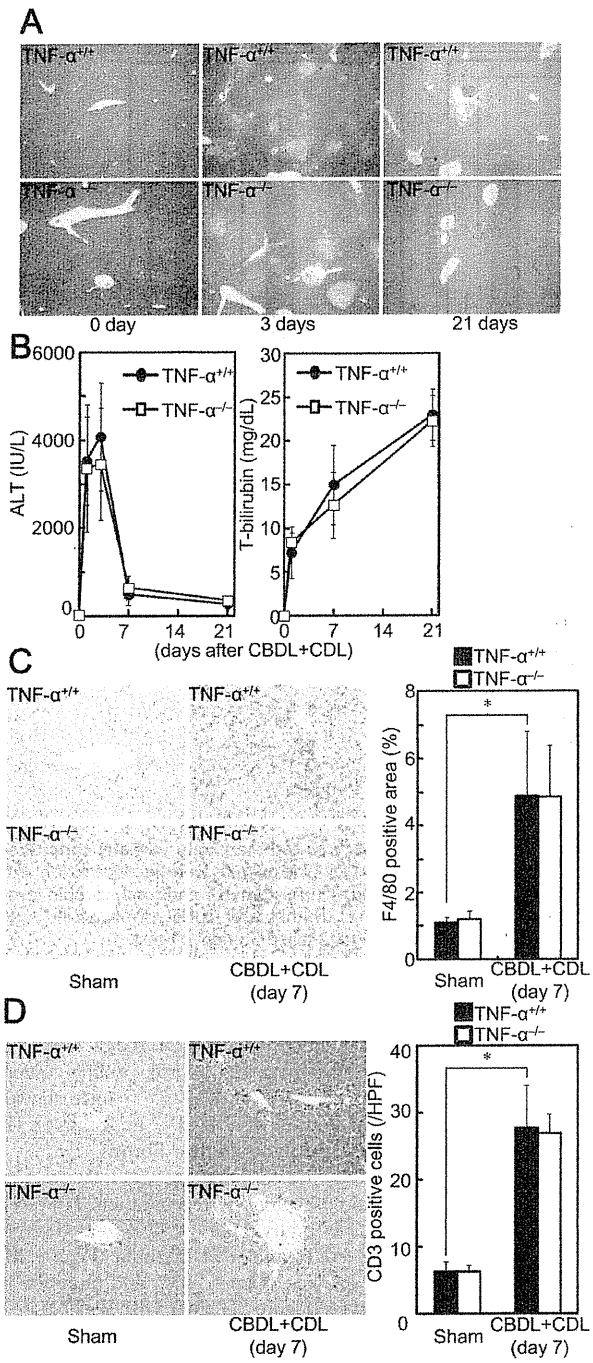

Figure 2. TNF-α deficiency did not affect CBDL+CDL-induced liver injury. TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received CBDL+CDL. The animals were sacrificed at the indicated times. (A) The injured lesion in the livers was assessed by H&E staining (original magnification: 40×). (B) Serum ALT and total bilirubin levels were compared at the indicated times. (C) Expression of F4/80 in the livers was examined by immunohistochemistry (original magnification: 200×). F4/80 positive area was compared (right panel). (D) $CD3^+$ cells in the livers were examined by immunohistochemistry (original magnification: 200×). Number of $CD3^+$ cells was compared. Data are mean ± SD from at least 5 independent experiments. *, $P<0.05$ vs. sham using a 2-tailed Student's t-test.

doi:10.1371/journal.pone.0065251.g002

Hydroxyproline Measurement

Hydroxyproline was measured for assessment of collagen content. The extracted liver protein was hydrolyzed in 6 M HCl (100°C, 24 h). The samples were neutralized with LiOH, and hydroxyproline was measured using a high-performance liquid chromatographic analyzer (Jasco, Hitachi, and Shimazu, Japan).

Isolation of Rat Primary HSC

Rat primary HSCs were isolated as previously described [29]. The liver was perfused via the portal vein with collagenase (Wako, Osaka, Japan) and pronase E (EMD Chemicals, Gibbstown, NJ, USA). After digestion, the cell suspension was filtered through nylon mesh and purified via 8.2% Nycodenz (Axis-Shield, Oslo, Norway) gradient centrifugation. The isolated HSCs were cultured in uncoated plastic dishes with DMEM (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum and antibiotic solution at 37°C in 5% CO_2. After plating for 4 h, the medium was changed to DMEM with 10% fetal bovine serum and antibiotics containing TNF-α (30 ng/ml, R&D Systems) for 72 h. The purity of HSCs was always 95% as determined by their typical starlike shape and abundant lipid droplets with vitamin A autofluorescence.

Western Blot

Electrophoresis of protein extracts and blotting were performed with anti-cyclin E (Santa Cruz Biotechnology, sc-481), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signaling Technology, Danvers, MA, USA, #2118), α-smooth muscle actin (αSMA) (Sigma-Aldrich, A2547), and TIMP-1 antibodies. Then, the membrane was incubated with the horseradish peroxidase-coupled secondary antibodies (Santa Cruz Biotechnology). Detection was performed with an ECL system (Amersham Biosciences, Buckinghamshire, UK), and the protein bands were quantified by densitometry using the ImageJ software.

Quantitative Real Time RT-PCR

RNA was extracted from liver tissue and cultured cells using the RNeasy and DNase Kits (Qiagen, Valencia, CA, USA) and was reverse-transcribed using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA). Quantitative real-time RT-PCR was performed using the SYBR Premix Ex Taq (Takara, Shiga, JAPAN) for mouse and rat TIMP-1 (forward; TGGGGAACCCATGAATTTAG, reverse; TCTGGCATCCTCTTGTTGC), rat collagen type I α1 (forward; TAGGCCATTGTGTATGCAGC, reverse; ACATGTTCAGCTTTGTGGACC), mouse αSMA (forward; GTTCAGTGGTGCCTCTGTCA, reverse; ACTGGGACGACATGGAAAAG), rat αSMA (forward; GTTCAGCGGCGCCTCCGTTA, reverse; ACTGGGACGACATGGAAAAG), rat and mouse desmin (forward; CTCGGAAGTTGAGAGCAGAGA, reverse; GTGAAGATGGCCTTGGATGT), mouse vimentin (forward; ACCGCTTTGCCAACTACAT, reverse; TTGTCCCGCTCCACCTC), and rat chemokine (C-C motif) ligand 5 (CCL5) (forward; CCACTTCTTCTCTGGGTTGG, reverse; GTGCCCACGTGAAGGAGTAT), and probe-primers sets (Applied Biosystems) for mouse procollagen type I α1 (Mm00801666g1), mouse transforming growth factor (TGF)-β1 (Mm00441724m1) and 18S ribosomal RNA (Hs99999901s1) with the LightCycler 480 (Roche Applied Science, Mannheim, Germany). The changes were normalized based on 18S rRNA values.

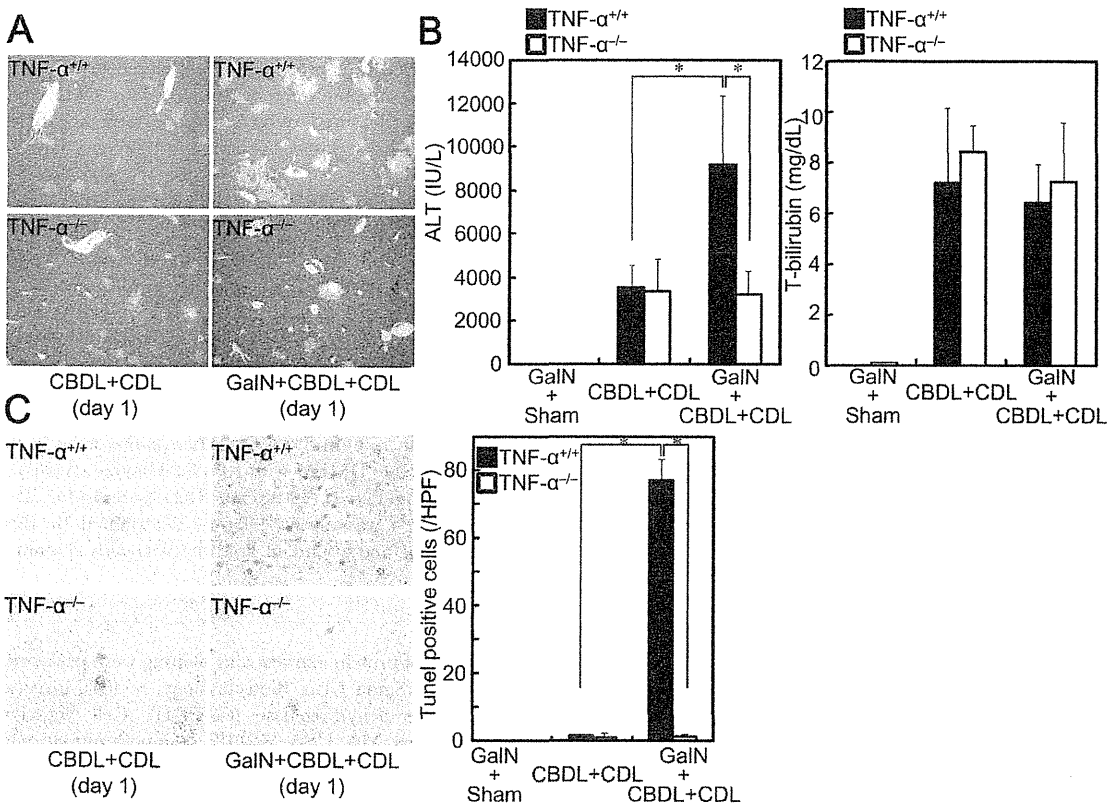

Figure 3. TNF-α-mediated increase of liver injury and hepatocyte apoptosis after CBDL+CDL was induced only in GalN sensitized mice. TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice were treated with or without GalN (20 mg) and subjected to CBDL+CDL. The animals were sacrificed 24 h after the surgery. (A) The injured lesions in the livers were assessed by H&E staining (original magnification: 40×). (B) Serum ALT and total bilirubin levels were compared. (C) Apoptotic nuclei were identified by TUNEL staining (original magnification: 400×). Numbers of TUNEL-positive cells were compared (right panel). Data are mean ± SD from at least 5 independent experiments. *, $P<0.05$ using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.g003

Gelatin Zymography

Gelatin zymography was performed with extracted proteins from the liver (50 μg) as described previously [30]. Frozen livers were homogenized in lysis buffer (20 mmol/L HEPES [pH 7.5], 150 mmol/L NaCl, 10 mmol/L CHAPS) and the homogenates were centrifuged at 20,000*g* for 20 min at 4°C. Proteins from supernatant were separated in 7.5% polyacrylamide gel containing 1 mg/mL of gelatin. The gels were equilibrated in developing buffer (50 mmol/L Tris [pH 7.4], 200 mmol/L NaCl, 10 mmol/L CaCl2, 0.02% sodium azaid). The gel was stained with 0.5% Coomassie Blue R-250, followed by destaining. Gelatinolytic activity was detected as clear bands on a dark blue background.

Statistical Analysis

Data are expressed as the mean ± SD of data collected from at least 5 independent experiments. Data between groups were analyzed by the 2-tailed Student's *t*-test. A *P* value of less than 0.05 was an indication of statistical significance.

Results

Deficiency of TNF-α Reduces CBDL+CDL-induced Liver Fibrosis

Serum TNF-α level was increased by CBDL+CDL in wild-type mice (Figure 1A), as previously reported [7]. To explore the roles of TNF-α on liver fibrosis, CBDL+CDL was performed on TNF-$\alpha^{-/-}$ mice. Fibrosis was induced in CBDL+CDL mice, as demonstrated by Sirius red staining and hydroxyproline content (Figure 1B, C). CBDL +CDL livers of TNF-$\alpha^{-/-}$ mice showed reduced fibrosis, compared to those of TNF-$\alpha^{+/+}$ mice (Figure 1B, C), suggesting that TNF-α contributes to liver fibrosis.

