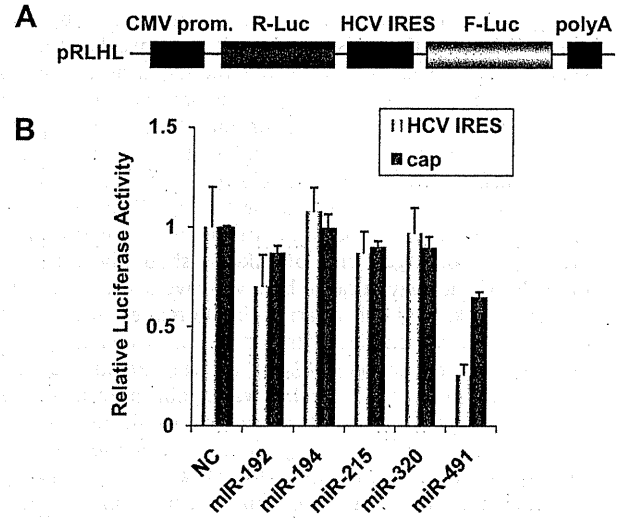

Fig. 2. Regulation of HCV IRES and cap-dependent translation by miRNAs. Huh-RepSI cells were transfected with a dicistronic vector, pRLHL (A), incubated for 24 h. The cells were seeded to 24-well plates and transfected with the miRNAs. After further incubation for 2 days, the cells were harvested and assayed for dual luciferase activity (B).

transfection (Fig. 2B). In this assay, activation of IRES was determined by the ratio of IRES-dependent luciferase activity to cap-dependent luciferase activity. Interestingly, none of the miRNAs could increase the HCV IRES activity. miR-491 suppressed cap-dependent translation and showed more suppression of HCV IRES activity. Thus, these results indicated that there was some mechanism upregulating HCV replication other than regulation of IRES activity.

Previous work demonstrated that HCV replication was affected by cell proliferation [14]. This led us to access the effects of the miRNAs on cell proliferation. Compared to negative control miRNA-transfected cells, however, none of the transfectants of the miRNAs, including those which increased HCV replication, revealed upregulation of cell proliferation, and miR-491 even suppressed it (Fig. 3). Therefore, regulation of cell proliferation was not the reason for the increase of HCV replication. The effect of miR-491 of suppressing cell growth was likely to be caused by inhibition of general translation as shown in Fig. 2B.

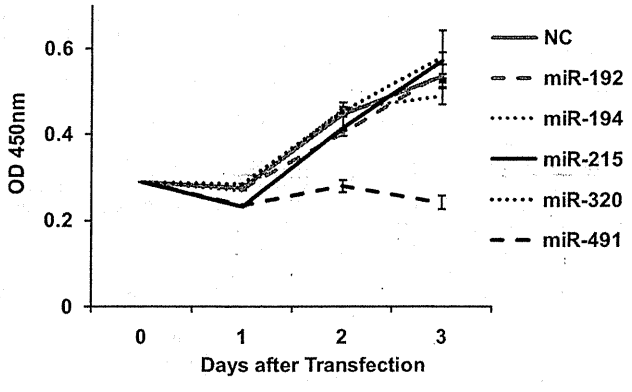

Fig. 3. Regulation of cell proliferation by miRNAs. Huh7 cells were seeded into 96-well plates, transfected with the miRNAs. At day 0, 1, 2, and 3 after transfection, the cells were subjected to WST-1 assay as described in Section 2.

3.4. Effect of miRNAs on intracellular signaling

To clarify the mechanism of the regulation of HCV replication, we next focused our investigation on intracellular signaling pathways. Previous studies have reported that HCV replication is regulated by intracellular signaling pathways, such as ERK [15], p38 [8], PI3 kinase/Akt [11], and smad [16], in addition to JAK/STAT. Since transfection of the miRNAs had no effect on the JAK/STAT signaling pathway (data not shown), we examined the phosphorylation of ERK and Akt. Because both showed a suppressing effect on HCV replication, suppression of the pathway was anticipated in cells in which HCV replication was enhanced. As shown in Fig. 4A, phosphorylation of Akt at Ser-473 was markedly suppressed in the cells transfected with miR-491, while no significant inhibition of ERK activity was observed. To further investigate the relevance of the PI3 kinase/Akt pathway to miR-491-induced upregulation of HCV replication, we used LY294002, a PI3 kinase inhibitor. When the PI3 kinase pathway was blocked by this reagent, the HCV RNA level was enhanced up to 2-fold. miR-491 transfection also resulted in an increase of HCV abundance, though the effect was less

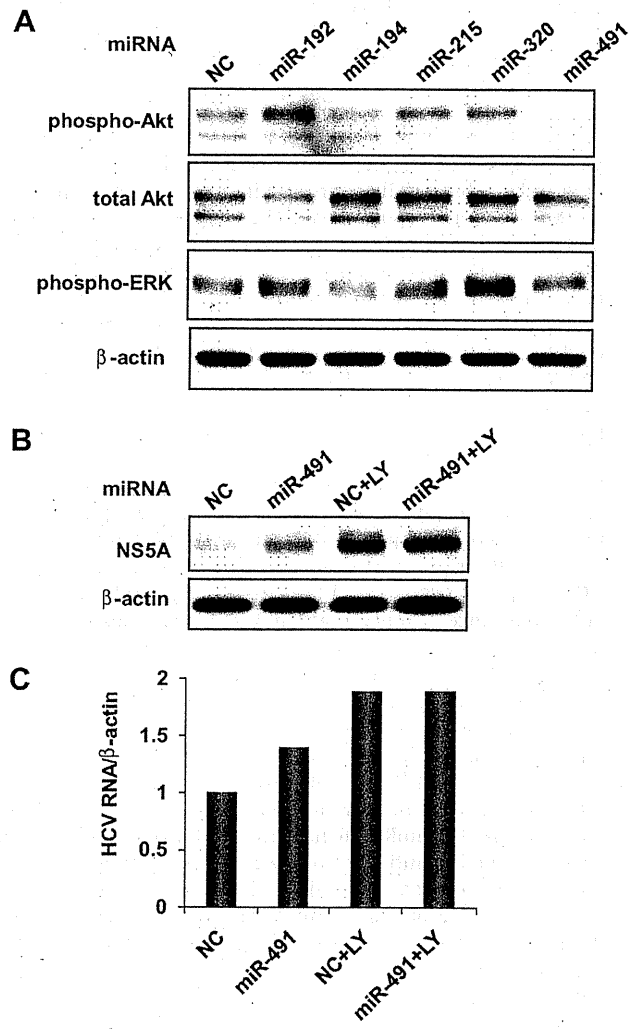

Fig. 4. Involvement of Akt suppression in miR-491-mediated upregulation of HCV replication. (A) Immunoblot analysis of miRNA-transfected HCV replicon cells using antibodies to Akt, phospho-Akt, phospho-ERK and β-actin. (B and C) HCV replicon cells were transfected with miR-491 or treated with Akt inhibitor, and assayed for NS5A protein abundance (B) or HCV RNA abundance (C). LY: LY294002.

than that of LY294002, presumably because of incomplete inhibition of Akt. When miR-491 transfected cells were cultured in the presence of LY294002, the HCV replication level was enhanced to the same extent as that in the LY294002-treated cells with negative control miRNA. Since no additive effect of miR-491 was observed under strong blockade of the PI3 kinase/Akt pathway, inhibition of this pathway was likely to be responsible for the miR-491-induced upregulation of HCV replication.

4. Discussion

In the present study, we tried to identify the miRNA(s) affected by HCV infection and establish how they influence HCV replication. Five miRNAs, miR-192, miR-194, miR-215, miR-320, and miR-491, were identified as HCV-regulated miRNAs by miRNA array analysis. Three upregulated miRNAs, miR-192, miR-194, and miR-215, were previously identified as p53-inducible miRNAs [12,13]. Two miRNA clusters which encode identical miR-194 sequences (i.e., the miR-194-2/miR-192 cluster on chromosome 11 and the miR-194-1/miR-215 cluster on chromosome 1) contain two closely related miRNAs, miR-192 and miR-215, suggesting that their expressions are regulated similarly which led to their simultaneous identification. miR-192/miR-194/miR-215 are known to act as tumor-suppressing miRNAs by inducing cell cycle arrest [12]. In Huh7 cells, however, the p53 function is believed to be abolished by a point mutation at codon 220. Therefore, the upregulation of miR-192/miR-194/miR-215 was likely to be exerted in a p53-independent manner. Since miR-192 and miR-194 are considered to be substantially expressed in human liver tissue [17] and there are several reports about the suppression of p53 function by HCV (reviewed in Ref. [18]), the result may not necessarily be the same if the investigation is conducted in human hepatocytes or in cells with intact p53 activity.

The downregulated miRNAs, miR-320 and miR-491, are considered to be relevant to carcinogenesis. miR-320 induces G1 arrest and suppresses cell proliferation by targeting CDK6 [19], CD71 [20], IGF1 [21] and induces apoptosis by suppressing Bcl-2 and Mcl-1 [22]. miR-491 is also capable of inducing apoptosis by targeting Bcl-xL [23], which is often upregulated in HCC tissues [24]. In this study, we showed that miR-491 inhibited the PI3 kinase/Akt pathway, which is one of the important pathways leading to cancerous properties. Importantly, miR-320 was identified as one of the significantly repressed miRNAs in CH-B, CH-C, and HCC compared with normal liver tissue [25]. Although the details of the relevance of miR320 and miR-491 to hepatocarcinogenesis have not yet been clarified, as these two miRNAs have a tendency to suppress genes related to carcinogenesis, their downregulation in HCV-infected cells may play some role in hepatocarcinogenesis.

Thus far, several miRNAs have been reported to regulate HCV replication. miR-122 was shown to be a direct activating factor for HCV replication [5], but alteration of this miRNA was not observed in response to HCV infection in this study. IFN-β-induced miRNAs, miR-196, miR-296, miR-351, miR-431 and miR-448, have been identified as anti-HCV miRNAs [6]. These miRNAs are able to regulate HCV replication by direct interaction with HCV genome RNA. In the case of miR-192/miR-215, there are several sites in the HCV genome sequence which show weak homology to the miRNAs (data not shown). Although the possibility of miR-192/miR-215 binding to the HCV genome and regulating replication cannot completely be excluded, this seems unlikely because the homologous sequence to miR-192/miR-215 cannot be found in the UTR region like miR-122 and direct binding to RNA usually suppresses the RNA function for protein synthesis. There is, however, a very rare case of miR-122-mediated facilitation of HCV replication by binding to two sites within the HCV genome.

Although the mechanism of miR-491-mediated suppression of the PI3 kinase pathway is not clear, it was speculated that some gene involved in Akt activation was the target of miR-491. However, the candidate of the target gene was not clearly found in the list of putative target genes of miR-491 revealed by in silico analysis. We tried to evaluate the mRNA levels of upstream genes of Akt, such as the genes which belong to the family of PI3 kinase, PTEN, growth factor receptors, using the RT-PCR method, but none of them was affected by miR-491 (data not shown). Nevertheless, investigation of target genes of miR-491 should be of interest for the field of oncology because here we have shown that miR-491 suppresses Akt, which is a factor closely related to various types of cancer via cell survival. Also, it has been demonstrated that miR-491 can induce apoptosis by ablating Bcl-xL [23]. Indeed, our observation that cell viability was significantly suppressed by forced expression of miR-491 presumably via decrease of Akt signaling suggests the anti-oncogenic feature of miR-491. Further study of the mechanism of miR-491, its target genes, and expression pattern in cancer tissue remain to be performed.

