an exacerbating factor,[16] while interferon α (IFNα) is a standard drug for treatment. Exogenously administered IFN activates the immune system *in vivo*. During treatment with IFN, iron dysregulation such as an increase in serum ferritin and hepcidin occurs especially when IFN therapy is effective.[17] Iron overload in chronic hepatitis C contributes to a poor response to IFN treatment, which lead to a poor prognosis.[18] It is therefore important to investigate iron metabolism during IFN treatment because it is unclear how exogenously administered IFN affects iron metabolism. Interesting findings in the relation between IFN and hepcidin have been reported; hepcidin expression was inhibited in patients with chronic hepatitis C,[19] and this inhibition was subsequently reduced by treatment with pegylated (PEG)-IFN with ribavirin, an antiviral agent.[20] However, it is still unclear whether IFN itself influences iron metabolism. We therefore studied the effect of IFN itself on iron metabolism using mouse models.

Materials and methods

Samples and tissues. Mouse IFNα (Miltenyi Biotec, Bergisch Gladbach, Germany) was subcutaneously administered to 6-week-old C57BL/6 mice (Clea Japan, Tokyo, Japan) at a dose of 10^4 IU/day or 10^5 IU/day for 3–7 days after which the mice were sacrificed, and blood, duodenum, spleen, bone marrow of thighbone, and liver were collected. Control mice were received subcutaneous injection of phosphate-buffered saline (PBS). *Ex vivo* culture of duodenums obtained from normal mice was performed, where the duodenums were washed with PBS and cultured with Dulbecco's modified Eagle's medium (DMEM) (Wako, Tokyo, Japan) supplemented with 10% fetal calf serum (FCS) and penicillin-streptomycin (Wako), 1000 IU/mL of mouse IFNα (Miltenyi Biotec), and 0.1 μg/mL of anti-mouse IFNα, β receptor antibody (eBioscience, San Diego, CA, USA) overnight. Protocols for animal experiments were approved by the Asahikawa Medical University Institutional Animal Care and Use Committee.

Measurements of markers involved in iron metabolism. Serum analysis for iron was performed using an automatic serum analyzer LAboSPECT008 (Hitachi, Tokyo, Japan). Quick auto neo Fe (Sino-test, Tokyo, Japan) was used in measuring serum iron. The iron contents of mouse liver, spleen, and bone marrow were measured by atomic absorption spectrometry (AAS), and the ratio of iron to dried tissues is calculated.

Human and mouse hepcidin standards were synthesized at Peptide Institute (Osaka, Japan). Mice hepcidin concentrations were measured by liquid chromatography (LC)-tandem mass spectrometry (MS) as previously reported.[21,22]

The concentrations of IL-6 in mouse serum were evaluated using commercially available multiplex bead-based sandwich immunoassay kits, Bio-Plex Pro Mouse Cytokine 23-plex Assay (Bio-Rad Laboratories, Hercules, CA, USA). Assays were performed following the manufacturer's instructions. Different sandwich immunocomplexes formed on distinct bead sets were measured and quantified using the Bio-Plex Suspension Array System (Bio-Rad Laboratories). Instrument control and data processing were performed using Bio-Plex Manager software (Bio-Rad Laboratories).

Real time reverse transcription–polymerase chain reaction. Total RNA was isolated from cells and tissues with the RNA purification system PureLink RNA Mini Kit (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with a high-capacity complementary DNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA). Real-time reverse transcription–polymerase chain reaction (RT-PCR) was then performed using ABI 7300 system (Applied Biosystems) with TaqMan probes. Probes (Applied Biosystems) used included human hepcidin, mouse hepcidin, mouse transferrin receptor 1, mouse transferrin receptor 2, ferritin heavy chain, divalent metal transporter 1, FPN, human IL-6, mouse IL-6. 18S ribosomal RNA was analyzed as an internal control (18 s rRNA, Applied Biosystems), and the ratio of hepcidin to 18S ribosomal RNA was calculated.

Immunofluorescent study for FPN expression by the duodenum. Tissue samples of duodenum were fixed with 3.7% formaldehyde for 24 h and processed for paraffin embedding. The paraffin sections were sequentially treated by deparaffinization, rehydration, and antigen retrieval before applying primary antibodies as follows: for antigen retrieval, sections were immersed in retrieval solution (pH 9.0) at 125°C for 15 min in a pressure boiler. The sections were then incubated with the anti-SLC40A1/FPN1 rabbit polyclonal antibody (Medical & Biological Laboratories, Nagoya, Japan) and then with Alexa Fluora 594 conjugated anti-rabbit immunoglobulin G (IgG) antibody (Invitrogen). This was followed by nuclear staining with 4′,6-diamidino-2-phenylindole.

Cell culture. Human hepatocellular carcinoma cell line HepG2 was obtained from ATCC (Rockville, MD, USA) and cultured in DMEM, supplemented with 10% FCS and penicillin-streptomycin. Mouse primary hepatocytes were isolated from male 6-week-old C57Bl/6 male mice by the collagenase perfusion method, and the hepatocytes with more than 95% viability were prepared by the Percoll centrifugation method. The cells were then plated on collagen-coated dishes, and cultured in Williams' E medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS, 0.1 mg/mL of epidermal growth factor, 10 mg/mL of insulin and penicillin-streptomycin. These cells were maintained at 37°C in a humidified atmosphere containing 5% CO_2 and 21% O_2. The HepG2 cells were treated with 0–500 IU/mL of human IFNα (Miltenyi Biotec), and primary cultured mouse hepatocytes were treated with 0–1000 IU/mL of mouse IFNα for 24 h. The concentrations of human and mouse IFN were previously reported.[23–26] In some experiments, 0.1 mg/mL of anti-human IFNα, β receptor antibody (Abcam, Cambridge, UK), or 0.1 μg/mL of anti-mouse IFNα, β receptor antibody (eBioscience) was added to inhibit IFN from binding to its receptor at the cell surface.

Western blot analysis. The cell samples were lysed in a Radioimmunoprecipitation assay buffer, separated in Mini-protean TGX precast polyacrylamide gel (Bio-Rad Laboratories) and electro-transferred to nitrocellulose membranes. After blocking the membranes with 5% non-fat dry milk in a PBST buffer (PBS containing 0.05% Tween-20), they were probed with

anti-SLC40A1/FPN1 rabbit polyclonal antibody (Medical & Biological Laboratories), anti-signal transducer and activator of transcription3 (STAT3) (Cell Signaling Technology, Danvers, MA, USA), anti-phosho-STAT3 (Cell signaling technology), and anti-actin antibody (BD Biosciences, Franklin Lakes, NJ, USA), followed by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibody (R&D Systems, Minneapolis, MN, USA). Antibody binding was then visualized with SuperSignal West Pico Chemiluminescent Substrate (Thermo Scientific, Waltham, MA, USA).

Statistical analysis. Statistical analysis was done using the Student paired *t*-test, and P values < 0.05 were considered to be statistically significant.

Results

The changes of serum iron and liver iron concentrations by IFNα in mice. To determine how IFNα affects iron metabolism, serum markers for iron metabolism and iron contents of the liver were evaluated in IFNα-treated mice. Serum iron was significantly decreased to 202.2 ± 28.6 μg/dL in 10^4 IU/day IFNα-treated mice compared with the 249.8 ± 25.8 μg/dL found in the control mice. Serum iron levels in 10^5 IU/day IFNα-treated mice however found to be 250.6 ± 12.5 μg/dL, showing no significant decrease as compared with the control mice (Fig. 1a). The liver iron contents determined by AAS were 231.6 ± 27.5 μg/dry g in control mice, 211.4 ± 38.8 μg/dry g in 10^4 IU/day IFNα-treated mice, and 200.7 ± 9.6 μg/dry g in 10^5 IU/day IFNα-treated mice; no significant differences between the IFNα-treated groups and control group were observed (Fig. 1b).

The effect of IFNα on the expressions of the molecules involved in iron metabolism. The messenger RNA (mRNA) expression of *TfR1*, *TfR2*, *ferritin heavy chain*, and *DMT1* in the liver were determined by real-time RT-PCR using TaqMan probes, and from the results obtained, there was no significant change in any of the genes expressed. The expression of *Hamp1* (the gene codes hepcidin) in the liver of the IFNα-treated mice, however, was found to be significantly upregulated. This effect was observed in both 10^4 and 10^5 IU/day IFNα-treated groups (Fig. 2).

Changes in serum hepcidin levels after IFNα treatment were determined using LC-tandem MS. Although there was no significant difference in serum hepcidin in the 10^4 IU/day IFNα-treated group, serum hepcidin was significantly upregulated in the 10^5 IU/day IFNα-treated mice (Fig. 3a).

The change of FPN expression in the duodenum by IFNα. Because the target molecule of hepcidin is FPN, the expression of FPN was determined to find out if IFNα-induced hepcidin upregulation can physiologically decrease FPN expression. Immunofluorescent study for FPN in the duodenal tissues showed a decrease in FPN expression in both the 10^4 and 10^5 IU/day IFNα-treated groups (Fig. 3b). Furthermore, neither the mRNA nor the protein levels of FPN were affected by the *ex vivo* duodenum culture with IFNα and/or anti-IFN receptor antibody (Fig. 3c,d). To elucidate the role of IFN-treatment in iron recycling from macrophages, tissue iron contents in spleen and bone marrow were evaluated; the results showed no significant changes in both tissue iron contents between controls and IFN-treatments (Fig. 3e).

