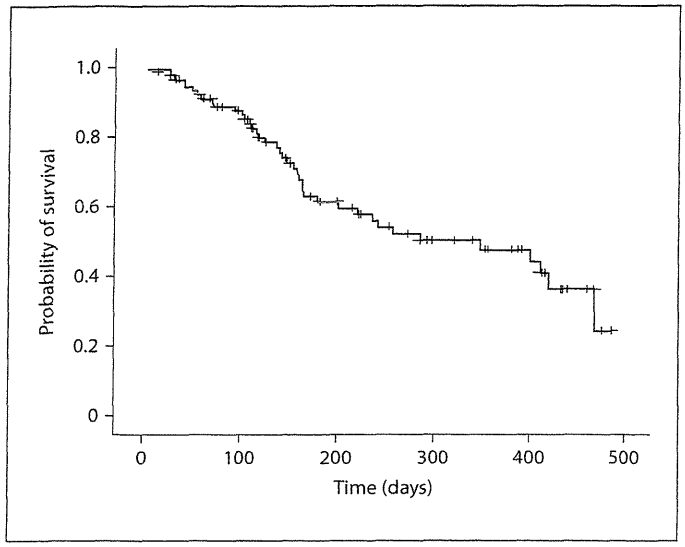


Fig. 1. Cumulative survival of 96 patients with advanced HCC treated with sorafenib. The MST of these patients was 11.6 months. The 1-year survival rate was 48%.

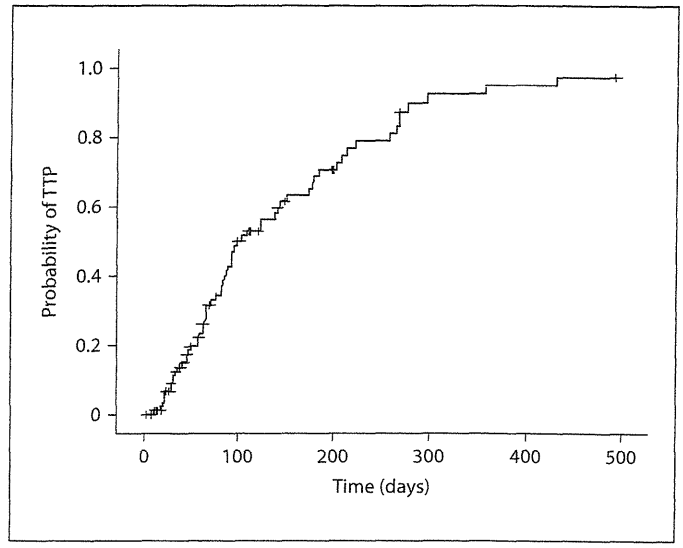


Fig. 2. Cumulative progression of 96 patients with advanced HCC treated with sorafenib. The median TTP of these patients was 3.2 months.

Table 2. Univariate and multivariate analyses of survival in patients with HCC

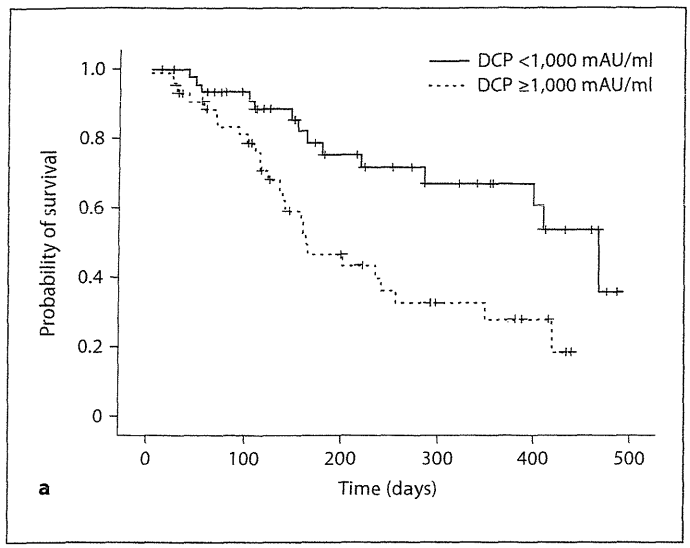
	Univariate		Multivariate	
	HR (95% CI)	p value	HR (95% CI)	p value
Age (≥70 years)	1.091 (0.581–2.050)	0.786		
Sex (male)	0.670 (0.320–1.403)	0.288		
Child-Pugh class (B)	2.273 (0.868–5.952)	0.094		
AFP (≥1,000 ng/ml)	1.953 (1.046–3.647)	0.036		
DCP (≥1,000 mAU/ml)	2.723 (1.394–5.316)	0.003	2.722 (1.369–5.412)	0.004
Daily average dosage (≥400 mg)	0.970 (0.503–1.870)	0.927		
Daily average dosage (≥600 mg)	1.042 (0.556–1.954)	0.898		
Duration of treatment (≥30 days)	0.403 (0.199–0.816)	0.012	0.407 (0.196–0.848)	0.016
Therapeutic effect (PD)	1.876 (0.991–3.549)	0.053		

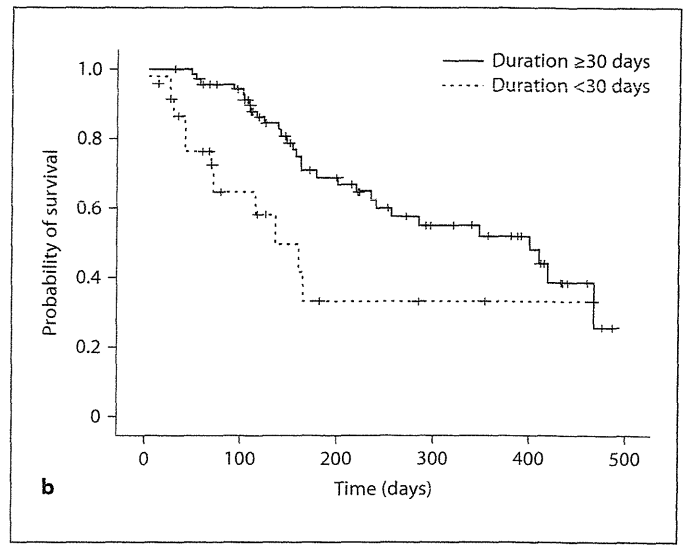
HR = Hazard ratio; 95% CI = 95% confidence interval.

ticular, interstitial pneumonia (n = 1; 1%) and tumor lysis syndrome (n = 1; 1%) were serious adverse events. The single case of interstitial pneumonia resulted in death.

Survival and Factors Associated with Outcome

The cumulative survival curve of 96 patients is shown in figure 1. The median survival time (MST) was 11.6 (range 0.1–16.2) months, with a 1-year survival rate of 48%. The median time to progression (TTP) was 3.2 (range 0.1–16.2) months (fig. 2). Cox proportional hazards regression analysis was performed to identify independent factors associated with survival (table 2). The results of univariate analysis showed that AFP serum level (≥1,000 ng/ml, p = 0.036), DCP serum level (≥1,000 mAU/ml, p = 0.003), and duration of treatment (>30 days, p = 0.012) were significant risk factors adversely impacting survival. Multivariate analysis showed that DCP serum level (≥1,000 mAU/ml, HR 2.722, 95% CI 1.369–5.412, p = 0.004) and duration of treatment (>30 days, HR 0.407, 95% CI 0.196–0.848, p = 0.016) were independent risk factors for decreased survival. Cumulative survival curves, plotted for DCP serum level and duration of treatment, are shown in figure 3.





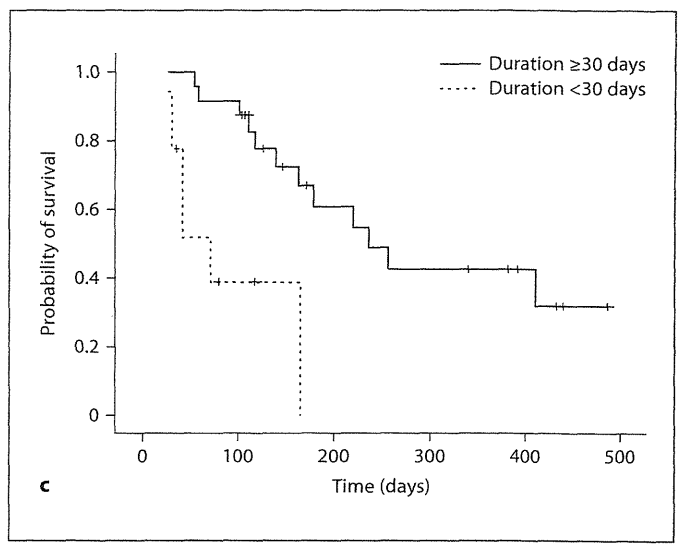


Fig. 3. **a** Cumulative survival of patients grouped by serum DCP levels. The MSTs of the group with DCP >1,000 and <1,000 mAU/ml were 5.4 and 15.6 months, respectively (p = 0.0023). **b** Cumulative survival of patients grouped by duration of treatment. The MSTs of >30 and <30 days of treatment were 13.3 and 4.5 months, respectively (p = 0.0091). **c** Cumulative survival of patients with PD grouped by duration of treatment. The MSTs with >30 and <30 days of treatment were 7.8 and 2.4 months, respectively (p = 0.0008).

Discussion

Sorafenib, an oral multikinase inhibitor, has recently become available as a new molecular targeted therapy for advanced HCC. A significant survival benefit and good tolerance was demonstrated with sorafenib treatment for patients with advanced HCC in 2 randomized phase III placebo-controlled trials [16, 18]. Consequently, sorafenib has become the standard treatment for advanced HCC in the United States, Europe, and many other countries, including Japan. This study prospectively assessed the efficacy and safety of sorafenib and identified the factors associated with survival in Japanese patients with advanced HCC. In this study, the TTP and MST of Japanese patients receiving sorafenib were 3.2 and 11.6 months, respectively. TTP in this study was shorter than that observed in the SHARP trial (5.5 months) and was similar to that observed in the Asia-Pacific study (2.8 months) [16, 18]. However, the MST in the current study was longer than that observed in the Asia-Pacific study (6.5 months) and was similar to that observed in the SHARP trial (10.7 months) [16, 18]. Compared with these 2 previous studies, the time between TTP and MST was longer in the current study, though the reason for this is unclear.

