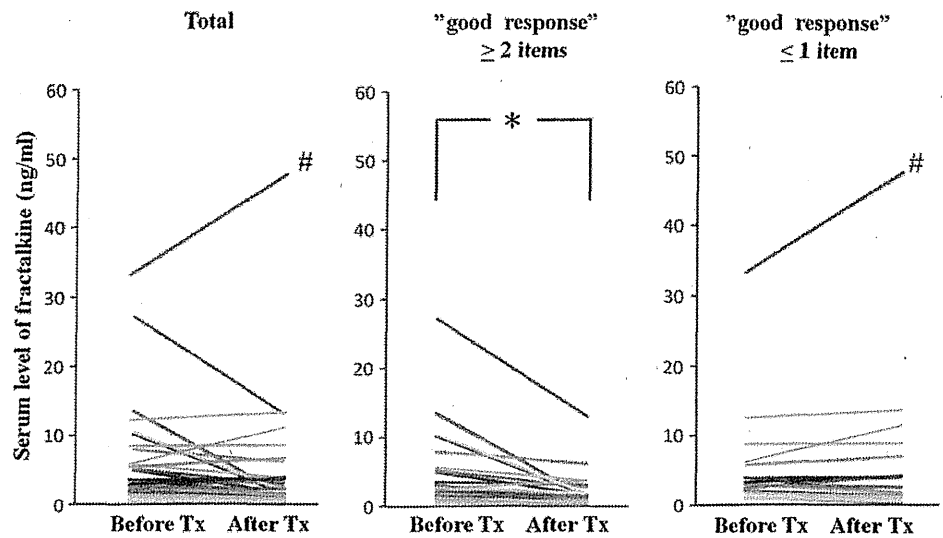

Fig. 4 Change in serum fractalkine levels before (*before Tx*) and 1 or 2 years after the beginning UDCA treatment (*after Tx*). *Total* is the data for all PBC cases. Among the three items defining the response to UDCA (serum ALP, ALT, and IgM levels), "*g, ≥2 itemsood response*" cases with high fractalkine levels (>5 ng/ml) before treatment showed a decrease after treatment, whereas "*good response, ≤1 item*" cases showed an increase

also detected in PBC patients [13–17]. Anti-gp210 and anti-sp100 antibodies are highly specific for PBC and useful for its diagnosis, particularly those who are negative for AMAs [13–17]. In addition, although AMAs are not associated with disease progression, ANAs are associated with disease severity and are therefore useful as a marker of poor prognosis [15–18]. Anti-gp210 antibodies are the strongest predictive factor among ANAs for progression to end-stage hepatic failure (i.e., hepatic failure type progression) [15–17]. Moreover, the presence of gp210 antibodies is a risk factor for more severe interface hepatitis and lobular inflammation defining hepatitis activity (HA). The present study revealed that most patients with high gp210 titers had low serum fractalkine levels, whereas most patients with high fractalkine levels had low gp210 titers. Moreover, the fractalkine levels in the CA3 cases were significantly higher than those with lower scores (CA0–CA2). In contrast, fractalkine levels in the HA3 cases were significantly lower than those with low scores (HA0–HA2). Therefore, a possible explanation of why high anti-gp210 antibody and fractalkine levels showed opposite trends with each other except in one unique case (Fig. 2b, #) is that the gp210 antibody and fractalkine are associated with marked hepatitic change (HA) and chronic cholangitis (CA), respectively, indicating that they may reflect two different pathogenetic mechanisms of PBC from the aspects of hepatitis and cholangitis, respectively. The unique case showing high gp210 antibody titers and fractalkine levels (Fig. 2b, #) had extensive chronic cholangitis (CA2) and hepatitic changes (HA3), as shown in Fig. 3. This PBC case was very rare and pathognomonic, and the patient rapidly progressed to stage 3 liver failure before the terminal stage without any improvement achieved with UDCA treatment. This indicates that the case was aggressive and had a poor prognosis, suggesting a case of atypical PBC.

As discussed above, fractalkine is speculated to be associated with the pathogenesis of chronic cholangitis in PBC, which is a characteristic feature of this disease. Moreover, the present study revealed that the cases with low fibrosis scores (0–1), and a score of 0 for Orcein-positive granules among the three histological findings defining histological stage, showed high fractalkine levels, similar to patients in early histological stages (stages 1–2). Therefore, fractalkine plays a role in the pathogenesis of chronic cholangitis in PBC, particularly in the early stages, indicating that fractalkine is an important factor in initial chronic cholangitis in PBC and also during the transition to chronic cholangiopathy in PBC without reference to hepatitic change. However, many PBC patients had low serum fractalkine levels, even in the early stages, indicating that the association of fractalkine in the pathogenesis of PBC might be varied in each case, and its significance differs between early and advanced stages, even in the same patient.

The present study demonstrated a correlation between serum fractalkine levels and response to UDCA; although patients with low fractalkine levels (<3 ng/ml) before UDCA treatment had low levels after treatment irrespective of the effectiveness of UDCA treatment, the cases with high levels (>5 ng/ml) showed a decrease or increase after UDCA treatment in good and poor biochemical responders, respectively. UDCA is a hydrophilic, nontoxic bile acid that contributes nearly 3 % to the normal bile acid pool in humans and has cytoprotective, anti-apoptotic, membrane stabilizing, antioxidative, and immunomodulatory effects [19]. In our previous study, we demonstrated that upregulation of fractalkine expression in biliary epithelial cells is

induced by the biliary innate immune response [9]. Therefore, it is possible that UDCA directly regulates the production of fractalkine in liver constituent cells, including biliary epithelial cells, but further study is needed to clarify the direct correlation between the effectiveness of UDCA and serum fractalkine levels.

The present study demonstrated that, among PBC patients, those with high serum fractalkine levels are characterized by low gp210 titers, extensive chronic cholangitis, limited hepatitic change, and early-stage PBC. Therefore, fractalkine is speculated to play a role in the initial pathogenesis of chronic inflammation in early-stage PBC and consequently chronic cholangitis. In addition to PBC, fractalkine is associated with chronic inflammation in other diseases, including RA and IBD, and an anti-fractalkine monoclonal antibody has been developed as a clinical molecular treatment. Collectively, our findings suggest that fractalkine-CX3CR1 signaling might be a useful molecular target for the treatment of PBC, particularly in UDCA-ineffective PBC cases with high serum fractalkine levels.

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Conflict of interest None.

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Review Article

Prevalence and risk factors of hepatocellular carcinoma in Japanese patients with primary biliary cirrhosis

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Primary biliary cirrhosis (PBC) tends to affect females more than males. PBC selectively damages intrahepatic small bile ducts, particularly interlobular bile ducts. The clinical presentation of PBC has changed according to recent advances in clinicobiological diagnosis and improvements in therapeutic effects and prognosis. In particular, we encounter PBC patients with hepatocellular carcinoma (HCC), and the number of these patients appears to have increased. The precise reason for the increased number of PBC patients with HCC in recent decades remains unknown, but recognizing the current status of carcinogenesis in PBC patients, identifying the associated clinicopathological risk factors and understanding how the pathogenesis of PBC is directly associated with HCC, is important. In this review, we summarize the data from two nationwide surveys undertaken in Japan as well as recent data from Japanese and international studies.

Key words: carcinogenesis, hepatocellular carcinoma, primary biliary cirrhosis, sex

INTRODUCTION

PRIMARY BILIARY CIRRHOSIS (PBC) is an autoimmune liver disease. It tends to affect females more than males. PBC selectively damages the intrahepatic small bile ducts, particularly interlobular bile ducts. Because of progressive loss of bile ducts, PBC develops into chronic cholestasis and finally biliary cirrhosis.

The clinical presentation of PBC has been changing over the years. In particular, the proportion of asymptomatic patients at diagnosis has increased. In contrast to other biliary diseases such as primary sclerosing cholangitis (PSC), the associated malignant tumor of PBC is hepatocellular carcinoma (HCC), although its incidence is low. The detailed clinicopathological significance and carcinogenesis of HCC associated with PBC remain unknown. In this review, recent data from Japan[1] and other countries are reviewed.

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COMPLICATION OF MALIGNANCY IN PATIENTS WITH PBC

SEVERAL STUDIES HAVE indicated that PBC may be associated with an increased risk of extrahepatic malignancies as well as HCC, although they represent a rare complication. By surveying 212 Greek patients with PBC, 10.8% patients were diagnosed with malignancy, 3.8% patients with HCC and 7.0% with extrahepatic malignancies.[2] Moreover, a meta-analysis using PubMed and EMBASE databases revealed that PBC is closely associated with a greater risk of overall cancer and HCC, but not with other cancers.[3] With respect to HCC, its incidence in patients with PBC varies from 0.76% to 5.9% depending on reports.[2,4–9] However, one report has stated that PBC is not a risk factor for HCC.[10] These divergent results may be because of the low prevalence of the association with HCC as well as geographical and environmental differences. However, the number of PBC patients associated with HCC has been recently increased, which may be due to the improvement of therapeutic effects and prognosis.[11–13]

National surveys of patients with PBC in Japan have been undertaken 15 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 surveys involved 8509 patients registered in the 1st–15th surveys performed between 1980 and 2012.[9,14,15] According to the 15th National Survey performed in 2012, the incidence of malignancy at the time of PBC diagnosis was 3.3%. Liver cancer was the most common (24%), followed by gastric cancer (16%), colon cancer (12%), breast cancer (10%), uterine cancer (5%), thyroid cancer (6%), hematopoietic cancer (5%), ovarian cancer (3%), lung cancer (3%) and others (16%).[15]