Liver fibrosis is induced by liver cell damage and inflammatory cell infiltration with impaired hepatocyte regeneration. However, TNF-$\alpha^{-/-}$ mice showed liver injury (Figure 2A), increased serum alanine aminotransferase (ALT) and total bilirubin (Figure 2B), infiltrated F4/80^{+} macrophages (Figure 2C), and number of infiltrated CD3^{+} lymphocytes (Figure 2D) after CBDL +CDL to a similar degree as was observed for TNF-$\alpha^{+/+}$ mice, suggesting that TNF-α is not related to liver injury and inflammatory cell infiltration in CBDL+CDL mice. To confirm the irrelevance of TNF-α to liver injury, the mice were pretreated with GalN, which increases sensitivity to TNF-α-induced liver damage and hepatocyte apoptosis [31], and subsequently received CBDL+CDL. GalN treatment alone did not induce liver injury or fibrosis (data not shown). Liver injury by CBDL+CDL was exacerbated in the GalN-pretreated mice (Figure 3A). Serum ALT levels were also higher in the GalN-pretreated mice than in the non-treated mice, although total bilirubin was comparable (Figure 3B), suggesting that hepatocyte cell death was exacerbated in the GalN-pretreated mice without increased cholestasis. Moreover, TUNEL-positive hepatocytes were increased in GalN-treated mice that received

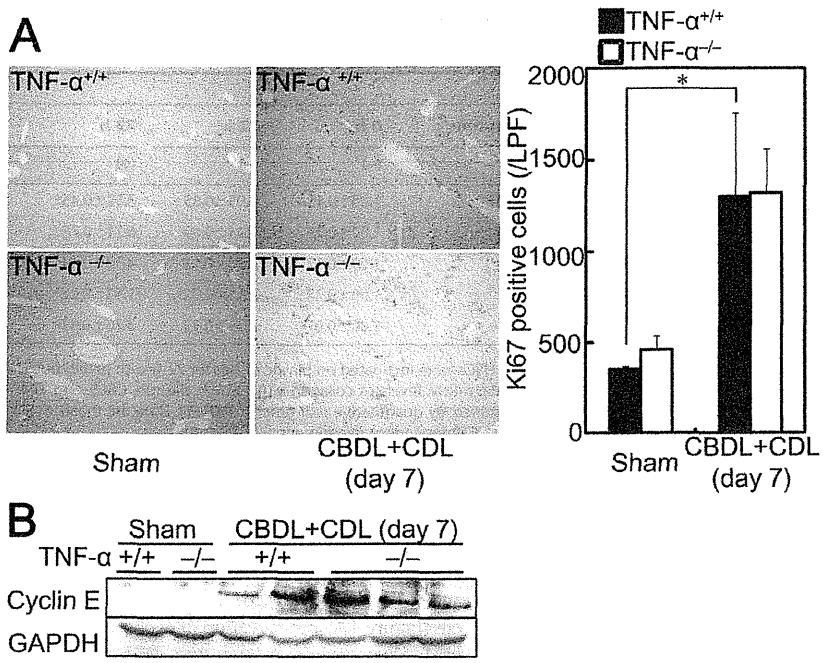

Figure 4. TNF-α deficiency did not affect hepatocyte regeneration after CBDL+CDL. TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received CBDL+CDL. The animals were sacrificed 7 days after the surgery. (A) Expression of Ki67 in the livers was examined by immunohistochemistry (original magnification: 40×). Number of $Ki67^{+}$ cells was compared (right panel). Data are mean ± SD from at least 5 independent experiments. *, $P<0.05$ using a 2-tailed Student's t-test. (B) The protein extracts from the livers were subjected to SDS-PAGE and immunoblotting was performed with anti-cyclin E and -GAPDH antibodies. The results shown are representative of at least 3 independent experiments.
doi:10.1371/journal.pone.0065251.g004

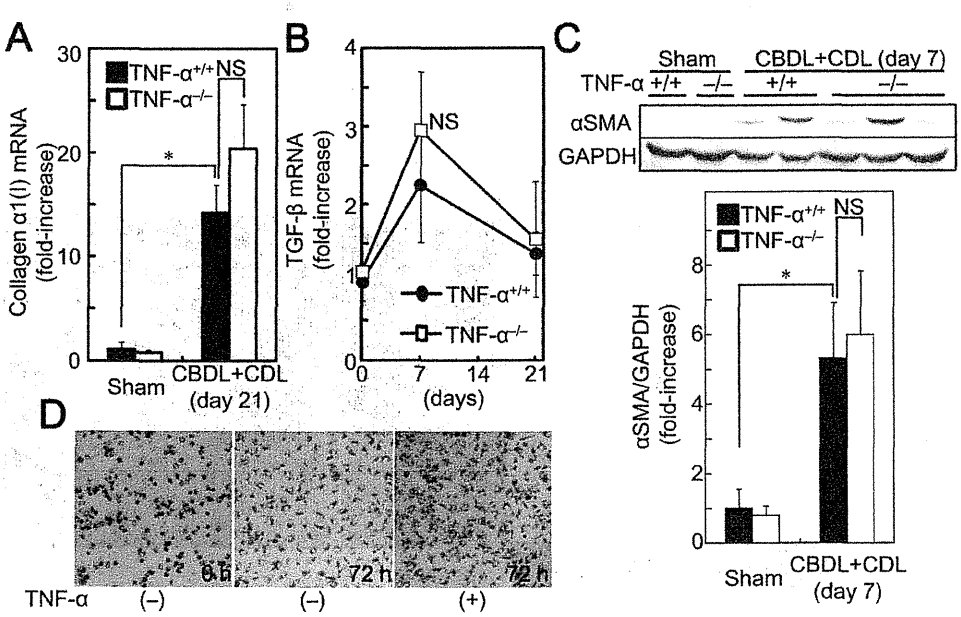

Figure 5. TNF-α deficiency did not affect collagen α1(I) mRNA expression and HSC activation in the livers of mice after CBDL+CDL. TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received CBDL+CDL. The animals were sacrificed at the indicated times. (A, B) mRNA levels of collagen α1(I) (A) and TGF-β1 (TGF-β) (B) in the livers were determined by quantitative real time RT-PCR. (C) The protein extracts from the livers were analyzed by SDS-PAGE, and immunoblotting was performed with anti-αSMA and -GAPDH antibodies. The results shown are representative of at least 5 independent experiments. Relative densitometric intensity of αSMA was determined for each protein band and normalized to GAPDH (bottom panels). (D) Primary rat HSCs were incubated on plastic dishes for 72 h with or without 30 ng/mL TNF-α. Bright field images of the HSCs are shown. Data are mean ± SD from at least 5 independent experiments. *, $P<0.05$ using a 2-tailed Student's t-test. NS, not significant.
doi:10.1371/journal.pone.0065251.g005

Table 1. Changes in the mRNA profiles of the liver after CBDL+CDL in TNF-$\alpha^{-/-}$ mice TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received CBDL+CDL.

	sham		CBDL+CDL	
	TNF-$\alpha^{+/+}$	TNF-$\alpha^{-/-}$	TNF-$\alpha^{+/+}$	TNF-$\alpha^{-/-}$
αSMA	1.00±0.64	1.36±0.25	5.43±0.98*	6.32±1.22
desmin	1.00±0.36	1.19±0.17	1.73±0.28*	1.76±0.69
vimentin	1.00±0.18	1.22±0.23	2.77±0.82*	3.66±0.77

The animals were sacrificed 7 days after the surgery. mRNA levels of αSMA, desmin, and vimentin in the livers were determined by quantitative real time RT-PCR. Results are presented as means ± SD of data collected from at least 5 independent experiments.
*P<0.05 versus sham-operated TNF-$\alpha^{+/+}$ mice using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.t001

Table 2. Effect of TNF-α on mRNA profiles of primary isolated rat HSCs.

Incubation time	0 h	72 h	72 h
TNF-α	(-)	(-)	(+)
collagen α1(I)	1.00±0.12	7.95±0.85	3.52±0.45*
αSMA	1.00±0.16	7.44±0.69	6.71±0.78
desmin	1.00±0.17	2.20±0.11	4.32±0.44*
CCL5	1.00±0.23	0.15±0.02	1.63±0.25*
TIMP-1	1.00±0.07	2.25±0.22	3.76±0.33*

Primary rat HSCs were incubated on plastic dishes for 72 h with or without TNF-α (30 ng/mL). mRNA levels of collagen α1(I), αSMA, desmin, CCL5, and TIMP-1 were determined by quantitative real time RT-PCR (E). Data are mean ± SD from at least 6 independent experiments.
*P<0.05 versus 72 h cultured HSCs without TNF-α using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.t002

CBDL+CDL (Figure 3C). These effects of GalN were blunted in TNF-$\alpha^{-/-}$ mice (Figure 3). TNF-α treatment alone does not induce hepatocyte cell death, and the sensitization of hepatocytes by GalN is required for TNF-α-induced liver injury in mice [11,12]. These previous findings, in conjunction with our results, suggest that the liver damage increments caused by GalN combination is induced by the TNF-α produced by CBDL+CDL; TNF-α causes liver damage only when the hepatocytes are sensitized by GalN in CBDL+CDL mice, and TNF-α does not contribute to liver damage in CBDL+CDL without GalN. Thus, this liver damage may be primarily induced by accumulated cytotoxic bile acids. Furthermore, CBDL+CDL-mediated induction of $Ki67^+$ cells (Figure 4A) and elevation of cyclin E (Figure 4B), which are makers for liver regeneration, in the livers of TNF-$\alpha^{-/-}$ mice was comparable to that in TNF-$\alpha^{+/+}$ mice, suggesting that liver regeneration after CBDL+CDL was not mediated by the

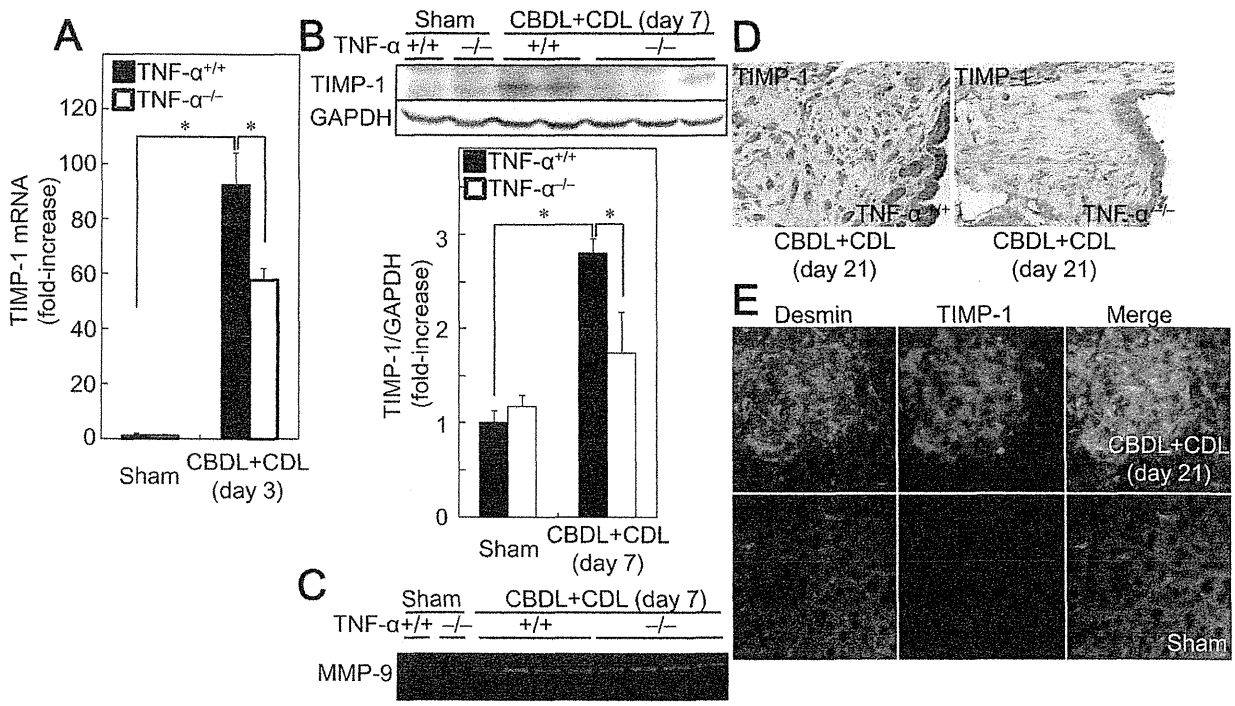

Figure 6. CBDL+CDL increased TIMP-1 in a TNF-α dependent manner. TNF-$\alpha^{+/+}$ and TNF-$\alpha^{-/-}$ mice received CBDL+CDL. The animals were sacrificed at the indicated times. (A) mRNA levels of TIMP-1 in the livers were determined by quantitative real time RT-PCR. (B) The protein extracts from the livers were analyzed by SDS-PAGE, and immunoblotting was performed with anti-TIMP-1 and -GAPDH antibodies. The results shown are representative of at least 5 independent experiments. Relative densitometric intensity of TIMP-1 was determined for each protein band and normalized to GAPDH (bottom panels). (C) Collagenase activities in the protein extracts were measured by gelatin zymography. (D) Expression of TIMP-1 in the livers was examined by immunohistochemistry (original magnification: 400×). (E) Expression of desmin (green) and TIMP-1 (red) around the interstitial space around dilated bile ducts was examined by immunofluorescent staining. The results shown are representative of at least 3 independent experiments. Data are mean ± SD from at least 5 independent experiments. *, P<0.05 using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.g006

