Table 1. Incidence of liver tumors in KO mice.

Age (yr)	Genotype	Tumor incidence
1.5		
	Bcl-xL$^{+/+}$	0% (0/10)
	Bcl-xL$^{-/-}$	88% (7/8)*
1		
	Bcl-xL$^{+/+}$	0% (0/4)
	Bcl-xL$^{-/-}$	27% (3/11)
1.5		
	Mcl-1$^{+/+}$	0% (0/22)
	Mcl-1$^{-/-}$	100% (16/16)*
1		
	Mcl-1$^{-/-}$ *Bak*$^{+/+}$	64% (14/22)
	Mcl-1$^{-/-}$ *Bak*$^{-/-}$	0% (0/7)*

*p <0.05 *vs.* control.

mitochondrial pathway of apoptosis, clearly suppresses hepatocyte apoptosis and liver carcinogenesis in *Mcl-1* KO mice. We also considered possible mechanisms involving oxidative stress that underlie elevated malignant transformation in the apoptosis-prone liver. The present study offers strong support for the hypothesis that chronically increased apoptosis in hepatocytes is carcinogenic. It also provides genetic evidence that inhibition of apoptosis may suppress liver carcinogenesis in chronic liver disease.

Materials and methods

Mice

Conditional *Bcl-xL* KO mice (*bcl-x*$^{flox/flox}$ *Alb-Cre*) and *Mcl-1* KO mice (*mcl-1*$^{flox/flox}$ *Alb-Cre*) were previously described [11]. We purchased *Bak* KO mice (*bak*$^{-/-}$) from the Jackson Laboratory (Bar Harbor, ME). We generated hepatocyte-specific Bak/Mcl-1 double KO mice (*bak*$^{-/-}$ *mcl-1*$^{flox/flox}$ *Alb-Cre*) by mating the strains. They were maintained in a specific pathogen-free facility and treated with humane care with approval from the Animal Care and Use Committee of Osaka University Medical School. Measurement of serum alanine aminotransferase (ALT) level, caspase-3/7 activity and histological analyses have been previously described [11].

Western blot analysis

For immunodetection, the following antibodies were used: anti-Bcl-xL antibody (Santa Cruz Biotechnology, Santa Cruz, CA), anti-Mcl-1 antibody (Rockland, Gilbertsville, PA), anti-Bak antibody (Millipore, Billerica, MA), anti-Bax antibody, anti-ERK antibody, anti-phospho-ERK antibody, anti-p38 antibody, anti-phospho-p38 antibody, anti-JNK antibody, anti-phospho-JNK antibody, anti-PCNA antibody (Cell Signaling Technology, Danvers, MA), and anti-beta-actin antibody (Sigma–Aldrich, Saint Louis, MO).

Real-time reverse-transcription PCR (RT-PCR)

The following TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA) were used: mouse-AFP (Mm00431715_m1), mouse-glypican-3 (Mm00516722_m1), mouse-IL-6 (Mm00446190_m1), mouse-TNF-α (Mm00443258_m1), mouse-MCP-1 (Mm00441242_m1), mouse-CD68 (Mm03047343_m1), mouse-CD4 (Mm00442754_m1), mouse-CD8 (Mm01182108_m1), mouse-heme oxygenase-1 (HO-1) (Mm00516005_m1), mouse-NAD(P)H:quinone oxidoreductase 1 (NQO1) (Mm00500821_m1), and mouse-Beta actin (Mm00607939_s1). All expression levels were corrected with the quantified expression level of beta actin.

Immunohistochemistry

8-Hydroxy-2′-deoxyguanosine (8-OHdG), cleaved caspase-9, PCNA, and ki-67 were labeled in paraffin-embedded liver sections using anti-8-OHdG antibody (Nikken Seil, Tokyo, Japan), anti-cleaved caspase-9 antibody, anti-PCNA antibody (Cell Signaling Technology), and anti-ki-67 antibody (Dako, Tokyo, Japan), respectively. Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) was performed according to a previously reported procedure [17].

Statistical analysis

Data are presented as mean ± SD. Differences between two groups were determined using the Student's *t*-test for unpaired observations. Carcinogenesis rates were analyzed using the Chi-square test. Multiple comparisons of *Bak/Mcl-1* double KO mice were performed by ANOVA followed by Scheffe *post hoc* correction. Fisher *post hoc* correction was used for the other multiple comparisons. A p <0.05 was considered statistically significant.

Results

Bcl-xL KO mice develop liver tumors in old age

We previously reported that hepatocyte-specific *Bcl-xL* KO mice developed spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1A) at as early as 1 month of age with a gradual increase in the liver fibrotic response from 3 to 7 months [10]. To examine the phenotypes at later time points, we sacrificed *Bcl-xL* KO mice and their control littermates at 1 and 1.5 years of age. Macroscopic tumors had developed in the liver of 27% and 88% of the KO mice, respectively, but not in the control littermates (Fig. 1A and Table 1). Most of the *Bcl-xL* KO mice had multiple tumors and the liver body-weight ratio for *Bcl-xL* KO mice was significantly higher than that of the control mice (Fig. 1B and C). Tumors were histologically defined as well-differentiated HCCs (Fig. 1D). To find out whether the *bcl-x* gene is really targeted in the tumors, we performed Western blot analysis for the expression of the Bcl-2 family proteins (Fig. 1E and Supplementary Fig. 2A). The tumors were confirmed to be deficient for Bcl-xL, excluding the possibility that transformed cells arising from hepatocytes in which the *bcl-x* gene was not deleted had expanded to form tumors. Interestingly, most of these tumors showed apparently higher levels of Mcl-1 expression than the wild-type liver or the non-cancerous surrounding tissues. Reciprocal overexpression of Mcl-1 may explain the possible survival advantage of these tumors. Tumors in *Bcl-xL* KO mice expressed higher levels of α-fetoprotein (Fig. 1F) and frequently showed activation of ERK and JNK (Fig. 1G), which are observed in human HCC [18,19].

Liver tumors in Mcl-1 KO mice show similar characteristics to human HCC

We have previously reported phenotypes of hepatocyte-specific *Mcl-1* KO mice, which display spontaneous hepatocyte apoptosis by the mitochondrial pathway (Supplementary Fig. 1B) and liver fibrotic responses at an early age [11]. Since our *Mcl-1* floxed mice differed from those of Weber *et al.* [12] in origin, we next examined the development of liver tumors in our hepatocyte-specific *Mcl-1* KO mice. All the *Mcl-1* KO mice, but none of the control littermates, developed liver tumors at 1.5 years of age, with a significant increase of liver body-weight ratio (Fig. 2A–C

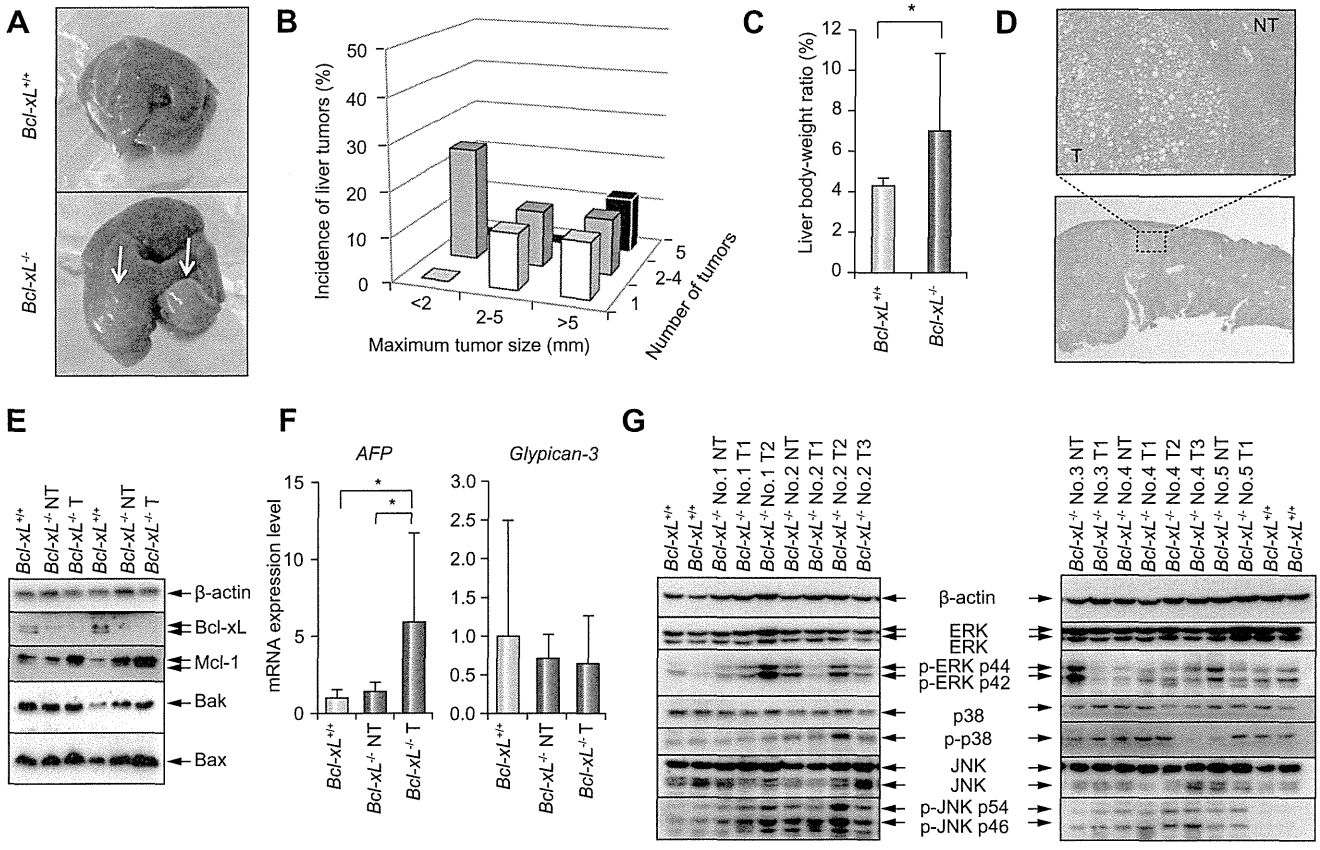

Fig. 1. Liver tumors in *Bcl-xL* KO mice. (A–E) Hepatocyte-specific *Bcl-xL*-deficient mice (*Bcl-xL*$^{-/-}$) (N = 8) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 10) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Bcl-xL* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Bcl-xL* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Bcl-xL* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 9 or 10 per group). (G) Expression and activation of mitogen-activated protein kinases. *p <0.05.

and Table 1). As in the case of tumors of *Bcl-xL* KO mice, liver tumors that developed in *Mcl-1* KO mice were deficient for Mcl-1 expression and, in most cases, reciprocally overexpressed Bcl-xL (Fig. 2E and Supplementary Fig. 2B). These tumors expressed higher levels of α-fetoprotein and glypican-3 (Fig. 2F) and frequently showed activation of ERK and JNK (Fig. 2G).