An exploratory multivariate analysis with the use of a Cox proportional hazards model identified 2 baseline patient characteristics that were prognostic indicators for overall survival: duration of treatment and serum DCP

level. In contrast, therapeutic effect and dosage of sorafenib were not significant risk factors adversely affecting survival in this study. In the SHARP trial and the Asia-Pacific study, administration of sorafenib was continued until the occurrence of both radiologic and symptomatic progression, or the occurrence of either unacceptable adverse events or death [16, 18]. In the current study, neither radiologic nor symptomatic progression were criteria for discontinuation. The difference in the discontinuation criteria may explain the gap between TTP and MST in this study. Even with tumor progression, the patients who continued on sorafenib may have had better survival potential compared to the patients in whom sorafenib was discontinued (fig. 3c). Therefore, this study suggests that sorafenib should be administered long-term in patients with advanced HCC independent of therapeutic effect and dosage.

Previous studies reported that for patients with HCC, high serum DCP levels are associated with vascular invasion, metastasis, and tumor recurrence [23]. Hypoxia has been reported to induce epithelial mesenchymal transition or cytoskeletal changes. Indeed, hypoxic stimulation induced hepatoma cell lines (HepG2 or PLC/PRF/5 cells) to undergo epithelial-to-fibroblastoid conversion or epithelial mesenchymal transition, and these cells produced DCP [23]. Therefore, DCP as an HCC tumor marker is more useful in larger tumors which are likely to be exposed to hypoxia during tumor development [23]. Thus, it is suggested that higher serum DCP levels represent a more advanced state of HCC, and, as a result, lead to reduced survival rates.

In this study, disease classification at the first radiologic assessment was PR for 12 (13%) patients, stable disease for 43 (45%) patients, and PD for 33 (34%) patients. Notably, the proportion of patients with PR in our study was higher compared to the SHARP trial (2%) and the Asia-Pacific study (3.3%). It is not clear why there appears to be a higher rate of PR in Japanese patients. Previous studies suggested that there may be racial differences in terms of gene mutations that may affect sorafenib treatment [24, 25]. Lynch et al. [26] reported that patients with non-small-cell lung cancer have specific mutations in the *EGFR* gene, which correlate with clinical responsiveness to the tyrosine kinase inhibitor gefitinib. Therefore, it is suggested that Japanese patients with advanced HCC may be more sensitive to sorafenib than Western and other Asian populations. To investigate the possible differences in the therapeutic effects of sorafenib, further studies with larger patient populations will be needed.

Treatment-related adverse events were a substantial issue impacting the continuation of treatment with sorafenib. In this study, although the overall incidence of treatment-related adverse events was high (90%), events were primarily controlled with medical treatment and/or sorafenib dose reductions. Adverse events leading to discontinuation of treatment included liver dysfunction (8%), HFSR (7%), and diarrhea (4%), which are commonly associated with sorafenib [27, 28]. However, in the SHARP trial, the overall incidence of treatment-related adverse events was 80% in the sorafenib group, and the most frequent adverse events leading to discontinuation of sorafenib treatment were gastrointestinal events (6%), fatigue (5%), and liver dysfunction (5%) [16]. HFSR is particularly well known as an early adverse event [29–31] associated with sorafenib therapy and the severity of HFSR depends on the duration of treatment, dosage, and accumulation of the drug [32]. Further effort put towards the effective control of adverse effects and management of sorafenib dosing, with a priority given to facilitating long-term administration, will lead to the most effective therapy for patients with HCC. Moreover, hepatic reserve is important for hepatic extraction and metabolism of sorafenib. In this study, liver dysfunction necessitating suspension or discontinuation of sorafenib occurred with similar frequency in patients with Child-Pugh class B and Child-Pugh class A disease. This result suggests that sorafenib can be used in patients with Child-Pugh class B, as well as in patients with Child-Pugh class A disease.

In conclusion, sorafenib was a safe and effective therapy in Japanese patients with advanced HCC. In addition, duration of treatment and serum level of DCP were independent risk factors negatively impacting survival in this study. The results of this study indicate that sorafenib should be administered as a long-term treatment for advanced HCC in patients regardless of therapeutic effect and dosage.

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Japanese Reference Panel of Blood Specimens for Evaluation of Hepatitis C Virus RNA and Core Antigen Quantitative Assays

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An accurate and reliable quantitative assay for hepatitis C virus (HCV) is essential for measuring viral propagation and the efficacy of antiviral therapy. There is a growing need for domestic reference panels for evaluation of clinical assay kits because the performance of these kits may vary with region-specific genotypes or polymorphisms. In this study, we established a reference panel by selecting 80 donated blood specimens in Japan that tested positive for HCV. Using this panel, we quantified HCV viral loads using two HCV RNA kits and five core antigen (Ag) kits currently available in Japan. The data from the two HCV RNA assay kits showed excellent correlation. All RNA titers were distributed evenly across a range from 3 to 7 log IU/ml. Although the data from the five core Ag kits also correlated with RNA titers, the sensitivities of individual kits were not sufficient to quantify viral load in all samples. As calculated by the correlation with RNA titers, the theoretical lower limits of detection by these core Ag assays were higher than those for the detection of RNA. Moreover, in several samples in our panel, core Ag levels were underestimated compared to RNA titers. Sequence analysis in the HCV core region suggested that polymorphisms at amino acids 47 to 49 of the core Ag were responsible for this underestimation. The panel established in this study will be useful for estimating the quality of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

Hepatitis C virus (HCV) is a major cause of chronic liver disease worldwide (15). There is no protective vaccine against this virus, and once an individual is infected, HCV often establishes persistent infection and leads to chronic hepatitis, cirrhosis, and hepatocellular carcinoma (9). The most widely used therapy for HCV infection is the combined administration of pegylated alpha interferon and ribavirin (29). However, this treatment is problematic, as it has limited efficacy, high cost, and severe adverse effects (8, 25). To estimate the outcome of antiviral therapy, and to understand the state of viral propagation, it is important to determine the HCV viral load in chronic hepatitis C patients by the use of accurate and reliable HCV quantitative assays (9, 14). For this purpose, several commercial assay kits for HCV RNA and core antigen (Ag) quantification are currently used in Japan. For quantification of HCV RNA levels, two real-time quantitative reverse transcription-PCR (qRT-PCR)-based assay kits are available, including the COBAS AmpliPrep/COBAS TaqMan HCV test (CAP/CTM-RNA; Roche Diagnostics, Tokyo, Japan) and the Abbott RealTime HCV test (ART-RNA; Abbott Japan, Tokyo, Japan). These assays are known to have high sensitivity and a wide dynamic range, but they require technical skill and attention to maintaining the specified conditions (4–6, 16, 24, 33–35). Alternatively, HCV viremia can be quantified by assessment of HCV core Ag level (1–3, 7, 10, 12, 13, 17–22, 27, 30–32). Five HCV core Ag assay kits are commercially available in Japan, including Architect HCV Ag (Architect-Ag; Abbott Japan), Lumipulse Ortho HCV Ag (Lumipulse-Ag; Fujirebio, Tokyo, Japan), Lumispot Eiken HCV Ag (Lumispot-Ag; Eiken Chemical, Tokyo, Japan), the Ortho HCV Ag ELISA test (ELISA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan), and the Ortho HCV Ag IRMA test (IRMA-Ag; Ortho Clinical Diagnostics, Tokyo, Japan). These assays have some disadvantages compared to those measuring HCV RNA (notably, low sensitivity and narrow range of quantification) but also have some advantages (including ease of use, reduced risk of contamination, reduced cost, and reliability even with samples stored at room temperature for extended periods of time [1, 32]). Although core Ag levels are thought to be related closely to HCV RNA titers, the correlation and linearity of core Ag levels have not yet been fully evaluated. In addition, these quantitative parameters are known to be affected by nucleotide and amino acid sequences at the target regions of the assays (5, 6, 28, 34), and this sequence variation depends on genotypes or predominant strains in specific geographical regions.

In this study, we established a Japanese reference panel of samples for evaluation of HCV RNA and core Ag levels by collecting donated blood specimens that tested positive for HCV RNA and anti-HCV antibodies. Using this reference panel, we evaluated the HCV loads in these specimens with two HCV RNA assay kits and five core Ag assay kits and assessed correlations among the data generated by these kits.

MATERIALS AND METHODS

Preparation of reference panel. To establish a reference panel for HCV quantitative assays, a total of 80 donated plasma samples were selected. All of these specimens, supplied by the Japanese Red Cross Blood Centers, tested positive for the presence of HCV RNA and anti-HCV antibodies. These samples, collected in Japan from May to September of 2007, were obtained from Japanese blood donor volunteers in various regions of

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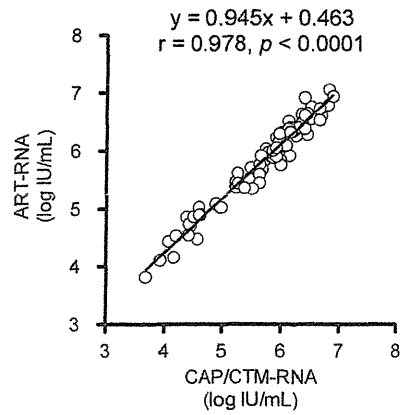

FIG 1 Correlation of HCV RNA titers as quantified by two commercial kits.

Japan. The samples were divided into 1-ml aliquots and stored at −80°C until use.

Quantification of HCV RNA and core Ag. The HCV RNA titer was measured with two real-time qRT-PCR kits, CAP/CTM-RNA (detection range, 1.5×10^1 to 6.9×10^7 IU/ml) and ART-RNA (detection range, 1.2×10^1 to 1.0×10^8 IU/ml). Additionally, samples were assessed using five HCV core Ag assay kits, including Architect-Ag (detection range, 3 to 20,000 fmol/liter), Lumipulse-Ag (detection range, 50 to 50,000 fmol/liter), Lumispot-Ag (detection range, 20 to 400,000 fmol/liter), ELISA-Ag (detection range, 44.4 to 3,600 fmol/liter), and IRMA-Ag (detection range, 20 to 20,000 fmol/liter). All assays were performed by the respective manufacturers at their research laboratories.