In conclusion, we showed altered expression profiles of miRNAs by HCV infection, and some of them were capable of regulating HCV replication, which may represent a complicated mechanism of HCV replication. A number of studies have demonstrated regulation of many cellular factors by miRNAs, which results in modulation of cellular functions including cell growth, apoptosis, cellular stresses, metabolism, and carcinogenesis. The miRNAs identified in this study may also be involved in changes in the phenotype of HCV-infected cells.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Efficacy of pegylated interferon plus ribavirin combination therapy for hepatitis C patients with normal ALT levels: a matched case–control study

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Abstract
Background The antiviral effect of pegylated interferon (Peg-IFN) plus ribavirin combination therapy in chronic hepatitis C (CHC) patients with normal alanine aminotransferase (ALT) levels (N-ALT) has been reported to be equivalent to that for patients with elevated ALT levels (E-ALT). However, the actual antiviral effect in N-ALT patients remains obscure because efficacy can be overestimated in patients with an advantageous background.
Methods In this study, 386 patients were extracted, for a matched case–control study, from 1320 CHC patients treated with Peg-IFN alpha-2b plus ribavirin combination therapy; 193 N-ALT patients [116 with hepatitis C virus genotype 1 (HCV-1), 77 with HCV genotype 2 (HCV-2)] were matched with 193 E-ALT patients by a propensity score method using the variables of age, sex, IFN treatment history, body mass index, and platelet counts.
Results On multivariate analysis for sustained virological response (SVR) in N-ALT patients, younger age, low HCV RNA level at baseline, and HCV-2 were significant factors. The matched case–control study showed that the SVR rates of N-ALT patients were equivalent to those of E-ALT patients; at 49 and 40% in the HCV-1 group ($P = 0.146$), and 78 and 81% in the HCV-2 group ($P = 0.691$). However, in N-ALT patients with non-SVR, approximately 40% showed ALT elevation at 24 weeks post-treatment.
Conclusion Our findings indicate that the antiviral effect of Peg-IFN plus ribavirin therapy in N-ALT patients is comparable to that for E-ALT patients irrespective of their advantageous background; however, the application of this therapy for N-ALT patients, especially for those with HCV-1, should be considered carefully.

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Keywords Hepatitis C virus · Normal alanine aminotransferase · Pegylated interferon plus ribavirin combination therapy · Propensity score method · Matched case–control study

Introduction

In patients with hepatitis C virus (HCV) infection, alanine aminotransferase (ALT) levels fluctuate and sometimes biochemical remission is maintained. Approximately 20% of patients with normal ALT levels (N-ALT) show ALT elevation and fibrosis progression within 3–5 years [1–5], and consequently, 70–80% of N-ALT patients have mild to moderate fibrosis on liver biopsy. N-ALT patients have been excluded from conventional interferon (IFN) therapy, because their sustained virological response (SVR) rates on conventional IFN monotherapy have been reported to be only 6–15% [6–9], and ALT levels were noted to increase during or after treatment in 47–62% of the patients. The incidence of ALT flares has raised concerns regarding the risk of conventional IFN therapy compared with a small benefit. However, a large randomized controlled trial has demonstrated that combination therapy with pegylated interferon (Peg-IFN) and ribavirin produced SVR rates in N-ALT patients with chronic hepatitis C (CHC) that were comparable to those of patients with elevated ALT levels (E-ALT) [10]. Thus, such treatment is now being considered for N-ALT patients with CHC [11].

Comparison of the characteristics of N-ALT and E-ALT patients has shown that the mean age of N-ALT patients was lower than that of E-ALT patients, and females and HCV genotype 2 patients were predominant among N-ALT patients [4, 7, 12–17]. In the American Association for the Study of Liver Disease guideline, the pretreatment predictors of achieving SVR with Peg-IFN plus ribavirin combination therapy for CHC patients are HCV genotype 2 or 3 infection, low viral load (<600 KIU/ml), female gender, and age less than 40 years [11]. Considering these characteristics, N-ALT patients with CHC can be said to have an advantageous background, and their response to antiviral therapy, including Peg-IFN plus ribavirin combination therapy, can be overestimated. Therefore, patient background, especially factors affecting the treatment efficacy of the combination therapy, needs to be matched between study groups in order to compare the treatment efficacies in N-ALT patients with CHC and E-ALT patients with CHC accurately. In this study, we evaluated, by a matched case–control study approach, whether the antiviral efficacy in N-ALT patients with CHC, reported to be equal to that in E-ALT patients with CHC, could be obtained without their advantageous background, and whether the factors contributing to SVR in N-ALT patients were the same as those in E-ALT patients. In addition, ALT flares after treatment in N-ALT patients without SVR were examined.

Patients and methods

Patient selection and study design

The subjects were 1320 consecutive CHC patients, 1015 with HCV genotype 1 (HCV-1) and 305 with HCV genotype 2 (HCV-2) who had undergone combination therapy with Peg-IFN alpha-2b (PEGINTRON; Schering-Plough, Kenilworth, NJ, USA) and ribavirin (REBETOL; Schering-Plough) at standard doses for 48 weeks (patients with HCV-1) or for 24 weeks (patients with HCV-2) at 30 medical institutions participating in the Osaka Liver Forum between December 2004 and December 2007. Peg-IFN alpha-2b and ribavirin dosages were based on body weight according to the manufacturer's instructions and were modified based on the manufacturer's instructions according to the severity of adverse hematologic effects. In the 1 month preceding treatment, none of the patients had received any IFN formulations or other types of drugs for liver supporting therapy. Before starting treatment, all patients had positive anti-HCV and a detectable level of HCV RNA according to a polymerase chain reaction (PCR)-based assay (COBAS Amplicor HCV Monitor Test v2.0; Roche Diagnostics, Branchburg, NJ, USA). None of the patients showed evidence of dual infection with hepatitis B virus or human immunodeficiency virus, or other forms of liver diseases such as alcoholic liver disorder, autoimmune hepatitis, or drug-induced liver injury.

In this study, a normal serum ALT level was defined as ALT $\leq$30 IU/l at the start of the combination therapy, as, in the guidelines for treatment of hepatitis C in N-ALT patients in Japan, ALT levels of $\leq$30 IU/l are regarded as an indicator of no or little inflammation in the liver, and patients whose ALT levels are $\leq$30 IU/l are recommended to be followed without antiviral therapy, especially if the platelet count is $\geq 15 \times 10^4/mm^3$.

Among the 1320 consecutive CHC patients, the antiviral effect in 193 N-ALT patients (116 with HCV-1, 77 with HCV-2) was compared with that in 193 E-ALT patients (116 with HCV-1, 77 with HCV-2) who were matched by a propensity score method based on age, sex, IFN treatment history, body mass index (BMI), and platelet counts. BMI was calculated as weight (kg)/[height (m)]^2.

HCV RNA was determined at week 4, week 12, end of treatment (EOT), and 24 weeks after EOT. HCV RNA was also determined at week 24 for HCV-1 patients. HCV RNA was monitored by the PCR Amplicor method with a detection limit of 50 IU/ml. (COBAS Amplicor HCV v2.0;

Roche Diagnostics). Complete early virological response (cEVR) and end-of-treatment response (ETR) were defined as undetectable HCV RNA at week 12 and EOT, respectively.

Written informed consent was obtained from each patient, and the study protocol was reviewed and approved according to the ethical guidelines of the 2004 Declaration of Helsinki by institutional review boards at the respective sites.

Propensity score

Propensity score methods are used to create balanced covariates and reduce selection bias in a matched case–control study. Propensity scores were calculated using a multivariate logistic regression model that had ALT levels as a dependent variable and other covariates as independent variables, and the model was utilized for matching between the N-ALT patients with CHC (the case group) and the E-ALT patients with CHC (the control group). Data analyses were conducted using SAS, version 9.2 (SAS Institute, Cary, NC, USA).

Statistical analysis

Continuous variables are reported as the mean with standard deviation (SD) or median levels, while categorical variables are shown as the count and proportion. Statistical significance was assessed by Student's t test (mean), the Mann–Whitney U test (median), and the χ^2 test for independent samples, and the paired t test for paired samples. For all tests, two-sided P values were calculated, and the results were considered statistically significant if $P < 0.05$. Variables that achieved statistical significance on univariate analysis were subjected to multivariate logistic regression analysis. Stepwise and multivariate logistic regression models were used to explore the independent factors that could be used to predict SVR. Statistical analysis was performed using the SPSS program for Windows, version 15.0 J (SPSS, Chicago, IL, USA).

Results

Baseline characteristics of all CHC patients according to HCV genotype and ALT levels before matching

The baseline characteristics of 1320 patients at the commencement of combination therapy with Peg-IFN and ribavirin are shown in Table 1, according to HCV genotype and ALT levels before matching. Of the 116 N-ALT patients with HCV-1 there were 36 males and 80 females (69%), with a mean age of 54 ± 11 years. Eighty-five (73%) were IFN-naïve. In terms of liver histology, 66 (73%) patients had

Table 1 Demographic characteristics of patients with normal ALT and patients with elevated ALT

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 899$)	P value	Normal ALT ($n = 77$)	Elevated ALT ($n = 228$)	P value
Sex: male/female	36/80	512/387	<0.001	32/45	121/107	0.081
Age (years)	54 ± 11	56 ± 10	0.136	51 ± 13	52 ± 13	0.423
Body mass index (kg/m^2)	22.9 ± 3.1	23.3 ± 3.2	0.131	23.0 ± 2.9	23.3 ± 3.2	0.424
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	547/352 (131/154)	0.011	58/19 (9/4)	175/53 (21/10)	0.876
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	296/338	<0.001	43/6	69/94	<0.001
Fibrosis: 0–1/2–4	67/24	330/304	<0.001	42/7	101/62	0.002
HCV RNA (KIU/ml)[c]	1800	1700	0.793	2200	1100	<0.001
White blood cell (/mm^3)	5220 ± 1507	5137 ± 1582	0.595	5538 ± 1687	5338 ± 1725	0.377
Neutrophil (/mm^3)	2770 ± 1074	2595 ± 1078	0.108	3017 ± 1180	2688 ± 1230	0.047
Hemoglobin (g/dl)	13.6 ± 1.5	14.2 ± 1.4	<0.001	13.8 ± 1.6	14.2 ± 1.4	0.071
Platelet (×10^4/mm^3)	19.9 ± 5.7	16.2 ± 5.3	<0.001	20.5 ± 4.5	17.8 ± 5.8	<0.001
ALT (IU/l)	24 ± 5	88 ± 62	<0.001	22 ± 5	97 ± 67	<0.001

ALT alanine aminotransferase, *HCV* hepatitis C virus, *IFN* interferon

[a] Status was unknown in 8 patients in the normal ALT group and 67 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 22 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 265 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and in 65 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

mild activity (activity, 0–1) and 67 (74%) had mild fibrosis (fibrosis, 0–1) by the METAVIR system. Mean white blood cell counts, hemoglobin levels, and platelet counts were 5220 ± 1570 /mm^3, 13.6 ± 1.5 g/dl, and 19.9 ± 5.7 × 10^4/mm^3. In 899 E-ALT patients compared to N-ALT patients, the proportions of female and IFN-naïve patients were significantly lower, at 43% ($P < 0.001$) and 61% ($P = 0.011$), respectively. Higher scores for activity ($P < 0.001$) and fibrosis ($P < 0.001$) were observed in E-ALT patients. E-ALT patients had higher hemoglobin levels and lower platelet counts than N-ALT patients, at 14.2 ± 1.5 g/dl ($P < 0.001$) and 16.2 ± 5.3 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 24 ± 5 IU/l in N-ALT patients and 88 ± 62 IU/l in E-ALT patients ($P < 0.001$).