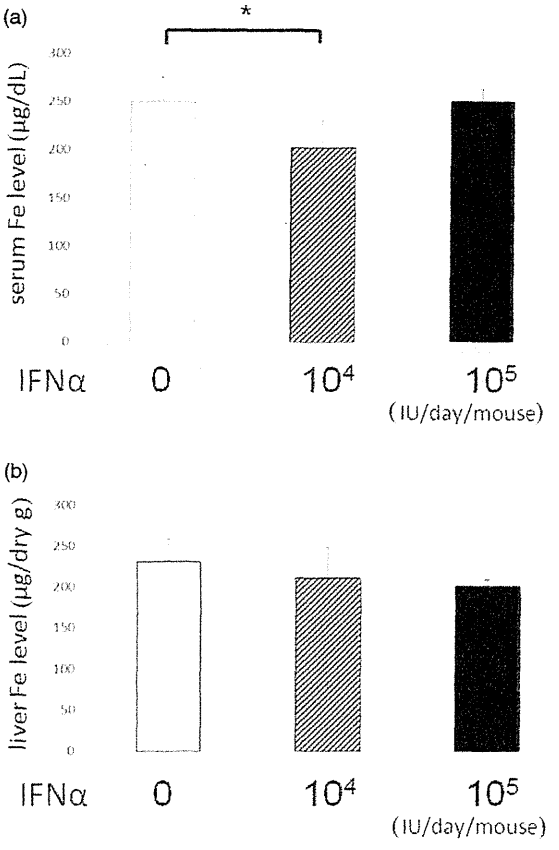

Figure 1 (a) Effect of interferon α (IFNα) on serum iron in mice. IFNα administration for 3 days at a dose of 10^4 IU/day significantly reduced serum iron level. However, no decrease in serum iron was observed in mice treated with 10^5 IU/day IFNα ($n = 5$ in each group). $^*P < 0.05$. (b) Effect of IFNα on liver iron content in mice. Liver iron content evaluated by atomic absorption spectrometry showed no significant change after administration of IFNα ($n = 5$ in each group).

Hepcidin is upregulated via direct and indirect mechanism. Because IL-6 is a strong inducer of hepcidin expression, we measured IL-6 during IFNα administration. Serum IL-6 levels were 5.3 ± 2.6, 17.4 ± 5.9, and 23.6 ± 7.8 pg/mL in the control mice, 10^4 IU/day, and 10^5 IU/day IFN-treated mice, respectively; a significant increase in serum IL-6 was observed after IFNα administration (Fig. 4). This observation suggests that IFNα may indirectly upregulate hepcidin via the induction IL-6.

We then determined if IFNα has a direct effect on hepcidin upregulation in hepatocytes. The changes in hepcidin mRNA expression after IFNα treatment was determined using primary culture hepatocytes derived from mice and human hepatoma cell line HepG2 cells. Hepcidin mRNA expression was observed to be significantly upregulated in both the primary hepatocytes

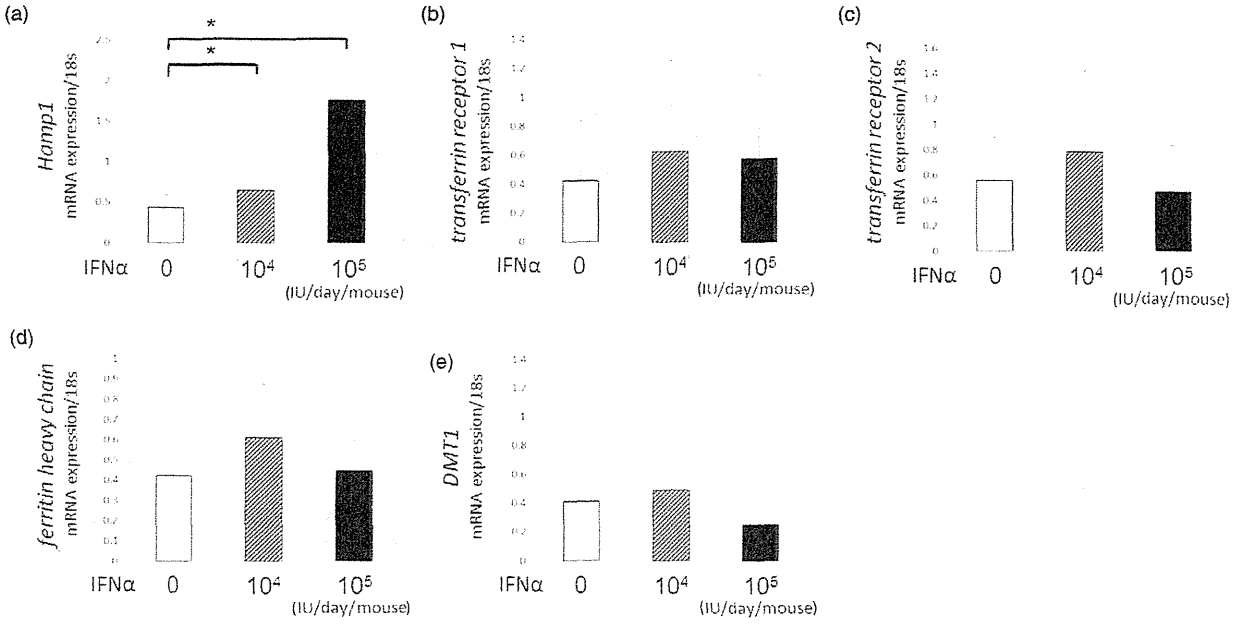

Figure 2 Changes in the expression of genes involved in iron metabolism in the liver of interferon α (IFNα)-treated mice. Quantitative polymerase chain reaction was performed to determine the expression levels of (a) *Hamp1*, (b) *transferrin receptor 1*, (c) *transferrin receptor 2*, (d) *ferritin heavy chain*, and (e) *divalent metal transporter 1 (DMT1)* using RNA extracted from the livers of mice treated with IFNα ($n = 5$ in each group). Relative expression levels were calculated by normalization using 18S expression. Only *Hamp1* expression was significantly increased by IFNα. $^*P < 0.05$.

and HepG2 cells, and this upregulation seemed to be IFNα concentration-dependent. The addition of a neutralizing antibody against IFN receptor prevented hepcidin upregulation (Fig. 5a,b).

Determination of the mechanism for direct upregulation of hepcidin by IFNα. We then focused on the direct mechanism by which IFNα may upregulate the expression of hepcidin. Commonly, binding of IFNα to IFN receptor on cell surfaces accelerates STAT1/STAT2 heterodimer formation via Janus kinase activation. However, there is no binding site for that heterodimer in the promoter region of the hepcidin gene. On the other hand, it has been reported that IFN can activate a STAT3 homodimer in human primary hepatocytes.[23] In addition, the promoter region of the hepcidin gene has a binding site for the STAT3 homodimer. Thus, it is possible that IFNα directly affects hepatocytes to enhance the transcription of the hepcidin gene via activated STAT3 homodimer. As hypothesized, phospho-STAT3 (Y705)/total STAT3 was observed to be significantly upregulated in both cultured primary mouse hepatocytes and HepG2 cells treated with IFNα (Fig. 6a,b). The addition of a neutralizing antibody against the IFN receptor cancelled STAT3 activation.

Although IL-6 is mainly produced by monocytes/macrophages and T-lymphocytes, hepatocytes are also known to produce IL-6; therefore, we determined if IFNα directly enhance IL-6 expression in hepatocytes. We determined if IFNα could induce *IL-6* mRNA expression in RNA extracted from mice liver (Fig. 7a) but found that no induction of *IL-6* mRNA expression after IFNα treatment. Because the liver contains not only hepatocytes but also other cells, such as Kupffer cells, we then examine the expression of IL-6 mRNA in primary cultured hepatocytes obtained from mice (Fig. 7b) and HepG2 cells (Fig. 7c) but did not observe any difference in *IL-6* mRNA expression with or without IFNα.

Discussion

Our results show that IFNα administration causes hepcidin upregulation in mice (Figs 2,3a). Serum IL-6 levels were increased in IFNα-treated mice (Fig. 4), so it is likely that IFNα-mediated IL-6 induction contributes to the upregulation of hepcidin as IL-6 is one of the most important inducible factors for hepcidin expression. We also confirmed that IFNα directly upregulates hepcidin expression in primary cultured mouse hepatocytes and HepG2 cells (Fig. 5a,b). IL-6 can be produced by hepatocytes; however, we excluded that possibility from the observation that IFNα treatment did not enhance IL-6 expression in hepatocytes (Fig. 7a–c). Together, IFNα directly affects hepatocytes to induce hepcidin expression and indirectly via IL-6 induction throughout the body.

