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Association between phospholipids and free cholesterol in high-density lipoprotein and the response to hepatitis C treatment in Japanese with genotype 1b

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SUMMARY. Pegylated interferon and ribavirin combination therapy is the standard treatment for patients with chronic hepatitis C (CHC), but treatment failure can be difficult to predict. We and others have reported a relation between lipid values and sustained viral responses in patients with CHC. However, the relationship between lipid values and treatment failure has not been previously reported. The present study investigated the association between the profiles of phospholipids and free cholesterol (FC), the main constitutive ingredients of the surface of lipoprotein, classified according to particle size and hepatitis C treatment, and determined the usefulness of these parameters for predicting the outcome of treatment. Fifty-five patients with CHC (33 men and 22 women) were included in the study. The serum total cholesterol, triglyceride, phospholipids, and FC levels in the lipoprotein subclasses were determined using high-performance liquid chromatography with gel permeation columns, enabling the lipoproteins to be classified into 13 subclasses according to particle size. According to a univariate analysis, the treatment failure group had a significantly higher serum phospholipid level overall in the high-density lipoprotein (HDL) and medium HDL fractions as well as a higher serum FC level in the HDL fraction and all HDL subclass fractions compared with the corresponding values in the non-nonvirological response group. Higher serum phospholipid and FC concentrations in the HDL subclasses were predictive of a failure to respond in patients with genotype 1b.

Keywords: free cholesterol, hepatitis C virus, high-performance liquid chromatography, interferon, nonvirological responses, phospholipid.

INTRODUCTION

About 200 million people worldwide are reportedly infected with hepatitis C virus (HCV), making HCV infection a major public health problem [1,2]. The virus cannot be eradicated in the majority of patients infected with HCV, and in some cases, HCV infection progresses to liver failure and hepatocellular carcinoma [3,4]. The distribution of major HCV genotypes varies according to geographical region, with genotype 1b being the most common in Japan. The HCV genotype has been identified as an important predictor of treatment efficacy, and no more than approximately 50% of patients with genotype 1b achieve a sustained viral response (SVR) after combined treatment with pegylated interferon plus ribavirin (PEG-IFN-RBV) [5,6]. Factors that may influence disease progression and response to therapy have been extensively investigated. These variables include age at the time of infection, sex, alcohol consumption, duration of infection, race, HCV genotype, viral load, and fibrotic stage. Modifications in lipid metabolism during the course of chronic hepatitis C (CHC) have also been studied [7–9].

Previously, we used computer-assisted high-performance liquid chromatography (HPLC) followed by the mathematical examination of chromatograms to examine the relation of cholesterol and triglyceride (TG) levels in lipoprotein subclasses among 44 patients with HCV infection [10]. This innovative method enables the serum lipid levels to be determined in each lipoprotein subfraction according to particle size [11–13]. We reported that higher serum

Abbreviations: CE, cholesteryl ester; CHC, chronic hepatitis C; FC, free cholesterol; HCV, hepatitis C virus; HDL, high-density lipoprotein; NVRs, nonvirological responses; PL, phospholipid; SVR, sustained viral response; VLDL, very low-density lipoprotein.

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cholesterol and TG concentrations in the lipoprotein subfractions were predictive of an SVR to therapy for infection with HCV (genotype 1b). No significant difference in the levels of TG and cholesterol in lipoprotein subfraction was observed between nonvirological responses (NVRs) to hepatitis C treatment and non-NVR. Most of the cholesterol and TG molecules are main constitutive ingredients of the inside of lipoprotein. The surface of lipoprotein contains phospholipid (PL), free cholesterol (FC), and apolipoproteins. In this study, we used computer-assisted HPLC to examine the levels of PL and FC, the main constitutive ingredients of the surface of lipoprotein. The aim of this study was to investigate the association between the levels of PL and FC in lipoprotein subclasses and hepatitis C treatment and to determine the clinical usefulness of the lipoprotein profiles for predicting a NVR to therapy for CHC prior to the start of interferon treatment.

PATIENTS AND METHODS

A total of 55 CHC Japanese patients with genotype 1b (mean ± standard deviation [SD] for age, 54.3 ± 12.4 years; age range, 25–72 years) were selected from among 131 consecutive patients who had received PEG-IFN-RBV combination therapy between April 2005 and October 2007 at Yokohama City University Hospital. The patients were selected based on the following criteria: (i) infection with HCV genotype 1b only; (ii) a high viral load (over 100 KIU/mL) according to a quantitative analysis [polymerase chain reaction (PCR)] of HCV-RNA (Cobas Amplicor HCV monitor v. 2.0, using the 10-fold dilution method; Roche K.K., Tokyo, Japan) performed within the 3 months preceding treatment; (iii) no severe fibrosis or cirrhosis visible at the time of liver biopsy [14]; (iv) no diabetes mellitus or renal disease; (v) no treatment with any lipid-lowering medication; (vi) no other forms of hepatitis, such as hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease, or autoimmune liver disease; (vii) no pregnant or lactating women; and (viii) an SVR assessed at least 24 weeks after the cessation of combined treatment. Each patient signed a consent form for the study protocol that had been approved by the Ethics Committee of Yokohama City University Hospital. In all the patients, plasma samples collected within 1 month before the start of treatment were available and were used to determine the fasting lipoprotein levels.

The PEG-IFNa-2b dose was adjusted according to body weight (60 μg for >35 and ≤45 kg, 80 μg for >45 and ≤60 kg, 100 μg for >60 and ≤75 kg, 120 μg for >75 and ≤90 kg, and 150 μg for >90 and ≤120 kg). The RBV dose was also adjusted according to body weight (600 mg for ≤60 kg, 800 mg for >60 and ≤80 kg, and 1000 mg for >80 kg). The patients received PEG-IFNa-2b at a median dose of 1.44 μg/kg (range, 0.91–1.72 μg/kg) subcutaneously each week and RBV orally at a median dose of 11.5 mg/kg (range, 8.0–14.0 mg/kg) daily for 48 weeks.

Patients who did not become negative for HCV-RNA during treatment were regarded as having an NVR. The patients were divided into NVR and non-NVR groups. The non-NVR group was composed of patients who relapsed and sustained negativity after the completion of treatment.

An HPLC system with two tandem gel permeation columns (Skylight Biotech, Inc., Akita, Japan) was used to evaluate the size distribution of the plasma lipoprotein particles [12,13,15]. Samples were diluted 20 times and analysed at a flow rate of 350 μL/min by monitoring the concentrations of choline-PL, total cholesterol, and TG, with an absorbance set at 585 nm for choline-PL and at 550 nm for cholesterol and TG. The particle sizes for individual subfractions were previously determined as 44.5–64 nm [large very low-density lipoprotein (VLDL)], 36.8 nm

Table 1 Basic clinical characteristics and major lipid profiles

Parameter	Total (n = 55)	NVR (n = 12)	Non-NVR (n = 43)	P
Basic clinical characteristics				
Age (years)	54.3 ± 12.4	56.8 ± 10.4	53.7 ± 13.2	0.461
Male sex (%)	60.0 ± 49.4	58.3 ± 51.5	60.5 ± 49.5	0.896
Body mass index (kg/m^2)	22.9 ± 3.1	23.9 ± 2.7	22.6 ± 3.2	0.232
Serum aspartate aminotransferase (IU/L)	57.1 ± 34.9	57.9 ± 28.7	56.9 ± 36.8	0.932
Serum alanine aminotransferase (IU/L)	73.4 ± 47.9	68.1 ± 36.9	74.9 ± 50.9	0.668
Hepatitis C virus -RNA load (KIU/mL)	1993 ± 1426	1916 ± 1454	2017 ± 1435	0.834
Major lipids				
Total cholesterol (mg/dL)	166.1 ± 30.1	163.2 ± 39.2	166.9 ± 27.5	0.708
High-density lipoprotein cholesterol (mg/dL)	50.9 ± 15.2	57.4 ± 22.5	49.0 ± 12.2	0.090
Low-density lipoprotein cholesterol (mg/dL)	32.9 ± 21.0	78.6 ± 22.5	84.0 ± 20.0	0.433
Triglycerides (mg/dL)	99.2 ± 29.4	97.4 ± 27.4	99.7 ± 30.2	0.809

The values are shown as the mean ± standard deviation (SD). NVR, nonvirological response to pegylated interferon plus ribavirin combination therapy.