Of the 77 N-ALT patients with HCV-2, 32 were males and 45, females (58%). Their mean age was 51 ± 13 years and 58 (75%) were IFN-naïve. In terms of liver histology, 43 (88%) patients had mild activity (activity, 0–1) and 42 (86%) had mild fibrosis (fibrosis, 0–1). Compared to the 228 E-ALT patients, the N-ALT patients had higher HCV RNA levels (median 2200 vs. 1100 KIU/ml, $P < 0.001$). Higher scores for activity ($P < 0.001$) and fibrosis ($P = 0.002$) were observed in E-ALT patients. Neutrophils and platelet counts in N-ALT patients were higher than those in E-ALT patients, at 3017 ± 1180 versus 2688 ± 1230 /mm^3 ($P = 0.047$) and 20.5 ± 4.5 × 10^4 versus 17.8 ± 5.8 × 10^4/mm^3 ($P < 0.001$), respectively. Mean ALT levels were 22 ± 5 IU/l in N-ALT patients and 97 ± 67 IU/l in E-ALT patients ($P < 0.001$).

Prognostic factors for SVR in the N-ALT patients

For all N-ALT patients (HCV-1, 116; HCV-2, 77), univariate analysis for factors associated with achieving SVR was performed for the following variables: sex, age, BMI, history of past IFN therapy, histology, baseline HCV RNA level, HCV genotype, white blood cell count, neutrophil count, hemoglobin level, platelet count, and ALT level (Table 2). The results indicated that age, fibrosis, baseline HCV RNA level, and HCV genotype contributed to SVR. Next, multivariate logistic regression analysis was performed for all N-ALT patients ($n = 193$), using these factors except for fibrosis, as there were many missing samples. The multivariate analysis showed that younger age [by 10-year increase: odds ratio (OR) 0.552; 95% confidence interval (CI) 0.404–0.756; $P < 0.001$] and lower baseline HCV RNA level (by 100-KIU/ml increase: OR 0.976; 95% CI 0.954–0.998; $P = 0.037$), as well as HCV genotype (genotype 2 vs. genotype 1: OR 3.724; 95% CI 1.859–7.463; $P < 0.001$) were independently associated with SVR (Table 3).

Table 2 Factors associated with SVR in patients with normal ALT—univariate analysis

Factor	SVR ($n = 117$)	Non-SVR ($n = 76$)	P value
Sex: male/female	43/74	25/51	0.645
Age (years)	50 ± 13	57 ± 9	<0.001
Body mass index (kg/m^2)	22.8 ± 3.3	23.1 ± 2.6	0.511
Past IFN therapy: naïve/experienced	88/29	55/21	0.737
Histology (METAVIR)[a]			
Activity: 0–1/2–3	67/14	42/17	0.148
Fibrosis: 0–1/2–4	69/12	40/19	0.022
HCV genotype: 1/2	57/60	59/17	<0.001
HCV RNA (KIU/ml)[b]	1700	2100	0.040
White blood cell (/mm^3)	5461 ± 1426	5170 ± 1798	0.213
Neutrophil (/mm^3)	2968 ± 1167	2709 ± 1032	0.126
Hemoglobin (g/dl)	13.7 ± 1.4	13.7 ± 1.6	0.970
Platelet (×10^4/mm^3)	20.4 ± 4.8	19.8 ± 5.8	0.388
ALT (IU/l)	23 ± 5	24 ± 5	0.384

SVR sustained virological response, *ALT* alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data missing in 36 patients in the SVR group and in 17 in the non-SVR group

[b] Values are expressed as medians

Table 3 Factors associated with SVR in patients with normal ALT—multivariate analysis

Factor	Category	Odds ratio	95% CI	P value
Age	By 10 years	0.552	0.404–0.756	<0.001
HCV genotype	1/2	3.724	1.859–7.463	<0.001
HCV RNA	By 100 KIU/ml	0.976	0.954–0.998	0.037

The number of patients used for this multivariate analysis was 193 (SVR, $n = 117$; non-SVR, $n = 76$)

SVR sustained virological response, *ALT* alanine aminotransferase, *CI* confidence interval, *HCV* hepatitis C virus

Comparison of patient characteristics between patients with normal ALT and those with elevated ALT matched by a propensity score method

The baseline characteristics of CHC patients matched by a propensity score method at the commencement of combination therapy with Peg-IFN and ribavirin were compared between N-ALT patients and E-ALT patients (see Table 4). There were 116 CHC patients with HCV-1 in each of the groups of N-ALT and E-ALT patients. The two groups were well matched by propensity score methods and there was no significant difference, except in ALT values (mean value, N-ALT, 24 ± 5 IU/l vs. E-ALT, 78 ± 53 IU/l, $P < 0.001$). Similarly, with CHC patients with HCV-2, there were no significant differences, except for ALT levels (mean value, N-ALT, 22 ± 5 IU/l vs. E-ALT, 80 ± 58 IU/l, $P < 0.001$), activity scores [0–1, N-ALT, 88% (43/49) vs. E-ALT, 49% (25/51), $P < 0.001$], and HCV RNA levels (median value, N-ALT, 2200 KIU/ml vs. E-ALT, 1000 KIU/ml, $P < 0.001$).

Treatment efficacy of combination therapy with Peg-IFN and ribavirin in CHC patients

Antiviral effects of the combination therapy with Peg-IFN and ribavirin were evaluated by rapid virological response (RVR), cEVR, ETR, SVR, and relapse rates, as shown in Table 5. Among patients with HCV-1 in the N-ALT and E-ALT patients, respectively, RVR rates were 6% (6/98) and 6% (6/102), cEVR rates were 53% (62/116) and 43% (50/116), and ETR rates were 72% (84/116) and 58% (67/116) ($P = 0.019$). SVR and relapse rates in N-ALT patients were 49% (57/116) and 32% (27/84). These rates in E-ALT patients were 40% (46/116) and 31% (21/67). In the patients with HCV-2, RVR, cEVR, ETR, SVR, and relapse rates were 68% (41/60), 90% (69/77), 96% (74/77), 78% (60/77), and 19% (14/74) for N-ALT patients, and 62% (36/58), 91% (70/77), 91% (70/77), 81% (62/77), and 11% (8/70) for E-ALT patients, respectively. Comparisons between N-ALT and E-ALT patients with HCV-1 or HCV-2 showed no significant differences in RVR, cEVR, ETR, SVR, and relapse rates, except in ETR rates in patients with HCV-1.

Changes in ALT levels during combination therapy and follow-up periods in N-ALT patients with SVR and those with non-SVR

Changes in ALT levels in N-ALT patients during the combination therapy and follow-up periods were evaluated according to the treatment response (Fig. 1). In patients with HCV-1, the mean baseline ALT level in the SVR

Table 4 Comparison of characteristics between patients with normal ALT and patients with elevated ALT matched by a propensity score method

	HCV genotype 1			HCV genotype 2		
	Normal ALT ($n = 116$)	Elevated ALT ($n = 116$)	P value	Normal ALT ($n = 77$)	Elevated ALT ($n = 77$)	P value
Sex: male/female	36/80	32/84	0.564	32/45	30/47	0.742
Age (years)	54 ± 11	55 ± 11	0.746	51 ± 13	50 ± 13	0.742
Body mass index (kg/m^2)	22.9 ± 3.1	22.6 ± 2.9	0.536	23.0 ± 2.9	22.8 ± 2.9	0.780
Past IFN therapy: naïve/experienced (relapser/non-responder)[a]	85/31 (18/5)	80/36 (13/18)	0.469	58/19 (9/4)	57/20 (7/4)	0.853
Histology (METAVIR)[b]						
Activity: 0–1/2–3	66/25	49/35	0.056	43/6	25/26	<0.001
Fibrosis: 0–1/2–4	67/24	59/25	0.736	42/7	36/15	0.068
HCV RNA (KIU/ml)[c]	1800	1700	0.896	2200	1000	<0.001
White blood cell (/mm^3)	5220 ± 1507	5329 ± 1626	0.569	5538 ± 1687	5530 ± 1780	0.977
Neutrophil (/mm^3)	2770 ± 1074	2702 ± 1094	0.641	3017 ± 1180	2755 ± 1189	0.189
Hemoglobin (g/dl)	13.6 ± 1.5	13.7 ± 1.4	0.542	13.8 ± 1.6	14.0 ± 1.4	0.592
Platelet (×10^4/mm^3)	19.9 ± 5.7	19.4 ± 7.1	0.562	20.5 ± 4.5	20.6 ± 5.5	0.911
ALT (IU/l)	24 ± 5	78 ± 53	<0.001	22 ± 5	80 ± 58	<0.001

ALT alanine aminotransferase, *IFN* interferon, *HCV* hepatitis C virus

[a] Data unknown in 8 patients in the normal ALT group and in 5 in the elevated ALT group with HCV genotype 1; and in 6 in the normal ALT group and 9 in the elevated ALT group with HCV genotype 2

[b] Data missing in 25 patients in the normal ALT group and in 32 in the elevated ALT group with HCV genotype 1; and in 28 in the normal ALT group and 26 in the elevated ALT group with HCV genotype 2

[c] Values are expressed as medians

Table 5 Antiviral effect for patients with normal ALT and those with elevated ALT according to HCV genotype

		Normal ALT	Elevated ALT	*P* value
HCV genotype 1		$n = 116$	$n = 116$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[a]	6% (6/98)	6% (6/102)	1.000
	At week 12 (cEVR)	53% (62/116)	43% (50/116)	0.287
	At week 48 (ETR)	72% (84/116)	58% (67/116)	0.019
	Post-24 weeks (SVR)	49% (57/116)	40% (46/116)	0.146
	Relapse rate	32% (27/84)	31% (21/67)	0.916
HCV genotype 2		$n = 77$	$n = 77$	
	Undetectable HCV RNA rate			
	At week 4 (RVR)[b]	68% (41/60)	62% (36/58)	0.563
	At week 12 (cEVR)	90% (69/77)	91% (70/77)	0.723
	At week 24 (ETR)	96% (74/77)	91% (70/77)	0.191
	Post-24 weeks (SVR)	78% (60/77)	81% (62/77)	0.691
	Relapse rate	19% (14/74)	11% (8/70)	0.212

ALT alanine aminotransferase, *HCV* hepatitis C virus, *RVR* rapid virological response, *cEVR* complete early virological response, *ETR* end-of-treatment response, *SVR* sustained virological response

[a] Data missing in 18 patients in the normal ALT group and in 14 in the elevated ALT group with HCV genotype 1

[b] Data missing in 17 patients in the normal ALT group and in 19 in the elevated ALT group with HCV genotype 2

group ($n = 57$) was similar to that in the non-SVR group ($n = 59$) (mean ± standard error of the mean (SEM): SVR group, 24.5 ± 0.6 IU/l; non-SVR group, 24.2 ± 0.7 IU/l; $P = 0.694$). Transitions of ALT levels were not significantly different between SVR and non-SVR groups during the therapy. However, in the SVR group, the ALT level fell to 15.1 ± 0.7 IU/l at 24 weeks after treatment completion ($P < 0.001$, compared to the baseline level), while in the non-SVR group, higher ALT levels were observed after treatment compared to the baseline level; the ALT level rose to the peak value of 36.2 ± 3.6 IU/l at post-12 weeks ($P = 0.001$), and slightly fell to 31.3 ± 2.6 IU/l at post-24 weeks ($P = 0.007$) (Fig. 1a). In comparison with the SVR group, the non-SVR group showed significant differences in mean ALT levels at post-4, -12, and -24 weeks ($P = 0.002$, <0.001, and <0.001, respectively). At post-48 weeks in the non-SVR group, the ALT level was 30.4 ± 2.9 IU/l, which was still higher than the baseline level ($P = 0.025$).

Similarly, in patients with HCV-2, baseline ALT levels in the SVR group ($n = 60$) and the non-SVR group ($n = 17$) were equivalent (mean ± SEM; SVR, 21.8 ± 0.7 IU/l; non-SVR, 22.5 ± 1.1 IU/l; $P = 0.622$), and there was no significant difference in transitions of the ALT levels during therapy. However, after treatment, in the non-SVR group, ALT levels tended to rise in comparison with those at baseline; they rose to 74.9 ± 26.9 IU/l at post-12 weeks ($P = 0.068$) and fell to 35.7 ± 10.2 IU/l at post-24 weeks ($P = 0.196$). On the other hand, in the SVR group, ALT levels fell significantly, to 16.4 ± 1.3 IU/l at post-12 weeks ($P < 0.001$) and 15.2 ± 1.2 IU/l at post-24 weeks ($P < 0.001$) (Fig. 1b). Comparison of ALT levels between the SVR and non-SVR groups after treatment showed that mean ALT levels in the non-SVR group tended to be high at post-4, -12 and, -24 weeks ($P = 0.045$, 0.051, and 0.066, respectively). At post-48 weeks in the non-SVR group, the ALT level was 32.4 ± 8.9 IU/l, which tended to be high compared with the baseline ALT level, although no significant difference was found ($P = 0.248$).

