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

Asako Murayama,[a] Nao Sugiyama,[a] Koichi Watashi,[a] Takahiro Masaki,[a] Ryosuke Suzuki,[a] Hideki Aizaki,[a] Toshiaki Mizuochi,[b] Takaji Wakita,[a] and Takanobu Kato[a]

Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan,[a] and Department of Research on Blood and Biological Products, National Institute of Infectious Diseases, Tokyo, Japan[b]

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.

epatitis 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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Address correspondence to Takanobu Kato, takato@nih.go.jp.
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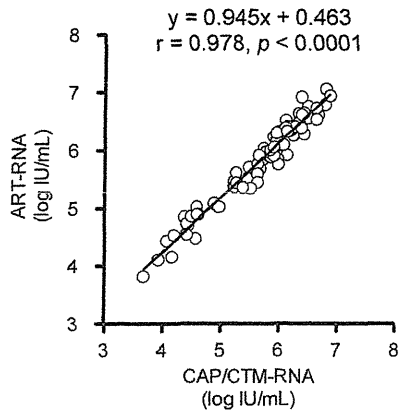

FIG 1 Correlation of HCV RNA titers as quantified by two commercial kits.

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,

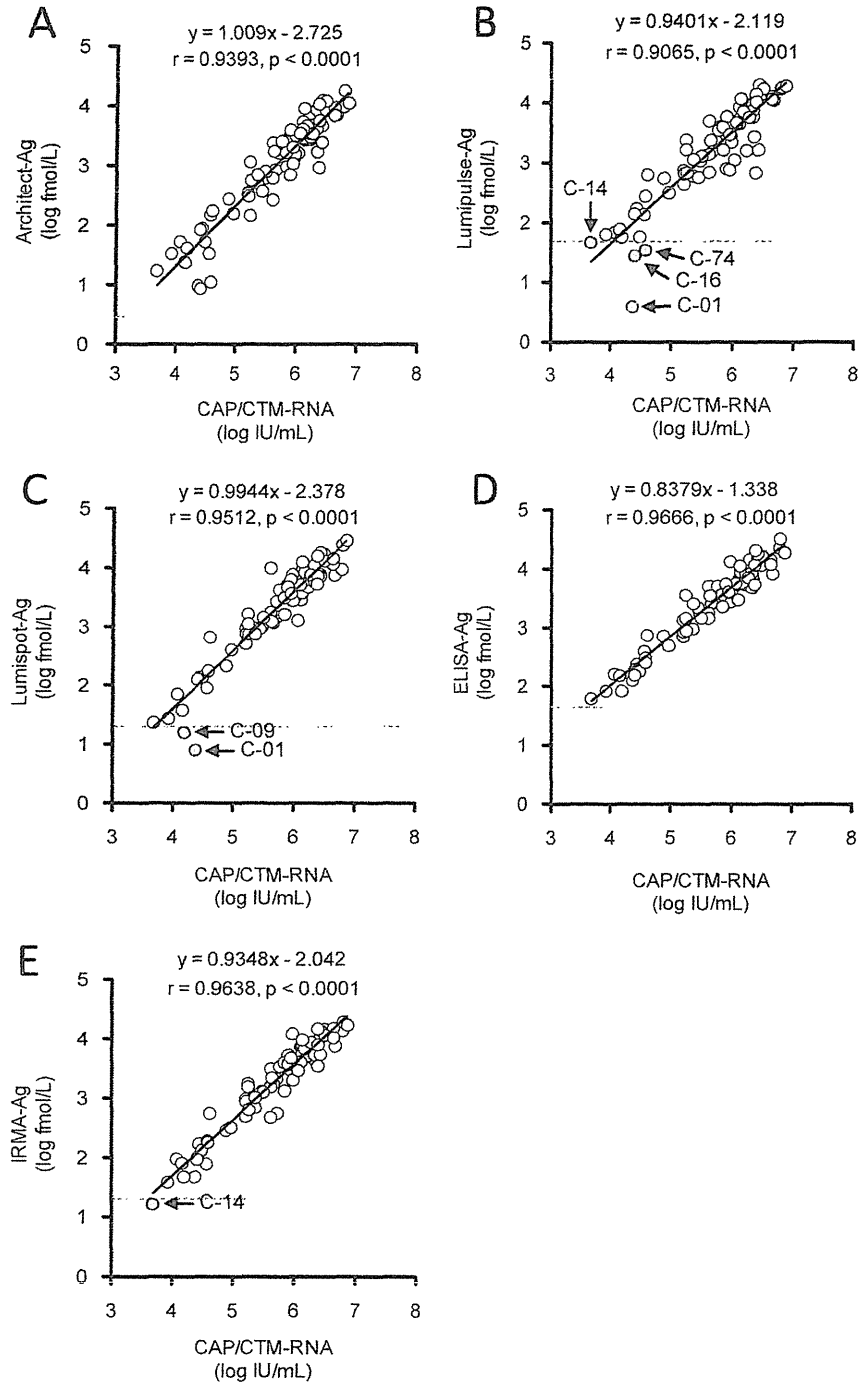

FIG 2 Correlation between CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. Data for core Ag levels were converted to log fmol/liter prior to analysis. In each plot, the lower limit of detection of the respective core Ag assay is indicated by a dotted line. Data for samples below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