Inflammatory response and oxidative stress occur in Bcl-xL- or Mcl-1-KO livers

To examine the molecular mechanism of tumor development, we examined gene expression in the livers of 6-week-old *Bcl-xL* or *Mcl-1* KO mice. Real-time RT-PCR analysis revealed increases of inflammatory cytokine TNF-α, but not IL-6, and chemokine MCP-1 in *Bcl-xL* and *Mcl-1* KO livers (Fig. 3A and B), despite overt histological inflammation (data not shown). Together with an increase of MCP-1, CD68 expression was significantly higher in KO livers than in control livers (Fig. 3C and D). In contrast, there was no difference in the expression of CD4 and CD8 between the groups. These findings suggest that activation or infiltration of myeloid-derived cells and production of TNF-α are characteristic of the *Bcl-xL* or *Mcl-1* KO liver. Together with the previous study reporting that TNF-α promotes cellular transformation [20], these results suggest that the increase in TNF-α may be one of the mechanisms of tumor development.

Since oxidative stress is also reported to cause carcinogenesis [21], we examined the expression of HO-1 and NQO1, inducible anti-oxidant enzymes, and 8-OHdG in the liver tissues. Real-time RT-PCR analysis revealed that HO-1 and NQO-1 expressions were significantly increased in *Mcl-1* KO livers at 6 weeks (Fig. 3E). 8-OHdG staining revealed that there were few 8-OHdG positive nuclei in both *Mcl-1* KO and the control liver at 6 weeks of age. However, scattered positive nuclei were observed in KO livers at 1.5 years of age, but not in the tumors, and the number of positive nuclei was significantly higher in KO livers than in control livers (Fig. 3F and Supplementary Fig. 3). Similarly, the number of 8-OHdG positive nuclei was significantly higher in *Bcl-xL* KO livers at 1.5 years of age than in control livers (Fig. 3G). These results suggest that oxidative stress may occur at as early as 6 weeks of age in KO livers and that oxidative injury arises at a later time point.

Bak deficiency significantly ameliorates hepatocyte apoptosis and reduces tumor development in Mcl-1 KO mice

Bak is a proapoptotic Bcl-2 family protein, which is able to oligmerize to form pores at the outer membrane of mitochondria. To understand whether inhibition of apoptosis could reduce the carcinogenic potential, we crossed *Mcl-1* KO mice and *Bak* KO mice and generated *Bak Mcl-1* double KO mice. As expected, *Bak* KO significantly suppressed hepatocyte apoptosis in *Mcl-1*

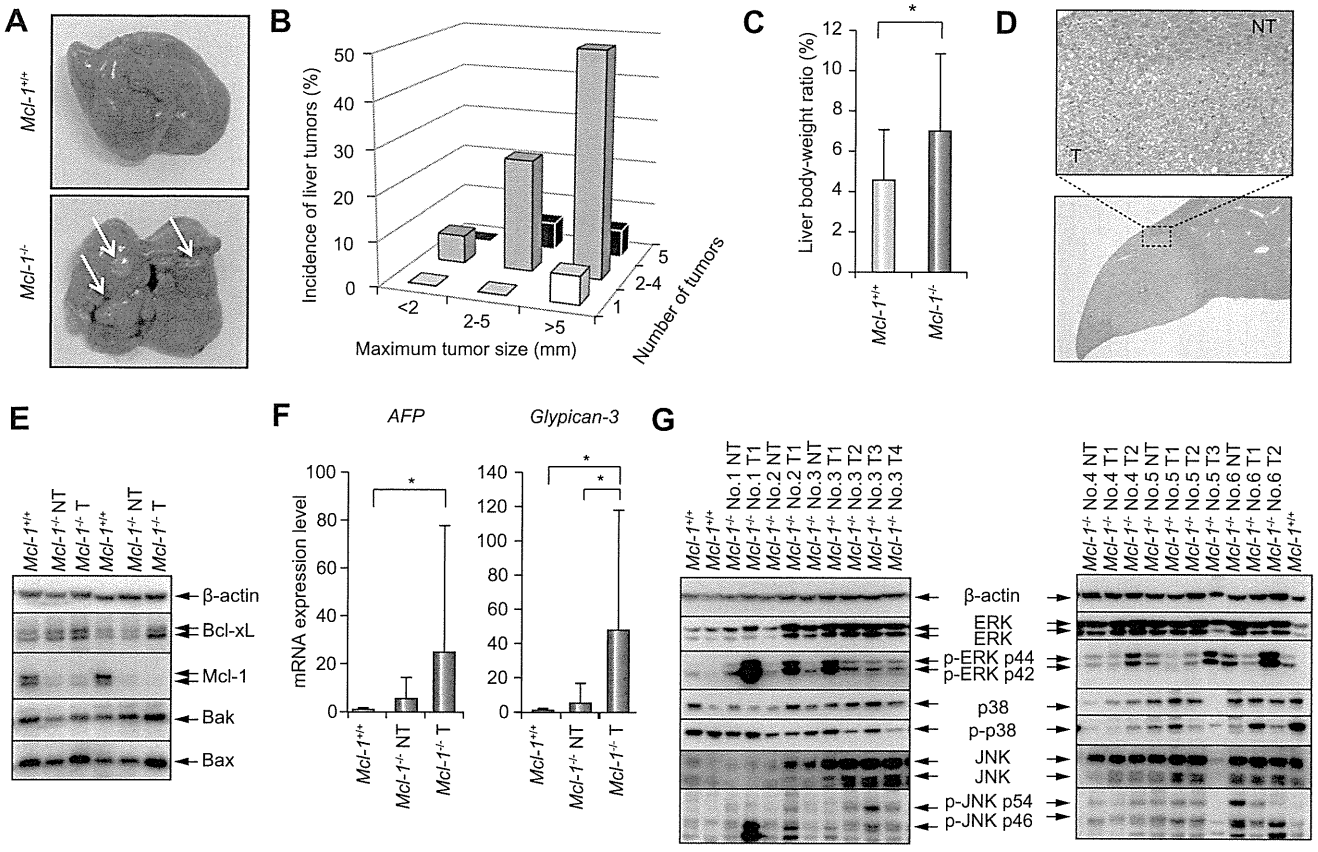

Fig. 2. Liver tumors in *Mcl-1* KO mice. (A–E) Hepatocyte-specific *Mcl-1*-deficient mice (*Mcl-1*$^{-/-}$) (N = 16) and their control littermates (*Mcl-1*$^{+/+}$) (N = 22) were sacrificed at 1.5 years of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors. (C) Liver body-weight ratio. (D) Representative histology of liver tumors in *Mcl-1* KO mice. (E) Western blot of the Bcl-2 family proteins in tumors (T) and surrounding non-cancerous livers (NT) of *Mcl-1* KO mice and livers of control mice. (F and G) Characteristics of liver tumors in *Mcl-1* KO mice. (F) Real-time RT-PCR analysis of the expression levels of α-fetoprotein (*AFP*) and *glypican-3* mRNA (N = 16 per group). (G) Expression and activation of mitogen-activated protein kinases. *p <0.05.

KO mice as evidenced by TUNEL staining of liver sections, serum ALT levels and caspase-3/7 activity at 6 weeks of age (Fig. 4A–C). Weber *et al.* [12] previously described hepatocyte regeneration in the *Mcl-1* KO liver. In agreement with this, *Mcl-1* KO livers showed higher expression of cell cycle markers PCNA and ki-67, than those from control littermates (Fig. 4A, B, and D and Supplementary Fig. 4). Importantly, the levels of PCNA and ki-67 expression decreased with a *Bak* KO background in *Mcl-1* KO mice. While *Mcl-1* KO livers show a mild fibrotic change [11], the levels of col1a1 expression at 6 weeks of age and Sirius red staining at 1 year of age decreased with a *Bak* KO background in *Mcl-1* KO livers (Fig. 4E and Supplementary Fig. 5). Bak deficiency also reduced expression levels of TNF-α, MCP-1, and CD68 at 6 weeks of age (Fig. 4F). Next, we examined the impact of apoptosis inhibition by Bak deficiency on oxidative stress markers, which were increased in *Mcl-1* KO livers. Real-time RT-PCR revealed that Bak deficiency reduced the levels of HO-1 and NQO1 expression at 6 weeks of age (Fig. 4G). Consistent with these observations, *Bak* KO significantly lowered the number of 8-OHdG-positive nuclei in *Mcl-1* KO livers at 1 year of age (Fig. 4H). These results suggested that inhibition of hepatocyte apoptosis reduced oxidative stress in the liver. Finally, to examine the impact of apoptosis inhibition on liver tumor development, we compared the carcinogenetic rates in *Mcl-1* KO mice with or without *Bak* KO background at 1 year of age and found that *Bak* KO significantly suppressed liver tumor development (Fig. 5A and B and Table 1).

Discussion

Mcl-1 was first identified as a gene induced during myeloid cell differentiation. Compared with other anti-apoptotic members such as Bcl-2, Bcl-xL, Bcl-w, and Bfl-1, Mcl-1 possesses a unique N-terminus containing two PEST domains, which are found in proteins displaying rapid turnover, and its expression is tightly regulated by growth factors and a variety of other stimuli. Mice systemically deficient for Bcl-xL suffered embryonic death due to massive apoptosis in hematopoietic organs and developing neurons [22]. On the other hand, systemic *Mcl-1* KO resulted in peri-implantation lethality, but *Mcl-1* KO embryos showed no alterations in the extent of apoptosis [23], suggesting that Mcl-1 may play a role early in development that is distinct from its anti-apoptotic functions. Indeed, *in vitro* studies have shown that Mcl-1 interacts with PCNA and Cdk1 in the nucleus and inhibits proliferation [13,14]. Recently, the early responding gene *IEX-1*