Next, the ALT levels in N-ALT patients were examined according to the treatment response at 24 weeks after completion of the combination therapy. In HCV-1 patients with SVR, ALT levels remained below the upper limit of normal (ULN) for this study (<30 IU/l) in 55 (98%) patients, and ALT elevation <2 × ULN occurred in only one (2%) patient (ALT 32 IU/l). On the other hand, in patients with non-SVR, ALT levels remained stable in 34 (60%) patients but increased to <2 × ULN in 20 (35%) patients, and to ≥2 × ULN in 3 (5%) patients (ALT 62, 79, and 135 IU/l). Similarly, in HCV-2 patients with SVR, ALT levels remained stable in 56 (95%) patients, and ALT elevation rarely occurred [<2 × ULN, 2 (3%) patients; ≥2 × ULN, one (2%) patient (ALT 68 IU/l)]. In contrast, in patients with non-SVR, ALT levels remained normal in 10 (67%) patients but increased to <2 × ULN in 4 (27%) patients and to ≥2 × ULN in one (6%) patient (ALT 174 IU/l).

Discussion

N-ALT patients with CHC are known to show demographic and virological features associated with higher

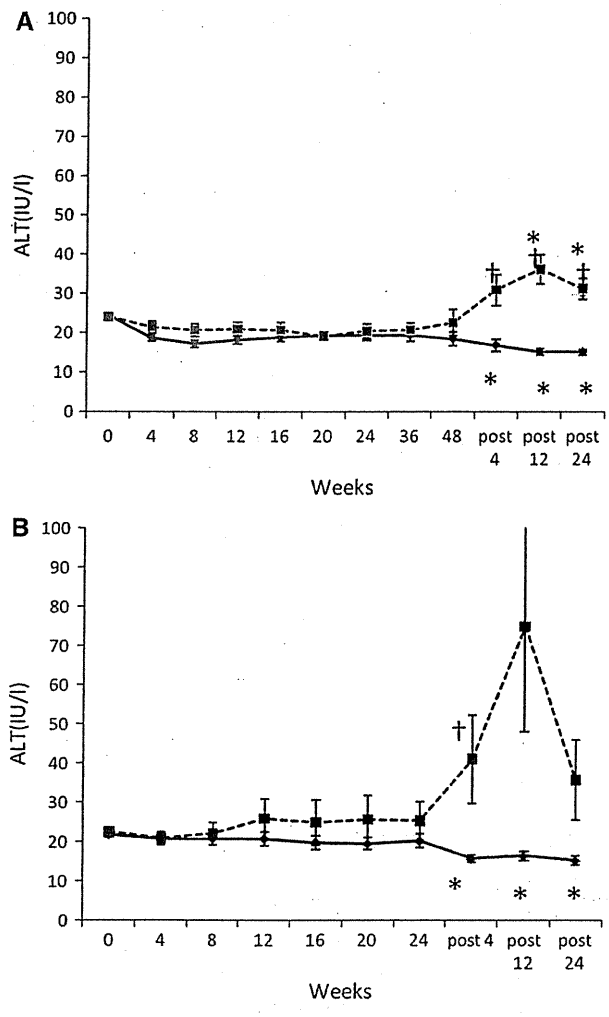

Fig. 1 Changes in serum alanine aminotransferase (*ALT*) levels (±standard error of the mean) according to response in patients with normal ALT levels with chronic hepatitis C treated with pegylated interferon and ribavirin. *Solid lines* show ALT levels in patients with a sustained virological response (SVR), and *dashed lines* show these levels in patients with a non-SVR. *Single-asterisks* denote a statistically significant difference ($P < 0.05$) in mean ALT levels between baseline and each time point of the follow-up period. *Daggers* denote a statistically significant difference between SVR and non-SVR groups. a Patients infected with hepatitis C virus genotype 1 (HCV-1). The number of patients was 57 in the SVR group and 59 in the non-SVR group. b HCV-2 patients. The number of patients was 60 in the SVR group and 17 in the non-SVR group

response rates to Peg-IFN and ribavirin combination therapy [4, 7, 12–17]. In the present study, N-ALT patients were younger and had higher platelet counts than E-ALT patients, thus giving N-ALT patients an advantage in antiviral efficacy in comparison with E-ALT patients in our cohort. However, the preponderance of females was greater in N-ALT patients with HCV-1 in this study, giving N-ALT patients a disadvantage. Accordingly, a direct comparison was made between these two patient groups after matching E-ALT patients with N-ALT patients using propensity score methods to reduce the bias due to differences in patient backgrounds. As a result, the efficacy of the combination therapy in N-ALT patients was revealed to be still equivalent to that in E-ALT patients, irrespective of their advantageous background. Moreover, in N-ALT patients with HCV-1, not only the ETR rate, but also the SVR rate tended to be higher than these rates in E-ALT patients (49% in N-ALT patients vs. 40% in E-ALT patients). Accordingly, N-ALT patients with HCV-1 can achieve a better treatment response in comparison with E-ALT patients, but further study is needed to clarify this.

In the present study, multivariate logistic regression analysis showed that achieving SVR was strongly influenced by HCV genotype and baseline HCV RNA level in N-ALT patients, which was consistent with findings of multicenter studies with E-ALT patients [18–21]. Therefore, decisions for treatment and the treatment regimen for N-ALT patients can mirror those recommended for E-ALT patients. The results of our multivariate analysis also revealed that patient age influenced the achievement of SVR in N-ALT patients. This offers support for the decision to offer antiviral treatment to younger N-ALT patients.

Among patients in our study who achieved SVR with the combination therapy, ALT levels after treatment decreased significantly, as shown in Fig. 1. However, approximately 40% of the non-SVR patients had increased ALT levels of up to $<2 \times$ ULN, and about 5% of patients had increased ALT levels of $\geq 2 \times$ ULN at 24 weeks after completion of the combination therapy, regardless of HCV genotype. When N-ALT patients are commencing the combination therapy, these patients should be told about the possibility of ALT exacerbation [6–9], although it is difficult to know whether this is drug-induced or due to the natural course. It is also difficult to state which patient characteristics make ALT elevation more likely to occur after the treatment.

Taking the findings obtained in the present study together, in N-ALT patients with HCV genotype 2, earlier treatment with Peg-IFN plus ribavirin combination therapy is desirable, as better efficacy was found for younger patients, with an SVR rate of approximately 80% being attained with this combination therapy, and few direct-acting antiviral agents (DAAs) have been developed for genotype 2. On the other hand, N-ALT patients with HCV genotype 1 should consider awaiting the DAAs, because SVR cannot be attained in about half of these patients, and the ALT level rises after treatment in about 40% of patients with non-SVR.

From the aspect of long-term prognosis, we need to verify, by prospective study, that viral eradication is really required for N-ALT patients because the incidence of hepatocellular carcinoma and liver-related mortality in

N-ALT patients has not been clarified. Deuffic-Burban et al. [22] calculated the impact of Peg-IFN plus ribavirin on morbidity and mortality in N-ALT patients using the Markov model and concluded that antiviral therapy in N-ALT patients would decrease morbidity and mortality rates. However, the treatment of N-ALT patients with CHC still remains an area of investigation, particularly with respect to the benefit-to-risk ratio of treatment. To help determine the indications for antiviral therapy in N-ALT patients, the liver histology should be evaluated before treatment. The presence of significant hepatic fibrosis (≥F2 by the METAVIR classification [23]) reflects continuous hepatic inflammation over a period of time and suggests a future risk of liver-related disease progression. Antiviral therapy may be appropriate for these patients. On the other hand, periodic follow up without antiviral therapy is recommended for patients in stages F0-1, because most of such patients show a low risk for progression to cirrhosis and the development of hepatocellular carcinoma [24].

This study had some limitations. First, the factors of viral mutation and host genetic mutation, which have been reported recently to affect the efficacy of Peg-IFN plus ribavirin combination therapy, could not be measured, and evaluation of the serum HCV RNA levels by a real-time PCR method, which is more sensitive to the measurement of serum HCV RNA levels, could not be done in the patients enrolled in this study, because we had few stored patient serum samples. Detailed examinations using the real-time PCR method in patients who are matched based on the factors of viral mutation and host genetic mutation as well as background factors will be needed for further study. Second, we excluded the factor of fibrosis from the multivariate analysis for factors associated with SVR in N-ALT patients, because data for fibrosis were lacking in 53 of the 193 patients in this study. Accordingly, the present study could not demonstrate whether fibrosis was associated with SVR in N-ALT patients. Finally, in this study, we investigated the antiviral efficacy of Peg-IFN plus ribavirin combination therapy for patients with N-ALT at the start of the therapy, not for patients with 'persistently' normal ALT. Accordingly, this study does not show the efficacy of this treatment in patients with persistently normal ALT. However, we believe that the results obtained in this study can be useful for pre-treatment prediction in outpatients who may not be followed by the reason of having normal ALT levels.

We have shown, in this matched case–control study using a propensity score method, that the therapeutic effect of combination therapy with Peg-IFN alpha-2b and ribavirin in N-ALT patients with CHC is comparable to that for E-ALT patients, irrespective of their advantageous background. Further work is needed to verify that HCV eradication can improve the prognosis of N-ALT patients.

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Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet

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Kamada Y, Kiso S, Yoshida Y, Chatani N, Kizu T, Hamano M, Tsubakio M, Takemura T, Ezaki H, Hayashi N, Takehara T. Estrogen deficiency worsens steatohepatitis in mice fed high-fat and high-cholesterol diet. *Am J Physiol Gastrointest Liver Physiol* 301: G1031–G1043, 2011. First published September 1, 2011; doi:10.1152/ajpgi.00211.2011.—Recent studies indicate an accelerated progression of nonalcoholic steatohepatitis (NASH) in postmenopausal women. Hypercholesterolemia, an important risk factor for NASH progression, is often observed after menopause. This study examined the effects of estrogen on NASH in ovariectomized (OVX) mice fed a high-fat and high-cholesterol (HFHC) diet. To investigate the effects of estrogen deficiency, OVX mice and sham-operated (SO) mice were fed normal chow or HFHC diet for 6 wk. Next, to investigate the effects of exogenous estrogen replenishment, OVX mice fed with HFHC diet were treated with implanted hormone release pellets (containing 17β-estradiol or placebo vehicle) for 6 wk. OVX mice on the HFHC diet showed enhanced liver injury with increased liver macrophage infiltration and elevated serum cholesterol levels compared with SO-HFHC mice. Hepatocyte monocyte chemoattractant protein-1 (MCP1) protein expression in OVX-HFHC mice was also enhanced compared with SO-HFHC mice. In addition, hepatic inflammatory gene expressions, including monocytes chemokine (C-C motif) receptor 2 (CCR2), were significantly elevated in OVX-HFHC mice. Estrogen treatment improved serum cholesterol levels, liver injury, macrophage infiltration, and inflammatory gene expressions in OVX-HFHC mice. Moreover, the elevated expression of liver CCR2 and MCP1 were decreased by estrogen treatment in OVX-HFHC mice, whereas low-density lipoprotein dose dependently enhanced CCR2 expression in THP1 monocytes. Our study demonstrated that estrogen deficiency accelerated NASH progression in OVX mice fed HFHC diet and that this effect was improved by estrogen therapy. Hypercholesterolemia in postmenopausal women would be a potential risk factor for NASH progression.

nonalcoholic steatohepatitis/nonalcoholic fatty liver disease, postmenopause

NONALCOHOLIC FATTY LIVER DISEASE (NAFLD) describes a broad spectrum of liver disorders ranging from benign simple steatosis to nonalcoholic steatohepatitis (NASH). NAFLD/NASH is considered the hepatic manifestation of the metabolic syndrome (Mets) and represents a growing medical problem in Western countries that is closely associated with obesity, insulin resistance, hypertension, and dyslipidemia (5, 40). The prevalence and severity of NASH reportedly differ with sex, with females showing an acceleration of disease progression after menopause (44, 52). Postmenopausal women also show a markedly accelerated accumulation of visceral fat and a rapid increase in the prevalence of Mets (27, 31). Moreover, the progression of atherosclerosis and dyslipidemia increases after menopause but can be improved by estrogen administration (17, 35). These findings implicate an important role for estrogen in the pathophysiology of NAFLD/NASH.

