Introduction

Nonalcoholic fatty liver disease (NAFLD) is a spectrum of liver disorders ranging from nonalcoholic steatosis to nonalcoholic steatohepatitis (NASH), which can develop to progressive disease including advanced liver fibrosis and hepatocellular carcinoma [1, 2]. Prolonged overnutrition causes accumulation of free fatty acid and triglycerides within the liver, which is referred to as steatosis. Simple steatosis leads to a predisposition for steatohepatitis, which exhibits inflammatory cell accumulation and fibrosis in the liver in addition to the steatosis [1, 2]. To transform from steatosis to steatohepatitis, several key biological responses such as oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and abnormal cytokine properties have been reported to be required [1–4]. However, the immunological aspect, in particular, that is involved in the development of steatosis/steatohepatitis remains to be fully elucidated.

Invariant natural killer T (iNKT) cells are characterized by the expression of surface markers of natural killer (NK) cells together with a single invariant T cell receptor (TCR) encoded by Vα14-Jα18 in mice and Vα24-Jα18 in humans [5]. These cells are included within the population of T cells expressing NK cell markers, also known as NKT cells [5, 6]. iNKT cells recognize glycolipid antigens presented in association with the major histocompatibility complex class Ib molecule CD1d [5], which is expressed on a variety of cells including dendritic cells, B cells, and stellate cells, as well as hepatocytes in the liver [5, 7, 8]. Following the recognition of antigens via TCR, iNKT cells have the ability to produce the T-helper (Th) 1 cytokine, interferon (IFN)-γ, and the Th2 cytokines, interleukin (IL)-4, -5, and -13, modulating subsequent immune responses [5, 6, 9]. These cells have been shown to play a proinflammatory role in some immune responses and an anti-inflammatory role in other immune responses [5, 6, 9]. iNKT cells most frequently reside in the liver in mice [10, 11]. Although humans appear to have proportionally fewer iNKT cells than mice, human iNKT cells also preferentially reside in the liver [12, 13]. Several lines of evidence indicate that the number of NKT cells is dysregulated in the development of NAFLD. Hepatic iNKT cells or NK1.1+ CD3+/TCRβ+ NKT cells, for instance, have been reported to decrease with the development of steatosis in wild-type (WT) as well as leptin-deficient ob/ob mice [14–17]. A reduced level of peripheral Vα24+ NKT cells has been associated with human NAFLD [18]. On the other hand, CD56+ CD3+ NKT cells have been recently reported to be increased in the livers of patients with NAFLD [19]. Also, the adoptive transfer of NK1.1+ CD3+ NKT cells has been shown to alleviate hepatic steatosis in ob/ob mice [20]. However, the precise role of NKT cells in the pathogenesis of NAFLD has not been investigated in the presence of a deficiency of these cells.

In the present study, we used iNKT cell-deficient as well as WT mice fed either a normal diet (ND) or a high-fat diet (HFD), and examined the role of these cells in the development of HFD-induced steatosis/steatohepatitis. We found that the lack of iNKT cells, together with the HFD, led to liver inflammation, which was characterized by the enhanced gene expression of inflammatory cytokines and chemokines and by T cell accumulation. We also found that prolonged liver inflammation in the absence of iNKT cells developed to liver fibrosis which was strongly enhanced by the HFD. This study delineated an immunoregulatory function of iNKT cells and their key role against liver inflammation progressing to fibrosis exacerbated by an HFD, which might represent a clinical aspect of human progressive NAFLD.

Materials and methods

Animals and animal care

Specific pathogen-free BALB/c WT mice were purchased from CLEA Japan (Tokyo, Japan) as needed. Breeding pairs of BALB/c Jα18-deficient (KO) mice [21, 22] were provided by Drs. Masaru Taniguchi and Ken-ichiro Seino (RIKEN, Yokohama, Japan). The KO mice were confirmed to have no iNKT cells by the use of mouse-CD1d tetramers loaded with α-galactosylceramide in the flow cytometry procedure described below (data not shown). These mice were kept in isolation facilities at the Institute of Experimental Animal Science, Osaka University. They were housed in groups of five in filter cages and were maintained in a temperature-controlled, specific-pathogen-free room on 12-h light and dark cycles with ad libitum access to water and diet as indicated.

Experimental protocol

Male mice used in the experiments were fed an irradiated HFD consisting of 56.7% of the calories from fat (HFD32; CLEA Japan) or an irradiated ND consisting of 14% of the calories from fat (CRF-1; Oriental Yeast, Osaka, Japan), starting from when the mice were 6–8 weeks old. In preliminary experiments, we monitored the body weight of the WT mice and KO mice fed the ND or HFD every 2 weeks after the initiation of feeding, because a gain of body weight usually parallels the level of hepatic steatosis as well as obesity. We did not observe a gain of body weight of more than 25% until 4 weeks after the initiation of feeding. In mice fed the HFD, the body weight gain reached a plateau around 14–16 weeks after the initiation

of feeding. These observations led us to set the time point for estimating liver steatosis and injury and inflammation or liver fibrosis during the course of feeding at week 5 or week 15, respectively. At the end of the indicated periods, the mice were weighed and anesthetized with pentobarbital sodium, and then their abdomens were opened. Following blood sampling via the inferior caval vein, the portal vein and inferior caval vein were cut to enable blood outflow and then the liver was removed, weighed, and processed for further analyses. All animal experimental protocols were approved by the Institute of Experimental Animal Science, Osaka University. To evaluate the levels of liver injury, serum alanine aminotransferase (ALT) activities were measured as previously described [23]. To determine the levels of steatosis, total lipids were extracted from the liver and then triglyceride content was measured as previously described [24].