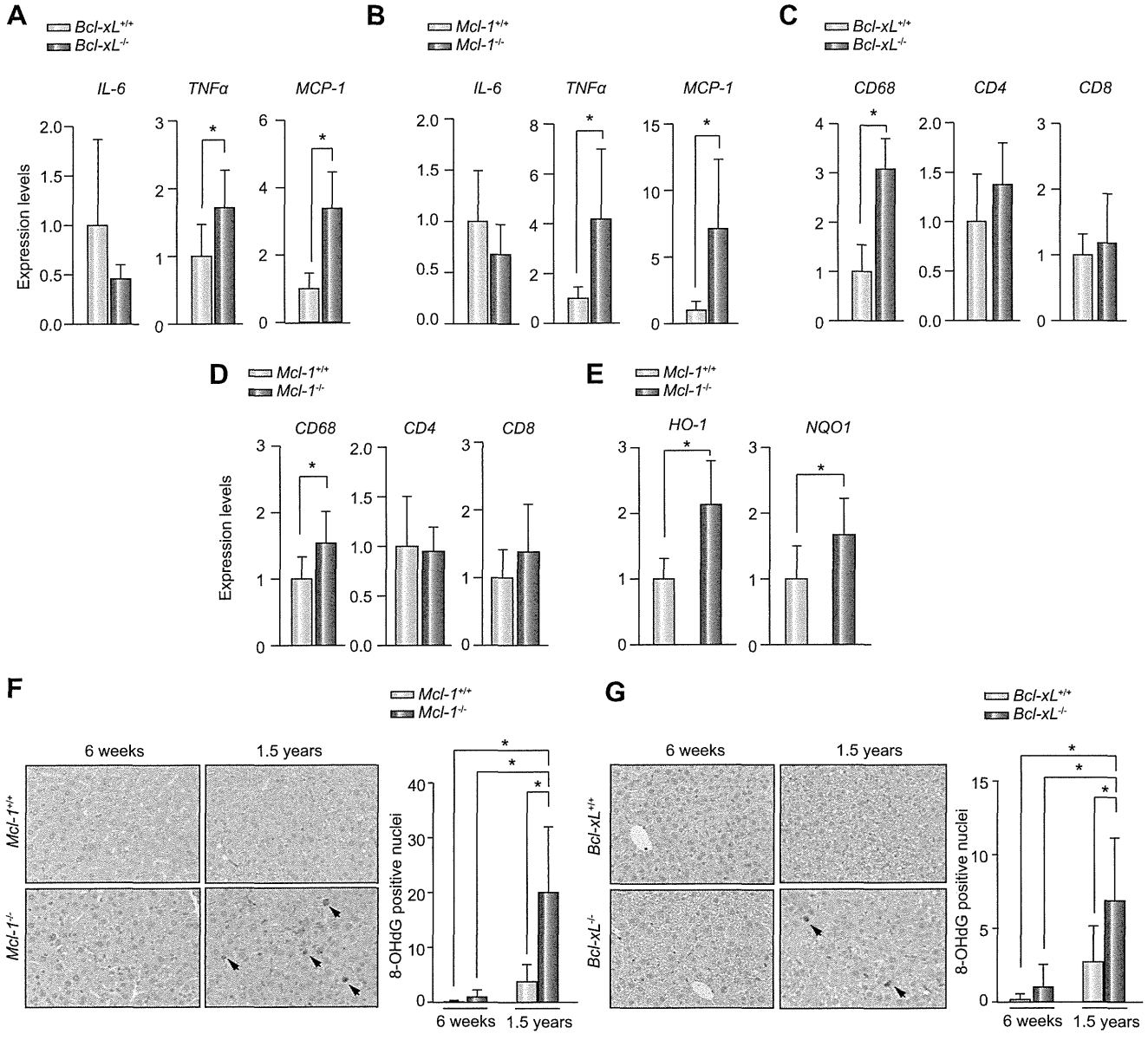

Fig. 3. Inflammatory response and oxidative stress in *Bcl-xL* or *Mcl-1* KO liver. (A–D) Inflammatory responses in KO livers. (A and C) Hepatocyte-specific *Bcl-xL* KO mice (*Bcl-xL*$^{-/-}$) and their control littermates (*Bcl-xL*$^{+/+}$) (N = 6 per group) as well as (B and D) hepatocyte-specific *Mcl-1* KO (*Mcl-1*$^{-/-}$) mice and their control littermates (*Mcl-1*$^{+/+}$) (N = 9 per group) were sacrificed at 6 weeks of age. Expression levels of (A and B) inflammatory molecules and (C and D) cell surface markers of immune cells were analyzed by real-time RT-PCR. (E–G) Oxidative injury in KO livers. (E) Real-time RT-PCR analysis of the expression levels of *HO-1* and *NQO1* of *Mcl-1* KO and control livers at 6 weeks of age (N = 9 per group). (F) Liver sections of *Mcl-1* KO or (G) *Bcl-xL* KO and the control liver at the indicated ages stained with anti-8-OHdG and statistics of the number of positive nuclei (N = 6 and more per group) (G). *p <0.05.

was found to be induced upon DNA damage and to be bound to and to transport Mcl-1 from the cytosol to the nucleus [15]. Mcl-1 was also reported to be induced upon DNA damage and to regulate the DNA damage response through activation of Chk1 [16]. These findings suggest that Mcl-1 possesses additional functions in cell cycle progression and the DNA damage response pathway. This raised concern as to whether the hepatocarcinogenesis observed in *Mcl-1* KO mice was actually related to increased apoptosis in the liver.

In the present study, we demonstrated that hepatocyte-specific destruction of Bcl-xL led to the development of liver cancer similarly to that in hepatocyte-specific *Mcl-1* KO mice. Although we could not completely exclude the possibility that Bcl-xL may have additional effects other than apoptosis, this finding clearly shows that hepatocarcinogenesis observed in the apoptosis-prone liver is not a specific finding of loss of Mcl-1 but is also observed with the knockout of other genes that are critically involved in hepatocyte integrity. Tumors observed in these murine livers frequently showed activation of ERK and JNK, similar to the activation observed in human HCC [18,19]. While 64% of *Mcl-1* KO mice (14/22) developed liver tumors within 1 year, only 27% of *Bcl-xL* KO mice (3/11) did so within 1 year (Table 1). These finding indicate that the incidence rate of carcinogenesis in *Bcl-xL* KO mice is lower than that of *Mcl-1* KO mice. This may be

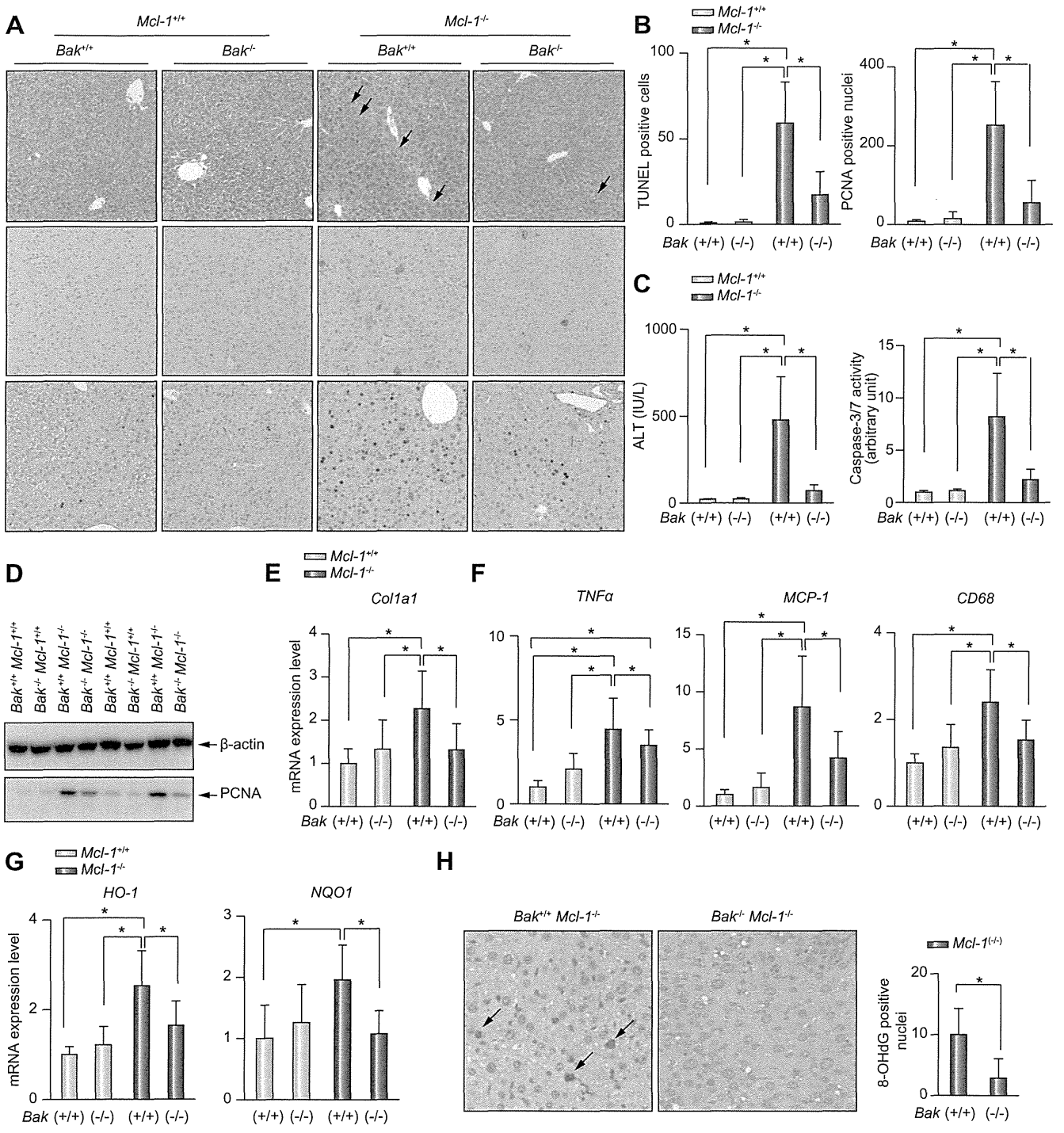

Fig. 4. Impact of Bak deficiency in *Mcl-1* KO mice. (A–G) *Bak*-deficient hepatocyte-specific *Mcl-1* KO mice ($Bak^{-/-}$ $Mcl\text{-}1^{-/-}$) were sacrificed at 6 weeks of age. (A) Representative pictures of hematoxylin–eosin with arrows indicating typical apoptotic cells (upper), TUNEL (middle) and PCNA staining (lower) and (B) statistics of TUNEL and PCNA staining of liver sections (N = 6 or 8 per group). (C) Serum levels of ALT and caspase-3/7 activity (N = 12 per group). (D) Western blot for PCNA expression. Real-time RT-PCR analysis for expression levels of (E) *Col1a1*, (F) *TNF-α*, *MCP-1*, *CD68*, (G) *HO-1* and *NQO1* in the livers at 6 weeks of age (N = 12 per group). (H) Liver sections of the *Bak*-deficient *Mcl-1* KO and control *Mcl-1* KO liver at 1 year of age stained with anti-8-OHdG. Representative images of liver sections stained with anti-8-OHdG (left) and statistics of the number of positive nuclei (N = 9 or 7 per group) (right). *p <0.05.

explained by the difference in levels of hepatocyte apoptosis and serum ALT, which are higher in *Mcl-1* KO mice than in *Bcl-xL* KO mice of the same age [10,11].

