV treatment were subjected to a univariate analysis in which the Wilcoxon test was used to analyze continuous factors and Pearson’s chi-squared test was used to analyze categorical factors. The results are presented as the mean ± SEM. P values <0.05 were considered to indicate statistical significance.

Results

Inter-individual differences in the serum 4β-hydroxycholesterol levels of CHC patients

Serum samples obtained from a total of 55 CHC patients at National Kyushu Medical Center and 113 healthy control subjects were used for the study. The control subjects were selected after matching for age and sex. After selection, the mean age and male/female ratio of the control and CHC groups did not differ to a statistically significant extent. In the control group, none of the subjects were obese (BMI > 25), and no patients exhibited hypercholesterolemia (total cholesterol >220 mg/dL), hypertriglyceridemia (TG >150 mg/dL), hypertension, or diabetes. However, in the CHC group, there were patients with hypertension and three patients with diabetes, and eight of the 55 CHC patients were classified as obese (BMI >25). The genotypes in the CHC group included viral genotype 1b (n=32), genotype 2a (n=13), genotype 2b (n=7), and unknown genotype (n=3).

As previously demonstrated (14), the total serum cholesterol concentrations were significantly lower in patients with CHC. It is possible that the serum oxysterol levels are affected by the serum cholesterol levels; however, the concentrations of 4βHC in CHC patients were significantly (+29%) higher than those in control subjects (15). Focusing on 4βHC, a number of subgroup analyses were performed: gender (male or female), age (over or under 50 years of age), and stage of liver fibrosis (according to New-Inuyama classification).

The serum 4βHC level of female control subjects (CTLs) was significantly higher in comparison to male CTLs (59.0±3.79 ng/mL in female CTLs vs. 43.7±2.31 ng/mL in male CTLs, p<0.01). This finding was consistent with previous reports, which demonstrated that the serum 4βHC level is elevated in the female population in comparison to the male population (16). Furthermore, whereas serum 4βHC level was significantly higher in CHCs in comparison to the CTLs, as mentioned previously, this gender difference was also seen among HCV-infected patients (61.7±3.7 ng/mL in male CHCs vs. 70.9±4.1 ng/mL in female CHCs, p<0.05) (Fig. 1A).

In terms of age differences, we divided each cohort into subjects of ≥50 years of age and those <50 years of age. There were no differences between the two groups in either CTL or CHC (data not shown), which supports the findings of a previous report, which indicated that an association between CYP3A activity and aging was unlikely (17). When
each group was divided again by gender, the serum 4βHC level in females was significantly higher in CHLs regardless of the age subgroup (male <50 years: female <50 year, 43.0 ±2.6 ng/mL: 56.9±3.2 ng/mL, p<0.05, male >50 years: female >50 years, 35.6±3.7 ng/mL: 55.4±5.3 ng/mL, p<0.05). On the other hand, in CHCs, gender difference was only seen in subgroups of patients who were ≥50 years of age (M:F, 57.7±4.8 ng/mL: 70.0±4.4 ng/mL, p<0.05), and not in the other subgroups (M:F, 64.0±7.0 ng/mL: 61.1±5.1 ng/mL, N.S.), suggesting increased CYP3A activity in older women with CHC (Fig. 1B).

It is possible that the progression of chronic liver disease affects the activity of hepatic enzymes, including CYP family members, due to the decrease in the number of hepatocytes; thus, the serum 4βHC levels of CHCs who had undergone percutaneous liver biopsy were compared. This revealed that the serum 4βHC levels among 43 patients who had undergone liver biopsy did not differ according to the fibrosis score (according to New-Inuyama criteria): F0 (n=5): 66.5±6.4, F1 (n=25): 63.2±3.8, F2 (n=10): 67.2±7.4, F3 (n=3): 73.6±13.7 ng/mL, respectively.

**OTV/PTV/r therapy and serum 4β-hydroxycholesterol**

In the cohort of patients treated with OTV/PTV/r, 1 of 16 patients showed treatment failure [earlier discontinuation due to an adverse event (systemic edema due to a co-administered calcium channel blocker)].