RISK FACTORS FOR HCC IN PATIENTS WITH PBC BASED ON DATA FROM JAPAN AND OTHER COUNTRIES

ACCORDING TO EPIDEMIOLOGICAL studies by single and multiple centers, cirrhosis, portal hypertension, advanced age, diabetes mellitus (DM), male sex, blood transfusion, smoking and excessive intake of alcohol are reported as risk factors for HCC (in addition to infection by the hepatitis virus), but these risk factors vary among reports.[4–7,12,16–18] Moreover, cases of patients with HCC arising from patients with non-cirrhotic PBC have been reported, and consideration of the carcinogenesis of HCC in PBC was noted. The Japanese data reported by Shibuya *et al.*[6] highlighted the importance of age at the time of PBC diagnosis, male sex and history of blood transfusion as independent risk factors associated with the development of HCC by proportional hazards analyses. In general, autoimmune diseases (including PBC), irrespective of the organs affected, preferably affect females more than males, but the incidence of HCC in PBC and autoimmune hepatitis is higher in males than in females.[13] This difference in incidence between the sexes has been reported and confirmed in studies outside Japan.[6,7,12,17,18]

NATIONAL SURVEY OF PBC IN JAPAN

ACCORDING TO THE 14th National Survey among patients with PBC with HCC in Japan, undertaken in 2009 among 2946 subjects (70 males, 2576 females) confirmed to either have or not have HCC as well as exclusion of hepatitis B virus (HBV) carriers and HB antigen positive and anti-hepatitis C virus antibody positive patients, the incidence of HCC during follow up was 2.4% (71/2946). This incidence was 5.1% (19/370) in males and 2.0% (52/2576) in females, and the proportion of males was 26.7%.[1,19] Moreover, according to a cohort study by the National Hospital Organization Study Group for Liver Disease in Japan, 20 cases (2.0%) (male/female = 5/15; proportion of males, 25%) among the 1007 patients with PBC registered in 1989–2011 had HCC,[20] supporting a similar value for the incidence revealed by the National Survey of PBC in Japan. Therefore, in Japan, the incidence of HCC in patients with PBC and the proportion of males have been speculated to be approximately 2% and 25%, respectively. Although the incidence of HCC is low, the incidence and mortality in patients with PBC are significantly higher than those in the general population of Japan based on detailed analyses using the standardized incidence ratio and standardized mortality ratio of HCC in patients with PBC.[21]

According to a comparative analysis of this population obtained from the National Surveys of patients with PBC in Japan (2009), male sex, old age, low serum albumin levels, low serum total cholesterol levels, advanced histological stage and symptomatic status at the time of PBC diagnosis were significant risk factors for HCC (Table 1).[1,22] 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 (Fig. 1). In particular, analyses of the incidence of HCC in patients aged 10–80 years revealed that male patients with PBC in their 40s and 50s had an increased risk of HCC compared with female patients with PBC in the same age groups. In multivariate analyses for risk factors of HCC, sex and histological stage were selected as the only significant factors among male sex, old age, low serum albumin levels, low serum total cholesterol levels, advanced histological stage and symptomatic status raised by comparative analyses. By multivariate analyses for risk factors of HCC by sex, histological stage at the time of PBC diagnosis was an independent risk factor for HCC in females (Table 2), whereas no significant independent factors were selected in males (Table 3). With respect to histological stage, there was no difference in the proportion of males and females who underwent histological staging at the time of PBC diagnosis (Fig. 2). The incidence of histological stages 3 and 4 was approximately 16.0% in male and female patients with PBC without HCC (Fig. 2), whereas it was 14.2% and 57.1% in male and female patients with PBC with HCC, respectively.[1,22] Advanced histological stage was a risk factor for HCC in females but not in males (Fig. 2, Tables 2,3). Therefore, male patients with PBC should be followed up to consider the possibility of complication with HCC in any PBC stage.

Table 1 Clinical characteristics of patients with PBC with or without HCC at the time of PBC diagnosis (comparative analysis)

	HCC (+)	HCC (−)	*P*
Case 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**
Total 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**
Total 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**
Use of UDCA (%)	89.7	91.8	0.5291
Clinical stage (asymptomatic : symptomatic)	38:33	2775/100	**<0.0001**

Bolding denotes statistically significant items.
†These are also selected as risk factors of HCC by multivariate analyses.
HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; SD, standard deviation; UDCA, ursodeoxycholic acid.

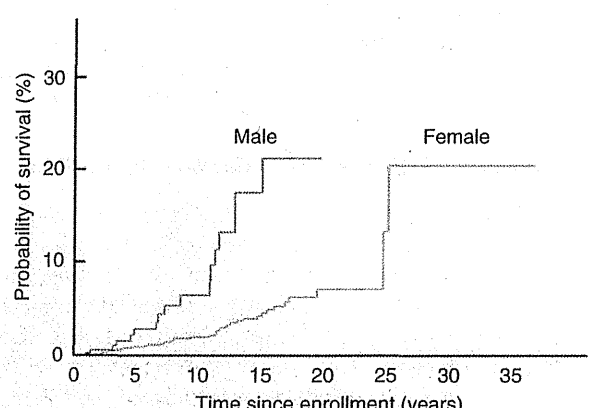

Figure 1 Cumulative rates for appearance of hepatocellular carcinoma in patients with primary biliary cirrhosis according to sex. There is a statistically significant difference between males and females.

SURVEY OF PATIENTS WITH PBC WITH HCC IN JAPAN

AT THE 47TH Annual Meeting of the Liver Cancer Study Group of Japan, the survey of 178 patients with PBC with HCC (100 fatalities in the past years and 78 patients followed up) revealed that the proportion of males was 27.5% (49 males and 129 females), which was similar to that from the National Survey of PBC in Japan. The average age at the time of PBC diagnosis was higher for males (68 years) than for females (62 years), but the time of HCC diagnosis was similar between males (73 years) and females (72 years; Fig. 3). Moreover, the duration between the diagnosis of PBC and that of HCC was shorter in males than in females (Fig. 3). HCC was simultaneously diagnosed during or before PBC diagnosis in 32.7% (16/49) of males and 14.7% (19/129) of females.

Clinicopathological data at the time of HCC diagnosis are shown in Table 4. There were more males with previous HBV infection and a history of alcohol

Table 2 Factors associated with the risk of HCC at the time of PBC diagnosis in female patients with PBC (multivariate analyses)

	Regression coefficient	SD	χ^2	Odds ratio	*P*
Age	−0.0130	0.0174	0.56	0.9870	0.4531
Total bilirubin	0.0817	0.1171	0.49	1.0851	0.4852
Albumin	−0.1771	0.3366	0.28	0.8376	0.5987
Total cholesterol	0.0038	0.0033	1.32	1.0038	0.2512
Histological stage (I/II/III/IV)	−1.0255	0.1964	27.25	0.3586	**<0.0001**
Use of UDCA (%)	−0.1607	0.3151	0.26	1.3791	0.6100
Clinical stage (asymptomatic : symptomatic)	0.4252	0.1913	4.94	0.4271	**0.0263**

Bolding denotes statistically significant items.
HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; SD, standard deviation; UDCA, ursodeoxycholic acid.

Table 3 Factors associated with risk of HCC at the time of PBC diagnosis in male patients with PBC (multivariate analyses)

	Regression coefficient	SD	χ^2	Odds ratio	*P*
Age	−0.0542	0.0319	2.89	0.9472	0.0893
Total bilirubin	−0.1018	0.1790	0.32	0.9032	0.5697
Albumin	0.5884	0.5591	1.11	1.8011	0.2926
Total cholesterol	0.0001	0.0020	0.00	1.0001	0.9511
Histological stage (I/II/III/IV)	0.2484	0.4096	0.37	1.2819	0.5443
Use of UDCA (%)	−0.5367	0.4254	1.59	2.9258	0.2071
Clinical stage (asymptomatic : symptomatic)	−0.3590	0.4635	0.60	2.0506	0.4385

HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis; SD, standard deviation; UDCA, ursodeoxycholic acid.

consumption than females. There were no differences with respect to the history of blood transfusion, diabetes mellitus, antimitochondrial antibody levels, antinuclear antibody levels, body mass index, serum triglyceride levels, serum total cholesterol levels associated with non-alcoholic fatty liver disease (including non-alcoholic steatohepatitis), and use of ursodeoxycholic acid (UDCA; Table 4) between males and females. However, an analysis excluding patients with previous HBV infection and a history of alcohol consumption revealed no difference in other clinical findings, although the proportion of males (male/female = 24/104, 18.5%) remained higher than that of the male patients with PBC (male/female = 370/2576, 12.6%). Moreover, in females, the incidence of HCC gradually increased with the histological stage, whereas that in males showed no trend or statistical significance. There was a significant difference in the distribution of the histological stage between males and females (Fig. 4). An analysis of patients with PBC with HCC according to the histological stage revealed no clinical findings (including previous HBV infection and alcohol consumption) that were significantly different between patients with and without cirrhosis at the time of HCC diagnosis, suggesting that previous HBV infection and alcohol consumption are not directly associated with progression to cirrhosis in patients with PBC with HCC.

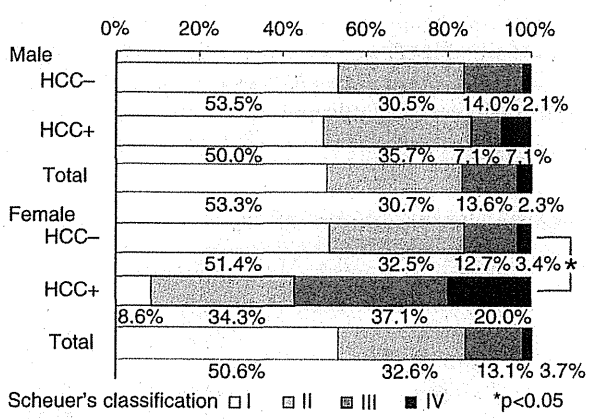

Figure 2 Histological stage of patients with primary biliary cirrhosis (PBC) with or without hepatocellular carcinoma (HCC) according to sex. There is a significant difference in the proportion of female PBC patients with HCC and those without.