Mcl-1 executes its anti-apoptotic function by either directly or indirectly inhibiting the pro-apoptotic functions of Bak and/or Bax [24]. In the present study, we have shown that deletion of the *bak*

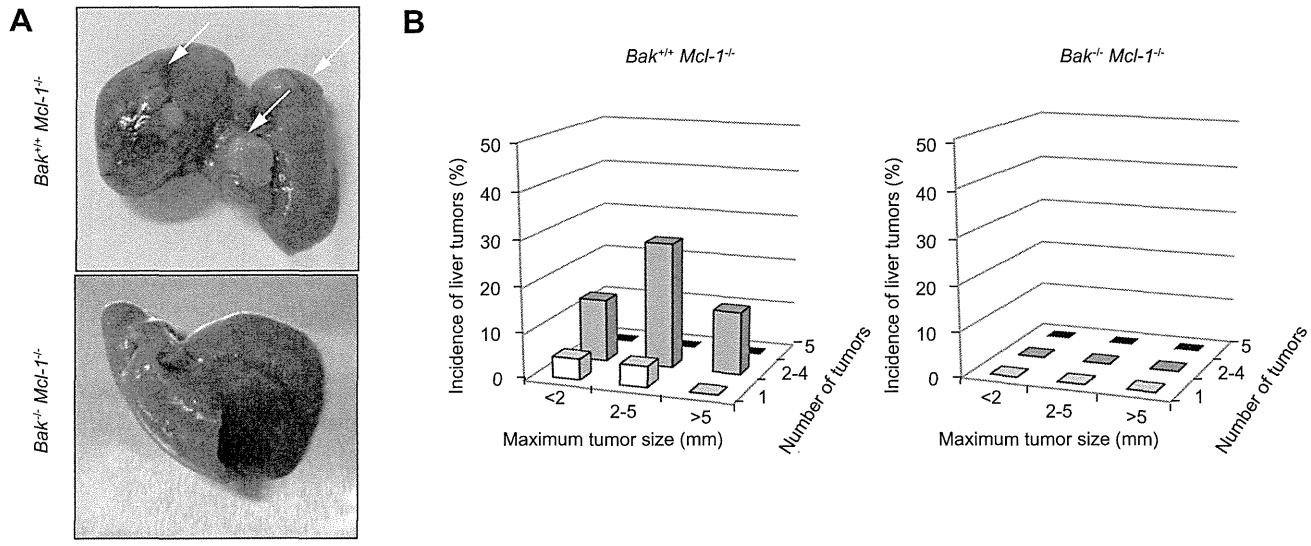

Fig. 5. Liver of aged *Bak/Mcl-1* double KO mice. (A and B) *Bak*-deficient *Mcl-1* KO mice ($Bak^{-/-}$ $Mcl\text{-}1^{-/-}$) (N = 7) and control *Mcl-1* KO mice ($Bak^{+/+}$ $Mcl\text{-}1^{-/-}$) (N = 22) were sacrificed at 1 year of age. (A) Representative macroscopic view of the livers with arrows indicating tumors. (B) Incidence of liver tumors separated by maximum tumor size and number of tumors.

gene resulted in a clear reduction in hepatocyte apoptosis in *Mcl-1* KO mice. Of importance is the finding that *bak* deletion leads to reduction of the liver regenerative response in *Mcl-1* KO mice. Bak is exclusively localized at the mitochondria in hepatocytes [25] and, upon exposure to apoptotic stimuli, undergoes oligomerization to form pores in the outer membrane of mitochondria, releasing cytochrome c, which in turn activates caspases. Since Bak is not involved in the activity of Mcl-1 in the nucleus, our present finding suggests that the regeneration observed in the *Mcl-1* KO liver is not due to loss of the Mcl-1 anti-proliferative effect but mainly to the compensatory regeneration of increased apoptosis. Most importantly, *bak* deletion clearly leads to reduced liver tumor incidence. This finding strongly suggests that the hepatocarcinogenesis observed in *Mcl-1* KO mice can be mostly ascribed to increased apoptosis in hepatocytes.

What does make hepatocytes undergo malignant transformation in the liver with increasing apoptosis? Regeneration is a physiological process in the liver like that in bone marrow or the intestine and compensatory liver regeneration itself is probably not sufficient to induce liver cancer [26]. The present study raised the possibility that TNF-α and oxidative stress are candidate factors responsible for the malignant transformation in the apoptosis-prone liver. TNF-α is reported to be a potent endogenous mutagen that promotes cellular transformation [20], and oxidative stress is reported to cause DNA damage leading to carcinogenesis [21]. Our results revealed that both TNF-α and oxidative stress were significantly increased in KO livers, and importantly, that inhibition of apoptosis by deletion of the *bak* gene reduced the levels of TNF-α and oxidative stress with a decrease in the tumorigenic rate. Some studies have shown that TNF-α induces oxidative stress in hepatocytes [27,28], while oxidative stress promotes production of inflammatory cytokines [29–31]. Taken together, oxidative stress and inflammatory cytokines may positively affect each other to turn healthy hepatocytes into malignant transformed hepatocytes in the liver of KO mice. Further studies are needed to examine the role of oxidative stress and inflammatory cytokines in apoptosis-induced hepatocarcinogenesis.

Apoptosis resistance has been established as a hallmark of cancer [32]. Indeed, accumulating evidence indicates that human HCC frequently overexpresses a variety of molecules which confer apoptosis resistance, such as anti-apoptotic Bcl-2 family proteins, Bcl-xL [33] and Mcl-1 [34,35]. Their overexpression was found to be associated with malignant phenotypes of tumors and poor prognosis of patients [36]. In the present study, tumors that developed in *Bcl-xL* or *Mcl-1* KO mice lacked expression of the respective proteins but reciprocally overexpressed Mcl-1 or Bcl-xL at high rates. We recently reported that conditional expression of Bcl-xL in tumor cells was translated into higher tumor growth in xenograft models [37], indicating that overexpression of anti-apoptotic Bcl-2 family proteins is important for tumor progression. Lack of Bcl-xL or Mcl-1 in hepatocytes generates persistent hepatocyte apoptosis leading to liver tumor development. On the other hand, reciprocal overexpression of Mcl-1 or Bcl-xL in the tumor of *Bcl-xL* or *Mcl-1* KO mice might be required for tumor progression.

Increasing evidence indicates that the serum level of ALT, a marker of hepatocyte apoptosis, is a risk factor for HCC in viral hepatitis [38] and non-alcoholic steatohepatitis [39]. A population-based study also revealed that elevated ALT levels raise the risk of liver cancer [40]. The present study provides evidence that spontaneous apoptosis in hepatocytes leads to liver cancer development and also offers genetic evidence that inhibition of apoptosis can help prevent liver cancer. Administration of caspase inhibitor was previously reported to lower serum ALT levels in patients with chronic hepatitis C [41]. It may be interesting and important, from a clinical point of view, to further determine whether pharmacological inhibition of apoptosis can be useful in preventing liver cancer development in *Bcl-xL* or *Mcl-1* KO mice.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jhep.2012.01.027.

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Difference in serum complement component C4a levels between hepatitis C virus carriers with persistently normal alanine aminotransferase levels or chronic hepatitis C

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Abstract. Certain hepatitis C virus (HCV) carriers exhibit persistently normal alanine aminotransferase (ALT) levels (PNALT) (≤30 IU/l) accompanied by normal platelet counts (≥15x10^4/μl); these individuals show milder disease activity and slower progression to cirrhosis. This study aimed to elucidate the characteristics of HCV carriers with PNALT using serum proteomics. The first group of subjects, who underwent clinical evaluation in the hospital, consisted of 19 HCV carriers with PNALT (PNALT-1) and 20 chronic hepatitis C (CHC-1) patients. The second group of subjects was part of a cohort study on the natural history of liver disease, and included 37 PNALT (PNALT-2) and 30 CHC (CHC-2) patients. Affinity bead-purified serum protein was subjected to matrix-assisted laser desorption ionization time-of-flight mass spectrometry analysis. Serum proteomics showed that 6 protein peaks with mass-to-charge ratios ranging from 1,000 to 3,000 differed significantly between the PNALT-1 and CHC-1 groups. Among these peaks, a 1738-m/z peak protein was identified as a fragment of complement component 4 (C4) and correlated significantly with serum C4a concentrations as determined by enzyme immunoassay. Serum C4a levels were also significantly higher in the PNALT-2 group compared to the CHC-2 group and healthy volunteers. Furthermore, in the PNALT-2 group, serum C4a levels negatively correlated with transaminase levels, but not with other biochemical tests, HCV core antigen levels, peripheral blood cell counts or serum hepatic fibrosis markers. This study indicates that host factors such as C4a not only differ between HCV carriers with PNALT and CHC, but that proteomic approaches could also contribute to the elucidation of factors in PNALT as more differences are discovered.

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Key words: hepatitis C virus, persistent normal alanine aminotransferase, complement component C4a, C4 fragment, serum proteomics

Introduction

Hepatitis C virus (HCV) infection, one of the main causes of chronic hepatitis, is estimated to affect 170 million people worldwide (1). The natural history of HCV infection is characterized by acute and eventually chronic infection, and may progress from a long-lasting asymptomatic condition to decompensated liver cirrhosis or hepatocellular carcinoma (HCC) (2). However, the long-term impact of HCV infection is highly variable; some patients with persistent HCV infection exhibit persistently normal alanine aminotransferase (ALT) levels (PNALT), which are associated with milder disease activity and slower progression to cirrhosis (3). In addition, the differences between PNALT patients and those with chronic hepatitis C (CHC) who exhibit elevated ALT levels have not yet been fully elucidated (4).

Persico *et al* reported that the grade of disease activity does not increase over a period of years, and that progression to cirrhosis is slow or absent in patients with HCV-related chronic hepatitis associated with PNALT (5). We have previously reported that the ALT level is a predictor of HCV-associated HCC incidence in a community-based population in Japan (6). In addition, a number of studies have shown that interferon (IFN)-based therapy reduces HCC in patients with CHC,

even in those in whom HCV RNA remains detectable (7,8). Continuous normalization of aminotransferase and α-feto protein (AFP) for more than 1 year during IFN therapy is associated with a reduced risk of HCC development following the termination of the IFN therapy (9).

A recent application of proteomic technology has identified a spectral pattern from the serum of patients with liver disease (10-12), and proteomic techniques will be able to identify serum biomarkers that are present in the serum of patients with PNALT. Furthermore, a biomarker or biomarker panel may also help to elucidate a possible mechanism for chronic hepatitis from PNALT and could perhaps lead to the development of more effective treatments for chronic hepatitis. However, proteomic approaches focused on PNALT have not been previously explored.

In this study, we verified differentially expressed protein in serum samples and showed that the level of the complement component 4a (C4a) in serum was higher in HCV carriers with PNALT compared to CHC patients or healthy volunteers. The present study reveals that C4a increases with HCV infection, but decreases with disease progression. Identification of these and other proteins will help clarify the underlying mechanisms and contribute to improved clinical outcomes for HCV carriers.