Flow cytometric analysis

Liver mononuclear cell populations were prepared as previously described [11, 23]. Cell surface staining of the prepared cells was performed as described [11, 23], using the following antibodies or tetramers: fluorescein isothiocyanate-conjugated anti-CD49b (DX5), phycoerythrin-conjugated anti-CD4 (H129.19), peridinin chlorophyll protein-conjugated anti-CD8α (53-6.7), and allophycocyanin-conjugated anti-TCRβ (H57-597) monoclonal antibody, or fluorescein isothiocyanate-conjugated anti-TCRβ, phycoerythrin-conjugated anti-CD4, peridinin chlorophyll protein-conjugated anti-CD45R/B220 (RA3-6B2) monoclonal antibody, and allophycocyanin-conjugated mouse-CD1d tetramers loaded with α-galactosylceramide. All antibodies were purchased from BD Biosciences (San Jose, CA, USA). Mouse CD1d tetramer was obtained from Proimmune (Oxford, UK) and the loading with α-galactosylceramide was performed following the manufacturer's protocol. The stained cells were analyzed with a FACScan (Becton Dickinson, Mountain View, CA, USA), and the data were processed using the CELLQuest program (Becton Dickinson). iNKT cells were detected on electronically gated CD45R/B220- TCRβ+ CD1d-tetramer-reactive cells.

RNA isolation and analysis

Total RNA was isolated from frozen liver tissues by using an RNeasy kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol. Complementary DNA was synthesized from isolated RNA using SuperScript III and random hexamer (Invitrogen, Carlsbad, CA, USA). Real-time reverse transcription polymerase chain reaction (RT-PCR) analysis was performed using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA) normalized to beta-actin.

Histological evaluation

The removed liver was partly fixed in 10% formalin for staining with hematoxylin–eosin (H&E), Sirius-Red, or Oil-red-O, or it was immediately embedded in Tissue-Tek OCT compound (Sakura Finetechnical, Tokyo, Japan) and frozen in liquid nitrogen for immunohistochemical staining. Sirius-Red staining was performed to assess liver fibrosis, which was quantified by the extent of the area, using image-analysis software, WinROOF (Mitani, Fukui, Japan). Intracellular lipid was stained with Oil-red-O. To evaluate the infiltration of CD4+ cells or CD8+ cells into the liver, acetone-fixed fresh-frozen tissue sections were immunostained with anti-mouse CD4 (H129.19) or anti-mouse CD8α (53-6.7) monoclonal antibody, respectively, using a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA, USA) following the manufacturer's protocol. The sections were developed with diaminobenzidine (DAB) substrate (Vector Laboratories) and then counterstained with hematoxylin. Antibody against CD4 or CD8 was purchased from BD Biosciences.

Statistical analysis

The statistical significance of differences between two groups was determined by applying the Mann–Whitney *U*-test. Statistical significance was defined as $P < 0.05$. All data are shown as mean ± standard error of the mean (SEM).

Results

Lipid accumulation in the liver induced by the HFD was independent of the presence or absence of iNKT cells

To investigate the role of iNKT cells in the development of diet-induced steatosis/steatohepatitis, we fed the ND or HFD to WT and KO mice for 5 weeks. The HFD increased the body weight by around 30% at week 5 in both WT and KO mice, while the ND increased it by around 14% (HFD-fed WT mice 31.6 ± 2.4%, HFD-fed KO mice 29.7 ± 5.6%, ND-fed WT mice 15.5 ± 0.6%, ND-fed KO mice 13.5 ± 1.1%; $n = 5$). The weight gains with the HFD or ND were not significantly different between WT and KO mice. Evaluation of the liver weight at week 5 showed that the HFD-fed WT or KO mice possessed significantly heavier livers than the ND-fed WT or KO mice, respectively, without any significant differences between the WT and KO mice (HFD-fed WT mice 1.95 ± 0.06 g, HFD-fed

KO mice 1.89 ± 0.07 g, ND-fed WT mice 1.52 ± 0.04 g, ND-fed KO mice 1.50 ± 0.06 g; $n = 5$).

We next performed Oil-red-O staining of liver sections from the mice to examine whether the absence of iNKT cells would affect the HFD-induced lipid accumulation in the liver. The staining showed that the HFD, compared with the ND, induced marked lipid retention in hepatocytes in both WT and KO mice (Fig. 1a). Evaluation of the liver triglyceride level demonstrated that the HFD, compared with the ND, clearly induced triglyceride accumulation in the livers of both WT and KO mice, without a significant difference between these groups of mice (Fig. 1b).

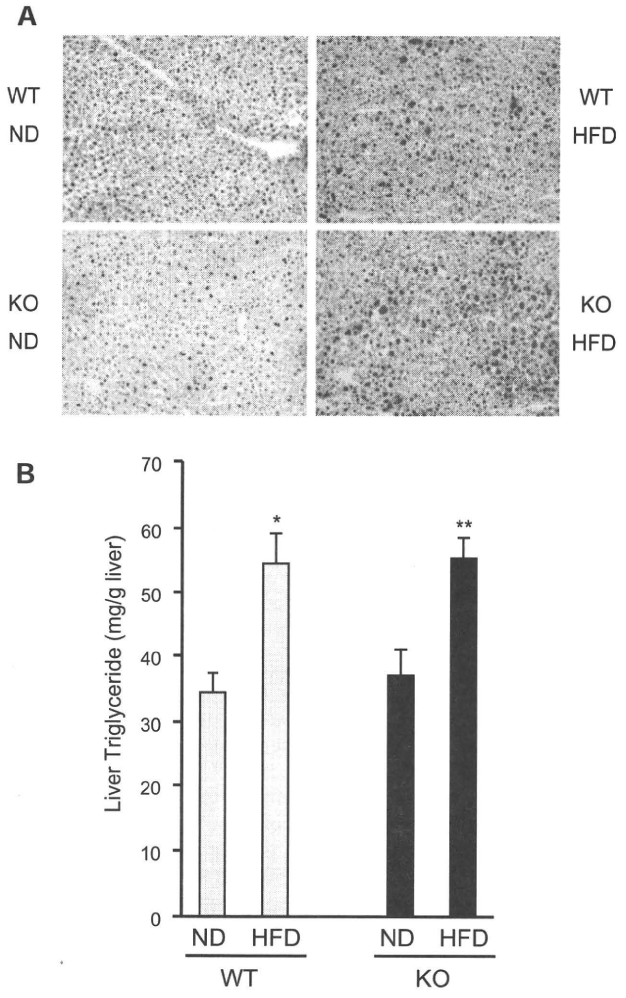

Fig. 1 Lipid accumulation in the liver induced by high-fat diet (*HFD*). Livers were obtained from BALB/c wild-type (*WT*) and BALB/c Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or an HFD for 5 weeks. **a** Lipid accumulation in liver sections was visualized by Oil-red-O staining. Representative images are shown (×200). **b** Hepatic triglyceride levels were quantified. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. *$P < 0.05$ versus WT fed ND. **$P < 0.05$ versus KO fed ND

Collectively, these results suggested that the absence of iNKT cells did not affect the level of HFD-induced steatosis.

