Acta Med. Okayama, 2015
Vol. 69, No. 3, pp. 137–144

Acta Medica Okayama
http://escholarship.lib.okayama-u.ac.jp/amo/

Original Article

Aberrant Expression of Keratin 7 in Hepatocytes as a Predictive Marker of Rapid Progression to Hepatic Failure in Asymptomatic Primary Biliary Cirrhosis

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A predictive marker of the rapid progression to hepatic failure is desired for patients with asymptomatic primary biliary cirrhosis (aPBC). We performed a systematic cohort analysis of 101 patients diagnosed as having aPBC and the rapid progression to liver failure in some, by focusing on cholestasis. Cholestasis was assessed by aberrant keratin7 (K–7) expressions in the patients' hepatocytes. Intralobular expressions of K–7 were found in 9 of the 101 patients. The grades of K–7 expression were significantly associated with the levels of alanine aminotransferase, alkaline phosphatase, and total bilirubin at the time of diagnosis, but not with bile duct loss or cholestasis. Stepwise logistic regression analysis revealed that high grades of K–7 expression correlated positively with high levels of total bilirubin. During the follow-up period, 8 patients developed jaundice, and the mean period until the development of jaundice was 5.2 years. The proportional hazards models for the risk of developing jaundice identified a high grade of aberrant K–7 expression in hepatocytes as the only significant risk factor. Aberrant K–7 expression in hepatocytes can be used as an additional marker to predict rapid progression to liver failure in patients with aPBC at the time of diagnosis.

Key words: primary biliary cirrhosis, keratin 7, hepatic failure

Primary biliary cirrhosis (PBC) is a chronic cholestatic disease characterized by the progressive destruction of small septal and interlobular bile ducts [1–3]. The recent emphasis on routine testing for biliary enzymes and anti-mitochondrial antibodies has increased the number of newly diagnosed cases of PBC without disease-related symptoms [4–6]. A significant proportion of these patients are in the early disease stage, and the prognosis of elderly asymptomatic patients is likely to be as good as that of age- and gender-matched controls from the general population [6]. Regarding the progression of PBC, at least 2 different types of progression in PBC have been proposed; one is a hepatic failure-type progression, in which the patients develop rapid progression to end-stage hepatic failure even when they are properly medicated with ursodeoxycholic acid and

Received September 12, 2014; accepted November 28, 2014.
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Conflict of Interest Disclosures: No potential conflict of interest relevant to this article was reported.

bezafibrate, and the other is a portal hypertension-type progression, in which patients develop complications of portal hypertension such as esophageal varices in the early disease stage without jaundice [7–9].

The histological staging of PBC patients has been reported by Scheuer *et al.* [10]. However, there have been some difficulties in the staging of PBC because the grading of cholestasis is not included in this classification. Cholestasis and inflammatory activity in the portal tract are independent events, and the progression of liver damage in PBC is caused by cholestasis and periportal inflammation. Nakanuma *et al.* proposed a new histological classification which separately evaluates the grade of cholestasis, the loss of bile ducts, and periportal inflammation in addition to the stage of fibrosis [11]. Cholestasis in PBC can be detected by the deposition of copper or copper-associated protein in hepatocytes [12]. We reported that the aberrant expression of bile duct-type keratin (K) 7 in hepatocytes is useful to evaluate the grade of cholestasis, and that the aberrant K–7 expression is more sensitive than the deposition of copper-associated protein [13].

In the present study, we focused on cholestasis in hepatocytes, and investigated the aberrant K–7 expression in hepatocytes from patients with aPBC. The patients were assessed for biochemical data, histological classifications, and disease prognosis in order to clarify whether the K–7 expression can be used as an additional marker to predict the rapid progression to liver failure in patients with aPBC at the time of diagnosis.