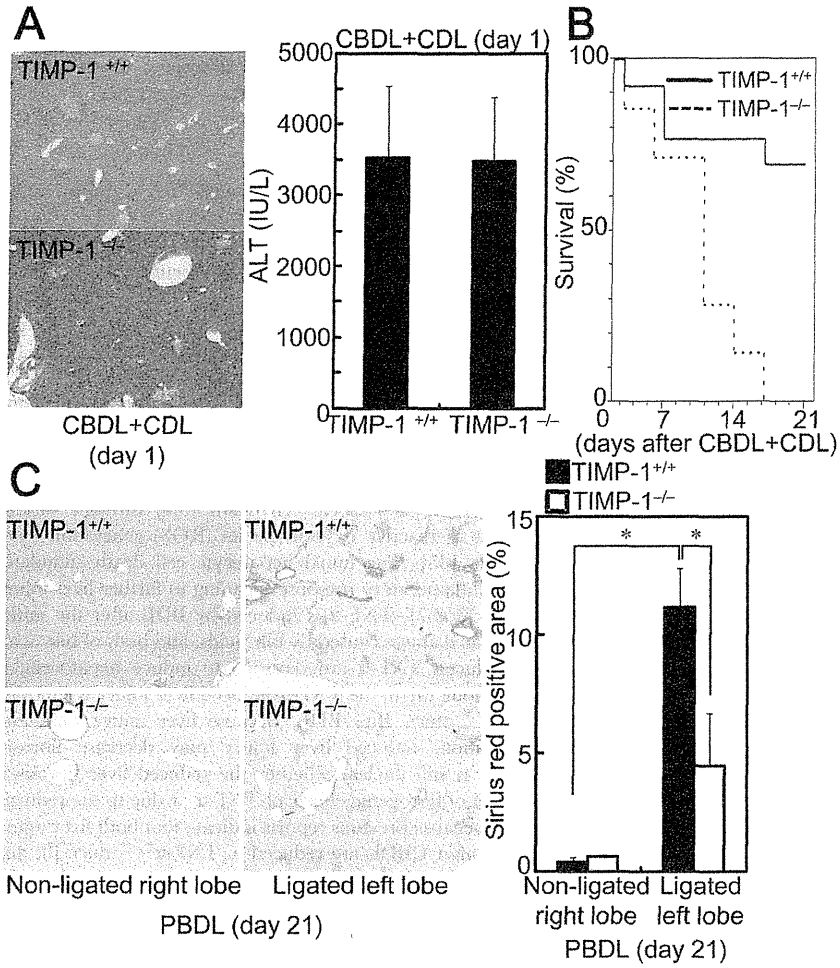

Figure 7. TIMP-1 deficiency reduced liver fibrosis after BDL. TIMP-1$^{+/+}$ and TIMP-1$^{-/-}$ mice received CBDL+CDL (A, B) or PBDL (C). The animals were sacrificed at the indicated times. (A) The injured lesions in the livers were assessed by H&E staining (original magnification: 40×, left panel). Serum ALT levels were compared (right panel). (B) Survival curves for animals with CBDL+CDL. (C) Collagen deposition in the ligated left robes was assessed by Sirius red staining (original magnification: 40×). Sirius red positive area was compared (right panel). Data are mean ± SD from at least 5 independent experiments. *, $P<0.05$ using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.g007

produced TNF-α. Thus, the reduced fibrosis in TNF-α$^{-/-}$ mice after CBDL+CDL was not related to the reduction of liver damage or the enhancement of liver regeneration.

TNF-α Decreases Collagen mRNA Expression in Isolated rat HSCs

Because fibrosis was decreased in TNF-α$^{-/-}$ mice, it is possible that TNF-α activates HSCs and increases their collagen production. Indeed, it is reported that TNF-α activates primary cultured HSCs by activation of p38 mitogen-activated protein kinase (MAPK) and c-jun N-terminal kinase (JNK) [32]. To compare the activity status of HSCs between TNF-α$^{+/+}$ and TNF-α$^{-/-}$ mice, we examined expression levels of collagen α1(I) mRNA, TGF-β mRNA, which is an activator of HSCs, desmin and vimentin mRNA, which are classical features of HSCs, and αSMA mRNA and protein, which is a maker for stellate cell activation. The increased CBDL+CDL-mediated expression levels of collagen α1(I) mRNA (Figure 5A), TGF-β mRNA (Figure 5B), αSMA mRNA, desmin mRNA, vimentin mRNA (Table 1), and αSMA protein (Figure 5C) in the livers of TNF-α$^{-/-}$ mice were comparable to those of TNF-α$^{+/+}$ mice, suggesting that TNF-α does not contribute to activation of HSCs and production of collagen in CBDL+CDL mice. To examine the direct effects of TNF-α on collagen expression in HSCs, primary HSCs were isolated from rats and were cultured on plastic dishes, on which the cells were automatically activated and proliferated (Figure 5D). According to the autoactivation, expression levels of collagen α1(I), αSMA, and desmin mRNA in HSCs were increased by culture on plastic dishes for 72 h (Table 2). Exogenous administration of TNF-α decreased the induction of collagen α1(I) mRNA as previously reported [15,16,17], although αSMA was not reduced by TNF-α. Moreover, TNF-α increased desmin and CCL5 mRNA in HSCs (Table 2) which is a mediator of HSC proliferation [33]. Thus, TNF-α decreases collagen α1(I) production without inhibition of activation and proliferation in primary cultured HSCs. These results further suggest that the reduced fibrosis in TNF-α$^{-/-}$ mice is not due to reduction of collagen synthesis in HSCs.

Table 3. Changes in the mRNA profiles of the liver after CBDL+CDL in TIMP-1$^{-/-}$ mice TIMP-1$^{+/+}$ and TIMP-1$^{-/-}$ mice received CBDL+CDL.

	sham		CBDL+CDL	
	TIMP-1$^{+/+}$	TIMP-1$^{-/-}$	TIMP-1$^{+/+}$	TIMP-1$^{-/-}$
αSMA	1.00±0.64	1.04±0.30	5.43±0.98*	4.01±1.72
desmin	1.00±0.36	1.39±0.18	1.73±0.28*	1.59±0.34
vimentin	1.00±0.18	1.19±0.29	2.77±0.82*	2.05±0.86

The animals were sacrificed 7 days after the surgery. mRNA levels of αSMA, desmin, and vimentin in the livers were determined by quantitative real time RT-PCR. Results are presented as means ± SD of data collected from at least 5 independent experiments.
*P<0.05 versus sham-operated TIMP-1$^{+/+}$ mice using a 2-tailed Student's t-test.
doi:10.1371/journal.pone.0065251.t003

TIMP-1 is Induced in BDL Livers in a TNF-α-dependent Manner

After finding that collagen mRNA was not decreased in TNF-α$^{-/-}$ mice, we hypothesized that the reduced fibrosis in the mice was due to post-transcriptional effects. To elucidate the mechanisms by which TNF-α contributes to BDL-mediated liver fibrosis, we focused on TIMP-1, which is an endogenous inhibitor of MMPs. Consistent with a report indicating that a TNF-α inhibitor can prevent the increase in rat hepatic TIMP-1 by CCl_4 treatment [34], we found that CBDL+CDL increased mRNA (Figure 6A) and protein (Figure 6B) expression of TIMP-1 in the liver, and the induction was lower in TNF-α$^{-/-}$ mice than in TNF-α$^{+/+}$ mice. In contrast, gelatin zymography showed that MMP-9 (Figure 6C) and MMP-2 (data not shown) were equally activated by CBDL+CDL in both TNF-α$^{+/+}$ and TNF-α$^{-/-}$ mice. TIMP-1 positive cells were induced in the increased interstitial space around the dilated bile ducts (Figure 6D) and most of the desmin^{+} cells (a marker of HSCs) showed double staining for TIMP-1 (Figure 6E), suggesting that TIMP-1 is expressed by HSCs. In addition, TIMP-1 mRNA expression in primary cultured rat HSCs was increased by TNF-α administration (Table 2), indicating that TNF-α stimulates TIMP-1 production, as previously reported [9,19,20]. These results suggest that the TNF-α produced by BDL induces TIMP-1 in HSCs.

Fibrosis is Reduced in TIMP-1$^{-/-}$ Mice

Induction of TIMP-1, but not MMPs, was reduced in TNF-α$^{-/-}$ mice. This led to the hypothesis that insufficient TIMP-1 expression leads to increased collagen removal by MMPs in TNF-α$^{-/-}$ mice. To investigate the roles of TIMP-1 on fibrosis, we performed BDL on TIMP-1$^{-/-}$ mice. TIMP-1$^{-/-}$ mice showed a similar degree of increased liver injury and serum ALT (Figure 7A) as TIMP-1$^{+/+}$ mice at 1 day after CBDL+CDL. In addition, the increased expression levels of αSMA, desmin, and vimentin mRNA in the livers of TIMP-1$^{-/-}$ mice at 7 days after CBDL+CDL were comparable to those in TIMP-1$^{+/+}$ mice (Table 3). These results suggest that TIMP-1 does not play a role in hepatocyte cell death induced by bile acid or in activation of HSCs in CBDL+CDL mice. Surprisingly, the mortality rate of TIMP-1$^{-/-}$ mice that received CBDL+CDL was extremely high; 0% of mice remained by 21 days after the surgery (Figure 7B). Mice treated with our established model, PBDL, only showed liver injury and fibrosis in the bile duct ligated left lobe of the liver, which improved the survival rate to 100% in both the TIMP-1$^{+/+}$ and TIMP-1$^{-/-}$ mice. Therefore, we used PBDL for the TIMP-1$^{-/-}$ mice. Sirius red staining showed a smaller positive area in the ligated left lobe of the TIMP-1$^{-/-}$ mice (Figure 7C), suggesting that fibrosis is reduced by a TIMP-1 deficiency. Thus, TIMP-1 may have fibrogenic effects due to inhibition of collagen removal by MMPs.

Discussion

The present study investigated the contribution of TNF-α to the progression of fibrosis after cholestatic liver injury. The results, which indicate that TNF-α increases liver fibrosis through TIMP-1 production from HSCs, suggest novel therapeutic possibilities for treating liver fibrosis.

TNF-α has been thought to be crucial for liver injury and fibrosis by BDL because those are reduced in TNF-α$^{-/-}$ mice [8] and TNFR1$^{-/-}$ mice [9]. Gabele et al. reported that TNF-α$^{-/-}$ mice display reduced levels of serum ALT, Sirius red positive area, collagen-I protein expression, αSMA positive cells, and TGF-β mRNA in the liver after BDL [8]. After BDL, hepatocytes are exposed to elevated concentrations of bile acid, and hydrophobic bile acids lead to hepatocyte cell death [3,4] through various factors, such as reactive oxygen species (ROS) generation from mitochondria [35]. The initial hepatocyte cell death stimulates subsequent inflammatory responses, leading to further liver injury and fibrosis [36,37]. TNF-α is induced by BDL after the initial hepatocellular damage caused by bile acids, and both of bile acids and the induced TNF-α are reported to induce hepatocellular damage. Among them, the TNF-α-induced liver injury is canceled in TNF-α$^{-/-}$ mice after BDL. Because liver injury stimulates HSC activation, reduced liver injury may decrease fibrosis. However, it is still unclear whether the reduced liver fibrosis is due to a lack of fibrogenic effects of TNF-α or due to the reduced liver injury because previous reports indicate that both liver injury and fibrosis after CBDL are reduced in TNF-α$^{-/-}$ mice [8] and TNFR1$^{-/-}$ mice [9]. In contrast to those reports, our study found a reduction of liver fibrosis after CBDL+CDL in TNF-α$^{-/-}$ mice without a reduction in liver injury. This can be attributed to this study's use of CBDL+CDL, which is composed of CBDL and CDL, in contrast to the usual CBDL procedure, in which only the common bile duct is ligated, used in the previous studies. In CBDL+CDL mice, congested bile did not remain in the gall bladder, leading to the post-surgical exposure of hepatocytes to high concentrations of bile acid, which induced severe hepatocyte cell death. CBDL+CDL induced same degree of liver damage in TNF-α$^{-/-}$ compared with TNF-α$^{+/+}$ suggesting that the liver injury induced by bile acid may be severe enough to conceal TNF-α-mediated liver injury. In actuality, pretreatment with GalN exacerbated the CBDL+CDL-induced liver injury that was blunted in TNF-α$^{-/-}$ mice, indicating that TNF-α has a minor role in liver injury in CBDL+CDL mice. TNF-α$^{-/-}$ mice showed reduced fibrosis after CBDL+CDL, without reduction of liver injury and inflammatory cell infiltration or enhancement of liver regeneration, indicating a direct contribution by TNF-α to liver fibrosis in CBDL+CDL mice.