PATHOLOGY OF HCC IN PATIENTS WITH PBC

WITH REGARD TO the pathological findings of HCC, approximately two-thirds of patients showed a solitary mass, and there was no difference in sex according to the National Survey at the 47th Annual Meeting of the Liver Cancer Study Group of Japan. The degree of differentiation in HCC was mostly well-differentiated and moderately differentiated, and there was no difference in sex. Therefore, the risk factors and carcinogenesis of HCC differ between males and females, but the features of complicated HCC are common between males and females (Table 5). As notable pathological findings, a survey of Japanese autopsy cases of PBC disclosed that fatty changes or bile plugs within tumors were frequently observed.[23] Mallory body clusters and focal copper-binding protein deposition were consistently found in cirrhotic liver and carcinoma tissues. Moreover, HCC in patients with PBC was speculated to evolve through multiple steps because of the presence of dysplastic nodules in the peripheries of liver tissues.[23]

CARCINOGENESIS OF HCC AND ITS RISK FACTORS

WHY DOES HCC develop in patients with PBC? PBC and PSC are typical biliary inflammatory diseases. PSC is a precursor lesion of cholangiocarcinoma,

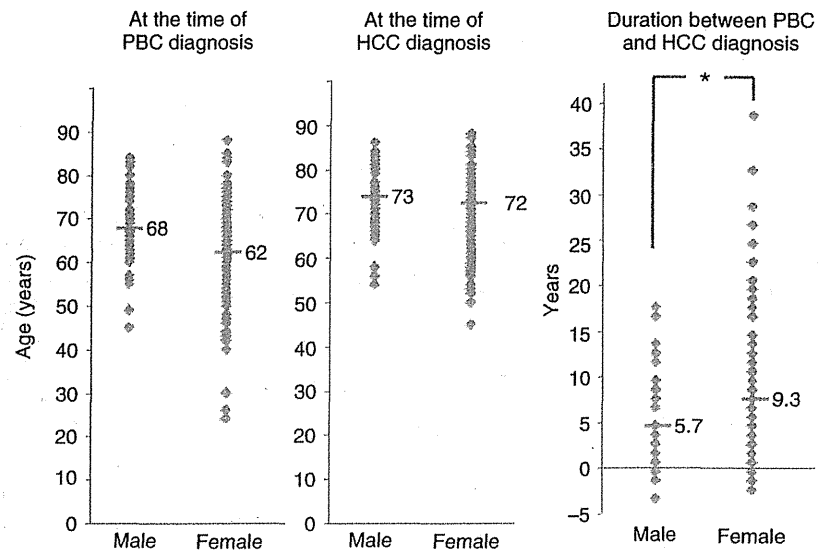

Figure 3 Average age at the time of diagnosis of primary biliary cirrhosis (PBC) and hepatocellular carcinoma (HCC), and the duration between the diagnosis of PBC and that of HCC. The duration between the diagnosis of PBC and that of HCC is shorter in males than in females.

although based on the national survey in 2003,[24] its incidence is relatively low in Japan (3.6%) compared with that in Europe and the USA (7–15%).[25] In contrast, HCC is the associated malignancy with PBC (but not cholangiocarcinoma), even though the etiology and carcinogenesis of HCC associated with PBC remain unknown. In PBC, hepatic changes as well as cholangitis are involved in its pathogenesis.[26] Therefore, this hepatic activity causing hepatocellular damage is speculated to be involved in the carcinogenesis of HCC in patients with PBC. Differing from the direct hepatocellular damage associated with virus and autoimmune reactions found in viral and autoimmune hepatitis, hepatocellular damage associated with chronic

Table 4 Clinical characteristics of patients with PBC at the time of 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 level	86%	82%	83%
ANA level	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%

Bolding denote statistically significant items.
*$P < 0.05$.
AMA, antimitochondrial antibody; ANA, antinuclear antibodies; BMI, body mass index; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; NAFLD, non-alcoholic fatty liver disease; PBC, primary biliary cirrhosis; SD, standard deviation; UDCA, ursodeoxycholic acid.

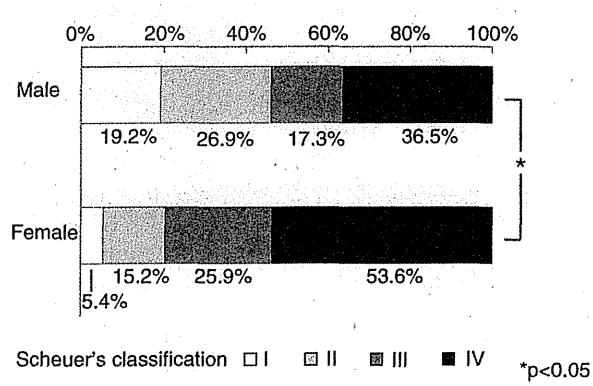

Figure 4 Histological stage according to sex at the time of hepatocellular carcinoma (HCC) diagnosis in patients with primary biliary cirrhosis. In females, the incidence of HCC gradually increases according to the histological stage, with a statistically significant difference.

Table 5 Pathological characteristics of HCC in patients with PBC with HCC

	Male	Female	Total
No. of HCC	(*n* = 49)	(*n* = 128)	(*n* = 178)
Solitary	65%	60%	62%
Multiple	35%	38%	37%
Unknown	0%	2%	2%
Differentiation of HCC	(*n* = 25)	(*n* = 41)	(*n* = 66)
Well	44%	37%	39%
Moderately	48%	56%	53%
Poorly	8%	7%	8%

HCC, hepatocellular carcinoma; PBC, primary biliary cirrhosis.

cholestasis and chronic inflammation (including interface hepatitis) may be associated with carcinogenesis of HCC in patients with PBC. In PBC, chronic cholestasis occurs from an early stage of PBC, and mitogenic factors in the bile could be directly associated with PBC carcinogenesis.[11,23,27,28]

Cirrhosis

The incidence and mortality rate of HCC in Japanese patients with PBC are significantly higher than those in the general Japanese population.[21] However, in patients with cirrhosis, the risk is considerably higher in PBC, and cirrhosis could be a risk factor of HCC irrespective of their etiologies. In Japan, PBC as an etiology of cirrhosis is observed in 2.4% cases.[29] In the cirrhotic state, stress to the hepatocytes could be associated with HCC carcinogenesis. Moreover, most female patients with PBC with HCC develop the advanced stage (including cirrhosis) at the time of HCC diagnosis (Fig. 3), supporting several reports stating that cirrhosis is a risk factor for HCC.[6,7,17,18] In contrast, Kuiper *et al.*[30] reported on the possibility that UDCA may protect against HCC. In UDCA-treated patients with PBC, the risk of HCC was relatively low, but the main risk factor for HCC was the absence of a biochemical response to UDCA and the development of cirrhosis. However, compared with females, the proportion of males with PBC with HCC was almost equally distributed among stages 1–4 (Fig. 4), suggesting that cirrhosis is a female-specific risk factor.

Males

PBC affects females more than males, but the rate of carcinogenesis is higher in males than in females. However, the male predominance of HCC is not exclusive to PBC, and is a common risk factor for developing HCC irrespective of its etiology. The reason for the rate of carcinogenesis being higher in males is speculated to be because of the inhibitory mechanism of estrogen in the carcinogenesis of HCC. The inflammatory cytokine interleukin (IL)-6 is produced by Kupffer cells and is associated with constitutive damage and malignant transformation of hepatocytes in the development of HCC. During this HCC carcinogenesis, estrogen inhibits the development of HCC by attenuating the IL-6 production from Kupffer cells.[31,32] Therefore, PBC affects females more than males, but with respect to the carcinogenesis of HCC, the estrogen deficiency-related HCC carcinogenesis is speculated to be closely associated with the high incidence of HCC in males.

PREVIOUS HBV INFECTION AND EXCESSIVE ALCOHOL INTAKE

ACCORDING TO THE 47th Annual Meeting of the Liver Cancer Study Group of Japan (2011), the time from the diagnosis of PBC to that of HCC is shorter in males than in females (Fig. 3). Moreover, the proportion of patients simultaneously diagnosed with PBC and HCC and with HCC before PBC was 32.7% in males and 14.7% in females, and the ratio of these cases in males was significantly higher than that in females (Fig. 3).[1] The reason for this significant difference is not because of the late diagnosis or underdiagnosis of HCC in males but because of the development of HCC from an early stage of PBC in males (Fig. 4). Moreover, the ratio of males with a history of HBV infection and excessive intake of alcohol was significantly higher than that of females (Table 4), suggesting that these risk factors could be associated with HCC carcinogenesis during the early stages in male patients with PBC. However, statistical analysis excluding these patients with these risk factors revealed that the incidence of HCC in males remained higher than that in females, suggesting that the male sex was not a confounding factor. Moreover, the ratio of previous HBV infection and excessive intake of alcohol was not significantly different between cirrhotic (Scheuer stage 4) and non-cirrhotic (Scheuer stages 1–3) patients, suggesting that at least these two factors are not associated with the development of cirrhosis in PBC patients with HCC.[1] Among patients with a previous HBV infection, integration of the HBV gene into the human genome has been reported to be associated with HCC carcinogenesis,[13] but the frequency and incidence of these patients among patients with PBC patients with HCC is not known.

CONCLUSION

THE DIFFERENCE BETWEEN the sexes with regard to the association of HCC among patients with PBC is an important risk factor in the HCC carcinogenesis in patients with PBC. However, it is not clear if HCC carcinogenesis is a specific mechanism for PBC. Moreover, in females, the development of cirrhosis is a risk factor for HCC in PBC. In males, HCC cases arising from an early PBC stage are not rare. Hence, male patients with PBC should be carefully followed up from an early stage to identify HCC.