There have been other reports on the effect of IFNα on hepcidin expression; for instance, Ryan *et al.* recently reported that IFNα treatment resulted in the expression of hepcidin.[27] However, they observed changes in hepcidin expression after IFNα treatment using monocytes, not hepatocytes. We used primary cultured hepatocytes from mice, thus providing more precise evidence regarding the direct effect of IFNα on hepcidin expression by hepatocytes. We then showed the cancellation effect of the anti-IFN receptor

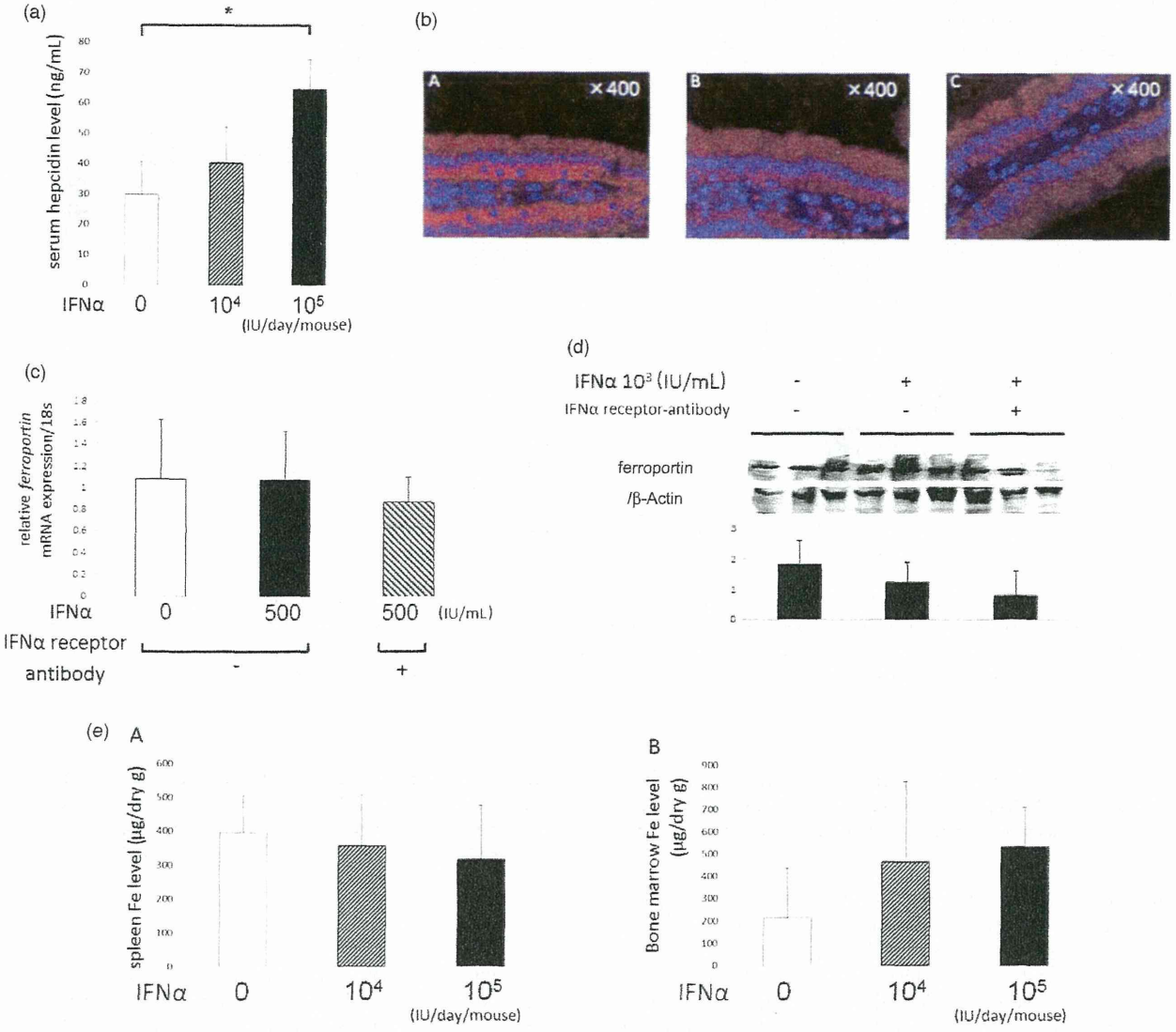

Figure 3 *(a) Concentrations of hepcidin in the serum of mice treated with interferon α (IFNα). Hepcidin levels were measured using liquid chromatography-tandem mass spectrometry (n = 5 in each group). Significant increases in hepcidin concentration were observed in mice treated with 10^5 IU/day of IFNα. * $P < 0.05$. (b) Immunofluorescent staining for ferroportin (FPN) in mouse duodenum tissue. The expression of FPN was observed on the basolateral side of the duodenum of (A) control mice, but disappeared after IFNα treatment (B) 10^4 IU/day and (C) 10^5 IU/day. (c) FPN mRNA expression and (d) FPN protein expression in mice duodenums treated with 10^3 IU/mL of IFNα and/or anti-mouse IFNα, β receptor antibody. (e) Tissue iron contents analyzed by atomic absorption spectrometry in spleens (A) and bone marrows (B).*

antibody on IFNα-induced hepcidin upregulation, indicating that the binding of IFNα to its own receptor on the cell surface of hepatocytes is necessary for increasing hepcidin expression. Binding of IFNα to its receptor usually induces the activation of the STAT1/STAT2 heterodimer, however, our results showed that IFNα-induced upregulation of activated STAT3 may be involved in hepcidin upregulation (Fig. 6a,b).

In the present study, we also confirmed that IFNα-induced hepcidin upregulation is physiologically functional, as the expression of FPN in duodenum was significantly reduced in the IFNα-treated mice (Fig. 3b). The possibility that IFNα directly decrease FPN expression seemed to be unlikely because we did not observe any significant changes of FPN expression in the duodenum treated with IFNα in the present study (Fig. 3c,d). FPN is expressed on the basolateral cell membrane of enterocytes and functions in iron transport from enterocytes to blood. FPN has also been reported to be internalized and degraded when hepcidin binds to FPN on the cell surface, resulting in a decrease in iron absorption from the gastrointestinal tract. The IFNα treatment may therefore result in a decrease in iron absorption. Besides, we observed

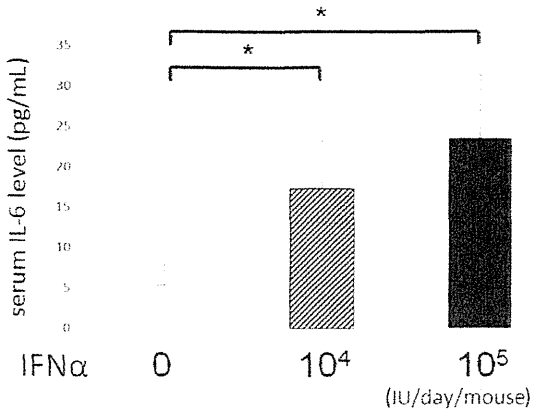

Figure 4 Change in interleukin (IL)-6 in the sera in interferon α (IFNα)-treated mice ($n = 5$ in each group). IFNα treatment significantly increased serum IL-6 levels with the administration of 10^5 IU/day of IFNα. $^*P < 0.05$.

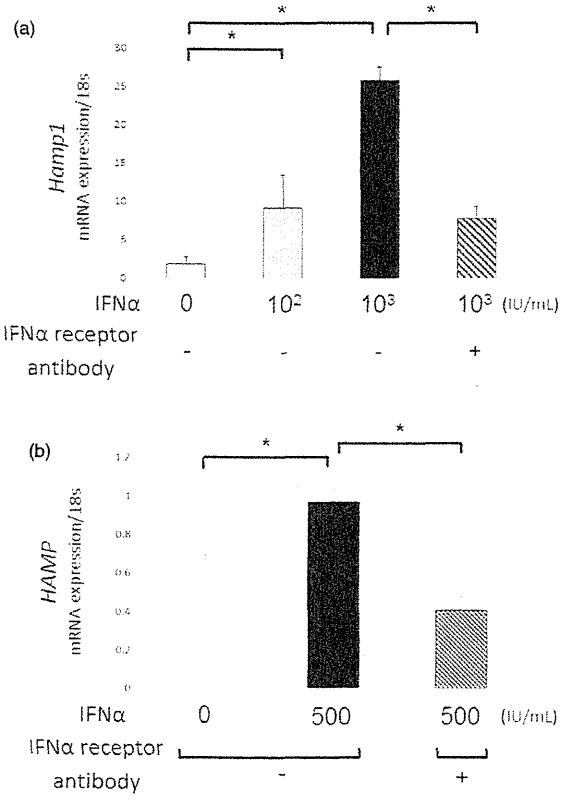

Figure 5 Expressions of hepcidin messenger RNA (mRNA) were evaluated in (a) mouse primary hepatocytes and (b) HepG2 cells. Quantitative polymerase chain reaction (PCR) was performed and the relative expression levels were calculated by normalization using 18S expression. In both cells, the expressions of hepcidin mRNA were significantly increased by the administration of interferon α (IFNα). These increases were cancelled by the neutralizing antibodies against IFNα receptors. $^*P < 0.05$.