(medium VLDL), 31.3 nm (small VLDL), 28.6 nm [large low-density lipoprotein (LDL)], 25.5 nm (medium LDL), 23 nm (small LDL), 16.7–20.7 nm (very small LDL), 13.5–15 nm [very large high-density lipoprotein (HDL)], 12.1 nm (large HDL), 10.9 nm (medium HDL), 9.8 nm (small HDL), and 7.6–8.8 nm (very small HDL) [12].

Data are expressed as the mean ± SD, unless indicated otherwise. The statistical analysis was conducted using SPSS 12.0 (SPSS, Chicago, IL, USA). For univariate comparisons between the patient groups, the *t*-test or Mann–Whitney's *U* test was used, where appropriate. $P < 0.05$ was considered significant.

RESULTS

The clinical characteristics and serum concentrations of the major lipoproteins for the 55 patients included in this study are shown in Table 1. The NVR group contained 12 patients (21.8%). No significant differences in age, sex, body mass index, serum aspartate aminotransferase, serum alanine aminotransferase, HCV-RNA load, total cholesterol, HDL-cholesterol, LDL-cholesterol, or TGs were observed between the SVR and non-SVR groups.

The serum PL concentrations in each of the lipoprotein subclasses are shown in Fig. 1b and Table 2. The NVR group exhibited significantly higher medium HDL-PL (31.6 ± 7.6 *vs* 27.0 ± 5.6 mg/dL, $P = 0.025$), total HDL-PL (137.9 ± 38.3 *vs* 111.7 ± 24.6 mg/dL, $P = 0.006$), and total PL (244.5 ± 54.7 *vs* 212.0 ± 31.1 mg/dL, $P = 0.010$) values than the non-NVR group.

The serum FC concentrations in each of the lipoprotein subclasses are shown in Fig. 1a and Table 2. The NVR group had significantly higher serum FC levels in the total HDL and all HDL subclasses.

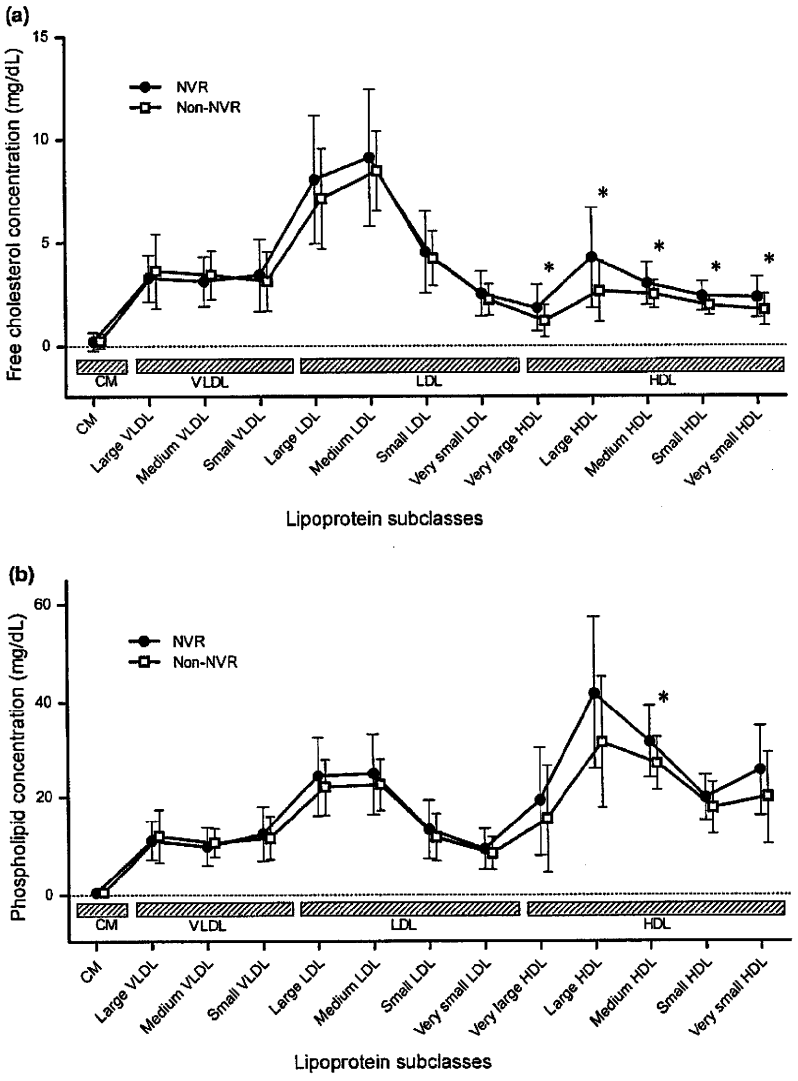

Fig. 1 Comparison of (a) free cholesterol and (b) phospholipid concentrations in lipoprotein subclasses between the nonvirological response (NVR) group and non-NVR group. The values are shown as the mean ± standard deviation (SD). CM, chylomicron (>70 nm in diameter); VLDL, very low-density lipoprotein (30–70 nm); LDL, low-density lipoprotein (16–30 nm); HDL, high-density lipoprotein (7–16 nm). *Subclass in which significant difference was observed between the NVR and non-NVR groups.

Table 2 Profiles of serum lipid levels in HDL subclasses

	Lipid concentrations (mg/dL)		
	NVR (n = 12)	Non-NVR (n = 43)	P
Phospholipid concentrations			
Very large HDL	19.3 ± 11.1	15.6 ± 10.8	0.306
Large HDL	41.6 ± 15.8	31.5 ± 13.7	0.070
Medium HDL	31.6 ± 7.6	27.0 ± 5.6	0.025
Small HDL	19.8 ± 4.6	17.7 ± 5.3	0.219
Very small HDL	25.6 ± 9.4	19.9 ± 9.4	0.050
Total HDL	137.9 ± 38.3	111.7 ± 24.6	0.006
Total lipoproteins	244.5 ± 54.7	212.0 ± 31.1	0.010
Free cholesterol concentrations			
Very large HDL	1.83 ± 1.12	1.20 ± 0.78	0.020
Large HDL	4.26 ± 2.43	2.63 ± 1.47	0.014
Medium HDL	3.01 ± 1.02	2.47 ± 0.65	0.031
Small HDL	2.39 ± 0.71	1.95 ± 0.47	0.013
Very small HDL	2.33 ± 0.96	1.73 ± 0.76	0.021
Total HDL	13.8 ± 5.1	10.0 ± 3.0	0.002
Total lipoproteins	48.1 ± 12.8	42.5 ± 7.39	0.056
Triglyceride concentrations			
Very large HDL	2.0 ± 1.0	1.9 ± 1.5	0.427
Large HDL	6.4 ± 3.6	5.6 ± 3.7	0.539
Medium HDL	5.0 ± 1.5	5.2 ± 1.9	0.684
Small HDL	3.2 ± 1.1	3.3 ± 1.1	0.852
Very small HDL	3.1 ± 0.8	3.0 ± 0.7	0.681
Total HDL	19.7 ± 6.2	19.6 ± 7.3	0.776
Total lipoproteins	97.4 ± 27.4	99.7 ± 30.2	0.935
Total cholesterol concentrations			
Very large HDL	5.2 ± 3.2	4.4 ± 2.7	0.367
Large HDL	17.2 ± 11.2	13.1 ± 7.2	0.229
Medium HDL	15.2 ± 5.6	13.7 ± 3.6	0.249
Small HDL	10.7 ± 4.1	10.0 ± 2.1	0.389
Very small HDL	9.0 ± 2.5	7.9 ± 1.6	0.071
Total HDL	57.4 ± 22.5	49.0 ± 12.2	0.165
Total lipoproteins	163.2 ± 39.2	166.9 ± 27.5	0.708

The values are shown as the mean ± standard deviation (SD). NVR, nonvirological response to pegylated interferon plus ribavirin combination therapy; HDL, high-density lipoprotein (7–16 nm in diameter).

No significant differences in the serum concentration of TG and the total cholesterol levels in all the lipoprotein subclasses were observed between the NVR and non-NVR groups (Table 2).