Estrogen controls energy homeostasis by regulating mitochondrial function (12). In aromatase knockout mice, which cannot synthesize endogenous estrogens, hepatic steatosis arises spontaneously in these mice due to the impaired hepatic fatty acid β-oxidation (38). The impairment in mitochondrial and peroxisomal fatty acid β-oxidation can be restored by estrogen supplementation in these mice (38). Compound heterozygous mutation of the aromatase gene in an adult humans presented as Mets characterized by abdominal obesity and NAFLD (32). Similarly, estrogen receptor (ER)-α knockout mice had hepatic steatosis (15, 30). Moreover, progressive hepatic steatosis among nonobese and nondiabetic patients with breast cancer treated with tamoxifen, a potent estrogen antagonist, induced NASH (39, 41). Thus estrogen deficiency could induce and exacerbate NAFLD/NASH progression in both human and mice.

Recent studies indicate that dietary cholesterol is an important risk factor for the progression of NASH in both human and rodents (34, 36, 50, 51) and that hypercholesterolemia and serum low-density lipoprotein (LDL) levels are independent predictors of human NASH severity (45). Lipidomic analysis indicated that hepatic free cholesterol content increased progressively from normal controls to patients with simple steatosis to NASH (42). Moreover, cholesterol-lowering drugs, such as statins and ezetimibe, demonstrably slowed NASH progression (1, 9, 19, 24). In the absence of estrogen action, mice developed progressive obesity and hypercholesterolemia (15, 16). Hypercholesterolemia and elevated serum LDL levels are also observed in postmenopausal women, and estrogen administration results in lowered serum levels of cholesterol and LDL (6, 17, 37).

Considering these findings, the estrogen deficiency itself and estrogen deficiency-induced hypercholesterolemia should accelerate the severity of NASH in postmenopausal women. However, the precise mechanisms underlying this progression are still unclear. To address this issue, we investigated the effects of estrogen on NASH progression in ovariectomized (OVX) mice fed high-fat and high-cholesterol (HFHC) diet.

MATERIALS AND METHODS

Experimental protocol. All experimental protocols and animal maintenance procedures used in this study were approved by the Ethics Review Committee for Animal Experimentation of Osaka

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University Graduate School of Medicine. Female C57BL/6J mice were purchased from Clea Japan (Tokyo, Japan) and maintained for 1 wk under the same conditions before starting the experiments. The animals were provided with unrestricted amounts of chow and water, housed in temperature- and humidity-controlled rooms, and maintained on a 12-h:12-h light/dark cycle. The HFHC diet (containing 1.25% cholesterol, 15% cocoa butter, and 0.5% cholic acid) was purchased from Oriental Yeast (Suita, Osaka, Japan).

To investigate the effects of estrogen deficiency, 28 female C57BL/6J mice (10-wk-old) were used. All mice were anesthetized for surgery, and either a bilateral ovariectomy (n = 14) or sham operation (SO) (n = 14) was performed. One week after the surgery, mice were divided into four groups (n = 7 for each groups) and fed with normal chow (NC) or the HFHC diet for 6 wk (SO-NC group, SO-HFHC group, OVX-NC group, OVX-HFHC group). SO mice fed with NC diet (n = 7) served as controls.

We next investigated the effects of exogenous estrogen replenishment, with the use of 12 OVX mice divided into two groups. All mice were implanted subcutaneously with 60-day release hormone pellets (Innovative Research of America, Toledo, OH) that contained 0.5 mg 17β-estradiol (E2) (n = 6) or placebo vehicle alone (n = 6). This estradiol dosage was shown to yield serum estrogen concentrations within the physiological range (46). One week after surgery, mice were fed for 6 wk on the HFHC diet [OVX-HFHC group (n = 6), OVX-HFHC+E2 group (n = 6)].

At the end of the each experimental period, mice were fasted overnight (10:00 PM-8:00 AM) but had free access to water and then euthanized after being weighed. Blood was collected aseptically from the inferior vena cava, centrifuged (13,000 g, 5 min, 4°C), and serum was collected. Mice spleens were harvested for isolating monocytes. The livers were also harvested and either fixed with 10% buffered formaldehyde for histological examination or immediately frozen in liquid nitrogen for mRNA extraction and lipid analysis. Hematoxylin-eosin staining of mice liver specimens was performed according to standard techniques. Samples were investigated and quantified according to NAFLD activity scoring (NAS) (25). Steatosis (0–3), lobular inflammation (0–2), and hepatocellular ballooning (0–2) were quantified, respectively.

Measurements of serum biochemical markers and liver lipid content. Serum alanine aminotransferase (ALT), glucose, and insulin concentrations were measured using the transaminase CII-test kit, glucose CII-test kit (Wako Pure Chemical Industries, Tokyo, Japan), and insulin ELISA kit (Morinaga, Yokohama, Japan), respectively, according to the manufacturers' instructions. Insulin resistance was calculated according to the homeostasis model of assessment of insulin resistance (HOMA-IR) using the formula, insulin (μU/ml) × blood glucose (mmol/l)/22.5.

Total lipids were extracted from the liver as described previously (22). Serum and liver triglyceride (TG), total cholesterol, and free fatty acid (FFA) were measured using the TG E-test kit, cholesterol E-test kit (Wako Pure Chemical Industries), and NESCAUTO NEFA Kit-U (Alfresa-Pharma, Osaka, Japan), respectively, according to the manufacturers' instructions. Serum lipoproteins were analyzed using an online dual-enzymatic method for the simultaneous quantification of cholesterol and TG using high-performance liquid chromatography (HPLC) at Skylight Biotech (Akita, Japan), according to a procedure described previously (49).

Mice hepatic gene expression analysis and quantitative RT-PCR. Forty-seven genes with links to NAFLD pathophysiology (cholesterol metabolism, endoplasmic reticulum stress, fatty acid metabolism, glucose metabolism, inflammation, macrophage, nuclear receptor) were chosen for analysis on a custom-made TaqMan Array Plate (Applied Biosystems, Foster City, CA). Total RNA was extracted from whole liver with the QIAshredder and the RNeasy Mini kit according to the instructions provided by the manufacturer (Qiagen, Hilden, Germany). Complementary DNAs (cDNAs) were reverse transcribed from total liver RNA samples using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) from SO-NC, SO-HFHC, OVX-NC, and OVX-HFHC mice liver. The cDNA of each mice group was mixed, and per individual assay 10 ng cDNA of each group mice liver was loaded together with TaqMan Gene Expression Master Mix (Applied Biosystems) and applied to single wells in a 96-well TaqMan Array Plate. This assay was performed on the Applied Biosystems 7900HT RT-PCR system (Applied Biosystems). Data were normalized to 18S expression.

Total RNA was extracted from whole liver or cells with the QIAshredder and RNeasy Mini kit according to the instructions provided by the manufacturer. The RNA was then transcribed into cDNA using a Quantitect Reverse Transcription kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed with the QuantiFast SYBR Green PCR kit using specific primers (Qiagen) on a LightCycler according to the instructions provided by the manufacturer (Roche Diagnostics, Indianapolis, IN). The primers used in this study were all purchased from Qiagen [monocyte chemoattractant protein-1 (MCP1) (QT00167832), monocytes chemokine

Table 1. *Body, liver, epididymal fat, and uterus weight and biochemical analysis of serum and liver extracts in mice*

	SO		OVX	
	NC	HFHC	NC	HFHC
n	7	7	7	7
Body weight, g	20.1 ± 0.55	19.6 ± 1.13	21.9 ± 1.41†	20.4 ± 1.32
Liver weight, g	0.92 ± 0.045	1.36 ± 0.074*	0.98 ± 0.045	1.53 ± 0.087‡
Epididymal fat weight, g	0.137 ± 0.025	0.137 ± 0.021	0.217 ± 0.04*	0.173 ± 0.026‡
Uterus weight, g	0.093 ± 0.012	0.087 ± 0.012	0.017 ± 0.006*	0.043 ± 0.029‡
Serum				
ALT, IU/l	16.1 ± 2.3	54.0 ± 10.0*	18.1 ± 5.1	89.6 ± 27.5‡
Total cholesterol, mg/dl	63.7 ± 7.6	98.2 ± 11.4*	73.8 ± 8.8	115.9 ± 17.4§
Triglyceride, mg/dl	84.0 ± 28.6	37.8 ± 9.5*	81.1 ± 16.4	35.1 ± 7.9
FFA, μEq/l	744.5 ± 221.7	651.0 ± 177.4	1000.3 ± 301.1	816.2 ± 209.7
Glucose, mg/dl	165.8 ± 43.5	203.2 ± 65.4	135.6 ± 32.7	137.9 ± 29.0§
Insulin, IU/l	11.0 ± 12.1	4.20 ± 2.36	12.4 ± 14.4	5.75 ± 2.85
HOMA-IR	3.21 ± 2.49	1.78 ± 0.99	3.52 ± 3.98	1.63 ± 0.77
Liver				
Triglyceride, mg/g	71.7 ± 19.2	86.6 ± 21.7	79.4 ± 9.56	67.0 ± 22.3§
Total cholesterol, mg/g	11.2 ± 3.50	35.3 ± 4.25*	10.8 ± 3.37	37.0 ± 1.89
FFA, μEq/g	14.6 ± 5.41	24.2 ± 7.23	20.3 ± 10.0	21.3 ± 9.76

Data are means ± SD. *$P < 0.01$; †$P < 0.05$ compared with sham-operated (SO)-normal chow (NC) group; ‡$P < 0.01$; §$P < 0.05$ compared with SO-high-fat high-cholesterol diet (HFHC) group. OVX, ovariectomized; ALT, alanine aminotransferase; FFA, free fatty acid; HOMA-IR, homeostasis model of assessment of insulin resistance.

(C-C motif) receptor 2 (CCR2) (QT02276813), TNF-α (QT00104006), IFN-γ (QT01038821), IL12 (QT01048334), nitric oxide synthase-2 (NOS2) (QT00100275), IL10 (QT00106169), IL4 (QT00160678), transforming growth factor-β1 (TGF-β1) (QT00145250), collagen Iα1 (QT00162204), cholesterol 7α-hydroxylase (CYP7A1) (QT00121569), LDL receptor (LDLR) (QT00109823), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) (QT01037848), CD36 (QT01058253), and GAPDH (QT00199388)]. The mRNA expression levels were normalized relative to GAPDH mRNA expression levels and expressed in arbitrary units.