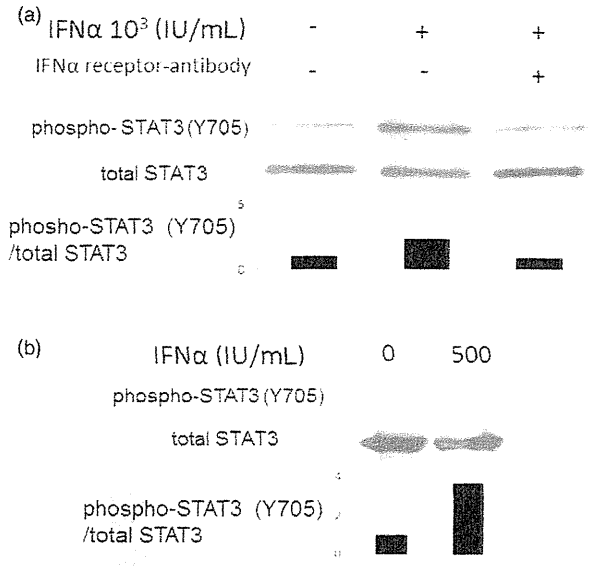

Figure 6 Phosphorylation of signal transducer and activator of transcription3 (STAT3) was determined in (a) mouse primary hepatocytes and (b) HepG2 cells treated with interferon α (IFNα). Densities of the bands corresponding to phosphorylated STAT3 levels were normalized by levels of total STAT3 shown. In both cell types, phosphorylated STAT3 was increased by IFNα treatment but cancelled using antibodies against IFNα receptors.

no significant accumulation of iron in the liver of the IFNα-treated mice (Fig. 1b), and this finding is supported by the absence of any increase in *TfR1* and *DMT1* mRNA in the liver (Fig. 2). Thus, IFN itself does not accelerate iron accumulation in the liver. However, further study will be necessary, as hepatocytes are thought to possess various pathways for iron uptake, including a transferrin receptor-independent route for the uptake of transferrin-bound iron.[28]

The decrease in FPN expression in the duodenum may explain the decrease in serum iron observed in the 10^4 IU/day IFNα-treated mice (Fig. 1a). There was a possibility that the inhibition of iron release from reticuloendothelial system contributed to the decrease of serum iron, but no significant changes of iron contents were observed in the spleen and the bone marrow (Fig. 3e). Ryan *et al.* reported that a single dose of PEG-IFNα/ribavirin resulted in a 50% reduction in serum iron and transferrin saturation over a 24-h period,[27] which is compatible with our findings in 10^4 IU/day IFNα-treated mice.

However, no significant reduction in serum iron was observed in the 10^5 IU/day IFNα-treated mice (Fig. 1a). It is however difficult to know the appropriate concentration of mouse IFNα that will have a substantially equal effect as the concentration of human IFN used in the treatment of diseases such as hepatitis. We therefore used two different concentrations of IFNα in the present study, but we have observed that the 10^5 IU/day IFNα may be too high. We also speculated that erythropoiesis may have been inhibited during the 10^5 IU/day IFNα treatment, resulting in reduced iron utilization in erythropoiesis decreased. A preliminary evaluation of cell numbers in 10^5 IU/day IFNα-treated mice thighbone

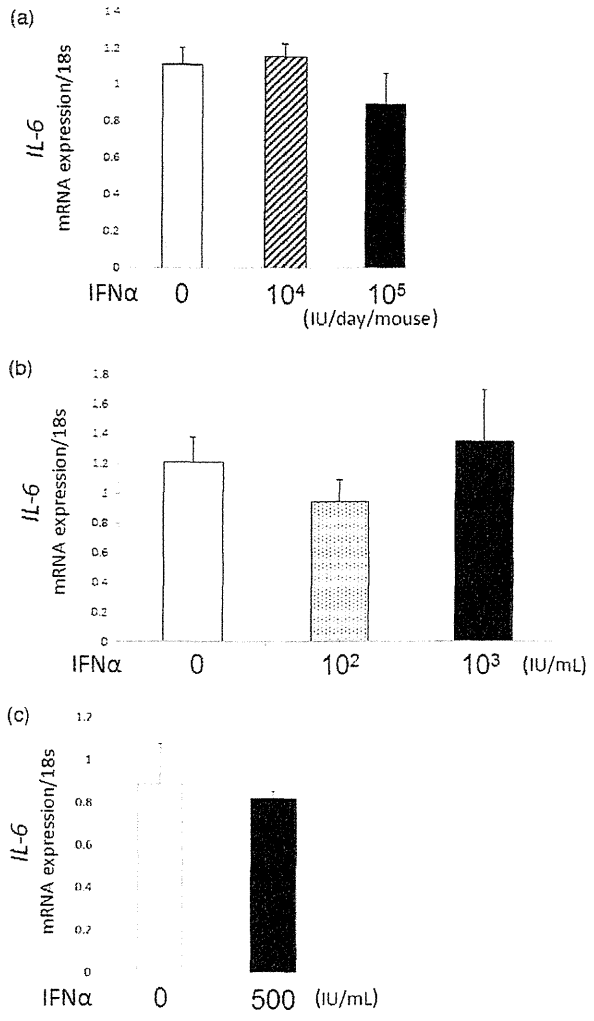

Figure 7 Interleukin-6 (*IL-6*) messenger RNA (mRNA) expression was determined in the (a) RNA extracted from the mouse liver, (b) mouse primary hepatocytes or (c) HepG2 cells treated with interferon α (IFNα) ($n = 5$ in each group). Expression levels of *IL-6* mRNA, shown as the relative expression levels calculated by normalization using 18S expression, and presented as *n*-fold changes. IFNα administration did not alter *IL-6* mRNA expression in either cell.

marrow showed a decrease in cell number (data not shown), suggesting that 10^5 IU/day IFNα may have been too high.

In addition, there might be another speculation. The gene expressions of *TfR1*, *TfR2*, *DMT1*, and *ferritin heavy chain* in the liver seemed to be slightly upregulated when 10^4 IU/day IFNα was administrated, although we did not observe any statistical significance (Fig. 2). Upregulation of TfR1, TfR2, and DMT1 might increase iron uptake, and the increase of ferritin heavy chain implies iron accumulation in the liver, arising the possibility that the accumulation of slight changes of several molecules involved in iron uptake may partly contribute to the decrease of serum iron when 10^4 IU/day IFNα was administrated. This should be only one possibility because we did not see any stainable iron overload in the liver even when 10^4 IU/day IFNα was administrated for 3 days. Further investigation should be needed to clarify if there is the increase of iron uptake by the liver when 10^4 IU/day IFNα was administered, although the change of stainable iron level cannot be detected.

In clinical situations, various drugs are also used with IFN. For instance, ribavirin is now widely used for treatment of chronic hepatitis C with IFN and is known to cause hemolysis, leading to an alteration of iron metabolism. However, our results showed that IFN itself increases hepcidin expression; therefore, it is important to evaluate iron homeostasis during treatment to decide which drug is responsible for the iron dysregulations that may occur. It is also important to take IFNα-induced hepcidin upregulation into consideration.

Acknowledgment

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Original Article

Survey of non-B, non-C liver cirrhosis in Japan

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Aim: The aim of this survey was to reveal clinical features for each etiology of non-B, non-C liver cirrhosis (NBNC LC) in Japan.

Methods: In a nationwide survey of NBNC LC in Japan at the 15th General Meeting of the Japan Society of Hepatology, 6999 NBNC LC patients were registered at 48 medical institutions. Epidemiological and clinical factors were investigated.

Results: The percentage of NBNC LC among LC patients was 26%. NBNC LC patients were categorized into 11 types according to etiological agents: non-alcoholic steatohepatitis (NASH), 14.5%; alcoholic liver disease (ALD), 55.1%; fatty liver disease (FLD), except NASH, ALD, and other known etiology, 2.5%; primary biliary cirrhosis, 8.0%; other biliary cirrhosis, 0.8%; autoimmune hepatitis, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5%. Compared with previous surveys, the percentage of ALD remained unchanged, whereas that of NASH increased. The mean age and percentage of females were significantly higher in NASH patients than in ALD and FLD patients. Prevalence of diabetes mellitus was significantly higher in NASH and FLD patients than in ALD ones. Prevalence of hepatocellular carcinoma (HCC) in NBNC LC patients was 35.9%. Among NASH, ALD and FLD patients, 50.9%, 34.3% and 54.5% had HCC, respectively. Positivity of hepatitis B core antibody was significantly higher in HCC patients than in those without HCC (41.1% vs 24.8%).

Conclusion: This survey determined the etiology of NBNC LC in Japan. These results should contribute new ideas toward understanding NBNC LC and NBNC HCC.