The profibrogenic effects of TNF-α in another fibrosis model, which uses CCl_4, have been reported previously, indicating that TNFR1 deficiency inhibits liver fibrosis after CCl_4 treatment, without any effects on liver injury [10,38]. TGF-β, one of the main fibrogenic factors, stimulates HSCs to induce collagen I α1 transcription [39]. However, the induction of TGF-β and collagen mRNAs after CBDL+CDL was similar in TNF-α$^{-/-}$ and TNF-α$^{+/+}$ mice. Moreover, exogenous administration of TNF-α decreased the induction of collagen α1(I) mRNA in primary cultured rat HSCs. These results suggest that TNF-α induces liver fibrosis post-transcriptionally. MMPs and their inhibitor, TIMP,

post-translationally modulate ECM remodeling, and the balance of their expression plays an important role in liver fibrosis [18]. Indeed, CBDL+CDL increased TIMP-1 expression, which can inhibit a broad range of MMPs and was attenuated by TNF-α deficiency. In addition, fibrosis in the ligated lobes of PBDL livers was reduced in TIMP-$1^{-/-}$ mice compared to that in TIMP-$1^{+/+}$ mice. Thus, insufficient TIMP-1 production is a possible explanation for the reduced fibrosis in TNF-$\alpha^{-/-}$ mice after CBDL+CDL. In contrast to our results, it has been reported that CCl_4-induced liver fibrosis and liver injury are increased in TIMP-$1^{-/-}$ mice [25]. In addition to its profibrogenic effects, TIMP-1 has a protective effect on hepatocytes [25]. Inhibition of MMPs blocks apoptosis of hepatocytes [40]. TIMP-$1^{-/-}$ mice demonstrate impaired liver injury and hepatocyte proliferation after hepatic ischemia and reperfusion [24]. Thus, deletion of TIMP-1 could accelerate liver fibrosis by increasing liver injury. Indeed, TIMP-$1^{-/-}$ mice that received CBDL+CDL showed an extremely high mortality rate, with an initial liver injury rate that was comparable to TIMP-$1^{+/+}$ mice, suggesting that TIMP-1 has roles in the BDL liver, in addition to MMP inhibition. However, our results using PBDL suggest that TIMP-1 has a profibrogenic role in cholestatic liver injury. TIMP-1 can act directly through cell surface receptors, in addition to indirectly directing cell fate through modulation of protease activity [41]. Both stimulatory [24] and inhibitory [42] effects of TIMP-1 on liver regeneration have been reported. Thus, further studies are required to investigate the multifunctional effects of TIMP-1 on liver injury and fibrosis. In addition, the mechanism of TIMP-1 up-regulation induced by TNF-α in the BDL liver is still unclear. It has been reported that activation of HSCs is accompanied by induction of TIMP-1 promoter activity and mRNA expression, and that AP-1, Pea3, and TIMP-1 element 1 are reported to be involved in transcriptional activity of the TIMP-1 promoter [43,44,45]. Moreover, TIMP-1 expression is also regulated by changes in mRNA stability [46]. Further studies are also needed to resolve this uncertainty.

In conclusion, we observed that TNF-α produced by cholestasis promoted liver fibrosis via TIMP-1 production from HSCs. Thus, targeting TNF-α and TIMP-1 may become a new therapeutic strategy for treating liver fibrosis and cholestatic liver injury.

Author Contributions

Conceived and designed the experiments: YO. Performed the experiments: YO MH. Analyzed the data: YO MH IY TS HM OK. Contributed reagents/materials/analysis tools: YO MH. Wrote the paper: YO.

References

1. Bataller R, Brenner DA (2001) Hepatic stellate cells as a target for the treatment of liver fibrosis. Semin Liver Dis 21: 437–451.
2. Park YJ, Qatanani M, Chua SS, LaRey JL, Johnson SA, et al. (2008) Loss of orphan receptor small heterodimer partner sensitizes mice to liver injury from obstructive cholestasis. Hepatology 47: 1578–1586.
3. Jang JH, Rickenbacher A, Humar B, Weber A, Raptis DA, et al. (2012) Serotonin protects mouse liver from cholestatic injury by decreasing bile salt pool after bile duct ligation. Hepatology 56: 209–218.
4. Sokol RJ, Devereaux M, Dahl R, Gumpricht E (2006) "Let there be bile"–understanding hepatic injury in cholestasis. J Pediatr Gastroenterol Nutr 43 Suppl 1: S4–9.
5. Higuchi H, Gores GJ (2003) Bile acid regulation of hepatic physiology: IV. Bile acids and death receptors. Am J Physiol Gastrointest Liver Physiol 284: G734–738.
6. Schwabe RF, Brenner DA (2006) Mechanisms of Liver Injury. I. TNF-alpha-induced liver injury: role of IKK, JNK, and ROS pathways. Am J Physiol Gastrointest Liver Physiol 290: G583–589.
7. Bemelmans MH, Gouma DJ, Greve JW, Buurman WA (1992) Cytokines tumor necrosis factor and interleukin-6 in experimental biliary obstruction in mice. Hepatology 15: 1132–1136.
8. Gabele E, Froh M, Arteel GE, Uesugi T, Hellerbrand C, et al. (2009) TNFalpha is required for cholestasis-induced liver fibrosis in the mouse. Biochem Biophys Res Commun 378: 348–353.
9. Tarrats N, Moles A, Morales A, Garcia-Ruiz C, Fernandez-Checa JC, et al. (2011) Critical role of tumor necrosis factor receptor 1, but not 2, in hepatic stellate cell proliferation, extracellular matrix remodeling, and liver fibrogenesis. Hepatology 54: 319–327.
10. Sudo K, Yamada Y, Moriwaki H, Saito K, Seishima M (2005) Lack of tumor necrosis factor receptor type 1 inhibits liver fibrosis induced by carbon tetrachloride in mice. Cytokine 29: 236–244.
11. Nagaki M, Sugiyama A, Osawa Y, Naiki T, Nakashima S, et al. (1999) Lethal hepatic apoptosis mediated by tumor necrosis factor receptor, unlike Fas-mediated apoptosis, requires hepatocyte sensitization in mice. J Hepatol 31: 997–1005.
12. Osawa Y, Nagaki M, Banno Y, Yamada Y, Imose M, et al. (2001) Possible involvement of reactive oxygen species in D-galactosamine-induced sensitization against tumor necrosis factor-alpha-induced hepatocyte apoptosis. J Cell Physiol 187: 374–385.
13. Nagaki M, Naiki T, Brenner DA, Osawa Y, Imose M, et al. (2000) Tumor necrosis factor alpha prevents tumor necrosis factor receptor-mediated mouse hepatocyte apoptosis, but not fas-mediated apoptosis: role of nuclear factor-kappaB. Hepatology 32: 1272–1279.
14. Yamada Y, Kirillova I, Peschon JJ, Fausto N (1997) Initiation of liver growth by tumor necrosis factor: deficient liver regeneration in mice lacking type I tumor necrosis factor receptor. Proc Natl Acad Sci U S A 94: 1441–1446.
15. Varela-Rey M, Fontan-Gabas L, Blanco P, Lopez-Zabalza MJ, Iraburu MJ (2007) Glutathione depletion is involved in the inhibition of procollagen alpha1(I) mRNA levels caused by TNF-alpha on hepatic stellate cells. Cytokine 37: 212–217.
16. Hernandez-Munoz I, de la Torre P, Sanchez-Alcazar JA, Garcia I, Santiago E, et al. (1997) Tumor necrosis factor alpha inhibits collagen alpha 1(I) gene expression in rat hepatic stellate cells through a G protein. Gastroenterology 113: 625–640.
17. Houglum K, Buck M, Kim DJ, Chojkier M (1998) TNF-alpha inhibits liver collagen-alpha 1(I) gene expression through a tissue-specific regulatory region. Am J Physiol 274: G840–847.
18. Arthur MJ (2000) Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. Am J Physiol Gastrointest Liver Physiol 279: G245–249.
19. Tomita K, Tamiya G, Ando S, Ohsumi K, Chiyo T, et al. (2006) Tumour necrosis factor alpha signalling through activation of Kupffer cells plays an essential role in liver fibrosis of non-alcoholic steatohepatitis in mice. Gut 55: 415–424.
20. Knittel T, Mehde M, Kobold D, Saile B, Dinter C, et al. (1999) Expression patterns of matrix metalloproteinases and their inhibitors in parenchymal and non-parenchymal cells of rat liver: regulation by TNF-alpha and TGF-beta1. J Hepatol 30: 48–60.
21. Roderfeld M, Weiskirchen R, Wagner S, Berres ML, Henkel C, et al. (2006) Inhibition of hepatic fibrogenesis by matrix metalloproteinase-9 mutants in mice. Faseb J 20: 444–454.
22. Parsons CJ, Bradford BU, Pan CQ, Cheung E, Schauer M, et al. (2004) Antifibrotic effects of a tissue inhibitor of metalloproteinase-1 antibody on established liver fibrosis in rats. Hepatology 40: 1106–1115.
23. Yoshiji H, Kuriyama S, Miyamoto Y, Thorgeirsson UP, Gomez DE, et al. (2000) Tissue inhibitor of metalloproteinases-1 promotes liver fibrosis development in a transgenic mouse model. Hepatology 32: 1248–1254.
24. Duarte S, Hamada T, Kuriyama N, Busuttil RW, Coito AJ (2012) TIMP-1 deficiency leads to lethal partial hepatic ischemia and reperfusion injury. Hepatology 56: 1074–1085.
25. Wang H, Lafdil F, Wang L, Yin S, Feng D, et al. (2011) Tissue inhibitor of metalloproteinase 1 (TIMP-1) deficiency exacerbates carbon tetrachloride-induced liver injury and fibrosis in mice: involvement of hepatocyte STAT3 in TIMP-1 production. Cell Biosci 1: 14.
26. Osawa Y, Seki E, Adachi M, Taura K, Kodama Y, et al. (2006) Systemic mediators induce fibrogenic effects in normal liver after partial bile duct ligation. Liver Int 26: 1138–1147.
27. Osawa Y, Seki E, Adachi M, Suetsugu A, Ito H, et al. (2010) Role of acid sphingomyelinase of Kupffer cells in cholestatic liver injury in mice. Hepatology 51: 237–245.
28. Osawa Y, Suetsugu A, Matsushima-Nishiwaki R, Yasuda I, Saibara T, et al. (2013) Liver acid sphingomyelinase inhibits growth of metastatic colon cancer. J Clin Invest.
29. Osawa Y, Uchinami H, Bielawski J, Schwabe RF, Hannun YA, et al. (2005) Roles for C16-ceramide and sphingosine 1-phosphate in regulating hepatocyte apoptosis in response to tumor necrosis factor-alpha. J Biol Chem 280: 27879–27887.
30. Osawa Y, Hannun YA, Proia RL, Brenner DA (2005) Roles of AKT and sphingosine kinase in the antiapoptotic effects of bile duct ligation in mouse liver. Hepatology 42: 1320–1328.

31. Osawa Y, Banno Y, Nagaki M, Nozawa Y, Moriwaki H, et al. (2001) Caspase activation during hepatocyte apoptosis induced by tumor necrosis factor-alpha in galactosamine-sensitized mice. Liver 21: 309–319.
32. Reeves HL, Dack CL, Peak M, Burt AD, Day CP (2000) Stress-activated protein kinases in the activation of rat hepatic stellate cells in culture. J Hepatol 32: 465–472.
33. Schwabe RF, Bataller R, Brenner DA (2003) Human hepatic stellate cells express CCR5 and RANTES to induce proliferation and migration. Am J Physiol Gastrointest Liver Physiol 285: G949–958.
34. Roderfeld M, Geier A, Dietrich CG, Siewert E, Jansen B, et al. (2006) Cytokine blockade inhibits hepatic tissue inhibitor of metalloproteinase-1 expression and up-regulates matrix metalloproteinase-9 in toxic liver injury. Liver Int 26: 579–586.
35. Yerushalmi B, Dahl R, Devereaux MW, Gumpricht E, Sokol RJ (2001) Bile acid-induced rat hepatocyte apoptosis is inhibited by antioxidants and blockers of the mitochondrial permeability transition. Hepatology 33: 616–626.
36. Jaeschke H (2002) Inflammation in response to hepatocellular apoptosis. Hepatology 35: 964–966.
37. Canbay A, Higuchi H, Bronk SF, Taniai M, Sebo TJ, et al. (2002) Fas enhances fibrogenesis in the bile duct ligated mouse: a link between apoptosis and fibrosis. Gastroenterology 123: 1323–1330.
38. Simeonova PP, Gallucci RM, Hulderman T, Wilson R, Kommineni C, et al. (2001) The role of tumor necrosis factor-alpha in liver toxicity, inflammation, and fibrosis induced by carbon tetrachloride. Toxicol Appl Pharmacol 177: 112–120.
39. Bataller R, Brenner DA (2005) Liver fibrosis. J Clin Invest 115: 209–218.
40. Wielockx B, Lannoy K, Shapiro SD, Itoh T, Itohara S, et al. (2001) Inhibition of matrix metalloproteinases blocks lethal hepatitis and apoptosis induced by tumor necrosis factor and allows safe antitumor therapy. Nat Med 7: 1202–1208.
41. Stetler-Stevenson WG (2008) Tissue inhibitors of metalloproteinases in cell signaling: metalloproteinase-independent biological activities. Sci Signal 1: re6.
42. Mohammed FF, Pennington CJ, Kassiri Z, Rubin JS, Soloway PD, et al. (2005) Metalloproteinase inhibitor TIMP-1 affects hepatocyte cell cycle via HGF activation in murine liver regeneration. Hepatology 41: 857–867.
43. Iredale JP, Benyon RC, Arthur MJ, Ferris WF, Alcolado R, et al. (1996) Tissue inhibitor of metalloproteinase-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. Hepatology 24: 176–184.
44. Bahr MJ, Vincent KJ, Arthur MJ, Fowler AV, Smart DE, et al. (1999) Control of the tissue inhibitor of metalloproteinases-1 promoter in culture-activated rat hepatic stellate cells: regulation by activator protein-1 DNA binding proteins. Hepatology 29: 839–848.
45. Trim JE, Samra SK, Arthur MJ, Wright MC, McAulay M, et al. (2000) Upstream tissue inhibitor of metalloproteinases-1 (TIMP-1) element-1, a novel and essential regulatory DNA motif in the human TIMP-1 gene promoter, directly interacts with a 30-kDa nuclear protein. J Biol Chem 275: 6657–6663.
46. Doyle GA, Saarialho-Kere UK, Parks WC (1997) Distinct mechanisms regulate TIMP-1 expression at different stages of phorbol ester-mediated differentiation of U937 cells. Biochemistry 36: 2492–2500.