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Alteration of energy metabolism in the pathogenesis of bile duct lesions in primary biliary cirrhosis

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ABSTRACT

Aim Primary biliary cirrhosis (PBC) is characterised by antimitochondrial antibody against the pyruvate dehydrogenase complex (PDC) and chronic non-suppurative destructive cholangitis (CNSDC). Pyruvate oxidation to acetyl-CoA by PDC is a key step in the glycolytic system. Oestrogen-related receptor-α (ORRα) is functionally activated by inducible coactivators such as peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α) and Bcl-3. Moreover, the PGC-1α–ORRα axis interrupts glycolytic metabolism through the upregulation of pyruvate dehydrogenase kinase, isozyme 4 (PDK4), which functionally inhibits PDC-E1α and stimulates fatty acid oxidation. In this study, we investigated the PGC-1α–ORRα axis to clarify PDC dysfunction in CNSDC of PBC.

Methods The expression of PGC-1α, Bcl-3, ORRα, PDK4 and PDC-E1α was examined by immunohistochemistry in liver sections from patients with PBC and controls. The expression of these molecules, the activity of mitochondrial dehydrogenase and PDC, and their alterations by starvation, a treatment used to induce PGC-1α expression, were examined in cultured human biliary epithelial cells (BECs).

Results The nuclear expression of PGC-1α, Bcl-3 and ORRα was exclusively observed in CNSDC of PBC. Moreover, the expression of PDK4 and PDC-E1α was enhanced in CNSDC of PBC. In cultured BECs, the amplification of Bcl-3 and PDK4 mRNAs by reverse-transcription-PCR and mitochondrial dehydrogenase activity were markedly increased but PDC activity was decreased according to the upregulation of PGC-1α.

Conclusions In CNSDC of PBC, the activation of the ORRα–PGC-1α axis was exclusively observed, suggesting the interference of PDC-related glycolytic function and the induction of the fatty acid degradation system. The switching of the cellular energy system is possibly associated with the pathogenesis of CNSDC in PBC.

INTRODUCTION

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease characterised by progressive inflammatory destruction and the disappearance of small intrahepatic bile ducts.[1 2] The most important serological feature of PBC is the presence of antimitochondrial antibodies (AMAs), which are detected in more than 95% of the PBC patients.[2] The mitochondrial components recognised by AMAs have been identified as distinct subunits of the mitochondrial 2-oxoacid dehydrogenase complexes consisting of the pyruvate dehydrogenase (PDH) complex (PDC), oxoglutaric dehydrogenase complex and branched-chain ketoacid dehydrogenase complex. These complexes loosely adhere to the inner mitochondrial membrane and consist of multiple copies of the E1, E2 and E3 subunits. Moreover, the E1 subunit of PDC exists as two forms (E1α and E1β). The dominant reactivity of AMA is against the dihydrolipoamide acetyltransferase component (E2) of PDC.[3] PDH catalyses the conversion of pyruvate to acetyl-CoA, and is an important control point in glucose and pyruvate metabolism in glycolytic metabolism.

PBC primarily affects middle-aged women, and the interlobular bile ducts are selectively damaged.[1 2] Although bile ducts have not been identified as a target organ of hormone regulation, several hormonal factors have been suggested to play important roles in the pathogenesis of PBC.[4] The oestrogen-related receptor α (ORRα) is a constitutively active nuclear hormone receptor that inhibits oestrogen receptor (OR)-dependent effects through competition with ORα.[5] ORRα is associated with mitochondrial fatty acid oxidation (β-oxidation), which includes electron transport, oxidative phosphorylation and mitochondrial biogenesis, and plays critical roles in the regulation of cellular energy metabolism.[6] Moreover, ORRα is functionally modulated by inducible coactivators such as the peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and Bcl-3, a cytokine-stimulated transcriptional regulator that synergises with PGC-1α to coactivate ORRα.[7 8] Therefore, the PGC-1α–ORRα axis induces fatty acid oxidation but simultaneously interrupts the glycolytic system through the upregulation of PDH kinase, isozyme 4 (PDK4), which binds to the E2 inner lipoyl domain (corresponding to the minimal T cell epitope) for its catalytic function and functionally inhibits PDC-E1α by phosphorylation.[9–12]

Although PBC is characterised by the presence of AMA against major autoantigens such as PDC, cellular energy metabolism that involves PDH, such as the conversion of pyruvate to acetyl-CoA in the glycolytic system, has not been observed in the bile ducts of PBC. In this study, we investigated the PGC-1α–ORRα axis to clarify the association of cellular energy metabolism in the pathogenesis of cholangiopathy in PBC.

MATERIALS AND METHODS

Patients and preparation of liver tissue

All tissue specimens were collected from the hepatobiliary file of our department. A total of 69 needle liver specimens were obtained from 26 patients with PBC (histological stage[13] I/II/III, 12/10/4;

mean age, 57 years; male/female, 3/23) and controls, which included 16 patients with hepatitis C virus-related chronic hepatitis (CH-C), five patients with primary sclerosing cholangitis and 22 patients with autoimmune hepatitis (AIH). The histopathological diagnoses were established by at least two pathologists who considered the clinical and laboratory data. All PBC patients were examined prior to ursodeoxycholic acid therapy, and AMA and/or M2 were detected in all PBC cases. All liver specimens consisted of neutral formalin-fixed paraffin-embedded tissues; 4-μm-thick sections were prepared for routine histological examinations and immunohistochemistry.

Immunohistochemistry

After deparaffinisation, the sections were pretreated in Target Retrieval Solution (Dako Japan Co, Tokyo, Japan) in a microwave oven or water bath at 95°C for 20 min to improve the antigenicity of the tissue prior to immunostaining.[14 15] Following endogenous peroxidase blocking in methanolic hydrogen peroxide for 20 min and incubation in normal goat serum (diluted 1:10; Vector Laboratories, Inc, Burlingame, California, USA) for 20 min, the sections were incubated at 4°C overnight with primary antibodies against PGC-1α (rabbit polyclonal, diluted 1:100, Bethyl Laboratories, Inc, Montgomery, Texas, USA), Bcl-3 (mouse monoclonal, clone 1E8, diluted 1:50, Abcam Japan, Tokyo, Japan), ORRα (mouse monoclonal, clone 1ERR87, 1 μg/mL, Santa Cruz Biotechnology, Inc, Santa Cruz, California, USA), PDK4 (mouse monoclonal, clone 1A10, 1 μg/mL, Abnova Corporation, Taipei City, Taiwan) and PDC-E1α (mouse monoclonal, clone 9H9AF5, 5 μg/mL, Abcam Japan). The CSA system (Dako Japan Co) was used for ORRα, and the Envision-HRP system (Dako Japan Co) was used for the others. After the benzidine reaction, the sections were weakly counterstained with haematoxylin. No positive staining was obtained when the primary antibodies were replaced with an isotype-matched, non-immunised immunoglobulin, which was used as a negative control for the staining procedures.

Histological examination

We primarily examined the interlobular bile ducts in these patients, including those with chronic non-suppurative destructive cholangitis (CNSDC), in this study because they are selectively affected in PBC.[1] For the PBC patients, in addition to evaluating their histological stages, the histological activity for chronic cholangitis was evaluated according to Nakanuma's system.[13] In brief, chronic cholangitis activity (CA) was categorised into four grades (CA0–3) according to the degree and distribution of chronic cholangitis. 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 showed evident