Key words: alcoholic liver disease, hepatocellular carcinoma, non-alcoholic steatohepatitis, non-B, non-C liver cirrhosis

INTRODUCTION

A NATIONWIDE SURVEY of liver cirrhosis (LC) for each etiology has been conducted as the main theme on four occasions at the national academic conference in Japan. Therefore, many registered patients have been surveyed on uniform diagnostic criteria.[1] The 15th General Meeting of the Japan Society of Hepatology was held in October 2011. In a featured session in this meeting, we conducted a nationwide survey of non-B, non-C LC (NBNC LC) in patients at medical institutions in Japan. NBNC LC was the main theme of the featured session in this meeting for two reasons. First, the prevalence of non-alcoholic fatty liver disease (NAFLD) is increasing and has been recently reported to be approximately 20% in adults in Japan. Approximately 1% of adults in Japan are estimated to have non-alcoholic steatohepatitis (NASH).[2,3] Thus, NASH is the most common chronic liver disease not only in Western countries but also in Japan. NASH patients can develop LC and even hepatocellular carcinoma (HCC), although there have been few investigations concerning the incidence of LC associated with NASH (NASH LC)

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in Japan. Second, the number of NBNC HCC patients has been rapidly increasing, and it has been recently reported to account for approximately 15% of all HCC patients in Japan.[4] Most NBNC HCC patients seem to have LC with alcoholic liver disease (ALD LC); however, NASH LC has been noted as a high-risk group of NBNC HCC. Nevertheless, HCC complicated with NBNC LC of an unknown cause has been occasionally reported. Therefore, it is important to investigate the clinical features of NBNC LC, which will lead to the development NBNC HCC. Based on these backgrounds, we report the characteristics of NBNC LC in Japan. This was one of the programs of the 15th General Meeting of the Japan Society of Hepatology in 2011.

METHODS

Patient database

AT 48 MEDICAL institutions (all investigators are listed in Appendix I) (Table 1), 6999 subjects were diagnosed with NBNC LC based on the negative results for serum hepatitis B surface antigen (HBsAg), anti-hepatitis C antibody and hepatitis C virus (HCV) RNA. The patients registered in this study were clinically (laboratory examinations and imaging studies) and histologically diagnosed with LC based on the criteria proposed by a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008).[1] The NBNC LC patients were categorized into 11 types according to etiology: (i) NASH; (ii) ALD; (iii) fatty liver disease (FLD); (iv) primary biliary cirrhosis (PBC); (v) other biliary cirrhosis (such as primary sclerosing cholangitis [PSC] and secondary biliary cirrhosis); (vi) autoimmune hepatitis (AIH) (including AIH-PBC overlap syndrome); (vii) metabolic disease (such as Wilson's disease, hemochromatosis and glycogen storage disease); (viii) congestive disease (including Budd–Chiari syndrome); (ix) parasitic disease (such as Japanese schistosomiasis); (x) other known etiology (such as sarcoidosis and drug-induced liver injury); and (xi) unknown etiology. The diagnosis of NASH was based on the following criteria: (i) absence of clinically significant alcohol consumption (intake of ≤20 g ethanol/day); (ii) appropriate exclusion of other liver diseases; (iii) complications with risk factors of steatosis such as obesity (in particular, visceral obesity), metabolic syndrome and diabetes mellitus; and (iv) the presence of steatosis on liver histology (histological

Table 1 Forty-eight medical institutions registered at the 15th General Meeting of the Japan Society of Hepatology on 2011

Akita University Graduate School of Medicine
Asahikawa-Kosei General Hospital
Asahikawa Medical University
(Division of Gastroenterology and Hematology/Oncology)
(Division of Metabolism and Biosystemic Science)
Asahikawa Red Cross Hospital
Chiba University
Dokkyo Medical University
Ehime Prefectural Central Hospital
Ehime University Graduate School of Medicine
Fukushima Medical University School of Medicine
Gunma University Graduate School of Medicine
Hyogo College of Medicine
Iwate Medical University
Jikei University School of Medicine, Katsushika Medical Center
Juntendo University School of Medicine
Kagawa University
Kanazawa Medical University
Keio University School of Medicine
Kumamoto University
Kurume University School of Medicine
Kyoto Second Red Cross Hospital
Mie University Graduate School of Medicine
Musashino Red Cross Hospital
Nagano Red Cross Hospital
Nara Medical University
National Center for Global Health and Medicine
Nihon University School of Medicine
Niigata Prefectural Central Hospital
Niigata University Medical and Dental Hospital
Oji General Hospital
Osaka City University
Osaka Police Hospital
Osaka Red Cross Hospital
Saiseikai Suita Hospital
Saitama Medical University
Sapporo City General Hospital
Sapporo-Kosei General Hospital
Shinshu University School of Medicine
Teikyo University School of Medicine
Teine-Keijinkai Hospital
Tokyo Medical and Dental University
Tokyo Medical University Ibaraki Medical Center
Tokyo Women's Medical University
Tottori University School of Medicine
University of Tokyo
University of Yamanashi
(First Department of Internal Medicine)
(First Department of Surgery)
Yamagata University Faculty of Medicine

diagnosis) or imaging studies (imaging diagnosis). The diagnosis of ALD was based on the proposed Diagnostic Criteria for Alcoholic Liver Disease by a Japanese study group for ALD (the Takada group).[5] The diagnosis of FLD was based on the following criteria: (i) alcohol consumption between that for NASH and ALD (i.e. intake of >20 g and <70 g ethanol/day); (ii) appropriate exclusion of other liver diseases; and (iii) the presence of steatosis on liver histology or imaging studies.

The following variables were used to investigate the clinical features of NBNC LC: age; sex; body mass index (BMI); prevalence of diabetes mellitus (DM), impaired glucose tolerance, hypertension and dyslipidemia; Child–Pugh classification; prevalence of gastroesophageal varices and HCC; and presence of hepatitis B core antibody (anti-HBc). In addition, the percentage of NBNC LC was investigated among all LC patients at each institution and was compared with previous reports. The ethics committees of the appropriate institutional review boards approved this study in accordance with the Declaration of Helsinki (2000).

Statistical analyses

Statistical tests were performed using the IBM SPSS Statistics ver. 21. The statistical significance of difference was determined using the χ^2-test, Mann–Whitney *U*-test and multivariate Cox's proportional hazard model as appropriate. $P < 0.05$ was considered statistically significant.

RESULTS

Percentage of NBNC LC among all LC patients

WE CALCULATED THE percentage of NBNC LC among all 25 020 LC patients at 37 registered institutions. The percentages of NBNC LC, hepatitis B virus (HBV)-related cirrhosis, HCV-related cirrhosis, and both HBV- and HCV-related cirrhosis were 26%, 12%, 60.9% and 1.1%, respectively. Compared with a previous nationwide survey (the 44th Annual Meeting of the Japan Society of Hepatology in 2008),[1] there was no significant difference between them (Table 2).

Frequency of each etiology among NBNC LC patients

We determined the frequency and percentage of each etiology among all 6999 NBNC LC patients at 48 registered institutions. The percentages of each etiology were as follows: NASH, 14.5%; ALD, 55.1%; FLD, 2.5%; PBC, 8.0%; other biliary cirrhosis, 0.8%; AIH, 6.8%; metabolic disease, 0.6%; congestive disease, 0.8%; parasitic disease, 0.2%; other known etiology, 0.2%; and unknown etiology, 10.5% (Table 3). Among 1015 NASH patients, 309 (30.4%) were diagnosed histologically, 402 (39.6%) were diagnosed by imaging studies and the method of diagnosis of 304 patients (30%) was not described in detail. Among 60 patients with other biliary cirrhosis, 71.7% had PSC and the rest had cholestatic diseases, except PBC and PSC (such as congenital biliary atresia and secondary biliary cirrhosis). Among 39 metabolic disease patients, 66.7% had Wilson's disease, 25.6% had hemochromatosis (glycogen storage disease, amyloidosis and citrullinemia in one patient each). All 12 cases of parasitic disease were Japanese schistosomiasis. Of 11 patients with other known etiology, two patients sarcoidosis, two post-liver transplantation, two post-hepatectomy, one drug-induced liver injury, one systemic lupus erythematosus-related liver injury and the diagnosis of the remaining patients was not described in detail.

Compared with the survey at the 44th Annual Meeting of the Japan Society of Hepatology in 2008,[1] the percentage of ALD among all NBNC LC patients did

Table 2 Percentage of NBNC LC among all patients with liver cirrhosis compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 25\,020$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 33\,379$)	*P*-value
NBNC LC	26.0%	24.0%	N.S.
HBV-related cirrhosis	12.0%	13.9%	N.S.
HCV-related cirrhosis	60.9%	60.9%	N.S.
both HBV- and HCV-related cirrhosis	1.1%	1.2%	N.S.

P-values were analyzed by χ^2-test.
HBV, hepatitis B virus; HCV, hepatitis C virus; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant.

Table 3 Frequency of each etiology among patients with NBNC LC compared with the 44th Annual Meeting of the Japan Society of Hepatology on 2008[1]

	The 15th General Meeting of the Japan Society of Hepatology on 2011 ($n = 6999$)	The 44th Annual Meeting of the Japan Society of Hepatology on 2008 ($n = 8011$)	*P*-value
NASH	14.5%	8.7%	$P < 0.001$
ALD	55.1%	56.3%	N.S.
FLD	2.5%	–	–
PBC	8.0%	9.9%	$P < 0.001$
Other biliary cirrhosis	0.8%	1.2%	$P < 0.001$
AIH	6.8%	7.9%	$P = 0.018$
Metabolic disease	0.6%	1.2%	$P < 0.001$
Congestive disease	0.8%	1.2%	$P = 0.013$
Parasites	0.2%	0.4%	$P = 0.011$
Other known etiology	0.2%	0.8%	$P < 0.001$
Unknown etiology	10.5%	12.4%	$P < 0.001$

P-values were analyzed by χ^2-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

not change (55.1% vs 56.3%), whereas that of NASH increased (14.5% vs 8.7%; $P < 0.001$) (Table 3).

