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Periostin, a matrix protein, has potential as a novel serodiagnostic marker for cholangiocarcinoma

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Abstract. Differentiating intrahepatic cholangiocarcinoma (CCA) from other hepatic malignancies is crucial in deciding on treatment modalities and predicting clinical outcomes in patients. Periostin is a secreted protein from stromal cells and regulates the development of cancer cells through interaction with the extracellular matrix. Given that proliferation of fibrous stromal cells is a pathological feature of CCA, we examined the potential use of periostin as a serodiagnostic marker for this disease. Our study enrolled a total of 79 patients including liver cirrhosis (n=26), hepatocellular carcinoma (HCC, n=24), CCA (n=8), other hepatic malignancies (n=13) and histologically normal livers (normal control, n=8). Periostin expression was evaluated using immunohistochemistry and serum periostin level was determined via enzyme-linked immunoassay. The diagnostic performance of serum periostin levels for distinguishing CCA patients from others was also assessed. Strong expression of periostin was noted only in the fibrous stroma of CCA tissue. Serum periostin levels (median) were significantly higher in patients with CCA (513 ng/ml) compared to those patients with normal liver, liver cirrhosis, HCC and other malignancies (120, 146, 155, 213 ng/ml, respectively, all P<0.05). The area under receiver operating characteristics curve of serum periostin level was 0.94 [95% confidence interval (CI), 0.85-1.00, P<0.001]. With optimal cut-off value of 302 ng/ml, diagnostic performances for CCA were as follows: sensitivity, 0.88 (95% CI, 0.47-0.99); specificity, 0.92 (0.83-0.96); accuracy, 0.91 (0.83-0.96); positive predictive value, 0.54 (0.25-0.81); negative predictive value, 0.98 (0.92-0.99); positive-likelihood ratio, 10.4 (4.8-13.4); and negative-likelihood ratio, 0.13 (0.03-0.49). We demonstrated increased expression of periostin in the stroma of CCA tissue. Serum periostin levels were significantly elevated in patients with CCA and enable distinction between CCA and other hepatic malignancies.

Introduction

Periostin is a newly emerged extracellular matrix protein belonging to the fasciclin family (1-3). Periostin enhances collagen fibrillogenesis by binds to collagen I, fibronectin, and tenascin-C (4-6) and by activating lysyl oxidase, a catalytic enzyme for cross-linking of collagen (7). Furthermore, periostin acts as a matricellular protein by binding to several integrin molecules, $\alpha_v\beta_1/\beta_3$, on cell surface, involved in tissue development and carcinogenesis (1,2,8). In malignant cells, periostin activates phosphatidylinositol 3-kinase and Akt pathways via integrin molecules, which are important for growth, migration, and epithelial-mesenchymal transition of the cells (9-11). Malignant cells themselves or stromal cells adjacent to malignant cells are sources of periostin (8).

Recent studies have reported that increased expression of periostin in some cancers with fibrous stromal cells including non-small cell lung carcinoma and breast cancer (12-14). The pathological feature of intrahepatic cholangiocarcinoma (CCA), but not hepatocellular carcinoma (HCC), is abundant in fibrous stromal cells (15), and that raised a possibility that expression of periostin is differently regulated in CCA and HCC. Indeed, it has been very recently shown that CCA-associated fibroblasts or CCA-containing tissues strongly

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Abbreviations: CCA, cholangiocarcinoma; HCC, hepatocellular carcinoma; CA19-9, carbohydrate antigen 19-9; CEA, carcinoembryonic antigen; AFP, α-fetoprotein; phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; ROC, receiver operating characteristics; PPV, positive predictive value; NPV, negative predictive value; LR, likelihood ratio; AUC, the area under the ROC curve; TGF-β, transforming growth factor β

Key words: periostin, cholangiocarcinoma, tumor marker, enzyme-linked immunosorbent assay, hepatocellular carcinoma

Table I. Patient characteristics.

	Reference value	Normal liver	Liver cirrhosis	HCC	Other hepatic malignancies	CCA	P-values[a]
Number	N/A	8	26	24	13	8	
Age (years)	N/A	56 (36-65)	59 (51-65)	70 (64-75)	61 (55-72)	59 (50-73)	0.090
Gender (male/female)	N/A	2/6	11/15	19/5	12/1	4/4	0.020
AFP (ng/ml)	<8.7	N/A	N/A	20 (4.1-173)	11.6 (5.5-76)	4.9 (3.4-5.9)	0.057
CEA (ng/ml)	<5.0	N/A	N/A	3.2 (2.1-3.4)[b]	3.3 (2.3-4.3)	3.8 (3.3-6.1)[b]	0.028[b]
CA19-9 (U/ml)	<37.0	N/A	N/A	47 (32-79)	47 (26-53)	96 (27-1323)	0.354
Periostin (ng/ml)	Unknown	120 (86-194)	146 (113-194)	155 (110-213)	213 (154-283)	513 (315-897)	<0.0001[c]