HFD augmented liver injury and inflammation in the absence of iNKT cells

To examine the levels of liver injury, we measured ALT activity in serum from WT and KO mice fed the ND or HFD at week 5 after the start of being fed the diets. The serum ALT level in the HFD-fed WT mice (35.8 ± 1.98 IU/l) was significantly higher than that in the ND-fed WT mice (25.2 ± 0.66 IU/l) (Fig. 2a). The serum ALT level in the HFD-fed KO mice (174.8 ± 61.2 IU/l) was also significantly higher than that in the ND-fed KO mice (36.4 ± 7.48 IU/l). It was also higher than that in the HFD-fed KO mice at week 2 (83.3 ± 16.5 IU/l). Of note is the finding that the magnitude of the increase in ALT level at week 5 was clearly much higher in KO (4.9-fold) than in

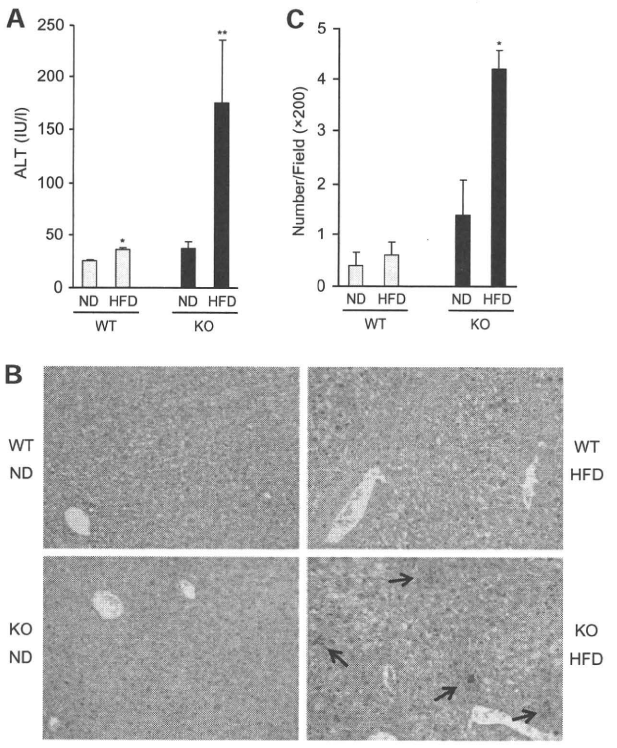

Fig. 2 Liver injury and inflammation exacerbated by HFD in the absence of invariant natural killer T (*iNKT*) cells. Serum and livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Serum alanine aminotransferase (*ALT*) levels were measured. *$P < 0.05$ versus WT fed ND. **$P < 0.05$ versus KO fed ND. **b** Liver tissues were stained with hematoxylin–eosin. Representative images are shown (×200). *Arrows* indicate the inflammatory foci. **c** The numbers of the foci were counted in five different fields per section. *$P < 0.05$ versus KO fed ND. All data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments

WT mice (1.5-fold), even though the serum ALT level in the ND-fed KO mice was modestly higher than that in the ND-fed WT mice.

We next conducted histological analyses of liver sections from the mice. H&E staining revealed that the livers from KO mice fed the HFD possessed not only steatotic areas but also scattered inflammatory foci composed of gathering nonparenchymal cells (Fig. 2b). Although inflammatory foci were also observed in the livers from KO mice fed the ND, a larger number of foci could clearly be seen in KO mice fed the HFD than in KO mice fed the ND (Fig. 2c). In contrast, WT mice fed the HFD, as well as those given the ND, showed few inflammatory foci. Taken together, these results indicated that the HFD augmented liver inflammation in KO mice but not in WT mice.

The HFD enhanced hepatic inflammation-related gene expression in the absence of iNKT cells

To understand the underlying mechanisms of the hepatic inflammation induced by the HFD in the absence of iNKT cells, we first examined the levels of several cytokines and chemokines in the livers from mice at week 5 after they had been started on the diets. Real-time RT-PCR analyses revealed that the messenger RNA expression of tumor necrosis factor (TNF)-α, IFN-γ, IL-10, chemokine (C–C motif) ligand (CCL) 2 and 4, and chemokine (C–X–C motif) ligand (CXCL) 9 and 10 were remarkably upregulated by the HFD, compared with the ND, in KO but not in WT mice (Fig. 3), although these values in KO mice fed the ND were modestly higher than those in WT mice fed the ND. In contrast, the messenger RNA expression of IL-4, -5, and -13 did not show any detectable levels in the livers from both WT and KO mice fed either the ND or HFD.

The HFD altered the proportions of subpopulations in liver mononuclear cells from KO mice, but not in those from WT mice

We next examined the phenotype of mononuclear cells in livers from mice at week 5 of feeding. Flow cytometric analyses demonstrated that the proportion of CD4+ TCRβ+ CD4 T cells was lower in KO mice fed the ND than in WT mice fed the ND (Fig. 4a), which might have resulted from a lack of iNKT cells partly composed of CD4+ cells [5]. The proportion of CD49b+ TCRβ− NK cells or CD8+ TCRβ+ CD8 T cells was higher in KO mice fed the ND than in WT mice fed the ND. The HFD did not lead to any significant changes in the proportion of hepatic CD4 T, CD8 T, NK, or iNKT cells in WT mice. In contrast, the HFD induced significant increases in the proportion of hepatic CD4 T cells and CD8 T cells, but not of NK cells, in KO mice. We then examined the distribution of these cells