Clinical features of NBNC LC patients

The male : female ratio for the NBNC LC patients was 1.93. The percentages of each etiology among 4608 male and 2391 female patients were as follows: NASH (9.5% and 24%), ALD (73.4% and 19.8%), FLD (3.4% and 0.9%), PBC (1.9% and 20%), other biliary cirrhosis (0.8% and 0.9%), AIH (1.5% and 17.1%), metabolic disease (0.5% and 0.8%), congestive disease (0.8% and 0.8%), parasitic disease (0.2% and 0.1%), other known etiology (0.1% and 0.2%) and unknown etiology (7.9% and 15.4%), respectively (Fig. 1). The male : female ratio for each etiology among the NBNC LC patients was as follows: NASH, 0.77; ALD, 7.12; FLD, 6.86; PBC, 0.18; other biliary cirrhosis, 1.73; AIH, 0.17; metabolic disease, 1.29; congestive disease, 2.17; parasitic disease, 5; other known etiology, 0.83; and unknown etiology, 0.99 (Table 4). Thus, the NASH patients were predominantly female as opposed to the ALD and FLD patients who were predominantly male.

The mean age at clinical diagnosis in the NBNC LC patients for NASH, ALD, FLD, PBC, other biliary cirrhosis, AIH, metabolic disease, congestive disease, parasitic disease, other known etiology and unknown etiology was 66.9, 60.3, 64.2, 63.6, 51.3, 64.5, 42.6, 52.7, 77.4, 56.1 and 68.8 years, respectively. In the patients with NASH, AIH, congestive disease and unknown etiology, the mean ages at clinical diagnosis of the male patients were lower than those of the female patients ($P < 0.001$). In contrast, in the ALD, FLD, PBC and metabolic disease patients, the mean ages at clinical diagnosis of the female patients were lower than those of the male patients ($P < 0.001$) (Table 5).

Regarding the risk factors of NASH, the following variables were investigated in the NASH, ALD and FLD patients: BMI and the prevalence of DM, impaired glucose tolerance (IGT), hypertension and dyslipidemia. BMI in the NASH, ALD and FLD patients was 27, 23.4 and 25 kg/m^2, respectively, and the differences among them were statistically significant. The prevalence of DM and IGT in the NASH and FLD patients (63% and 57%, respectively) was significantly higher compared with that in the ALD patients (31%) ($P < 0.001$). The prevalence of dyslipidemia in the NASH and FLD patients (25% and 29%, respectively) was significantly higher compared with that in the ALD patients (14%) ($P < 0.001$). The prevalence of hypertension in the NASH patients (52%) was significantly higher compared with that in the ALD and FLD patients (28% and 35%, respectively) ($P < 0.001$) (Table 6).

The levels of hepatic functional reserve based on the Child–Pugh classification for each etiology are summarized in Table 7. The percentages of moderate-to-low hepatic reserve (Child–Pugh class B and C) in the ALD and AIH patients (52.9% in both) were significantly higher compared with those in the NASH and FLD patients (35.8% and 27%, respectively) ($P < 0.001$).

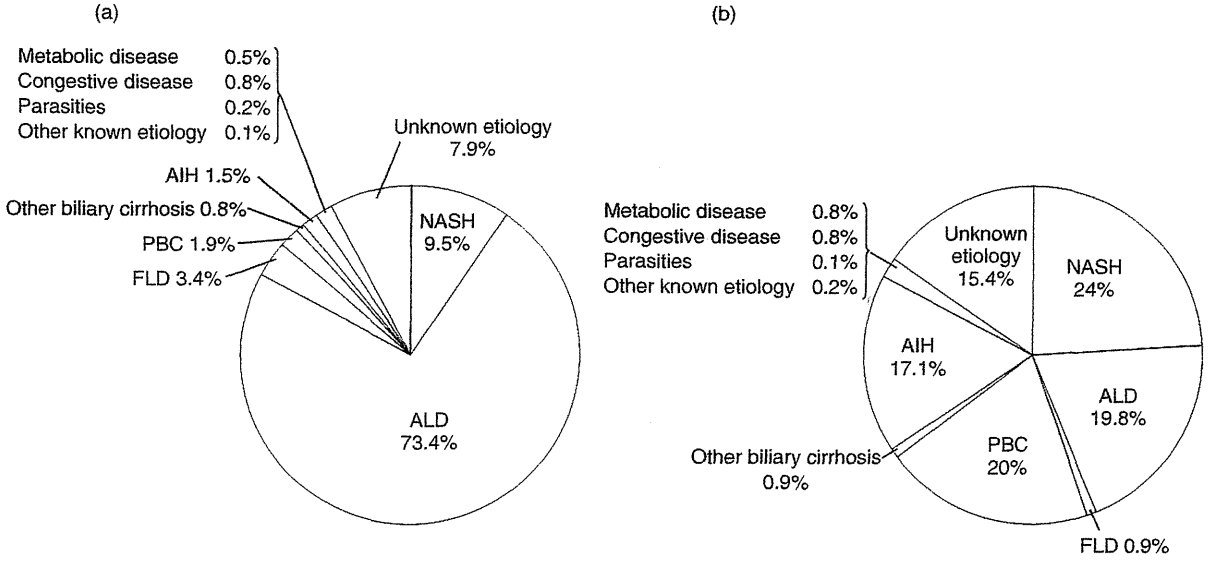

Figure 1 Frequency of each etiology among male or female patients with NBNC LC. (a) Male, (b) female. AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

To determine the frequency of complicated portal hypertension patients, the prevalence of gastroesophageal varices was calculated. The prevalence in the ALD and PBC patients (54.5% and 61.9%, respectively) was significantly higher compared with that in the patients with NASH, FLD, AIH and unknown etiology (40.8%, 40.7%, 48.2% and 45.9%, respectively) ($P < 0.05$). Considering only patients with Child–Pugh class A, the prevalence of gastroesophageal varices in PBC patients was highest among all etiologies. ALD had significantly higher prevalence than NASH, the histology of which was very similar (Table 8).

Table 4 Male : female ratio of each etiology

	Male ($n = 4608$)	Female ($n = 2391$)	Male : female ratio
NASH	440	575	0.77
ALD	3381	475	7.12
FLD	151	22	6.86
PBC	87	477	0.18
Other biliary cirrhosis	38	22	1.73
AIH	69	409	0.17
Metabolic disease	22	19	1.29
Congestive disease	39	18	2.17
Parasites	10	2	5.00
Other known etiology	5	4	0.83

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; N.S., not significant; PBC, primary biliary cirrhosis.

The prevalence of HCC in the NBNC LC patients was 35.9%. Among 2438 NBNC HCC patients, 51.9% were diagnosed with HCC simultaneously with the diagnosis of NBNC LC, 25.6% were diagnosed after, 1.4% were diagnosed before the diagnosis of NBNC LC and the diagnosis of the remaining patients was not described in detail. The male : female ratio for the NBNC HCC patients was 3.06. The percentage of each etiology among the HCC patients was as follows: NASH, 19.9%; ALD, 53.4%; FLD, 3.7%; PBC, 3.2%; other biliary cirrhosis, 0.2%; AIH, 4.9%; metabolic disease, 0.1%; congestive disease, 0.7%; parasitic disease, 0.1%; other known etiology, 0%; and unknown etiology, 13.8%. The percentage of NASH among the NBNC HCC patients was significantly higher than that among the NBNC LC patients (19.9% vs 14.5%, $P < 0.001$). The clinical diagnosis of HCC was made at a mean age of 67.2 years in all patients. The mean age of onset of HCC was 70.8, 64.8 and 68.4 years in the NASH, ALD and FLD patients, respectively, and the differences among them were significant ($P < 0.001$). The prevalence of

Table 5 The mean ages at clinical diagnosis in the patients with NBNC LC

	Total ($n = 6999$)	Male ($n = 4608$)	Female ($n = 2391$)	*P*-value (M vs F)
NASH	66.9 ± 11.6	64.8 ± 13.2	68.5 ± 9.8	$P < 0.001$
ALD	60.3 ± 11.0	60.9 ± 10.7	55.7 ± 12.1	$P < 0.001$
FLD	64.2 ± 11.8	64.7 ± 11.3	61.2 ± 15.0	$P < 0.001$
PBC	63.6 ± 12.1	66.0 ± 11.3	63.2 ± 12.0	$P < 0.001$
Other biliary cirrhosis	51.3 ± 20.7	52.0 ± 22.0	50.0 ± 19.0	$P < 0.001$
AIH	64.5 ± 12.2	63.3 ± 14.2	66.0 ± 11.7	$P < 0.001$
Metabolic disease	42.6 ± 18.2	44.0 ± 18.0	40.7 ± 19.0	$P < 0.001$
Congestive disease	52.7 ± 20.4	50.5 ± 20.7	57.4 ± 19.6	$P < 0.001$
Parasites	77.4 ± 5.9	76.5 ± 6.1	81.5 ± 2.1	$P < 0.001$
Other known etiology	56.1 ± 19.1	53.0 ± 18.7	58.7 ± 20.8	$P < 0.001$
Unknown etiology	68.8 ± 11.9	67.9 ± 13.0	69.8 ± 10.7	$P < 0.001$