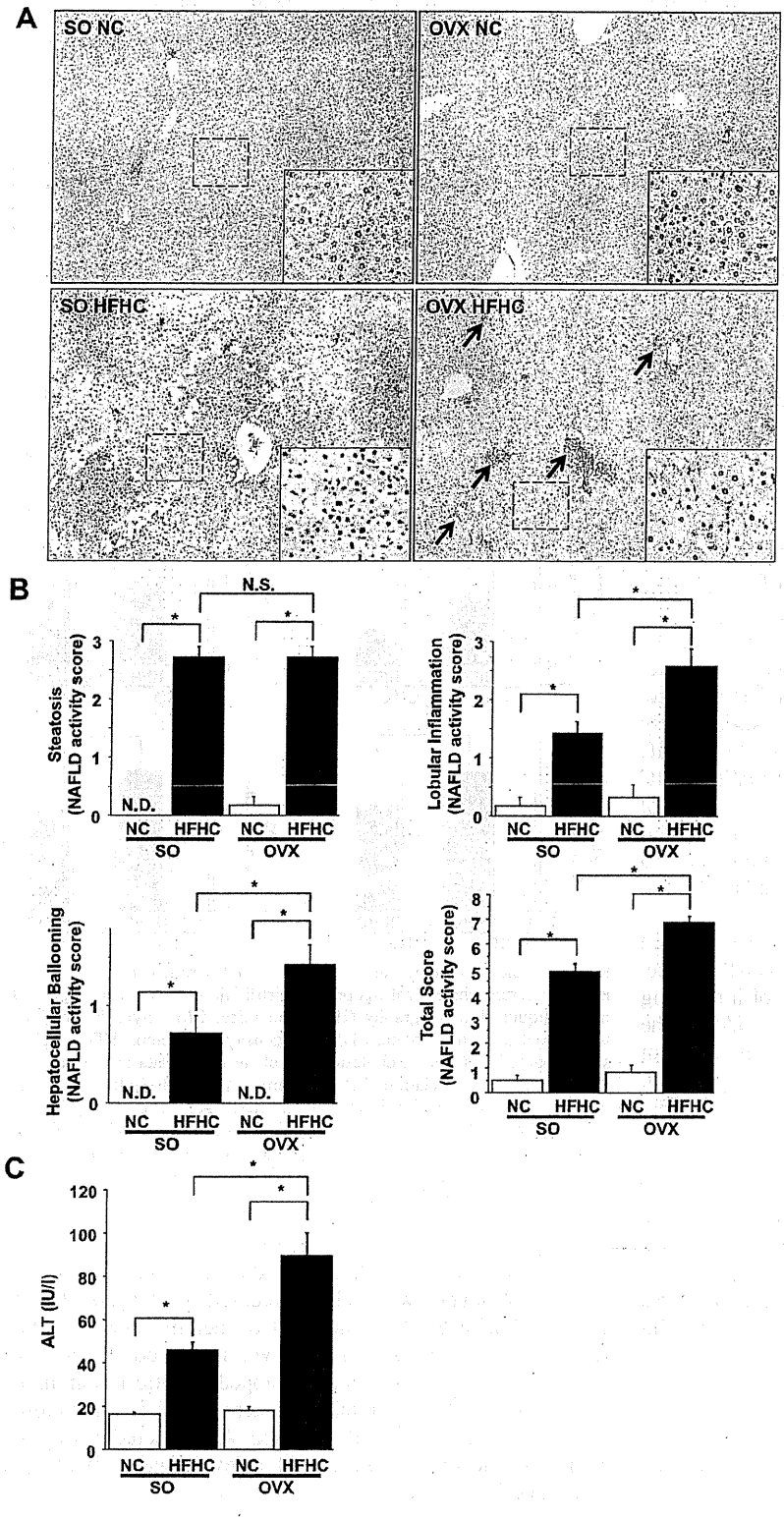

Fig. 1. Ovariectomy (OVX) exacerbated liver injury in mice fed high-fat and high-cholesterol (HFHC) diet. *A*: hematoxylin-eosin (H&E) staining of mice liver sections. Mice received a sham operation (SO) or bilateral. 1 wk after surgery, mice were fed with either a normal chow (NC) diet or HFHC diet for 6 wk. Each group contained 7 mice. The arrows indicate infiltration of the inflammatory cells in the hepatic parenchyma. *Insets*: magnified views of typical hepatocytes in mice (dashed squares). Original magnification, ×100. *B*: nonalcoholic fatty liver disease (NAFLD) activity scores in mice. The graphs represent the scores of hepatic steatosis, lobular inflammation, hepatocellular ballooning, and total NAFLD activity. *C*: serum alanine aminotransferase (ALT) levels of mice. Open bars indicate mice fed with NC diet, and solid bars indicate mice fed with HFHC diet. Data are means ± SD. $*P < 0.01$, $**P < 0.05$. N.S., not significant.

Picrosirius red staining and immunohistochemistry. Picrosirius red (Sigma, St. Louis, MO) staining was used to detect collagen fibrils in mice specimens. The fibrosis area stained by picrosirius red in this study was quantified by using Adobe Photoshop (Adobe Systems, San Jose, CA) as described previously (23).

Mice liver tissue sections were subjected to immunohistochemical staining using a monoclonal antibody against rat anti-mouse F4/80 (1:100) (Serotec, Oxford, UK) or MCP1 (1:250) (Cell Signaling, Beverly, MA).

CCR2 expression in monocytes. Monocytes were isolated from mice spleens of each group by magnetic cell sorting using magnetic beads (MACS; Miltenyi Biotec, Gladbach, Germany) with CD11b antibody according to the manufacturer's protocol. RNA was extracted from the spleen monocytes of mice from each group for quantitative real-time RT-PCR analysis. THP1 cells, a human monocytic cell line, were obtained from American Type Culture Collection (ATCC) (Manassas, VA). Growth medium consisted of RPMI-1640/10% FCS. To examine the effect of LDL (Sigma) on monocyte CCR2 expression, THP1 monocytes were cultured in RPMI medium with or without individual concentrations of LDL (0, 5, 50 μg/ml) for 18 h, and RNA was extracted for quantitative real-time RT-PCR analysis.

Statistical analysis. Statistical analysis was conducted using JMP 8.0 software (SAS Institute, Cary, NC). The results are presented as means ± SD. Differences between groups were examined for statistical significance using ANOVA with Fisher's paired least-significant difference test. Statistical significance was defined as $P < 0.05$.

RESULTS

Ovariectomy enhances liver injury in HFHC diet-fed mice. Body weight was significantly elevated in OVX-NC mice compared with SO-NC mice (Table 1). Liver weight was significantly higher in OVX-HFHC mice than in SO-HFHC mice, whereas epididymal fat weight was significantly higher in OVX mice than in SO mice with both NC and HFHC diet feeding. Serum ALT and cholesterol levels were significantly increased in OVX-HFHC mice compared with SO-HFHC mice, whereas liver TG levels were decreased in OVX-HFHC mice compared with SO-HFHC controls, and liver cholesterol contents were elevated in HFHC diet-fed mice compared with NC diet-fed mice with or without ovariectomy. There were no differences in liver cholesterol contents between the HFHC groups.

There were no changes in liver histology between SO and OVX mice on NC diets (Fig. 1*A*). In HFHC diet-fed mice, ovariectomy significantly increased the numbers of infiltrating inflammatory cells in liver. We next assessed the NAS in the mice livers (Fig. 1*B*). The liver steatosis score was increased in HFHC diet-fed mice compared with those fed an NC diet, but there was no change in hepatic steatosis between the HFHC diet-fed groups. However, both lobular inflammation and hepatocyte ballooning scores were significantly elevated in OVX-HFHC mice compared with SO-HFHC mice. Consequently, the total NAS was significantly higher in OVX-HFHC mice than in SO-HFHC mice, as were serum ALT levels (Fig. 1*C*).

Impact of ovariectomy and diet on serum lipoprotein and cholesterol levels. To investigate whether dyslipidemia was induced by ovariectomy, we checked serum lipoprotein and cholesterol levels in the mice (Fig. 2, *A–C*). Cholesterol lipoprotein distribution by HPLC showed that HFHC diet feeding elevated serum very-low-density lipoprotein (VLDL) and LDL cholesterol levels in SO-HFHC mice compared with SO-NC mice, and this increase was exacerbated in the OVX-HFHC mice. In contrast, high-density lipoprotein (HDL) cholesterol

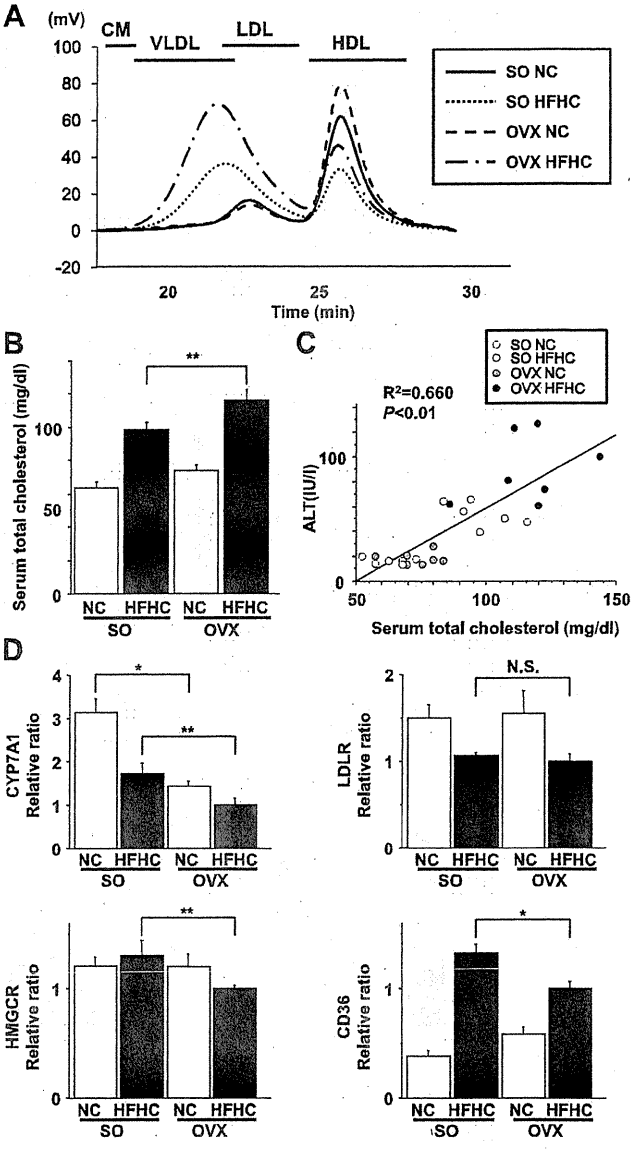

Fig. 2. Cholesterol lipoprotein distribution and serum cholesterol levels in mice. *A*: serum cholesterol lipoprotein distribution analyzed by high-performance liquid chromatography (HPLC) in mice. CM, chylomicron; VLDL, very-low-density lipoprotein; LDL, low-density lipoprotein; HDL, high-density lipoprotein. *B*: serum cholesterol levels in mice. Data are means ± SD. $^{**}P < 0.05$. *C*: correlations between serum ALT and cholesterol levels in mice. *D*: hepatic cholesterol-metabolism related gene expression changes in mice. Liver GAPDH-normalized cholesterol 7α-hydroxylase (CYP7A1), LDL receptor (LDLR), 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR), and CD36 gene expressions. Data are means ± SD. $^{*}P < 0.01$, $^{**}P < 0.05$. N.S.; not significant.

levels were decreased in HFHC diet-fed mice. Serum total cholesterol levels were also increased in SO-HFHC mice compared with SO-NC mice and further increased in OVX-HFHC mice. A consistent finding was increased serum cholesterol levels in the mice that developed hepatic inflammation, suggesting that serum total cholesterol level is an important determinant of hepatic inflammation. As expected, serum total cholesterol levels and serum ALT levels were significantly correlated in this study.

