proteins with a molecular weight ranging from 14·9 to 26·7 kD. IL-32α is the most abundant transcript. IL-32 exhibits several properties typical of proinflammatory cytokines [16]. For example, it stimulates the secretion of proinflammatory cytokines and chemokines such as IL-1α, tumour necrosis factor (TNF)-α, IL-6, IL-8 and vascular endothelial growth factor (VEGF) through the activation of nuclear factor-κB (NF-κB) and p38 mitogen-activated protein kinases (MAPKs) [15,17,18]. In contrast, the production of IL-32 is induced or enhanced by the presence of proinflammatory cytokines, including IL-1β, IFN-γ and TNF-α via the activation of caspase 1 [17,19,20]. IL-32 has been implicated in inflammatory disorders such as rheumatoid arthritis, inflammatory bowel diseases, chronic obstructive pulmonary diseases, atopic dermatitis and allergic rhinitis [14,19–22].

Although human hepatocytes and hepatoma cells express IL-32 in hepatitis C virus (HCV)-associated chronic hepatitis and this expression is regulated by proinflammatory stimuli [23], the pathophysiological role of IL-32 in innate immune-related biliary diseases, including BA, remains unclear. We therefore investigated the IL-32 expression in the inflamed bile ducts of BA patients and the effect of innate immune stimulation by ligands of TLR-3 and cytokines on IL-32 expression in cultured human BECs. Our results provide evidence that biliary epithelial cells are sufficient sources of IL-32 for the biliary inflammation at sites of BA, and IL-32 may therefore play a role in the pathophysiology of BA.

Materials and methods

Patients and tissue preparations

A total of 21 patients with BA (surgical specimens; average age 1·7 months; age range 0·7–12 months; nine male/12 female) and age-matched control patients consisting of one neonatal hepatitis (giant cell hepatitis; wedge biopsy; 3 months; male) and six non-hepatobiliary diseases (congenital heart anomalies; autopsied specimens; average age 2·5 months; three male/three female) were examined. Resected common bile ducts and wedge liver biopsy specimens were obtained from patients with BA using the Kasai procedure. These specimens had been fixed in 10% neutral-buffered formalin and embedded in paraffin; 4 μm-thick sections were prepared for histological observation and immunohistochemistry.

Immunohistochemistry and immunocytochemistry

For immunocytochemistry using cultured BECs, formalin-fixed, paraffin-embedded sections of cell blocks were prepared according to the protocol reported by Mayall *et al.* [24]. The deparaffinized and rehydrated sections were heated in 10 mM citrate buffer for 20 min in a microwave oven. Following the blocking of endogenous peroxidase, these sections were incubated at 4°C overnight with antibody against the C-terminus of IL-32 [rabbit polyclonal immunoglobulin (Ig)G, 1 μg/ml; Lifespan, Seattle, WA, USA], TLR-3 (rabbit polyclonal IgG, 1 μg/ml; Santa Cruz, Santa Cruz, CA, USA) and caspase 1 (rabbit monoclonal IgG, diluted 1:1000; Abcam, Tokyo, Japan) and then at room temperature for 1 h with anti-rabbit immunoglobulins conjugated to a peroxidase-labelled dextran polymer (Simple Staining Kit; Nichirei, Tokyo, Japan). After a benzidine reaction, sections were counterstained lightly with haematoxylin. As a negative control, normal rabbit IgG was used as the primary antibody; no staining was obtained.

For semiquantitative evaluation of the immunohistochemistry, intrahepatic bile ducts and extrahepatic common bile ducts were chosen in each section for assessment and IL-32 immunoreactivity in these bile ducts was graded semiquantitatively as follows: score 0, absence of expression; score 1, low constitutive expression; score 2, intermediate expression; and score 3, high expression.

In addition, simultaneous detection of IL-32 and cytokeratin (CK)19 was performed using double immunohistochemical staining. After IL-32 immunostaining, CK19 antibody (mouse monoclonal IgG1kappa, 0·45 μg/ml; Dako Japan, Tokyo, Japan) was applied overnight at 4°C, followed by immunoglobulins conjugated with alkaline phosphatase labelled-dextran polymer (Nichirei). Colour development of IL-32 and CK19 was achieved with diaminobenzidine (brown) and Vector blue (Vector Laboratories, Burlingame, CA, USA), respectively.

Cultured human BECs and stimulation with PAMPs and proinflammatory cytokines

A cultured cell line of human intrahepatic BECs was established from the explant liver of a 24-year-old male with BA who had already received the Kasai procedure during the newborn period, and cultured as reported previously [25]. The cultured BECs were incubated with a culture medium composed of Dulbecco's modified Eagle's medium (DMEM)/F-12 (Invitrogen, Tokyo, Japan), 5% newborn calf serum (Invitrogen), 0·18 mM adenine (Sigma, St Louis, MO, USA), hydrocortisone (0·4 μg/ml), cholera toxin (10 ng/ml), tri-iodo-thyronine (1·3 μg/l), ITS+ (Becton Dickinson, Franklin Lakes, NJ, USA), 25 mM sodium bicarbonate (Sigma), 1% antibiotics anti-mycotic, human epidermal growth factor (20 ng/ml) (Invitrogen) and human hepatocyte growth factor (10 ng/ml) (Invitrogen). The cells were grown as monolayers in a humidified incubator with 5% CO_2 at 37°C. More than 95% of the cells were confirmed to be biliary epithelial cells by the expression of a biliary-type cytokeratin (CK19). The cultured BECs were used between passages 4 and 9. Informed consent for human research was obtained from the patient prior to surgery. This study was approved by the Kanazawa Univer-

sity Ethics Committee. Moreover, as control cultured cells, a commercially available cell line derived from human hepatocellular carcinoma, HepG2, was obtained from the Health Science Research Resources Bank (Osaka, Japan).

These cultured cells were stimulated with a TLR-3 ligand, polyinosinic–polycytidylic acid [poly(I:C), a synthetic analogue of viral dsRNA, 25 μg/ml; Invivogen, San Diego, CA, USA] and recombinant cytokines [IL-1β, IFN-γ, TNF-α, transforming growth factor (TGF)-β1 and IL-10, 1000 U/ml; PeproTech, London and IL-32, 1000 U/ml; R&D Systems, Minneapolis, MN, USA] for 3 h (molecular analysis) and 48 h (protein analysis by immunocytochemistry and Western blotting analysis).

Isolation of RNA, reverse transcription–polymerase chain reaction (RT–PCR) and real-time PCR

For evaluation of mRNA of IL-32, caspase 1, TLR-3, IL-1β and IL-6 in cultured BECs, isolation of RNA from BECs and reverse transcription were performed using the RNeasy Total RNA System (Qiagen, Hilden, Germany) and ReverTra Ace (Toyobo, Osaka, Japan). First, to examine the presence of target molecules and the validity of the newly designed primers, conventional PCR was performed. Specific primers for IL-32, caspase 1, TLR-3 and glyceraldehyde 3 phosphate dehydrogenase (GAPDH, positive control) were designed: IL-32 forward: 5′-AGCTGGAGGACGAC TTCAAA-3′, reverse: 5′-TTGAGGATTGGGGTTCAGAG-3′ [predicted size, 258 base pairs (bp)]; TLR-3 forward: 5′-CCATTCCAGCCTCTTCGTAA-3′, reverse: 5′-GGATGT TGGTATGGGTCTCG-3′ (predicted size, 505 bp); caspase 1 forward: 5′-CCACAATGGGCTCTGTTTTT-3′, reverse: 5′-CATCTGGCTGCTCAAATGAA-3′ (predicted size, 117); IL-1β forward: 5′-CCAGGGACAGGATATGGAGCA-3′, reverse: 5′-TTCAACACGCAGGACAGGTACAG-3′ (predicted size, 129 bp); IL-6 forward: 5′-AGTGAGGAACAA GCCAGAGC-3′, reverse: 5′-AAGCTGCGCAGAATGAGAT-3′ (predicted size, 189 bp); and GAPDH forward: 5′-GG CCTCCAAGGAGTAAGACC-3′, reverse: 5′-AGGGGTCTA CATGGCAACTG-3′ (predicted size, 147 bp). The reaction profile consisted of initial denaturation at 94°C for 3 min followed by 25–40 cycles with 30 s of denaturation at 94°C, 30 s of annealing of primers at 55°C and a 60-s extension at 72°C. Next, to carry out relative quantification, real-time quantitative PCR was performed according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan). Relative gene expression was calculated using the comparative cycle threshold method and adjusted based on expression of the housekeeping gene (GAPDH). Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase and DNase free water.

Western blotting

Cell lysates of poly(I:C)-stimulated or unstimulated cultured cell lines (10 μg protein/lane) and the culture medium were subjected to sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Recombinant IL-32 protein (0·1 μg; R&D) was used as a positive control. Separated proteins were transferred onto a nitrocellulose membrane; the membrane was blocked in 5% bovine serum albumin, then probed for 1 h with a primary antibody against human IL-32 (0·1 μg/ml). After washing, the membrane was incubated for 1 h with the Simple Staining Kit, and visualized with the benzidine reaction. The band density was evaluated quantitatively using NIH images.

Statistical analysis

Data were analysed using the paired *t*-test or Welch's *t*-test; $P < 0{\cdot}05$ was considered statistically significant.