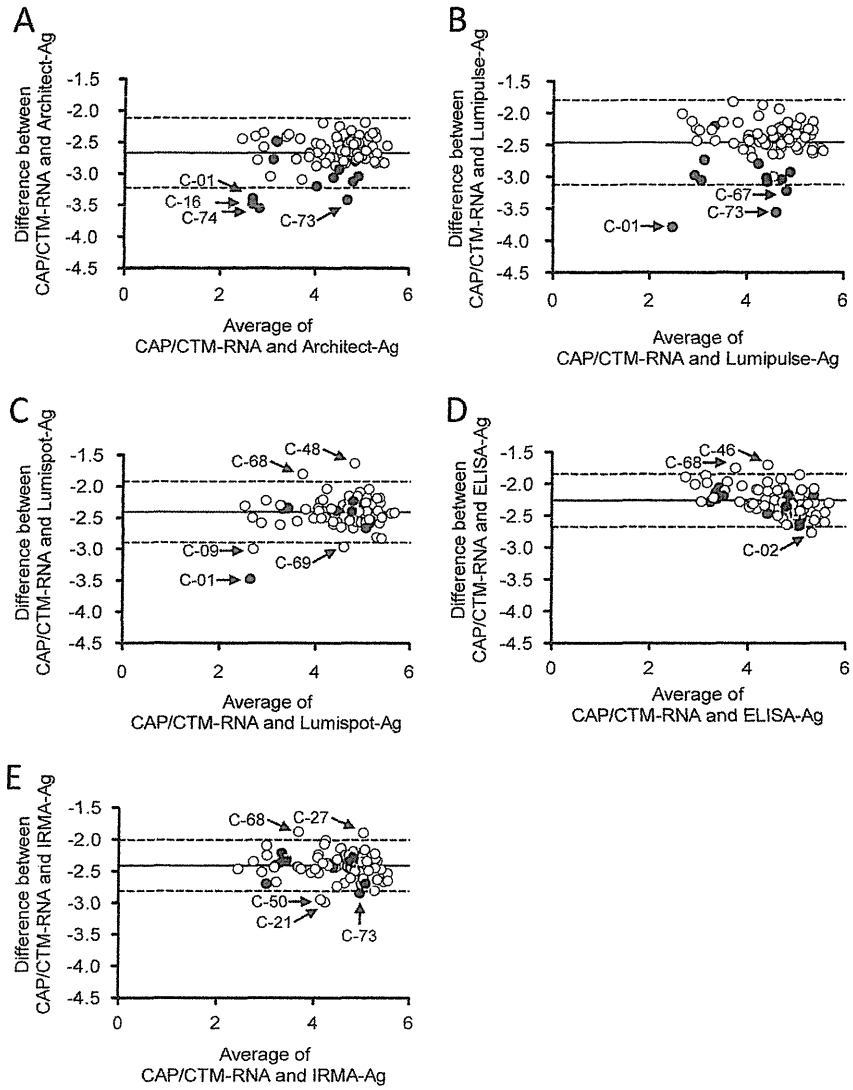

FIG 3 Bland-Altman plot analysis of CAP/CTM-RNA and core Ag levels as quantified by five commercial kits. These plots show the difference between the values of HCV RNA and core Ag as a function of the average of these two values. Data for core Ag levels were converted to log fmol/liter prior to analysis. 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

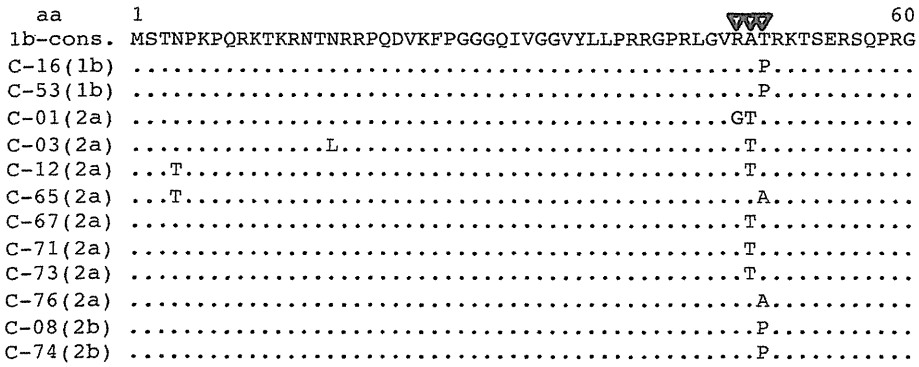

FIG 4 Alignment of the first 60 amino acids of the HCV core region of strains with polymorphisms at amino acid residues 47 to 49. The position numbers are given at the top. Dots indicate identical amino acids. The consensus sequence of 1b strains (1b cons.) isolated in this study was determined and used 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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REFERENCES