J Gastroenterol (2013) 48:1051–1060
DOI 10.1007/s00535-012-0704-y

ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Noninvasive scoring systems in patients with nonalcoholic fatty liver disease with normal alanine aminotransferase levels

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Received: 16 August 2012 / Accepted: 14 October 2012 / Published online: 27 November 2012

Abstract
Background The severity of liver fibrosis must be estimated to determine the prognosis, for surveillance, and for optimal treatment of nonalcoholic fatty liver disease (NAFLD). However, the severity of hepatic fibrosis tends to be underestimated in patients with normal ALT.
Methods We investigated histological data and scoring systems (FIB-4 index, NAFLD fibrosis score, BARD score, and AST/ALT ratio) of 1,102 liver-biopsy-confirmed NAFLD patients.
Results A total of 235 NAFLD patients with normal ALT were estimated to exist. The ratio of advanced fibrosis (stage 3–4) was seen in 16.1 % of subjects with normal ALT. Scoring systems, especially the FIB-4 index and NAFLD fibrosis score, were clinically very useful (AUROC >0.8), even in patients with normal ALT. Furthermore, with resetting of the cutoff values, the FIB-4 index (>1.659) and NAFLD fibrosis score (>0.735) were found to have a higher sensitivity and higher specificity for the prediction of advanced fibrosis, and all of these scoring systems (FIB-4 index, NAFLD fibrosis score, BARD score, and AST/ALT ratio) had higher negative predictive values (>90.3 %). By using the resetting cutoff value, liver biopsy could have been avoided in 60.4 % (FIB-4), 66.4 % (NAFLD fibrosis score), 51.9 % (BARD score), and 62.1 % (AST/ALT ratio).

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Conclusions We reset the cutoff values of numerous non-invasive scoring systems to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal ALT, and these non-invasive scoring systems with the reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

Keywords NAFLD · NASH · Normal ALT · Scoring systems

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUROC	Area under the receiver-operating characteristic curve
BMI	Body mass index
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
NPV	Negative predictive value
PPV	Positive predictive value
AAR	AST/ALT ratio

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an important clinical subtype of chronic liver disease in many countries around the world [1]. The histological changes range over a wide spectrum, extending from simple steatosis, which is generally non-progressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, liver failure, and sometimes even hepatocellular carcinoma [2–5]. The severity of liver fibrosis must be estimated to determine the prognosis, for surveillance, and for optimal treatment of NAFLD, similar to the case for other liver diseases [6]. Liver biopsy is recommended as the gold standard for the diagnosis and staging of fibrosis in patients with NASH [1, 2, 7]. This procedure, however, is invasive and is associated with a high risk of complications [8]. Approximately 24.6 % of all patients complain of pain during/after the biopsy procedure [9], and the estimated risk of severe complications is 3.1 per 1,000 procedures [10]. Furthermore, it is impossible to enforce liver biopsy in all NAFLD patients, because the estimated number of NAFLD patients has reached 80–100 million in the US and over 20 million in Japan [11]. These considerations underscore the need for the development of simple non-invasive methods for assessing the severity of fibrosis.

Numerous non-invasive panels of tests have been developed for the staging of liver disease consisting of combinations of clinical and routine laboratory parameters, as well as specialized tests, such as direct markers of fibrosis and elastography [12–15]. Especially serum alanine aminotransferase (ALT) has long been used as a surrogate marker of liver injury [16, 17] and has been used in many scoring systems for various liver diseases, including NAFLD, such as the aspartate aminotransferase (AST)-to-ALT ratio (AAR) [18], NAFLD fibrosis score [19], BARD score [20], and FIB-4 index [21]. It is, however, well known that both fatty liver and NASH may exist without elevation of the serum ALT value [22, 23]. It is also well known that the serum ALT values may not always be well correlated with the severity of liver disease [17].

The purpose of this study was to compare the distribution of histological fibrosis stage and scoring systems in various serum ALT levels and to investigate the clinical usefulness of established clinical scoring systems for detecting the presence of advanced liver fibrosis (bridging fibrosis or cirrhosis) and resetting the reported cutoff values, as appropriate, in a large retrospective cohort of Japanese patients with NAFLD patients with normal ALT levels.

Patients and methods

Patients

A total of 1,102 patients with liver-biopsy-confirmed NAFLD between 2002 and 2011 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following ten hepatology centers in Japan: Nara City Hospital, Yokohama City University, Hiroshima University, Kochi Medical School, Saga Medical School, Osaka City University, Kyoto Prefectural University of Medicine, Asahikawa Medical College, Kurume University, and Saiseikai Suita Hospital. We performed liver biopsy for the purpose of diagnosis and staging of NASH. The principal indications for liver biopsy were a persistent decrease of the platelet count and increase in the serum levels of the direct markers of fibrosis (type IV collagen 7s and hyaluronic acid) according to the consensus of the Japan Society of Hepatology (JSH). In addition, older age, presence of diabetes, obesity, a prolonged history of steatosis, and the results of elastography were also considered on an individualized basis. The histological criterion used for the diagnosis of NAFLD was the presence of macrovesicular fatty changes in the hepatocytes, with displacement of the nuclei to the edges of the cells [24]. The criteria for exclusion from this study included a history of hepatic disease, such as chronic hepatitis C or concurrent active hepatitis B (seropositive for hepatitis B

surface antigen), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, as well as a current or past history of consumption of more than 20 g of alcohol daily. Informed consent was obtained from each patient included in the study, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Anthropometric and laboratory evaluation

The weight and height of the patients were measured using a calibrated scale after requesting the patients to remove their shoes and any heavy clothing. Venous blood samples were obtained in the morning after the patients had fasted overnight for 12 h. Laboratory evaluations in all patients included determination of the blood cell counts, and measurement of the serum levels of AST, ALT, γ-glutamyl transpeptidase (GGT), cholinesterase (ChE), albumin, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, fasting immunoreactive insulin (IRI), hyaluronic acid and type IV collagen 7s domain, and fasting plasma glucose. All of the parameters were measured using standard techniques.

Based on the previous study, the upper normal limit of the serum ALT was set at 40 IU/l [25, 26]. The FIB-4 index was calculated as age × AST (IU/l)/platelet count ($\times 10^9$/l)/$\sqrt{}$ALT (IU/l) [21]. The NAFLD fibrosis score was calculated according to the following formula: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+$ 1.13 × impaired fasting glycemia or diabetes (yes = 1, no = 0) + 0.99 × AST/ALT ratio − 0.013 × platelet (x10^9/l) − 0.66 × albumin (g/dl) [19]. The BARD score was estimated as the weighted sum of three variables (BMI >28 = 1 point, AST/ALT ratio >0.8 = 2 points, diabetes = 1 point) [20]. AAR was calculated as AST/ALT [18].

Histologic evaluation

All patients enrolled in this study had undergone a percutaneous liver biopsy under ultrasound guidance. Fatty liver was defined as the presence of >5 % steatosis, while steatohepatitis was defined as the presence of steatosis, inflammation, and hepatocyte ballooning [27–29]. The degree of steatosis was assessed based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, 5–33 % hepatocytes containing macrovesicular fat droplets; grade 2, 33–66 % hepatocytes containing macrovesicular fat droplets; grade 3, >66 % hepatocytes containing macrovesicular fat droplets. The individual parameters of fibrosis were scored independently according to the NASH Clinical Research Network (CRN) scoring system developed by the NASH CRN [30]. Advanced fibrosis was classified as stage 3 or 4 (bridging fibrosis or cirrhosis).

Statistical analysis

Statistical analysis was conducted using SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data were represented as numbers, with the percentages indicated within parentheses. The statistical significances of differences in the quantitative data were determined using the t test or Mann-Whitney's U test. Because the variables were often not normally distributed, group comparisons of more than two independent groups were performed using the Kruskal-Wallis test. The percentage of cases with advanced fibrosis was compared between the ALT ≤40 and ALT >40 groups using Fisher's exact test. The diagnostic performances of the scoring systems were assessed by analyzing the receiver-operating characteristic (ROC) curves. The probabilities of a true-positive (sensitivity) and true-negative (specificity) assessment were determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cutoff points. Differences were considered to be statistically significant at $p < 0.05$.

Results

Patient characteristics

Using a multicenter database, 1,102 biopsy-proven cases of NAFLD were investigated. Of these, the serum ALT levels were more than 40 IU/l in 867 (78.7 %) patients and less than or equal to 40 IU/l in 235 (17.4 %) patients. In NAFLD patients with serum ALT levels ≤40 IU/l, steatosis grade, inflammatory activity, and fibrosis stage were not correlated with the serum ALT levels ($p = 0.4536$, 0.6238, and 0.1158 respectively by Kruskal-Wallis analysis). The distribution of histological fibrosis stage in various serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) is shown in Table 1. The distribution of the fibrosis stage in ALT level ≤40 was as follows: stage 0, $n = 91$ (38.7 %); stage 1, $n = 65$ (27.7 %); stage 2, $n = 41$ (17.4 %); stage 3, $n = 21$ (8.9 %); stage 4, $n = 17$ (7.2 %). The ratio of advanced fibrosis was 16.1 % (ALT ≤40 IU/l), 24.5 % (ALT 41–60 IU/l), 16.2 % (ALT 61–80 IU/l), 27.9 % (ALT 81–100 IU/l), and 25.0 % (≥101 IU/l) (Table 1). The percentage of cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l was

Table 1 The distribution of histological fibrosis stage in serum ALT levels

ALT range	Patients no.	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Advanced fibrosis ratio (%)
All patients	1,102	240 (21.8 %)	339 (30.8 %)	290 (26.3 %)	196 (17.8 %)	46 (4.2 %)	22.0
≤40	235	91 (38.7 %)	65 (27.7 %)	41 (17.4 %)	21 (8.9 %)	17 (7.2 %)	16.1
41–60	229	48 (21.0 %)	76 (33.2 %)	49 (21.4 %)	41 (17.9 %)	15 (6.6 %)	24.5
61–80	172	35 (20.3 %)	53 (30.8 %)	56 (32.6 %)	23 (13.4 %)	5 (2.9 %)	16.2
81–100	122	18 (14.8 %)	41 (33.6 %)	38 (31.1 %)	31 (25.4 %)	3 (2.5 %)	27.9
≥101	344	48 (14.0 %)	104 (30.2 %)	106 (30.8 %)	80 (23.3 %)	6 (1.7 %)	25.0

significantly lower than that among the NAFLD patients with serum ALT levels >40 IU/l, as evaluated by Fisher's exact test ($p = 0.0163$).

The demographic and laboratory characteristics of NAFLD patients with serum ALT levels ≤40 IU/l and the clinical and laboratory features of the subjects with no or mild fibrosis (stage 0–2) compared with those of the patients with advanced fibrosis (stage 3–4) are shown in Table 2. In the patient group with serum ALT levels ≤40 IU/l, comparison of the characteristics of the subjects with no or mild fibrosis with those of subjects with advanced fibrosis revealed significantly higher values of age, serum AST, serum HDL cholesterol, fasting plasma glucose, serum fasting IRI, HOMA-IR, serum hyaluronic acid and serum type IV collagen 7s domain, and lower values of serum cholinesterase, serum albumin, hemoglobin, and platelet count in subjects with advanced fibrosis. In addition, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR were all significantly higher in patients with advanced fibrosis as compared with the values in the patients with no or mild fibrosis in the patient group with serum ALT levels ≤40 IU/l (Table 2).