Results

Expression of IL-32, caspase 1 and TLR-3 in extrahepatic bile ducts of BA

Immunohistochemistry revealed the expression of IL-32 in BECs, infiltrating inflammatory cells and endothelial cells at various intensities. In particular, damaged common bile ducts showing cholangitis in BA expressed IL-32 strongly, accompanying many IL-32-positive inflammatory cells and vessels (Fig. 1a–c). As shown in Fig. 1f, double immunohistochemistry highlighted that CK19-postive bile ducts clearly expressed IL-32. However, non-damaged biliary epithelium found at the margin of resected common bile ducts did not express IL-32 (Fig. 1g,h). In wedge liver biopsies, hepatocytes were also positive for IL-32 in addition to small bile ducts (interlobular bile ducts), but the intensity was lower than that in damaged common bile ducts (Fig. 1i,j). Moreover, congestive bile in intrahepatic bile ducts was also strongly positive for IL-32 (Fig. 1j). In contrast, BECs in common bile ducts and intrahepatic bile ducts of age-matched controls expressed only weakly or lacked IL-32 (Fig. 2a,d). The semiquantitative analysis for immunoreaction confirmed that the expression of IL-32 in damaged common bile ducts of BA was up-regulated significantly, compared with those in non-damaged/normal bile ducts of BA and age-matched controls (Fig. 3). Caspase 1 and TLR-3 were expressed constantly in BECs of extrahepatic bile ducts in both the BA and control patients (Fig. 2b,c).

Induction of IL-32 expression by PAMPs and cytokines in cultured BECs

To examine the presence of target molecules and the validity of the newly designed primers, RT–PCR at 40 cycles was

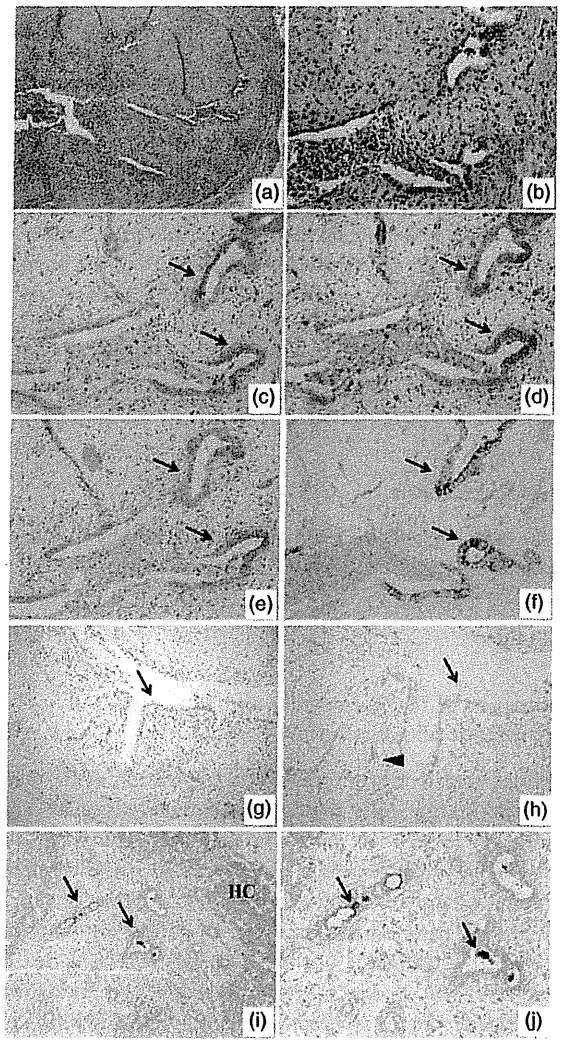

Fig. 1. Histology and immunohistochemistry for interleukin (IL)-32, Toll-like receptor (TLR)-3 and caspase 1 in biliary atresia (BA). (a,b) Transverse sections of biliary remnants. Damaged extrahepatic bile ducts line inconsistently by desquamated columnar epithelium and surrounding fibroplasia with an inflammatory cell infiltrate; (b) a higher magnification of (a). Haematoxylin and eosin (H&E) staining. Original magnification (a) ×100 and (b) ×400. Immunohistochemistry for IL-32 (c), TLR-3 (d) and caspase 1 (e). The strong expression of IL-32, TLR-3 and caspase 1 was found in biliary epithelial cells (arrows) of damage bile ducts. Original magnification ×400. (f) Double immunohistochemistry for CK19 and IL-32 highlighted the CK19-positive bile ducts (blue) clearly expressed IL-32 (brown) (arrows). Original magnification ×400. (g,h) Immunohistochemistry for IL-32. Undamaged extrahepatic bile duct located at the resected margin in BA. IL-32-positive neovascular structures (arrowhead) were found, but undamaged biliary epithelium lacked IL-32 expression (arrows); (h) is higher magnification of (g). Original magnification (g) ×200 and (h) ×400. (i,j) Immunohistochemistry for IL-32 using wedge liver specimens of BA. Interlobular bile ducts (arrows in i) and hepatocytes (HC in i) expressed IL-32. Moreover, condensed bile in dilated bile ducts was also strongly positive for IL-32 (arrows in j); (j) is a higher magnification of (i). Original magnification (e) ×200 and (f) ×400.

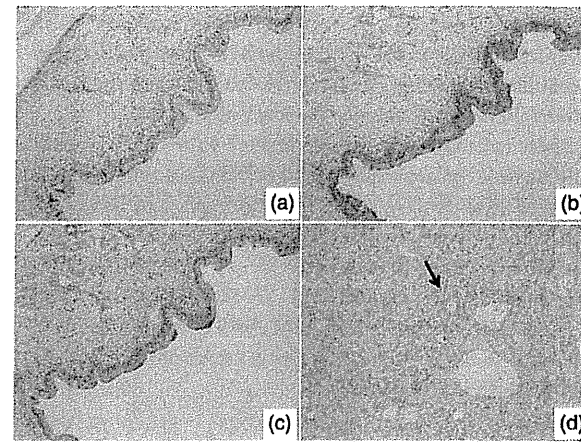

Fig. 2. Immunohistochemistry for interleukin (IL)-32 (a,d), Toll-like receptor (TLR)-3 (b) and caspase 1 (c) in age-matched controls. (a–c) Biliary epithelial cells in common bile ducts of non-hepatobiliary diseases (congenital heart anomalies) expressed TLR-3 (b) and caspase 1 (c), but lacking or faintly expressed IL-32 (a) was faint or negative. Original magnification ×200. (d) Interlobular bile duct in neonatal hepatitis was negative for IL-32 (arrow). Original magnification ×400.

performed and an amplification of all molecules could be detected as a single band from cultured BECs at the expected size. Moreover, the BECs constantly expressed the mRNA of TLR-3 and caspase 1, which is necessary for the recognition of poly(I:C) and the production of functional IL-32 protein, respectively. The real-time PCR analysis revealed that TLR-3 ligand, poly(I:C) and proinflammatory cytokines (IL-1β, IFN-γ and TNF-α), but not regulatory cytokines (TGF-β1 and IL-10), enhanced the mRNA expression of IL-32, the increases being statistically

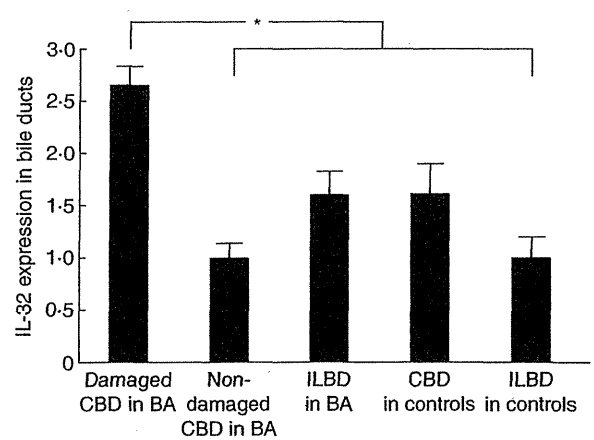

Fig. 3. Semiquantitative analysis of immunohistochemistry for interleukin (IL)-32. The expression of IL-32 in damaged common bile ducts (CBD) of biliary atresia (BA) was up-regulated significantly compared with those of non-damaged CBD and interlobular bile ducts (ILBD) in BA, and of CBD and ILBD in age-matched controls. *P<0·05.

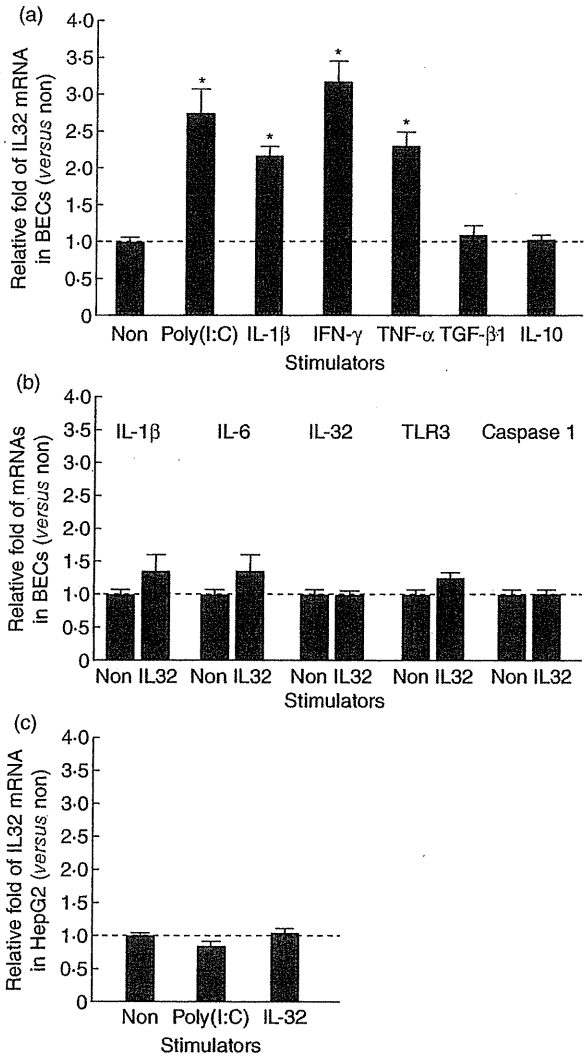

Fig. 4. (a) Induction of interleukin (IL)-32 expression by Toll-like receptor (TLR)-3 ligand (poly I:C) and cytokines in cultured biliary epithelial cells (BECs). Quantitative analysis using real-time polymerase chain reaction (PCR) revealed that a TLR-3 ligand, poly(I:C) and proinflammatory cytokines [interleukin (IL)-1β, interferon (IFN)-γ and tumour necrosis factor (TNF)-α], but not regulatory cytokines [transforming growth factor (TGF)-β1 and IL-10], up-regulated significantly the mRNA expression of IL-32. (b) Detection of BEC-producing cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs. The stimulation with IL-32 did not up-regulate the expression of any cytokines, TLR-3 or caspase 1 significantly. (c) Detection of IL-32 in a control cell line, HepG2. Induction of IL-32 expression was not found by stimulation with poly(I:C) or IL-32. Results were obtained from three independent experiments and shown as relative mRNA expression compared with the level without any treatments (Non). Bars indicate the mean ± standard error of the mean. $^*P < 0{\cdot}05$.

significant (Fig. 4a). In contrast, stimulation with IL-32 did not up-regulate significantly the expression of BEC-producing cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs (Fig. 4b). Although the control cell line, HepG2, also expressed IL-32 mRNA, up-regulation of IL-32 was not significant by stimulation with poly(I:C) or IL-32 (Fig. 4c).