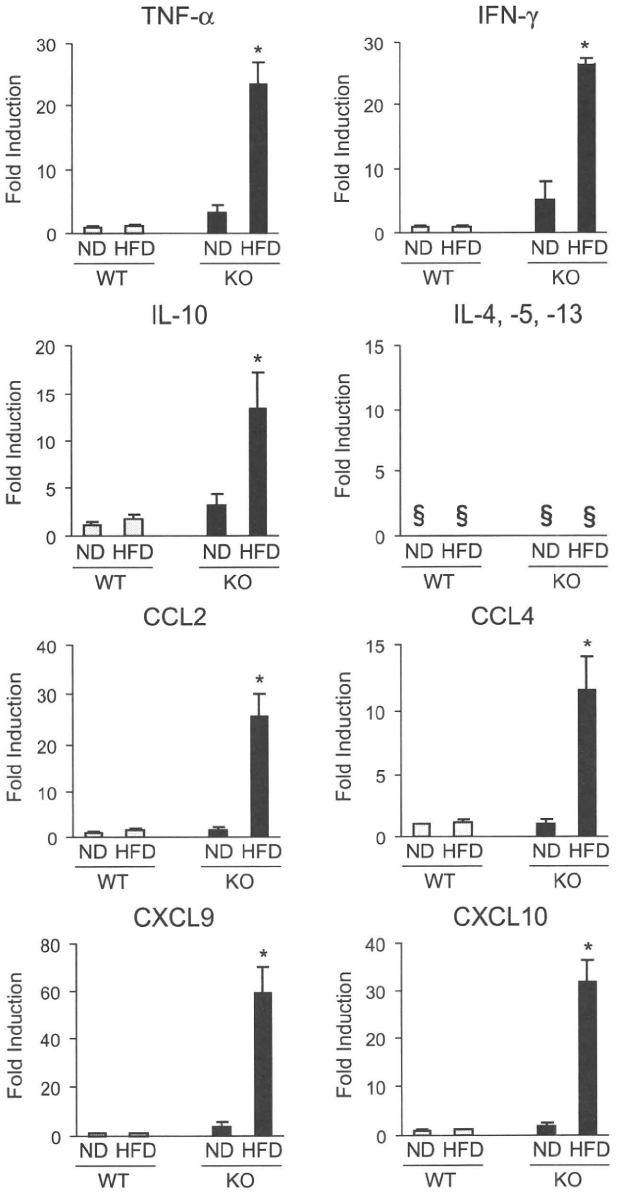

Fig. 3 Inflammatory cytokine and chemokine gene expression in the liver. Liver tissues were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. Liver RNA levels of the indicated genes and beta-actin as a control were analyzed using real-time reverse transcription polymerase chain reaction (RT-PCR). Data are shown as the fold increase of HFD-fed WT, ND-fed KO, or HFD-fed KO compared with ND-fed WT mice, with means ± SEM from five mice in each group. Data are representative of more than four independent experiments. *TNF-α* Tumor necrosis factor alpha, *IFN-γ* interferon gamma, *IL* interleukin, *CCL* chemokine (C–C motif) ligand, *CXCL* chemokine (C–X–C motif) ligand. §, not detected. $^*P < 0.05$ versus KO fed ND

in the liver. Immunohistochemical examination revealed that CD4+ cells and CD8+ cells formed foci surrounding hepatocytes in the livers of KO mice (Fig. 4b), which partly corresponded to the inflammatory foci observed in the

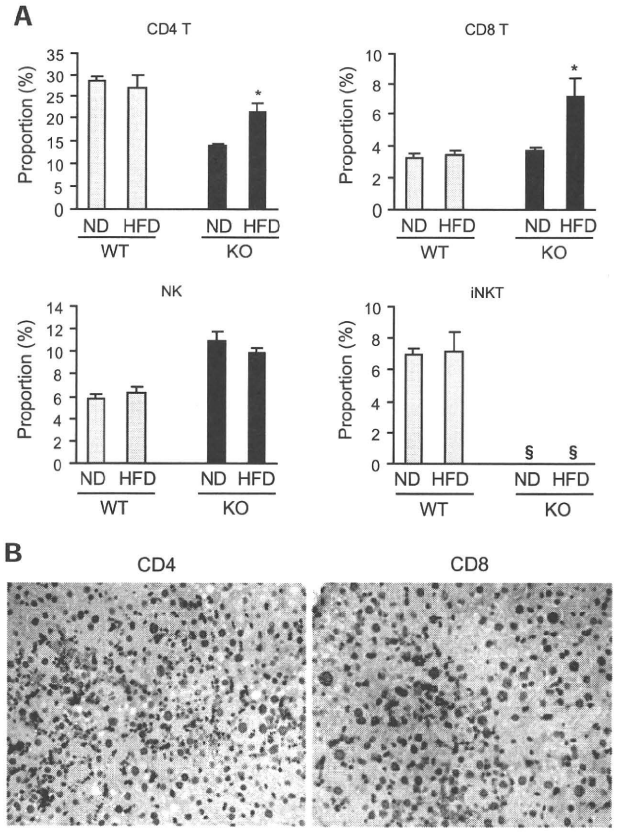

Fig. 4 Involvement of CD4 T and/or CD8 T cells in liver inflammation. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 5 weeks. **a** Prepared mononuclear cells from the livers were stained with cell markers indicated in "Materials and methods". Proportions of the indicated cell population were analyzed by flow cytometry. Data shown are means ± SEM from five mice in each group. Data are representative of more than four independent experiments. §, not detected. $*P < 0.05$ versus KO fed ND. **b** Liver sections were analyzed by immunohistochemical staining for CD4- or CD8-positive cells. Representative images are shown (×200)