1. **Alvarez M, Planelles D, Vila E, Montoro J, Franco E.** 2004. Prolonged hepatitis C virus seroconversion in a blood donor, detected by HCV antigen test in parallel with HCV RNA. Vox Sang. **86**:266–267.
2. **Aoyagi K, et al.** 1999. Development of a simple and highly sensitive enzyme immunoassay for hepatitis C virus core antigen. J. Clin. Microbiol. **37**:1802–1808.
3. **Aoyagi K, et al.** 2001. Performance of a conventional enzyme immunoassay for hepatitis C virus core antigen in the early phases of hepatitis C infection. Clin. Lab. **47**:119–127.
4. **Bossler A, et al.** 2011. Performance of the COBAS(R) AmpliPrep/COBAS TaqMan(R) automated system for hepatitis C virus (HCV) quantification in a multi-center comparison. J. Clin. Virol. **50**:100–103.
5. **Chevaliez S, Bouvier-Alias M, Brillet R, Pawlotsky JM.** 2007. Overestimation and underestimation of hepatitis C virus RNA levels in a widely used real-time polymerase chain reaction-based method. Hepatology **46**: 22–31.
6. **Elkady A, et al.** 2010. Performance of two real-time RT-PCR assays for quantitation of hepatitis C virus RNA: evaluation on HCV genotypes 1–4. J. Med. Virol. **82**:1878–1888.
7. **Enomoto M, et al.** 2005. Chemiluminescence enzyme immunoassay for monitoring hepatitis C virus core protein during interferon-alpha2b and ribavirin therapy in patients with genotype 1 and high viral loads. J. Med. Virol. **77**:77–82.
8. **Feld JJ, Hoofnagle JH.** 2005. Mechanism of action of interferon and ribavirin in treatment of hepatitis C. Nature **436**:967–972.
9. **Feld JJ, Liang TJ.** 2006. Hepatitis C—identifying patients with progressive liver injury. Hepatology **43**:S194–S206.
10. **Icardi G, et al.** 2001. Novel approach to reduce the hepatitis C virus (HCV) window period: clinical evaluation of a new enzyme-linked immunosorbent assay for HCV core antigen. J. Clin. Microbiol. **39**:3110–3114.
11. **Ikeda K, et al.** 1996. Hepatitis C virus subtype 3b infection in a hospital in Japan: epidemiological study. J. Gastroenterol. **31**:801–805.
12. **Kaiser T, et al.** 2008. Kinetics of hepatitis C viral RNA and HCV-antigen during dialysis sessions: evidence for differential viral load reduction on dialysis. J. Med. Virol. **80**:1195–1201.
13. **Leary TP, et al.** 2006. A chemiluminescent, magnetic particle-based immunoassay for the detection of hepatitis C virus core antigen in human serum or plasma. J. Med. Virol. **78**:1436–1440.
14. **Liang TJ.** 1998. Combination therapy for hepatitis C infection. N. Engl. J. Med. **339**:1549–1550.
15. **Liang TJ, Rehermann B, Seeff LB, Hoofnagle JH.** 2000. Pathogenesis, natural history, treatment, and prevention of hepatitis C. Ann. Intern. Med. **132**:296–305.
16. **Matsuura K, et al.** 2009. Abbott RealTime hepatitis C virus (HCV) and Roche Cobas AmpliPrep/Cobas TaqMan HCV assays for prediction of sustained virological response to pegylated interferon and ribavirin in chronic hepatitis C patients. J. Clin. Microbiol. **47**:385–389.

17. **Mederacke I, et al.** 2009. Performance and clinical utility of a novel fully automated quantitative HCV-core antigen assay. J. Clin. Virol. 46:210–215.
18. **Medici MC, et al.** 2011. Hepatitis C virus core antigen: analytical performances, correlation with viremia and potential applications of a quantitative, automated immunoassay. J. Clin. Virol. 51:264–269.
19. **Miedouge M, et al.** 2010. Analytical evaluation of HCV core antigen and interest for HCV screening in haemodialysis patients. J. Clin. Virol. 48: 18–21.
20. **Morota K, et al.** 2009. A new sensitive and automated chemiluminescent microparticle immunoassay for quantitative determination of hepatitis C virus core antigen. J. Virol. Methods 157:8–14.
21. **Moscato GA, et al.** 2011. Quantitative determination of hepatitis C core antigen in therapy monitoring for chronic hepatitis C. Intervirology 54: 61–65.
22. **Nubling CM, Unger G, Chudy M, Raia S, Lower J.** 2002. Sensitivity of HCV core antigen and HCV RNA detection in the early infection phase. Transfusion 42:1037–1045.
23. **Ogata N, Alter HJ, Miller RH, Purcell RH.** 1991. Nucleotide sequence and mutation rate of the H strain of hepatitis C virus. Proc. Natl. Acad. Sci. U. S. A. 88:3392–3396.
24. **Park Y, Lee JH, Kim BS, Kim DY, Han KH, Kim HS.** 2010. New automated hepatitis C virus (HCV) core antigen assay as an alternative to real-time PCR for HCV RNA quantification. J. Clin. Microbiol. 48:2253–2256.
25. **Pawlotsky JM.** 2006. Therapy of hepatitis C: from empiricism to eradication. Hepatology 43:S207–S220.
26. **Robertson B, et al.** 1998. Classification, nomenclature, and database development for hepatitis C virus (HCV) and related viruses: proposals for standardization. Arch. Virol. 143:2493–2503.
27. **Ross RS, et al.** 2010. Analytical performance characteristics and clinical utility of a novel assay for total hepatitis C virus core antigen quantification. J. Clin. Microbiol. 48:1161–1168.
28. **Saeed M, et al.** 2009. Evaluation of hepatitis C virus core antigen assays in detecting recombinant viral antigens of various genotypes. J. Clin. Microbiol. 47:4141–4143.
29. **Seeff LB, Hoofnagle JH.** 2002. National Institutes of Health Consensus Development Conference: management of hepatitis C: 2002. Hepatology 36:S1–S2.
30. **Takahashi M, Saito H, Higashimoto M, Atsukawa K, Ishii H.** 2005. Benefit of hepatitis C virus core antigen assay in prediction of therapeutic response to interferon and ribavirin combination therapy. J. Clin. Microbiol. 43:186–191.
31. **Tanaka E, et al.** 2000. Evaluation of a new enzyme immunoassay for hepatitis C virus (HCV) core antigen with clinical sensitivity approximating that of genomic amplification of HCV RNA. Hepatology 32:388–393.
32. **Tanaka Y, et al.** 2003. High stability of enzyme immunoassay for hepatitis C virus core antigen-evaluation before and after incubation at room temperature. Hepatol. Res. 26:261–267.
33. **Vermehren J, et al.** 2008. Differences between two real-time PCR-based hepatitis C virus (HCV) assays (RealTime HCV and Cobas AmpliPrep/Cobas TaqMan) and one signal amplification assay (Versant HCV RNA 3.0) for RNA detection and quantification. J. Clin. Microbiol. 46:3880–3891.
34. **Vermehren J, et al.** 2011. Development of a second version of the Cobas AmpliPrep/Cobas TaqMan hepatitis C virus quantitative test with improved genotype inclusivity. J. Clin. Microbiol. 49:3309–3315.
35. **Vermehren J, et al.** 2011. Multi-center evaluation of the Abbott RealTime HCV assay for monitoring patients undergoing antiviral therapy for chronic hepatitis C. J. Clin. Virol. 52:133–137.