DISCUSSION

This study demonstrated that the serum PL and FC levels in the HDL fraction were significantly elevated in the NVR group compared with the values in the non-NVR group, before PEG-IFN-RBV combination therapy. HDL plays a key role in transporting cholesterol from the peripheral tissues to hepatocytes via scavenger receptor class B type I (SR-BI), a receptor for both HDL and HCV. Several laboratories have reported the infectivity of retroviral pseudoparticles bearing HCV E1E2 gps (HCVpp) [16–18]. HDL reportedly stimulates HCVpp entry into human hepatocarcinoma target cells [19–24], and this enhancement of HCVpp infection involves a complex interplay between the hypervariable region of HCV E2 protein, SR-BI, and HDL [19,23]. Nevertheless, the mechanism by which HDL increases HCV cell entry is not fully understood. Several studies have examined the enhancement of HCV infectivity by the apolipoproteins that constitute HDL [24–26]. Lipid-free apoA-I and apoA-II proteins, the major HDL apolipoproteins, had no effect on HCVpp entry despite their weak but real binding capacity to SR-BI [27,28]. The functional role of HDLs in lipid transfer is dependent on the proper orientation of apoA-I associated with lipids [29]. No reports have discussed the direct association between HCV infectivity and the lipid component of

HDL. We speculated that the lipids constituting HDL are necessary for the HDL/SR-BI-mediated facilitation of HCVpp entry.

In this study, the serum PL and FC levels in HDL were significantly elevated in the NVR group. However, no statistically significant differences in the HDL-cholesterol and the HDL-TG concentrations were observed between the NVR and non-NVR groups, as observed in previous reports [30–33]. Few cholesteryl ester (CE) and TG molecules are apparently present on the surface of HDL. Instead, the surface of HDL contains PL, FC, and apolipoproteins. Ligands of SR-BI include discoidal reconstituted HDL (rHDL) containing apoA-I and other apolipoproteins [28,34–36] and spherical HDL [28,34]. Larger spherical HDL is more tightly bound to SR-BI than smaller HDL [28]. However, our results were unable to demonstrate this difference in affinity according to HDL size. In this study, the NVR group had a significantly higher serum PL level in the medium HDL fraction and tended to have higher levels in the other HDL subclasses compared with the levels in the non-NVR group. The serum FC levels were significantly higher in all the HDL subclasses in the NVR group compared with the levels in the non-NVR group. We suspected that the number of patients in this study was insufficient to reveal a difference in affinity according to HDL size. However, we speculated that differences in the surface structures of HDL, consisting of PL, FC, and apolipoproteins, influence the affinity between SR-BI and HDL and affect the HDL/SR-BI-mediated facilitation of HCV entry.

FC, in particular, is the principal ingredient of the cholesterol that is included in the cell membrane. Local changes in the lipid composition of the cell membrane induced by HDL/SR-BI might potentially affect HCV infectivity [37]. The SR-BI-mediated uptake of HDL-CE is followed by hydrolysis to FC by a neutral CE hydrolase. SR-BI-mediated lipid uptake leads to an increase in the cholesterol content of the target cell membrane [38–40]. Local cholesterol enrichment may facilitate binding [41] and/or conformational changes [42] within the HCV glycoproteins that are required for membrane fusion processes. Because HCVpp internalization has been shown to be specifically accelerated by HDL [21], HCVpp entry is more rapid than virus neutralization.

In agreement with these previous reports and the present assumptions, our data suggest that PL-rich and/or FC-rich HDL with SR-BI promotes HCV infectivity in cells and ultimately makes patients resistant to antiviral treatments. Nevertheless, the present study had a retrospective and small-scale design. Larger prospective studies should enable further analysis of the lipoprotein subclasses and the relationship between lipoprotein and HCV *in vivo*.

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Association between lipoprotein subfraction profile and the response to hepatitis C treatment in Japanese patients with genotype 1b

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SUMMARY. Pegylated interferon and ribavirin combination therapy is the standard treatment for patients with chronic hepatitis C (CHC). Some groups have reported a relation between lipid values and response while others have reported that microsomal triglyceride transfer protein, a key enzyme in the assembly and secretion of lipoproteins, was related to hepatitis C virus (HCV). The aim of this study was to investigate the association between the lipoprotein profiles, classified according to size, and hepatitis C treatment and the usefulness for predicting the outcome of treatment. Forty-four patients with CHC (27 men and 17 women) were included in the study. The serum cholesterol and triglyceride (TG) levels in the lipoprotein subclasses were determined using high-performance liquid chromatography with gel permeation columns, which classified lipoproteins into 20 subfractions based on particle size. According to a univariate analysis, those who achieved an sustained viral response (SVR) had a significantly higher serum total cholesterol level, higher cholesterol levels in the low-density lipoprotein subfraction (25.5 nm in diameter) and the very low-density lipoprotein (VLDL) subfraction (44.5 and 36.8 nm), and a higher serum TG level in the VLDL subfraction (44.5 nm), compared with the corresponding values in the non-SVR group. Higher serum cholesterol and TG concentrations in the lipoprotein subfractions were predictive of an SVR to therapy for HCV infection with genotype 1b prior to the start of interferon treatment.

Keywords: genotype 1b, hepatitis C virus, high-performance liquid chromatography, interferon, lipoprotein, sustained virological responses.

INTRODUCTION

About 200 million people worldwide are reportedly infected with hepatitis C virus (HCV), making HCV infection a major public health problem [1,2]. The majority of HCV-infected patients fail to eradicate the virus and, in some cases, HCV infection progresses to liver failure and hepatocellular carcinoma [3,4]. The distribution of major HCV genotypes varies according to geographical region, with genotype 1b being the most common in Japan. HCV genotype has been identified as an important predictor of treatment efficacy, and no more than approximately 50% of genotype 1b patients achieve a sustained viral response (SVR) after combined treatment with pegylated interferon (PEG-IFN) plus ribavirin (PEG-IFN–RBV) [5,6]. The ability to predict the success of combination therapy before the start of actual treatment would be very useful for both patients and their physicians. While many groups have reported various patient and viral factors that help to predict the response to PEG-IFN therapy, no single pretreatment factor can precisely predict individual treatment outcome [7,8].

Several groups have reported a relation between HCV and lipid metabolism [9]. Reportedly, serum low-density lipoprotein (LDL), total cholesterol and LDL-cholesterol (LDL-C) levels are all associated with the outcome of hepatitis C

Abbreviations: CHC, chronic hepatitis C; HCV, hepatitis C virus; HPLC, high-performance liquid chromatography; LDL-C, LDL-cholesterol; LDL, low-density lipoprotein; LDL-r, LDL receptor; MTP, microsomal triglyceride transfer protein; PEG-IFN, pegylated interferon; PEG-IFN–RBV, pegylated interferon plus ribavirin; PCR, polymerase chain reaction; SVR, sustained viral response; TG, Triglyceride; VLDL, very low-density lipoprotein; VLDL-C, very low-density lipoprotein cholesterol.

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treatment [10–12]. However, minor discrepancies in the lipid characteristics associated with hepatitis C treatment were noted.

In this study, we used computer-assisted high-performance liquid chromatography (HPLC) followed by the mathematical examination of chromatograms to examine the relation of cholesterol and triglyceride (TG) levels in lipoprotein subclasses in patients with HCV infection [13]. This innovative method provides the serum TG levels in each lipoprotein subfraction based on differences in particle size [14,15]. The aim of this study was to investigate the association between the lipoprotein profiles classified according to size and hepatitis C treatment and to determine the clinical usefulness of lipoprotein profiles for predicting an SVR to chronic hepatitis C (CHC) therapy prior to the start of interferon treatment.