Patients and methods

Study population. Anti-HCV seropositive subjects with detectable HCV core antigen (HCVcAg) or HCV RNA were considered to be persistently infected with HCV and were classified as HCV carriers. ALT levels >30 IU/l and platelet counts $<15x10^4/\mu l$ were considered to be abnormal. HCV carriers exhibiting persistently normal ALT levels accompanied by normal platelet counts during the observation period were defined as the PNALT group in this study (13). Subjects who underwent oral or intravenous administration of medical herbs or other palliative therapies were not excluded from this study, but those who had received IFN therapy were excluded. All subjects were negative for hepatitis B virus surface antigen (HBsAg).

The first group of subjects, who were undergoing hospital-based clinical evaluation, consisted of 39 HCV carriers. Of these, 19 with PNALT (PNALT-1 group) and 20 with CHC and abnormal ALT levels (CHC-1 group) were enrolled. HCV carriers with PNALT (PNALT-1 group) were defined as those who had normal serum ALT levels (≤30 U/l) over a 12-month period and on at least 3 different occasions, and platelet counts of $\geq 15x10^4/\mu l$. Blood samples from the PNALT-1 and CHC-1 groups were obtained during the last observation period.

The second group of subjects was part of a larger cohort being followed-up as part of a study on the natural history of liver disease; data on these individuals were acquired from 1994 through 2005 (14). An analysis was conducted of HCV carriers who had undergone at least 3 independent ALT measurements obtained during annual general health examinations or liver disease screenings. In total, 37 HCV carriers with persistently normal ALT levels and platelet counts $\geq 15x10^4/\mu l$ (PNALT-2 group) and 30 HCV carriers with persistently abnormal ALT levels and platelet counts $<15x10^4/\mu l$ (CHC-2) were investigated. Blood samples from PNALT-2 or CHC-2 subjects were obtained during the last observation period in this study from 2002 to 2005. Serum samples were also obtained from healthy volunteers without HCV infection (n=12).

After the blood samples were collected, serum was stored at -80°C. Written informed consent was obtained from each subject and the study protocol was approved by the Ethics Committee of Kagoshima University Hospital; the Faculty of Medicine, University of Miyazaki and Kyoto Prefectural Medical School.

Serum pre-treatment with ClinProt magnetic beads. Serum samples (5 μl) were purified and concentrated using magnetic bead-based weak cation exchange chromatography resins (WCX) (Bluker Daltonics, Bremen, Germany). 2-Cyano-4-hydroxycinnamic acid (CHCA) matrix solution (Bluker Daltonics) was diluted to 0.3 g/l in an ethanol:acetone (2:1) solution. Purified serum samples and diluted CHCA solutions were mixed (1:9), and 1 μl of the solution was applied onto a matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) AnchorChip.

Mass spectrometry (MS) and peptide identification. The AnchorChip target plate was placed in an AutoFlex II TOF/TOF mass spectrometer (Bluker Daltonics). Spectra were acquired in the positive linear mode in a molecular mass range from 1,000 to 3,000 Da. The MALDI-TOF MS spectrum was subjected to a Mascot database search (Matrix Science, Boston, MA, USA) using the SwissProt database.

Serum markers. The presence of serum anti-HCV antibody (Ab) was determined using a commercially available third-generation enzyme-linked immunosorbent assay (ELISA). Serum levels of HCVcAg were determined by a chemiluminescence enzyme immunoassay (HCV core protein; SRL, Tokyo, Japan), with a detection threshold of 20 fmol/l. The serologically defined HCV genotype (HCV serotype) was tested with a serological genotyping assay kit (Immunocheck F-HCV Grouping, International Reagents Co., Tokyo, Japan). In some patients, the HCV genotype was examined (HCV Core Genotype, SRL, Tokyo, Japan). HCV genotype 1b was included with serotype I, and genotypes 2a and 2b with serotype II. No other HCV genotype was detected in this study population.

The serum concentration of C4a was determined using a C4a ELISA kit (Human C4a ELISA kit, BD Biosciences, San Diego, CA, USA).

Statistical analysis. The results are presented as the means ± standard deviation (SD). All spectra in MALDI-TOF MS were analyzed using Bluker Daltonics FlexAnalysis 2.2 software and ClinProTools 2.0 software. Statistical analysis of other clinical data was performed using StatView 4.5 software (Abacus Concepts, Berkeley, CA, USA) or SPSS software (SPSS Inc., Chicago, IL, USA). Differences were evaluated by the Mann-Whitney U test, the Fisher's exact test or the Chi-square test as appropriate. Any probability value <0.05 was considered to indicate a statistically significant difference.

Table I. Patient characteristics in the hospital-based group.

Characteristics	PNALT-1 (n=19)	CHC-1 (n=20)	P-value[a]
Age	55.2±15.1	52.3±11.7	0.17
Gender (male/female)	3/16	7/13	0.27
HCV core antigen (fmol/l)	1163±803	1072±669	0.72
HCV serotype (I/II/UD)	9/5/5	11/5/4	0.87
Platelet count (x10^4/μl)	21.3±5.5	14.8±4.0	<0.001
AST (IU/l)	25.4±3.9	73.6±37.5	<0.001
ALT (IU/l)	23.5±5.1	90.9±54.8	<0.001
γ-GTP (IU/l)	18.6±8.5	89.4±77.9	<0.001
Total cholesterol (mg/dl)	200.0±24.4	180.1±27.4	0.03
Albumin (g/dl)	4.5±0.2	4.5±0.4	0.76

PNALT, persistently normal ALT; CHC, chronic hepatitis C; n, number of patients; HCV, hepatitis C virus; UD, undetermined; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase. Data are presented as the means ± standard deviation or number. [a]Differences between mean values were evaluated using either the Fisher's exact test, the Chi-square test or the Mann-Whitney U test, as appropriate.

Table II. Protein peaks expressed differentially between HCV carriers with persistent normal ALT levels (PNALT) and chronic hepatitis C (CHC) patients with abnormal ALT levels.

	Peak intensity		
m/z	PNALT-1 (n=19)	CHC-1 (n=20)	P-value[a]
1738	109.4±67.1	83.9±54.0	<0.01
1896	105.0±64.8	111.3±63.3	<0.05
1943	191.3±149.5	139.6±73.6	<0.01
2858	104.0±34.3	85.3±25.6	<0.001
2928	31.8±9.7	64.0±28.9	<0.001
2947	59.3±34.9	80.7±36.2	<0.001

Data are presented as the means ± standard deviation. [a]Differences between mean values were evaluated using the Mann-Whitney U test ALT, alanine aminotransferase.

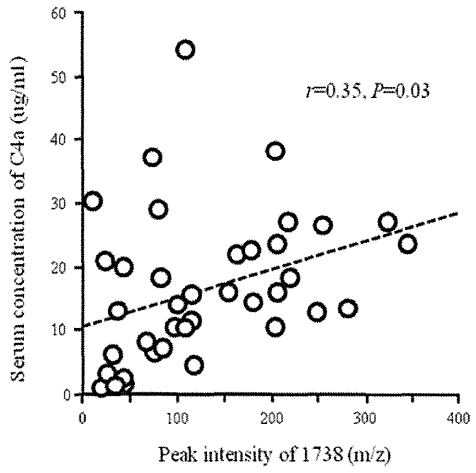

Figure 1. Association between the peak intensity of 1738 m/z (C4 fragment) and the serum level of C4a as determined by enzyme-linked immunosorbent assay. The peak intensity of 1738 m/z correlated with serum C4a levels (r=0.35, P=0.03). C4a, complement component 4a.

Results

Profiling sera from patients with PNALT and chronic hepatitis C using MALDI-TOF MS analysis. In the first hospital-based group, serum levels of ALT, aspartate aminotransferase (AST), and γ-glutamyltranspeptidase (γ-GTP) were lower and platelet counts and total cholesterol were higher in PNALT-1 patients than in CHC-1 patients (Table I). In this group, the sera of patients were analyzed to identify protein peaks that differed most between patient subsets. Serum proteomics revealed that 6 serum protein peaks with mass-to-charge ratios ranging from 1,000 to 3,000 differed significantly between PNALT-1 and CHC-1 groups (Table II). In these protein peaks, a 1738-m/z peak protein was identified as a fragment of C4, with the sequence NGFKSHALQLNNRQI.

Correlation between the protein peak of 1738 m/z and serum levels of C4a determined by ELISA. Although the identified C4 fragment is part of C4c, serum concentrations of C4c could not be determined by commercially available methods, such as ELISA. By contrast, the recalibrated peak intensity of this fragment significantly correlated with the serum level of C4a, which could be determined with a commercially available assay kit (Fig. 1). In addition, serum concentrations of C4a were significantly higher in PNALT-1 subjects [means ± SD (μg/ml), 20.6±11.9] compared with those in CHC-1 subjects (12.2±10.2) (P=0.01).

Serum levels of C4a determined by ELISA in the second group. In the cohort-based population, age, the prevalence of females and of serotype II, platelet counts, and serum albumin

Table III. Patient characteristics in cohort-based population with HCV infection.

Characteristics	PNALT-2 (n=37)	CHC-2 (n=30)	P-value[a]
Age	75.6±6.5	70.4±6.6	<0.01
Gender (male/female)	8/29	15/15	0.02
HCV core antigen (fmol/l)	6,042±4,295	4,553±3,546	0.27
HCV serotype (I/II)	14/23	24/6	<0.001
Platelet count (x$10^4/\mu$l)	22.3±5.3	11.8±3.8	<0.001
AST (IU/l)	28.3±8.0	100.1±81.8	<0.001
ALT (IU/l)	19.5±6.0	96.9±81.8	<0.001
γ-GTP (IU/l)	15.1±10.6 (n=31)	56.2±46.7 (n=20)	<0.001
T-Cho (mg/dl)	181.8±29.3 (n=31)	158.8±27.1 (n=29)	0.02
Albumin (g/dl)	4.4±0.3 (n=33)	4.1±0.5 (n=29)	<0.01

HCV, hepatitis C virus; PNALT, persistently normal alanine aminotransferase; CHC, chronic hepatitis C; AST, aspartate aminotransferase; ALT, alanine aminotransferase; γ-GTP, γ-glutamyltranspeptidase; T-Cho, total cholesterol; n, number of patients or the number of samples analyzed. Data are presented as the means ± standard deviation or number. [a]Differences between mean values were evaluated using either the Fisher's exact test or the Mann-Whitney U test, as appropriate.

Table IV. Correlation between serum C4a levels and blood laboratory parameters in PNALT subjects.