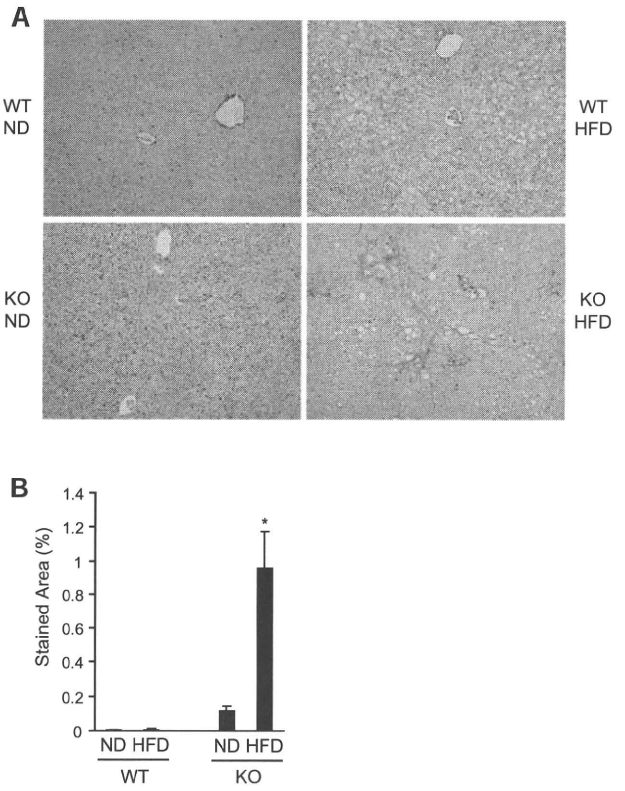

Fig. 5 Liver fibrosis following inflammation in the absence of iNKT cells. Livers were obtained from wild-type (*WT*) and Jα18-deficient (*KO*) mice fed either a normal diet (*ND*) or a high-fat diet (*HFD*) for 15 weeks. **a** Liver tissues were stained with Sirius-Red to assess liver fibrosis. Representative images are shown (×200). **b** The stained areas were evaluated in five different fields per section. Data shown are means ± SEM from five mice in each group. Data are representative of more than two independent experiments. $*P < 0.05$ versus KO fed ND

H&E-stained liver sections of KO mice. WT mice fed either the ND or HFD did not display such foci consisting of stained cells in the livers. Collectively, these results suggested that CD4 T and/or CD8 T cells played a role in the HFD-enhanced liver inflammation in KO mice.

The HFD led to the development of liver fibrosis in the absence of iNKT cells

Persistent hepatic inflammation causes fibrotic changes in the liver [25]. To investigate whether inflammation with steatosis due to the HFD in KO mice would induce fibrosis in the liver, we fed the ND or HFD to WT and KO mice for a longer period of 15 weeks. H&E staining and Oil-red-O staining showed that KO mice fed the HFD possessed the inflammatory foci, together with lipid retention in the liver, at week 15, as well as showing these findings at week 5, and that WT mice fed the HFD showed lipid retention, but few inflammatory foci, in the liver at week 15 (data not shown). Sirius-Red staining revealed clear fibrosis in the livers from KO mice fed the HFD and also in the livers from KO mice fed the ND, but to a much lesser extent (Fig. 5a). In contrast, the staining showed no obvious fibrosis in the livers from WT mice fed the HFD or in those given the ND. Quantitative analyses to evaluate the stained areas also showed that the HFD-fed KO mice possessed significantly greater areas of hepatic fibrosis than the ND-fed KO mice, while WT mice fed either the ND or HFD had few fibrotic areas (Fig. 5b). Taken together, these results indicated that the HFD led to the development of liver fibrosis accompanied by steatohepatitis in KO mice.

Discussion

An increasing amount of evidence suggests that iNKT cells play a role in immune responses in the liver [12], although

the exact implication of that role is controversial. iNKT cells, for instance, have been reported to play a critical role in animal models of liver injury induced by concanavalin A, α-galactosylceramide, or salmonella infection [26–28], suggesting a proinflammatory role of these cells. On the other hand, iNKT cells have been very recently implicated in the suppression of liver damage in a mouse model of cholestasis [29], suggesting an anti-inflammatory role of these cells. The present study, using iNKT cell-deficient mice fed an HFD, demonstrated that the HFD led to the development of steatohepatitis with fibrosis in the absence of iNKT cells, while the HFD led to steatosis but not steatohepatitis in the presence of these cells. This suggests that iNKT cells play a critical role in suppressing the development of inflammation and fibrosis in the steatotic liver.

Our real-time RT-PCR analyses demonstrated that CCL2, CCL4, CXCL9, and CXCL10 were remarkably upregulated by the HFD in KO mice but not in WT mice (Fig. 3). CCL2 or CCL4 has the ability to attract predominantly Th1 cells via chemokine (C–C motif) receptor 2 or 5, respectively. CXCL9 and CXCL10 also attract predominantly Th1 cells via chemokine (C–X–C motif) receptor 3 [30, 31]. Indeed, Th1 cytokines such as TNF-α and IFN-γ were remarkably upregulated by the HFD in KO mice but not in WT mice. Although IL-10, which is one of the anti-inflammatory cytokines, was also upregulated by the HFD in KO mice but not in WT mice, the upregulation of IL-10 may have counteracted the upregulation of the proinflammatory Th1 cytokines TNF-α and IFN-γ. Our flow cytometric analyses and immunohistochemical analyses showed that the proportions of CD4 T and CD8 T cells were increased (Fig. 4a) and that these cells also accumulated to form foci (Fig. 4b) in the livers of KO mice fed the HFD. Bigorgne et al. [32] reported that HFD-induced obesity in leptin-deficient ob/ob mice rendered hepatic mononuclear cells, particularly CD4 T and CD8 T cells, sensitive to chemokines such as CXCL12 and CXCL13, which attract T cells, suggesting an important role of chemokines in liver inflammation with steatosis. Although the sources of the chemokines upregulated in our model were not clear, these chemokines presumably play an important role in the infiltration of proinflammatory cells in the liver of the KO mice fed the HFD. iNKT cells suppress the production of these chemokines directly or indirectly; thus, they may prevent steatohepatitis induced by an HFD.

The liver can be anatomically exposed to gut-derived contents, such as food antigens and bacterial products, via the portal vein [33, 34]. Once these entities flow into the liver, they can activate a variety of cells in the liver, which may be associated with certain types of liver disease [33, 34]. Gut-derived food-antigens can activate T cells [33] and gut-derived bacterial products can stimulate all resident cells in the liver, such as hepatocytes, Kupffer cells, stellate cells, and dendritic cells, via toll-like receptors [33–36]. Moreover, fat itself, particularly saturated fatty acids, stimulates an immune response in the liver [37, 38]. On the other hand, the liver is an immune-tolerogenic organ, in which immune-suppressive cells may play a critical role to keep this organ immunologically silent [33]. The present study demonstrated that liver inflammation was greatly exacerbated—where CD4 T and/or CD8 T cells infiltrated to form foci surrounding damaged hepatocytes—by an HFD in the absence of iNKT cells. This suggests a suppressive role of iNKT cells in the development of liver inflammation with steatosis. Thus, iNKT cells may play an important role in keeping the liver immunologically silent, and the absence of iNKT cells together with steatosis may elicit a break of hepatic immune tolerance, resulting in the activation of CD4 T and/or CD8 T cells to provoke liver inflammation. Consistent with this speculation is the observation that the absence of iNKT cells, even without steatosis, caused modest liver inflammation.