Patients and Methods

Patients. The diagnosis of PBC was made at Okayama University Hospital from 1980 to 2012, based on the following criteria: (1) positive test for anti-mitochondrial antibody or antinuclear antibody, (2) biochemical evidence of cholestasis, and (3) liver biopsy compatible with the diagnosis [14]. Patients with overlapping PBC and autoimmune hepatitis with severe lobular inflammation were excluded. Patients who were serum-positive for hepatitis B surface antigen or anti-hepatitis C virus antibodies, and those with daily ethanol use of more than 80 grams were also excluded. The study was performed in accordance with the Helsinki Declaration and was approved by the ethical committee of the institution. All patients provided informed consent.

Of these 150 patients, histological cirrhosis, jaundice and/or symptoms of portal hypertension were observed in 49 patients at the time of diagnosis. Thus, a total study population of 101 patients who had aPBC without histological cirrhosis, or disease-related symptoms of jaundice or portal hypertension was used for further analysis. Jaundice was defined as total bilirubin > 2.0 mg/dL, and portal hypertension as meeting one of the following criteria: (1) esophageal or gastric varices, (2) splenomegaly, or (3) platelet counts $< 100{,}000$/dL. Anti-gp210 antibodies were detected as previously described [8].

Histological evaluation. Laparoscopy-assisted liver biopsy was done for all patients at the time of diagnosis in order to accurately rule out cirrhotic patients. The histological stage was determined according to the criteria of Scheuer *et al.* [10]. We also used a new histological classification proposed by Nakanuma *et al.* [11]. Briefly, chronic cholangitis with mild periductal lymphoplasmacytic infiltration, including chronic nonsuppurative destructive cholangitis, and the combined activity of interface hepatitis and lobular hepatitis were categorized into 4 grades on the basis of their degree and distribution (CA0–3 and HA0–3, respectively). For staging, cholestasis (evaluated with orcein staining), fibrosis and bile duct loss were independently scored from 0 to 3 on the basis of their degrees, and a final stage score was created from the sum.

Immunohistochemistry of keratin. Immunohistochemical analysis was performed using formalin-fixed paraffin-embedded samples at the time of diagnosis. Five-micron sections were cut and deparaffinized. The sections were treated with 0.05% protease (type XXV, Sigma Aldrich Japan, Tokyo, Japan) in a 50–mM Tris-HCl solution for 10 min at room temperature for antigen retrieval. The intrinsic peroxidase activity was blocked by immersing sections in a methanol solution containing 0.3% hydrogen peroxide. The sections then underwent a reaction with a monoclonal antibody for K–7 (OV-TL 12/30, Dako, Glostrup, Denmark) after nonspecific binding was blocked with casein. The Envision detection system was equipped with DAB (Dako). For controls, the first monoclonal antibody was omitted from the reaction process. In the normal liver, K–7 expresses only in bile duct epithe-

lial cells, whereas livers of PBC patients show varying degrees of K-7 expression in proliferated bile ductules and hepatocytes as well as bile duct epithelial cells. Proliferated bile ductules were markedly present at the border between the portal tract and liver parenchyma.

As previously described [13], K-7 expression patterns were classified into the following 4 grades (Fig. 1): Grade 1, K-7 expression in bile duct epithelial cells and proliferated bile ductules; Grade 2, periportal hepatocytes positive for K-7; Grade 3, intralobular hepatocytes also positive for K-7; Grade 4, K-7 diffusely stained throughout the lobules.

Survival. All patients underwent a physical exam at least every 3 to 6 months and an endoscopic check-up every year of the follow-up period. Patients who had not visited one of our hospitals in the previous 6 months were contacted by letter or telephone and asked to provide details about recent medications and disease-related symptoms. Other hospitals that study participants visited were asked for physical examination results and medical data. When patients died, the date and cause of death were recorded.