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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

Tadashi Ikegami · Hideyuki Hyogo · Akira Honda · Teruo Miyazaki · Katsutoshi Tokushige · Etsuko Hashimoto · Kazuo Inui · Yasushi Matsuzaki · Susumu Tazuma

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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

T. Ikegami · A. Honda · Y. Matsuzaki (✉)
Division of Gastroenterology and Hepatology, Tokyo Medical University Ibaraki Medical Center, 3-20-1 Chuo, Ami-machi, Inashiki-gun, Ibaraki 300-0395, Japan
e-mail: ymatsuzaki-gi@umin.ac.jp

H. Hyogo
Department of Medicine and Molecular Science, Graduate School of Biochemical Science, Hiroshima University, Hiroshima, Japan

A. Honda · T. Miyazaki
Center for Collaborative Research, Tokyo Medical University Ibaraki Medical Center, Inashiki-gun, Japan

T. Miyazaki · Y. Matsuzaki
Department of Development for Community Medicine, Tokyo Medical University, Tokyo, Japan

K. Tokushige · E. Hashimoto
Department of Medicine and Gastroenterology, Tokyo Women's Medical University, Tokyo, Japan

K. Inui
Department of Internal Medicine, Fujita Health University School of Medicine, Toyoake, Japan

S. Tazuma
Department of General Medicine, Graduate School of Biochemical Science, Hiroshima University, Hiroshima, Japan

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,

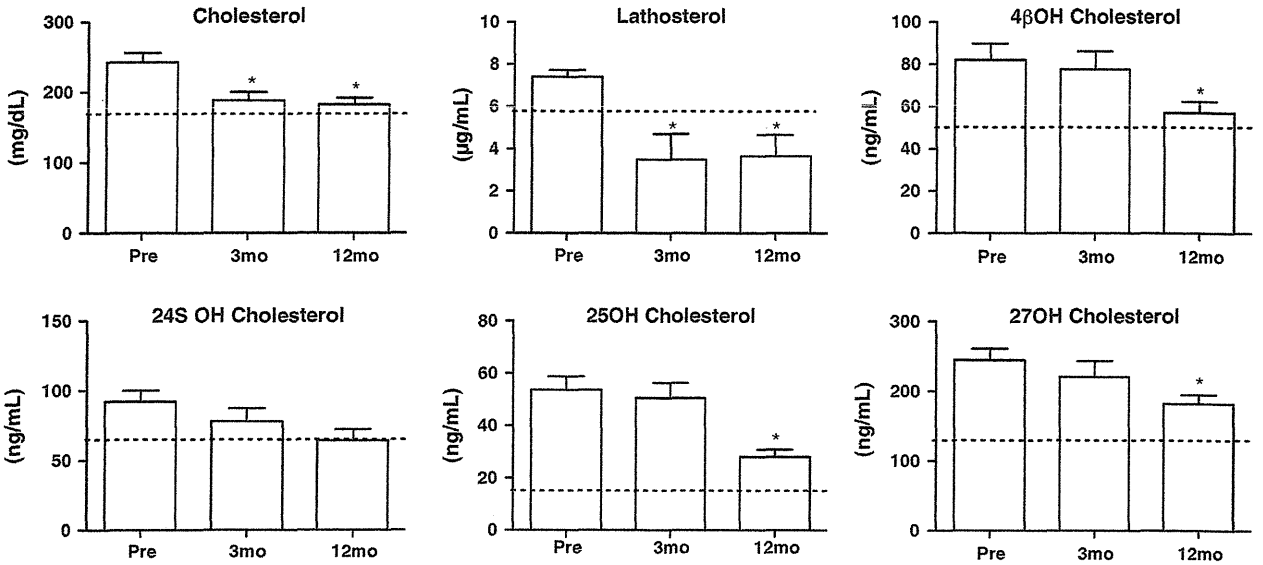

Fig. 1 Effects of pitavastatin treatment on serum absolute concentrations of total cholesterol, lathosterol, and oxysterols. *Pre* basal level before pitavastatin treatment, *3mo* serum concentrations at 3 months from initiation of therapy, *12mo* serum concentrations at 12 months from initiation of 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