The AUROC of the platelet count and serum level of the type IV collagen 7s domain for detecting cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l

Because significant differences in the platelet count and type IV collagen 7s domain were observed between patients with advanced fibrosis and those with no or mild fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l, the AUROCs of the platelet count and serum level of type IV collagen 7s domain for detecting fibrosis stages ≥ stage 3 were calculated. The AUROC for estimating the diagnostic performance of the platelet count for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.786 (optimal cutoff value 19.3 × 10^4/μl, sensitivity 81.6 %, specificity 65.5 %) (Fig. 1a). The AUROC for estimating the diagnostic performance of the serum level of type IV collagen 7s for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.794 (optimal cutoff value 5.0 ng/ml, sensitivity 63.2 %, specificity 84.3 %) (Fig. 1b).

The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

In order to investigate the diagnostic accuracy of the scoring systems in NALFD with various serum ALT levels, the AUROC for detecting fibrosis stages ≥ stage 3 was calculated in various distributions of serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) (Table 3). The AUROCs were calculated for the FIB-4 index (0.706–0.878), NAFLD fibrosis score (0.657–0.843), BARD score (0.517–0.684), and AAR (0.684–0.804). The diagnostic accuracy of each scoring system was more than equivalent also in case of ALT ≤40 IU/l. Furthermore, concerning the AUROC of the FIB-4 index, the NAFLD fibrosis score had the highest value in case of ALT ≤40 IU/l.

In NAFLD patients in the ALT >40 group, the optimal cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were 1.499, 0.502, 2, and 0.723, which were close to the cutoff values reported before [19, 20, 31, 32].

Prediction of the presence of advanced liver fibrosis and resetting of the cutoff value in NAFLD with normal ALT levels

In order to investigate the diagnostic accuracy of the scoring systems, ROC curves were constructed (Fig. 2). Then, the AUROC, optimal cutoff value, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined for each of the scoring systems. The FIB-4 index ranged from 0.305 to 12.482 in the NAFLD group with serum ALT values ≤40 IU/l. The FIB-4 index values stratified by the fibrosis stage were as follows: stage 0, 1.233 ± 0.711; stage 1, 1.570 ± 1.167; stage 2, 1.870 ± 1.496; stage 3, 3.071 ± 1.703; stage 4, 6.010 ± 3.704. Thus, in the NAFLD group with serum ALT values ≤40 IU/l, the FIB-4 index increased with increasing histological severity of the

Table 2 Characteristics of the NAFLD patients with serum ALT values ≤40 IU/l

Variable	Total ($n = 235$)	No or mild fibrosis (stage 0–2)	Advanced fibrosis (stage 3, 4)	p value
Age (years)	59.9 ± 12.1	58.6 ± 11.3	66.7 ± 8.4	0.0112
Body mass index (kg/m^2)	26.9 ± 4.0	26.5 ± 4.0	28.7 ± 4.7	0.0564
AST (IU/l)	24.7 ± 10.2	23.3 ± 6.93	31.9 ± 8.60	<0.001
ALT (IU/l)	23.7 ± 7.0	23.8 ± 4.56	23.0 ± 5.38	0.5293
Alkaline phosphatase (IU/l)	271.9 ± 124.4	264.3 ± 123.3	311.3 ± 205.3	0.2206
GGT (IU/l)	52.7 ± 74.1	51.9 ± 43.3	56.9 ± 54.1	0.6927
Cholinesterase (IU/l)	345.6 ± 90.6	366.3 ± 91.0	238.1 ± 104.4	<0.001
Albumin (g/dl)	4.28 ± 0.79	4.39 ± 1.06	3.71 ± 0.45	0.0177
Total cholesterol (mg/dl)	202.6 ± 37.9	203.5 ± 34.9	198.1 ± 43.7	0.6152
LDL cholesterol (mg/dl)	124.7 ± 31.3	127.1 ± 29.2	112.5 ± 31.6	0.1426
HDL cholesterol (mg/dl)	53.9 ± 13.5	52.4 ± 13.3	61.9 ± 16.2	0.0300
Triglyceride (mg/dl)	140.4 ± 72.2	146.2 ± 74.8	110.1 ± 60.8	0.0934
FPG (mg/dl)	123.3 ± 48.2	117.7 ± 40.1	152.1 ± 93.0	0.0176
Fasting insulin (μU/ml)	11.4 ± 8.31	10.6 ± 9.00	15.7 ± 9.01	0.0901
HOMA-IR	3.96 ± 3.56	3.39 ± 3.63	6.96 ± 7.77	0.0127
HbA1c (%)	5.96 ± 0.94	6.07 ± 0.80	5.42 ± 0.78	0.0885
Hemoglobin (g/dl)	13.5 ± 1.61	13.7 ± 1.38	12.2 ± 2.51	0.0010
Platelet count (×10^4/μl)	21.1 ± 6.90	22.7 ± 6.56	12.6 ± 6.18	<0.001
Hyaluronic acid (ng/ml)	87.3 ± 119.3	76.9 ± 128.9	141.0 ± 86.3	0.1302
Type IV collagen 7s (ng/ml)	4.74 ± 1.65	4.29 ± 1.38	7.05 ± 2.26	<0.001
Dyslipidemia	150 (63.8 %)	132 (67.0 %)	18 (47.3 %)	–
Diabetes mellitus	108 (46.0 %)	88 (44.7 %)	20 (52.6 %)	–
Steatosis grade (1/2/3)	124/84/27	94/79/24	30/5/3	–
Inflammatory grade (0/1/2/3)	59/138/38/0	57/117/23/0	2/21/15/0	
Fibrosis stage (0/1/2/3/4)	91/65/41/21/17	–	–	–
FIB 4 index	2.03 ± 1.93	1.47 ± 1.09	4.92 ± 3.42	<0.001
AST/ALT	1.07 ± 0.34	1.01 ± 0.32	1.41 ± 0.30	<0.001
NAFLD fibrosis score	−0.69 ± 1.81	−1.18 ± 1.63	1.82 ± 1.41	<0.001
BARD score	2.46 ± 1.23	2.28 ± 1.09	3.39 ± 0.65	0.0006

Values are mean ± SD. p values from Student's t test, Mann-Whitney test or χ^2 test, as appropriate

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *FPG* fasting plasma glucose, *HOMA-IR* homeostasis model assessment-insulin resistance

hepatic fibrosis ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the FIB-4 index for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT ≤40 IU/l was 0.878 (Fig. 2a) (optimal cutoff value 1.659, sensitivity 89.5 %, specificity 71.1 %). Using the previously published cutoff value proposed by Shah et al. [31] (>2.67), the sensitivity of this index for the detection of advanced fibrosis was calculated as 63.2 % and the specificity as 88.3 % (Table 4).

The NAFLD fibrosis score ranged from −6.304 to 4.639 in the NAFLD patients with serum ALT values ≤40 IU/l. The NAFLD fibrosis scores stratified by the fibrosis stage were as follows: stage 0, −1.439 ± 1.538; stage 1, −1.290 ± 1.592; stage 2, −0.762 ± 1.591; stage 3, 0.256 ± 1.400; stage 4, 2.110 ± 1.332; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the NAFLD fibrosis score for hepatic fibrosis stages ≥ stage 3 in the NAFLD patients with serum ALT values ≤40 IU/l was 0.843 (Fig. 2b) (optimal cutoff value 0.735, sensitivity 68.4 %, specificity 88.3 %). Using the previously published cutoff point proposed by Angulo et al. [19] (>0.676), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 68.4 % and the specificity as 87.8 % (Table 4).

The BARD score ranged from 0 to 4 in the NAFLD patients with serum ALT values ≤40 IU/l. The BARD scores stratified by the fibrosis stage were as follows: stage 0, 2.000 ± 1.200; stage 1, 1.967 ± 1.303; stage 2, 2.100 ± 1.215; stage 3, 2.316 ± 1.057; stage 4, 3.333 ± 0.724. The AUROC calculated to estimate the

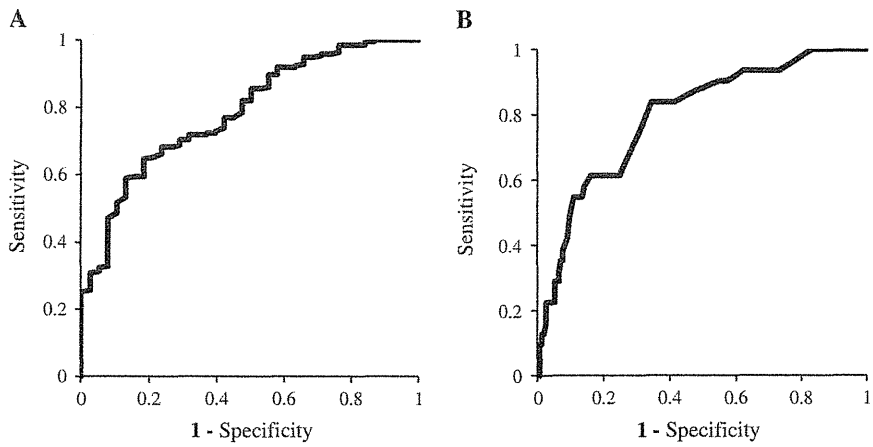

Fig. 1 Receiver-operating characteristic (ROC) curves for detecting advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** The platelet count, **b** type IV collagen 7s

Table 3 The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

ALT levels (IU/l)	FIB4 index	NAFLD fibrosis score	BARD score	AST/ ALT
≤40	0.878	0.843	0.671	0.794
41–60	0.818	0.726	0.684	0.804
61–80	0.706	0.654	0.663	0.737
81–100	0.752	0.657	0.517	0.684
≥101	0.773	0.670	0.578	0.728

diagnostic performance of the BARD score for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.671 (Fig. 2c) (optimal cutoff value 3, sensitivity 65.8 %, specificity 59.9 %). Using the previously published cutoff point proposed by Harrison et al. [20] (>2), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 86.8 % and the specificity as 32.5 % (Table 4).

AAR ranged from 0.448 to 2.630 in the NAFLD patients with serum ALT values ≤40 IU/l. The AAR values stratified by the fibrosis stage were as follows: stage 0, 0.869 ± 0.221; stage 1, 0.942 ± 0.269; stage 2, 0.998 ± 0.424; stage 3, 1.232 ± 0.423; stage 4, 1.359 ± 0.361; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the AAR for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.794 (Fig. 2d) (optimal cutoff value 0.975, sensitivity 78.9 %, specificity 70.1 %). Using the previously published cutoff point proposed by McPherson et al. [32] (>0.8), the sensitivity of this ratio for the detection of advanced fibrosis was calculated as 89.5 % and the specificity as 37.1 % (Table 4).

Discussion

The incidence of NAFLD is rising rapidly in both adults and children because of the currently ongoing epidemics of obesity and type 2 diabetes [33]. Thus, development of a rapid and non-invasive method for the detection of fibrosis in NAFLD patients is of major clinical interest. In recent years, Shah et al. [31] reported, from a multicenter trial, the usefulness of scoring systems for NAFLD patients. In their study, they evaluated 541 NAFLD patients and concluded that the AUROC values calculated to estimate the diagnostic performances of FIB4, the NAFLD fibrosis score, and AAR in which the serum ALT is included for hepatic fibrosis stages ≥ stage 3 were 0.802, 0.768, and 0.720, respectively. We also previously validated these scoring systems in 576 biopsy-proven Japanese NAFLD patients [34]. Furthermore, in this study, the cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were close to the cutoff values reported before [19, 20, 31, 32].