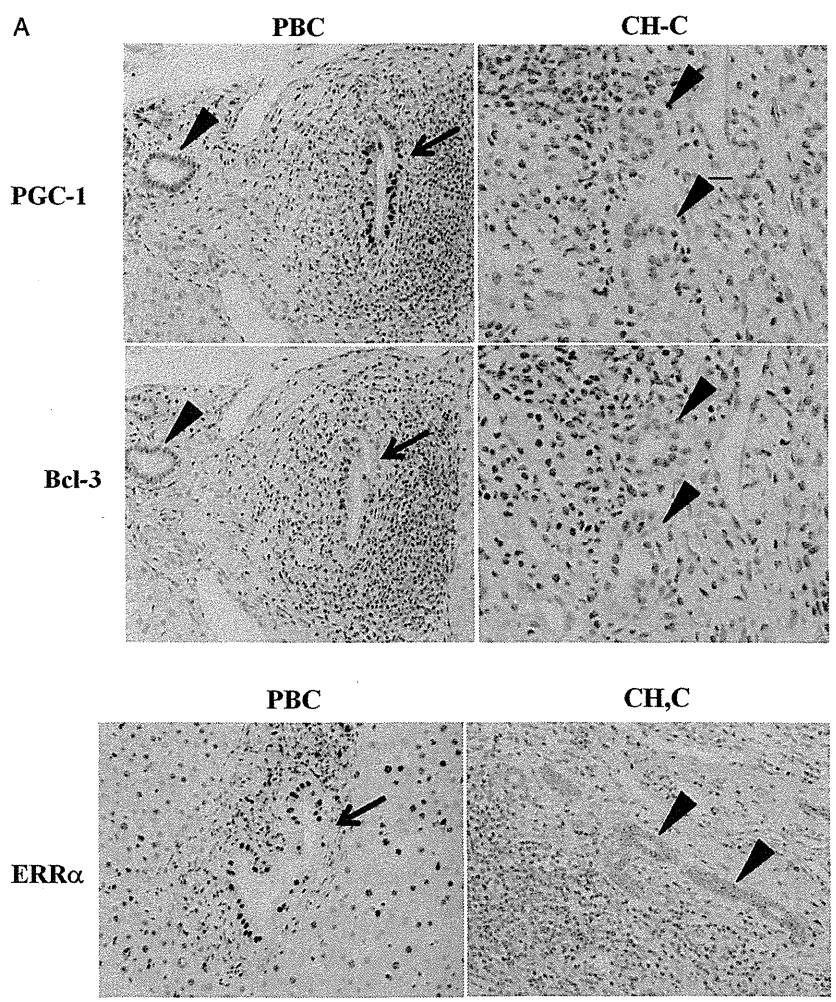


Figure 1 The expression of peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, Bcl-3 and oestrogen-related receptor α (ORRα) in liver tissue and its correlation with chronic cholangitis activity (CA). (A) Immunohistochemistry for PGC-1α, Bcl-3 and ORRα. Biliary epithelial cells with chronic non-suppurative destructive cholangitis of primary biliary cirrhosis (PBC) expressed PGC-1α and ORRα strongly in the nucleus and weakly in the cytoplasm and expressed Bcl-3 in the nucleus (arrows). However, undamaged (almost normal) bile ducts with PBC and bile ducts with hepatitis C virus-related chronic hepatitis (CH-C) were negative for these molecules (arrowheads). (B) There was good correlation between the expression of PGC-1α and Bcl-3 compared with the degree of chronic cholangitis (CA) in PBC. PGC-1α; r=0.69, p value=0.001, Z value=3.20, Z (0.975)=1.95. Bcl-3; r=0.66, p value=0.0009, Z value=3.11, Z (0.975)=1.95. (C) Semiquantitative analyses revealed that the expression of these molecules in bile ducts was higher in PBC than that in controls. PGC-1α, p value=0.002; Bcl-3, p value<0.001; ORRα, p value<0.001. Moreover, the statistical analysis excluding positive reactive cases (+) also revealed that the ratio of strong positive cases (++) were significantly higher in PBC than those in controls. PGC-1α, p value=0.004; Bcl-3, p value<0.001; ORRα, p value<0.001.

chronic cholangitis. In CA3 (marked activity), at least one damaged bile duct showed CNSDC and/or granulomatous cholangitis. For the semiquantitative evaluations of the immunohistochemistry, representative portal tracts that contained the interlobular bile ducts, including those with CNSDC, were chosen in each section for assessment. The immunoreactivity in the bile ducts was semiquantitatively graded as follows: negative (−), weakly positive (+) or strongly/enhanced positive (++).

Cultured human BECs and the induction of PGC-1α

Two cultured human biliary epithelial cell (BEC) lines were isolated from the explanted liver of two PBC patients. Informed consents to conduct research were obtained from both patients. These cells were grown as monolayers in a standard medium containing 20% fetal calf serum (Life Technologies Japan, Tokyo, Japan) in a 5% CO_2-humidified incubator at 37°C.[16] The cell lines were confirmed to be BECs by the expression of the biliary-type cytokeratins CK7 and CK19 (>99%) and also aquaporin 1 (>90%). BECs were used between passages 6 and 10 for this study. To induce the expression of PGC-1α, the cultured BECs were treated by starvation (1% fetal calf serum),[17 18] and the following examinations were performed.

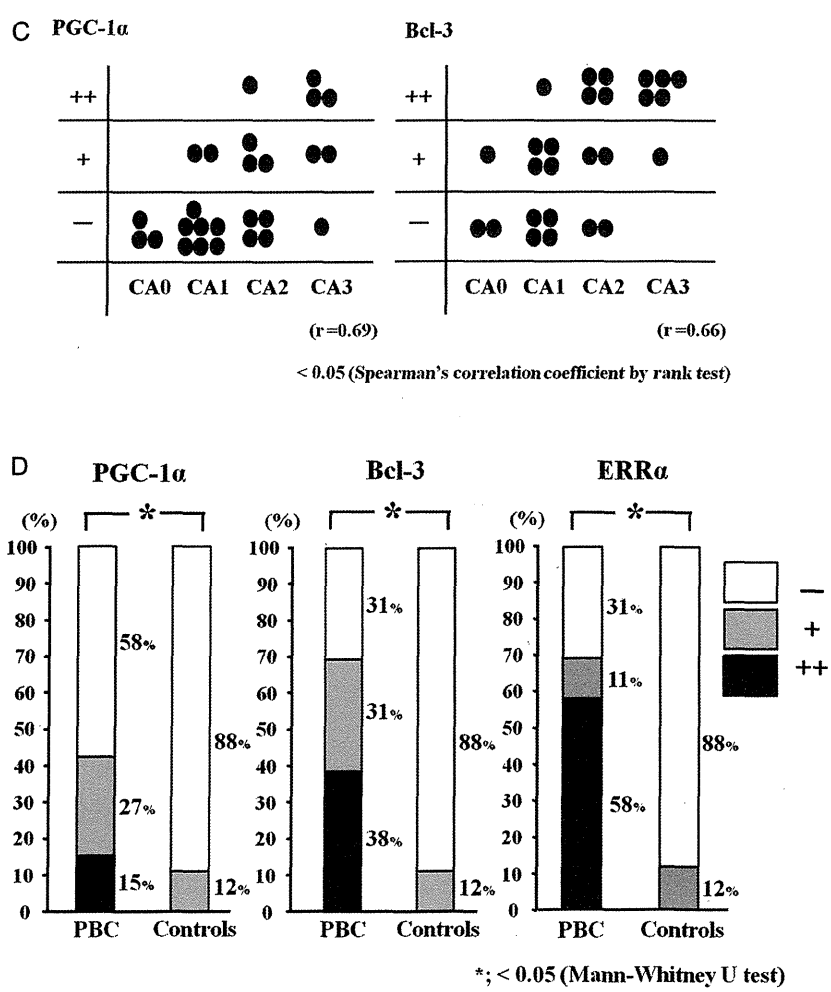
Assessment of cell viability and mitochondrial dehydrogenase activity

Approximately 1×10^4 cells/well placed into 96-well plates were treated by starvation to induce PGC-1α. Cell counts and mitochondrial dehydrogenase activity were measured 24 h later with a microplate reader using the DNA-IdU Labeling and Detection Kit (Takara Bio Inc, Otsu, Japan) and the tetrazolium salt WST-1 assay (Roche Diagnostics GmbH, Penzberg, Germany) according to the manufacturer's instructions. Mitochondrial dehydrogenase activity per cell was presented as a relative ratio of the optical density in WST-1/DNA-IdU labelling assays.

Isolation of RNA and real-time RT-PCR

The basal levels of expression of ORRα, PGC-1α, Bcl-3, PDK4 and PDC-E1α mRNAs and their alterations by starvation were examined by reverse-transcription-PCR (RT-PCR) and real-time PCR, respectively. Total RNA was extracted from cultured BECs using the RNeasy Total RNA System (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Following this, 1 μg of total RNA was reverse transcribed with an oligo-(dT) primer and ReverTra Ace (Toyobo Co, Osaka, Japan) to synthesise a cDNA template for PCR. For 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 (Agilent Technologies, Tokyo, Japan), and the relative levels of gene expression were calculated using the comparative cycle threshold method. The specific primers were as follows: PGC-1α: forward, 5′-TTGGTAACCGAACTGGTGCT-3′ and reverse, 5′-GTGCAAAGTTCCCTCTCTGC-3′; Bcl-3: forward, 5′-CCC

Figure 1 (Continued)



TATACCCCATGATGTGC-3′ and reverse, 5′-GGTGTCTGCC GTAGGTTGTT-3′; ORRα: forward, 5′-TGGCTACCCTC TGTGACCTC-3′ and reverse, 5′-CTCATCCTGCAGTGGCA GT-3′; PDK4: forward, 5′-GGCCTAGTGTTGTGGTGCTT-3′ and reverse, 5′-GAGCTGGACTCCCACCATTA-3′; PDC-E1α: forward, 5′-GTCAGTGCTTCAAGCCAACA-3′ and reverse, 5′-TTAAACTGCAGCCTGCCTTC-3′; and glyceraldehyde 3 phosphate dehydrogenase (internal positive control): forward, 5′-GGCCTCCAAGGAGTAAGACC-3′ and reverse, 5′-AGGGG TCTACATGGCAACTG-3′. The results were obtained from two independent experiments and are presented as the relative levels of mRNA expression compared with the levels without any treatments. Negative controls were obtained by replacing the reverse transcriptase or cDNA samples with RNase- and DNase-free water.

Quantitative and functional assessments of PDK4 expression and PDH activity

The levels of expression of PDK4 and PDH activity and the alterations in them resulting from the induction of PGC-1α by starvation in cultured BECs were measured using the PDK4 Human ELISA Kit and the PDH Enzyme Activity Microplate Assay Kit, respectively, according to the manufacturer's instructions.

Statistical analysis

The data were analysed using Mann–Whitney U test, paired t test, Wilcoxon signed-ranks test and Spearman's correlation coefficient by rank test. p Values <0.05 were considered statistically significant.