Detection of intracytoplasmic and secreted IL-32protein

To investigate secretion of the IL-32 protein, Western blotting was performed using the cell lysate and culture medium of BECs. IL-32 was detected in the medium as well as lysate from the poly(I:C)-stimulated BECs (Fig. 5a). Semiquantitative analysis using NIH image analysis revealed that the band density was up-regulated in cell lysate and culture medium by stimulation with poly(I:C) (Fig. 5a). Moreover, immunocytochemistry also demonstrated that IL-32 protein was expressed strongly in poly(I:C)-stimulated BECs, compared with non-stimulated BECs (Fig. 5b).

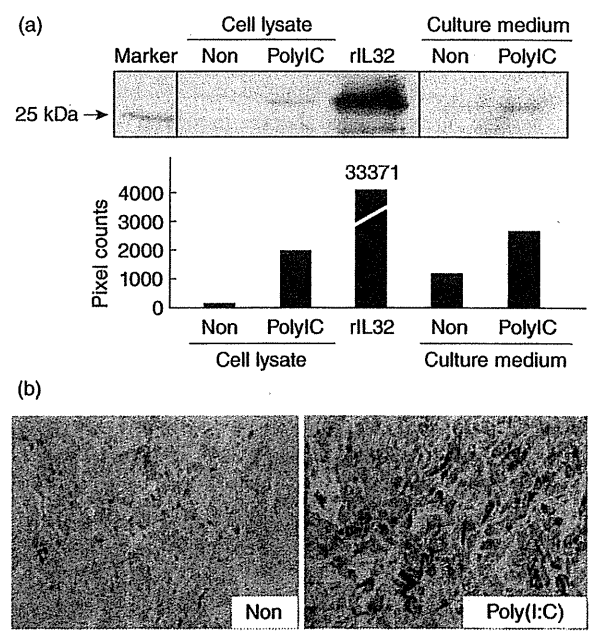

Fig. 5. Detection of intracytoplasmic and secreted interleukin (IL)-32 protein in cultured biliary epithelial cells (BECs). (a) Western blotting revealed that the culture medium as well as cell lysate of poly(I:C)-treated cultured cells contained IL-32 protein, but the level was faint in untreated cells (Non). As a positive control, recombinant IL-32 (rIL-32, 0·1 μg) was used. Semiquantitative analysis using NIH image analysis confirmed that the density of bands was up-regulated in cell lysate and culture medium by stimulation with poly(I:C). (b) Immunocytochemistry also demonstrated that IL-32 was expressed strongly in the poly(I:C)-stimulated BECs compared with unstimulated BECs (Non). Original magnification ×400.

Discussion

BA is characterized initially by periductal inflammation and fibrosis and the obstruction of common bile ducts, known as fibrosclerosing cholangitis. Recruitment of inflammatory cells results in the release of other proinflammatory cytokines and chemokines, sustaining the cholangitis associated with the biliary innate immune response and promoting chronic cholangitis associated with the subsequent acquired immune response in a later phase [26]. IL-32 is a recently described cytokine that is a strong inducer of proinflammatory cytokines whose expression is increased markedly in several inflammatory disorders, including rheumatoid arthritis (RA) and inflammatory bowel disease (IBD), and correlated with the severity of these diseases [14,19]. In the present study, human BECs were demonstrated to be the local source of IL-32. Immunohistochemical analysis showed a cytoplasmic distribution of IL-32 in BECs of the damaged common bile ducts in BA cases, although BECs of common bile ducts in age-matched controls were negative or only weakly positive for IL-32, suggesting that IL-32 is associated closely with the histogenesis of periductal inflammation in BA. However, IL-32 production in BECs is not specific to BA alone. In fact, we confirmed the expression of IL-32 in bile ducts of adult biliary diseases such as primary biliary cirrhosis, but its intensity was lower than those in the damaged common bile ducts of BA. Therefore, we speculated that the induction of IL-32 by unique factors such as viral infections in BA was stronger than those in other biliary diseases. Inflammasomes are multi-protein cytoplasmic complexes that mediate the activation of inflammatory caspase-1. For example, caspase-1 cleaves pro-IL-1β to the active form of IL-1β. In this manner, caspase-1 controls the maturation of some of the proinflammatory cytokines, and IL-32 also depends upon caspase 1 activation [17,20]. Therefore, the presence of caspase 1 is necessary for the functional expression of IL-32 in BECs. In the present study, BECs constantly expressed caspase 1 *in vitro* and *in vivo*, suggesting the expression of functional IL-32 in BECs.

Recent studies have focused upon the role of innate immunity associated with Reoviridae (reovirus and rotavirus) in the pathogenesis of BA. Having a dsRNA genome, Reoviridae in particular are characterized by epithelial tropism [1,3,4,9,10,27,28]. The initial sensing of innate immunity is mediated by the recognition of PAMPs through TLRs. IL-32 also appears to play an important role in the host defence against invading micro-organisms [23,29,30]; that is, IL-32 is described as a proinflammatory cytokine that enhances host immunity against various microbial pathogens. The present study revealed that stimulation with poly(I:C), a mimic of Reoviridae, enhanced IL-32 expression in cultured BECs, suggesting that the biliary innate immune response directly induces the production of IL-32 in BECs. A control cell line used in this study, HepG2, also expressed IL-32 mRNA, but the up-regulation of IL-32 was not significant by stimulation with poly(I:C). It has already been reported that IL-32 expression is induced in peripheral blood mononuclear cells and monocytes by *Mycobacterium tuberculosis* [31] but, to our knowledge, this is the first description concerning the production of IL-32 in epithelial cells such as BECs via an innate immune response.

IL-1β, IFN-γ and TNF-α were reported to be inducers of IL-32 expression [16,19]. However, the regulatory mechanism of these proinflammatory cytokines remains unclear. In this study, we found that all these proinflammatory cytokines are potent stimulators of IL-32 expression in cultured BECs. In contrast, the aforementioned results suggest that the secretion of IL-32 could stimulate periductal inflammatory and/or immune cells to secrete proinflammatory cytokines and contributes to the deterioration of periductal inflammation. Because these inflammatory cytokines and an innate immunity play important roles in the immune-mediated histogenesis of BA, the inflammatory responses and innate immune response in the affected bile ducts of BA patients may be amplified by constant IL-32-induced secretion of proinflammatory cytokines from BECs and periductal inflammatory cells, suggesting that IL-32 plays a central role in the inflammatory responses involved in BA pathogenesis. However, IL-32 itself could not up-regulate the expression of inflammatory cytokines (IL-1β, IL-6 and IL-32), TLR-3 and caspase 1 in cultured BECs, suggesting that IL-32 produced by BECs was unlikely to be involved in direct reciprocal signalling resulting in up-regulation of inflammatory cytokines and of susceptibility to virus in BECs.

In this study, we demonstrate that stimulation with poly(I:C) induced the transcription of IL-32 mRNA in BECs and also confirmed the presence of the protein in the culture medium as well as cell lysate. Moreover, immunohistochemistry also revealed that a condensed bile in intrahepatic small bile ducts was positive for IL-32. These findings suggest the secretion of IL-32 from IL-32-expressing BECs. Therefore, IL-32 is speculated to be secreted extracellularly in periductal tissue fluids and into bile in BA. As mentioned above, the secreted IL-32 induces the production of proinflammatory cytokines in inflammatory and/or immune cells, resulting in marked amplification of the inflammatory cytokine milieu, and these responses may contribute to the aggravation of BA. Moreover, it was suggested recently that IL-32 acts as a cytoplasmic protein: IL-32 was expressed at high levels in human epidermal keratinocytes after stimulation with IFN-γ and TNF-α, but was not secreted by keratinocytes [21]. Moreover, it was also shown that the up-regulation of cytoplasmic IL-32 expression induces apoptosis [21,32]. In IBD, the apoptosis of damaged colonic cells by accumulated intracellular IL-32 can be considered a host defence mechanism against invading microorganisms, by which damaged epithelial cells are

eliminated efficiently along with invading microorganisms and further invasions of microorganisms can be blocked [19,33]. In BA, our previous study found that biliary apoptosis was enhanced in the damaged common bile ducts and associated closely with bile duct loss in BA, which was caused by the production of an apoptosis-inducer, TRAIL, in BECs via the biliary innate immune response to a TLR-3 ligand, poly(I:C) [10]. However, this TRAIL-mediated biliary apoptosis is only partially involved in the poly(I:C)-induced mechanism, and other possible mechanisms could also exist [10]. Therefore, the IL-32-mediated mechanism is also likely in poly(I:C)-induced biliary apoptosis, and might be associated with enhanced biliary apoptosis in the damaged common bile ducts of BA.

In conclusion, we have demonstrated that IL-32 expression is enhanced in the damaged common bile ducts of BA patients. Expression of IL-32 in BECs was induced by the innate immune response to dsRNA [poly(I:C)] and proinflammatory cytokines (IL-1β, IFN-γ and TNF-α). This study has identified IL-32 as an important inflammatory cytokine involved in the cholangitis of BA. So far, anti-IL-32 treatment has been studied in only a few diseases, such as rheumatoid arthritis [34,35]. The regulation of IL-32 expression may form the basis of a new strategy for the treatment of BA.