NAFLD often presents as abnormal liver enzyme levels in the absence of markers of other common liver diseases, e.g., hepatitis C. The severity of hepatic fibrosis tends to be underestimated in patients with serum ALT values within normal limits, even though normal serum ALT values do not guarantee the absence of advanced fibrosis in patients with NAFLD [23]. It is not uncommon for patients to present with complications of previously unrecognized cirrhosis despite being under long-standing medical care, because these patients often do not manifest the classical physical changes associated with cirrhosis. At present,

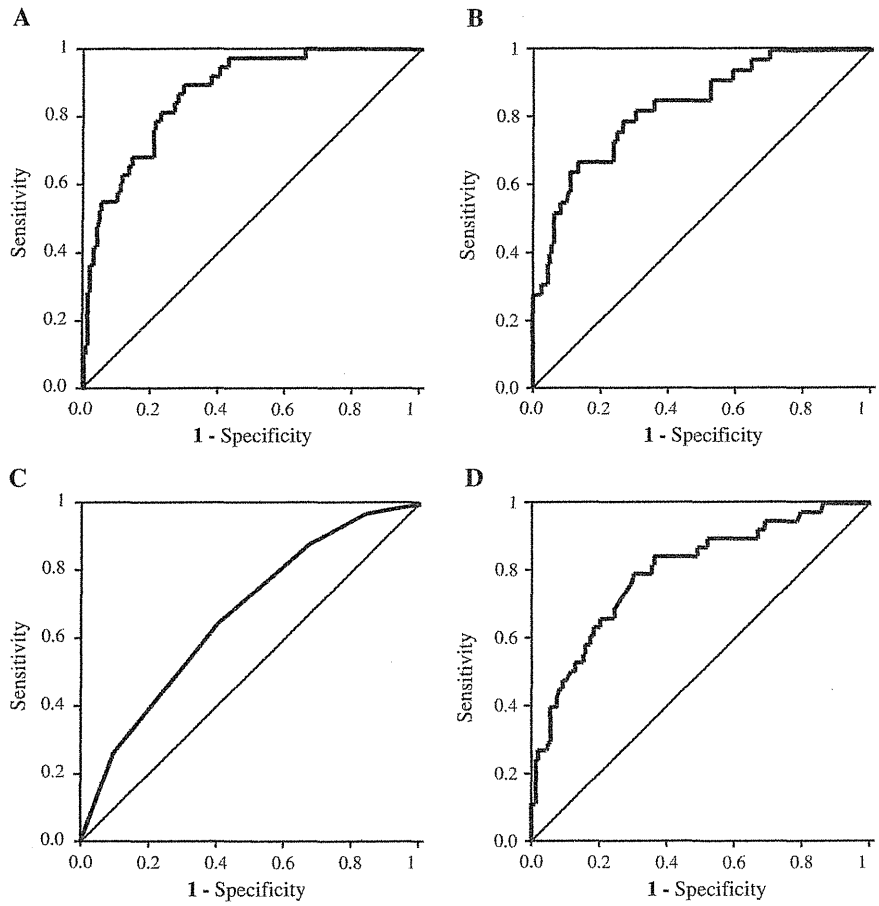

Fig. 2 Receiver-operating characteristic (ROC) curves for the noninvasive scores for a diagnosis of advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** FIB-4 index, **b** NAFLD fibrosis score, **c** BARD score, **d** AST/ALT ratio (AAR)

Table 4 Comparison of the performance of each of the scoring systems for the diagnosis of advanced fibrosis in 235 NAFLD patients with serum ALT values under 40 IU/l using reported cutoff and reset cutoff values

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	References
Fib-4						
Reported by Shah et al.	2.67	63.2	88.3	51.1	92.6	[31]
Re-setup	1.659	89.5	71.1	37.4	97.2	
NAFLD fibrosis score						
Reported by Angluo et al.	0.676	68.4	87.8	52.0	93.5	[19]
Re-setup	0.735	68.4	88.3	53.0	93.5	
BARD score						
Reported by Harrison et al.	2	86.8	32.5	19.9	92.8	[20]
Re-setup	3	65.8	59.9	24.0	90.1	
AST/ALT (AAR)						
Reported by McPherson et al.	0.8	89.5	37.1	21.5	94.8	[32]
Re-setup	0.975	78.9	70.1	30.7	94.5	

PPV positive predictive value, *NPV* negative predictive value

NAFLD patients with normal serum ALT values are very rarely investigated or subjected to liver biopsy. Mofrad et al. [23] and Fracanzani et al. [25] found that the histological features of NAFLD sometimes progress even in persons with normal serum ALT values and that the liver histology in these persons is not very different from that in

patients with high serum ALT levels; in addition, a low or normal serum ALT level does not serve as a reliable criterion to exclude the need for liver biopsy in NAFLD patients [23, 25]. Fracanzani et al. [25] reported that a persistent increase of the serum ferritin level, persistent evidence of severe steatosis on ultrasonography, and a persistent increase of the serum GGT levels were the main reasons for liver biopsy in patients with normal serum ALT levels. Mofrad et al. [23] reported that the principal indications for liver biopsy in patients with normal ALT levels were persistent hepatomegaly, donor evaluation for living donor liver transplantation, elevated serum ferritin levels, abnormal imaging characteristics of the liver suggestive of parenchymal liver disease, baseline biopsy to initiation of methotrexate therapy, and clinical features of portal hypertension without other evidence of liver disease.

A first finding in our study is the ratio of advanced fibrosis (stage 3–4) in various distributions of ALT. Advanced fibrosis was seen in 16.1 % of subjects with serum ALT levels ≤40 IU/l. Thus, caution must be exercised in evaluating the disease severity in NAFLD patients with normal serum ALT values. While the platelet count and serum level of the collagen 7s domain were reported to be useful for predicting the presence of advanced fibrosis in NAFLD patients [35, 36], it appears that they may also be useful for predicting severe fibrosis in cases of NAFLD with normal serum ALT levels. However, the specificity of the platelet count and sensitivity of type IV collagen 7s were slightly low. So far, no previous studies have investigated the usefulness of the available tests for the prediction of liver fibrosis in NAFLD patients with normal serum ALT values, because the small sample size of NAFLD subjects with normal serum ALT levels hampers any attempt to construct scoring systems for predicting NASH or fibrosis [25]. Thus, the previous scoring systems, especially their cutoff values, seem to be insufficient for the diagnosis of fibrosis in the NAFLD patients with normal ALT.

A second finding of this study was that the scoring systems investigated in NALFD with normal ALT. Of these, especially the FIB-4 index and NAFLD fibrosis score were clinically very useful (AUROC >0.8) even in patients with normal serum ALT values. Furthermore, with resetting of the cutoff values, they were found to have a higher sensitivity and higher specificity for the prediction of advanced fibrosis in a retrospective cohort of NAFLD patients with normal serum ALT values. The BARD score failed to detect the outstanding sensitivity and specificity in all the ALT groups. Consistent with the present study, Fujii and colleagues [37] reported significantly poorer applicability of the BARD score in Japanese patients with NAFLD compared to Caucasian subjects. It has been suggested that the BARD score is less predictive of advanced fibrosis in Japanese NAFLD patients because they are less obese that those in western countries.

As a third finding of this study, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR all had high NPVs (>90.1 %) for advanced fibrosis in the cohort of patients with NAFLD. This suggests that these scoring systems could be used clinically to exclude advanced fibrosis in subjects with NAFLD. For example, using the FIB-4 index (<1.659) to exclude advanced fibrosis, liver biopsy could have been avoided in 60.4 % of the patients in our cohort of patients with serum ALT values ≤40 IU/l. Similarly, prediction of the presence/absence of fibrosis based on the NAFLD fibrosis score (<0.735), BARD score (<3), and AAR allowed avoidance of liver biopsy in 66.4, 51.9, and 62.1 % of patients, respectively. Given the large numbers of NAFLD patients with normal serum ALT values, use of these non-invasive tests with reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

As a fourth finding of this study, in contrast to the NPVs, the PPVs of the tests did not have sufficient accuracy for the diagnosis of advanced fibrosis. It would, therefore, seem appropriate to consider liver biopsy in all patients with values above the cutoff of the selected index. We previously reported, for the first time in the world, that transient elastography and acoustic radiation force impulse (ARFI) elastography can be used to measure the severity of fibrosis in patients with NAFLD [15, 38]. It is possible that a combination of transient elastography and one of the aforementioned scoring systems may provide better performance than each of them used alone, although this needs to be verified in future studies.

This study had several limitations. First, the proportion of subjects with advanced fibrosis was small. Second, the patients were recruited from hepatology centers in Japan with a particular interest in the study of NAFLD; therefore, the possibility of some referral bias cannot be ruled out. Patient selection bias could also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. The findings may thus not represent those of the NAFLD patients in the community at large. The question remains as to whether the revised cutoff values of the various scoring systems might be useful in real clinical practice. Another limitation is that the supposedly normal range of ALT values is incorrect. The public health implications and clinical usefulness of reducing the upper limits of the normal value for the serum ALT continue to be under debate, and the currently proposed cutoff values for the upper limits of the serum ALT levels are 30 IU/l for men and 19 IU/l for women [39]. Recently, the upper limit of the normal range of serum ALT levels in the Asian population was reported as 35 IU/l for men and 26 IU/l for patients with a normal liver

histology [40]. According to our preliminary data, the AUROC calculated for detecting advanced fibrosis was 0.907 (FIB4 index), 0.916 (NAFLD fibrosis score), 0.793 (BARD), and 0.859 (AAR) in 127 biopsy-proven NAFLD patients with ALT ≤30 (data not shown). We also acknowledge that the pathologic diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling errors and/or inter-observer variability [41, 42].

In conclusion, the issue of development of a non-invasive method for the assessment of disease severity remains crucial in patients with NAFLD given the high number of subjects with steatosis and normal serum ALT values in the general population. We reset the cutoff values of numerous non-invasively determined indices to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal serum ALT values. In the absence of biopsy or of an adequate score capable of identifying subjects at risk, these patients could miss being included in the list for careful follow-up and might be scarcely motivated to adopt lifestyle modifications that could potentially cure their liver disease. Clinicians should be aware of the importance of complete clinical evaluation for early diagnosis and treatment of liver diseases. Non-invasive scoring systems, especially the FIB-4 index and the NAFLD fibrosis score showed high sensitivity and specificity, and they can be reliably used to exclude advanced fibrosis in NAFLD subjects with normal serum ALT levels.

Acknowledgments This work was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan (22790660 to MY and 22590741 to YE), by a Grant from the Chiyoda Mutual Life Foundation to YS, and by a Thrust Area Research Grand from Osaka City University to HF and NK.

Conflict of interest The authors have no conflicts of interest to disclose.

References

1. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;18:1221–31.
2. Liou I, Kowdley KV. Natural history of nonalcoholic steatohepatitis. J Clin Gastroenterol. 2006;40(Suppl 1):S11–6.
3. Browning JD, Szczepaniak LS, Dobbins R, Nuremberg P, Horton JD, Cohen JC, et al. Prevalence of hepatic steatosis in an urban population in the United States: impact of ethnicity. Hepatology. 2004;40:1387–95.
4. Wanless IR, Lentz JS. Fatty liver hepatitis (steatohepatitis) and obesity: an autopsy study with analysis of risk factors. Hepatology. 1990;12:1106–10.
5. Neuschwander-Tetri BA, Caldwell SH. Nonalcoholic steatohepatitis: summary of an AASLD single topic conference. Hepatology. 2003;37:1202–19.
6. National Institutes of Health. National Institutes of Health Consensus Development Conference statement: management of hepatitis C 2002 (June 10–12, 2002. Hepatology. 2002;36(5 supple 1):S3–20.
7. Angulo P, Keach JC, Batts KP, Lindor KD. Independent predictors of liver fibrosis in patients with nonalcoholic steatohepatitis. Hepatology. 1999;30:1356–62.
8. Cadranel JF. Good clinical practice guidelines for fine needle aspiration biopsy of the liver: past, present and future. Gastroenterol Clin Biol. 2002;26:823–4.
9. Saadeh S, Cammell G, Carey WD, Younossi Z, Barnes D, Easley K. The role of liver biopsy in chronic hepatitis C. Hepatology. 2001;33:196–200.
10. Poynard T, Ratziu V, Bedossa P. Appropriateness of liver biopsy. Can J Gastroenterol. 2003;14:543–8.
11. Eguchi Y, Hyogo H, Ono M, Mizuta T, Ono N, Fujimoto K, et al. Prevalence and associated metabolic factors of nonalcoholic fatty liver disease in the general population from 2009 to 2010 in Japan: a multicenter large retrospective study. J Gastroenterol. 2012 (Epub ahead of print).
12. Pinzani M, Vizzutti F, Arena U, Marra F. Technology insight: noninvasive assessment of liver fibrosis by biochemical scores and elastography. Nat Clin Pract Gastroenterol Hepatol. 2008;5:95–106.
13. Ratziu V, Massard J, Charlotte F, Messous D, Imbert-Bismut F, Bonyhay L, CYTOL study group, et al. Diagnostic value of biochemical markers (FibroTest-FibroSURE) for the prediction of liver fibrosis in patients with non-alcoholic fatty liver disease. BMC Gastroenterol. 2006;6:6.
14. Guha IN, Parkes J, Roderick P, Chattopadhyay D, Cross R, Harris S, Kaye P, et al. Noninvasive markers of fibrosis in nonalcoholic fatty liver disease: validating the European Liver Fibrosis Panel and exploring simple markers. Hepatology. 2008;47:455–60.
15. Yoneda M, Yoneda M, Mawatari H, Fujita K, Endo H, Iida H, et al. Noninvasive assessment of liver fibrosis by measurement of stiffness in patients with nonalcoholic fatty liver disease (NAFLD). Dig Liver Dis. 2008;40:371–8.
16. Karmen A. Transaminase activity in human blood. J Clin Invest. 1955;34:126–33.
17. Kallei L, Hahn A, Roeder V, Zupanic V. Correlation between histological findings and serum transaminase values in chronic disease of the liver. Acta Med Scand. 1964;175:49–56.
18. Williams AL, Hoofnagle JH. Ratio of serum aspartate to alanine aminotransferase in chronic hepatitis. Relationship to cirrhosis. Gastroenterology. 1988;95:734–9.
19. Angulo P, Hui JM, Marchesini G, Bugianesi E, George J, Farrell GC, et al. The NAFLD fibrosis score: a noninvasive system that identifies liver fibrosis in patients with NAFLD. Hepatology. 2007;45:846–54.
20. Harrison SA, Oliver D, Arnold HL, Gogia S, Neuschwander-Tetri BA. Development and validation of a simple NAFLD clinical scoring system for identifying patients without advanced disease. Gut. 2008;57:1441–7.
21. Sterling RK, Lissen E, Clumeck N, Sola R, Correa MC, Montaner J, et al. Development of a simple noninvasive index to predict significant fibrosis in patients with HIV/HCV coinfection. Hepatology. 2006;43:1317–25.
22. Pagano G, Pacini G, Musso G, Gambino R, Mecca F, Depetris N, et al. Nonalcoholic steatohepatitis, insulin resistance, and metabolic syndrome: further evidence for an etiologic association. Hepatology. 2002;35:367–72.
23. Mofrad P, Contos MJ, Haque M, Sargeant C, Fisher RA, Luketic VA, et al. Clinical and histologic spectrum of nonalcoholic fatty liver disease associated with normal ALT values. Hepatology. 2003;37:1286–92.
24. Sanyal AJ. AGA technical review on nonalcoholic fatty liver disease. Gastroenterology. 2002;123:1705–25.