In this cohort, we determined the change in the serum 4βHC levels during the OTV/PTV/r treatment period in the 10 patients who were available, all of whom achieved SVR12. In the pre-treatment period, the serum 4βHC concentrations varied (Mean: 48.4±8.5 pg/mL). With the exception of 1 case, all cases showed a significant reduction of the serum 4βHC level immediately after the initiation of DAA treatment. At 2 weeks from the initiation of OTV/PTV/r, a 36.3% reduction of 4βHC was observed (p<0.01 in comparison to the pre-treatment level); at 4 weeks this reduction reached 46.1% (p<0.05, in comparison to the level at 2 weeks). After 4 weeks, the 4βHC levels plateaued or slightly increased, then returned to approximately 10.0% less than the pre-treatment level at 8 weeks after the end of treatment (Fig. 2).
**Table 1. Background of ASV/DCV Cohort.**

<table>
<thead>
<tr>
<th></th>
<th>SVR12 (n=76)</th>
<th>Virological Failure (n=4)</th>
<th>Terminate due to AE (n=3)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender (M:F)</td>
<td>32:26</td>
<td>1:3</td>
<td>2:1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Age (mean±SEM)</td>
<td>67.2±1.0</td>
<td>70.3±2.5</td>
<td>66.5±1.5</td>
<td>N.S.</td>
</tr>
<tr>
<td>CH:LC</td>
<td>58.1±8</td>
<td>1.3</td>
<td>2.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>T-Chol (mg/dL)</td>
<td>158.4±3.9</td>
<td>153.4±10.1</td>
<td>150.0±2.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>eGFR (mL/min/1.73m²)</td>
<td>68.1±4.5</td>
<td>67.2±4.8</td>
<td>65.1±3.3</td>
<td>N.S.</td>
</tr>
<tr>
<td>BMI</td>
<td>23.9±0.7</td>
<td>23.6±1.3</td>
<td>21.6±0.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>RAVs with direct sequence</td>
<td>0/76</td>
<td>0/4</td>
<td>0/3</td>
<td></td>
</tr>
<tr>
<td>RAVs with Invader assay</td>
<td>12/36</td>
<td>4/4</td>
<td>0/3</td>
<td></td>
</tr>
</tbody>
</table>

T-Chol: serum total cholesterol, eGFR: estimated GFR, BMI: body mass index, RAVs: resistance associated variants

These parameters were determined at pre treatment period.

**Table 2. Pre-treatment RAVs Detected in Cases with Virological Failure.**

<table>
<thead>
<tr>
<th>Gender</th>
<th>Age</th>
<th>CH:LC</th>
<th>RAVs detected by Invader assay</th>
<th>Treatment outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>F</td>
<td>71</td>
<td>LC</td>
<td>Y93H (&lt;20%)</td>
<td>Viral breakthrough</td>
</tr>
<tr>
<td>F</td>
<td>71</td>
<td>CH</td>
<td>L31V/M (≥20%)</td>
<td>Viral breakthrough</td>
</tr>
<tr>
<td>F</td>
<td>76</td>
<td>LC</td>
<td>D168V (≥20%)</td>
<td>Null response</td>
</tr>
<tr>
<td>M</td>
<td>64</td>
<td>LC</td>
<td>D168V (&lt;20%)</td>
<td>Null response</td>
</tr>
</tbody>
</table>

**ASV/DCV therapy and serum 4β-hydroxycholesterol**

In the cohort of patients treated with ASV/DCV, 7 of 83 patients showed treatment failure, 4 due to viral breakthrough during the treatment period or a relapse of HCV-RNA after treatment, and 3 due to adverse events [alanine aminotransferase (ALT) elevation to >3 fold the upper limit of normal (n=2), reduction of eyesight (n=1)]. In four patients with virologic failure, direct sequencing did not detect RAVs during the pretreatment period, while the prevalence of RAVs, as detected by the Invader assay, was low (Tables 1 and 2).

The pretreatment serum 4βHC levels of the patients were determined according to the outcomes of treatment. The serum 4βHC levels of patients with virologic failure (n=4) during ASV/DCV treatment (85.1±11 ng/mL) were significantly higher in comparison to patients with an SVR but in whom the Invader assay detected minor RAVs during the pretreatment period (n=10, 60.1±10 ng/mL, p<0.05). In addition, the analysis of the serum 4βHC level during the treatment period showed that during treatment the 4βHC level did not change to a statistically significant extent in either group (Fig. 3A).