Sequencing and genotyping of HCV in reference panel samples. Viral RNA was extracted with the QIAamp viral RNA kit (Qiagen, Valencia, CA) from 140 μl of each plasma sample. HCV RNA was amplified by RT-PCR with primers corresponding to the 5′ untranslated region (UTR) (43S-IH, 5′-CCTGTGAGGAACTACTGTCTTC-3′; c/s17-ssp, 5′-CCGGGAGAGCCATAGTGGTCTGCG-3′) and the E1 region (1323R-IH, 5′-GGCGACCAGTTCATCATCAT-3′); the amplified products were sequenced directly. HCV genotypes of the isolated strains were assigned by phylogenetic analysis using an alignment with a representative strain of each genotype.

Statistical analysis. The correlations of obtained quantitative data were assessed by Pearson's correlation coefficient analysis, and values for r and P were calculated. A P value of <0.05 was considered to indicate statistical significance. Analysis was performed using Prism 5 software (GraphPad Software, Inc., La Jolla, CA).

Nucleotide sequence accession numbers. The accession numbers of C-01 to C-80 are AB705312 to AB705391, respectively.

RESULTS

Quantification of HCV RNA levels. The reference panel established in this work was used to measure HCV RNA levels with the CAP/CTM-RNA and ART-RNA kits. The correlation of the data obtained with the two kits is shown in Fig. 1. The RNA titers of these samples were distributed evenly, and all values were within the dynamic ranges of both assays. The HCV titers ranged from 3.68 to 6.88 and 3.82 to 7.08 log IU/ml in CAP/CTM-RNA and ART-RNA, respectively, and the correlation was significant ($r = 0.978$; $P < 0.0001$).

Quantification of HCV core Ag levels. HCV core Ag levels were measured using Architect-Ag, Lumipulse-Ag, Lumispot-Ag, ELISA-Ag, and IRMA-Ag kits. Among the 80 specimens in the reference panel, core Ag levels could be measured in all samples using Architect-Ag and ELISA-Ag kits, whereas core Ag levels were below the detection limit in 4, 2, and 1 samples using Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag kits, respectively (Fig. 2; also, see Fig. S1 in the supplemental material). Significant correlations were observed between assays of HCV core Ag and HCV RNA ($r = 0.9065$ to 0.9666 and $P < 0.0001$ compared with CAP/CTM-RNA data [Fig. 2]); $r = 0.8877$ to 0.9552 and $P < 0.0001$ compared with ART-RNA data [see Fig. S1 in the supplemental material]). The theoretical lower limits of detection of these assays were calculated by use of these correlation formulas and were 3.2 and 3.4 log IU/ml for Architect-Ag, 4.2 and 4.2 log IU/ml for Lumipulse-Ag, 3.7 and 3.9 log IU/ml for Lumispot-Ag, 3.6 and 3.8 log IU/ml for ELISA-Ag, and 3.6 and 3.8 log IU/ml for IRMA-Ag (compared to CAP/CTM-RNA and ART-RNA, respectively). These calculated detection limits were substantially higher than those for the RNA quantitative assays (1.18 and 1.08 log IU/ml for CAP/CTM-RNA and ART-RNA, respectively).

In addition, we found that several samples showed considerable deviation from the linear regression (Fig. 2; also, see Fig. S1 in the supplemental material). To identify the deviating samples, we used Bland-Altman plot analysis (Fig. 3; also, see Fig. S2 in the supplemental material). This plot shows the difference between the titer values of HCV RNA and core Ag as a function of the average of these two values. Several samples demonstrated discordance between the measured HCV RNA and core Ag levels. Among these samples, we focused on samples with discordant results in multiple core Ag assays compared to both RNA quantitative assays. For sample C-01, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and Lumispot-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag, Lumipulse-Ag, Lumispot-Ag, and IRMA-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Likewise, for sample C-73, core Ag levels were underestimated when measured with Architect-Ag, Lumipulse-Ag, and IRMA-Ag in comparison with CAP/CTM-RNA (Fig. 3) and when measured with Architect-Ag and Lumipulse-Ag in comparison with ART-RNA (see Fig. S2 in the supplemental material). Thus, sample-specific underestimation was observed in several HCV core Ag kits.

Nucleotide sequences in core region of reference panel samples. To clarify the sources of these underestimates of HCV core Ag levels, HCV RNA was extracted from each of the samples in the reference panel, and the nucleotide sequences of core regions were determined. Phylogenetic analysis with these sequences permitted classification of the individual strains by genotype. Of 80 samples in the reference panel, 1 (1.3%) was genotype 1a, 35 (43.8%) were genotype 1b, 26 (32.5%) were genotype 2a, and 18 (22.5%) were genotype 2b (Table 1; also, see Fig. S3 in the supplemental material). These strains were distributed evenly among reference strains of each genotype and cover the sequence diversity of strains isolated in Japan (see Fig. S3 in the supplemental material). The genotypes of samples associated with underestimated core Ag values (samples C-01 and C-73) were both classified as genotype 2a.

Predicted amino acid sequences of HCV core protein were aligned with the consensus core protein sequence for the genotype 1b strains obtained in this study (see Fig. S4 in the supplemental material). Excluding the genotype-specific sequence variations, a specific amino acid polymorphism was identified at amino acid (aa) residue 48 (Ala to Thr) in samples C-01 and C-73. Sample C-01, which yielded underestimated values in most core Ag assays, also possessed an additional polymorphism in the same region,

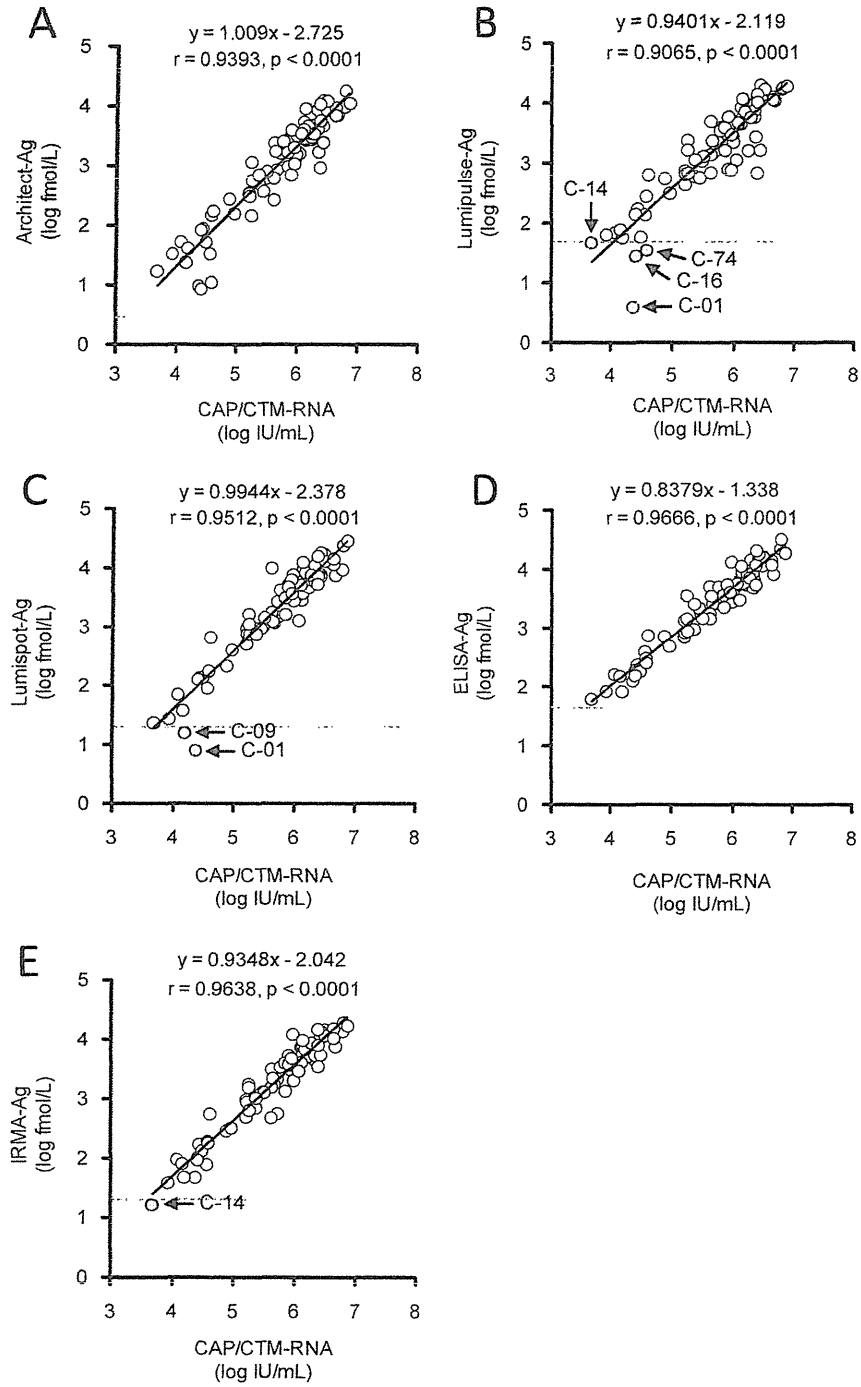

FIG 2 Correlation between CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. Data for core Ag levels were converted to log fmol/liter prior to analysis. In each plot, the lower limit of detection of the respective core Ag assay is indicated by a dotted line. Data for samples below the lower detection limit of each assay are indicated by shaded circles labeled with the respective sample designations.

specifically an Arg-to-Gly substitution at aa 47. We suspected that these polymorphisms altered the antigenicity of the core protein, thereby reducing detected core Ag levels and leading to underestimation of values by the core Ag quantification kits. To assess the correlation of these polymorphisms with the underestimation of core Ag values, strains containing polymorphisms in this region (at aa 47 to 49 [Fig. 4]) were identified in Bland-Altman plots of HCV RNA and core Ag (Fig. 3; also, see Fig. S2 in the supplemental material). A total of 12 strains exhibited polymorphisms at these positions, including 2 strains of genotype 1b, 8 of genotype 2a, and 2 of genotype 2b (Table 1). In the Bland-Altman plot of CAP/CTM-RNA and Architect-Ag, 4 of 12 values (for samples C-01, C-16, C-73, and C-74) were located under the line of the lower 95% limit of agreement (Fig. 3A). Likewise, in the plot of CAP/CTM-RNA and Lumipulse-Ag, 3 of 12 values (those for samples C-01, C-67, and C-73) were located under the line of the lower