In conclusion, iNKT cells suppress liver inflammation progressing to fibrosis that is exacerbated by HFD-induced steatosis, thus contributing to the maintenance of immune homeostasis in the liver. This study has shed some light on iNKT cells as immunoregulatory cells and their key role in the pathogenesis of NAFLD.

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Characteristics of patients with nonalcoholic steatohepatitis who develop hepatocellular carcinoma

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Abbreviations: AFP, α-fetoprotein; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index; DCP, des-γ-carboxy prothrombin; γ-GTP, γ-glutamyl transpeptidase; HCC, hepatocellular carcinoma; HDL, high-density lipoprotein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis.

Authors' contributions:
Kohichiroh Yasui: acquisition of data; analysis and interpretation of data; drafting of the manuscript; statistical analysis.
Etsuko Hashimoto, Yasuji Komorizono, Kazuhiko Koike, Shigeki Arii, Yasuharu Imai, Toshihide Shima, Yoshihiro Kanbara, Toshiji Saibara, Takahiro Mori, Sumio Kawata, Hirofumi Uto, Shiro Takami, Yoshio Sumida, Toshinari Takamura, and Miwa Kawanaka: acquisition of data.
Takeshi Okanoue: study concept and design; critical revision of the manuscript for important intellectual content; obtained funding.

Abstract

Background & Aims: Nonalcoholic steatohepatitis (NASH) can progress to hepatocellular carcinoma (HCC). We aimed to characterize the clinical features of NASH patients with HCC.

Methods: In a cross-sectional multicenter study in Japan, we examined 87 patients (median age 72 years, 62% male) with histologically proven NASH who developed HCC. The clinical data were collected at the time HCC was diagnosed.

Results: Obesity (body mass index ≥25 kg/m2), diabetes, dyslipidemia, and hypertension were present in 54 (62%), 51 (59%), 24 (28%), and 47 (55%) patients, respectively. In non-tumor liver tissues, the degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (i.e., liver cirrhosis) in 44 (51%). The prevalence of cirrhosis was significantly lower among male patients (21 of 54; 39%), compared with female patients (23 of 33; 70%) (P = 0.008).

Conclusions: Most patients with NASH who develop HCC are men; the patients have a high rates of obesity, diabetes, and hypertension. Male patients appear to develop HCC at a less advanced stage of liver fibrosis than female patients.

Key words: Liver cancer, incidence, sex, retrospective study

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third leading cause of cancer mortality [1]. HCC largely occurs within an established background of chronic liver disease and cirrhosis. Although the risk factors for HCC, including infection with hepatitis B and C viruses as well as alcohol consumption, are well defined, 5 to 30% of patients with HCC lack a readily identifiable risk factor for their cancer. It has been suggested that a more severe form of nonalcoholic fatty liver disease (NAFLD), namely nonalcoholic steatohepatitis (NASH), may account for a substantial portion of cryptogenic cirrhosis and HCC cases [2].

NAFLD is one of the most common causes of chronic liver disease in the world [3, 4]. NAFLD is associated with obesity, diabetes, dyslipidemia, and insulin resistance, and is recognized as a hepatic manifestation of metabolic syndrome. The spectrum of NAFLD ranges from a relatively benign accumulation of lipid (simple steatosis) to progressive NASH associated with fibrosis, necrosis, and inflammation. Despite its common occurrence and potentially serious nature, relatively little is known about the natural history or prognostic significance of NAFLD. Although prospective studies on the natural history of NAFLD and NASH using a larger cohort are awaited, these studies may be limited by the long and asymptomatic clinical course of these diseases, by their high prevalence in the general population, and by the lack of serological markers for NASH. The evidence suggesting that NASH can progress to HCC comes from (1) case reports and case-series [5-8]; (2) retrospective studies [9-12] and (3) prospective studies [13-17]. These studies generally examined limited numbers of cases and follow-ups; therefore, the incidence of HCC and risk factors for HCC in NASH patients remain unclear.

The Japan NASH Study Group (representative: Takeshi Okanoue) [18] was established in 2008 by the Ministry of Health, Labour and Welfare of Japan to address unmet research needs in the area of liver diseases. As a part of this mandate, the study group conducted a cross-sectional multicenter study to characterize the clinical features of histologically proven NASH patients who developed HCC.

Methods

Patients

We retrospectively identified and reviewed 87 Japanese patients with NASH, who developed HCC between 1993 and 2010, at 15 hepatology centers that belong to the Japan NASH Study Group [18] and their affiliated hospitals in Japan. The diagnosis of NASH was based on (1) the histological features of steatohepatitis (see histological examination section below), (2) negligible alcohol consumption, and (3) exclusion of liver diseases of other etiology. To determine alcohol consumption as accurately as possible, we reviewed medical records in our institutions and, when patients had been transferred from other institutions, we also reviewed a summary of medical records from those institutions. According to the medical records, alcohol consumption was assessed based on a detailed history that was obtained by physicians and by interviewing family members. Exclusion criteria included consumption of more than 20 g of alcohol per day, positivity for hepatitis B virus surface antigen, positivity for anti-hepatitis C virus antibody, the presence of other types of liver diseases (e.g., primary biliary cirrhosis, autoimmune hepatitis, Wilson's disease, or hemochromatosis), previous treatment with drugs known to produce hepatic steatosis, and a history of gastrointestinal bypass surgery. The sections of non-tumor liver tissues were re-analyzed by experienced hepatopathologists (T.O. and E.H.) who were blinded to the laboratory parameters and clinical data. We excluded patients whose histological diagnosis of NASH was not confirmed by central review and patients with insufficient or inconclusive information concerning alcohol consumption, body mass index, and laboratory data including fasting glucose and lipid.

Of the 87 patients, 14 patients had been previously diagnosed as NAFLD or NASH and had been followed at our institutions; 73 patients had been transferred from other institutions to our institutions for investigation and treatment of HCC. Most patients had been identified as having HCC during screening, which included ultrasound and/or computerized tomography (CT) of the liver and α-fetoprotein testing.