Parameter	Correlation coefficient	P-value[a]
HCV core antigen	0.06	0.73
White blood cell	-0.06	0.72
Hematocrit	-0.06	0.12
Platelet	0.12	0.51
Albumin	0.03	0.88
γ-globulin	-0.05	0.77
AST	-0.39	0.02
ALT	-0.47	<0.01
Total-bilirubin	-0.07	0.69
Total cholesterol	0.05	0.76
Ferritin	-0.18	0.30
Hyaluronic acid	-0.23	0.17
Type IV collagen	-0.04	0.82
α-fetoprotein	-0.29	0.09
DCP	-0.07	0.69

PNALT, persistently normal alanine aminotransferase; HCV, hepatitis C virus; AST, aspartate aminotransferase; ALT, alanine aminotransferase; DCP, des-γ-carboxy prothrombin. [a]P-values were assessed by Spearman's rank correlation analysis.

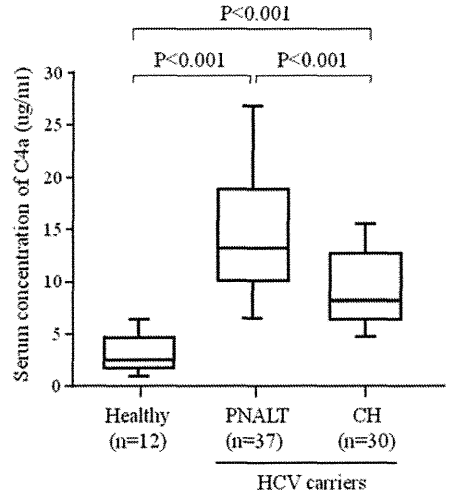

Figure 2. Serum concentrations of C4a determined by enzyme-linked immnosorbent assay in HCV carriers and healthy controls. Serum C4a levels were significantly higher in HCV carriers with PNALT than in HCV carriers with chronic hepatitis or healthy controls (P<0.001). Boxes indicate the median ± 25th percentile, the lower bar indicates the 10th percentile, and the upper bar indicates the 90th percentile. HCV, hepatitis C virus; PNALT, persistently normal alanine aminotransferase; CH, chronic hepatitis; C4a, complement component 4a.

and total cholesterol levels were significantly higher in the PNALT-2 group than in the CHC-2 group. By contrast, serum AST, ALT and γ-GTP levels were lower in the PNALT-2 group (Table III). In the cohort-based population, serum concentrations of C4a, as determined by ELISA, were significantly higher in the PNALT-2 group than in the CHC-2 group and healthy controls (Fig. 2).

Serum C4a levels in the PNALT-2 group correlated significantly with serum AST and ALT levels, but not with HCVcAg levels or other blood laboratory parameters (Table IV). In addition, a significant negative correlation between serum C4a and ALT levels was observed in the population as a whole (Fig. 3, r=-0.35, P=0.03) and in PNALT patients

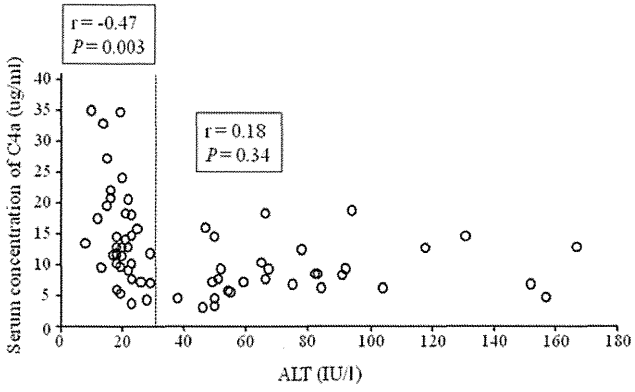

Figure 3. Association between serum C4a and ALT levels in all HCV carriers (including PNALT and chronic hepatitis). Serum C4a levels negatively correlated with serum ALT levels (r=-0.35, P=0.03). This correlation was observed in subjects with normal ALT (≤30 IU/l) (r=-0.47, P=0.003) but not in those with abnormal ALT (>30 IU/l). C4a, complement component 4a; HCV, hepatitis C virus; ALT, alanine aminotransferase; PNALT, persistently normal ALT.

(Table IV; ALT ≤30 IU/l; Fig. 3), but not in CHC-2 patients (ALT >30 IU/l; Fig. 3).

Discussion

HCV is not thought to be directly cytopathic to hepatocytes, and a T helper (Th)1-type or cytotoxic T lymphocyte (CTL) response is critically involved in HCV-mediated liver injury (15). Therefore, it is conceivable that various suppressor mechanisms exist against Th1-type immune responses in HCV carriers with PNALT, which may be distinct from those in CHC patients with active liver inflammation (16). However, few studies have focused on PNALT using a serum proteomic approach. In this study of HCV carriers, a number of proteins were detected which were differentially expressed between PNALT and CHC patients. Of these, the C4 fragment was identified by peptide mass fingerprint (PMF) methods following a Mascot search, and serum levels of C4a, which correlated with the protein peak of the identified C4 fragment, were higher in PNALT than in CHC patients, as determined by ELISA. In addition, serum C4a levels correlated with ALT levels in the PNALT but not CHC patients.

Following acute HCV infection, approximately 70% of individuals remain positive for both anti-HCV Ab and HCV RNA, and are defined as HCV carriers. By contrast, approximately 30% of acutely infected individuals clear the HCV and remain positive for anti-HCV Ab but negative for HCV RNA. We confirmed that serum C4a levels in those who cleared the virus were similar to healthy controls (data not shown). Serum C4a levels were higher in CHC patients and individuals with PNALT compared to healthy controls. Therefore, serum C4a levels appear to be at least elevated by existing HCV infection, although serum C4a levels did not correlate with serum HCVcAg levels in individuals with PNALT (Table IV). It was previously reported that serum L-ficolin levels were increased in HCV patients, and that this protein only recognized and bound to glycoproteins E1 and E2 of the HCV envelope, but also activated the complement lectin pathway-mediated cytolytic activity in HCV-infected hepatocytes (17). In the lectin pathway, mannose-binding lectin (MBL)-associated serine protease-2 (MASP-2) cleaves C4, releasing C4a and generating C4b (18). Thus, C4a levels should increase in HCV carriers compared to healthy controls by post-translational mechanisms.

Previous studies have reported decreased serum C4 levels in patients with CHC (19,20). Recently, the HCV core protein and non-structural 5A protein (NS5A) were reported to transcriptionally downregulate C4 expression by modulating the expression of upstream stimulating factor 1 and IFN regulatory factor 1, respectively (21). Thus, serum C4 protein levels are decreased in HCV patients compared to healthy controls as a result of altered transcriptional regulation (22). Although the mechanism of C4a variation in HCV carriers has not been elucidated, our study suggests that serum C4a levels in HCV carriers with PNALT should be dominantly affected by post-translational mechanisms, but patients with CHC may be affected by both translational (downregulation) and post-translational (upregulation) mechanisms.

In CHC, decreased specific C4 activity without C3 consumption suggests complement activation leading to the N-terminal cleavage of C4 with the production of C4a (20). Another study demonstrated increased C4a levels in CHC patients without a significant increase in the levels of C3a (23). Avirutnan *et al* reported that flaviviruses, such as dengue virus, use their non-structural protein, NS1, to attenuate complement activation by directly interacting with C4, leading to viral persistence (24). Although the mechanisms responsible for HCV persistence or PNALT in HCV carriers are not well understood, the interactions between HCV and the host immune system are thought to play a pivotal role in patients with HCV infection.

The majority of individuals with PNALT have minimal or mild inflammation and absent or minimal fibrosis, and follow-up studies have shown disease stability with minimal fibrosis progression over the years, leading to a favorable prognosis. However, cirrhosis and HCC are occasionally observed in HCV carriers with normal ALT levels (25). In addition, some patients with PNALT may develop ALT elevation over time (4), and these individuals may be at increased risk of the significant progression of fibrosis. Long-term observation or liver biopsy has not always been performed; serum C4a levels may be a diagnostic marker for advanced fibrosis or a predictor for ALT elevation in PNALT. These issues should be subject to further analysis.

It has been reported that in HCV carriers with PNALT or normal ALT levels, IFN-based therapy is safe and efficacious (26,27). However, the decision whether or not to treat HCV carriers should be made with the specific clinical setting in mind (28). In addition, it is recommended that serum ALT levels be kept <30 IU/l to prevent the occurrence of HCC (29). If serum C4a levels are an indicator of disease prognosis, HCV carriers with low serum C4a levels may have to be treated despite ALT elevation. More significantly, elucidating the mechanism underlying the association between serum C4a and ALT levels should lead to new approaches to the treatment of HCV carriers with PNALT.

In conclusion, host factors such as C4a differ between HCV carriers with PNALT and CHC patients with elevated ALT levels. Proteomic approaches could greatly contribute to elucidate the host factor in PNALT patients as more differ-

ences are discovered. Identification of these and other proteins will help clarify the mechanism and may improve clinical outcomes of HCV carriers.

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Clinical Features of Hepatitis C Virus Carriers With Persistently Normal Alanine Aminotransferase Levels

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ABSTRACT

Hepatitis C virus (HCV) infection causes chronic hepatitis, which frequently leads to hepatic fibrosis and hepatocellular carcinoma (HCC). Alanine aminotransferase (ALT) is a biomarker of hepatocyte injury and is associated with the progression of hepatic fibrosis. Advanced hepatic fibrosis also predisposes HCV carriers to a risk of HCC. In contrast, some cases with persistent HCV infection have normal ALT levels that persist for a long time, and these HCV carriers have no or mild hepatitis and hepatic fibrosis. These HCV carriers are defined as persistent normal ALT (PNALT) cases and their risk of HCC is low compared to HCV carriers with abnormal ALT. However, there are various definitions of normal ALT and PNALT, and advanced hepatic fibrosis may be missed without a liver biopsy. In addition, there is also a risk of ALT elevation in HCV carriers with PNALT, which increases the risk of progression to hepatic fibrosis and HCC. Most HCV carriers with PNALT have asymptomatic or nonspecific symptoms. HCV carriers with PNALT are also considered to be responsive to interferon-based treatment. Thus, assessment of hepatic fibrosis is important in HCV carriers, and the eradication of HCV infection is more likely in HCV carriers with evidence of hepatic fibrosis, regardless of their ALT levels.

▸ *Implication for health policy/practice/research/medical education:*
Epidemiological studies have suggested a linkage between alanine aminotransferase (ALT) levels and hepatic fibrosis or hepatocellularcarcinoma (HCC) in hepatitis C virus (HCV) carriers. However, clinical features of HCV carriers with persistent normal ALT levels (PNALT) are not fully elucidated. This review focuses on the PNALT in HCV carriers and clinical significance of hepatic fibrosis in these carriers in order to bring out some common opinions on how to manage such HCV carriers with PNALT.