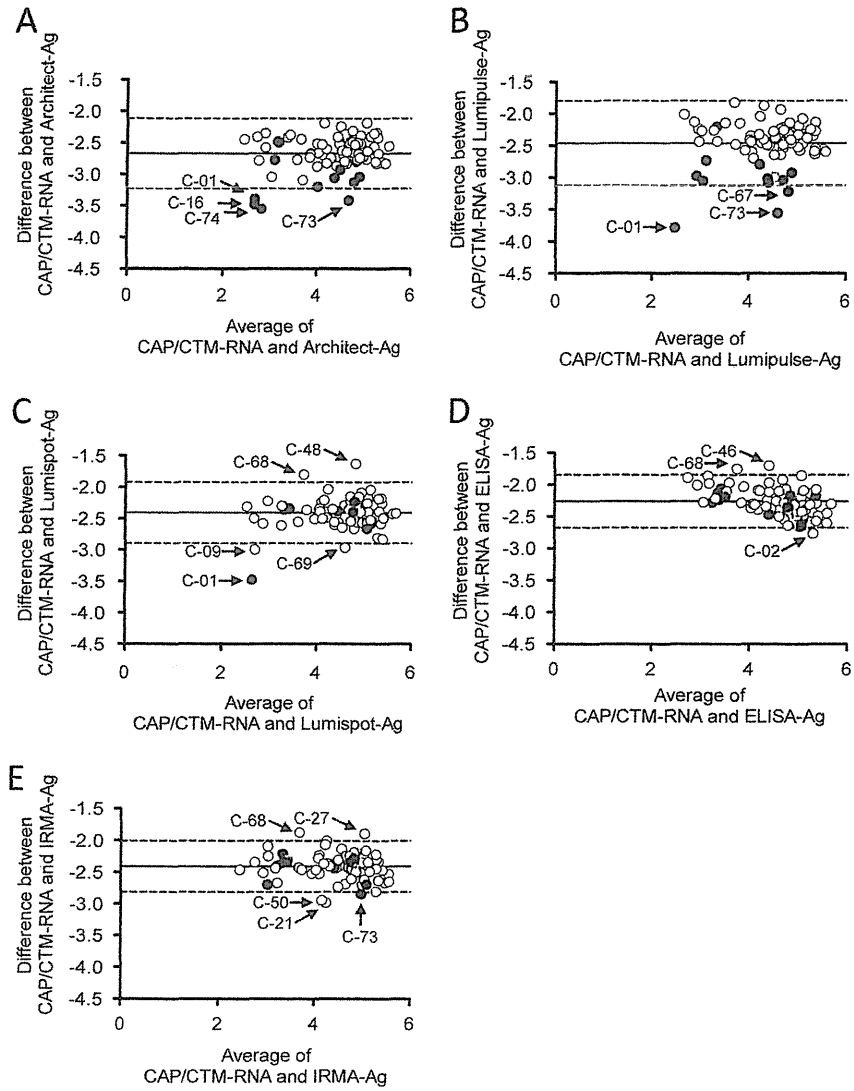

FIG 3 Bland-Altman plot analysis of CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. These plots show the difference between the values of HCV RNA and core Ag as a function of the average of these two values. Data for core Ag levels were converted to log fmol/liter prior to analysis. The bias and 95% limits of agreements are indicated by solid and dashed lines, respectively. Data for samples with polymorphisms at amino acid residues 47 to 49 are indicated by solid circles. Data points outside the 95% limits are indicated by arrows labeled with the sample designations.

95% limit of agreement (Fig. 3B). In these plots, underestimation for samples that lacked these polymorphisms (at aa 47 to 49) was not detected. In the plot of CAP/CTM-RNA and Lumispot-Ag, only 1 sample (C-01) was located under the line of the lower 95% limit of agreement, but this sample exhibited the most discordant value (Fig. 3C). In the plot of CAP/CTM-RNA and ELISA-Ag, no correlation between polymorphisms at these positions and underestimation was observed (Fig. 3D). In the plot of CAP/CTM-RNA and IRMA-Ag, sample C-73 was located under the line of the lower 95% limit of agreement, as were other samples that lacked polymorphisms at aa 47 to 49 (Fig. 3E). Similar trends were observed in comparison with ART-RNA levels (see Fig. S2 in the supplemental material). Based on these results, the levels of HCV core Ag measured with Architect-Ag and Lumipulse-Ag seem to be more strongly affected by single polymorphisms at these positions. In the case of Lumispot-Ag, underestimation may be limited to specimens with multiple polymorphisms at these positions.

TABLE 1 Number of reference panel strains with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains	
	Total	With polymorphisms
1a	1	0
1b	35	2 (5.7)
2a	26	8 (30.8)
2b	18	2 (11.8)
Total	80	12 (15.0)

DISCUSSION

The quantification of HCV viral load is essential for selecting an appropriate antiviral strategy and for monitoring the efficacy of treatment. Since HCV is known to be highly variable and rapidly

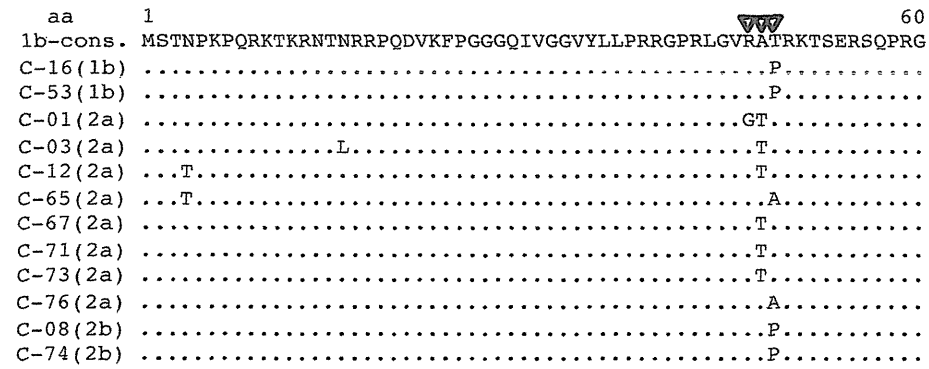

FIG 4 Alignment of the first 60 amino acids of the HCV core region of strains with polymorphisms at amino acid residues 47 to 49. The position numbers are given at the top. Dots indicate identical amino acids. The consensus sequence of 1b strains (1b cons.) isolated in this study was determined and used as a reference sequence. Genotypes of strains are given in parentheses. Positions of polymorphisms are indicated by inverted triangles above the sequence alignment.

evolving (23, 26), the assays for quantifying this virus should be unaffected by sequence polymorphisms. In this study, we established a reference panel with HCV-positive samples and evaluated the correlation among multiple assays for HCV RNA and core Ag quantification.

Using this reference panel, we found that the results from two HCV RNA assay kits, CAP/CTM-RNA and ART-RNA, correlated with excellent agreement ($r = 0.978$, $P < 0.0001$ [Fig. 1]), although discrepancies for values generated by these two assays have been reported for strains of genotypes 1, 2, and 4 (5, 6, 34). In Japan, the prevalent genotypes are 1b, 2a, and 2b (11); no genotype 4 sample was included in our reference panel (Table 1). In quantification with CAP/CTM-RNA, underestimation of HCV RNA titer has been reported for French genotype 2 samples (5). In our panel, no underestimation was observed for data from genotype 2 samples compared to values obtained using ART-RNA. Therefore, underestimation in quantification with CAP/CTM-RNA is expected to be rare in Japanese samples, and the two assays for HCV RNA quantification should be considered accurate and reliable, at least for Japanese samples. Additionally, the prepared reference panel appears to be suitable for the evaluation of HCV quantification assays, because genotypes of samples in this panel are representative of those found in Japan and viral loads are distributed evenly across the range of expected titers.

The quantification of HCV core Ag is an alternative test for HCV infection and viral load. However, in this study, several core Ag quantitative assays failed to provide accurate results for all of the samples in the reference panel (Fig. 2). Some quantified values were below the kits' detection limits. This shortcoming was mainly attributable to the lower sensitivity of the core Ag assay kits; increased sensitivity is urged in the future development of HCV core Ag kits. Among the kits tested here, Architect-Ag assay exhibited the highest sensitivity and was sufficient for quantifying the viral load in all samples. However, even in the case of Architect-Ag, theoretical lower limits of detection, calculated by correlation formula using CAP/CTM-RNA and ART-RNA, were 3.2 and 3.4 log IU/ml, respectively; these detection limits still exceeded the lower limits of the HCV RNA quantification assays. Therefore, the sensitivity of the available HCV core Ag assays is still insufficient to detect low-titer HCV infections. Core Ag kits therefore may be unsuitable for the detection of breakthrough hepatitis during antiviral therapy or for the detection of HCV infection in a window period.

Comparison between HCV RNA and core Ag assays revealed good correlations, with r coefficients ranging from 0.8877 to 0.9666 and P values being less than 0.0001 (Fig. 2; also, see Fig. S1 in the supplemental material). Therefore, the HCV core Ag levels may serve as an alternative to HCV RNA levels when titers remain within the detection ranges of the core Ag kits. However, several discordances were detected when core Ag levels were compared with those of HCV RNA. For one sample in our panel (sample C-01), core Ag levels were lower than expected when quantified using any of the three core Ag kits (Architect-Ag, Lumipulse-Ag, and Lumispot-Ag) (Fig. 3; also, see Fig. S2 in the supplemental material). Another sample (C-73) also yielded lower-than-expected levels when assayed with Architect-Ag and Lumipulse-Ag kits. Sequence analysis of the core region revealed that polymorphisms at aa 47 and 48 correlated with these underestimates by core Ag kits (see Fig. S4 in the supplemental material). These results are consistent with our previous study, which suggested that core Ag levels of HCV strain JFH-1 were underestimated by the Lumipulse-Ag kit in comparison to the ELISA-Ag assay (28). Strain JFH-1 harbors an Ala-to-Thr substitution at aa 48; conversion of Thr to Ala at this position in JFH-1 was sufficient to overcome this underestimation. This region of the core Ag presumably corresponds to one of the epitopes recognized by the monoclonal antibodies used in the Lumipulse-Ag kit, such that polymorphisms at this position affected the antigenicity of the core protein. In this study, we found that the presence of other polymorphisms in this region (aa 47 to 49) correlated with reduced core Ag levels as detected by Lumipulse-Ag, as well as by other assays (Architect-Ag and Lumispot-Ag). Sample C-01 demonstrated a drastic deviation from expected core Ag levels in these assays (Fig. 3; also, see Fig. S2 in the supplemental material). The HCV strain in this sample contains two polymorphisms (Arg to Gly at aa 47 and Ala to Thr at aa 48); the multiple polymorphisms may impair antibody binding more severely and therefore result in underestimation of core Ag levels. Interestingly, this sample exhibited reasonable core Ag levels when assayed using ELISA-Ag. Thus, the underestimation of core protein levels in this sample was kit dependent, suggesting the targeting of distinct epitopes by the antibodies used in each of these kits. This hypothesis could not be confirmed, because the identity of the epitopes targeted by each kit is proprietary.