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Disclosure

The authors declare no conflicts of interest.

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Incidence of and Risk Factors for Hepatocellular Carcinoma in Primary Biliary Cirrhosis: National Data from Japan

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Primary biliary cirrhosis (PBC) primarily affects females and is rarely complicated by hepatocellular carcinoma (HCC). Although the HCC incidence in PBC patients is low, several characteristics and risk factors associated with its development have been reported. In this study, national data concerning the current status of carcinogenesis in PBC patients in Japan are reviewed. Using data from two national questionnaire surveys, we investigated the clinicopathological findings associated with HCC in PBC patients. According to the data of all reviewed PBC patients, the HCC incidence was 2.4% (71/2946). The HCC incidence by gender was 5.1% (19/370) in males and 2.0% (52/2576) in females, and the proportion of males was 26.7%. Prognosis was significantly poorer in the PBC patients with HCC than in those without. Multivariate analysis of risk factors associated with HCC by gender revealed histological stage at the time of PBC diagnosis as an independent risk factor associated with the development of HCC in females, but not in males. Furthermore, data from another national survey of 178 PBC patients with HCC (male/female = 49/129; proportion of males 27.5%) revealed that the duration between the diagnosis of PBC and that of HCC was significantly shorter in males than in females. In addition, histological stage at the time of HCC diagnosis was an independent risk factor for HCC in females, whereas no risk factors were identified in males. *Conclusion:* these data indicate that males are at risk of developing HCC at any histological stage of PBC. Therefore, male PBC patients in particular should be carefully screened for HCC from the early stages of PBC. (HEPATOLOGY 2013;57:1942-1949)

Primary biliary cirrhosis (PBC) primarily affects middle-aged females. Histologically, the interlobular bile ducts are primarily damaged and show characteristic findings such as chronic nonsuppurative destructive cholangitis (CNSDC) followed by progressive bile duct loss.[1,2] A terminal feature of PBC is irreversible biliary cirrhosis, and liver transplantation is the sole treatment for hepatic failure.[3] Although hepatic failure defines the prognosis in most PBC patients, hepatocellular carcinoma (HCC) is also reported to occur in 0.76%-5.9% of PBC patients.[4-9] Recently, however, the incidence of PBC complicated by HCC has been gradually increasing with improvements in PBC treatment and survival.

In general, HCC is typically encountered in the terminal stage, when irreversible biliary cirrhosis sets in. Moreover, the hepatitis virus is a major risk factor for HCC development in patients with chronic hepatitis B virus (HBV) or hepatitis C virus (HCV) infections. In PBC patients, however, no carcinogenic factors directly associated with HCC have been identified. The proposed risk factors for HCC arising from PBC-affected livers include the hepatitis virus, cirrhosis, older age, diabetic mellitus, and male gender.[4,5,10-13] However, epidemiologic studies are limited and provide conflicting results, perhaps because of the low prevalence of the disease and geographical and environmental differences.

Abbreviations: AMA, antimitochondrial; CNSDC, chronic nonsuppurative destructive cholangitis; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; PBC, primary biliary cirrhosis; PDH, pyruvate dehydrogenase.

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In the present study we evaluated data from two nationwide surveys performed in Japan. Our aim was to clarify the current status of carcinogenesis in PBC patients, identify the associated clinicopathological risk factors, and understand how the pathogenesis of PBC is directly associated with HCC.

Materials and Methods

Setting and Patient Selection

Survey of PBC in Japan (National Survey by the Intractable Hepato-Biliary Diseases Study Group). National surveys of PBC patients in Japan have been performed 14 times biennially or triennially by the Intractable Hepato-Biliary Diseases Study Group for Research on Measures for Intractable Disease, which is supported by Health Labor Sciences Research Grants in Japan. The subjects included 7,376 patients registered in the 1st-14th surveys performed between 1980 and 2009.[9,14] Of the 7,376 patients, the absence or presence of HCC was confirmed during follow-up in 2,946 (70 males, 2,576 females), who were then investigated in the current study. HBV carriers and HB antigen- and anti-HCV antibody-positive patients were excluded.

Survey of PBC Patients with HCC in Japan (National Survey by the Liver Cancer Study Group of Japan). This project was set up at the 47th Annual Meeting of the Liver Cancer Study Group of Japan (President, Professor Ichida), and it was executed in 2011. Questionnaires were sent to 340 hospitals or institutions included in the Liver Cancer Study Group of Japan. Eighty-six of the 340 hospitals responded, and data from 178 PBC patients with HCC from 39 hospitals or institutions were eventually included. HBV carriers and HB antigen- and anti-HCV antibody-positive patients were excluded. The cooperating institutions are listed in the Appendix.

PBC Diagnosis

PBC was diagnosed according to criteria established by the Intractable Hepato-Biliary Diseases Study Group of Japan. Patients whose condition met one of the following criteria were diagnosed as having PBC: (1) histologically confirmed CNSDC with laboratory findings positive for PBC; (2) positivity for antimitochondrial (AMA) and/or anti-pyruvate dehydrogenase (PDH) antibodies, absence of histological findings of CNSDC, and presence of histological findings compatible with PBC; and (3) no histological examination, but positivity for AMA and/or anti-PDH antibodies and clinical findings and course indicative of PBC. PBC symptoms were defined as pruritus, overt jaundice, esophageal varices, ascites, and hepatic encephalopathy.[15] Histological findings were classified according to Scheuer's system.[16]

Statistical Analysis

The Mann-Whitney U and chi-square tests test were used as nonparametric and independence tests, respectively. Logistic regression analysis was used for the multivariate analysis of prognostic factors. Survival rate was obtained by the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Results

HCC Incidence in the Japanese PBC Population. The current status of and risk factors for HCC in PBC patients in Japan were analyzed on the basis of data from the national survey conducted by the Intractable Hepato-Biliary Diseases Study Group. The total number of PBC patients was 2,946. Of these, 2,100 cases available for analysis of histological stage of PBC at diagnosis underwent liver biopsy. HCC incidence during follow-up was 2.4% (71/2,946). This incidence was 5.1% (19/370) in males and 2.0% (52/2,576) in females, and the proportion of males was 26.7%. The mean ± standard deviation and median values for the observation period were 80.1 ± 70.8 (range, 1-443) and 58 months, respectively. The mean value for males was 65.1 ± 57.2 (range, 1-237; median, 45) months, while that for females was 82.2 ± 72.2 (range, 1-443; median, 60) months.

A comparative analysis of PBC patients with and without HCC revealed male gender, old age, low serum albumin levels, low serum total cholesterol levels, advanced histological stage, and symptomatic status at the time of PBC diagnosis as significant risk factors for HCC (Table 1). There was no difference in total bilirubin levels and the presence or absence of

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Table 1. Clinical and biological characteristics of PBC patients with or without HCC at PBC diagnosis

	HCC(+)	HCC(−)	P
Number	71	2875	
Sex (M:F)	19:52	351:2524	0.0003
Age (Mean ± SD)	60.5 ± 10.4	56.4 ± 11.2	0.0023
T-Bilirubin (Mean ± SD)	1.37 ± 1.63	0.99 ± 1.52	0.1061
Albumin (Mean ± SD)	3.81 ± 0.58	4.05 ± 0.51	0.0002
T-cholesterol (Mean ± SD)	201.3 ± 60.5	217.4 ± 86.7	0.0397
Histological stage (I/II/III/IV)	10/17/14/8	1060/662/263/66	<0.0001
UseofUDCA(%)	89.7	91.8	0.5291
Clinical stage (asvmptomatic: symptomatic)	38:33	2775/100	<0.0001

ursodeoxycholic acid (UDCA) treatment between the two groups (Table 1). Prognosis was significantly poorer in the PBC patients with HCC than in those without (Fig. 1). The cumulative incidence of carcinogenesis was 6.5% in males and 2.0% in females during the 10 years after PBC diagnosis; the difference between males and females was statistically significant ($P < 0.0001$) (Fig. 2). In particular, analyses of HCC incidence in patients aged 10-80 years revealed that male PBC patients in their 40s and 50s had an increased risk of HCC compared with female PBC patients in the same age groups (data not shown). In multivariate analysis for risk factors of HCC, gender and histological stage were selected as significant factors ($P < 0.00001$) (Table 2). There was no difference in the proportion of males and females who underwent histological staging at PBC diagnosis. The incidence of histological stages 3 and 4 was ~16.0% in both male and female PBC patients without HCC (Fig. 3), whereas it was 14.2% and 57.1% in male and female PBC patients with HCC, respectively.

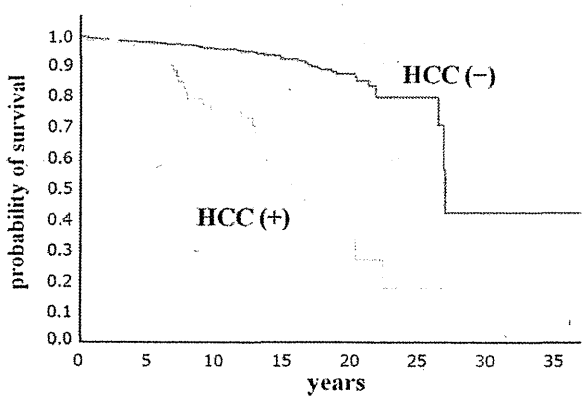

Fig. 1. Kaplan-Meier curve for survival in patients with PBC with (+) or without (−) HCC. There is a statistically significant difference between the curves ($P < 0.05$).