The diagnosis of HCC was based on liver histology, and, in the absence of histology, on typical features of HCC as assessed by dynamic CT or magnetic resonance imaging (MRI) (i.e., hypervascular with washout in the portal/venous phase) [19]. Of the 87 patients, 49 patients were diagnosed as HCC following hepatic resection; 21 patients were diagnosed following ultrasound-guided tumor biopsy and 17 patients were diagnosed by dynamic CT or MRI.

The Ethics Committees of each participating center approved this study. Informed

consent was obtained from each patient in accordance with the Declaration of Helsinki.

Clinical assessment and Laboratory tests

The clinical and laboratory data were collected at the time HCC was diagnosed. Body mass index (BMI) was calculated using the following formula: weight in kilograms/(height in meters)^2. Obesity was defined as a BMI $\geq$25 kg/m^2 according to the criteria of the Japan Society for the Study of Obesity [20]. Diabetes was defined as a fasting plasma glucose concentration of $\geq$126 mg/dL or a 2-h plasma glucose concentration of $\geq$200 mg/dL during an oral glucose (75 g) tolerance test or by the use of insulin or oral hypoglycemic agents to control blood glucose [21]. Hypertension was defined as a systolic blood pressure $\geq$130 mmHg or a diastolic blood pressure $\geq$85 mmHg or by the use of antihypertensive agents [22]. Dyslipidemia was defined as serum concentrations of triglycerides $\geq$150 mg/dL or high-density lipoprotein (HDL) cholesterol <40 mg/dL and <50 mg/dL for men and women, respectively, or by the use of specific medication [22].

Venous blood samples were taken in the morning after a 12-h overnight fast. The laboratory evaluation included a blood cell count and measurement of serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), fasting plasma glucose, HbA1c, total cholesterol, HDL cholesterol, triglyceride, ferritin, hyaluronic acid, α-fetoprotein (AFP), and des-γ-carboxy prothrombin (DCP). These parameters were measured using standard clinical chemistry techniques.

Histopathological examination

Non-tumor liver tissues were obtained from all 87 patients to diagnose the background liver tissue at the time HCC was diagnosed. In 49 patients who underwent hepatic resection for HCC, we examined non-tumor liver tissues that were surgically resected. In 21 patients who underwent ultrasound-guided tumor biopsy, non-tumor liver tissues far from HCC tumors were separately biopsied. In 17 patients who were diagnosed as HCC by dynamic CT or MRI and did not undergo either hepatic resection or tumor biopsy, only non-tumor liver tissues far from HCC tumors were obtained by ultrasound-guided biopsy.

The specimens were fixed in formalin, embedded in paraffin, and stained with hematoxylin and eosin, with Masson's trichrome, and by silver impregnation. NASH was defined as steatosis with lobular inflammation, hepatocellular ballooning, and Mallory's hyaline (Mallory's body) or fibrosis [23-25]. The necroinflammatory grade

and the degree of fibrosis was evaluated and scored according to the criteria proposed by Brunt et al. [26].

Statistical Analysis

Results are presented as numbers with percentages in parentheses for qualitative data or as the medians and ranges (25th–75th percentiles) for quantitative data. Comparisons were made using a chi-square test for qualitative factors or a Mann-Whitney U test on ranks for quantitative factors with non-equal variance. *P* values below 0.05 from two-sided tests were considered to be significant. All statistical analyses were performed using SPSS 15.0 software (SPSS Inc., Chicago, IL, USA).

Results

The characteristics of the 87 NASH patients who developed HCC are summarized in Table 1. The median age was 72 years (25th percentile, 75th percentile: 69, 75); the mean age (SD) was 71.2 (6.7) years. There were 54 male patients (62%) and 33 female patients (38%); the male : female ratio was 1.6:1. The median BMI was 26.0 kg/m^2, and 54 patients (62%) were obese (BMI ≥25 kg/m^2). Diabetes, dyslipidemia, and hypertension were present in 51 (59%), 24 (28%), and 47 (55%) patients, respectively.

The diagnosis of NASH was proven by histological examination of non-tumor liver tissues at the time HCC was diagnosed. The degree of steatosis was grade 1 (5-33%) in 60 patients (69%), grade 2 (34-66%) in 19 (22%), and grade 3 (>66%) in 7 (8%). One patient who showed less than 5% steatosis was diagnosed as "burn-out" NASH, because a previous liver biopsy that was performed before development of HCC had demonstrated typical histological features of NASH. The necroinflammatory grade was mild (grade 1) in 31 patients (35%), moderate (grade 2) in 45 (52%), and severe (grade 3) in 11 (13%). The degree of fibrosis was stage 1 in 10 patients (11%), stage 2 in 15 (17%), stage 3 in 18 (21%), and stage 4 (i.e., liver cirrhosis) in 44 (51%).

The median diameter of HCC tumors was 3.0 cm (25th percentile, 75th percentile: 2.0, 4.0). A single HCC lesion was present in 65 of 87 patients (75%).

Data were stratified according to sex (Table 1). Compared to female patients, male patients had significantly less hypertension, lower HDL cholesterol and AFP, higher ferritin, and a less advanced stage of fibrosis. The prevalence of cirrhosis was significantly lower in male patients (21 of 54; 39%) than in female patients (23 of 33; 70%) ($P = 0.008$).

Discussion

In this cross-sectional multicenter study in Japan, we show the clinical features of a relatively large number ($n = 87$) of NASH patients with HCC. The male : female ratio was 1.6:1. Males have higher HCC rates than females in almost all populations, with male : female ratios usually averaging between 2:1 and 4:1 [2]. In the latest nationwide survey of HCC in Japan [27], this ratio was 2.5:1. The reasons underlying higher rates of HCC in males may relate to sex-specific differences in exposure to risk factors. Men are more likely to be infected with hepatitis B and C viruses, consume alcohol, smoke cigarettes, and have increased iron stores [2]. Moreover, androgens are considered to influence the development of HCC. With regards to the male : female ratio of HCC associated with NASH, a male : female ratio of 1.3:1 was reported in a summary of 16 published cases of HCC associated with NASH [28]. Ratios of 2.8:1 and 0.67:1 were reported in two retrospective studies of HCC arising from cryptogenic cirrhosis in Italy ($n = 44$) [10] and the United States ($n = 30$) [9], respectively, and a ratio of 1.6:1 was reported for 36 cases of NASH-associated HCC from a single center in Japan [15]. Overall, NASH patients with HCC are more often men. However, these male : female ratios may be lower than the ratios for HCC of other etiologies, including viral hepatitis and alcohol consumption.