The serum concentrations of ASV/DCV were determined by LC-MS/MS. In the outpatient setting, it is difficult to obtain serum samples in an adjusted sampling period after the administration of these agents; thus, serum samples obtained at three points during the treatment period (2, 4, and 8 weeks after the start of treatment) were used, and the average concentrations of these samples were shown. As shown in Fig. 3B and C, the concentrations of ASV or DCV among the patients who showed virologic failure (n=4) tended to be lower in comparison to the SVR group (n=13); however, the difference was not statistically significant.

The factors that were previously reported to be associated with the outcomes of ASV/DCV treatment were selected and evaluated by a univariate analysis. The factors selected in the present study were as follows: gender, age, liver cirrhosis or chronic hepatitis, aspartate aminotransferase (AST) level, ALT level, platelet count, estimated glomerular filtration rate (eGFR), presence of Y93 mutation in the Invader assay, presence or absence of a rapid virological response (RVR), past history of IFN-based regimens, and the pretreatment serum 4βHC level (Table 3). The absence of an RVR, and a pre-treatment serum 4βHC level of >60 ng/mL were found to be significantly associated with a non-SVR outcome. However, no factors remained associated with an SVR12 in the multivariate analysis, probably due to the smaller sample size.

**Discussion**

Although some CYP3A test substrates, including midazolam, erythromycin, alprazolam, and nifedipine, have been proposed (18), the selection of an appropriate CYP3A phenotyping substrate and metric remains a matter of discussion. However, the administration of these drugs is sometimes difficult because the administration itself may have a negative impact on patient safety. Some reports have shown that polymorphisms of CYP3A4 and CYP3A5 were associated with drug concentrations (19). This might be an attractive approach; however, it is unknown whether this approach...
Table 3. Factors Associated with SVR and NonSVR by Treatment with ASV/DCV.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SVR group (n=66)</th>
<th>NSVR group (n=4)</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>66.7±24.4</td>
<td>67.8±21.2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Male Gender (%)</td>
<td>42.8</td>
<td>25.0</td>
<td>N.S.</td>
</tr>
<tr>
<td>LC (%)</td>
<td>35.7</td>
<td>64.3</td>
<td>N.S.</td>
</tr>
<tr>
<td>Previous IFN treatment (%)</td>
<td>45.2</td>
<td>52.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>AST level</td>
<td>52.0±34.6</td>
<td>44.0±19.7</td>
<td>N.S.</td>
</tr>
<tr>
<td>ALT level</td>
<td>47.9±38.9</td>
<td>35.5±21.1</td>
<td>N.S.</td>
</tr>
<tr>
<td>Platelet number (×10^4/μL)</td>
<td>14.9±7.6</td>
<td>8.7±6.4</td>
<td>N.S.</td>
</tr>
<tr>
<td>eGFR value</td>
<td>63.2±12.9</td>
<td>68.1±12.9</td>
<td>N.S.</td>
</tr>
<tr>
<td>4βHC level &lt;60 ng/mL (%)</td>
<td>100.0</td>
<td>57.1</td>
<td>0.01</td>
</tr>
<tr>
<td>Presence of RVR (%)</td>
<td>64.7</td>
<td>0</td>
<td>0.006</td>
</tr>
<tr>
<td>Y93 mutation (%)</td>
<td>14.3</td>
<td>25.0</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

Male Gender (%): ratio of male subjects in the group. LC (%): ratio of cirrhotic subjects in the group. Previous IFN treatment (%): ratio of patients having previous IFN treatment. 4βHC level<60ng/mL (%): ratio of patients with serum 4βHC below 60 ng/mL at pre-treatment period. Presence of RVR (%): ratio of patients with RVR (rapid virological response). Y93H mutation (%): ratio of patients having Y93 mutation detected by Invader assay at pre-treatment period. Wilcoxon test for continuous factors and Pearson’s chi-square test for categorical factors were used.