Statistical analysis. Data are expressed as the mean ± standard deviation or the median with range. Patient characteristics at the time of diagnosis were compared among groups using the Wilcoxon rank sum test or Fisher exact probability test. Factors associated with the grade of cholestasis were analyzed by a stepwise logistic regression analysis. Receiver operating characteristic curves were constructed for clinical characteristics with correlations of the grades of aberrant K-7 expression in hepatocytes. The best cut-off values were determined based on these results, and used for further analyses. Proportional hazards models were used to estimate the factors associated

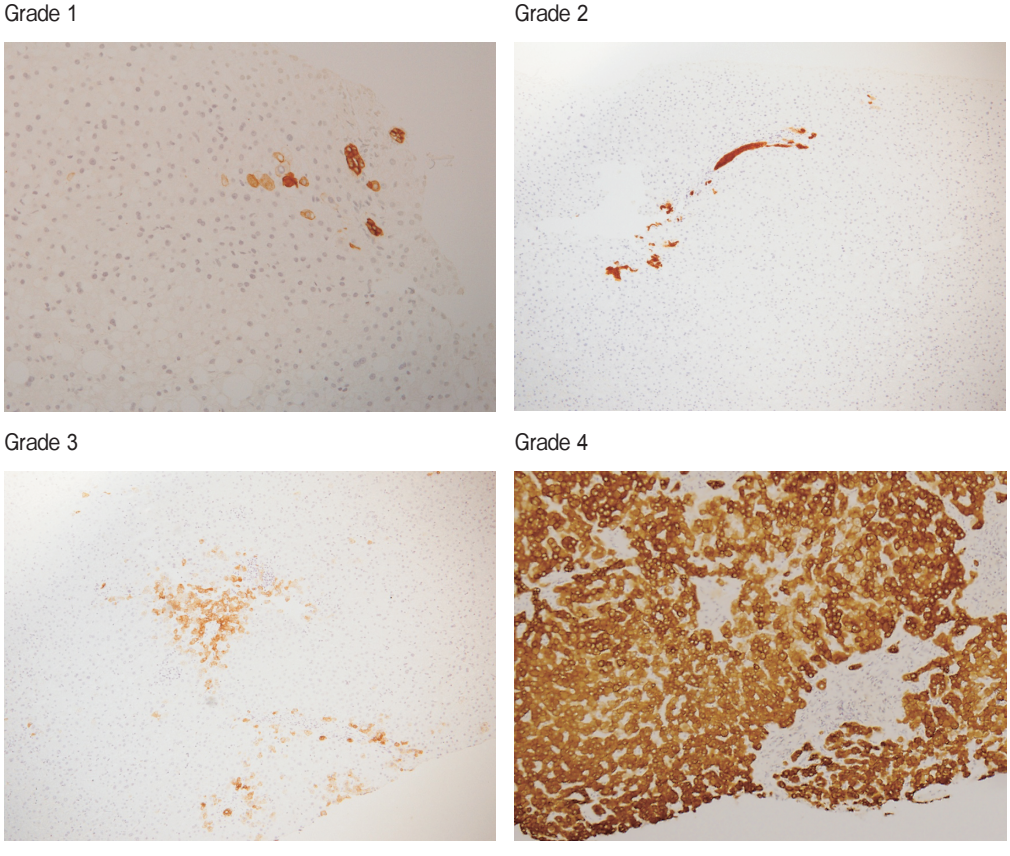

Fig. 1 The grades of aberrant K-7 expression in hepatocytes. Grade 1: K-7 is expressed only in proliferated bile ductules at the interface between the portal tract and the parenchyma. Grade 2: K-7 is expressed in periportal hepatocytes as well as in proliferated bile ductules in the portal tract. Grade 3: K-7 is expressed in intralobular hepatocytes. Grade 4: K-7 is expressed diffusely in hepatocytes.

with the development of jaundice during the follow-up period in the patients with aPBC, and the factors were selected in a stepwise manner among the significant factors in univariate analysis for the multivariate analysis. The incidence of jaundice was determined using the Kaplan-Meier method, and the results were compared using the log rank test. Statistical analyses were performed using JMP 9.0 software (SAS Institute, Cary, NC, USA). P-values < 0.05 were considered significant.