Of 12 samples with amino acid polymorphisms in this region, 2 (5.7%) were of genotype 1b, 8 (30.8%) were of genotype 2a, and

TABLE 2 Number of strains in the sequence database[a] with polymorphisms at amino acid residues 47 to 49 of the HCV core region

Genotype	No. (%) of strains				
	Tested	With polymorphism			
		At aa 47 (R/C, G)	At aa 48 (A/T, P)	At aa 49 (T/A, P, L)	Total
1b	543	2 (0.36)	4 (0.74)	16 (2.96)	22 (4.1)
2a	24	0	6 (25.0)	1 (4.2)	7 (29.2)
2b	39	0	0	2 (6.9)	2 (6.9)

[a] http://s2as02.genes.nig.ac.jp/.

2 (11.8%) were of genotype 2b (Table 1). Searches of the Hepatitis Virus Database (http://s2as02.genes.nig.ac.jp/) revealed that corresponding amino acid polymorphisms were observed in 22 of 543 strains (4.1%) of genotype 1b, 7 of 24 strains (29.2%) of genotype 2a, and 2 of 39 strains (6.9%) of genotype 2b (Table 2). These percentages were consistent with our observations in the proposed reference panel. These data (our results and those from the database) clearly indicate that genotype 2a strains are the most frequent source of underestimation of core Ag levels. Notably, our search of the sequence database did not yield any HCV strain with multiple polymorphisms in the region from aa 47 to 49, as we saw in our sample C-01. Therefore, strains with such multiple polymorphisms are rare so far, but detection of this isolate among donated blood specimens suggests that such HCV strains could be emerging in clinical samples. For patients harboring such strains, HCV viral load may be underestimated if measurement of HCV viral load is performed by core Ag assay. Such underestimates may result in erroneous selection of therapy, adversely affecting patient outcome. Thus, this shortcoming in HCV core Ag assay kits needs to be addressed.

There is a growing need for evaluation of clinical assay kits with domestic specimen reference panels, since the performance of these kits may be affected by the genotypes or polymorphisms of predominant strains in different geographic regions. To our knowledge, such an investigation of HCV clinical assay kits with domestic specimens has not previously been conducted in Japan. The Japanese HCV reference panel described here was generated with plasma samples collected from Japanese volunteers. Each sample was divided into small aliquots, and the panel was prepared in multiple sets. The samples in our HCV reference panel represent the predominant strains and genotypes seen in Japan. We expect that this reference panel will be of use for the development, evaluation, and optimization of HCV assay kits for the Japanese clinical market.

In conclusion, we have established a Japanese reference panel for evaluation of HCV quantification assays. Using this reference panel, we found that two assay kits for HCV RNA could quantify HCV titers concordantly. We also found that the data generated by HCV core Ag assay kits correlated with the results of HCV RNA assays. However, the nominal core Ag levels measured by several kits underestimated actual levels for HCV samples with polymorphisms at aa 47 to 49 of the core Ag. The panel established in this study is expected to be useful for estimating the accuracy of currently available and upcoming HCV assay kits; such quality control is essential for clinical usage of these kits.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Increased serum liver X receptor ligand oxysterols in patients with non-alcoholic fatty liver disease

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Abstract
Background This study is a post-hoc analysis of a subset of patients who participated in our multi-institutional case-control study that evaluated the effects of pitavastatin in patients with non-alcoholic fatty liver disease (NAFLD) with hypercholesterolemia.
Methods Serum samples of fifteen patients with biopsy-proven NAFLD with dyslipidemia were investigated. Serum markers of lipid metabolism were quantified by liquid chromatography-mass spectrometry (LC–MS)/MS. These data were then compared with those of 36 sex- and age-matched healthy controls. In addition, changes in these markers produced by treatment with pitavastatin were evaluated.
Results Serum non-cholesterol sterols, reflecting intestinal cholesterol absorption, were significantly lower in the NAFLD patients compared to the controls, and the cholesterol synthesis marker, the ratio of lathosterol to cholesterol, was not significantly different between the two groups. Serum proportions of liver X receptor α (LXRα) ligand oxysterols (ratios to cholesterol) were significantly elevated in the NAFLD patients compared to the controls. The sum of oxysterols relative to cholesterol and the homeostasis model assessment as an index of insulin resistance (HOMA-IR) were significantly correlated. The marker representing cholesterol synthesis was significantly suppressed by pitavastatin treatment, from 3 months after initiation of the treatment, and the suppression remained significant during the observation period. The markers representing cholesterol absorption were unchanged at 3 months, but had significantly increased at 12 months. Serum oxysterol levels relative to cholesterol maintained high values and did not change significantly during the 12-month period of treatment.
Conclusions: We speculate that serum LXRα ligand oxysterol levels (relative to cholesterol) could be surrogate markers of insulin resistance, and that high oxysterol levels in the circulation may play an important role in the development of hepatic and peripheral insulin resistance followed by NAFLD.

Keywords NAFLD · Cholesterol metabolism · Oxysterol · Bile acids

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is characterized by an increase in intra-hepatocellular tryglycerides that is not due to alcohol or other known causes [1]. NAFLD could be called "insulin resistance-associated steatosis" because all components of the metabolic syndrome correlate with liver fat independent of the body mass index (BMI) [2]. In addition, hepatic insulin resistance is also closely correlated with the amount of fat in the liver both in non-diabetic [3] and type 2 diabetic [4] subjects. Recent studies have implicated several important hepatic cellular processes and signaling pathways that are affected by abnormal lipid metabolism, resulting in the specific biochemical, histological, and clinical changes associated with NAFLD.

Biological samples contain a large number of oxysterols [5] and some of them are important molecules to preserve lipid homeostasis in the body [6]. In particular, 4β-hydroxycholesterol, 22*R*-hydroxycholesterol, 24*S*-hydroxycholesterol, 24*S*,25-epoxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol are reported to be endogenous ligands of a nuclear receptor, liver X receptor α (LXRα; NR1H3) [7, 8]. When LXRα is activated by these oxysterols, the fatty acid biosynthetic pathway is stimulated through the up-regulation of sterol regulatory element-binding protein 1c (SREBP1c) [9], and an up-regulated SREBP1c and fatty acid biosynthetic pathway has actually been observed in NAFLD [10, 11]. Furthermore, a recent report by Kotronen et al. [12] showed that diacylglycerols might contribute to hepatic insulin resistance in NAFLD. Thus, oxysterols appear to play an important role in the development of hepatic insulin resistance and NAFLD.

Oxysterols are synthesized from cholesterol, and the total body pool of cholesterol is enlarged by endogenous synthesis or by dietary absorption [13]. The use of plasma sterol biomarkers for cholesterol synthesis and fractional absorption [14] clarified that obesity [15, 16], the metabolic syndrome [17], type 2 diabetes [18], and NAFLD [19] were all characterized by low efficiency of dietary cholesterol absorption and high cholesterol biosynthesis.

These data suggest that inhibition of the cholesterol biosynthetic pathway by statins, which are inhibitors of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, may be an effective way to reduce LXRα ligand oxysterols in the bodies of NAFLD patients. In fact, statins have already been used in patients with NAFLD and/or non-alcoholic steatohepatitis (NASH) complicated with dyslipidemia or metabolic syndrome [20–22]. Most reports have demonstrated certain advantages of statin therapy in NAFLD/NASH patients with dyslipidemia, but their effectiveness is still controversial. For instance, in a randomized placebo-controlled trial using simvastatin in the treatment of NASH, Nelson et al. [22] concluded that simvastatin did not seem to be an effective treatment for NASH. We conducted a multi-institutional case-control study to evaluate the efficacy of pitavastatin, a newly developed statin, for the treatment of NAFLD with hypercholesterolemia [23]. In that study, we demonstrated that the alanine aminotransferase (ALT) and gamma-glutamyl transpeptidase (GGT) levels, and serum lipid profiles (including total cholesterol, low-density lipoprotein [LDL]-cholesterol and triglyceride), were significantly improved by 12-month treatment with pitavastatin. However, there was no significant difference in insulin resistance before and after pitavastatin treatment in this cohort [23].

The present study is a post-hoc analysis of a subset of patients who participated in the multi-institutional case-control study mentioned above, in which we evaluate the effects of pitavastatin in NAFLD patients with hypercholesterolemia. The aims of the study were to identify the characteristic features of serum oxysterol profiles, which could be a clue to an understanding of their biological roles in the cholesterol metabolism of NAFLD patients. We found that NAFLD patients had a significantly elevated level of certain LXRα ligand oxysterols in their serum, and pitavastatin did not reverse this elevation in spite of its strong reducing effect on serum cholesterol levels.