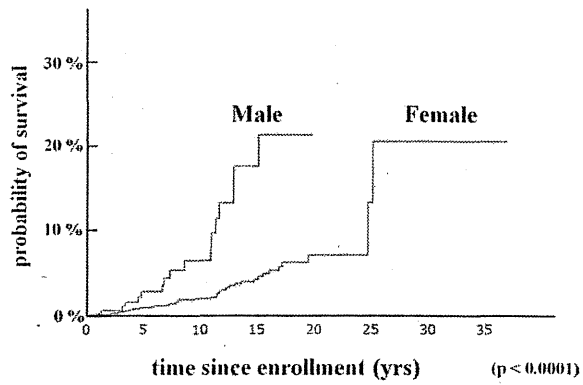

Fig. 2. Cumulative appearance rates of HCC in patients with PBC by gender. There is a statistically significant difference between males and females.

Advanced histological stage was a risk factor for HCC in females ($P < 0.0001$; Fig. 3; Table 2). Multivariate analysis for risk factors of HCC by gender revealed that histological stage at the time of PBC diagnosis was an independent risk factor for HCC in females (Supplemental Table 1), whereas no significant independent factors were revealed for males (Supplemental Table 2). Moreover, although we assessed PBC patients with HCC according to histological stage, we found no difference in any clinical or biological characteristics between patients with and without cirrhosis at PBC diagnosis (Supplemental Table 3).

PBC Patients with HCC in Japan. From the data of the national survey specially set up at the 47th Annual Meeting of the Liver Cancer Study Group of Japan, we collected and investigated those for 178 PBC patients with HCC from a total of 39 hospitals included in the study group. These cases included 100 fatalities in the past years as well as 78 patients followed up from each hospital or institute as of June, 2011. Among the followed-up patients, four

Table 2. Factors associated with increased risk of HCC in PBC patients (multivariate analysis)

	regression coefficient	standard deviation	χ^2	odds ratio	P value
Sex (M:F)	−0.5646	0.1737	10.56	3.0932	0.0012
Age	−0.0242	0.0149	2.63	0.9760	0.1050
T-Bilirabin	0.0302	0.0880	0.12	1.0307	0.7313
Albumin	0.0274	0.3087	0.01	1.0277	0.9292
T-cholesterol	0.0021	0.0026	0.65	1.0021	0.4210
Histological stage (I/II/III/IV)	−0.7294	0.1661	19.27	0.4821	<0.0001
UseofUDCA(%)	−0.2823	0.2473	1.3	1.7590	0.2537
Clinical stage (asymptomatic: symptomatic)	0.2990	0.1674	3.19	0.5498	0.0741

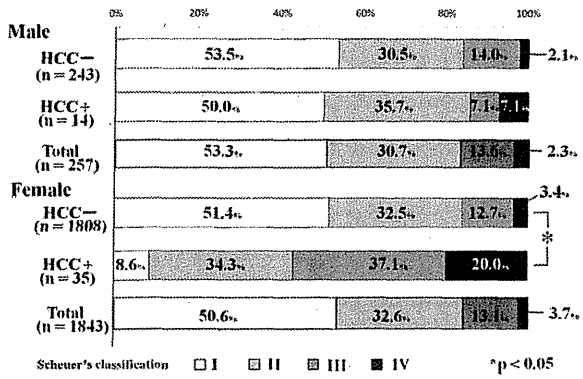

Fig. 3. Histological stage at the diagnosis of PBC in patients with or without HCC by gender. The proportion of patients with histological stages 3 and 4 at the time of PBC diagnosis is ~16.0% for both male and female PBC patients without HCC. However, the proportion of patients with histological stages 3 and 4 is 14.2% in male and 57.1% in female PBC patients with HCC. Moreover, there is a significant difference in the proportion of female PBC patients with HCC and that without. The parentheses identify the number of patients examined.

underwent liver transplantation, which was performed at the time of HCC discovery in three and 3 years after HCC discovery in one. There were 49 male and 129 female PBC patients with HCC, and the proportion of males was 27.5%, which was similar to that from the previously described national survey of PBC. Although the average age at the time of PBC diagnosis was slightly higher for males (68 years) than for females (62 years), that at the time of HCC diagnosis was similar between males (73 years) and females (72 years; Fig. 4). Moreover, the duration between the diagnosis of PBC and that of HCC was shorter in males than in females. HCC was diagnosed simultaneously with or prior to the diagnosis of PBC in 32.7% (16/49) males and 14.7% (19/129) females (Fig. 4).

Table 3. Clinical and biological characteristics of male and female PBC patients at HCC diagnosis

	Male (n=49)	Female (n=129)	Total (n=178)
Blood transfusion	9%	8%	9%
past HBV infection*	33%	18%	22%
Alcohol intake*	27%	2%	9%
Diabetes mellitus	24%	23%	24%
AMA levels	86%	82%	83%
ANA levels	41%	49%	47%
BMI (>25%)	25%	31%	29%
Triglyceride (>150)	8%	9%	9%
Total cholesterol (>220)	15%	9%	11%
associated with NAFLD	0%	4%	3%
Use of UDCA	84%	84%	84%

(*p<0.05)

Pathological examination for HCC and background liver tissue assessment by biopsy or hepatectomy was conducted for 66 and 82 patients, respectively. Clinicopathological data at the time of HCC diagnosis are shown in Table 3. There were more males with prior HBV infection and a history of alcohol consumption compared with females. There were no differences in the history of blood transfusion, diabetes mellitus, AMA levels, anti-nuclear antibody levels, body mass

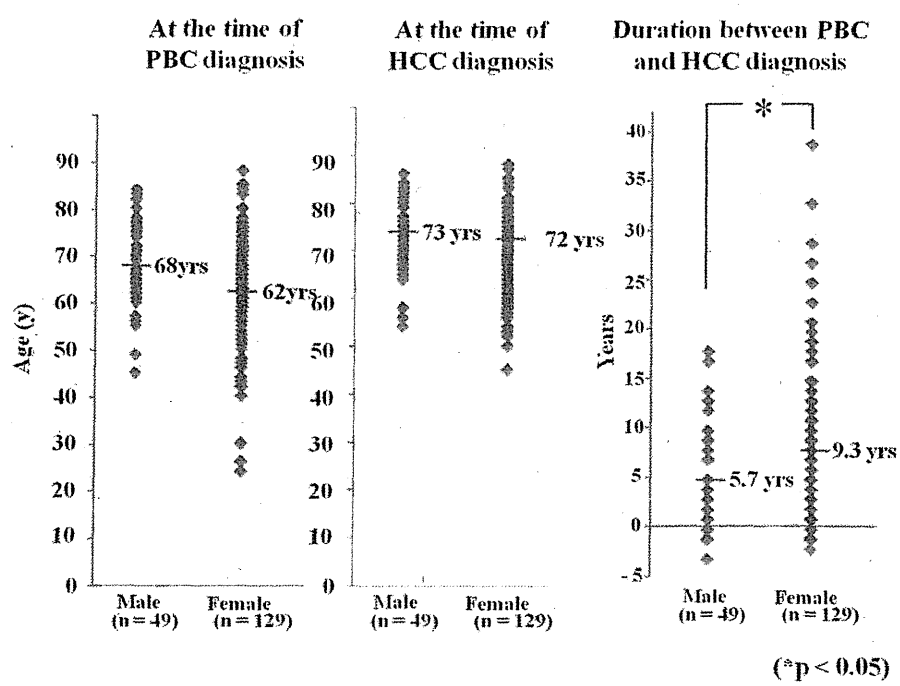

Fig. 4. Average age at the time of diagnosis of PBC and HCC, and the duration between the diagnosis of PBC and that of HCC. The duration between the diagnosis of PBC and that of HCC is shorter in males than in females ($P < 0.05$). The parentheses identify the number of patients examined.

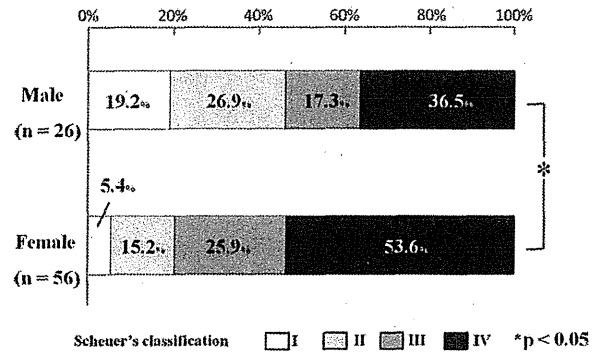

Fig. 5. Histologic stage by gender at the time of HCC diagnosis in patients with PBC. In females the HCC incidence gradually increases according to histological stage, with a statistically significant difference ($P < 0.05$). The parentheses indicate the number of patients examined.

index, serum triglyceride levels, serum total cholesterol levels associated with nonalcoholic fatty liver disease (including nonalcoholic steatohepatitis), and use of UDCA between males and females (Table 3). However, an analysis excluding patients with past HBV infection and a history of alcohol consumption revealed that there was no difference in other clinical findings, although the proportion of males (male/female = 24/104, 18.5%) remained higher than that of the total PBC male patients (male/female = 370/2,576, 12.6%; $P < 0.05$; Supplemental Table 4). Moreover, in females the HCC incidence gradually increased with histologic stage, while the incidence in males showed no trend or statistical significance. There was a significant difference in the distribution of histological stage between males and females (Fig. 5). An analysis of PBC patients with HCC according to histological stage revealed no clinical findings (including past HBV infection and alcohol consumption) that were significantly different between patients with and without cirrhosis at HCC diagnosis (Supplemental Table 5). There was also no significant difference in tumor number and differentiation between males and females (Supplemental Table 6).