Results

Associations of patient characteristics with aberrant K-7 expression. The present study enrolled 101 patients who were diagnosed as having aPBC without histological cirrhosis, jaundice or portal hypertension. The clinical characteristics and pathological features of the patients are listed in Table 1. The majority of the patients were females (97 females and 4 males) in their 50s, which is consistent with previous studies [6]. High grades of aberrant K-7 expression in hepatocytes (grades 3 and 4) were found in 9 patients (9%). As shown in Table 2, the comparisons of clinical characteristics between the patient groups with high and low grades of aberrant K-7 expression in hepatocytes revealed that the grades of aberrant K-7 expression in hepatocytes were not significantly associated with the histological classifications such as the PBC stages in the classifications by Scheuer or Nakanuma, or the detailed parameters of bile duct loss or chronic cholestasis ($p = 0.31$, 0.20, 0.23, and 1.0, respectively, the Fisher exact probability test).

Table 1 Clinical characteristics of the patients at the time of diagnosis

Patient characteristics	
Age (years)	52 (29–76)[a]
Gender (female/male)	97/4
Follow-up period (years)	11 ± 6.5[c]
Liver histology (Scheuer, 1/2/3/4)	38/14/49/0
Liver histology (Nakanuma, 1/2/3/4)[b]	28/50/23/0
Chronic cholangitis activity (0/1/2/3)[b]	5/12/2/82
Hepatic activity (0/1/2/3)[b]	58/26/10/7
Fibrosis (0/1/2/3)[b]	47/23/31/0
Bile duct loss (0/1/2/3)[b]	54/21/16/10
Cholestasis score (0/1/2/3)[b]	68/14/5/4
Aberrant K-7 expression (1/2/3/4)	84/8/7/2
Anti-mitochondrial antibody (% positive)	77/96 (80%)
Antinuclear antibody (% positive)	63/96 (66%)
Aspartate aminotransferase (IU/L)	46 ± 28[c]
Alanine aminotransferase (IU/L)	52 ± 40[c]
Alkaline phosphatase (IU/L)	297 ± 262[c]
γ-glutamyl transpeptidase (IU/L)	223 ± 277[c]
Total bilirubin (mg/dL)	0.7 ± 0.3[c]
Platelet count ($\times 10^4$/mm^3)	23 ± 5.9[c]
Immunoglobulin G (mg/dL)	1808 ± 505[c]
Immunoglobulin M (mg/dL)	379 ± 204[c]
Anti-gp210antibody (% positive)	8/48 (17%)

[a]median (range). [b]Histological stage classified by Nakanuma *et al.* [11]. [c]mean ± standard deviation.

Patients with high grades of aberrant K-7 expression in hepatocytes had significantly higher levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin in the serum compared to those with low grades of aberrant K-7 expression in hepatocytes ($p = 0.0030$, 0.0060, 0.020, and 0.0015, respectively, the Wilcoxon rank sum test). We also evaluated the positivity of anti-gp210 antibody of 56 of the PBC patients in the present study, because the positivity of anti-gp210 antibody has been reported to be a useful marker for detecting PBC patients with poor prognosis. Patients with higher grades of aberrant K-7 expression in hepatocytes were 66% positive for the anti-gp210 antibody while those with lower grades of aberrant K-7 expression were only 13% positive ($p = 0.068$, the Fisher exact probability test).

Correlations of grades of aberrant K-7 expression in hepatocytes with clinical characteristics are listed in Table 3. Serum liver enzyme levels of aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and total bilirubin showed significant positive correlations with aberrant K-7 expression in hepatocytes by univariate logistic regression analysis. Multivariate analysis revealed that high grades of aberrant K-7 expression in hepatocytes were significantly positively correlated with levels of total bilirubin ($p = 0.0020$, the stepwise logistic regression analysis), although the cut-off value of total bilirubin was as low as 0.8 mg/dL.