PATIENTS AND METHODS

A total of 44 CHC Japanese patients with genotype 1b [mean ± standard deviation (SD) for age, 53.8 ± 12.4 years; age range 29–71 years) were selected from among 131 consecutive patients who had received PEG-IFN–RBV combination therapy between April 2005 and June 2007 at Yokohama City University Hospital. The patients were selected based on the following criteria: (1) infection with HCV genotype 1b only; (2) a high viral load (over 100 KIU/mL) according to a quantitative analysis [polymerase chain reaction (PCR)] of HCV-RNA (Cobas Amplicor HCV monitor v. 2.0, using the 10-fold dilution method; Roche Diagnostics K.K., Tokyo, Japan) performed within the 3-months preceding treatment; (3) no severe fibrosis or cirrhosis visible at the time of liver biopsy [16], (4) no diabetes mellitus or renal disease; (5) no treatment with any lipid-lowering medication; (6) no other forms of hepatitis, such as haemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease or autoimmune liver disease; (7) no pregnant or lactating women and (8) SVR assessed at least 24 weeks after the cessation of combined treatment. Each patient signed a consent form for the study protocol that had been approved by the Ethics Committee of Yokohama City University Hospital. In all the patients, plasma samples collected within 1 month before the start of treatment were available and were used to determine the fasting lipoprotein levels.

The PEG-IFNa-2b dose was adjusted according to body weight (60 μg for >35 and <45 kg, 80 μg for >45 and <60 kg, 100 μg for >60 and <75 kg, 120 μg for >75 and <90 kg and 150 μg for >90 and <120 kg). The RBV dose was also adjusted according to body weight (600 mg for <60 kg, 800 mg for >60 and <80 kg and 1000 mg for >80 kg). The patients received PEG-IFNa-2b at a median dose of 1.44 μg/kg (range 0.91–1.72 μg/kg) subcutaneously each week and orally took RBV at a median dose of 11.5 mg/kg (range 8.0–14.0 mg/kg) daily for 48 weeks. Patients who achieved HCV-RNA negativity based on an HCV-RNA quantitative PCR analysis at 24 weeks after the completion of treatment were defined as SVR. We divided the patients into SVR and non-SVR groups. The non-SVR group was composed of patients who did not achieve an SVR.

An HPLC system with two tandem gel permeation columns (Skylight Biotech, Inc., Akita, Japan) was used to evaluate the size distribution of the plasma lipoprotein particles [13,17,18]. Samples were diluted 20 times and analysed at a flow rate of 350 μL/min by monitoring the concentrations of choline-phospholipid, total cholesterol and TG, with an absorbance at 585 nm for choline-phospholipid and at 550 nm for total cholesterol and TG.

Data are expressed as the mean ± SD, unless indicated otherwise. The statistical analysis was conducted using SPSS 12.0 (SPSS, Chicago, IL, USA). For univariate comparisons between the patient groups, a *t*-test was used, where appropriate. *P* values of <0.05 were considered significant.

RESULTS

The clinical characteristics and serum concentrations of the major lipoproteins for the 44 patients included in this study are shown in Table 1. An SVR was achieved by 19 of the 44 (43.1%) patients. The SVR group was significantly younger ($P = 0.014$), had a higher serum aspartate aminotransferase level ($P = 0.029$) and had a higher total cholesterol level ($P = 0.032$), compared with the non-SVR group. On the contrary, no differences in the LDL-C, high-density lipoprotein cholesterol and total TG concentrations were observed between the SVR and non-SVR groups.

The serum cholesterol concentrations in each of the 20 lipoprotein subfractions were measured using computer-assisted HPLC followed by mathematical treatment; the results for the SVR and non-SVR groups are summarized in Fig. 1a. The SVR group had significantly higher serum levels of very low-density lipoprotein cholesterol (VLDL-C) with a diameter of 44.5 nm (10.7 ± 6.9 *vs* 6.7 ± 3.8 mg/dL, respectively; $P = 0.019$), VLDL-C with a diameter of 36.8 nm (10.5 ± 3.1 *vs* 8.5 ± 3.4 mg/dL; $P = 0.049$) and LDL-C with a diameter of 25.5 nm (33.9 ± 7.7 *vs* 29.1 ± 7.6 mg/dL; $P = 0.041$; Table 2).

The serum TG concentrations in each of the 20 lipoprotein subfractions were measured using computer-assisted HPLC followed by mathematical treatment; the results for the SVR and non-SVR groups are summarized in Fig. 1b. The SVR group had a significantly higher level of VLDL-TG with a diameter of 44.5 nm (18.9 ± 9.3 *vs* 14.1 ± 6.1 mg/dL, respectively; $P = 0.045$; Table 2). No other significant differences in the subfractions were observed between the SVR and non-SVR groups.

DISCUSSION

The present study demonstrated that the serum TG levels in the VLDL subfraction, classified according to size, and the serum cholesterol levels in the VLDL subfraction and LDL

Table 1 Basic clinical characteristics and major lipid profiles

Parameter	Total (n = 44)	SVR (n = 19)	non-SVR (n = 25)	P-value
Basic clinical characteristics				
Age (years)	53.8 + 12.4	48.7 + 13.4	57.8 + 10.2	0.014
Male sex (%)	61.4 + 49.3	68.4 + 47.8	56.0 + 50.7	0.413
Body mass index (kg/m^2)	22.8 + 3.2	21.8 + 3.84	23.5 + 2.42	0.087
Serum aspartate aminotransferase (IU/L)	61.8 + 37.3	47.8 + 17.9	72.4 + 44.5	0.029
Serum alanine aminotransferase (IU/L)	78.3 + 50.2	72.4 + 45.6	82.9 + 53.9	0.498
HCV-RNA load (KIU/mL)	2012 + 1441	2132 + 1580	1916 + 1348	0.632
Major lipid				
Total cholesterol (mg/dL)	163.1 + 30.2	174.1 + 28.4	154.6 + 29.3	0.032
High-density lipoprotein cholesterol (mg/dL)	49.0 + 14.7	50.6 + 10.2	47.7 + 17.4	0.514
Low-density lipoprotein cholesterol (mg/dL)	81.3 + 21.5	86.7 + 22.0	77.1 + 20.5	0.143
Triglycerides (mg/dL)	100.7 + 29.8	103.8 + 31.5	98.3 + 28.8	0.547

The values are shown as the mean ± standard deviation (SD). SVR, sustained viral response with pegylated interferon plus ribavirin combination therapy.

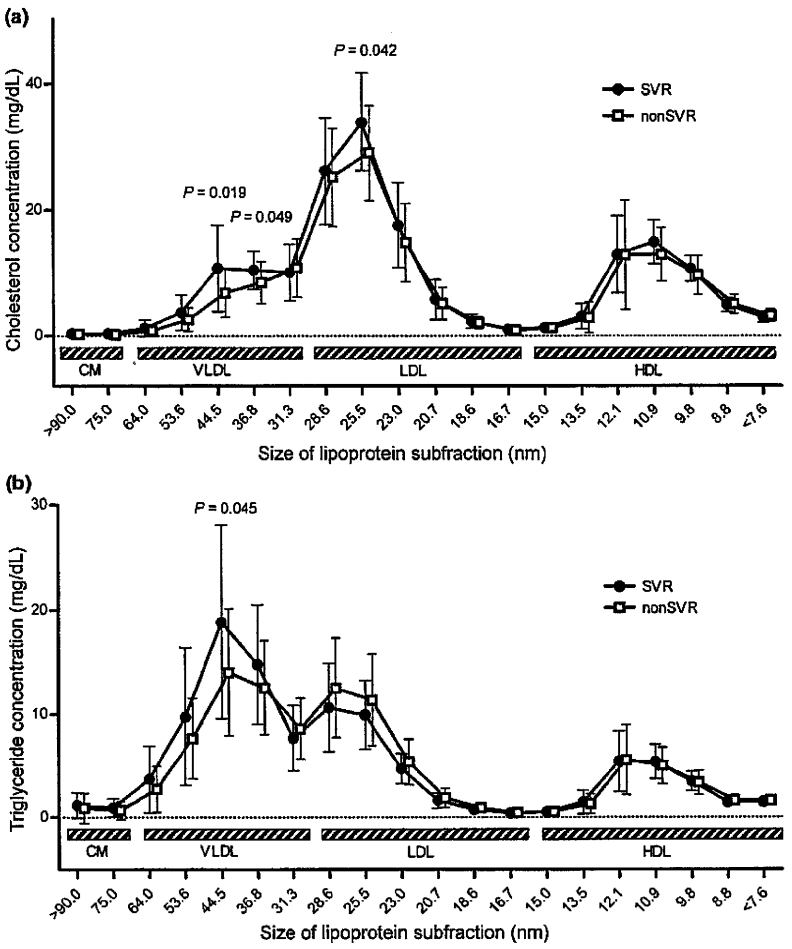

Fig. 1 Comparison of (a) cholesterol and (b) triglycerides concentrations in lipoprotein subfraction between the SVR and non-SVR patients. The values are shown as the mean ± standard deviation (SD). SVR, sustained viral response with pegylated interferon plus ribavirin combination therapy; CM, chylomicron (>70 nm in diameter); VLDL, very low-density lipoprotein (30–80 nm); LDL, low-density lipoprotein (16–30 nm); HDL, high-density lipoprotein (7–16 nm).