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1. Context

Hepatitis C virus (HCV) causes chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) at high rates (1-3). However, some cases remain asymptomatic with normal levels of alanine aminotransferase (ALT) after HCV infection and detection of the infection in these cases may only occur through screening, such as with an anti-HCV antibody test. The ALT level usually rises in hepatitis (4), but it is normal in approximately 20-30% of HCV carriers (5, 6). Previous studies have shown that elevated ALT levels predict an increased rate of HCV-associated HCC in a community-based population and that serial measurements to identify persistent ALT abnormality may be useful in determining the HCC risk (7, 8). Thus, HCV carriers with normal ALT levels were previously not indicated for antiviral therapy. In contrast, it has been shown that hepatic inflammation and fibrosis are histologically present in many HCV carriers, even though their ALT level is normal, and these HCV carriers are candidates for antiviral therapy. These conflicting issues may arise from ambiguity around the definition of normal ALT and with the natural course of HCV carriers with persistent normal ALT (PNALT). In this review, which is based on a search for articles using terms such as persistent ALT, normal ALT, and HCV, we describe the characteristics, natural course, and treatment in HCV carriers with PNALT.

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2. Evidence Acquisition

2.1. Epidemiologic Features of HCV Carriers

Many HCV carriers are asymptomatic. According to American Association for the Study of Liver Diseases (AASLD) practice guidelines, drug users, patients with a high risk for HCV infection (patients with HIV infection or hemophilia treated with coagulation factor preparations before 1987, and patients with hemodialysis or ALT abnormality), patients who received blood transfusion or organ transplantation before July 1992, children born to HCV-infected mothers, medical workers exposed to needles used for HCV-infected patients or to the mucosa of HCV-infected patients, and people who have had a sexual relationship with an HCV carrier should be screened for a HCV infection (9). More than 170 million people worldwide are thought to be infected with the HCV. HCV infection progresses to a chronic state in 60-85% of infected people and may develop into liver cirrhosis and HCC after 20-35 years (3, 10). Poynard *et al.* reported that 33% of patients with a HCV infection had an expected median time to cirrhosis of less than 20 years without treatment (11). Other studies have also shown a rate of progression to cirrhosis of between 1.5 and 16 years following blood transfusion in approximately 20% of cases (12). The rate of progression to HCC was reported to be approximately 2% per year (13, 14). In addition, about 1.4 million people (approximately 1% of HCV carriers) are estimated to die annually due to liver cirrhosis or HCC (1, 3). We previously reported that 70 out of 758 HCV carriers died due to a liver-related cause over an average of 8.2 years of follow-up (a rate of 1% per year) (8). The important point is that those rates may be mainly due to the diversity in the design of the studies themselves. Host factors and environmental factors affect the progression of hepatic inflammation and fibrosis. Host factors include; advanced age at the time of infection, male gender, excess alcohol consumption, excess iron intake, cigarette smoking, obesity, complications with diabetes, fatty liver and metabolic syndrome, and coinfection with human immunodeficiency virus (HIV), hepatitis B virus (HBV), and *Schistosomiasis* (3, 15). In addition, hepatic fibrosis in liver grafts shows rapid progression after liver transplantation in hepatitis C patients (16, 17), although the mechanism for this remains unclear. In contrast, the progression of hepatic fibrosis may not be associated with HCV genotype or viral load (11, 18).

2.2. The Concept of ALT Normality and Definition for PNALT

Since ALT is released into the blood when the hepatocyte membrane is impaired or hepatocytes are destroyed, elevation of the serum ALT level is a useful marker indicating hepatocellular impairment (15). According to UpToDate ver. 19.3 (UpToDate, Inc. Waltham, MA), HCV infection with a normal ALT level is defined by the following (5); detectable HCV RNA and serum ALT concentration that is persistently within the normal range. The definition of PNALT includes the idea of "persistence", indicating maintenance of an ALT level that is below the upper limit of the normal value over a long period of time (e.g., over a six-month period). However, this definition has varied among the many reports on PNALT, which have used different periods of observation and upper limits of normal ALT that are often set at the highest value of the measurement instrument. Moreover, the ALT level varies depending on; age, gender, race, and body mass index (BMI) (9). Prati *et al.* investigated 6,835 blood donors with ALT levels within the standard range (male: ≤ 40 IU/l, female: ≤ 30 IU/l) who were negative for infections (HBV, HCV, and HIV), had no history of medication, with blood glucose, cholesterol, and triglyceride levels lower than the upper limits of the standard ranges, and had a BMI < 25 kg/m^2. The findings showed that 30 and 19 IU/l were appropriate as the upper limits of the normal ALT range in males and females, respectively (20). However, according to the AASLD Practice guidelines, PNALT is present when ALT is measured 2-3 times over 1-6 months and all values are < 40 IU/l (9). Thus, the standard ALT level is unclear and the criteria for PNALT vary among the different reports. Therefore, it is important to pay attention to the definition of a normal ALT level in order to evaluate the natural course of HCV carriers with PNALT.

2.3. Clinical Manifestations in HCV Carriers With PNALT

Most HCV carriers with PNALT are discovered incidentally when anti-HCV antibodies are found following a blood donation. Most of these patients have asymptomatic disease or nonspecific symptoms including; fatigue, weakness, and upper right quadrant pain (20). The frequency and severity of these symptoms have been reported to be similar among anti-HCV positive patients with normal serum ALT, mildly elevated values (< 2 times normal), and more marked elevation (> 2 times normal) (21). In contrast, Jamal *et al.* have suggested that there is a trend towards depression being more common in HCV carriers with abnormal ALT compared to those with normal ALT, although they found that there was no statistically significant difference in depressive symptoms (20). Therefore, depression, though nonspecific, might be an important clinical marker of a more severe disease.

2.4. Epidemiology, Clinical Course and Management in HCV Carriers With PNALT

The ALT level is within the normal range in about 30% of HCV carriers and lower than 2-fold the normal upper limit in 40% (5, 6), although what constitutes a normal ALT value is not completely clear. The frequency of PNALT is reported to be higher in HCV genotype 2 carriers and females (6, 22). Inflammation and fibrosis were shown to be histologically mild in approximately 70% of PNALT cases (23); however, fibrosis of stage 2 (F2) or more severe fibrosis was noted in 22% of histologically evaluated PNALT cases in another report (22). Therefore, severe fibrosis can be present in some cases in which their ALT level is

normal. This may be because ALT levels occasionally normalize in patients with liver cirrhosis. The rate of progression of hepatic fibrosis in patients with chronic hepatitis C is reported to be 0.1-0.13 units/year (11, 24). PNALT cases have been found to show only a slight histological progression over a 10-year follow-up period (25), with a reported rate of progression of fibrosis of 0.05 units per year (26, 27). These results suggest that fibrosis progresses more slowly in PNALT cases. During the long-term follow-up of patients with PNALT, ALT levels rose in 21.5% of cases 3-18 months after PNALT was judged to be present (6). Platelet count is thought to be a simple marker for hepatic fibrosis, and PNALT patients with severe fibrosis can be identified using the criterion of a platelet count ≤ 150,000/μL. In patients selected using this criterion and an ALT level of ≤30 IU/L for 5 years, Okanoue *et al.* showed that ALT ≤ 30 IU/L was maintained in only 14% of patients (28). In our study, 101 HCV carriers with PNALT (defined as ALT ≤ 34 IU/l) were surveyed between 1993 and 2000, and ALT levels rose in 31.8% of these patients over a 5-year observation period (29). Thus, there is a risk of ALT elevation in HCV carriers with PNALT, even if the ALT level has been continuously normal over several years and if the definition of PNALT includes the platelet count, in addition to the ALT level. Furthermore, we found that an ALT level of 20-34 IU/l [odds ratio (OR): 5.6], a serum ferritin level of ≥ 90 ng/ml (OR: 3.1), and a minor allele of the HFE gene (H63D, OR: 4.8) were independent risk factors for ALT elevation (29). Shiffman *et al.* found that the ALT level was maintained at about 20 IU/L in PNALT cases (30). Therefore, when the ALT level is ≤ 19 IU/L, it is unlikely to rise, and this suggests that 19 IU/L may be an appropriate upper limit for ALT in the definition of PNALT.

2.5. ALT levels in HCV Carriers with End Stage Renal Failure or HIV Coinfection

The population of patients with fibrosis progression, despite having ALT levels within the normal range, typically includes dialysis patients with HCV infection and HCV carriers with HIV coinfection. Dialysis patients are a high-risk group for HCV infection, with an HCV prevalence of about 13.5% among these patients (31). Dialysis patients also generally have a low ALT level, and we also found low mean ALT levels of 13.2 and 18.5 IU/l in 238 HCV-negative and HCV-positive dialysis patients (32). A comparison of ALT levels with histologically severe inflammation and fibrosis showed that the ALT levels were lower in patients with end-stage renal failure who were under dialysis, compared to patients with normal renal function (33). However, the cause of the low ALT level in end-stage renal failure patients is unclear. Among HIV-infected patients, 25% are reported to be infected with HCV (34), and the incidence of fibrosis progression in PNALT cases is nearly 2 times higher in patients with HIV-HCV coinfection than in those with a HCV infection alone (35). Thus, although histological progression is generally mild in HCV carriers with PNALT, hepatic fibrosis is more progressive in those patients with end-stage renal failure or an HIV coinfection.

2.6. Immunopathogenesis of HCV Carriers With PNALT

The mechanism for ALT elevation in HCV carriers is not yet fully elucidated, but may be associated with functional abnormalities of the immune cells, such as activated lymphocytes and NK cells in patients with chronic hepatitis C (36, 37). Dendritic cells (DCs) play a central role in the activation of immunocytes, and the numbers of myeloid DCs (mDCs) and plasmacytaid DCs (pDCs) are smaller in patients with chronic hepatitis C than in subjects without a HCV infection. A comparison of PNALT cases and chronic hepatitis C patients showed no difference in the number of mDCs or pDCs, but a reduced DC function was associated with a higher ALT level (38). In contrast, many immunosuppressive regulatory T cells

Table 1. Diagnostic Accuracy of the Models for Predicting Liver Fibrosis Using Examination of Peripheral Blood and Serum Chemistry

Model Name (Reference)	Variables	Etiology	Fibrosis Stage	Cut off Values	AUROC [a]
APRI [a] (44)	AST [a], Plt [a]	HCV	Ishak score 0-2 Ishak score 3-6	< 0.5 ≥1.5	0.80-0.88
FIB-4 (45)	Age, AST, Plt, ALT [a]	HCV/HIV	Ishak score 0-3 Ishak score 4-6	<1.45 ≥3.25	0.765
Forns Index (46)	Plt, GGT [a], age, cholesterol	HCV	F0-1 F2-4	> 4.2 > 6.9	0.81-0.86
Fibro Index (47)	Plt, AST, γ globulin	HCV	F0-1 F2-3	≤1.25 ≥2.25	0.83
FibroTest (48)	α2-MC [a], α2 globulin (or haptoglobin), γ-globulin, apolipoprotein A1, GGT, Total bilirubin	HCV	F0-1 F2-4	0-0.1 0.6-1.0	0.836-0.870
SHASTA Index (49)	HA [a], AST, albumin	HCV/HIV	Ishak score 0-2 Ishak score 3-6	< 0.3 > 0.8	0.878
Hepascore (50)	HA, α2-MC, GGT, age, gender	HCV	F0-2 F3-4	< 0.5 ≥ 0.5	0.82-0.90