RESULTS

Exclusive expression of PGC-1a, Bcl-3 and ORRα in the damaged bile ducts of PBC

The expression of PGC-1α and ORRα was detected in the cytoplasm and nucleus of positive cells, and the nuclear expression indicated the activated forms. This was considered to be functional positivity. The expression of Bcl-3 was limited to the nucleus of positive cells. In the controls, strong nuclear expression of PGC-1α, Bcl-3 and ORRα was not detected in any interlobular bile ducts, including mildly injured bile ducts (hepatic bile duct injury) in CH-C and AIH. The bile ducts that exhibited almost normal or mild cholangitis also lacked these molecules in PBC; however, the damaged bile ducts that showed moderate to severe

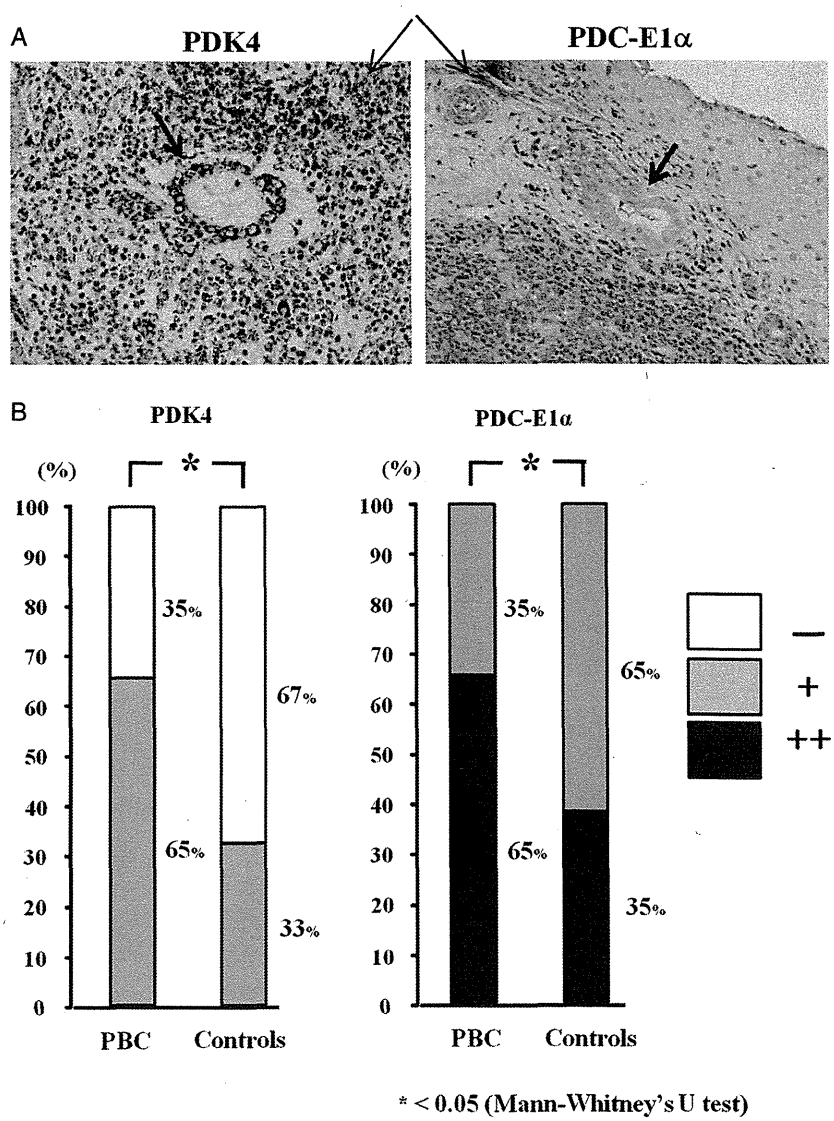


Figure 2 The expression of pyruvate dehydrogenase kinase isozyme 4 (PDK4) and pyruvate dehydrogenase complex (PDC)-E1α in liver tissue. (A) Tissue from patients with chronic non-suppurative destructive cholangitis in primary biliary cirrhosis (PBC) weakly expresses PDK4 and strongly expresses PDC-E1α in cytoplasmic patterns. (B) Semiquantitative analyses revealed that the expression of PDK4 was negative or weakly positive in the interlobular bile ducts, and the frequency of weakly positive bile ducts was higher in PBC patients than that in controls. PDC-E1α was basically expressed (weakly or strongly positive) in the interlobular bile ducts, and the frequency of strongly positive bile ducts was higher in PBC patients than that in controls. PDK4, p value=0.008; PDC-E1α, p value=0.015.

cholangitis, including CNSDC in PBC, preferentially expressed PGC-1α, Bcl-3 and ORRα in a nuclear pattern. As shown in figure 1, the nuclear expression of PGC-1α, Bcl-3 and ORRα in the bile ducts was exclusive in PBC, and the levels of expression of PGC-1α and Bcl-3 correlated well with the degree of CA activity in PBC.

Expression of PDK4 and PDC-E1α in bile ducts

The expression of PDK4 was basically negative or weakly positive in the interlobular bile ducts, and bile ducts exhibiting strong positivity were not observed (figure 2). However, the frequency of weakly positive bile ducts was higher in PBC compared with that in controls (figure 2). In contrast, PDC-E1α was consistently expressed in all interlobular bile ducts (figure 2). Strong expression was more frequent in the bile ducts of PBC patients (figure 2).

Effects of the induction of PGC-1α in cultured BECs on mitochondrial dehydrogenase activity

To investigate the alterations in mitochondrial dehydrogenase activity by the induction of PGC-1α in cultured BECs, we analysed the relative cell counts and mitochondrial dehydrogenase activities in starved BECs using WST-1 and DNA-IdU labelling assays, respectively. As shown in figure 3, the induction of PGC-1α by starvation downregulated the cell number; however, it did not affect the degree of total mitochondrial dehydrogenase activity. Therefore, the mitochondrial dehydrogenase activity per cell that was presented as the relative ratio of optical density values was increased in the starved BECs by approximately twofold compared with that in non-starved (static) BECs (figure 3).

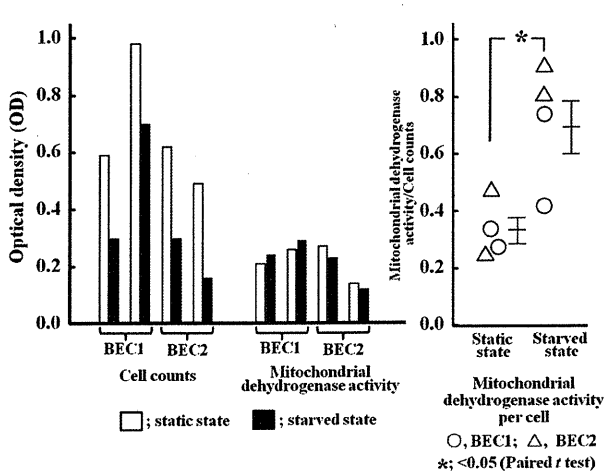


Figure 3 Mitochondrial dehydrogenase activity in cultured biliary epithelial cells (BECs). The number of cells that were starved was decreased; however, the degree of mitochondrial dehydrogenase activity remained significantly unchanged in total. Mitochondrial dehydrogenase activity per cell, which is presented as the relative ratio of optical density, was significantly higher in starved BECs by 0.69±0.10-fold (mean±SEM) than that in untreated (static) BECs (0.32±0.04-fold). p Value=0.013. Bars indicate mean and SEM. In the induction of peroxisome proliferator-activated receptor γ coactivator-1α expression by the starvation of cultured BECs, cell counts and mitochondrial dehydrogenase activity were measured with WST-1 and DNA-IdU labelling assays, respectively. The results were obtained from two independent experiments with two cell lines of cultured BECs.

Expression of Bcl-3, ORRα, PDK4 and PDC-E1α and their alterations by the induction of PGC-1α in cultured BECs

RT-PCR analysis revealed ~~that~~ the amplification of all mRNAs of PGC-1α, ORRα, PDK4 and PDC-E1α in cultured human BECs (figure 4A). Moreover, real-time PCR analysis demonstrated that the levels of amplification of mRNAs of PGC-1α, ORRα and PDK4 were statistically increased according to the upregulation of PGC-1α by starvation, although the degrees of increase varied (paired t test or Wilcoxon signed-ranks test) (figure 4B).

Assessment of PDK4 expression and PDH activity

PDK4 ELISA demonstrated that the levels of expression of PDK4 were significantly upregulated by the induction of PGC-1α by starvation in cultured BECs. In contrast, PDH enzyme activity was decreased in the starved state (figure 4C).

DISCUSSION

In contrast to OR, ORRα and its related family members, ORRβ and ORRγ, do not have known ligands; therefore, they are called orphan nuclear receptors. ORRα plays key roles in the gene regulatory control of the mitochondrial energetic systems. ORRα has been identified as a novel PGC-1α-binding partner, and the ORR family members must interact with PGC-1 coactivators to be transcriptionally active.[19] Bcl-3 synergises with PGC-1α to coactivate ORRα, and the complex of ORRα, PGC-1α and Bcl-3 is found on an ORRα-responsive element within the PDK4 gene promoter.[8] In mitochondrial energy systems, ORRα regulates fatty acid degradation (β-oxidation), which is an aerobic and more effective energy metabolic system compared with the anaerobic glycolytic system. Therefore, the PGC-1α–ORRα axis plays a key role in various aspects of cellular energy homeostasis, including mitochondrial biogenesis, thermal regulation and glucose metabolism.[10 20] Consistent with this function, ORRα is prominently expressed in tissues that have a high capacity for the β-oxidation of fatty acids, such as the heart, brown fat and skeletal muscle.[21] In the liver, ORRα and PGC-1α are expressed at low levels; however, they are induced in fasting animals.[18] Although these metabolic studies have been conducted in liver hepatocytes,[18 22] there have been no reports regarding the metabolic state of BECs and the dysregulations of BECs in human biliary diseases, including PBC. The present study revealed that the activated expression of ORRα, PGC-1α and Bcl-3 in a nuclear pattern was exclusively observed in the CNSDC of PBC. Although bile ducts that are similarly damaged from hepatic bile duct injury or hepatitis-associated bile duct injury has been observed in AIH and CH-C, no or weak ORRα expression in the nuclei was observed in these damaged bile ducts. However, the expression of PGC-1α and Bcl-3 correlated well with the degree of chronic CA in PBC, indicating that the cooperative expression of the ORRα–PGC-1α axis was closely associated with the energy metabolic responses in the pathogenesis of CNSDC in PBC.