Changes in macrophage infiltration and hepatic inflammatory gene expression are enhanced in OVX-HFHC mice liver. To investigate the mechanism of accelerated steatohepatitis in the OVX-HFHC mice, we analyzed the expressions of 47 genes associated with NAFLD pathophysiology using a TaqMan Array Plate (Tables 2 and 3). The gene expression of CYP7A1, a rate-limiting enzyme in bile acid production from cholesterol, was significantly decreased in OVX mice liver compared with SO-NC and SO-HFHC mice (Fig. 2*D*). The gene expression of HMGCR was decreased in OVX-HFHC mice compared with SO-HFHC mice, but there were no significant differences in the gene expression of LDLR among mice groups. CD36, a major scavenger receptor, was downregulated in the liver of OVX-HFHC mice compared with SO-HFHC mice. There were no obvious expression changes in other genes related to cholesterol metabolism, fatty acid metabolism, glucose metabolism, or nuclear receptors between the HFHC diet-fed groups (Table 2). However, genes related to inflammation and macrophage infiltration were highly upregulated in OVX-HFHC mice compared with SO-HFHC mice, including TNF-α, MCP1, CCR2, F4/80, and NOS2 (Table 3).

Table 2. *Expression of liver metabolism-related genes, gene links to NAFLD pathophysiology*

Classification	Gene	SO-NC	SO-HFHC	OVX-NC	OVX-HFHC
Cholesterol metabolism					
	Abca1	1	1.981	1.146	2.012
	Abcg5	1	3.726	0.893	3.424
	Apoa1	1	0.979	0.877	1.012
	Cyp7a1	1	0.691	0.738	0.424
	Hmgcr	1	0.998	0.925	0.77
	Ldlr	1	0.661	1.008	0.68
	Scarb1	1	1.272	0.845	1.398
	Soat2	1	1.141	0.956	1.004
	Srebf1	1	5.266	0.977	4.587
	Srebf2	1	0.663	0.921	0.641
Fatty acid metabolism					
	Acaca	1	1.054	1.11	1.039
	Acox1	1	0.948	1.295	0.909
	Apob	1	0.847	1.256	0.817
	Cpt1a	1	0.768	1.088	0.655
	Fasn	1	1.045	1.075	1.116
	Lipc	1	0.818	1.275	0.837
	Mttp	1	0.791	1.181	0.677
	Ppara	1	1.129	1.184	0.893
	Scd1	1	6.575	1.451	7.095
Glucose metabolism					
	Adipor1	1	1.016	0.974	1.041
	Adipor2	1	0.818	1.201	0.769
	Gys2	1	0.789	1.058	0.621
	Irs2	1	0.822	1.132	0.526
	Mlxipl	1	1.315	1.277	1.132
	Pck1	1	0.666	1.161	0.612
	Pparg	1	1.69	1.417	1.663
	Ppargc1a	1	1.164	1.23	0.881
	Prkaa2	1	1.162	1.13	0.955
	Slc2a2	1	9.863	1.179	10.404
Nuclear receptor					
	Nr0b2	1	1.3	1.099	1.259
	Nr1 h3	1	0.945	0.909	0.843
	Nr1 h4	1	0.808	0.956	0.662

Gene abbreviations and classification are based on function. Expression is shown as fold change compared with levels of SO-NC mice. NAFLD, nonalcoholic fatty liver disease.

Table 3. *Expression of liver inflammatory-related genes, gene links to pathophysiology of NAFLD*

Classification	Gene	SO-NC	SO-HFHC	OVX-NC	OVX-HFHC
Inflammation					
	Cat	1	0.78	1.14	0.774
	Il6	1	0.419	1.726	0.8
	Socs3	1	0.881	1.01	1.268
	Stat3	1	0.867	1.265	1.066
	Tnf	1	5.464	1.127	9.49
Macrophage					
	Arg1	1	0.86	0.716	0.99
	Ccl2	1	10.847	2.462	17.58
	Ccr2	1	2.944	0.842	4.891
	Emr1	1	2.068	0.756	3.066
	Il10	1	1.996	1.12	2.067
	Ncf1	1	2.185	0.875	2.68
	Nos2	1	4.954	0.71	52.744
Endoplasmic reticulum stress					
	Ddit3	1	1.827	1.23	1.63
	Hspa5	1	0.87	1.218	0.787
	Xbp1	1	1.051	1.04	0.98

Gene abbreviations and classification are based on function. Expression is shown as fold change compared with levels of SO-NC mice.

Gene expression profiling indicated enhanced inflammatory macrophage infiltration in OVX-HFHC mice livers. We therefore assessed liver macrophage infiltration using F4/80 immunohistochemistry (Fig. 3*A*). In NC diet-fed mice, the degree of liver macrophage infiltration was not different with or without ovariectomy. In contrast, massive macrophage infiltration was seen in OVX-HFHC mice liver.

To examine the expressions of inflammatory cytokines and chemokines, we performed quantitative real-time RT-PCR (Fig. 3*B*). The gene expressions of hepatic MCP1 and its receptor CCR2 were significantly elevated in OVX-HFHC mice compared with SO-HFHC mice. Inflammatory cytokine genes for TNF-α, IFN-γ, and NOS2 were also significantly upregulated in OVX-HFHC mice livers. In contrast, there were no obvious changes in anti-inflammatory Th2 cytokine expressions, including IL10 and IL4.

We next assessed the relationships between mice serum cholesterol levels and hepatic inflammatory gene expressions levels (Fig. 3*C*). Interestingly, the expression of all the relevant factors (MCP1, CCR2, TNF-α, IFN-γ, IL12, and NOS2) correlated significantly with serum cholesterol levels.

Higher hepatic MCP1 protein expression in OVX-HFHC mice than SO-HFHC mice. It was previously reported that dietary cholesterol enhances hepatic MCP1 expression (48), and hepatic MCP1 gene expression was elevated in the OVX-HFHC mice in this study. Accordingly, we also examined the levels of hepatic MCP1 protein expression in mice using immunohistochemistry (Fig. 3*D*). There were no differences in liver MCP1 staining in NC-fed mice with or without ovariectomy. HFHC diet feeding increased the number of MCP1-positive hepatocytes, and this number was further increased in OVX-HFHC mice.

Enhanced liver fibrosis in OVX-HFHC mice compared with SO-HFHC mice. Picrosirius red staining revealed increased hepatic fibrosis in OVX-HFHC mice (Fig. 4). The expressions

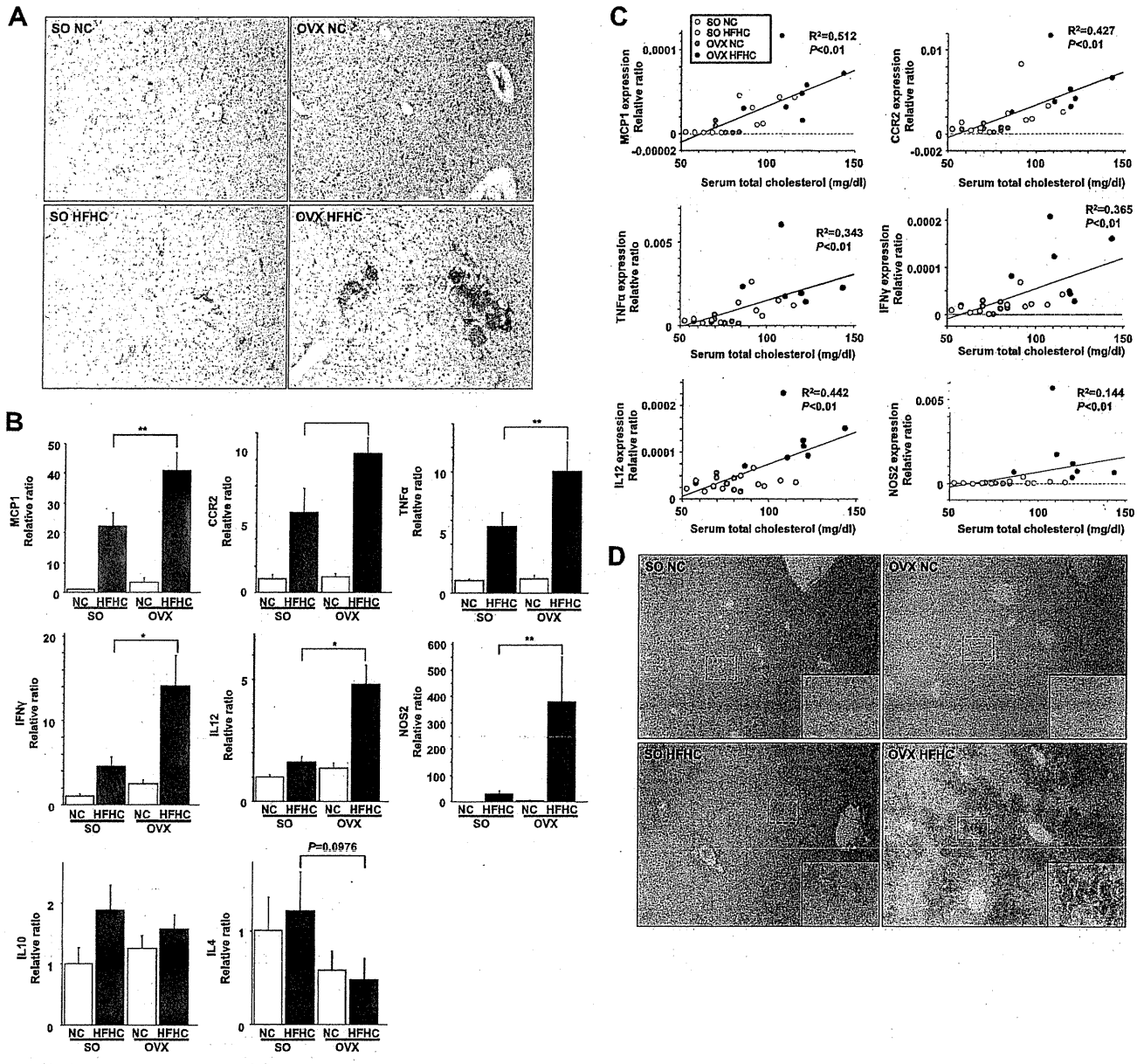

Fig. 3. OVX exacerbated liver macrophage infiltration in mice fed HFHC diet with enhanced inflammatory gene expression. *A*: F4/80 immunohistochemistry of mice livers. Original magnification, ×200. *B*: hepatic gene expression changes in mice livers. Liver GAPDH-normalized monocyte chemoattractant protein-1 (MCP1), monocytes chemokine (C-C motif) receptor 2 (CCR2), TNF-α, IFN-γ, IL12, nitric oxide synthase 2 (NOS2), IL10, and IL4 gene expression determined by real-time RT-PCR. Data are means ± SD. $*P < 0.01$, $**P < 0.05$. *C*: correlations between serum cholesterol levels and liver inflammation-related gene expressions. *D*: MCP1 immunohistochemistry of mice livers. *Insets*: magnified views of mice livers (dashed squares indicate the magnified areas). Original magnification, ×100.

of fibrogenic genes, TGF-β1 and collagen Iα1, were also increased in SO-HFHC mice compared with SO-NC mice and further increased in OVX-HFHC mice. In addition, the gene expressions levels of TGF-β1 and collagen Iα1 were significantly correlated with serum cholesterol levels.

Administration of estrogen ameliorates liver injury in OVX-HFHC mice. To investigate the therapeutic effects of estrogen following ovariectomy, we next investigated another set of OVX-HFHC mice implanted with estrogen pellets (OVX-HFHC+E2). Body weight was significantly decreased in OVX-HFHC+E2 mice compared with OVX-HFHC controls (Table 4 and Fig. 5), whereas liver weight was not different. In addition, epididymal fat weight, serum ALT level, and serum cholesterol level (total as well as VLDL and LDL cholesterol) were significantly decreased in OVX-HFHC+E2 mice compared with OVX-HFHC mice. Serum cholesterol levels in these groups of mice correlated significantly with serum ALT levels (Fig. 6). There were no significant changes in liver lipid contents. Assessment of liver histology using NAS revealed that estrogen ameliorated the liver inflammation and hepatocyte ballooning in treated mice compared with OVX-HFHC mice.