All results are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

HCC in the patients with NASH, FLD and unknown etiology (50.9%, 54.5% and 47.5%, respectively) were significantly higher compared with that in the ALD, PBC and AIH patients (34.3%, 14.4% and 26.0%) ($P < 0.0001$). The percentage of moderate-to-low hepatic reserve (Child–Pugh class B and C) in HCC in AIH patients was significantly higher than those in the patients with NASH, FLD and unknown etiology

Table 6 Risk factors of NASH in the patients with NASH, ALD and FLD

Variable	NASH ($n = 1015$)	ALD ($n = 3856$)	FLD ($n = 173$)	*P*-value
Body mass index (kg/m^2)	27.0 ± 4.3	23.4 ± 6.4	25.0 ± 3.7	$P < 0.001$*,**
Diabetes mellitus or Impaired glucose tolerance	62.5%	37.5%	56.5%	$P < 0.001$*
Dyslipidemia	25.0%	13.5%	29.4%	$P < 0.001$* $P = 0.01$**
Hypertension	52.0%	28.2%	34.7%	$P < 0.001$*,**

ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.
Results of body mass index are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test and χ^2-test.
*NASH vs ALD, **NASH vs FLD.

Table 7 Levels of hepatic functional reserve based on the Child–Pugh classification

Child–Pugh classification	Class A	Class B	Class C	Percentages of both class B and C	*P*-value
NASH ($n = 783$)	503	222	58	35.8%	
ALD ($n = 2710$)	1276	867	567	52.9%	$P < 0.001$*
FLD ($n = 89$)	65	18	6	27.0%	
PBC ($n = 355$)	204	105	46	42.5%	
AIH ($n = 295$)	139	106	50	52.9%	$P < 0.001$** $P = 0.01$***
Unknown etiology ($n = 515$)	300	150	65	41.7%	

AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.
P-values were analyzed by χ^2-test.
*vs NASH, FLD, PBC and unknown etiology; **vs NASH and FLD; ***vs PBC and unknown etiology.

Table 8 Prevalence of patient with gastroesophageal varices

	Total	Child–Pugh classification		
		Class A	Class B	Class C
NASH ($n = 686$)	40.8% (280/686)*	31.8% (138/434)**	56.1% (111/198)	57.4% (31/54)
ALD ($n = 2365$)	54.5% (1289/2365)***	44.2% (486/1099)†	59.0% (447/757)	69.9% (356/509)
FLD ($n = 81$)	40.7% (33/81)	36.5% (23/63)	50.0% (7/14)	75.0% (3/4)
PBC ($n = 331$)	61.9% (205/331)††	53.5% (100/187)††	70.6% (72/102)	78.6% (33/42)
AIH ($n = 278$)	48.2% (134/278)	39.7% (52/131)	53.5% (53/99)	60.4% (29/48)
Unknown etiology ($n = 401$)	45.9% (184/401)	42.9% (94/219)	47.2% (60/127)	54.5% (30/55)

P-values were analyzed by Fisher's exact test or χ^2-test.
*$P < 0.05$, vs ALD, PBC and AIH; **$P < 0.01$, vs ALD, PBC and unknown etiology; ***$P < 0.05$, vs NASH, FLD and unknown etiology; †$P < 0.0001$ vs NASH; ††$P < 0.05$ vs NASH, ALD, FLD, AIH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; FLD, fatty liver disease; NASH, non-alcoholic steatohepatitis.

($P < 0.0001$). BMI in the NASH, ALD and FLD patients was 26.8, 24.0 and 25.8 kg/m^2, respectively, and the differences among them were statistically significant. (Table 9).

Table 10 shows the analysis of the risk factors associated with HCC in patients with ALD LC. Obesity and complication of DM were the risk factors of hepatic carcinogenesis in ALD LC patients as well as male sex and being older. Conversely, portal hypertension and anemia of ALD LC patients without HCC were worse than those with HCC. Accordingly, we investigated the comparison of the clinical features between the two ALD LC groups divided based on BMI (Table 11). Although the mean age was similar in these two groups, the prevalence of HCC in the ALD LC patients with obesity (BMI, ≥25 kg/m^2) was significantly higher compared with that in those without obesity (BMI, <25 kg/m^2) (48.3% vs 35.7%, $P < 0.001$) and similar to that in the NASH LC patients (48.3% vs 50.9%, not significant).

Of the NBNC LC patients, 31.3% were anti-HBc positive. Anti-HBc positivity was 30.7%, 30.8%, 34.7% and 43% in the patients with NASH, ALD, FLD and unknown etiology, respectively. The positivity was significantly higher in the patients with unknown etiology compared with the NASH, ALD and FLD patients ($P < 0.001$). Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC (41.1% vs 24.8%, $P < 0.001$).

DISCUSSION

THIS NATIONWIDE SURVEY revealed the following clinical features in the NBNC LC patients:

1 Compared with the previous nationwide survey,[1] the percentage of ALD among the NBNC LC patients remained unchanged, whereas that of NASH increased.
2 The NASH LC patients were significantly older, predominantly female, heavier, hypertensive and more likely to have DM and HCC.
3 The ALD LC patients were significantly younger, predominantly male, had low hepatic reserve and were more likely to have portal hypertension than NASH LC.
4 The FLD LC patients were observed at an age between that of the NASH and ALD patients, were predominantly male (similar to the ALD patients) and were more likely to have DM and HCC similar to the NASH patients.
5 Approximately 10% of the NBNC LC patients still had an unknown etiology, and these patients were more likely to have HCC similar to both the NASH and FLD patients.
6 Anti-HBc positivity was significantly higher in the HCC patients than in those without HCC.

Although the natural history of NASH is not completely understood, Matteoni *et al.* reported that 23% of NASH patients progressed to cirrhosis within 10–15 years.[6] In addition, Starley *et al.* recently stated that approximately 26–37% of NASH patients demonstrate the progression of fibrosis over time periods up to 5.6 years, with up to 9% patients progressing to cirrhosis.[7] BMI and DM have been found to be independent risk factors associated with the progression of fibrosis in NASH patients.[8] Therefore, it is thought that the NASH LC patients in the present study had significantly more severe disease and were more likely to have DM. Conversely, the prevalence of NAFLD in Japan appears to be twice as high in males than in females;[9] however, the NASH LC patients in the present study were

Table 9 Clinical features of patients with HCC

	Percentage (%)	Prevalence of HCC (%)	Age of onset of HCC (years)	M : F ratio	Child–Pugh classification (A/B/C, %)	BMI	Platelet (10^3/mm^3)
Total (n = 2438)	100	35.9	67.2 ± 10.1	3.06	62.6/28.8/8.6	24.6 ± 4.0	127 ± 66
NASH (n = 485)	19.9	50.9*	70.8 ± 9.0**	1.06	66.0/28.9/5.1	26.8 ± 4.3‡‡	128 ± 61
ALD (n = 1302)	53.4	34.3	64.8 ± 9.4†	19.05††	60.3/29.8/9.9	24.0 ± 3.8	126 ± 66
FLD (n = 91)	3.7	54.5*	68.4 ± 8.8***	17.20††	82.5/15.9/1.6	25.8 ± 4.0‡‡‡	120 ± 61
PBC (n = 79)	3.2	14.4	68.0 ± 10.4***	0.32†††	53.2/35.9/10.9	22.3 ± 3.0	110 ± 54
Other biliary cirrhosis (n = 4)	0.2	6.8	–	–	–	–	–
AIH (n = 119)	4.9	26.0	68.8 ± 8.7**	0.23†††	42.5/42.5/15.0‡	24.3 ± 4.1	107 ± 60§
Metabolic disease (n = 2)	0.1	5.1	–	–	–	–	–
Congestive disease (n = 16)	0.7	32.0	52.0 ± 16.6	1.67	57.2/21.4/21.4	23.6 ± 3.2	127 ± 72
Parasites (n = 3)	0.1	30.0	–	–	–	–	–
Unknown etiology (n = 337)	13.8	47.5*	70.9 ± 10.9**	1.57	70.8/22.4/6.8	23.6 ± 3.7	143 ± 76

Results of age are expressed as mean ± standard deviation. P-values were analyzed by Mann–Whitney U-test and χ^2-test as appropriate.
*P < 0.0001, vs ALD, PBC and AIH; **P < 0.0001, vs ALD and congestive disease; ***P < 0.001, vs ALD and congestive disease; †P < 0.001, vs congestive disease; ††P < 0.0001, vs NASH, PBC, AIH and unknown etiology; †††P < 0.0001, vs NASH, ALD, FLD and unknown etiology; ‡P < 0.0001, vs NASH, FLD and unknown etiology; ‡‡P < 0.0001, vs ALD, PBC, AIH and unknown etiology; ‡‡‡P < 0.0001, vs ALD, PBC and unknown etiology; §P < 0.001, vs NASH and unknown etiology.
AIH, autoimmune hepatitis; ALD, alcoholic liver disease; BMI, body mass index; FLD, fatty liver disease; HCC, hepatocellular carcinoma; NASH, non-alcoholic steatohepatitis; NBNC LC, non-B, non-C liver cirrhosis; PBC, primary biliary cirrhosis.