The PDC-E1 enzyme is a heterotetramer of two α and two β subunits. The E1α subunit, which contains the E1 active site, plays a key role in the function of PDC. PDK decreases PDH activity through the phosphorylation of PDC-E1α. Three serine phosphorylation sites on PDC-E1α are targeted by PDKs, and the phosphorylation of PDC-E1α completely inhibits the activity of PDH.[23] There is increased phosphorylation of PDC in the heart and skeletal muscle in cases of starvation and diabetes, and this allows pyruvate to be conserved while mitochondrial fatty acid oxidation is increased.[24] Four PDK isoenzymes (PDK-1, -2, -3 and -4) have been identified. The expression of

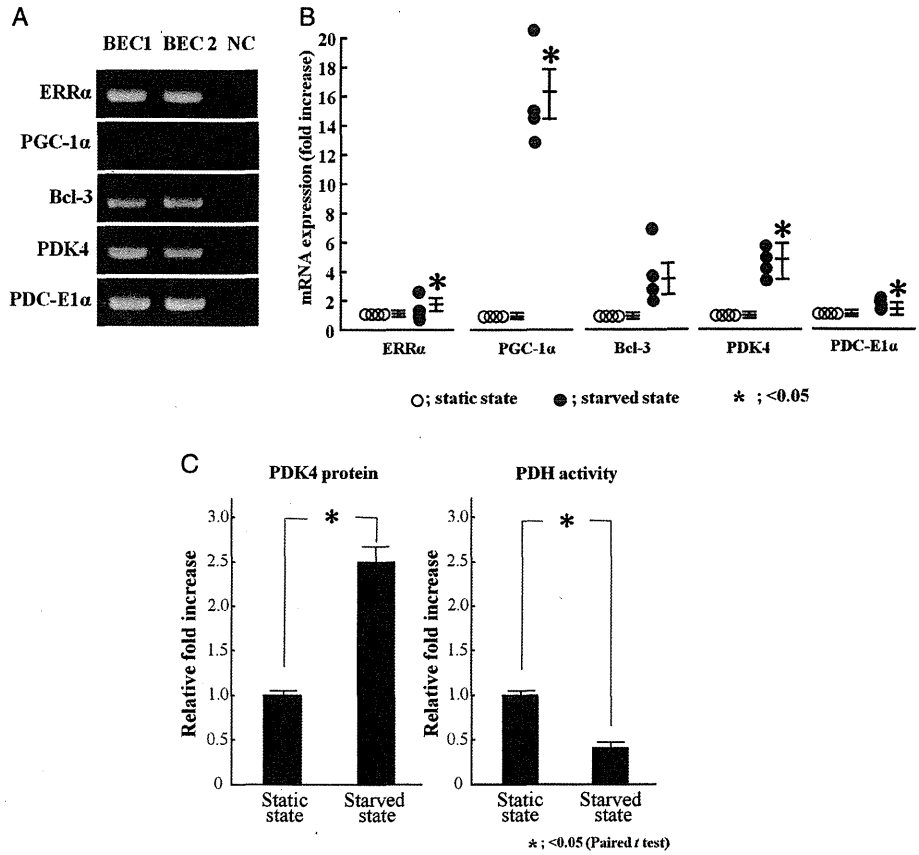


Figure 4 The expression of Bcl-3, oestrogen-related receptor α (ORRα), pyruvate dehydrogenase kinase isozyme 4 (PDK4), and pyruvate dehydrogenase complex (PDC)-E1α and pyruvate dehydrogenase (PDH) activity in cultured biliary epithelial cells (BECs). (A) RT-PCR was performed for 40 cycles, and amplification of ORRα, peroxisome proliferator-activated receptor γ coactivator (PGC)-1α, Bcl-3, PDK4 and PDC-E1α was detected as a single band from cultured BECs at the expected sizes. Negative controls were obtained by replacing the reverse transcriptase with RNase- and DNase-free water for the reverse transcription. (B) Real-time PCR analyses demonstrated that the fold-increase of ORRα, PGC-1α, Bcl-3, PDK4 and PDC-E1α by starvation were 1.7±0.2- (mean±SEM, p value=0.048), 16.3±1.8- (p value=0.003), 3.5±1.3- (p value=0.19), 4.6±0.4- (p=0.004) and 1.3±0.1 (p=0.030)-fold, respectively. (C) An ELISA for PDK4 demonstrated that the expression of PDK4 was significantly upregulated by 2.5±0.2-fold by the induction of PGC-1α (starved state) in cultured BECs (p=0.01). In contrast, PDH enzyme activity was decreased by 0.4±0.01-fold in the starved state (p<0.001). The results were obtained from two independent experiments with two cell lines and are shown as the relative levels of expression compared with the levels without any treatments (static state).

PDK4 is suppressed under basal conditions in most tissues; however, its expression is increased by starvation, glucocorticoids, diabetes, a high-fat diet and extended exercise through the PGC-1α–ORRα axis, particularly in the heart, skeletal and other muscle tissues, kidney, and liver. PDK4 overexpression prevents glucose oxidation.[10 24 25] The present study revealed the expression of PDK4 and its increases according to the induction of PGC-1α by starvation in cultured human BECs. Moreover, functional analysis confirmed the decrease in PDH function. These findings suggested that the functions of PDH in human BECs are regulated by the ORRα–PGC-1α axis and that in CNSDC, the exclusive expression of the PGC-1α–ORRα axis and the enhanced expression of PDK4 result in PDH dysfunction through PDK4.

Although samples with CNSDC have reactive findings such as enlargement compared with the original size of the bile duct and increased mitochondria,[1 26 27] these damaged bile ducts finally undergo disappearance (bile duct loss),[28] mainly through biliary apoptosis. This unique finding concerning the histogenesis of CNSDC in PBC can be explained by metabolic alterations. In other words, switching from the utilisation of glucose to that of fatty acids as an energy source results in a more effective energy system, indicating that metabolic switching by ORRα/PGC-1α increases the metabolic activity of CNSDC in PBC. The in vitro study of cultured BECs demonstrated that the induction of PGC-1α by starvation caused an increase in mitochondrial dehydrogenase activity per cell. Although the oxidative phosphorylation of fatty acids is a vital part of metabolism, it produces reactive oxygen species such as superoxides and hydrogen peroxide, which lead to the propagation of free radicals and result in damaged and apoptotic cells and contribute to several diseases. In fact, several reports have already demonstrated that increased oxidative stress and enhanced biliary apoptosis are observed in the bile ducts of PBC patients.[28–31] Therefore, metabolic switching from glycolytic systems to fatty acid oxidation has been speculated to cause an increased susceptibility to the apoptotic induction of CNSDC in PBC through oxidative stress.

ORRs share homology with ORs; however, ORRs do not bind to oestrogen or other known physiological ligands. Therefore, ORRs inhibit OR-dependent oestrogen effects through competition with ORα. ORs form homodimers or heterodimers that consist of ORα and ORβ, which bind to an oestrogen-response element and affect cell proliferation and the promotion of apoptosis and differentiation, respectively.[32] Both

ORα and ORβ are observed in bile ducts in the early stages of PBC; however, the disappearance of ORα in bile ducts during the cirrhotic stage and an oestrogenic deficiency have been speculated to accompany the evolution of PBC toward ductopenia.[4] In addition to this loss of ORα, the present study revealed that the activated expression of ORRα in a nuclear pattern was exclusively found in CNSDC of PBC, indicating that the activation of ORRα inhibits the cell-proliferating function of ORα and induces the bile duct loss caused by regenerative failure in CNSDC of PBC.

In conclusion, we demonstrated the activation of the ORRα–PGC-1α axis and the upregulation of PDK4 in CNSDC of PBC, suggesting an interference in PDH function and the switching from glycolytic to fatty acid oxidation. Moreover, the dysfunction of PDH which are major epitopes for AMA suggests any associations with the pathogenesis of AMA. Further studies are required to clarify the aetiology and mechanisms underlying the activation of the ORRα–PGC-1α axis in vivo and the production mechanism of AMA. Although compensatory responses to some bile duct injuries have been suggested, metabolic switching was possibly associated with the pathogenesis of CNSDC and the consequent bile duct loss in PBC. The enzymes conducting the correction of the metabolic system in BECs may also be the target of drugs in PBC.

Take home messages

- In chronic non-suppurative destructive cholangitis (CNSDC) of primary biliary cirrhosis (PBC), the activation of the oestrogen-related receptor α–peroxisome proliferator-activated receptor γ coactivator-1α axis is exclusively observed.
- The interference of pyruvate dehydrogenase complex-related glycolytic function and the induction of the fatty acid degradation system are speculated in CNSDC of PBC.
- The switching of the cellular energy system is possibly associated with the pathogenesis of CNSDC in PBC.

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今月の話題

原発性胆汁性肝硬変の新しい病期分類・活動度分類

原田憲一*，中沼安二*

はじめに

原発性胆汁性肝硬変 primary biliary cirrhosis (PBC) は隔壁胆管から小葉間胆管レベルの肝内小型胆管を標的とする自己免疫疾患で，慢性非化膿性破壊性胆管炎 chronic non-suppurative destructive cholangitis (CNSDC) と胆管消失が基本病態である．血清学的にはPBC患者の90%以上に抗ミトコンドリア抗体 antimitochondrial antibody (AMA) が検出される．現在，AMAの主要対応抗原である pyruvate dehydrogenase complex の E_2 成分 (PDC-E_2) などのリコンビナント蛋白を用いた高感度かつ特異性の高いAMAの検出が行われるようになり，血清学的診断技術の向上および病態の解析が進んだ．一方，治療に関してもウルソデオキシコール酸 ursodeoxycholic acid (UDCA) やベザフィブレートによる薬物治療の有効性が確認されている．同時にPBCの病態の多様性も明らかとなり，PBCの診断のみならず治療選択のための病態の把握が，PBCの病理診断に求められつつある．

本稿では，2012年，厚生労働省難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班「PBCの診断ガイドライン」で推奨されている，PBCの新しい組織学的病期および活動度分類を紹介する[1-4]．