Subjects, materials, and methods

Subjects

In this prospective study we evaluated 15 patients diagnosed with biopsy-proven NAFLD with hypercholesterolemia between 2006 and 2009 . Written informed consent was obtained from each enrolled patient, and the study was conducted in accordance with the ethical guidelines of the Declaration of Helsinki and was approved by the ethics and research committees of each institute participating in this study (Tokyo Medical University Ibaraki Medical Center, Hiroshima University, Tokyo Women's Medical University, and Fujita Health University School of Medicine). In all patients, current and past daily alcohol consumption was less than 20 g per week; detailed information regarding alcohol consumption was obtained independently by at least 2 physicians and confirmed by close family members. Exclusion criteria other than alcohol consumption of more than 20 g per week were: evidence of pregnancy, treatment with corticosteroid, and hormone replacement therapy. Subjects using lipid-lowering medication or food enriched with functional plant stanols or sterols were excluded from the study. Subjects with positive test results for the following disorders were also excluded: secondary causes of

steatohepatitis and drug-induced liver injury, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, α-1-antitrypsin deficiency, hemochromatosis, Wilson's disease, and biliary obstruction. After registration of the study, all patients received 2 mg/day of pitavastatin (Livalo; Kowa Pharmaceuticals, Tokyo, Japan) for 12 months. In addition, all patients were given standard weight-loss counseling and encouraged to follow a low-fat and low-carbohydrate diet before and during treatment. Venous blood samples were taken in the morning(following a 12-h overnight fast) at baseline and 3 and 12 months after the initiation of pitavastatin treatment. Some serum samples were utilized for various laboratory tests, and the remaining sera were stored at −20 °C until later analysis.

Fasting sera of 60 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 obtained, and samples were selected from 36 sex- and age-matched subjects, and used as the control group. The control serum samples were stored and handled as mentioned above.

Quantification of serum lipid biomarkers

Serum non-cholesterol sterols (lathosterol, campesterol, and sitosterol) and LXRα ligand oxysterols were quantified by liquid chromatography-mass spectrometry (LC–MS)/MS as described in our previous papers [24–26]. Briefly, coprostanol and deuterated oxysterols were added to 10 μl of serum as internal standards, and alkaline hydrolysis was carried out in 1 N ethanolic KOH with butylated hydroxytoluene at 37 °C for 1 h. Sterols were extracted with *n*-hexene, derivatized to the picolinyl esters, and injected into an LC–electron spray ionization (ESI)–MS/MS system consisting of a TSQ Vantage triple stage quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) equipped with an HESI-II probe and a Prominence ultra fast liquid chromatography (UFLC) system (Shimazu, Kyoto, Japan).

Serum concentrations of 7α-hydroxy-4-cholesten-3-one (C4), a biomarker of CYP7A1 activity, were determined by LC–MS/MS without alkaline hydrolysis [27]. Deuterium-labeled C4 was added to 20 μl of serum and C4 was extracted with acetonitrile. After derivatization into the picolinyl ester, it was injected into the LC–ESI–MS/MS system described above.

Serum malonic acid (MA), a marker of lipogenesis, was quantified by our previously described method [28]. After the addition of [$^{13}C_3$]MA as an internal standard, MA was extracted with acetonitrile from 20 μl of serum, derivatized into di-(1-methyl-3-piperidinyl)-MA and determined by the LC–ESI–MS/MS system described above.

Serum acetylcarnitine (ACT), a marker of fatty acid β-oxidation, was quantified by the method of Ghoshal et al. [29] with some modifications. In brief, 50 ng of [$^2H_3$]ACT HCl was added to 10 μl of serum and ACT was extracted with 100 μl of acetonitrile–water (19:1, v/v). The extract was evaporated to dryness and redissolved in 150 μl of water, and an aliquot (2 μl) was analyzed by LC–ESI–MS/MS. Chromatographic separation was performed using a Hypersil GOLD aQ column (150 × 2.1 mm, 3 μm; Thermo Fisher Scientific) at 40 °C. The mobile phase was comprised of 0.2 % formic acid in water and was used at a flow rate of 200 μl/min.

Determination of serum fibroblast growth factor 19 (FGF19)

Serum FGF19 levels were determined by using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (Quantikine Human FGF19 Immunoassay; R&D systems, Minneapolis, MN, USA), following the manufacturer's instructions.

Statistical analysis

Statistical analyses were performed either by one-way analysis of variance or with the two-tailed Student's *t*-test, using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). Results are shown as means ± SEM, and *P* values of less than 0.05 were considered statistically significant.

Results

Characteristics of the study groups

The sera of 15 NAFLD patients were available for the determination of lipid biomarkers. The mean ages and ratios of male/female subjects were not significantly different between the control and NAFLD groups. In the control group, none of the subjects were obese (BMI >25), and none had hypercholesterolemia (total cholesterol >220 mg/dl), hypertriglycemia (triglyceride [TG] >150 mg/dl), hypertension (systolic blood pressure [BP] >120 mmHg), or diabetes, while in the NAFLD group there were 3 subjects with hypertension, 3 with diabetes, and 10 of the 15 were obese (BMI >25) (Table 1).

Cholesterol, bile acid, and fatty acid metabolism in NAFLD patients

As shown in Table 2, serum total cholesterol concentrations were significantly higher in the patients with with

Table 1 Characterization of the study population

Characteristics	Control ($n = 36$)	NAFLD ($n = 15$)
Sex (male/female)	20:16	10:05
Age, years (range)	42.2 (20–49)	43.7 (25–53)
BMI (kg/m^2)		
<25	36 (100 %)	5
25–29	0	7
>30	0	3
Hypercholesterolemia	0	15
Hypertension	0	3
Diabetes mellitus	0	3

NAFLD non-alcoholic fatty liver disease, *BMI* body mass index

Table 2 Serum biomarkers representing lipid metabolism

	Control ($n = 36$)	NAFLD ($n = 15$)	P
Total cholesterol (mg/dl)	177.7 ± 4.46	242.8 ± 13.3	<0.001
Cholesterol absorption			
Sitosterol (μg/mg Chol)	1.90 ± 0.08	0.73 ± 0.06	<0.0001
Campesterol (μg/mg Chol)	2.34 ± 0.11	0.75 ± 0.07	<0.0001
Cholesterol synthesis			
Lathosterol (μg/mg Chol)	3.29 ± 0.17	3.03 ± 0.31	NS
Bile acid synthesis			
C4 (ng/mg Chol)	14.68 ± 1.65	13.68 ± 2.91	NS
Bile acid absorption			
FGF19 (pg/ml)	345.8 ± 48.6	195.1 ± 21.1	NS
Fatty acid synthesis			
Malonic acid (ng/ml)	63.0 ± 22.6	68.7 ± 4.7	NS
Fatty acid β-oxidation			
Acetylcarnitine (ng/ml)	1059.0 ± 195.3	1011.0 ± 134.4	NS

Chol cholesterol, *C4* 7α-hydroxy-4-cholesten-3-one, *NS* not significant, *FGF19* fibroblast growth factor 19

NAFLD than in controls. Serum non-cholesterol sterols, reflecting intestinal cholesterol absorption (ratio of sitosterol or campesterol to cholesterol), were significantly lower in NAFLD patients compared to controls ($P < 0.0001$), and the cholesterol synthesis marker, the ratio of lathosterol to cholesterol, was not significantly different between the two groups.

Bile acid metabolism was investigated by determination of the concentrations of a biomarker reflecting bile acid synthesis (ratio of C4 to cholesterol) and FGF19. FGF19 is an enterokine that is synthesized and released when bile acids are taken up into the ileum. Serum FGF19 inhibits the expression of hepatic CYP7A1, the rate-limiting enzyme in the major bile acid biosynthetic pathway. In NAFLD patients, neither serum C4 levels nor FGF19 concentrations were significantly different from those in controls (Table 2).

Fatty acid synthesis and β-oxidation in the subjects with NAFLD were studied by the quantification of serum MA and ACT levels, respectively. However, these markers did not show any differences between NAFLD patients and controls.

Table 3 Serum LXRα ligand oxysterol levels

	Control ($n = 36$)	NAFLD ($n = 15$)	P
4βOH-Chol (ng/mg Chol)	27.3 ± 1.1	34.5 ± 3.5	<0.05
22*R*OH-Chol (ng/mg Chol)	Trace	Trace	
24*S*OH-Chol (ng/mg Chol)	34.0 ± 0.9	36.8 ± 2.5	NS
24*S*,25-epoxy-Chol (ng/mg Chol)	Trace	Trace	
25OH-Chol (ng/mg Chol)	7.9 ± 0.6	23.9 ± 2.6	<0.0001
27OH-Chol (ng/mg Chol)	73.1 ± 2.3	101.1 ± 7.7	<0.0005

LXRα liver X receptor α

Serum LXRα ligand oxysterol levels in NAFLD patients

The serum levels of LXRα ligand oxysterols (ratios to cholesterol) are shown in Table 3. The levels of 4β-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol were significantly elevated, at 26 % ($P < 0.05$), 303 % ($P < 0.0001$), and 38 % ($P < 0.0005$), respectively, in NAFLD patients compared with controls, although the 24*S*-hydroxycholesterol level in NAFLD patients was not significantly different from that in the controls. 22*R*-hydroxycholesterol and 24*S*, 25-epoxycholesterol are reported to be effective ligands of LXRα, but only trace amounts of these oxysterols were detected by our present method. The absolute concentrations of these oxysterols were also elevated in the NAFLD patients. Not only 4β-hydroxycholesterol (+58 %, $P < 0.001$), 25-hydroxycholesterol (+200 %, $P < 0.001$), and 27-hydroxycholesterol (+80 %, $P < 0.001$), but also 24*S*-hydroxycholesterol was increased (33 %, $P < 0.05$) in NAFLD patients.