subfraction were significantly elevated in the SVR group, compared with the values in the non-SVR group, after PEG-IFN–RBV combination therapy. These results suggest that the lipoprotein subfraction profiles classified according to size are associated with the outcome of treatment for HCV infection and could be a useful predictor of the response to

Table 2 Profiles of serum triglyceride and cholesterol levels in lipoprotein subfractions

Parameter	Total (n = 44)	SVR (n = 10)	non-SVR (n = 19)	*P*-value
Cholesterol in lipoprotein subfraction				
44.5 nm in diameter lipoprotein cholesterol (mg/dL)	8.7 + 5.7	10.7 + 6.9	6.7 + 3.8	0.019
36.8 nm in diameter lipoprotein cholesterol (mg/dL)	9.3 + 3.4	10.5 + 3.1	8.5 + 3.4	0.049
25.5 nm in diameter lipoprotein cholesterol (mg/dL)	31.2 + 7.9	33.9 + 7.7	29.1 + 7.6	0.042
Triglycerides in lipoprotein subfraction				
44.5 nm in diameter lipoprotein triglycerides (mg/dL)	16.1 + 7.9	18.9 + 9.3	14.1 + 6.1	0.045

The values are shown as the mean ± standard deviation (SD). SVR, sustained viral response with pegylated interferon plus ribavirin combination therapy.

treatment. Our study also suggested that the serum total cholesterol levels were significantly elevated in the SVR group, as has been previously reported [12,19,20]. On the contrary, our results showed that the serum total TG levels and the LDL-C levels were similar in the SVR and non-SVR groups.

Contradicting reports have been published: one group reported that the serum total TG level was a good predictor of response to IFN treatment [20], whereas other groups have reported that this factor is not a good predictor [11,12]. Similarly, some [11,12,21,22], not all [20,23], previous studies have reported that the serum total cholesterol and LDL-C levels are predictors of SVR in patients with CHC. Differences in study designs, inadequate numbers of participants, variability in patient selection methods, as well as differences in the methodologies of laboratory determinations may account for these discrepancies.

Triglyceride-rich VLDL are secreted by hepatocytes and circulate in the blood. Within the bloodstream, VLDL acquires ApoE, and three enzymes modify its content, causing it to become enriched in cholesteryl esters and depleted in TG [24]. We speculated that the lipoprotein subfraction profiles, classified according to size, might be useful indicators of lipid metabolism in patients with HCV, as VLDL is converted to lipoproteins with higher densities and large sizes and these processes are dramatically influenced by individual differences. Until now, quantifying lipoproteins on the basis of differences in specific gravity using the sedimentation velocity method has been used for research on HCV [25,26]. HPLC using gel permeation columns is an alternative method for classifying and quantifying lipoproteins based on differences in particle size [13,27]. This is the first report to discuss the relation between lipoprotein and HCV using data obtained with this new method.

The present study demonstrated that serum TG levels in the VLDL subfraction of particles with a diameter of 44.5 nm was significantly elevated in SVR patients, compared with the value in non-SVR patients, before PEG-IFN–RBV combination therapy. Some groups performing basic research have reported that HCV inhibits microsomal triglyceride transfer protein (MTP) activity, which has been shown to be essential for hepatic lipoprotein assembly and secretion in a transgenic mouse model [28]. Another clinical study reported that liver MTP activity was significantly reduced in patients with HCV genotype 3 [29]. We speculated that a difference in the concentration of VLDL-TG with a diameter of 44.5 nm between SVR and non-SVR groups was caused by a reduction of MTP activity as a result of HCV genotype 1b infection [30–32].

There are reports that HCV production is dependent on assembly and secretion of VLDL [33] and that the particles of HCV exist in binding form with beta-lipoproteins (i.e. LDL and VLDL) in the human sera of patients with HCV infection [34,35]. There are some conflicting data, not only in our study, regarding the use of serum lipoprotein levels as predictors of response to HCV treatment [10–12,20–23]. We speculate that many factors are responsible for this discrepancy: the complexity of VLDL production and metabolism regulation [36,37], the impact of the serum lipid on immune regulation and HCV cell entry [38,39], and the existence of serum lipoprotein unbinding with HCV. The examination from many aspects is necessary to solve this contradiction.

The mechanism whereby high levels of serum LDL-C enhance the response to PEG-IFN–RBV is unclear. Some groups have reported that HCV may enter into the cell through the CD81 and LDL receptor (LDL-r) [27,40,41]. Another group has stated that the intracellular cholesterol level modulates LDL-r expression, and thus a high LDL-C expression could down-regulate LDL-r and diminish the spread of HCV infection to other hepatocytes. Thus, the correlation between treatment efficacy and LDL-C may be explained by the role of LDL-C in transporting the HCV–LDL complex into hepatocytes [27,42].

A better understanding of the mechanisms underlying HCV infection and the efficacy of interferon treatment may suggest other advances to improve the outcome of therapy. The potential involvement of VLDL secretion and the LDL-r in HCV infection provides a new approach to therapy in the future. Such advances may include the use of agents blocking VLDL secretion and LDL receptor-blocking analogues that may slow viral replication and disease progres-

sion or improve the rate of SVR. Because the number of patients in whom the antiviral response was analysed in our retrospective study was relatively small, the present results should be considered as preliminary findings.

In conclusion, higher serum TG concentrations in large VLDL and middle VLDL lipoprotein fractions were predictive of an SVR to therapy for HCV infection with genotype 1b prior to the start of interferon treatment. Nevertheless, the present study had a retrospective design, and the presently reported results should be confirmed in prospective studies. Larger prospective clinical trials are needed to establish the diagnostic significance of our proposed parameter, the serum level of VLDL-TG with a diameter of 44.5 nm, for identifying patients with an increased likelihood of an SVR after combined PEG-IFN–RBV therapy.

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ORIGINAL ARTICLE

Serum Ferritin Is a Clinical Biomarker in Japanese Patients with Nonalcoholic Steatohepatitis (NASH) Independent of *HFE* Gene Mutation

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Abstract Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury. The spectrum of NAFLD is broad, extending from simple steatosis through nonalcoholic steatohepatitis (NASH). Iron is regarded as a putative element that interacts with oxygen radicals, and high rates of hyperferritinemia and increased hepatic iron stores have been demonstrated in NASH. We investigated serum ferritin concentrations, *HFE* gene mutations, and insulin resistance in Japanese NASH patients and the diagnostic utility of serum ferritin concentrations as a means of distinguishing NASH. Serum ferritin concentrations were measured in 86 patients with histopathologically verified NAFLD (24 with steatosis and 62 with NASH) and 20 control subjects, they were tested for *HFE* gene mutations and their insulin resistance was measured. The serum ferritin concentration was significantly higher in the NASH patients than in the patients with simple steatosis ($P = 0.006$). There was no significant difference between the groups in HFE gene mutation (C282Y, H63D, and S65C), and the serum ferritin level was related with insulin resistance. The area under the ROC curve was 0.732 for distinguishing NASH from simple steatosis ($P = 0.005$; 95% CI, 0.596–0.856). In conclusion high serum ferritin concentrations are a distinguishing feature of Japanese NASH patients independent of *HFE* gene mutations.