Ⅰ．PBCの新しい病期分類

PBCの組織学的病期分類としてScheuer分類[5]が長らく親しまれてきた．簡便な方法ではあるが，CNSDC (florid duct lesion) や細胆管反応 (細胆管増生) などの単一の組織所見に基づく分類法であるがゆえに，病期を特徴づける所見が重複し判定に苦慮する症例があり，また肝針生検ではサンプリングエラーも起こりやすい．一方，PBC新病期分類は複数の組織所見を基に評価するため，病期分類を評価する際の矛盾やサンプリングエラーの問題を回避することができる．PBCの病期を規定する所見として胆管消失，肝線維化，銅沈着を因子解析により選出し[1]，**表1**に示すごとく，線維化，胆管消失，さらにオルセイン染色標本がある場合にはオルセイン陽性顆粒沈着 (銅沈着) の程度を各々スコア0～3に評価し，2組織所見または3組織所見のスコアの合計点を基にStage 1 (no progression)，Stage 2 (mild progression)，Stage 3 (moderate progression)，Stage 4 (advanced progression) の4段階に病期分類する．なお，オルセイン染色は染色方法が難しいこともあり汎用されていない施設が多いが，当教室での染色プロトコールと代表的な染色結果を示す (**図1**)．

Ⅱ．新病期分類とScheuer病期分類との比較

PBC肝針生検材料を用いて新病期分類とScheuer分類との比較を，2つの異なった母集団を対象に解析を行った[3,4]．いずれの解析結果も，Scheuer分類ではStage 1症例が最も割合が高かったが，新分類ではStage 2が最も割合が高く，病初期症例の分布に大きな違いがみられた．新病期分類の大きな特徴の一つはStage 1を「no progression」として定義した点である．PBCの極めて病初期の段階と解釈しうる群であり，新分類Stage 1症例の10年後生存率は100%という結果も得られている[3,4]．また，Scheuer分類Stage 3相当で肝移植が施行された症例をしばしば経験する．このような症例は前硬変肝であるが，高度の胆汁うっ滞をきたした肝不全の状態であり，新病期分類では胆管消失やオルセイン陽性顆粒沈着が高スコアとなりStage 4に分類される．単に肝硬変期と定義するScheuer分類Stage 4とは異なり，新分類ではこのような肝移植適応となる肝不全症例がStage 4と分類され，臨床的にも意義のある分類である．また，予後調査結果に基づく生存解析においても，Scheuer分類と比べて新病期分類では予後と良好な相関が得られ，新病期分類の予後予測に関する有用性，さらに病期分類としての妥当性が証明されている．

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1. 脱パラフィン	
2. 流水水洗	5分
3. 0.25%過マンガン酸カリウム 0.2%硫酸混合液(酸化液)	1分
4. 流水水洗	5分
5. 2%シュウ酸(還元液)	1分
6. 流水水洗	5分
7. 蒸留水	1分
8. オルセイン液*	2〜3時間
9. 70%エタノール	1分
10. 脱水	
11. 透徹	
12. 封入	

*：オルセイン液

オルセイン(メルク)	0.5 g
70%エタノール	100 mL
濃塩酸	0.8 mL

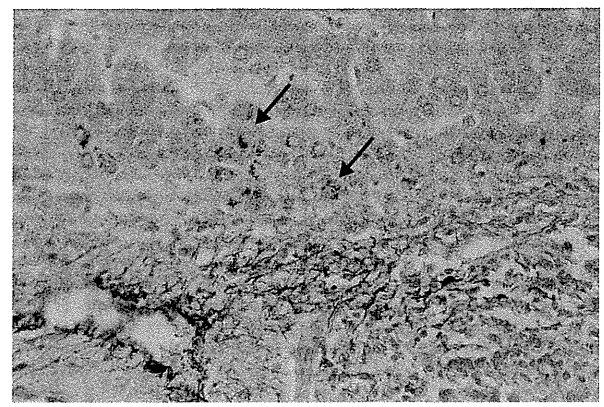

図1 当教室でのオルセイン染色 門脈域周辺の多数の肝細胞にオルセイン陽性顆粒(銅関連蛋白)の沈着(矢印)を認める.

表1 PBCの組織学的新病期分類

線維化のスコア	Score 0	門脈域での線維化がないか，あるいは線維化が門脈域に限局	
	Score 1	門脈域周囲の線維化あるいは不完全な線維性隔壁を伴う門脈域線維化	
	Score 2	種々の小葉構造の乱れを伴う架橋性線維化	
	Score 3	再生結節と高度の線維化を伴う肝硬変	
胆管消失のスコア	Score 0	胆管消失がない	
	Score 1	1/3以下の門脈域で胆管消失をみる	
	Score 2	1/3〜2/3の門脈域で胆管消失をみる	
	Score 3	2/3以上の門脈域で胆管消失をみる	
オルセイン陽性顆粒沈着のスコア	Score 0	陽性顆粒の沈着なし	
	Score 1	1/3以下の門脈域周辺の肝細胞(少数)に陽性顆粒の沈着をみる	
	Score 2	1/3〜2/3 の門脈域周辺の肝細胞(種々の程度)に陽性顆粒の沈着をみる	
	Score 3	2/3以上の門脈域周辺の肝細胞(多数)に陽性顆粒の沈着をみる	
上記組織病変スコアの合計による病期診断	Stage	3因子スコアの合計(線維化＋胆管消失＋オルセイン陽性顆粒沈着)	2因子スコアの合計(線維化＋胆管消失)
	Stage 1 (no progression)	0	0
	Stage 2 (mild progression)	1〜3	1〜2
	Stage 3 (moderate progression)	4〜6	3〜4
	Stage 4 (advanced progression)	7〜9	5〜6

Ⅲ. PBCの活動度分類

近年，ウイルス性慢性肝炎に加えて非アルコール性脂肪性肝炎 non-alcoholic steatohepatitis (NASH) においても，病勢を反映する壊死炎症性変化を評価した活動度分類が用いられており，患者の病態を把握したり，治療方針を決定するための一助となっている[6]．PBCの活動性を反映する所見としてCNSDCを含む胆管炎，インターフェイス肝炎，小葉炎を因子解析により抽出し，慢性胆管炎 (CA) および肝炎 (HA) の程度を個別に0 (no activity)，1 (mild activity)，2 (moderate activity)，3 (marked activity) の4段階にスコア化し評価する[1,2] (**表2**)．慢性胆管炎の活動度 (CA) に関しては，明瞭な慢性胆管炎とCNSDCの所見の有無と程度を基に評価し，肝炎の活動度 (HA) に関してはインターフェイス肝炎と小葉炎の有無と程度を基に評価する．PBC肝針生検を用いて本活動度分類に従って評価した結果，肝炎の活動度 (HA) はHA0〜HA1の低いスコア症例が多い．門脈域炎と小葉炎からなる慢性活動性肝炎様の所見はPBCの診断価値が低い所見であるが，PBCの肝炎性の病勢を反映し病期進展に加担することが明らかとなってきている．

PBCとAIH (autoimmune hepatitis) の病像が同時に，あるいは異時性に共存する病態はオーバーラップ症候群と呼ばれ，現在Chazouillèresら[7]によって診断基準が提唱されている．しかし，その病態は未だ不明であり，肝炎性変化の目立つPBC (肝炎型PBC) として病態を解釈する知

表2　PBCの組織学的活動度分類

慢性胆管炎の活動度 chronic cholangitis activity (CA)	CA0 (no activity) CA1 (mild activity) CA2 (moderate activity) CA3 (marked activity)	・胆管炎がない，あるいは軽度の胆管上皮障害をみる． ・明瞭な慢性胆管炎を1ヵ所にみる． ・明瞭な慢性胆管炎を2ヵ所以上にみる． ・CNSDC を少なくとも1ヵ所にみる．
肝炎の活動度 hepatitis activity (HA)	HA0 (no activity) HA1 (mild activity) HA2 (moderate activity) HA3 (marked activity)	・インターフェイス肝炎がない．小葉炎はないか，軽微． ・1ヵ所の門脈域または線維性隔壁周囲の肝細胞10個程度にインターフェイス肝炎をみる．軽度～中等度の小葉炎をみる． ・2ヵ所以上の門脈域または線維性隔壁周囲の肝細胞10個程度にインターフェイス肝炎をみる．軽度～中等度の小葉炎をみる． ・半数以上の門脈域周囲の肝細胞20個程度にインターフェイス肝炎をみる．中等度の小葉炎，あるいは架橋性や帯状壊死をみる．

CNSDC：chronic non-suppurative destructive cholangitis.

見もあり[8]，PBC活動度分類でHA3 (marked activity) を示すPBC症例はオーバーラップ症候群の指標の一つになる可能性もある．また，オーバーラップ症候群はUDCAに加えて副腎皮質ステロイドなどの免疫抑制剤が奏効するという報告が多いが，どのような症例にステロイドを投与すべきかについての投与基準は未だ定められていない．本邦の33例のオーバーラップ症候群症例を用いた解析では，PBCに比べHAスコアが高く，AIH的要素が強い．現在，AIHの診断には国際AIHグループが提唱している新AIH国際診断基準 (Simplified criteria，2008年)[9]が使われているが，病理組織所見の項目をHAスコアに置き換えた“modified”simplified AIHスコアによって，比較的高い特異度でステロイド治療が必要となるオーバーラップ症候群症例を抽出できる可能性が示されている（詳細は文献[10]，または厚生労働省難治性疾患克服研究事業「難治性の肝・胆道疾患に関する調査研究」班「PBCの診断ガイドライン」を参照）．PBCの活動度評価は病態や病勢の把握，さらに治療効果や治療選択，予後の判定に有用な指標になるものと思われる．

おわりに

今月の話題として，2012年本邦の「PBCの診断ガイドライン」で推奨されているPBCの新しい組織学的病期分類および活動度分類を紹介した．なお，本分類は組織学的診断基準ではなく，あくまでも臨床病理学的にPBCと診断された症例に対しての病期/活動度分類であることを明記したい．したがって，病期や活動度を規定する組織所見のみを観察するのではなく，肉芽腫，小細胞性ディスプラジア様の変化，nodular regenerative hyperplasia等などのPBCに特徴的な所見も注目し，診断する必要がある．

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