Effects of pitavastatin treatment on the serum markers

The marker representing cholesterol synthesis (lathosterol to cholesterol) was significantly suppressed by pitavastatin treatment, from 3 months after the initiation of the treatment ($P < 0.01$) and its level remained significantly suppressed during the observation period (12 months after

Table 4 Changes in serum biomarkers produced by pitavastatin treatment

	Pre-treatment	3 Months	12 Months
Total cholesterol (mg/dl)	242.8 ± 13.3	188.3 ± 12.1*	182.7 ± 9.29*
Cholesterol absorption			
Sitosterol (μg/mg Chol)	0.73 ± 0.06	0.91 ± 0.08	1.16 ± 0.12*
Campesterol (μg/mg Chol)	0.75 ± 0.07	0.94 ± 0.09	1.24 ± 0.15*
Cholesterol synthesis			
Lathosterol (μg/mg Chol)	3.03 ± 0.31	1.44 ± 0.27*	1.72 ± 0.23*
Bile acid synthesis			
C4 (ng/mg Chol)	13.68 ± 2.91	10.57 ± 2.27	17.23 ± 3.44
Bile acid absorption			
FGF19 (pg/ml)	195.1 ± 21.1	172.4 ± 36.3	166.3 ± 23.1
Fatty acid synthesis			
Malonic acid (ng/ml)	68.7 ± 18.4	82.0 ± 5.6	61.3 ± 5.9
Fatty acid β-oxidation			
Acetylcarnitine (ng/ml)	1011.0 ± 134.4	914.2 ± 98.2	1318.0 ± 172.9

Chol cholesterol, *C4* 7α-hydroxy-4-cholesten-3-one

*$P < 0.01$ compared to pre-treatment

Table 5 Changes in serum LXRα ligand oxysterol levels produced by pitavastatin treatment

	Pre-treatment	3 Months	12 Months
4βOH-Chol (ng/mg Chol)	34.5 ± 3.5	33.8 ± 4.3	32.9 ± 3.0
22*R*OH-Chol (ng/mg Chol)	Trace	Trace	Trace
24*S*OH-Chol (ng/mg Chol)	36.8 ± 2.5	31.9 ± 3.3	36.1 ± 3.2
24*S*,25-epoxy-Chol (ng/mg Chol)	Trace	Trace	Trace
25OH-Chol (ng/mg Chol)	23.9 ± 2.61	22.3 ± 3.2	16.2 ± 1.7
27OH-Chol (ng/mg Chol)	101.1 ± 7.7	94.8 ± 11.1	105.0 ± 6.4

initiation of treatment, $P < 0.05$) (Table 4). On the other hand, the markers representing cholesterol absorption (sitosterol or campesterol to cholesterol) were unchanged at 3 months after the initiation of pitavastatin administration, but were significantly increased at 12 months ($P < 0.01$). However, the markers for bile acid and fatty acid metabolism and the levels of oxysterols (relative to cholesterol) were not changed significantly by pitavastatin treatment (Tables 4, 5).

Figure 1 shows a comparison of the effects of pitavastatin treatment on serum absolute concentrations of total cholesterol, lathosterol, and oxysterols. Total cholesterol and lathosterol concentrations were markedly decreased at 3 months, and no further reduction was observed at 12 months. In contrast, the concentrations of oxysterols, except for 24*S*-hydroxycholesterol, were significantly decreased at 12 months after treatment initiation, but not at 3 months. The 24*S*-hydroxycholesterol concentration tended to be decreased by pitavastatin treatment, but the difference from the pre-treatment level was not statistically significant.

Serum oxysterol levels and insulin resistance

The association between serum oxysterol levels and insulin resistance, calculated by the homeostasis model assessment as an index of insulin resistance; HOMA-IR (=fasting serum insulin (μU/ml) × fasting blood glucose (mg/dl)/405), was determined. As shown in Fig. 2, the sum of oxysterols (27-hydroxycholesterol, 25-hydroxycholesterol, and 4β-hydroxycholesterol: relative to serum cholesterol) and HOMA-IR were significantly correlated ($r^2 = 0.2762$, $P < 0.001$). Among the oxysterols determined in the present study, 24*S*-hydroxycholesterol was excluded in this calculation because a significant increase of 24*S*-hydroxycholesterol relative to cholesterol was not observed in the NAFLD patients, as mentioned above. Pitavastatin treatment did not improve the insulin resistance over the treatment period, similar to results reported in our previous study [23].

Discussion

This is the first report that demonstrates a significant elevation of serum concentrations of LXRα ligand oxysterols in NAFLD patients. Most serum oxysterols are found in the LDL and high-density lipoprotein (HDL) fractions [30], suggesting that oxysterols are transported in serum with cholesterol. Accordingly, the hypercholesterolemia found in NAFLD patients may lead to the overestimation of oxysterol production in their body. However, we found that 4β-hydroxycholesterol, 25-hydroxycholesterol,

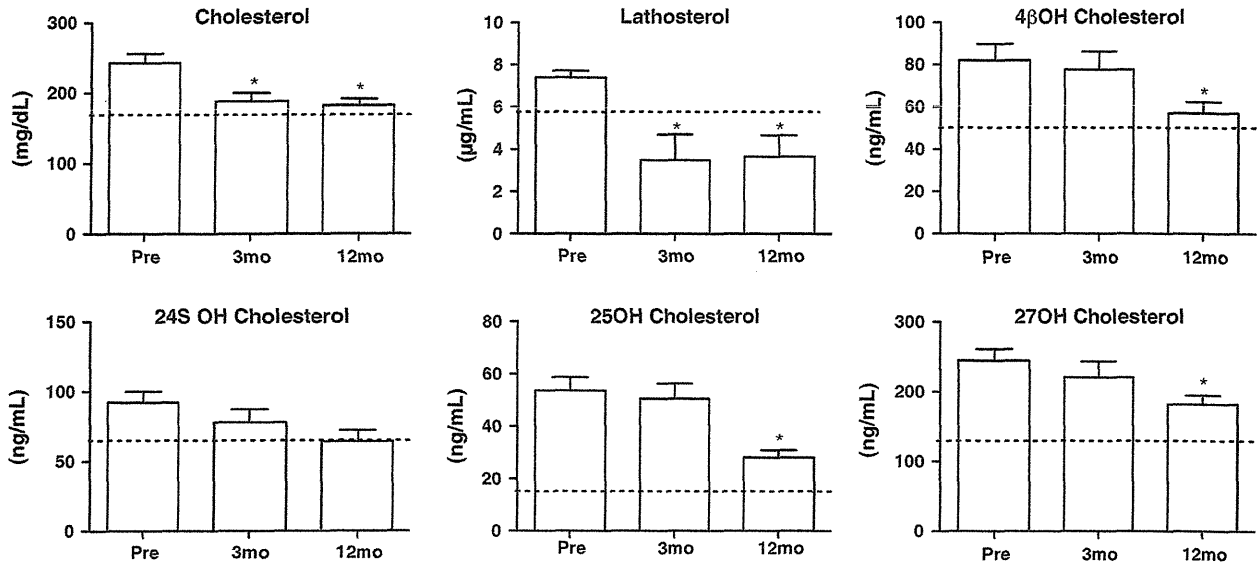

Fig. 1 Effects of pitavastatin treatment on serum absolute concentrations of total cholesterol, lathosterol, and oxysterols. *Pre* basal level before pitavastatin treatment, *3mo* serum concentrations at 3 months from initiation of therapy, *12mo* serum concentrations at 12 months from initiation of therapy, *OH* hydroxy. *Dotted lines* represent the mean value of each sterols in control subjects. $^*P < 0.01$

and 27-hydroxycholesterol levels expressed relative to cholesterol, but not those of 24*S*-hydroxycholesterol,were significantly increased in NAFLD patients compared with controls (Table 3). Thus, the increased production of LXRα ligand oxysterols appears to be a characteristic feature of NAFLD. Because most oxysterols are formed from cholesterol by enzymatic oxidation or autoxidation [25], it may be reasonable to assume that oversaturation of the tissue cholesterol concentration results in augmented oxysterol production. It was intriguing that only 24*S*-hydroxycholesterol levels expressed relative to cholesterol were not elevated in NAFLD patients. While serum 4β-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol are produced by ubiquitously expressed CYP3A4 [31] and CYP27A1 [32], 24*S*-hydroxycholesterol is synthesized by a brain-specific CYP46A1 [33]. Therefore, our results suggest that, in NAFLD, the cholesterol metabolism in the brain is not affected as much as that in other organs.

Cholesterol balance in our NAFLD patients was studied by the determination of serum markers for intestinal absorption (sitosterol/cholesterol and campesterol/cholesterol), biosynthesis (lathosterol/cholesterol), and catabolism to bile acids (C4/cholesterol). The results showed that cholesterol absorption was significantly reduced, while cholesterol and bile acid syntheses were not altered in NAFLD patients compared to controls. The reduced cholesterol absorption in NAFLD was consistent with the findings of a previous Finnish study [19], but the unchanged cholesterol synthesis was not consistent with the findings of that study. There are no definitive data to explain this inconsistency; however, differences in the patients' backgrounds in the two studies should be noted. First, the severity of liver damage in the study subjects needs to be considered. In the Finnish study, although subjects were recruited based on strict exclusion and inclusion criteria, the diagnosis of NAFLD was based on the measurement of liver fat content by [1] proton magnetic resonance spectrometry (H-MRS) without liver biopsy. In contrast, all NAFLD patients in our cohort were diagnosed by liver biopsy, and elevation of ALT was greater in our patient cohort (average ALT level at baseline in the NAFLD group was 102.1 U/l in our study, while the level was 39.5 U/l in the Finnish study [19]). Although severe fibrosis was not seen in any of our enrolled subjects, it is possible that sustained inflammation acted upon cholesterol synthesis. Second, the inclusion of patients with hypercholesterolemia in the present study may explain the differences in the cholesterol synthesis findings. Because of the use of pitavastatin, NAFLD patients with hypercholesterolemia were enrolled in the present cohort. It is possible that, in the present study cohort, cholesterol synthesis was already suppressed due to increased tissue cholesterol concentration. Third, differences in genetic background between Finnish and Japanese may also be discussed. However, it is assumed that increased cholesterol synthesis is not a major observation in hypercholesterolemic NAFLD patients in Japan.