Discussion

Recently, we encountered PBC patients with HCC during routine pathological assessments, and the number of these patients appears to have increased according to reports from other institutes.[11,17,18] In most patients, HCC is detected during follow-up for PBC, whereas some patients are simultaneously diagnosed with PBC and HCC or diagnosed with HCC prior to PBC. Although prognosis has improved with advances in treatment for PBC, the precise reason for the increased number of PBC patients with HCC in recent decades remains unknown. Therefore, we analyzed data from Japanese PBC patients and those with PBC and HCC who were independently surveyed by two different study groups. One set of data was from a national survey of PBC patients performed 14 times between 1980 and 2009, while the other was from PBC patients with HCC who were evaluated as a special project of the Annual Meeting of the Liver Cancer Study Group of Japan in 2011. Both surveys collected data through questionnaires administered to foundation hospitals or specialized hospitals for hepatology in Japan. Therefore, although the investigative hospitals/institutions and objectives did not match, it is speculated that most PBC patients with HCC overlapped. Moreover, the proportion of males among PBC patients with HCC almost coincided in these two independent studies (26.7% versus 27.5%), validating the use of these studies together as representative of the situation in Japan.

Although some studies have reported that PBC patients do not have an increased risk of developing HCC,[19] others showed that the HCC incidence was high in PBC patients.[5,7,20] The HCC incidence among PBC patients is reportedly low, at 0.76%-5.9%, according to previous reports.[4-9] In this study, we investigated the incidence of and risk factors for HCC in Japanese PBC patients. According to data from the nationwide survey by the Intractable Hepato-Biliary Diseases Study Group, the HCC incidence was 2.4%. As for risk factors associated with HCC in PBC patients, several conflicting results have been reported.[4,5,11-13] In general, male gender, advanced stage, HCV infection, and a history of blood transfusion were reported to be associated with HCC in PBC patients.[5,7,20] In a proportional hazards analysis of patients with PBC in Japan, Shibuya et al.[5] reported three factors to be independently associated with HCC development: age at the time of diagnosis, male gender, and history of blood transfusion. While autoimmune liver disease, including PBC, is more common in females than in males, HCC incidence in PBC patients was higher in males than in females. In agreement with previous reports from Japan, Europe, and the US,[4,5,11-13] gender was identified as a risk factor associated with HCC in the nationwide survey of PBC patients conducted by the Intractable Hepato-Biliary Diseases Study Group. HCC incidence was 5.1% in males and 2.0% in females (proportion of males, 26.7%), indicating that male PBC patients had a 2.1-fold higher risk of HCC compared with female PBC

patients. The proportion of males among the PBC patients with HCC was consistent with that in the nationwide survey by the Liver Cancer Study Group of Japan (27.5%). Moreover, the cumulative HCC incidence was 6.5% in males and 2.0% in females during the 10 years after PBC diagnosis, and male PBC patients had a 3.3-fold higher risk of HCC compared with females. In general, during the carcinogenesis of HCC, estrogen can protect hepatocytes from malignant transformation by way of downregulation of interleukin (IL)-6 release from Kupffer cells, indicating that estrogen-mediated inhibition of IL-6 production by Kupffer cells potentially decreased the risk of HCC in females.[21,22] Therefore, although PBC primarily affects females, HCC may be more common in male PBC patients because of a lack of estrogen-mediated prevention. The national survey by the Liver Cancer Study Group of Japan revealed that the duration between the diagnosis of PBC and that of HCC was shorter in males than in females and that the diagnosis of HCC was performed simultaneously at or prior to the diagnosis of PBC in 32.7% males and 14.7% females. Several reasons may be responsible for the delayed diagnosis of PBC and carcinogenesis in the early stage in males, but the details remain unspecified. Moreover, the rate of past HBV infection and alcohol consumption was significantly higher in males than in females, indicating that these factors also possibly affect the increased HCC incidence in male PBC patients. Watanabe et al.[18] reported that past HBV infection is an important factor in the association of HCC with PBC. In a patient with HBV infection, HBV-DNA possibly integrates into the human genome, but the frequency of this integration in prior HBV-infected PBC patients with HCC remains unknown. Moreover, because the distribution of past HBV infection by gender in the whole PBC population could not be obtained, the extent to which previous infection with HBV is directly associated with HCC carcinogenesis in male PBC patients remains debatable. However, analysis excluding cases with past HBV infection and a history of alcohol consumption revealed that the proportion of males with HCC in PBC patients with HCC remained high compared with that of all PBC male patients. In addition, analysis according to histological stage (noncirrhosis versus cirrhosis) suggested that past HBV infection and alcohol consumption were not directly associated with progression to cirrhosis in PBC patients with HCC.

In addition to male gender, the national survey by the Intractable Hepato-Biliary Diseases Study Group demonstrated that old age, low serum albumin levels, low total cholesterol levels, advanced histological stage, and symptomatic status at the time of PBC diagnosis were statistically significant in PBC patients with HCC compared to those without HCC. However, multivariate analysis by gender revealed that histological stage at the time of diagnosis of PBC was an independent risk factor for HCC in females, but not in males. In addition to the time of diagnosis of PBC, and that of HCC, histological stage is associated with HCC by the national survey for PBC with HCC patients. However, there was no difference in any clinical or biological characteristics between PBC patients with HCC with or without cirrhosis at HCC diagnosis. In females, the HCC incidence gradually increased according to histological stage, indicating that the terminal stage of PBC, which is a cirrhotic state, may be a risk factor for HCC development in females, whereas males are likely to develop HCC at any stage. The carcinogenesis of HCC in PBC patients should be further clarified. PBC is pathologically characterized by CNSDC, and the main inflammatory lesions associated with PBC are not hepatocytes but cholangiocytes, which may be one of the reasons why the HCC incidence in PBC patients is relatively low compared with the incidence of sustained hepatic diseases such as chronic viral hepatitis and autoimmune hepatitis. Male PBC patients with HCC are thought to be a good model because they lack estrogen-mediated prevention of HCC. Unlike that in hepatic diseases, intrahepatic cholestasis is found from the early stage in PBC,[1,23] and some mitogenic factors in the bile of PBC patients presumably participate in the carcinogenesis of HCC from an early stage.[17,24] However, this hypothesis remains a matter of speculation, and further study is required to clarify the molecular mechanism involved in the carcinogenesis of HCC in PBC patients.

In conclusion, we investigated the risk factors for HCC using data from two nationwide surveys of PBC patients in Japan. Because male PBC patients are at risk of developing HCC at any histologic stage, they should be carefully screened for HCC from an early stage of PBC, irrespective of histological stage.

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Appendix

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ORIGINAL ARTICLE

Clinicopathological Significance of Serum Fractalkine in Primary Biliary Cirrhosis

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Abstract
Background Primary biliary cirrhosis (PBC), characterized by cholangitis and loss of intrahepatic small bile ducts, predominantly affects middle-aged females. We have reported that fractalkine expression associated with chronic inflammation is observed in the damaged bile ducts and periductal vessels of PBC patients, which is closely associated with chronic cholangitis.
Aims We investigated the association between serum fractalkine levels and clinicopathological findings in PBC patients.
Methods Liver biopsy specimens before ursodeoxycholic acid treatment and serum samples at the time of liver biopsy and 1 and 2 years after treatment were obtained from 68 PBC patients (M/F = 14/54). Serum fractalkine levels were measured by enzyme-linked immunosorbent assay, and their association with clinicopathological findings (liver function data, autoantibodies, cholangitis activity, hepatitis activity, fibrosis, bile duct loss, and orcein-positive granules) was analyzed.
Results Serum fractalkine levels were in the range of 0.1–33.2 ng/ml (average, 3.2 ng/ml). They were increased in PBC patients with high degrees of cholangitis activity, a mild degree of hepatitis activity, fibrosis, orcein-positive granules, and early stages. In cases with high serum fractalkine levels, those who exhibited good biochemical responses to treatment mostly showed improved serum fractalkine levels after treatment.
Conclusion Serum fractalkine levels of PBC patients were high in cases with marked cholangitis activity at early stages. In addition, they closely correlated with the effect of therapy, indicating that fractalkine plays a role in the pathogenesis of initial cholangitis in early stage PBC and consequent chronic cholangitis. Thus, our results suggest that fractalkine is a good candidate for molecular-targeted treatment.

Keywords Primary biliary cirrhosis · gp210 · Fractalkine · Pathology · Cholangitis

Abbreviations

ADAM	A disintegrin and metalloprotease
AMA	Anti-mitochondrial antibody
CNSDC	Chronic nonsuppurative destructive cholangitis
IBD	Inflammatory bowel disease
PBC	Primary biliary cirrhosis
RA	Rheumatoid arthritis
TLR	Toll-like receptor
UDCA	Ursodeoxycholic acid
ULN	Upper limit of normal

Introduction

Fractalkine (CX3CL1) plays an important role in leukocytes migration to target sites under both physiological and pathological conditions. Unlike other chemokines, fractalkine is

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a membrane-bound protein that can be shed in soluble chemotactic form following cleavage by a disintegrin and metalloprotease (ADAM) 10 and ADAM17 [1]. Soluble fractalkine is known to be a potent chemoattractant for CD8-positive and CD4-positive T cells, CD16-positive natural killer cells, and macrophage/monocytes expressing its receptor (CX3CR1), and promotes strong adhesion of these leukocytes in an integrin-independent manner. Therefore, fractalkine signaling is thought to be involved in the development of chronic inflammation, as has been reported in cases of rheumatoid arthritis (RA), atherosclerosis, inflammatory bowel disease (IBD), and rejection of implanted organs, and genetic polymorphisms of fractalkine have been speculated to increase disease susceptibility [2]. Moreover, fractalkine was recently noted as a molecular target of therapeutic agents for RA and IBD, and anti-inflammatory treatments using anti-CX3CR1 antibody have been developed for RA and an animal model of heart transplantation [2, 3].

Primary biliary cirrhosis (PBC) mainly affects middle-aged females; histologically, the interlobular bile ducts are primarily damaged with characteristic findings such as chronic nonsuppurative destructive cholangitis (CNSDC) followed by progressive loss of bile ducts [4]. There is considerable evidence that bile duct damage is mediated by autoreactive or cytotoxic T cells [5–8], and the molecular mechanisms responsible for the migration of pathogenic T cells around or within bile ducts have been clarified over the past several years. We previously reported that the level of fractalkine is significantly elevated in the sera of PBC patients and in small bile ducts, particularly those that are damaged. In addition, vascular endothelial cells expressing fractalkine is increased in PBC [9], suggesting that fractalkine is an important mediator associated with the continuous portal, particularly periductal, inflammation of PBC. Moreover, the expression of fractalkine in bile ducts is reported to be associated with innate immunity via Toll-like receptor (TLR) 3 and TLR4 in vascular endothelial cells, infiltrating mononuclear cells, and biliary epithelial cells [10, 11].