[a] Abbreviations: α2-MC, α2-macroglobulin; ALT, alanine aminotransferase; AUROC, area under receiver operating characteristic curve; AST, aspartate aminotransferase APRI, AST-platelet ratio index; GGT, gamma-glutamyltransferase;HA, hyaluronic acid; Plt, platelet

Table 2. Diagnostic Accuracy for Predicting Liver Fibrosis Using Transient Elastography or Acoustic Radiation Force Impulse

Technique	(Reference)	Etiology	Fibrosis Stage	Cut off Values	AUROC [a]
TE [a]	(51)	HCV [a]	F≥2	8.7	0.79
			F≥3	9.56	0.91
			F=4	14.52	0.99
	(52)	HCV	F≥2	7.1	0.88
			F≥3	9.5	0.95
			F=4	12.5	0.95
	(53)	CLD [a]	F=4	14.6	0.95
	(54)	HCV	F≥2	7.1	0.88
			F≥3	9.6	0.90
			F=4	11.6-16.9	0.90
	(55)	HCV	F≥1	5.2	0.902
			F≥2	8.1	0.941
			F≥3	9.6	0.926
			F=4	13.1	0.945
ARFI [a]	(56)	HCV	F≥2	1.215	0.902
			F≥3	1.54	0.993
			F=4	1.94	0.993
	(57)	CLD [a]	F≥2	1.34	0.94
			F≥3	1.44	0.94
			F=4	1.80	0.96
	(55)	HCV	F≥1	1.19	0.709
			F≥2	1.34	0.851
			F≥3	1.61	0.869
			F=4	2.00	0.911

[a] Abbreviations: ARFI, acoustic radiation force impulse; AUROC, area under receiver operating characteristic curve; CLD, chronic liver disease; HCV Hepatitis C virus; TE, transient elastography

(Treg) are present in HCV carriers and the inhibitory activity is stronger in PNALT cases than in patients with active hepatitis (39). These results indicate that therapy to regulate the functional abnormalities of immune cells may be valuable in patients with chronic hepatitis C in order to reduce the ALT level or hepatic inflammation. In addition, the frequency of DR13 in HCV-infected patients with a normal ALT level was significantly higher than that of HCV-infected patients with elevated ALT (42% *vs.* 4%, Pc < 0.003) (40). Thus, immunological analysis in HCV carriers with PNALT may lead to new therapies for patients with chronic hepatitis C.

2.7. Noninvasive Evaluation of Fibrosis in HCV Carriers With PNALT

In HCV carriers, the risk of HCC increases with the progression of hepatic fibrosis (41). Liver biopsy is the standard test for hepatic fibrosis, but it is invasive and causes complications at a rate of 1-3% and has a mortality rate of 1/10000-12000 (42). Moreover, sampling errors leading to the underestimation of liver cirrhosis have been reported in 14.5% of cases (43). Noninvasive tests for the evaluation of fibrosis have also been described, these include; combinations of peripheral blood and serum chemistry tests (44-48) and fibrosis markers (49, 50) (*Table 1*); elastography using transient elastography (TE FibroScan®) and acoustic radiation force impulse (ARFI) (51-57) (*Table 2*). Accuracy increases when several tests are used in combination, but the utility of these tests for the evaluation of hepatic fibrosis in PNALT cases remains uncertain.

2.8. PNALT and the Risk for HCC in HCV Carriers

Tanaka *et al.* investigated HCC development in 1,927 HCV antibody-positive blood donors and found that the incidence of HCC was lower in subjects with a low ALT level compared to those with a high ALT level (58). In our study, subjects were followed for an average of 8 years before HCC development, and a strong association between the ALT level and HCC development was found, with a significant association between a 20 IU/L higher ALT level and the subsequent incidence of HCC being observed [hazard ratio (HR) = 1.2] (7). The HCC risk was also much lower in the PNALT cases than in subjects with a persistent abnormal ALT level. Among 551 subjects with at least 4 repeated measurements of ALT, those with persistently abnormal ALT levels (n = 118) had a significantly increased rate of HCC compared to those with persistently normal ALT levels (n = 296) (HR = 23.2) (7). Kumada *et al.* also found that a high ALT level and low platelet count were strongly associated with HCC development (59). When a low ALT level persists for a prolonged period and the platelet count does not decrease, the HCC complication rate may be very low despite the persistence of a HCV infection. Collectively, these results show that the risk for HCC in subjects with PNALT is low among HCV carriers, including patients with chronic hepatitis and liver cirrhosis.

2.9. Insulin Resistance and Fatty Liver in HCV Carriers

In liver cirrhosis, insulin sensitivity decreases in peripheral tissue, for which pancreatic β cells compensate through the secretion of excess insulin, inducing hyperinsulinemia. Allison *et al.* found that the rate of diabetes complications was higher in liver cirrhosis associated with a HCV infection, than in that induced by other causes (60). The homeostasis model assessment-insulin resistance (HOMA-IR) value, which is an index of insulin resistance, was found to be significantly higher in stage 0 or 1 chronic hepatitis C patients with mild hepatic fibrosis than in healthy subjects, and HOMA-IR served as a predictor of the progression of hepatic fibrosis (61). Animal studies also suggest that the HCV core protein acts on the insulin signal transmission pathway and induces insulin resistance (62). However, it is unclear whether insulin resistance is present in PNALT cases without hepatic fibrosis. A high rate of fat deposition in the liver is caused by HCV infection (63), and fatty changes in the liver and insulin resistance are induced in transgenic mice expressing the HCV core protein (64). Castera *et al.* performed a liver biopsy at mean intervals of 48 months and observed that; male gender, histological stage, and the presence of advanced fatty changes were significant risk factors in promoting hepatic fibrosis, and that fatty change in the liver was an independent risk factor in multivariate analysis (65). Fatty change in the liver is particularly marked with genotype 3 viruses (66), and fatty change in the liver and insulin resistance have also been associated with the negative effects of interferon (IFN)-based therapy (67). Thus, insulin resistance and fat deposition in the liver, which are associated with hepatic fibrosis, should be less severe in PNALT cases compared to HCV patients with abnormal ALT, and this may produce a more favorable outcome for IFN-based treatment. However, the antiviral effect of IFN-based therapy in patients with normal ALT is comparable to that for patients with abnormal ALT, regardless of their background advantages (68, 69). Therefore, further studies of insulin resistance and fat deposition in the liver are needed in PNALT cases.

3. Results

3.1. Improved Outcomes With Antiviral Treatment for HCV Carriers

Treatment for HCV carriers has improved markedly in recent years. Combination therapy with pegylated (PEG) interferon (IFN) plus ribavirin achieves a sustained viral response (SVR) in approximately 50% of the most intractable genotype 1 patients and high viral load cases. The therapeutic effect is determined by; host, viral, and drug factors, and host factors include; age, gender, severity of hepatic fibrosis, fatty liver changes, and insulin resistance. In 2009, a study performed to identify single nucleotide polymorphisms (SNPs) that determine the effect of IFN-based therapy in chronic hepatitis C patients revealed the importance of a SNP near the IL28B gene (70). IL28B (also called IFNλ3) is thought to induce antiviral activity via the JAK-STAT pathway through phosphorylation of STAT1/STAT2 and the subsequent induction of the IFN-stimulating gene (ISG) (71). In addition to the genotype and viral load of HCV RNA, mutations of amino acids in the core (72) and the interferon sensitivity-determining region (ISDR) (73) have been reported as viral factors that determine the effect of IFN-based therapy. In multivariate analysis of host and viral factors, a high platelet count with mild hepatic fibrosis (F0-1), ≥ 2 ISDR mutations, and the IL28B SNP as the major allele were identified as factors associated with SVR (74). A lower γGTP level, and milder inflammation, fibrosis, and fatty changes were found in patients with the IL-28B SNP as the major allele, compared to those with this SNP as the minor allele (74). This suggests that the IL-28B SNP is involved in the progression of hepatitis C pathology, as well as in the therapeutic effect. These results indicate that SVR is likely in PNALT cases. However, there has been no report of an IL-28B SNP associated with PNALT. Drug factors that influence the therapeutic effect include; the type of IFN and the dose and duration of administration of IFN or ribavirin. Novel drugs, such as protease and polymerase inhibitors, will soon become available. In combination therapy with PEG-IFN, ribavirin and protease inhibitor, the overall SVR rate should increase to more than 60% in patients with genotype 1b and a high viral load. In addition, SVR was achieved in 84% of cases with IL-28B SNP as the major allele and glutamine or histidine at position 70 in the core protein, and in 50% of cases with IL-28B SNP as the minor allele and arginine at position 70 (75). These viral factors and host factors require further investigation in HCV carriers with PNALT.

3.2. Antiviral Treatment in HCV Carriers With PNALT

The treatment of chronic hepatitis C has improved significantly over the last few years. Detailed analysis of host factors including the IL28B SNP and viral factors may improve the accuracy of predicting IFN-based therapy effects. For example, this may be based on a prediction of the therapeutic effects using data mining of the blood test results (74, 76). Establishment of tailor-made therapies may be possible with further investigation of SNPs and other data for prediction of IFN-based treatments, including those for PNALT cases. Antiviral therapy for PNALT cases has been estimated to reduce the prevalence of liver cirrhosis and complications by 22% and 16%, respectively, and to decrease the mortality rate by 14% (77). The AASLD practice guidelines suggest that a decision to use PEG-IFN plus ribavirin therapy should be made based on; a histological evaluation, the possibility of adverse effects, prediction of therapeutic effects, and the presence of complications, regardless of the ALT level (9). In addition, Okanoue *et al.* reported that the combination of ALT and platelet counts is useful for evaluating the fibrosis stage in HCV carriers with normal serum ALT levels, and that most patients with platelet counts < 150000/

μL are candidates for antiviral therapy, especially those with ALT levels ≥ 31 U/L when the focus is placed on the inhibition of HCC development (27). Puoti *et al.* have also found that a rapid virological response was a predictor of sustained response in HCV carriers with PNALT (69). Thus, although further studies are required to determine whether antiviral therapy should be given in all PNALT cases, it seems likely that many HCV carriers with PNALT should be treated with IFN-based therapy. Tailor-made therapies may also be possible based on the accumulation of data for the prediction of IFN-based treatment efficacy in PNALT cases.

4. Conclusion

In this review, we have described the clinical characteristics of HCV carriers with PNALT. For HCV carriers, it is important to minimize complications such as liver cirrhosis and HCC through careful observation and treatment at the appropriate time. For PNALT cases, the course and prognosis should also be monitored and treatment should be considered at an early stage.

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