Figure 3. The serum 4βHC concentrations and ASV/DCV concentration in the ASV/DCV-treated cohort. (A) The serum 4βHC concentrations in the SVR group (n=10, with minor RAVs detected by the Invader assay), and patients with virologic failure during or after ASV/DCV treatment (n=4, with minor RAVs detected by the Invader assay), was determined. The concentrations of 4βHC were determined in the pretreatment period, at 12 weeks from the initiation of therapy, and at the end of treatment. *p<0.05. (B & C) The average concentrations (± S.D.) of ASV (3-B), and DCV (3-C) were plotted on the x-axis and the serum 4βHC level in the pre-treatment period (± S.D.) was plotted on the y-axis.

The serum 4βHC level was suggested as a potent surrogate marker for surveying the pretreatment CYP3A activity in various clinical situations, because its relatively long half-life means that intra-individual variation is relatively small (6). As shown in the present study, treatment with OTV/PTV/r resulted in a gradual decrease of the 4βHC level (Fig. 3). This finding suggests that this surrogate marker can be applied to determine the CYP3A activity in DAA-treated CHC patients. In addition, the serum 4βHC level in CHC patients was significantly higher in comparison to healthy control subjects, suggesting that pharmacokinetics and pharmacodynamics, which were mainly assayed in healthy subjects, seemed to be equivocal in the clinical setting (Fig. 1).

Direct sequencing revealed no pretreatment RAVs in the four ASV/DCV-treated cases that showed virologic failure. In the clinical setting, this is the method that is most frequently used to detect RAVs. However, minor RAVs were detected by the more sensitive Invader assay (Table 3). On
the contrary, some patients in whom minor RAVs were only detectable by the Invader assay showed a favorable treatment outcome. Indeed, 48% of the patients achieved an SVR in a large clinical study, even when RAVs were present (20), suggesting possible inter-individual differences in drug bioavailability. Among the host factors associated with drug bioavailability, inter-individual differences in the activity of drug metabolizing enzymes needs to be considered. It is especially worthwhile to pay attention to CYP3A activity, since the majority of DAAs, such as ASV and DCV, are potent CYP3A4 substrates. Thus, understanding the inter-individual differences in CYP3A activity may provide valuable information for predicting the outcome of treatment. The present study demonstrated that a higher pre-treatment serum 4βHC level, and in turn increased CYP3A4 activity, is disadvantageous in ASV/DCV therapy to overcome minor pretreatment RAVs.

In addition to the treatment efficacy, these inter-individual differences in the serum 4βHC level are important for understanding the possible drug-drug interactions occurring with the administration of DAAs. As previously mentioned, the majority of Japanese CHC patients, who are predominantly elderly, already use multiple medications for comorbidities; thus, DAA, which affects the CYP3A4 activity, may also influence the bioavailability of the pre-administered drug. Administration with OTV/PTV/r resulted in a gradual decrease in the serum 4βHC level, which reached a plateau at 4 weeks from the initiation of therapy, suggesting that patients should be carefully monitored - at least until 4 weeks from initiation of OTV/PTV/r therapy - for adverse effects, which mainly occur due to drug-drug interactions with other medications or supplements. This finding also suggested that patient with lower serum 4βHC level at pre-treatment period may have higher susceptibility to adverse event occurred by drug-drug interactions.

It has been reported that the replication of HCV is closely associated with the cholesterol metabolism in hepatocytes (21). Epidemiological studies have also shown that patients with HCV infection tend to have lower serum cholesterol levels (14). In addition, the serum cholesterol has been shown to increase after treatment (with either IFN-based or IFN-free regimens) (22, 23). Thus, the difference of the serum cholesterol level, the substrate of oxysterol, could affect the serum 4βHC level. However, despite the possible increase in serum cholesterol during the treatment period, the 4βHC concentration declined in patients who were treated with OTV/PTV/r: In addition, the 4βHC levels of patients who received another DAA combination, ASV/DCV, did not change during the treatment period (Fig. 3). In addition, we also discovered that the behavior of other oxysterols such as 7αHC, 24HC and 27HC - during treatment period with OTV/PTV/r differed from that of 4βHC (data not shown). Taken together, it is strongly suggested that the change of 4βHC during OTV/PTV/r is mainly caused by ritonavir.