Fractalkine is an important chemokine closely associated with the pathogenesis of cholangiopathy, and is likely involved in the continuous inflammation of chronic cholangitis in PBC. In this study, we investigated serum fractalkine levels in PBC patients and their association with clinicopathological findings.

Methods

Subjects and Clinical Information

Sixty-eight patients with PBC were selected from registered files of the National Hospital Organization (NHO) Nagasaki Medical Center. The patients included 14 males and 54 females with average ages of 53 and 59 years, respectively. Serum samples were obtained at the diagnosis of PBC or before ursodeoxycholic acid (UDCA) treatment for PBC and 1 and 2 years after starting UDCA treatment, and liver function data [aspartate transaminase (AST), alkaline phosphatase (ALP) levels], IgM levels, and levels of autoantibodies [anti-mitochondrial antibody (AMA), anti-centromere antibody, and anti-gp210 antibody] were analyzed at each time point. The reserved serum samples were used for the measurement of soluble fractalkine with an enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN, USA). Liver biopsy specimens from all cases were also obtained at the time PBC was diagnosed, before treatment.

Diagnosis of PBC and Criteria of UDCA Effect

The diagnosis of PBC and biochemical response to UDCA were defined following criteria established by the Intractable Hepato-Biliary Disease Study Group in Japan. Patients whose condition meets one of the following criteria were diagnosed with PBC: (1) histologically observed chronic nonsuppurative destructive cholangitis (CNSDC) and laboratory findings not contradicting PBC; (2) positive AMA and/or anti-pyruvate dehydrogenase (PDH) antibody, CNSDC not histologically observed but histological findings compatible with PBC; or (3) histological examination not performed, but positive AMA or anti-PDH antibodies and clinical findings and course indicating PBC. Biochemical response to UDCA was as follows: good: normalization of serum ALP, ALT, and IgM within 2 years of starting UDCA treatment; fair: serum ALP, ALT, and IgM within <1.5 upper limit of normal (ULN) 2 years after starting UDCA treatment; and poor: serum ALP, ALT, and IgM within ≥1.5 ULN 2 years after starting UDCA treatment.

Histological Examination

Sections greater than 10 4 μm thick were prepared from each paraffin-embedded block; several were stained with hematoxylin–eosin (HE), Gomori's reticulum, and Orcein stain for histological diagnosis, grading, and staging.

The grading system and new staging system proposed by Nakanuma [12] were used to evaluate disease activity and stage. In summary, chronic cholangitis activity (CA) was categorized into four grades (CA0–3) according to the degree and distribution. CA0 (no activity) was defined as absent or ambiguous bile duct damage. In CA1 (mild activity), one bile duct showed evident chronic cholangitis. In CA2 (moderate activity), two or more bile ducts were affected. In CA3 (marked activity), at least one damaged bile duct showed CNSDC and/or granulomatous

cholangitis. Evident chronic cholangitis was defined as a damaged bile duct entirely surrounded by mild to moderate, duct-oriented lymphoplasmacytic inflammation. Hepatitis activity (HA) was also categorized into four grades (HA0–3) according to the presence and degree of interface hepatitis and lobular hepatitis. In HA0 (no activity), interface hepatitis was not present. The presence of interface hepatitis affecting at least 10 continuous hepatocytes at the interface of one portal tract or fibrous septum was categorized as HA1 (mild activity) and in two or more portal tracts or fibrous septa as HA2 (moderate activity). In HA3 (marked activity), interface hepatitis affecting at least 20 continuous hepatocytes at the limiting plate in more than half of the portal tracts or fibrous septa was present throughout the specimen, with entrapment of hepatocytes in the expanded portal tracts. Although no or minimum lobular hepatitis was found in HA0, mild to moderate lobular hepatitis was observed in HA1 and HA2, and moderate lobular hepatitis in HA3. Occasional zonal necrosis and bridging necrosis was regarded as HA3.

Three factors were evaluated for the new staging system: fibrosis, bile duct loss, and Orcein-positive granule deposition. These three items were scored as follows. For fibrosis, a score of 0 indicated almost no fibrosis or fibrosis limited to the portal tracts, a score of 1 indicated fibrosis extending beyond the portal area with occasional incomplete septal fibrosis, a score of 2 indicated completely connecting septal fibrosis or bridging fibrosis with variable lobular distortion, and a score of 3 was assigned for cirrhosis (extensive fibrosis with regenerative nodules). For bile duct loss, interlobular bile ducts were evaluated in well-formed portal tracts with evident hepatic arterial branches and portal vein branches. A score of 0 meant interlobular bile ducts were distinguishable in all portal tracts in specimens. Scores of 1 and 2 meant that bile duct loss was evident in <1/3 and in 1/3–2/3 of portal tracts, respectively. A score of 3 indicated that bile ducts were absent in >2/3 of portal tracts. For Orcein-positive granule deposition, a score of 0 meant no deposition in periportal hepatocytes, a score of 1 indicated deposition in some periportal hepatocytes in <1/3 of portal tracts, and a score of 3 was given for patients with deposition in many hepatocytes of >2/3 portal tracts or fibrous septa. Samples intermediate between 1 and 3 were assigned a score of 2. After each of these items was scored, they were summed: a total score of 0 indicated stage 1 (no or minimum progression), 1–3, stage 2 (mild progression); 4–6, stage 3 (moderate progression); and 7–9, stage 4 (advanced progression).

Statistical Analysis

Data were analyzed using Welch's t tests, paired t tests, and Spearman rank correlation coefficient; $p < 0.05$ was considered statistically significant for all analyses.

Results

Serum Fractalkine Levels and Their Relationship with Serological Data

Serum fractalkine levels in PBC patients ranged from 0 to 33.2 ng/ml before UDCA treatment, and the mean was 3.2 ng/ml. However, most values were 0–3.0 ng/ml, and 14 cases showed high levels (>5 ng/ml) (Fig. 1). This trend confirmed the findings in our previous report [9], where serum fractalkine levels in all controls including healthy controls and patients with extrahepatic biliary obstruction and HCV-related chronic hepatitis were <3.0 ng/ml [9]. Next, the correlation between fractalkine levels and liver function data (ALT and ALP levels) and IgM levels was examined. The correlation coefficients between fractalkine levels and ALT, ALP, and IgM levels were 0.12, 0.06, and 0.10, respectively (Fig. 2a). With regard to autoantibodies, none showed significant positive correlation with serum fractalkine levels (Fig. 2b). However, patients with high gp210 titers had low fractalkine levels, whereas patients with high serum fractalkine levels had low gp210 titers, except one case in which both levels were high (fractalkine, 33.2 ng/ml; gp210, 132.4 times) (Fig. 2b). This patient (Fig. 2b, #) was a 35-year-old male positive for both AMA and ANA and had increased IgG and IgM levels. His condition rapidly deteriorated despite all treatment, and he promptly underwent liver transplantation, suggesting the existence of other factors such as autoimmune hepatitis exacerbating liver injury. Therefore, we considered this case as an outlier and performed the subsequent statistical analysis excluding it. Consequently, the serum fractalkine level of 1.5 ± 0.2 ng/ml (mean ± standard error of mean) in patients with high gp210 titers (>5 times) was significantly lower (5.5 ± 1.5 ng/ml) than that in patients with low gp210 titers (≤5 times) (Welch's t test, $p < 0.05$). Moreover, gp210 titers (1.7 ± 0.3 times) in patients with high serum fractalkine levels (>3 ng/ml) were significantly lower (35.1 ± 11.1 times) than those in patients with low fractalkine levels (<3 ng/ml) (Welch's t test, $p < 0.05$).

Correlation Between Serum Fractalkine Levels and Histological Activity and Staging of PBC

Fractalkine levels in PBC patients were analyzed according to the histological activity of chronic cholangitis (CA) and hepatitic change (HA). As shown in Fig. 3, fractalkine levels in CA3 cases were significantly higher than those in cases with other scores (CA0–CA2) and, in contrast, the fractalkine levels in HA3 cases were significantly lower than those in cases with other scores (HA0–HA2) (Welch's t test, $p < 0.05$) with the exception of one case (Fig. 3a, b).

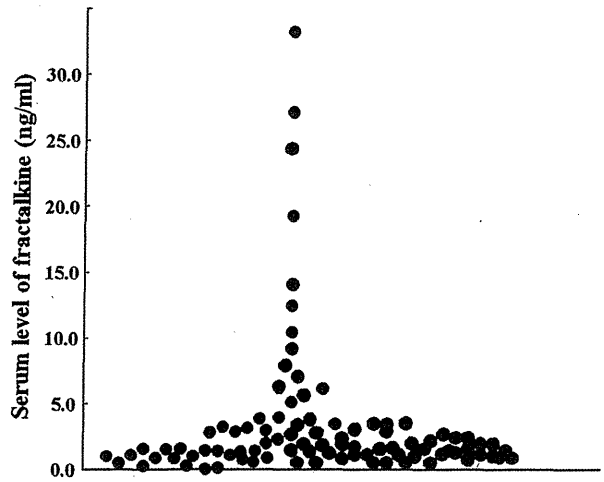

Fig. 1 Distribution of serum fractalkine levels in PBC patients before UDCA treatment. Levels ranged from 0 to 33.2 ng/ml, and the mean was 3.2 ng/ml. Fourteen cases showed high fractalkine levels (>5 ng/ml)

As for the three histological findings defining histological stage, fibrosis, bile duct loss, and Orcein-positive granules, the cases with low scores (0–1) of fibrosis and a score of 0 for Orcein-positive granules showed significantly higher fractalkine levels than in cases with a score of 2–3 for fibrosis and 1–3 for Orcein-positive granules (Fig. 3d, f) (Welch's t test, $p < 0.05$). Moreover, the cases with early histological stages (stages 1–2) showed higher fractalkine levels than those with advanced stages (stages 3–4) (Fig. 3c).