Although it is well known that male gender is a risk factor for HCC in patients infected with hepatitis B and C viruses [2], it remains unclear whether male gender is a factor associated with the development of HCC in NASH patients. It is now suspected that there is an even distribution of NASH among males and females [29]. In another study by our group [30], the male : female ratio was 0.85:1 in 342 NASH patients without cirrhosis and HCC. The male : female ratio (1.6:1) of NASH patients with HCC in the present study is higher than this ratio. In agreement with our observations, a case-control study showed that the male : female ratio was 1.6:1 in 34 NASH patients with HCC, while the ratio was 0.69:1 in 348 NASH patients without HCC [15]. A recent prospective study indicated that an older age and alcohol consumption were independent risk factors for the development of HCC in patients with NASH-cirrhosis; and that male gender tended to be associated with the development of HCC, although this trend did not reach statistical significance [17].

The median age of our patients was 72 years. There was no significant difference in age between males and females. Although the global age distribution of HCC varies by geographic region, sex, and etiology, in almost all areas the peak female age group in HCC patients is 5 years older than in male HCC patients [2]. In a nationwide survey of

HCC in Japan [27], the mean ages were 65.5 years for males and 69.4 years for females. The male patients in the present study are slightly older than the mean ages reported in these previous studies.

Consistent with the literature [9-12], more than half of our patients displayed obesity, diabetes, and hypertension. Obesity constitutes a significant risk factor for cancer mortality in general and is an increasingly recognized risk factor for HCC in particular [31, 32]. In the present study, body weight was measured at the time HCC was diagnosed. Since advanced HCC may cause weight loss, it is likely that our patients were obese before the development of HCC. Diabetes has also been proposed as a risk factor for HCC [2]. Thus, HCC shares two major risk factors, obesity and diabetes, with NASH.

Once cirrhosis and HCC are established, it is difficult to identify pathologic features of NASH. As NASH progresses to cirrhosis, steatosis tends to disappear; so called "burn-out NASH" [5]. As expected, the grade of steatosis was mild in most of our cases. It was possible to diagnose one case without steatosis as "burn-out NASH", because a previous liver-biopsy specimen (liver biopsy was performed 25 years prior) was preserved and available. It is likely that many cases of NASH-associated HCC may have been missed because of loss of the telltale sign of steatosis.

Most HCC arises on a background of cirrhosis. It is less clear whether cirrhosis is a necessary predisposition for the development of HCC in patients with NASH. Case reports of HCC arising from NAFLD and NASH patients without fibrosis or cirrhosis have been accumulating [33-36]. Cirrhosis (fibrosis stage 4) was present in 51% of cases, and advanced stages of fibrosis (stage 3 or 4) were found in 72% of cases in the present study. Indeed, cirrhosis or advanced fibrosis appeared to be the predominant risk factors for HCC development. However, in the remaining 28% of cases, HCC developed in patients with less fibrosis (stage 1 or 2). Interestingly, male patients developed HCC at a less advanced stage of fibrosis than female patients and the prevalence of cirrhosis was significantly lower in males (39%) than in females (70%). Although the reason for the sex differences is unclear, these findings indicate that screening for HCC is needed, not only in NASH patients with advanced fibrosis, but also in those with less fibrosis, particularly if they are males. Further studies are needed to confirm this potentially important observation. Paradis et al. reported that, in patients whose only risk factors for chronic liver disease are features of metabolic syndrome, HCC usually occurs in the absence of significant liver fibrosis [37]. Additionally, they found that some of these HCCs developed on preexisting liver cell adenomas. However, no preexisting adenomas were observed in the present cases.

Compared to female patients, male patients had significantly higher serum ferritin. The normal value for ferritin varies according to the age and gender of the individual. Adult males have serum ferritin values averaging approximately100 ng/mL (range, 75-250), while adult females have levels averaging approximately 30 ng/mL (range, 20-75) [38]. Thus, normal males have higher ferritin levels than females. Elevation of ferritin levels is associated with NASH [39]. Because we excluded patients with alcohol consumption as rigorously as possible, we believe that alcohol consumption did not contribute to the elevation of ferritin levels in our patients.

The median diameter of the HCCs in the present study was 3.0 cm, which is equal to, or smaller than, the size of previously reported HCCs [9, 10, 12, 28, 37]. This is probably because most of our patients had been identified as having HCC during screening. A single HCC lesion was present in 75% of patients. For early detection of NASH associated-HCC, vigilant screening is important [9] and the development of serological markers for NASH is necessary.

The mechanisms of carcinogenesis in NASH remain to be elucidated. Possible mechanisms include hyperinsulinemia caused by insulin resistance in NASH, increased levels of insulin-like growth factor which promotes tumor growth, increased susceptibility of the steatotic liver to lipid peroxidation, production of reactive oxygen species and subsequent DNA mutations, disordered energy and hormonal regulation in obesity, and aberrations in regenerative processes occurring in cirrhosis [25].

Certain limitations should be considered in the interpretation of our findings. First, the cross-sectional study design hinders the ability to draw inferences regarding the causality of NASH in HCC. Second, the study did not include a control group of HCC patients with other liver diseases. Third, there may be a bias in patient selection, because patients were retrospectively identified as having NASH-associated HCC. Finally, although our patients were negative for hepatitis B virus surface antigen, it is still possible that occult hepatitis B virus infection may be associated with the development of HCC in some of our cases.

In summary, we show here the clinical features of NASH patients with HCC. NASH patients with HCC were more often men, and frequently displayed obesity, diabetes, and hypertension. Our results suggest that male patients may develop HCC at a less advanced stage of fibrosis than female patients. Further prospective studies with a longer follow-up time and larger cohorts are needed to determine the causal association of NASH with HCC and to identify risk factors for the development of HCC in NASH patients.

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