The mechanism(s) underlying the basal-level elevation of 4βHC in CHC patients is unknown, but several possibilities can be suggested. We previously reported that various serum oxysterols (i.e., 7αHC, and 25HC) were also increased in patients with CHC (15). We hypothesized that the increase of these oxysterols was the result of increasing oxidative stresses in CHC, since interferon treatment - which has a potent anti-inflammatory action - dramatically attenuated the serum levels of these oxysterols regardless of the presence or absence of an early viral response (EVR) (15), whereas IFN-free, ASV/DCV treatment did not alter their levels, as was mentioned in the present study. However, this mechanism is not enough to explain why 4βHC, an oxysterol that is predominantly synthesized by an enzymatic reaction - and not by autoxidation - is increased in CHC patients. One possible explanation for the increasing CYP3A activity might be a compensatory mechanism to detoxify highly toxic xenobiotics and/or endogenous substances such as hydrophobic bile acids. Indeed, our previous study demonstrated that the serum 4βHC levels were elevated in patients with primary biliary cholangitis (PBC), which is associated with the retention of cytotoxic bile acids, such as lithocholic acid (LCA) (24). Our present findings suggests the possibility that toxic substances, the levels of which may be increased, need to be catabolized by CYP3A in CHC. In addition, our findings, which demonstrated no significant changes in the serum 4βHC levels among the stages of differential fibrosis (F0-F3). This suggests the possibility that the serum 4βHC level and, in turn, the CYP3A4 activity, is maintained even in relatively advanced stages of CHC. Although the change of the serum 4βHC level in patients with very advanced-stage cirrhosis was not investigated in the present study, it would be useful to investigate whether it is maintained at the same level in such patients. In the treatment for patients with decompensated liver cirrhosis, the dose control of DAAs according to changes in drug metabolism should be a matter of discussion. Further studies are needed to elucidate the mechanism underlying the elevation of 4βHC in CHC patients and its association with the progression of the disease, including hepatocarcinogenesis.

The present study was associated with some limitations, including the relatively small study population. In addition, another system should be used to define the bioavailability of these medications (i.e., inter-individual differences in OATP-1B1 or 1B3, which are thought to be involved in the uptake of DAAs) should be analyzed in future studies. Furthermore, previous reports suggested that inter-racial differences in 4βHC concentrations are associated with differences in CYP3A4 activity (16). Asian people have been shown to have higher 4βHC concentrations in comparison to Caucasian and African people (16). Thus, the fact that the study population was limited to Japanese patients is considered to be a limitation.

In summary, we demonstrated, for the first time that the serum 4βHC level (a marker of CYP3A activity) was elevated in CHC patients and that it varied in patients with different backgrounds. The results obtained from samples from patients treated with OTV/PTV/r suggest that it is a suro-
gate marker that can be used to monitor the CYP3A4 activity in CHC patients under DAA treatment. Furthermore, the changes in the serum concentration of ASV/DCV due to inter-individual differences in the CYP3A4 activity of CHCs could partially explain the different outcomes of ASV/DCV therapy in patients with minor pre-existing RAVs.

We are currently in an era in which global HCV eradication is possible. It has been reported that newly available DAAs, such as grazoprevir/elbasvir, are also metabolized by CYP3A4. In clinical trials, these new agents have been characterized by a very low rate of adverse events, and higher efficacy, even in patients with pre-existing RAVs (25). Thus, there is an opinion that variation in CYP3A4 activity may no longer be a critical issue in treatment efficacy. However, the mean age of the HCV-infected population and the number of elderly patients with more advanced liver diseases as well as concomitant medications are gradually increasing worldwide. Thus, predicting markers to avoid serious adverse events due to drug-drug interactions still needs to be considered, especially for patients in this group. In this setting, the evaluation of the CYP3A4 activity by measuring 4βHС before treatment may provide additional information that is useful in the selecting a safe, potentially cost-effective and treatment strategy with optimal efficacy.

The authors state that they have no Conflict of Interest (COI).

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References
25. Lawitz E, Gane E, Pearlman B, et al. Efficacy and safety of 12 weeks versus 18 weeks of treatment with grazoprevir (MK-5172) and elbasvir (MK-8742) with or without ribavirin for hepatitis C virus genotype 1 infection in previously untreated patients with...
cirrhosis and patients with previous null response with or without cirrhosis (C-WORTHY): a randomised, open-label phase 2 trial. Lancet 385: 1075-1086, 2015.

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