Correlation Between Serum Fractalkine Levels and UDCA Effects

The changes in serum fractalkine levels before (before treatment) and 1–2 years after the beginning of UDCA treatment (after treatment) are shown in Fig. 4. Most cases with low fractalkine levels (<3.0 ng/ml) before the treatment retained low levels. However, 14 cases with high

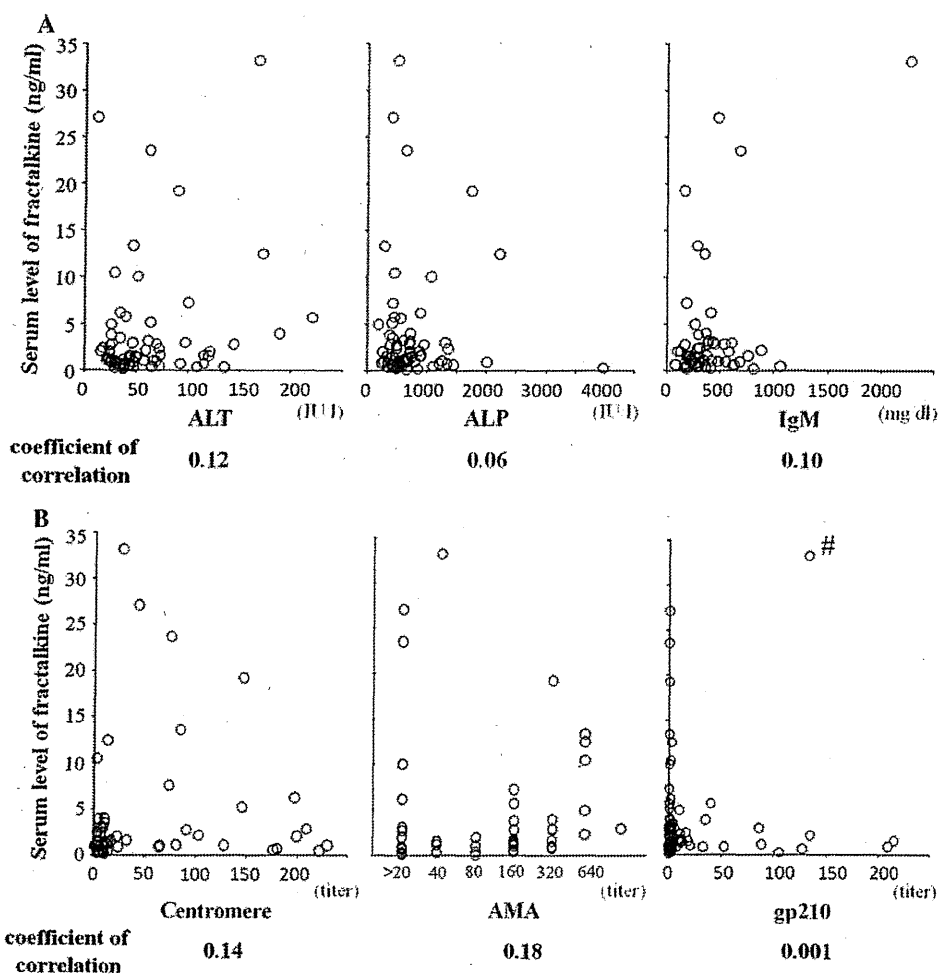

Fig. 2 **a** Correlation between serum fractalkine levels and liver function data (ALT and ALP levels) and IgM levels at the time of PBC diagnosis before UDCA treatment. The correlation coefficients between fractalkine levels and ALT, ALP, and IgM levels were 0.12, 0.06, and 0.10, respectively, which were not significant. **b** Correlation between serum fractalkine levels and autoantibodies at the time of PBC diagnosis before UDCA treatment. The correlation coefficients between fractalkine levels and levels of anti-centromere antibody, AMA, and gp210 antibody were 0.14, 0.18, and 0.001, respectively, which were not significant. However, a clear trend between fractalkine levels and the gp210 antibody titers was found; patients with high gp210 titers had low fractalkine levels, and patients with high fractalkine levels had low gp210 titers except one case (#). This exceptional case (#) had a high fractalkine level (33.2 ng/ml) and a high gp210 titer (132.4) and was clinically unique

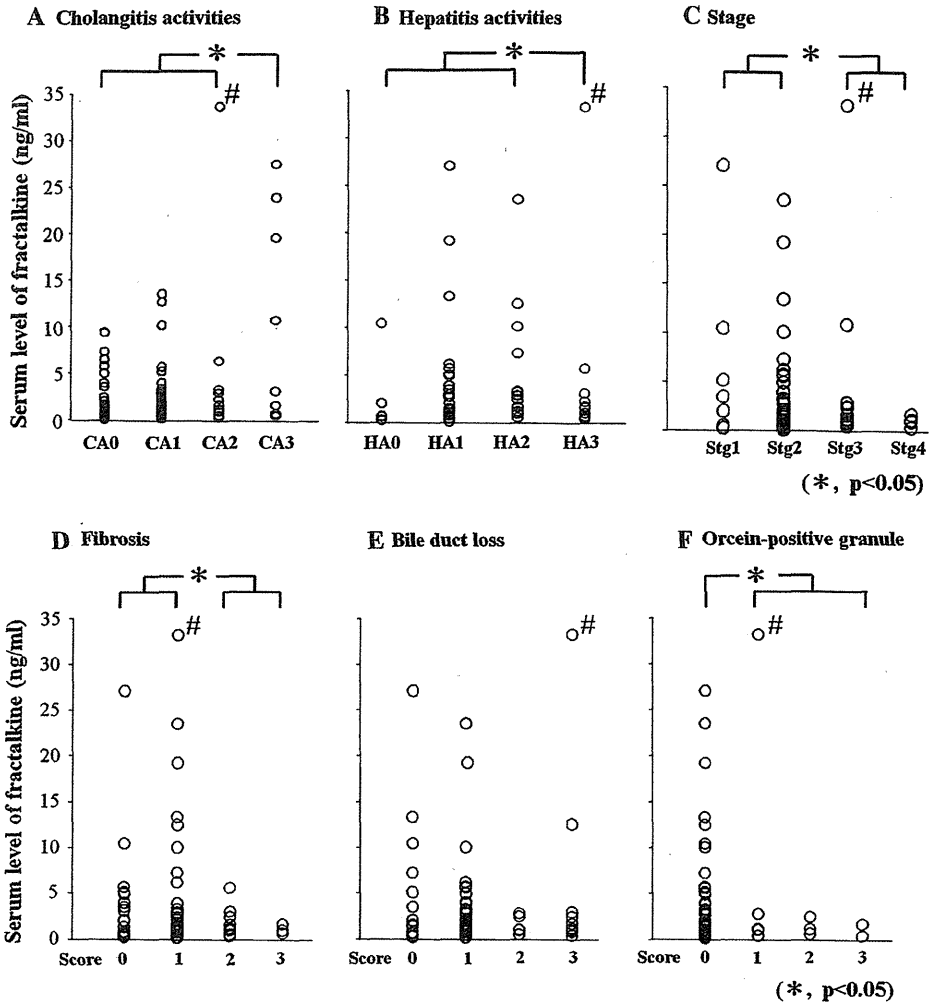

Fig. 3 Correlation between serum fractalkine levels and histological activation, stage, and three findings defining stage at the time of diagnosis of PBC before UDCA treatment. Serum fractalkine was significantly higher in cases with marked chronic cholangitis activity (*CA3*) than those with lower CA scores (*CA0–CA2*). In contrast, it was lower in cases with marked hepatitic activity (*HA3*) compared with those with lower HA scores (*HA0–HA2*). Among the three histological findings of fibrosis, bile duct loss, and Orcein-positive granules, the cases with low scores (0–1) for fibrosis, a score of 0 for Orcein-positive granules, and also early stages (stage 1–2) showed significantly higher fractalkine levels than those with a scores of 2–3 for fibrosis and 1–3 for Orcein-positive granules, and advanced stages (stage 3–4). The statistical analysis was performed without the unique case (#)

fractalkine levels (>5 ng/ml) showed changes over time. Among serum ALP, ALT, and IgM defining the biochemical response to UDCA, the patients with >2 items of good response or <1 item of good response were evaluated separately. The patients with high fractalkine levels (>5 ng/ml) before treatment showed a decrease after treatment in the patients with >2 items of good response, but those with high fractalkine levels showed an increase in those with <1 item of good response. Statistical analysis demonstrated that in patients with >2 good response items, fractalkine level was significantly decreased after treatment (2.9 ± 1.1 ng/ml) compared with before treatment (4.6 ± 1.6 ng/ml) (paired *t* test, $p < 0.05$).

Discussion

Fractalkine chemoattracts CXCR1-expressing cells in target organs and maintains continuous inflammation, ultimately inducing chronic inflammation. We previously reported that damaged bile ducts and vessels in PBC express fractalkine and that the induction of fractalkine expression in biliary epithelial cells is closely associated with biliary and periductal innate immunity [9, 10]. In the present study, we measured serum fractalkine levels using different PBC patients' sera and obtained a similar average and distribution. Most cases had levels <3 ng/ml, but 14 patients had high levels (>5 ng/ml). These high levels were only observed in PBC patients; levels in the healthy population and patients with other liver diseases were <3 ng/ml [9]. Therefore, in PBC patients with high fractalkine levels, intrahepatic fractalkine produced by liver constituent cells, including biliary epithelial cells, is the likely cause of the increase in serum fractalkine. This indicates that fractalkine-mediated inflammation is associated with chronic cholangitis and portal inflammation.

Although a serological hallmark of PBC is the presence of AMA, which is found in >90 % of patients, ANAs are