The reason for the reduced cholesterol absorption in NAFLD has not been clarified. However, LXRα ligand oxysterols may up-regulate the expression of the

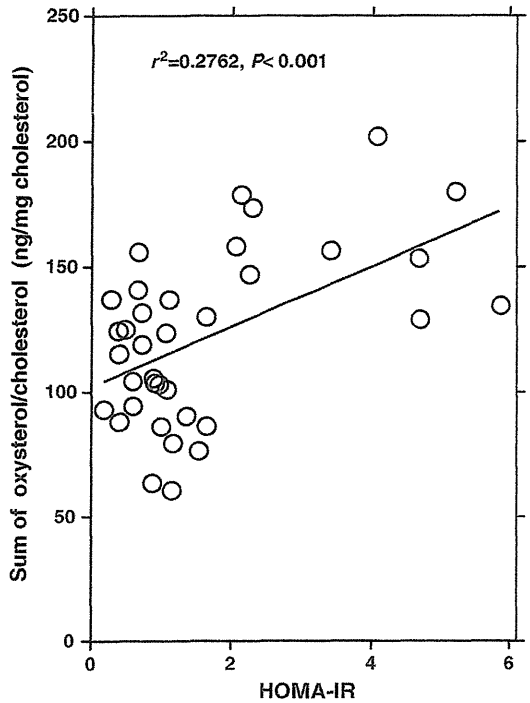

Fig. 2 The association between serum oxysterol levels and insulin resistance, calculated by the homeostasis model assessment as an index of insulin resistance (*HOMA-IR*; =fasting serum insulin (μU/ml) × fasting blood glucose (mg/dl)/405), was determined. The sum of oxysterol levels relative to cholesterol (25-hydroxycholesterol, 27-hydroxycholesterol, and 4β-hydroxycholesterol) were plotted against HOMA-IR

ATP-binding cassette transporters G5 and G8 (ABCG5/G8) through the activation of LXRα [34]. These transporters are present in the ileal brush-border membrane as well as in the hepatic apical membrane, and are responsible for the efflux of cholesterol into the intestinal lumen and bile duct. Net cholesterol absorption from the intestine depends on the competing activities of three membrane proteins: Niemann-Pick C1-like 1 (NPC1L1), ABCG5, and ABCG8 [35–37]. Pharmacological induction or overexpression of ABCG5/G8 in mice decreases fractional cholesterol absorption from the intestine [38, 39]. In addition, Nakamuta et al. [10] have reported the overexpression of LXRα and ABCG5 genes in the livers of NAFLD patients. Thus, up-regulation of ABCG5/G8 due to the activation of LXRα could contribute to the decreased cholesterol absorption in NAFLD, as demonstrated in the schematic figure shown in Fig. 3. We have previously reported the importance of the serum 27-hydroxycholesterol level (relative to cholesterol) for predicting the effects of a high-cholesterol diet on plasma LDL cholesterol concentrations [40]. In subjects with high serum 27-hydroxycholesterol levels (more than 80 ng/mg cholesterol), serum LDL cholesterol concentrations tended to increase after cholesterol ingestion (750 mg/day for 4 weeks) compared with findings in those with low serum 27-hydroxycholesterol levels (<80 ng/mg cholesterol). These results suggest that ABCG5/G8 proteins were fully up-regulated before cholesterol loading in the subjects with high 27-hydroxycholesterol levels, so that they might have been unable to adapt to a high-cholesterol diet.

The above cholesterol loading study showed that the relative 27-hydroxycholesterol levels were quite stable and were not influenced by the cholesterol loading itself or by the change of serum cholesterol concentrations after the cholesterol loading [40]. Stability of oxysterol levels was also observed during the treatment with pitavastatin in the present study. In NAFLD patients, pitavastatin markedly reduced serum cholesterol concentrations in parallel with the inhibition of cholesterol biosynthesis (Table 4), and serum total cholesterol concentrations became normal and were not significantly different from those in untreated control subjects after 3 months of treatment. However, levels of LXRα ligand oxysterol expressed relative to cholesterol remained high and did not change significantly during the 12-months period of pitavastatin treatment (Table 5). As shown in Fig. 1, the stability of oxysterol levels is due to the extremely slow reduction of absolute oxysterol concentrations compared with cholesterol and lathosterol. This finding also supported our hypothesis that the increase of oxysterol found in NAFLD is not only due to an increase of tissue cholesterol but is also due to other factors. All enrolled NAFLD patients in the present study cohort had insulin resistance according to HOMA-IR, and pitavastatin treatment did not improve the insulin resistance over the treatment period [23]. Plots of each determined oxysterol/cholesterol ratio and HOMA-IR demonstrated the significant associations between serum oxysterols and insulin resistance (Fig. 2). We speculate that serum LXRα ligand oxysterol levels (relative to cholesterol) could be a surrogate marker of insulin resistance, and that high oxysterol levels in the circulation may play an important role in the development of hepatic and peripheral insulin resistance followed by NAFLD. A study by Biddinger et al. [41] demonstrated the increased expression of ABCG5/G8 in the insulin-resistant liver, associated with increased biliary cholesterol excretion, and increased susceptibility to cholesterol gallstones. This finding is also suggestive of the involvement of oxysterol in increasing the expression of ABCG5/G8 in conditions with insulin resistance. The precise mechanism of how insulin resistance and oxysterol are linked should be elucidated in future studies.

In the present study, the C4-to-cholesterol ratio (C4/Chol; a biomarker representing bile acid synthesis) and FGF19 were not significantly different between the NAFLD patients and controls. In addition, the administration of pitavastatin exerted no effect on C4/Chol despite

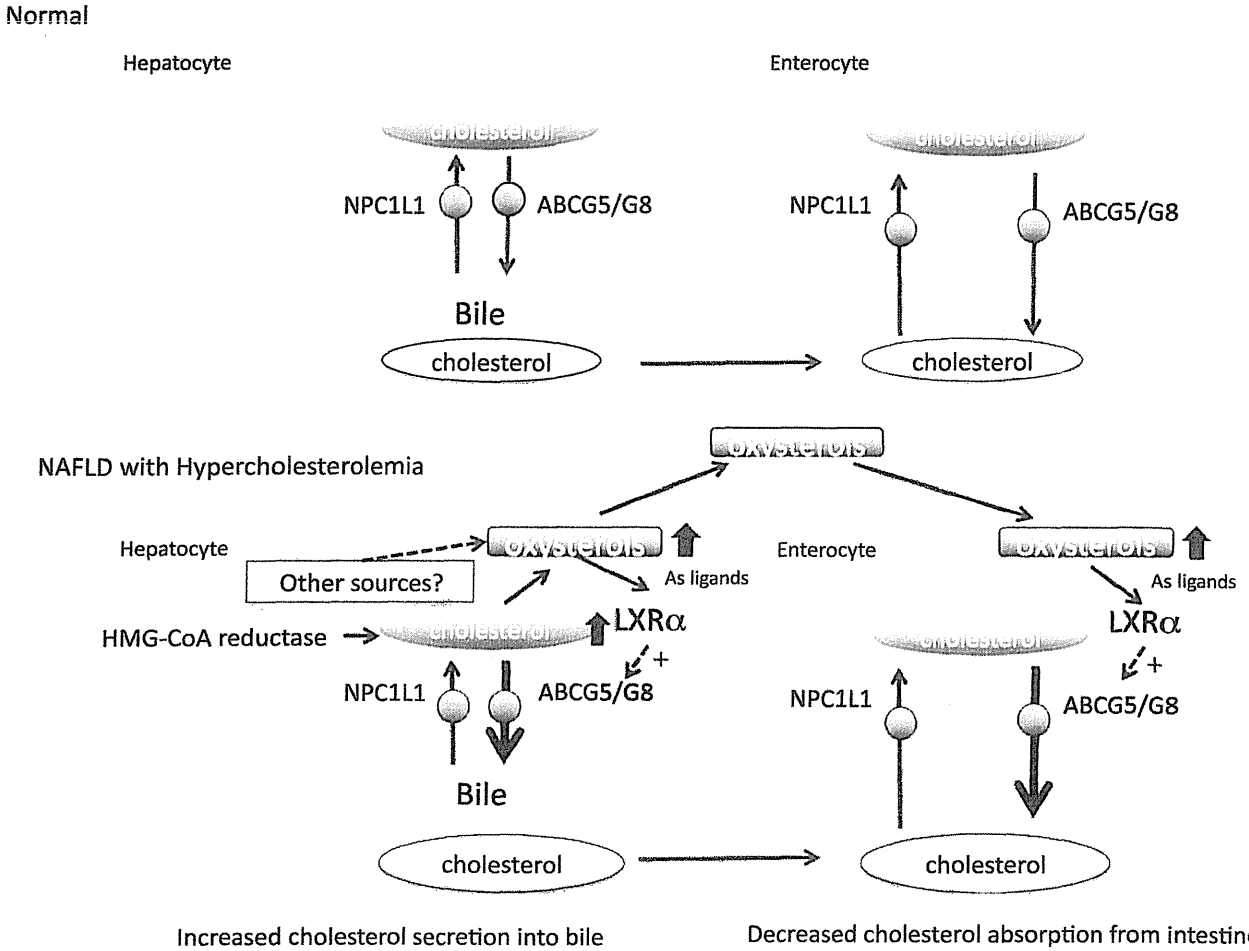

Fig. 3 Schematic figure demonstrating the hypothesis that up-regulation of ABCG5/G8 due to the activation of liver X receptor α (*LXRα*) could contribute to the decreased cholesterol absorption in non-alcoholic fatty liver disease (*NAFLD*). *HMG-CoA* 3-hydroxy-3-methylglutaryl-coenzyme A

producing a marked decrease in the serum cholesterol level. These findings suggest a lack of enhanced bile acid synthesis and secretion in NAFLD patients with hypercholesterolemia despite their increased cholesterol concentrations. A recent report also suggests that the hepatic response to FGF19 is impaired in patients with NAFLD and insulin resistance [42]. As poor adjustment of the bile acid synthesis system for an increased cholesterol level in humans may be a potential risk for metabolic syndrome, it has been suggested in recent studies that the alteration of bile acid signaling and/or hepatic flux may contribute to the pathogenesis of NAFLD and metabolic disorders [43]. This idea is consistent with the report by Yang et al., which demonstrated that the levels of FXR protein and mRNA were decreased in patients with NAFLD, whereas those of LXR were increased [44]. Hence, further clinical studies of bile acid metabolism in NAFLD should also be performed.

In conclusion, NAFLD patients showed significantly elevated levels of LXRα ligand oxysterols, 4β-hydroxycholesterol, 25-hydroxycholesterol, and 27-hydroxycholesterol in their sera. The reduced intestinal cholesterol absorption in NAFLD seemed to be caused by the upregulation of ABCG5/8 through the activation of LXRα by the oxysterols. The inhibition of cholesterol biosynthesis by pitavastatin normalized serum cholesterol concentrations in 3 months, but the abnormal oxysterol levels (relative to cholesterol) had not recovered by the end of the 12 months of treatment.

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Conflict of interest All authors declare that they have no conflict of interest.

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