Development of jaundice among the patients with aPBC stages at diagnosis. During the follow-up period, 8 patients developed jaundice, and the mean period until the development of jaundice was 5.2 years (1 to 12 years), as shown in Fig. 2. Table 4 shows the proportional hazards model results for the

Table 2 Clinical characteristics of patients stratified by grades of aberrant K-7 expression in hepatocytes

	Aberrant K-7 expression		
	Grades 1, 2 N = 92	Grades 3, 4 N = 9	*P*
Age (year)	52 ± 9[a]	51 ± 10[a]	0.47
Liver histology (Scheuer, 1, 2/3, 4)	49/43	3/6	0.31
Liver histology (Nakanuma, 1, 2/3, 4)[b]	73/19	5/4	0.20
Chronic cholangitis activity (0, 1/2, 3)[b]	15/77	3/6	0.36
Hepatic activity (0, 1/2, 3)[b]	76/16	8/1	0.58
Fibrosis (0, 1/2, 3)[b]	65/27	5/4	0.45
Bile duct loss (0, 1/2, 3)[b]	70/22	5/4	0.23
Chronic cholestasis (0, 1/2, 3)[b]	74/8	8/1	1.0
Aspartate aminotransferase (IU/L)	42 ± 20[a]	91 ± 56[a]	0.0030
Alanine aminotransferase (IU/L)	47 ± 33[a]	101 ± 68[a]	0.0060
Alkaline phosphatase (IU/L)	279 ± 255[a]	477 ± 285[a]	0.020
γ-glutamyl transpeptidase (IU/L)	218 ± 283[a]	276 ± 205[a]	0.12
Total bilirubin (mg/dL)	0.7 ± 0.3[a]	1.1 ± 0.5[a]	0.0015
Platelet count ($\times 10^4$/mm^3)	23 ± 6[a]	21 ± 6[a]	0.70
Immunoglobulin M (mg/dL)	377 ± 207[a]	408 ± 160[a]	0.53
Anti-gp210 antibody (positive/negative)	6/45	2/3	0.068

[a]mean±standard deviation. [b]Histological stage classified by Nakanuma *et al*. [11].

Table 3 Correlations of aberrant K-7 expression in hepatocytes with patient characteristics

	Univariate analysis		Multivariate analysis	
	Odds ratio	*P*	Odds ratio	*P*
Age (< 52 years)	2.0 (0.5–8.9)	0.49		
Liver histology (Scheuer 3, 4)	2.3 (0.5–9.7)	0.31		
Liver histology (Nakanuma 3, 4)[a]	3.1 (0.75–13)	0.23		
Chronic cholangitis activity (2, 3)[a]	0.58 (0.1–3.2)	0.62		
Hepatic activity (2, 3)[a]	Not assessed	1.0		
Fibrosis (2, 3)[a]	1.9 (0.48–7.7)	0.45		
Bile duct loss (2, 3)[a]	2.4 (0.59–9.7)	0.24		
Chronic cholestasis (2, 3)[a]	2.1 (0.39–11)	0.33		
Aspartate aminotransferase (≥40 IU/L)	10.0 (1.2–83)	0.014		
Alanine aminotransferase (≥41 IU/L)	9.5 (1.1–79)	0.016	2.9 (0.29–71)	0.38
Alkaline phosphatase (≥224 IU/L)	9.5 (1.1–79)	0.016	4.0 (0.43–98)	0.24
γ-glutamyl transpeptidase (≥149 IU/L)	2.2 (0.51–9.3)	0.32		
Total bilirubin (≥0.8 mg/dL)	20.3 (2.4–171)	0.0006	14.9 (2.4–289)	0.0020
Platelet count (≥22 $\times 10^4$/mm^3)	0.95 (0.12–7.1)	1.0		
Immunoglobulin M (≥331 mg/dL)	1.0 (0.19–5.2)	1.0		
Anti-gp210 antibody (positive)	13.3 (1.0–171)	0.015		

[a]Histological stage classified by Nakanuma *et al*. [11].

risk of developing jaundice. High grades of aberrant K-7 expression in hepatocytes, and higher levels of aspartate aminotransferase, alanine aminotransferase, and alkaline phosphatase at the time of diagnosis showed significant associations with the development of jaundice in the univariate analysis. A high grade of aberrant K-7 expression in hepatocytes was the only significant risk factor in the multivariate analysis (hazards ratio 8.7, $p = 0.019$). Actually, the patient groups of different grades of aberrant K-7 expression in hepatocytes revealed significant differences in the incidence of jaundice by the Kaplan-Meier method (p