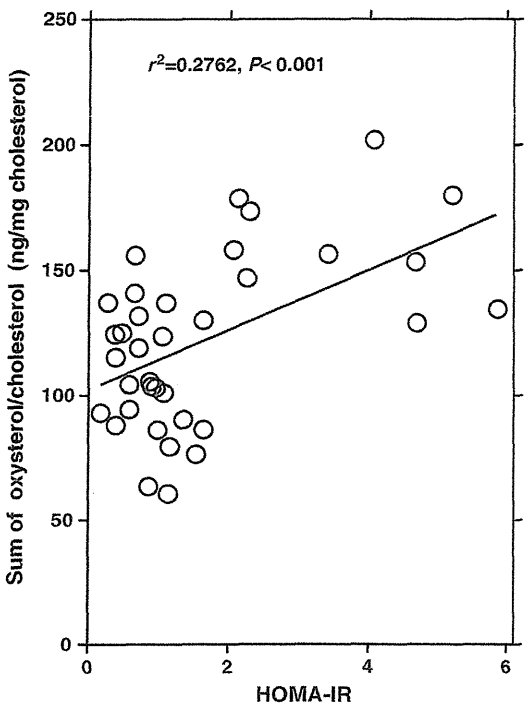

Fig. 2 The association between serum oxysterol levels and insulin resistance, calculated by the homeostasis model assessment as an index of insulin resistance (*HOMA-IR*; =fasting serum insulin (μU/ml) × fasting blood glucose (mg/dl)/405), was determined. The sum of oxysterol levels relative to cholesterol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