25. Fracanzani AL, Valenti L, Bugianesi E, Andreoletti M, Colli A, Vanni E, Bertelli C, et al. Risk of severe liver disease in nonalcoholic fatty liver disease with normal aminotransferase levels: a role for insulin resistance and diabetes. Hepatology. 2008;48:792–8.
26. Prati D, Taioli E, Zanella A, Della TE, Butelli S, Del Vecchio E, et al. Updated definitions of healthy ranges for serum alanine aminotransferase levels. Ann Intern Med. 2002;137:1–10.
27. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology. 1999;116:1413–9.
28. Sanyal AJ, Brunt EM, Kleiner DE, Kowdley K, Chalasani N, Lavine J, et al. Endopoints and clinical trial design for nonalcoholic steatohepatitis. Hepatology. 2011;54:344–53.
29. Brunt EM, Tiniakos DG. Histopathology of nonalcoholic fatty liver disease. World J Gastroenterol. 2010;16:5286–96.
30. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.
31. Shah AG, Lydecker A, Murray K, Tetri BN, Contos MJ, Sanyal AJ. Comparison of noninvasive markers of fibrosis in patients with nonalcoholic fatty liver disease. Clin Gastroenterol Hepatol. 2009;7:1104–12.
32. McPherson S, Stewart SF, Henderson E, Burt AD, Day CP. Simple non-invasive fibrosis scoring systems can reliably exclude advanced fibrosis in patients with non-alcoholic fatty liver disease. Gut. 2010;59:1265–9.
33. Charlton M. Nonalcoholic fatty liver disease: a review of current understanding and future impact. Clin Gastroenterol Hepatol. 2004;2:1048–58.
34. Sumida Y, Yoneda M, Hyogo H, Itoh Y, Ono M, Fujii H, et al. Validation of the FIB4 index in a Japanese nonalcoholic fatty liver disease population. BMC Gastroenterol. 2012;12:2.
35. Yoneda M, Fujii H, Sumida Y, Hyogo H, Itoh Y, Ono M, et al. Platelet count for predicting fibrosis in nonalcoholic fatty liver disease. J Gastroenterol. 2011;46:1300–6.
36. Yoneda M, Mawatari H, Fujita K, Yonemitsu K, Kato S, Takahashi H, et al. Type IV collagen 7s domain is an independent clinical marker of the severity of fibrosis in patients with nonalcoholic steatohepatitis before the cirrhotic stage. J Gastroenterol. 2007;42:375–81.
37. Fujii H, Enomoto M, Fukushima W, Tamori A, Sakaguchi H, Kawada N. Applicability of BARD score to Japanese patients with NAFLD. Gut. 2009;58:1566–7.
38. Yoneda M, Suzuki K, Kato S, Fujita K, Nozaki Y, Hosono K, et al. Nonalcoholic fatty liver disease: US-based acoustic radiation force impulse elastography. Radiology. 2010;256:640–7.
39. Kaplan MM. Alanine aminotransferase: what's normal? Ann Intern Med. 2002;137:49–51.
40. Lee JK, Shim JH, Lee HC, Lee SH, Kim KM, Lim YS, et al. Estimation of the healthy upper limits for serum alanine aminotransferase in Asian populations with normal liver histology. Hepatology. 2010;51:1577–83.
41. Ratziu V, Charlotte F, Heurtier A, Gombert S, Giral P, Bruckert E, et al. Sampling variability of liver biopsy in nonalcoholic fatty liver disease. Gastroenterology. 2005;128:1898–906.
42. Merriman RB, Ferrell LD, Patti MG, Weston SR, Pabst MS, Aouizerat BE, et al. Correlation of paired liver biopsies in morbidly obese patients with suspected nonalcoholic fatty liver disease. Hepatology. 2006;44:874–80.

Hepatology Research

Hepatology Research 2013 doi: 10.1111/hepr.12139

Original Article

Clinical efficacy of combination therapy with ME3738 and pegylated interferon-alpha-2a in patients with hepatitis C virus genotype 1

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Aim: ME3738, a derivative of soyasapogenol B, enhances the anti-hepatitis C virus (HCV) effect of interferon in an *in vitro* replication system and an *in vivo* mouse model of HCV infection. ME3738 plus pegylated interferon (PEG IFN)-α-2a treatment for 12 weeks decreased HCV RNA levels in enrolled late virus responder (LVR) patients with relapsed HCV. Half of the patients reached undetectable HCV RNA level. The present clinical study of ME3738 was conducted in naïve chronic hepatitis C patients to investigate the sustained virological response (SVR) and safety of 48-week treatment with ME3738 plus PEG IFN-α-2a.

Methods: Subjects ($n = 135$) with genotype 1b chronic hepatitis C with high viral loads were divided into three groups (ME3738 50 mg b.i.d., 200 mg b.i.d. or 800 mg b.i.d.). ME3738 was administrated p.o. and PEG IFN-α-2a (180 μg/week) s.c. for 48 weeks, and SVR was assessed at 24 weeks of treatment-free follow up.

Results: The viral disappearance rates at 12 and 48 weeks were 23.0% and 48.9%, respectively. SVR was seen in 5.9% of subjects. ME3738 did not worsen the adverse reactions generally seen with PEG IFN-α-2a treatment, and any adverse reactions specific to ME3738 were not observed.

Conclusion: ME3738 plus PEG IFN-α-2a treatment to naïve chronic hepatitis C patients showed an antiviral effect and a good safety profile up to 48 weeks. However, HCV RNA was again detected in many subjects after treatment termination. Even though ME3738 is not enough to suppress HCV reproduction in this treatment. ME3738 was concurrently used with PEG IFN-α-2a treatment; however, a clear additional effect on SVR was not confirmed.

Key words: clinical efficacy, hepatitis C virus, ME3738, pegylated interferon-alpha-2a

INTRODUCTION

HEPATITIS C VIRUS (HCV) causes chronic hepatitis through persistent infection and is a major cause of liver cancer, particularly in Japan.[1,2] The standard therapy for chronic hepatitis C is co-administration of pegylated interferon (PEG IFN) and ribavirin (RBV), with a reported efficacy rate of approximately 50% in refractory cases with genotype 1b and high levels of plasma HCV RNA.[3] In Japan, many patients with chronic hepatitis C are elderly at present, and this standard therapy in these patients can result in unavoidable treatment discontinuation, low therapeutic effect due to a high incidence of adverse reactions, the need for dose reduction of PEG IFN because of decreased neutrophil count, or the need for dose reduction of RBV because of anemia.[4,5]

ME3738 is a derivative of soyasapogenol B derived from soybeans and ME3738 has been shown to

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Received 27 January 2013; revision 11 April 2013; accepted 15 April 2013.

suppress liver inflammation in various hepatitis mouse models.[6,7] In patients with chronic hepatitis C with elevated alanine aminotransferase (ALT) values, ME3738 monotherapy showed a tendency toward suppression of liver inflammation, and the only major adverse reactions were digestive symptoms and abnormal laboratory data such as 10% of the patients enrolled in frequency.

ME3738 has also shown to enhance the suppressive effect of IFN on HCV in an *in vitro* replication system of HCV and in an *in vivo* HCV-infected mouse model (chimeric mice).[8,9]

A clinical study of ME3738 was conducted in patients with chronic hepatitis C to confirm viral disappearance with 12 weeks of treatment.[10] Patients were those with chronic hepatitis C who had received combination therapy with PEG IFN-α-2b and RBV. Among the patients who achieved viral disappearance after 12–24 weeks of treatment (late virus responders: LVR), those who showed viral reactivation after 48 weeks of treatment were enrolled.

ME3738 was administrated p.o. twice a day at a dose of 50 mg/day or 800 mg/day, and PEG IFN-α-2a was administrated s.c. at a dose of 180 μg once a week. This combination therapy was administrated for 12 weeks. In all subjects enrolled in this study, viral disappearance had not been achieved by previous therapy (co-administration of PEG IFN-α-2b and RBV) within 12 weeks of treatment (HCV RNA, ≥1.7 log IU/mL). On the other hand, when ME3738 was co-administrated, viral disappearance was achieved in approximately 50% of subjects within 12 weeks of treatment. Therefore, it was suggested that ME3738 co-administrated with PEG IFN would contribute to early viral disappearance.

The present clinical study of ME3738 was conducted in naïve chronic hepatitis C patients to investigate the sustained virological response (SVR) and safety of 48-week treatment with ME3738 plus PEG IFN-α-2a.

METHODS

Study design and dosing

THIS STUDY WAS conducted as a multicenter, randomized, single-blind, comparative phase II study (protocol no. ME3738-11).

ME3738 used in the study was provided by Meiji Seika Pharma (Tokyo, Japan).

ME3738 doses were selected as 50 mg/day and 800 mg/day, the safety of which was confirmed in a previous study using 12-week combined administration. In addition, 200 mg/day was selected as an intermediate dose to confirm a dose-dependent effect on SVR, and a total of three doses were selected.

Subjects were divided into three groups, with ME3738 administrated p.o. twice a day (after breakfast and dinner) at a dose of 50 mg/day, 200 mg/day or 800 mg/day. PEG IFN-α-2a 180 μg was administrated s.c. to all subjects once a week. This combination therapy was administrated for 48 weeks. Before starting treatment, subjects were assigned to each of the three groups at the central registration center, so that the number of subjects in each group was identical.

Patients were assigned doses based on an allocation chart that was preliminarily prepared in a central registration center (Moss Institute), which was a party independent of the investigative group.

In subjects who achieved disappearance of HCV RNA at the completion of the 48-week treatment (end of treatment: EOT), follow-up observation was performed for an additional 24 weeks (24-week follow-up after EOT) to confirm SVR.

During the study period, blood cell counts were measured every week before administration of PEG IFN-α-2a, and the dose of PEG IFN-α-2a was reduced to 90 μg or withdrawn according to the following criteria:

- Dose reduction: neutrophil count less than 750/mm^3 or platelet count less than 50 000/mm^3
- Dose withdrawal (suspension): neutrophil count less than 500/mm^3, platelet count less than 25 000/mm^3 or hemoglobin less than 8.5 g/dL.

The following study discontinuation criteria were set. When the HCV RNA determined after 12 weeks of treatment was not lower than the baseline level by at least 2.0 Log IU/mL, the study was to be discontinued in the subject concerned. When HCV RNA was detected after 48 weeks of treatment or during the follow-up period, the study was also to be discontinued in the subject concerned at the time point of detection.

Subjects

The study population included patients with naïve chronic hepatitis C.

The target patients were aged 20 years or more, with HCV genotype 1b and a high viral load (≥5.0 log IU/mL).

Patients meeting the following conditions were excluded from this study: patients positive for hepatitis B surface antigen, patients with hepatitis other than chronic hepatitis C, patients with liver cirrhosis or liver cancer, patients with a FibroIndex[11] not less than 2.25 (F4), and patients with a baseline neutrophil count less than 1500/mm^3, a baseline platelet count less