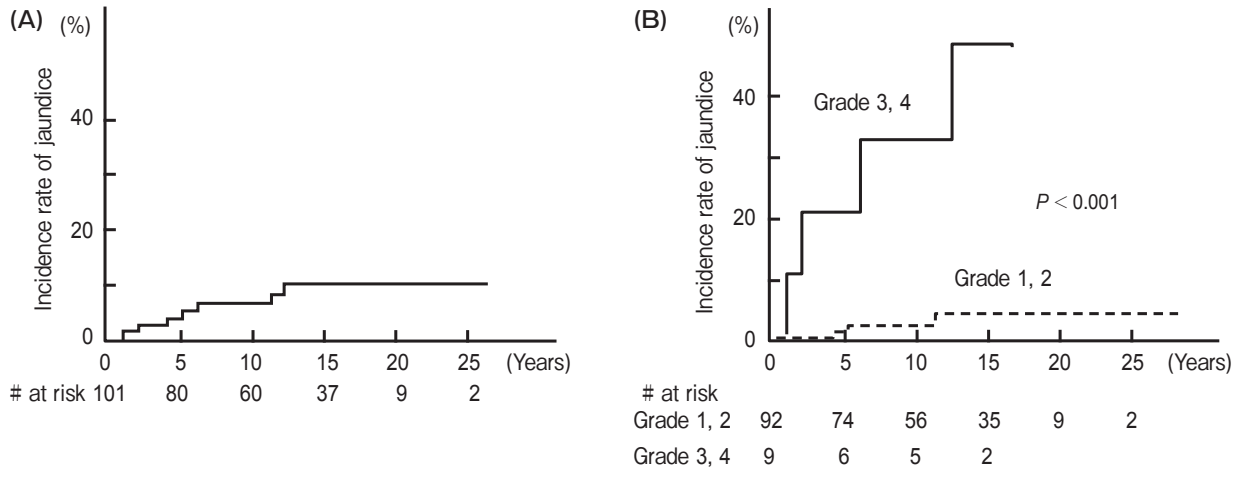

Fig. 2 Development of jaundice in patients with aPBC. The incidence rate of jaundice during follow-up care was assessed by the Kaplan-Meier method for patients with aPBC at the time of diagnosis (**A**). The incidence rate of jaundice was calculated for the patient groups stratified by grade of aberrant K-7 expression in hepatocytes (**B**).

Table 4 Proportional hazards model for the development of jaundice in the patients with early PBC

Factors	Univariate analysis		Multivariate analysis	
	Hazard ratio (range[a])	*P*	Hazard ratio (range[a])	*P*
Age (< 52 year)	5.7 (0.93–104)	0.060	4.3 (0.68–87)	0.13
Liver histology (Scheuer 3, 4)	1.4 (0.30–6.9)	0.69		
Liver histology (Nakanuma 3, 4)[b]	1.3 (0.17–5.5)	0.77		
Chronic cholangitis activity (2, 3)[b]	1.0 (0.16–19)	0.99		
Hepatic activity (2, 3)[b]	0.73 (0.04–4.3)	0.78		
Fibrosis (2, 3)[b]	0.84 (0.12–3.9)	0.84		
Bile duct loss (2, 3)[b]	2.0 (0.39–9.0)	0.38		
Chronic cholestasis (2, 3)[b]	1.7 (0.09–10)	0.63		
Aspartate aminotransferase (≥40 IU/L)	6.9 (1.2–130)	0.031		
Alanine aminotransferase (≥41 IU/L)	6.6 (1.1–125)	0.035	1.9 (0.20–46)	0.63
Alkaline phosphatase (≥224 IU/L)	7.6 (1.3–144)	0.020	2.8 (0.35–63)	0.37
γ-glutamyl transpeptidase (≥149 IU/L)	6.2 (1.1–117)	0.043		
Total bilirubin (≥0.8 mg/dl)	4.5 (0.97–31)	0.055	1.1 (0.11–14)	0.92
Platelet count ($\geq 22 \times 10^4/mm^3$)	1.6 (0.26–12)	0.62		
Immunoglobulin M (≥331 mg/dl)	3.3 (0.5–65)	0.23		
Anti-gp210 antibody (positive)	10.5 (1.0–225)	0.050		
K-7 expression (3, 4)	15.7 (3.4–79)	0.0008	8.7 (1.4–85)	0.019

[a]95% confidence interval. [b]Histological stage classified by Nakanuma *et al*. [11].