Keywords NASH · NAFLD · Hyperferritinemia · HFE · Insulin resistance

Abbreviations

NASH	Nonalcoholic steatohepatitis
NAFLD	Nonalcoholic fatty liver disease
IR	Insulin resistance
PCR	Polymerase chain reaction
VFA	Visceral fat area
BMI	Body mass index
SFA	Subcutaneous fat area
HOMA-IR	Homeostasis model assessment for insulin resistance

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver injury in many countries around the world [1, 2]. NAFLD represents a spectrum of conditions that are histologically characterized by macrovesicular hepatic steatosis, and the diagnosis is made in patients who have not consumed alcohol in amounts considered harmful to the liver. The histological changes range over a wide spectrum, extending from simple steatosis,

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which is generally nonprogressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, and liver failure, and sometimes even hepatocellular carcinoma [3, 4]. We have previously reported that the serum type IV collagen 7s concentration [5], high-sensitivity C-reactive protein [6], plasma Pentraxin3 [7], and transient elastography [8, 9] are useful for making the diagnosis of NASH. Differentiation between NASH and nonprogressive NAFLD has remained difficult based on the clinical and imaging findings alone [10]. The invasive diagnostic technique of liver biopsy is currently recognized as the only means of examining the liver for the presence and extent of specific necroinflammatory changes and fibrosis necessary to make the diagnosis of NASH, and a noninvasive method of diagnosing NASH is required.

A commonly accepted model of the pathogenesis of NASH is the so-called "two hit" hypothesis, wherein the first "hit" leads to accumulation of hepatic free fatty acids resulting in a histological picture of macrovesicular steatosis [11]. A subsequent "hit" associated with oxidative stress may result in liver injury, which over time may lead to eventual development of hepatic fibrosis and possible progression to cirrhosis. Iron is regarded as a putative element that interacts with oxygen radicals to induce liver damage and fibrosis [12], and serum ferritin is reported to predict severe fibrosis in NASH patients [13]. However, the relationship between NASH and iron metabolism/overload is still controversial. Some studies suggest an increased prevalence of common mutations of the *HFE* gene associated with hereditary hemochromatosis in patients with NASH [12, 14] and insulin resistance (IR) [15]. Furthermore the fact that there have been no reports on the clinical usefulness of measuring serum ferritin concentrations to differentiate between NASH and nonprogressive simple steatosis prompted us to investigate the diagnostic utility of measuring serum ferritin concentrations to distinguish NASH patients from simple steatosis patients.

In this study we investigated serum ferritin concentrations, *HFE* gene mutations, and IR in Japanese NASH patients and the diagnostic utility of serum ferritin concentrations as a means of distinguishing between NASH and steatosis.

Patients and Methods

Patients

A total of 86 Japanese NAFLD patients (62 NASH and 24 simple steatosis) and 20 healthy control subjects were recruited. All control subjects were confirmed to have normal liver function and no viral hepatitis infection. It was also confirmed they were not alcoholics. All of the 86 NAFLD patients underwent liver biopsy. This study was conducted with the approval of the Ethics Committee of Yokohama City University. The study was restricted to men and postmenopausal women to eliminate the influence of menstruation, pregnancy, and female hormone replacement therapy and to reduce possible confounding by iron deficiency and iron supplementation. Other exclusion criteria were: history of hepatic disease, such as chronic hepatitis C or concurrent active hepatitis B (serum positive for hepatitis B surface antigen), autoimmune hepatitis, primary biliary cirrhosis (PBC), sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, hepatic injury caused by substance abuse, and current or past consumption of more than 20 g alcohol daily. None of the patients had any clinical evidence of hepatic decompensation, for example hepatic encephalopathy, ascites, variceal bleeding, or elevation of the serum bilirubin level to more than twofold the upper limit of normal. A detailed history was obtained and physical examination of all 86 patients was performed.

Clinical and Laboratory Evaluation

The weight and height of the patients were measured with calibrated scales after requesting the patients to remove their shoes and any heavy clothing. Venous blood samples for measurement of serum AST, ALT, iron, ferritin, glucose, insulin, type IV collagen 7s, hyaluronic acid concentrations, cell counts of erythrocytes, hematocrit, and hemoglobin were obtained after the patients had fasted overnight (12 h). Serum insulin levels were measured by radioimmunoassay. Other laboratory biochemical characteristics were measured with a conventional automated analyzer.

IR was calculated by the modified homeostasis model assessment of insulin resistance (HOMA-IR), using the formula: HOMA-IR = fasting insulin (μU/ml) × plasma glucose (mg/dl)/405. HOMA-IR was originally reported by Matthews and has since been modified [16]. This index has been shown to correlate well with results from the euglycemic–hyperinsulinemic clamp method of determining insulin resistance in type 2 DM patients.

Determination of Visceral Fat Area (VFA) and Subcutaneous Fat Area (SFA)

The abdominal fat distribution of the subjects was determined by computed tomography (CT) with the subject in the supine position in accordance with a previously described procedure [17]. SFA and intra-abdominal VFA were measured at the level of the umbilicus, in terms of the CT number, by a standardized method. In brief, a region of interest was defined in the subcutaneous fat layer by tracing its contour on each scan, and the attenuation range for fat

tissue was measured in terms of the CT number (in Hounsfield units).

Pathology

Liver specimens were obtained with an 18-gauge needle biopsy apparatus (Pro-Mag, Medical Device Technologies, Gainsville, FL, USA). The biopsy specimens were stained with hematoxylin–eosin, reticulin, and Masson trichrome stains, and the histopathological findings were scored by two pathologists. Macrovesicular steatosis affecting at least 5% of the hepatocytes was observed in all cases, and the cases were classified as having steatosis or steatohepatitis. In addition to steatosis, the minimum criteria for diagnosis of steatohepatitis included the presence of lobular inflammation and either ballooning of cells or perisinusoidal/pericellular fibrosis in zone three of the hepatic acini [18]. In all cases the severity of fibrosis was scored according to the method of Brunt [19]. The degree of steatosis was assessed as follows, based on the percentage of hepatocytes containing macrovesicular fat droplets: grade 0, no steatosis; grade 1, <33% hepatocytes containing macrovesicular fat droplets; grade 2, 33–66% hepatocytes containing macrovesicular fat droplets; grade 3, >66% hepatocytes containing macrovesicular fat droplets. The severity of fibrosis was expressed on the following four-point scale: 0 = none; 1 = perivenular and/or perisinusoidal fibrosis in zone three; 2 = combined pericellular portal fibrosis; 3 = septal/bridging fibrosis; and 4 = cirrhosis.

Mutation Analyses of the HFE Gene

For the mutation analysis, genomic DNA was isolated from whole peripheral blood with the QIAamp DNA Blood Mini Kit (Qiagen, Chatsworth, CA, USA). DNA fragments were amplified by PCR using the primers [20, 21] 5′-TGGC AAGGGTAAACAGATCC-3′ and 5′-CTCAGGCACTCC TCTAACC-3′ for C282Y (rs1800562) and 5′-ACATGG TTAAGGCCTGTTGC-3′, and 5′-GCCACATCTGGCTTG AAATT-3′ for H63D (rs1799945). Because the DNA changes underlying H63D and S65C (rs1800730) analysis are both located in *HFE* exon 2, primers and PCR conditions previously used to amplify *HFE* exon 2 for H63D analysis were used [22]. Thirty-five amplification cycles were performed, with each cycle consisting of denaturation at 96°C for 30 s, annealing at 56°C for C282Y and 60°C for H63D for 60 s, and extension at 72°C for 60 s. Genotyping for *HFE* S65C was performed using the same conditions as for *HFE* H63D. Restriction fragment length analysis was then performed by digesting the PCR products with *Sna*B1 (for C282Y), *Bcl*I (for H63D), and *Hinf*I (S65C) for 2 h under the buffer and temperature conditions recommended by the manufacture. The digested samples were then electrophoresed on a 10% Poly (NAT) precast gel and visualized by ethidium bromide staining.

Statistical Analysis

Results are expressed as means ± SD, unless otherwise indicated. Statistical analysis was performed with SPSS 12.0 software (SPSS, Chicago, IL, USA). The *t*-test or Wilcoxon rank sum test, as appropriate, was used for univariate comparisons between patient groups. Because many of the variables were not normally distributed, the Kruskal–Wallis test was used for comparisons of more than two independent groups. The diagnostic performance of serum ferritin was assessed by analysis of receiver operating characteristic (ROC) curves. The ROC curve is a plot of sensitivity versus 1 − specificity for all possible cut-off values. The most commonly used index of accuracy is the area under the ROC curve (AUROC), with values close to 1.0 indicating high diagnostic accuracy. Calculations of correlation coefficients and linear regression analysis were used to test for associations between the variables. P values <0.05 were considered significant.