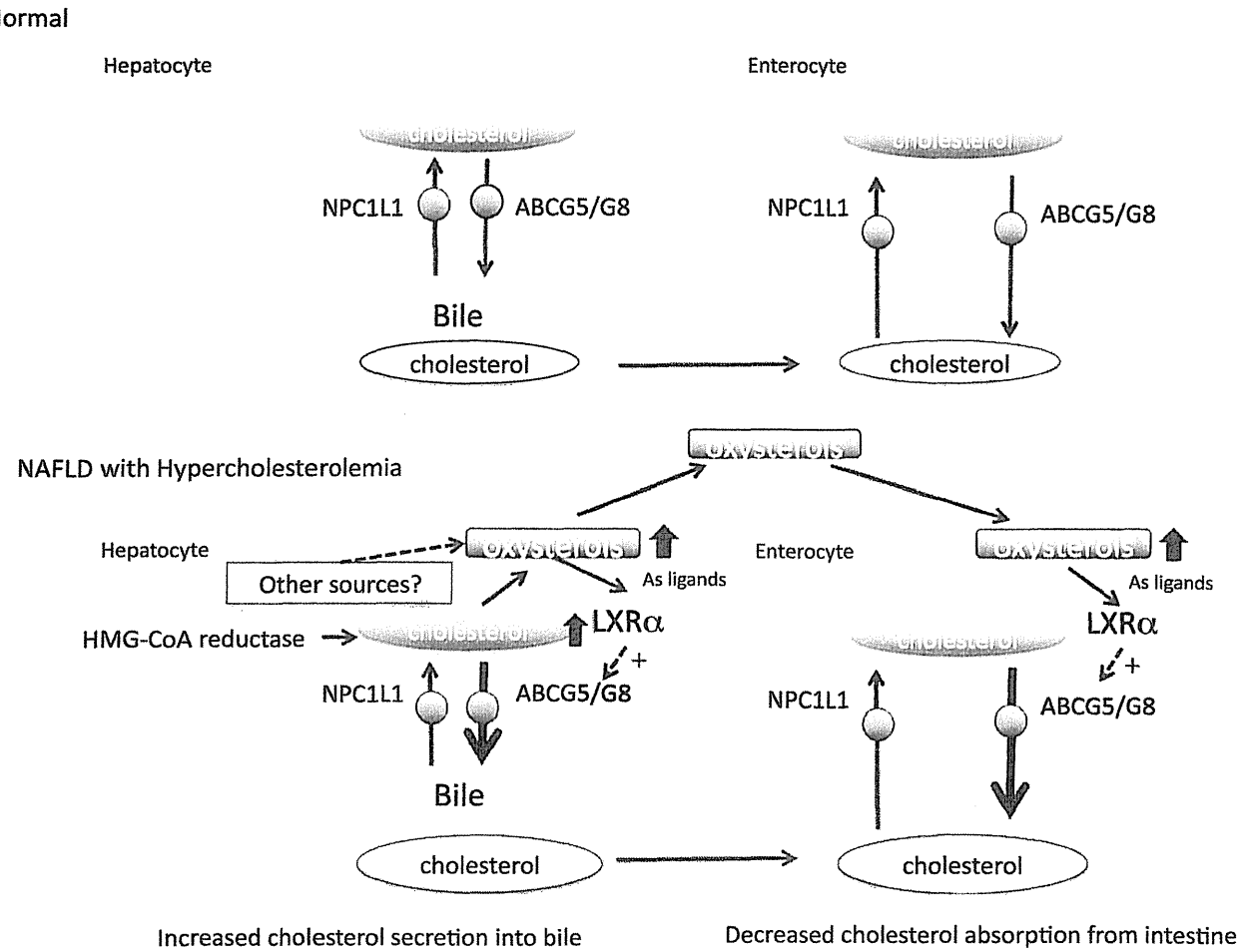

Fig. 3 Schematic figure demonstrating the hypothesis that up-regulation of ABCG5/G8 due to the activation of liver X receptor α (*LXRα*) could contribute to the decreased cholesterol absorption in non-alcoholic fatty liver disease (*NAFLD*). *HMG-CoA* 3-hydroxy-3-methylglutaryl-coenzyme A

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.