< 0.001, the log rank test, Fig. 2).

Discussion

Among asymptomatic PBC patients without histological cirrhosis, jaundice, or portal hypertension, a certain portion of patients progress rapidly to end-stage hepatic failure. Since the progression to hepatic failure is caused by cholestasis and periportal inflammation, the present study focused on cholestasis in hepatocytes at the time of diagnosis by studying aberrant K-7 expression in hepatocytes. Our results

revealed that high grades of aberrant K-7 expression in hepatocytes at the time of diagnosis were significantly associated with the development of jaundice during the follow-up period for the patients with aPBC; *i.e.*, PBC without histological cirrhosis, jaundice, or portal hypertension. This predictive importance of aberrant K-7 expression in hepatocytes is independent from biochemical parameters and histological classifications. Aberrant K-7 expression in hepatocytes can be used as an additional marker to predict a rapid progression to liver failure in patients with aPBC at the time of diagnosis.

Keratin is an intermediate filament and is divided into several subtypes. The type of keratin is specific to the cell lineage. In the normal liver, hepatocytes and bile duct epithelial cells express a distinct type of keratin [15-17]. Bile duct epithelial cells and reactive ductule cells express keratins 7 and 19. A recent report by Khan *et al.* demonstrated that K-19 expression could detect loss of the canals of Hering, and they suggested that this "minimal change" feature may support a clinical diagnosis of PBC even in the absence of characteristic, granulomatous, duct-destructive lesions [18].

On the other hand, in certain disease conditions such as various cholestatic diseases including PBC and alcoholic liver disease, groups of hepatocytes express K-7 and K-19 [15]. These aberrant expressions of K-7 and K-19 in hepatocytes are dependent on the grade and duration of cholestasis, and are associated with the progression of cholestasis [19]. Aberrant K-7 expression in hepatocytes is shown to be more sensitive than that of K-19 for this purpose. We therefore used K-7 for the evaluation of cholestasis in the present study.

Our results revealed significant positive correlations of the grade of aberrant K-7 expression in hepatocytes with the serum levels of total bilirubin and alanine aminotransferase. Since none of the patients enrolled in the present study had jaundice with total bilirubin < 2.0 mg/dL, aberrant K-7 expression in hepatocytes might be used as a sensitive marker of cholestasis. Further assessments of bilirubin levels might provide a clinical cut-off for estimating cholestasis in hepatocytes of PBC patients. However, in the present study the serum levels of alkaline phosphatase did not show significant associations with the grade of aberrant K-7 expression in hepatocytes. We reported that a high level of alkaline phosphatase at the time of diagnosis suggests portal inflammation in patients with early PBC [9]. Serum levels of alkaline phosphatase are indirectly associated with cholestasis, and might not necessarily represent the grade of cholestasis.

Several drugs have been used to slow the disease progression in PBC patients, with variable results. Our present data are not sufficient to assess the benefits of those drugs for suppressing the development of jaundice. However, our results suggest that patients with aberrant K-7 expression in hepatocytes need more aggressive therapy to avoid rapid progression to liver failure. Further study on the efficacy of medication to improve cholestasis in the liver is needed for patients with aPBC.

In conclusion, aberrant K-7 expression in hepatocytes may provide additional information for the precise histological grading of PBC and can be used as an additional marker to predict rapid progression to liver failure in patients with aPBC at the time of diagnosis.

Acknowledgments. This work was supported in part by the Research Program of Intractable Disease provided by the Ministry of Health, Labor, and Welfare of Japan, and by a Grant-in-aid (22590733) for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (to Y.I.). We thank Taiko Kameyama for technical assistance.

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