Results

Characteristics of the Patients

The histological findings in the liver biopsy specimens of the subjects with simple steatosis ($n = 24$) and steatohepatitis (NASH) ($n = 62$) are shown in Table 1. The clinical and biochemical characteristics of the NASH patients and simple steatosis patients are shown in Table 2. Marked serum ferritin elevations were observed in the NASH patients in comparison with the patients with simple steatosis ($P = 0.0060$), and healthy control subjects ($P < 0.0001$). Furthermore, there was a significant difference between

Table 1 Histopathological findings in steatohepatitis patients and steatosis patients

	Steatohepatitis (NASH) ($n = 62$)	Steatosis ($n = 24$)
Steatosis grade		
1	40 (65%)	17 (71%)
2	19 (31%)	4 (17%)
3	3 (5%)	3 (13%)
Fibrosis stage		
0	2 (3%)	NA
1	27 (44%)	
2	17 (27%)	
3	14 (24%)	
4	2 (3%)	

Table 2 Clinical and biochemical characteristics of NASH patients and steatosis patients

	Steatosis patients	NASH patients	*P* value
Age (years)	48.0 ± 18.1	51.7 ± 12.7	0.3059
BMI (kg/m^2)	26.8 ± 3.2	28.2 ± 5.2	0.2455
Cell counts of erythrocytes (×10^4/μl)	463.4 ± 45.9	473.4 ± 42.1	0.4159
Hematocrit (%)	41.4 ± 4.0	43.0 ± 3.9	0.1542
Hemoglobin (g/dl)	14.0 ± 1.4	14.4 ± 1.3	0.1235
VFA (cm^2)	118.8 ± 45.6	133.7 ± 51.6	0.3000
SFA (cm^2)	161.5 ± 54.6	219.6 ± 90.8	0.0178*
AST (U/ml)	30.0 ± 11.1	52.7 ± 33.4	0.0031**
ALT (U/ml)	50.4 ± 27.6	83.2 ± 59.7	0.0171*
FBS (mg/dl)	109.8 ± 16.0	123.0 ± 35.7	0.1043
IRI (Ul/ml)	11.7 ± 7.9	14.3 ± 11.5	0.3612
HOMA-IR	3.21 ± 2.30	4.13 ± 3.96	0.3321
HDL cholesterol (mg/l)	51.7 ± 13.9	48.3 ± 11.2	0.2741
LDL cholesterol (mg/l)	117.8 ± 29.8	131.0 ± 35.5	0.1381
Triglyceride (mg/l)	164.2 ± 62.8	167.2 ± 78.6	0.8764
Iron (ng/ml)	118.9 ± 48.8	113.1 ± 37.5	0.6008
Ferritin (ng/ml)	164.9 ± 95.5	278.6 ± 156.3	0.0060**
Hyaluronic acid (ng/dl)	30.9 ± 26.6	51.0 ± 51.5	0.1007
Type IV collagen 7s (ng/dl)	4.21 ± 0.88	4.62 ± 1.04	0.1277

Data are expressed as means ± SD

VFA visceral fat area, *SFA* subcutaneous fat area, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *FBS* fasting blood sugar, *IRI* immunoreactive insulin, *HOMA-IR* homeostasis model assessment of insulin resistance

* $P < 0.05$, ** $P < 0.01$

simple steatosis and healthy control subjects ($P = 0.0053$) (Fig. 1). Significant differences between the NASH patients and patients with simple steatosis were also observed in subcutaneous fat area ($P = 0.0178$), AST ($P = 0.0031$), ALT ($P = 0.0171$), and high-sensitivity CRP values ($P < 0.0001$) (Table 2).

Frequency of HFE Gene Mutations

The frequency of *HFE* gene mutations in the patients with simple steatosis and in the NASH patients is shown in Table 3. Neither C282Y nor S65C hetero or homomutations were observed in either group. Only one patient with NASH had an H63D heteromutation, and none of the patients with simple steatosis had an H63D heteromutation. No H63D homomutations were observed in either group (Table 3).

Relationship Between Serum Ferritin Values and IR

To determine the relationship between serum ferritin levels and IR we assessed the NASH patients for an association

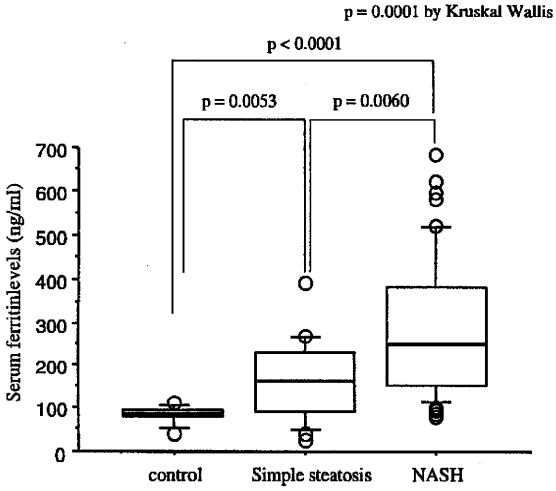

Fig. 1 Serum ferritin levels in healthy control subjects, simple steatosis patients, and NASH patients. Box plots of plasma PTX3 levels showing interquartile range (*box*), median range (*thick line*), full range (*thin lines*), and outliers (*circles*). The length of the box represents the interquartile range within which 50% of the values were located. Serum ferritin levels were significant among the three groups ($P = 0.0001$ by Kruskal–Wallis test.). Marked serum ferritin elevations were observed in the NASH patients in comparison with the patients with simple steatosis ($P = 0.0060$), and healthy control subjects ($P < 0.0001$). A significant difference was further found between simple steatosis and healthy control subjects ($P = 0.0053$)

Table 3 Frequency of *HFE* gene mutations in patients with simple steatosis and NASH patients

Mutation status	Simple steatosis	NASH
C282Y heteromutation	0/20	0/58
C282Y homomutation	0/20	0/58
H63D heteromutation	0/20	1/58
H63D homomutation	0/20	0/58
S65C heteromutation	0/20	0/58
S65C homomutation	0/20	0/58

between HOMA-IR and serum ferritin levels. The results showed no significant correlation between the subjects' serum ferritin levels and fasting blood glucose levels ($r = 0.079$, $P = 0.5150$) (Fig. 2a) or fasting plasma insulin levels ($r = 0.212$, $P = 0.080$) (Fig. 2b); a significant correlation was found between the serum ferritin levels and HOMA-IR ($r = 0.240$, $P = 0.0487$) (Fig. 2c).

Receiver Operating Characteristic (ROC) Curves for Differentiating Between NASH and Simple Steatosis Based on the Serum Ferritin Level

The area under the ROC curve for distinguishing between NASH and steatosis based on serum ferritin levels was 0.732 (Fig. 3). We assessed the diagnostic accuracy of serum