predominantly female. Yasui *et al.* reported that NASH HCC patients were predominantly male, although the prevalence of cirrhosis among these patients was significantly lower in male patients compared with that in female patients.[10] These studies suggest that sex is implicated in the progression of fibrosis in NASH patients in Japan. In addition, the prevalence of HCC in the NASH LC patients in the present study was significantly higher compared with that in the previous nationwide survey (50.9% vs 31.5%, $P < 0.001$).[1] The incidence of NASH and NASH HCC has been gradually increasing in Japan, contrary to the decreased incidence of virus-related HCC.[4] Starley *et al.* found that as many as 4–27% of cases of NASH transform to HCC after the development of cirrhosis, and that the prevalence of HCC in NAFLD is 0–0.5%, whereas that of HCC in NASH is 0–2.8% over time periods of up to 19.5 years.[7] Yatsuji *et al.* reported the prospective evaluation of NASH LC and HCV-related LC (LC-C). They reported that NASH LC followed a course similar to that of LC-C, namely, complications of cirrhosis developed, including HCC (the 5-year cumulative rate of HCC development was 11.3% for NASH LC and 30.5% for LC-C).[11] Therefore, NASH LC patients need to be followed up carefully with respect to the occurrence of HCC, similar to LC-C patients.

Alcoholic liver disease remains the most prevalent cause of NBNC LC in Japan, accounting for approximately 55% of all NBNC LC cases. In the present study, the prevalence of HCC was significantly lower in the ALD LC patients than in the NASH LC patients, whereas the ALD LC patients were significantly younger and had a lower hepatic reserve. Regarding the comparison of outcomes with LC-C, Toshikuni *et al.* reported that the risk of HCC was lower in ALD LC than in LC-C, whereas the risk of hepatic decompensation and mortality was the same.[12] It is estimated that there are approximately 2.4 million heavy drinkers in Japan, and the number of ALD patients has been increasing because of increased alcohol consumption.[13] Therefore, ALD LC patients need to be followed up carefully with respect to the occurrence of hepatic decompensation, similar to LC-C patients. Obesity appears to be involved in the progression of ALD LC.[13] Accordingly, we investigated the risk factors associated with HCC and clarified that obesity and complication of DM could be the risk for hepatic carcinogenesis in ALD LC patients. The comparison of the clinical features between the two ALD LC groups divided based on BMI revealed that the prevalence of HCC in the ALD LC patients with obesity was significantly higher compared with that in those without obesity. Horie *et al.* also reported similar results.[14] Thus,

Table 10 Factors associated with HCC in patients with ALD

Factors	HCC (–), ($n = 2494$)	HCC (+), ($n = 1303$)	Univariate analysis, *P*-value	Multivariate analysis, *P*-value
Sex (M : F)	83.7%:16.3%	95.0%:5.0%	<0.0001	<0.0001
Age (years)	57.9 ± 11.0	64.8 ± 9.4	<0.0001	<0.0001
Body mass index (kg/m^2)	22.8 ± 3.8	24.0 ± 3.8	<0.0001	<0.0001
Hypertension (– : +)	77.4%:22.6%	61.9%:38.1%	<0.0001	0.068
Dyslipidemia (– : +)	87.0%:13.0%	81.6%:18.4%	<0.0001	0.482
Diabetes mellitus (– : +)	67.2%:32.8%	50.2%:49.8%	<0.0001	<0.0001
Child–Pugh classification (A : B + C)	38.5%:61.5%	60.3%:39.7%	<0.0001	0.188
Esophageal varices (– : +)	42.3%:57.7%	57.9%:42.1%	<0.0001	<0.0001
Ascites (– : +)	57.1%:42.9%	76.5%:23.5%	<0.0001	<0.0001
WBC (/mm^3)	6014 ± 3465	5532 ± 3484	0.001	0.547
Hemoglobin (g/dL)	11.3 ± 2.6	12.7 ± 2.2	<0.0001	<0.0001
Platelet (×10^3/mm^3)	114.6 ± 67.1	126.1 ± 65.5	<0.0001	0.104
AST (IU/L)	93 ± 209	65 ± 71	<0.0001	0.974
ALT (IU/L)	51 ± 118	45 ± 43	0.159	0.786
Bilirubin (mg/dL)	2.8 ± 3.9	1.6 ± 2.4	<0.0001	0.006
Albumin (g/dL)	3.3 ± 1.0	3.5 ± 0.7	<0.0001	0.281
PT%	69 ± 22	79 ± 19	<0.0001	0.628

Results of age are expressed as mean ± standard deviation. *P*-values were analyzed by Mann–Whitney *U*-test, χ^2-test and multivariate Cox's proportional hazard model as appropriate.
ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; HCC, hepatocellular carcinoma; PT, prothrombin time; WBC, white blood cells.

obesity appears to be involved in the progression of HCC in ALD LC. Therefore, ALD LC patients with obesity need to be followed up carefully with respect to the occurrence of HCC, similar to NASH LC and LC-C patients. Not only abstinence from alcohol but also improvement in lifestyle is important to decrease the progression of ALD LC.

In the present study, we established a new clinical etiologic criterion: FLD. According to previous clinical etiologic criteria in Japan, mild drinkers (intake of >20 g and <70 g of ethanol/day) with steatohepatitis were not diagnosed with both NASH and ALD. The prevalence of minor homozygote or heterozygote type of the aldehyde dehydrogenase-2 gene (*ALDH2*), which oxidizes acetaldehyde to acetate and is a key enzyme in alcohol metabolism, is very high in Asian countries. The enzyme activity of a minor homozygote of *ALDH2* is completely defective. Moreover, the enzyme activity of a heterozygote is only 1/16th. Our survey is the first to reveal that these FLD LC patients were observed in 2.5% of NBNC LC patients. Considering the frequencies of mild drinkers and obese people in Japan, it is thought that the frequency of FLD LC is lower than that of LC with unknown etiology. This is because there were many patients whose amounts of daily alcohol intake were unknown; therefore, some were diagnosed as having an

Table 11 Clinical features of patients with ALD LC

	BMI <25 ($n = 1915$)	BMI ≥25 ($n = 749$)	*P*-value
Sex (M : F)	1644:317 (83.4%:16.6%)	692:57 (92.4%:7.6%)	$P < 0.001$
Age	60.2 ± 11.1	61.0 ± 10.2	N.S.
Diabetes mellitus	35.1%	43.9%	$P < 0.001$
HCC	35.7%	48.3%	$P < 0.001$

Results of age are expressed as mean ± standard deviation, *P*-values were analyzed by by Mann–Whitney *U*-test and χ^2-test as appropriate.
ALD, alcoholic liver disease; BMI, body mass index; HCC, hepatocellular carcinoma; N.S., not significant.

unknown etiology. Interestingly, the clinical features of the FLD LC patients overlapped with those of the NASH LC and ALD LC patients. Because the mean age of the FLD LC patients was between that of the NASH and ALD patients, the FLD LC patients were predominantly male, similar to the ALD LC patients, and they were more likely to have DM and HCC similar to the NASH LC patients. Horie *et al.* described a category such as FLD as overlap steatohepatitis.[13,14] The most important clinical feature in FLD LC patients was that the prevalence of HCC was high, similar to that in the NASH LC patients. This finding suggests that steatohepatitis per se is a potent risk factor of HCC, irrespective of alcohol consumption.

The LC patients with unknown etiology (or cryptogenic LC) were approximately 10% of the NBNC LC patients and were more likely to have HCC similar to the NASH and FLD patients. Some FLD LC patients whose daily alcohol intake was unknown may have been included in this group, and some "burnt-out" NASH LC patients whose liver showed complete disappearance of steatosis[15] may have also been included in this group. In addition, some patients who had been HBV carriers but had become HBsAg negative or those with occult HBV may have also been included in this group. Anti-HBc positivity was significantly higher in this group than in the NASH, ALD and FLD LC groups. Several studies have suggested a high prevalence of occult HBV among cryptogenic LC and NBNC HCC patients and also the participation of occult HBV in the progression to cirrhosis and occurrence of HCC.[16,17] In the present study, anti-HBc positivity was significantly higher in the NBNC LC patients with HCC than in those without HCC; however, the role of occult HBV in the progression to cirrhosis and carcinogenesis remains unclear. Occult HBV is defined as the presence of HBV DNA in the liver (with or without detectable HBV DNA in serum) for patients testing HBsAg negative.[18] Because of the lack of a HBV DNA assay in the present study, the impact of occult HBV on carcinogenesis could not be evaluated. Thus, a HBV DNA assay in the liver is needed for the evaluation of occult HBV on carcinogenesis. Although NBNC LC seemed to include varied etiology, occult HBV should be taken into account in the prediction of future HCC development in NBNC LC.

Our nationwide survey determined the etiology of NBNC LC in Japan. Future changes in etiology must be considered for the establishment of precise diagnostic strategies. We hope that these results contribute new ideas toward understanding NBNC LC and NBNC HCC.

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APPENDIX I

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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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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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