References

1. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD Single Topic Conference. Hepatology. 2003;37:1202–19.
2. Kotronen A, Westerbacka J, Bergholm R, Pietilainen KH, Yki-Jarvinen H. Liver fat in the metabolic syndrome. J Clin Endocrinol Metab. 2007;92:3490–7.
3. Seppala-Lindroos A, Vehkavaara S, Hakkinen AM, Goto T, Westerbacka J, Sovijarvi A, et al. Fat accumulation in the liver is associated with defects in insulin suppression of glucose production and serum free fatty acids independent of obesity in normal men. J Clin Endocrinol Metab. 2002;87:3023–8.
4. Ryysy L, Hakkinen AM, Goto T, Vehkavaara S, Westerbacka J, Halavaara J, et al. Hepatic fat content and insulin action on free fatty acids and glucose metabolism rather than insulin absorption are associated with insulin requirements during insulin therapy in type 2 diabetic patients. Diabetes. 2000;49:749–58.
5. Dzeletovic S, Breuer O, Lund E, Diczfalusy U. Determination of cholesterol oxidation products in human plasma by isotope dilution-mass spectrometry. Anal Biochem. 1995;225:73–80.
6. Gill S, Chow R, Brown AJ. Sterol regulators of cholesterol homeostasis and beyond: the oxysterol hypothesis revisited and revised. Prog Lipid Res. 2008;47:391–404.
7. Janowski BA, Willy PJ, Devi TR, Falck JR, Mangelsdorf DJ. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha. Nature. 1996;383:728–31.
8. Reschly EJ, Ai N, Welsh WJ, Ekins S, Hagey LR, Krasowski MD. Ligand specificity and evolution of liver X receptors. J Steroid Biochem Mol Biol. 2008;110:83–94.
9. DeBose-Boyd RA, Ou J, Goldstein JL, Brown MS. Expression of sterol regulatory element-binding protein 1c (SREBP-1c) mRNA in rat hepatoma cells requires endogenous LXR ligands. Proc Natl Acad Sci USA. 2001;98:1477–82.
10. Nakamuta M, Fujino T, Yada R, Yada M, Yasutake K, Yoshimoto T, et al. Impact of cholesterol metabolism and the LXRalpha-SREBP-1c pathway on nonalcoholic fatty liver disease. Int J Mol Med. 2009;23:603–8.
11. Higuchi N, Kato M, Shundo Y, Tajiri H, Tanaka M, Yamashita N, et al. Liver X receptor in cooperation with SREBP-1c is a major lipid synthesis regulator in nonalcoholic fatty liver disease. Hepatol Res. 2008;38:1122–9.
12. Kotronen A, Seppanen-Laakso T, Westerbacka J, Kiviluoto T, Arola J, Ruskeepaa AL, et al. Hepatic stearoyl-CoA desaturase (SCD)-1 activity and diacylglycerol but not ceramide concentrations are increased in the nonalcoholic human fatty liver. Diabetes. 2009;58:203–8.
13. Matthan NR, Lichtenstein AH. Approaches to measuring cholesterol absorption in humans. Atherosclerosis. 2004;174:197–205.
14. Miettinen TA, Tilvis RS, Kesaniemi YA. Serum plant sterols and cholesterol precursors reflect cholesterol absorption and synthesis in volunteers of a randomly selected male population. Am J Epidemiol. 1990;131:20–31.
15. Miettinen TA. Cholesterol production in obesity. Circulation. 1971;44:842–50.
16. Miettinen TA, Gylling H. Cholesterol absorption efficiency and sterol metabolism in obesity. Atherosclerosis. 2000;153:241–8.
17. Gylling H, Hallikainen M, Kolehmainen M, Toppinen L, Pihlajamaki J, Mykkanen H, et al. Cholesterol synthesis prevails over absorption in metabolic syndrome. Transl Res. 2007;149:310–6.
18. Bennion LJ, Grundy SM. Effects of diabetes mellitus on cholesterol metabolism in man. N Engl J Med. 1977;296:1365–71.
19. Simonen P, Kotronen A, Hallikainen M, Sevastianova K, Makkonen J, Hakkarainen A, et al. Cholesterol synthesis is increased and absorption decreased in non-alcoholic fatty liver disease independent of obesity. J Hepatol. 2011;54:153–9.
20. Gomez-Dominguez E, Gisbert JP, Moreno-Monteagudo JA, Garcia-Buey L, Moreno-Otero R. A pilot study of atorvastatin treatment in dyslipemid, non-alcoholic fatty liver patients. Aliment Pharmacol Ther. 2006;23:1643–7.
21. Hyogo H, Tazuma S, Arihiro K, Iwamoto K, Nabeshima Y, Inoue M, et al. Efficacy of atorvastatin for the treatment of nonalcoholic steatohepatitis with dyslipidemia. Metabolism. 2008;57:1711–8.
22. Nelson A, Torres DM, Morgan AE, Fincke C, Harrison SA. A pilot study using simvastatin in the treatment of nonalcoholic steatohepatitis: a randomized placebo-controlled trial. J Clin Gastroenterol. 2009;43:990–4.
23. Hyogo H, Ikegami T, Tokushige K, Hashimoto E, Inui K, Matsuzaki Y, et al. Efficacy of pitavastatin for the treatment of non-alcoholic steatohepatitis with dyslipidemia: an open-label, pilot study. Hepatol Res. 2011;41:1057–65.
24. Honda A, Yamashita K, Miyazaki H, Shirai M, Ikegami T, Xu G, et al. Highly sensitive analysis of sterol profiles in human serum by LC–ESI–MS/MS. J Lipid Res. 2008;49:2063–73.
25. Honda A, Yamashita K, Hara T, Ikegami T, Miyazaki T, Shirai M, et al. Highly sensitive quantification of key regulatory oxysterols in biological samples by LC–ESI–MS/MS. J Lipid Res. 2009;50:350–7.
26. Honda A, Miyazaki T, Ikegami T, Iwamoto J, Yamashita K, Numazawa M, et al. Highly sensitive and specific analysis of sterol profiles in biological samples by HPLC–ESI–MS/MS. J Steroid Biochem Mol Biol. 2010;121:556–64.
27. Honda A, Yamashita K, Numazawa M, Ikegami T, Doy M, Matsuzaki Y, et al. Highly sensitive quantification of 7alpha-hydroxy-4-cholesten-3-one in human serum by LC–ESI–MS/MS. J Lipid Res. 2007;48:458–64.
28. Honda A, Yamashita K, Ikegami T, Hara T, Miyazaki T, Hirayama T, et al. Highly sensitive quantification of serum malonate, a possible marker for de novo lipogenesis, by LC–ESI–MS/MS. J Lipid Res. 2009;50(2124):30.
29. Ghoshal AK, Guo T, Soukhova N, Soldin SJ. Rapid measurement of plasma acylcarnitines by liquid chromatography-tandem mass spectrometry without derivatization. Clin Chim Acta. 2005;358:104–12.
30. Babiker A, Diczfalusy U. Transport of side-chain oxidized oxysterols in the human circulation. Biochim Biophys Acta. 1998;1392:333–9.
31. Honda A, Miyazaki T, Ikegami T, Iwamoto J, Maeda T, Hirayama T, et al. Cholesterol 25-hydroxylation activity of CYP3A. J Lipid Res. 2011;52:1509–16.
32. Cali JJ, Russell DW. Characterization of human sterol 27-hydroxylase. A mitochondrial cytochrome P-450 that catalyzes multiple oxidation reaction in bile acid biosynthesis. J Biol Chem. 1991;266:7774–8.
33. Lund EG, Guileyardo JM, Russell DW. cDNA cloning of cholesterol 24-hydroxylase, a mediator of cholesterol homeostasis in the brain. Proc Natl Acad Sci USA. 1999;96:7238–43.
34. van der Veen JN, van Dijk TH, Vrins CL, van Meer H, Havinga R, Bijsterveld K, et al. Activation of the liver X receptor stimulates trans-intestinal excretion of plasma cholesterol. J Biol Chem. 2009;284:19211–9.
35. Altmann SW, Davis HR Jr, Zhu LJ, Yao X, Hoos LM, Tetzloff G, et al. Niemann–Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. Science. 2004;303:1201–4.
36. Berge KE, Tian H, Graf GA, Yu L, Grishin NV, Schultz J, et al. Accumulation of dietary cholesterol in sitosterolemia caused by mutations in adjacent ABC transporters. Science. 2000;290:1771–5.