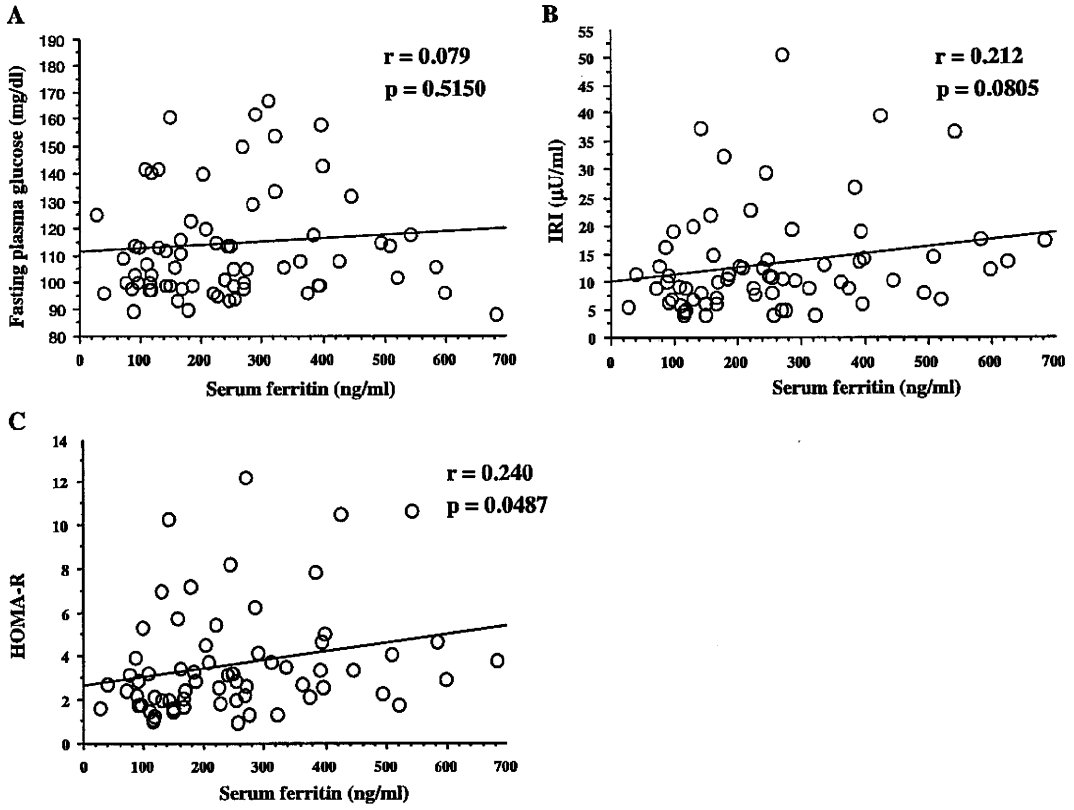

Fig. 2 Serum ferritin levels and insulin resistance. **a** Correlation between serum ferritin concentrations and fasting plasma glucose values ($r = 0.079$, $P = 0.5150$). **b** Correlation between serum ferritin concentrations and IRI ($r = 0.212$, $p = 0.0805$). **c.** Correlation between serum ferritin concentrations and HOMA-IR ($r = 0.240$, $P = 0.0487$)

ferritin levels of the NASH patients and calculated their sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV). The optimal cutoff value was 196 ng/ml ($P = 0.005$; 95% CI, 0.596–0.856), and the results for their sensitivity, specificity, PPV, and NPV were 64.2%, 76.5%, 88.9%, and 43.1%, respectively.

Discussion

The results of this study demonstrated markedly elevated serum ferritin concentrations in Japanese NASH patients in comparison with patients with simple steatosis.

There has been much interest in the role of iron in viral hepatitis during the past few years. Iron is considered a putative element that interacts with oxygen radicals in inducing liver damage and fibrosis [12]. High rates of hyperferritinemia and increased hepatic iron stores have been demonstrated in NASH patients [12], and removal of excess iron by repeated phlebotomy may be of therapeutic benefit for both chronic hepatitis C patients [23] and NASH patients [24]. In steatotic livers, saturation of β-oxidation by excess free fatty acids will ultimately lead to generation of hydrogen peroxide, which in turn can be converted to highly reactive hydroxyl radicals in the presence of free iron [13, 25]. There is strong evidence from in-vitro and in-vivo studies that iron overload enhances oxidative stress [26, 27]. Iron can also promote fibrosis through hepatocellular necrosis (the so-called sideronecrosis) and inflammation with activation of Kupffer cells which release profibrogenic mediators, as a direct fibrogenic promoter acting as a paracrine activator of hepatic stellate cells or as a cofactor in fibrogenesis in conjunction with other hepatotoxins [28]. Hereditary hemochromatosis, an inborn error of iron metabolism, is the most common autosomal recessive disorder of iron metabolism and affects 1 in 250–400 individuals of Northern European descent [29]. Three missense mutations in a gene (designated *HFE*) that are responsible for hereditary hemochromatosis have recently been identified. One results in a substitution of tyrosine for a highly conserved cysteine residue in the *HFE* protein (C282Y) [20]. The role of the second mutation, which results in replacement of the histidine at residue 63 by aspartic acid (H63D) in hereditary hemochromatosis is still a matter of controversy [20, 30]. The third mutation in the *HFE* gene is predominantly localized in the intron

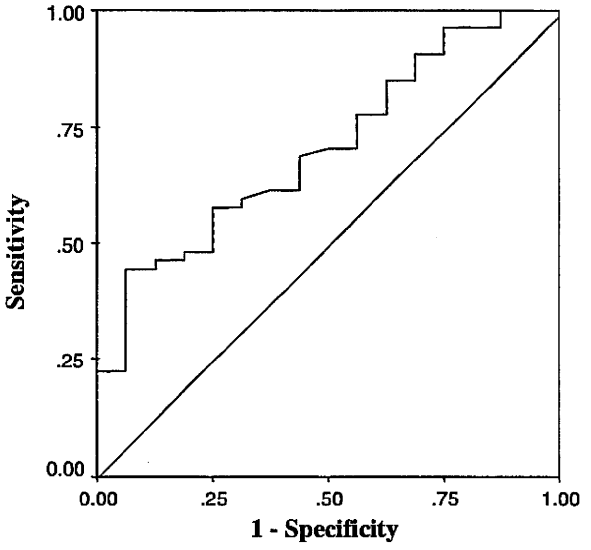

Fig. 3 ROC curve for differentiating simple steatosis and NASH according to serum ferritin levels. The ROC curve is a plot of sensitivity versus 1 − specificity for all possible cutoff values. The most commonly used index of accuracy is the area under the ROC curve (AUROC) for differentiating between steatosis and NASH on the basis of the serum ferritin concentration (area under the curve = 0.732). The optimal cutoff value was 196 ng/ml ($P = 0.005$; 95% CI, 0.596–0.856)

sequences except for one, namely 193A → T, which leads to a serine-to-cysteine substitution (S65C) localized in exon 2 in the vicinity of H63D [31]. Hepatic iron overload thought to be associated with *HFE* gene mutations [10, 11]. A significantly higher prevalence of *HFE* mutations in NASH patients has been reported as a factor responsible for liver fibrosis by increasing hepatic iron deposition [14, 32], but recent studies have failed to confirm this [33–35]. In our study the prevalence of hyperferritinemia was significantly higher in the NASH patients than in patients with simple steatosis. Our results were consistent with a previous report indicating low frequencies of *HFE* mutations in the Japanese population (C282Y, 0%; H63D, 0.99%; S65C unknown) [36], in that none of the patients in this study had *HFE* mutations. Because *HFE* gene mutations have been found almost exclusively in Caucasians of North-European descent, it is not an important contributor to NASH in other populations, including the Japanese population. Thus, hyperferritinemia and hepatic iron deposition seem to be characteristic conditions in NASH, but *HFE* mutations are not associated with the pathogenesis of NASH, at least in Japanese patients.

The mechanisms leading to the elevation of iron indices in NASH patients remain unknown. In recent years there has been growing interest in understanding the mechanisms leading to the accumulation of excessive fat in the liver of obese type 2 DM patients and in the role it might play in the pathogenesis of IR [37]. Experimental and clinical data have demonstrated that mild iron overload contributes to IR [15, 38, 39]. Insulin resistance (IR) is considered an essential requirement for the development of NASH [40] and has been reported to cause hepatic iron overload [15]. We therefore assessed the relationship between IR and both the homeostasis model assessment (HOMA-IR) and serum ferritin concentration in Japanese NASH patients. The results revealed a significant correlation between the serum ferritin concentrations and HOMA-IR.

We then investigated the diagnostic utility of measuring serum ferritin concentrations to distinguish NASH patients from simple steatosis patients. The area under the curve for serum ferritin was 0.732, and its specificity and PPV were high, indicating that high serum ferritin concentrations are a clinical biomarker for NASH in Japanese patients. These findings indicated that measurement of serum ferritin concentrations is of clinical value, because serum ferritin concentration determinations have become routine clinical laboratory tests. However, their sensitivity and NPV were not high enough to rule out normal or low serum ferritin concentrations in NASH. Thus, it seems that iron contributes to the pathogenesis of NASH only in individuals with excess serum and hepatic iron levels. Other factors may play a part in the pathogenesis of NASH in patients with no abnormal serum and hepatic iron values.

In conclusion, we investigated the clinical utility of the measurement of serum ferritin concentrations to distinguish NASH from simple steatosis. Hyperferritinemia may be a distinctive feature in NASH when compared with simple steatosis, but not *HFE* gene mutations, but IR was considered to influence the development of hyperferritinemia in Japanese NASH patients.

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