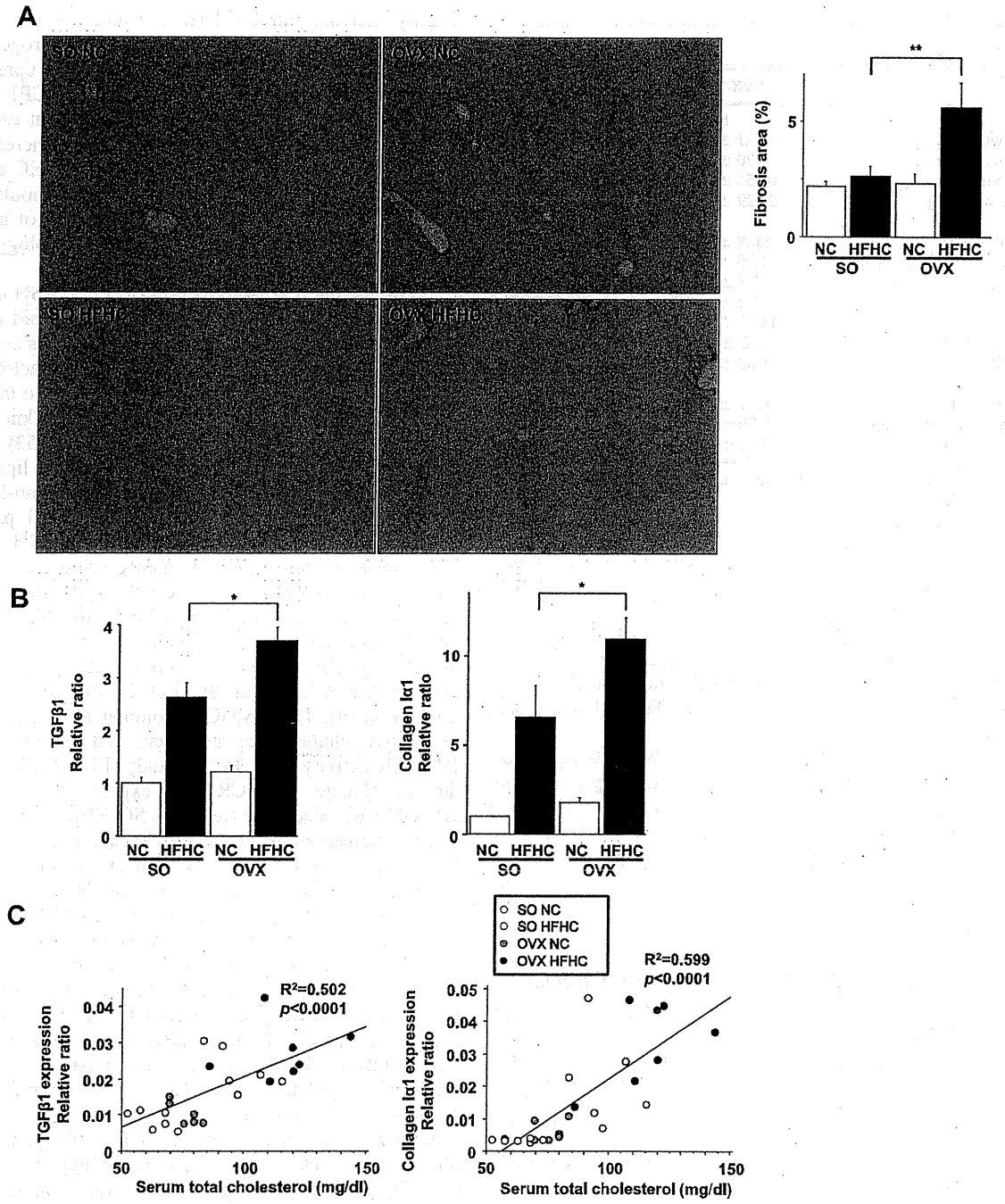

Fig. 4. Hepatic fibrosis was exacerbated in OVX-HFHC mice. *A*: picrosirius red staining of mice livers. Original magnification, ×100. Histographic representation of quantified data. The fibrosis areas were measured as described in MATERIALS AND METHODS. The degree of fibrosis was expressed as the percentage of the total area measured. *B*: gene expression changes related to fibrosis. Liver GAPDH-normalized TGF-β1, and collagen Iα1 gene expression was determined by real-time RT-PCR. Data are means ± SD. $*P < 0.01$, $**P < 0.05$. *C*: correlations between serum cholesterol levels and fibrosis-related gene expressions (TGF-β1, collagen Iα1).

F4/80 immunohistochemistry revealed that both estrogen treatment decreased the hepatic macrophage infiltration observed in OVX-HFHC mice (Fig. 7*A*), with a concomitant downregulation of hepatic inflammatory gene expressions compared with untreated OVX HFHC mice (Fig. 7*B*). Hepatic gene expressions of CYP7A1 and LDLR were elevated in mice after estrogen treatment compared with OVX-HFHC mice (Fig. 6*D*). Hepatic HMGCR gene expression was higher in OVX-HFHC+E2 mice than OVX-HFHC mice. The levels of hepatic inflammatory genes, MCP1, CCR2, TNF-α, IFN-γ, and NOS2, were significantly decreased with the reduction in serum cholesterol levels in mice treated with estrogen (Fig. 7*C*). MCP1 immunohistochemistry revealed that estrogen treatment decreased hepatocyte MCP1 expression in OVX HFHC mice liver (Fig. 7*D*).

Table 4. *Biochemical analysis of serum and liver extracts of mice*

	OVX-HFHC	OVX-HFHC+E2
n	6	6
Body weight, g	20.71 ± 0.854	18.69 ± 0.99*
Liver weight, g	1.70 ± 0.122	1.59 ± 0.083
Epididymal fat weight, g	0.151 ± 0.034	0.1 ± 0.02*
Uterus weight, g	0.029 ± 0.027	0.15 ± 0.01*
Serum		
ALT, IU/l	90.8 ± 25.2	47.7 ± 23.5*
Total cholesterol, mg/dl	117.6 ± 30.7	82.6 ± 24.9†
Triglyceride, mg/dl	35.5 ± 8.26	35.6 ± 14.8
FFA, μEq/l	815.2 ± 318.7	858.8 ± 281.0
Glucose, mg/dl	143.9 ± 40.7	114.6 ± 18.3
Insulin, IU/l	4.65 ± 2.33	5.26 ± 3.0
HOMA-IR	1.62 ± 1.05	1.54 ± 0.96
Liver		
Triglyceride, mg/g	67.1 ± 28.0	80.5 ± 19.0
Total cholesterol, mg/g	37.0 ± 9.43	43.5 ± 3.41
FFA, μEq/g	21.3 ± 8.02	19.7 ± 2.20

Data are means ± SD. *$P < 0.01$; †$P < 0.05$ compared with OVX HFHC group. E2, 17β-estradiol.

Picrosirius red staining revealed that estrogen treatment improved liver fibrosis in OVX-HFHC mice (Fig. 8). The gene expression of liver collagen Iα1 was also improved by estrogen treatment, but hepatic TGF-β1 expression showed no changes. Hepatic collagen Iα1 gene expression decreased in accordance with the reduction in serum cholesterol although there was no statistical correlation between mice liver TGF-β1 and serum cholesterol levels.

Cholesterol loading induces monocyte CCR2 gene expression. We examined CCR2 gene expression in mice spleen monocytes from all five groups in this study (Fig. 8*D*). HFHC diet feeding elevated CCR2 expression in SO-HFHC mice compared with SO-NC mice, and ovariectomy further increased the CCR2 expression in OVX-HFHC mice. This CCR2 upregulation was in turn ameliorated by estrogen treatment. These changes in monocyte CCR2 expression were similar to the changes in serum cholesterol concentrations, which is consistent with previous studies that showed marked increase in monocyte CCR2 expression in hypercholesterolemic patients, and LDL loading resulted in increased monocyte CCR2 expression (13, 14). We also found that LDL dose dependently increased the CCR2 expression in THP1 monocytes, one of the human monocyte cell lines (Fig. 8*E*). These data could indicate that elevated serum cholesterol in OVX-HFHC mice induced monocyte CCR2 expression, leading to enhanced hepatic macrophage infiltration.

DISCUSSION

Recent studies have demonstrated accelerated progression of both NASH and hypercholesterolemia after menopause. Here, we showed that ovariectomy enhanced NASH progression with increased hepatic macrophage infiltrations in mice fed HFHC diet. Serum cholesterol levels were higher in OVX-HFHC mice than in SO-HFHC mice, and serum cholesterol levels significantly correlated with serum ALT levels, liver injury, and changes in the expression levels of various hepatic inflammatory genes. Estrogen replenishment decreased serum cholesterol levels and attenuated liver injury in HFHC diet-fed mice. Moreover, hepatic MCP1 protein expression was significantly increased in OVX-HFHC mice compared with SO-HFHC mice, and this effect was rescued by estrogen treatment. In an in vitro study, LDL dose dependently upregulated the gene expression of CCR2, known as the MCP1 receptor, in THP1 monocytes. These results indicated that estrogen deficiency enhanced NASH progression through increased hepatic macrophage infiltration in mice fed with HFHC diet. Hypercholesterolemia in postmenopausal women should accelerate NASH progression, suggesting that treatment of hypercholesterolemia would improve NASH pathophysiology in women after menopause.

Dyslipidemia is commonly observed in NASH patients, and it has been postulated that abnormalities in lipid metabolism, such as increased serum LDL cholesterol levels and decreased HDL cholesterol levels, may be contributing factors to NASH development (26). Accordingly, hyperlipidemic mice develop diet-induced NASH, including not only LDLR knockout mice, but also apolipoprotein E-deficient mice (50, 53). Unlike humans, mice fed an NC diet carry most of their lipids as HDL. However, mice fed an HFHC diet adopt a human-like lipoprotein profile and thus could serve as a useful physiological animal model to study the progression of NASH.

Cholesterol homeostasis is mainly achieved by regulating the enzymes involved in cholesterol synthesis, uptake, and clearance (3), and estrogen plays important roles in regulating cholesterol homeostasis. For example, estrogen stimulates expression of the LDLR gene through the ER-α, which can activate transcription of the LDLR promoter through Sp1 interaction (4). The HMGCR promoter also has an estrogen-responsive element-like sequence, and estradiol stimulates HMGCR activity (10). In this study, LDLR gene expression did not change, HMGCR gene expression was reduced in OVX-HFHC mice compared with SO-HFHC mice, and estrogen supplementation elevated both gene expressions in mice liver. Hepatic cholesterol levels were similar despite the higher serum cholesterol levels between the HFHC mice groups, which might reflect a reduced liver cholesterol uptake by LDLR and a reduced hepatic de novo cholesterol production by HMGCR. In addition, the cholesterol clearance rate would be interrupted in the estrogen-deficient state (16). CYP7A1 catalyzes the rate-limiting step of cholesterol conversion into bile acid and is encoded by the CYP7A1 gene. Estrogen administration increased CYP7A1 activity in baboon in vivo, and estradiol upregulated CYP7A1 gene expression in rat hepatocytes in vitro (8, 28). Aromatase knockout mice, which cannot synthesize endogenous estrogen and present age-progressive obesity and hepatic steatosis, develop hypercholesterolemia (16). These mice show significantly reduced hepatic CYP7A1 expression compared with wild-type mice, and the reduced cholesterol clearance attributable to the decreased CYP7A1 activity increased serum cholesterol levels. In our study, hepatic CYP7A1 gene expression was also decreased in OVX mice as expected, and estrogen treatment restored this hepatic expression. The interrupted cholesterol clearance in OVX mice should therefore induce enhanced hypercholesterolemia in the HFHC mice in our study.

Recent studies point to the importance of dietary cholesterol as a toxic lipid in liver inflammation and NASH both in rodents and humans (33, 34, 36, 48, 50, 51). Mari et al. (33) reported that high-cholesterol feeding causes lipotoxicity that sensitizes rat livers to develop hepatic inflammation after exposure to