37. Lee MH, Lu K, Hazard S, Yu H, Shulenin S, Hidaka H, et al. Identification of a gene, ABCG5, important in the regulation of dietary cholesterol absorption. Nat Genet. 2001;27:79–83.
38. Yu L, Hammer RE, Li-Hawkins J, Von Bergmann K, Lutjohann D, Cohen JC, et al. Disruption of Abcg5 and Abcg8 in mice reveals their crucial role in biliary cholesterol secretion. Proc Natl Acad Sci USA. 2002;99:16237–42.
39. Yu L, Li-Hawkins J, Hammer RE, Berge KE, Horton JD, Cohen JC, et al. Overexpression of ABCG5 and ABCG8 promotes biliary cholesterol secretion and reduces fractional absorption of dietary cholesterol. J Clin Invest. 2002;110:671–80.
40. Hirayama T, Mizokami Y, Honda A, Homma Y, Ikegami T, Saito Y, et al. Serum concentration of 27-hydroxycholesterol predicts the effects of high-cholesterol diet on plasma LDL cholesterol level. Hepatol Res. 2009;39:149–56.
41. Biddinger SB, Haas JT, Yu BB, Bezy O, Jing E, Zhang W, et al. Hepatic insulin resistance directly promotes formation of cholesterol gallstones. Nat Med. 2008;14:778–82.
42. Schreuder TC, Marsman HA, Lenicek M, van Werven JR, Nederveen AJ, Jansen PL, et al. The hepatic response to FGF19 is impaired in patients with nonalcoholic fatty liver disease and insulin resistance. Am J Physiol Gastrointest Liver Physiol. 2010;298:G440–5.
43. Trauner M, Claudel T, Fickert P, Moustafa T, Wagner M. Bile acids as regulators of hepatic lipid and glucose metabolism. Dig Dis. 2010;28:220–4.
44. Yang ZX, Shen W, Sun H. Effects of nuclear receptor FXR on the regulation of liver lipid metabolism in patients with non-alcoholic fatty liver disease. Hepatol Int. 2010;4:741–8.

Hepatology

Anticholestatic Effects of Bezafibrate in Patients with Primary Biliary Cirrhosis Treated with Ursodeoxycholic Acid

Akira Honda,[1,2] Tadashi Ikegami,[1] Makoto Nakamuta,[3] Teruo Miyazaki,[2] Junichi Iwamoto,[1] Takeshi Hirayama,[1] Yoshifumi Saito,[1] Hajime Takikawa,[4] Michio Imawari,[5] and Yasushi Matsuzaki[1]

From the [1]Department of Gastroenterology, and the [2]Joint Research Center, Tokyo Medical University Ibaraki Medical Center, Ami, Ibaraki, Japan; the [3]Department of Gastroenterology, Kyushu Medical Center, National Hospital Organization, Fukuoka, Japan; the [4]Department of Medicine, Teikyo University School of Medicine, Tokyo, Japan; and the [5]Division of Gastroenterology, Department of Medicine, Showa University School of Medicine, Tokyo, Japan.

Keywords: bile acid synthesis; CYP3A4; HepaRG cells; peroxisome proliferator-activated receptors (PPARs); pregnane X receptor (PXR).

Address reprint requests to: Yasushi Matsuzaki, MD. PhD.

Department of Gastroenterology, Tokyo Medical University Ibaraki Medical Center, 3-20-1 Chuoh, Ami, Inashiki, Ibaraki 300-0395, Japan

Phone:+81-29-887-1161 Fax:+81-29-887-9113

e-mail: ymatsuzaki-gi@umin.ac.jp

Abbreviations: PBC, primary biliary cirrhosis; UDCA, ursodeoxycholic acid; BSEP, bile salt export pump; MDR, multidrug resistance protein; ABC, ATP-binding cassette transporter; MRP, multidrug resistance-associated protein; FXR, farnesoid X receptor; PPAR, peroxisome proliferator-activated receptor; NF-κB, nuclear factor-κB; PXR, pregnane X receptor; C4, 7α-hydroxy-4-cholesten-3-one; FGF, fibroblast growth factor; 4β-HC, 4β-hydroxycholesterol; 24S-HC, 24S-hydroxycholesterol; 27-HC, 27-hydroxycholesterol; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; CA, cholic acid; LCA, lithocholoc acid; LXRα, Liver X receptor α; PGC1α, peroxisome proliferator-activated receptor-γ coactivator-1α; NTCP, Na^+/taurocholate cotransporting polypeptide; HMGCR, HMG-CoA reductase; CAR, constitutive androstane receptor; HNF4α, hepatocyte nuclear factor 4α.

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Abstract

Bezafibrate is a widely used hypolipidemic agent and is known as a ligand of the peroxisome proliferator-activated receptors (PPARs). Recently this agent has come to be recognized as a potential anticholestatic medicine for the treatment of primary biliary cirrhosis (PBC) that does not respond sufficiently to ursodeoxycholic acid (UDCA) monotherapy. The aim of this study was to explore the anticholestatic mechanisms of bezafibrate by analyzing serum lipid biomarkers in PBC patients and by cell-based enzymatic and gene expression assays. Nineteen patients with early-stage PBC and an incomplete biochemical response to UDCA (600 mg/day) monotherapy were treated with the same dose of UDCA plus bezafibrate (400 mg/day) for 3 months. In addition to the significant improvement of serum biliary enzymes, IgM, cholesterol and triglyceride concentrations in patients treated with bezafibrate, reduction of 7α-hydroxy-4-cholesten-3-one (C4), a marker of bile acid synthesis, and increase of 4β-hydroxycholesterol, a marker of CYP3A4/5 activity, were observed. *In vitro* experiments using human hepatoma cell lines demonstrated that bezafibrate controlled the target genes of PPARα, as well as those of the pregnane X receptor (PXR); downregulating CYP7A1, CYP27A1 and sinusoidal Na^+/taurocholate cotransporting polypeptide (NTCP), and upregulating CYP3A4, canalicular multidrug resistance protein 3 (MDR3), MDR1 and multidrug resistance-associated protein 2 (MRP2). ***Conclusion***: Bezafibrate is a dual PPARs/PXR agonist with potent anticholestatic efficacy in early-stage PBC patients with an incomplete biochemical response to UDCA monotherapy.