Data are expressed as median (25th to 75th percentile of interquartile range) or number of patients. HCC, hepatocellular carcinoma; CCA, cholangiocarcinoma; N/A, not-applicable; AFP, α-fetoprotein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9. [a]P-values are calculated using the Kruskal-Wallis test and Fisher's exact test. [b]Multiple pairwaise comparisons revealed that serum CEA level in the CCA group was significantly higher than that in HCC group (Bonferroni correction, P=0.024). [c]For serum periostin level, see Fig. 3 and description.

express periostin compared to non-tumorigenic liver fibroblasts or liver tissues containing HCC (16,17). Furthermore, an *in vitro* study found that periostin enhanced cell proliferation and invasion. CCA is one of the most devastating malignancies and differentiating CCA from HCC is crucial in deciding treatment modalities and predicting clinical outcomes, and patient counseling (18-21). Periostin is a secretory protein (11,22); however, it remains unclear whether expression of periostin in CCA tissues reflects serum level of periostin and whether measurement of serum level of periostin can be applied to differential diagnosis between CCA and HCC.

The aims of this study were to examine the tissue expression of periostin in CCA and the efficacy of periostin as a serodiagnostic marker for CCA.

Patients and methods

Patients. We retrospectively enrolled a total of 79 patients with liver cirrhosis (n=26), HCC (n=24), CCA (n=8), other hepatic malignancies (n=13), including combined type of HCC (n=7), scirrhous type of HCC (n=3) and HCC with sarcomatous change (n=3), or histologically normal livers obtained from hepatic resection or liver diagnostic biopsy for benign liver lesions (n=8), such as focal nodular hyperplasia or angiomyolipoma. All of the patients were hospitalized for diagnostic liver biopsy or radical hepatic resection for liver tumor. All of the diagnoses were based on clinical, serological, imaging and histological evidence and characteristics of enrolled patients are shown in Table I. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki 2008 (23) as reflected in prior approval by the Ethics Committee of our institution. All patients gave the written informed consent.

Serum tumor markers. Venous blood samples were taken in the morning after a 12-h overnight fast. Serum α-fetoprotein (AFP), carcinoembryonic antigen (CEA), and CA19-9 levels were measured in all patients with malignant neoplasm (n=45) by using standard clinical methods (Department of Clinical Laboratory, Kurume University Hospital).

Histological diagnosis. For each patient, a liver specimen was fixed in 10% formalin buffer and stained with hematoxylin-eosin. All of histological diagnoses were performed by two experienced pathologist who were unaware of the patients' clinical and laboratory data.

Establishment of anti-periostin monoclonal and polyclonal antibodies (Abs). We newly established anti-periostin mAbs as previously reported (6). Briefly, 6-10-week-old Crj:Wistar rats (Charles River Japan, Inc., Kanagawa, Japan) were injected in footpads two or more times with 20 μg of recombinant human periostin emulsified in TiterMax Gold adjuvant (TiterMax USA, Norcross, GA). Three days after the last injection, lymphocytes from popliteal, inguinal and iliac lymph nodes were fused with a Sp2/O myeloma cell line. Rat anti-periostin mAbs were purified from culture supernatant of the hybridomas using a protein G affinity chromatography column. Specific pathogen-free rabbits were immunized with recombinant human periostin, and antiserum was obtained. Purified rabbit anti-human periostin polyclonal Ab was generated from the antiserum as previously reported (6).

Immunohistochemistry. Immunohistochemical analysis was performed as previously described (6,24-27). Paraffin-embedded liver sections were deparaffinized and were washed three times for 5 min each in phosphate-buffered saline (PBS) (pH 7.4, 130 mmol/l NaCl, 2 mmol/l NaH_2PO_4, and 7 mmol/l Na_2HPO_4) and then blocked with 10% skim milk in PBS for 30 min. Sections were incubated overnight at 4°C with the rat anti-human periostin mAbs (clone no. SS19B or SS5D) diluted 1:100 in PBS. After several washes with PBS, the sections were incubated with the secondary antibodies, biotin-labeled goat anti-rat IgG diluted 1:100 in PBS at room temperature for 1 h. Subsequently, the sections were washed with PBS and Positive reactivity was identified using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA) and developed with 3,3'-diaminobenzidine (Dako, Kyoto, Japan).

Establishment of enzyme-linked immunosorbent assay (ELISA). Serum were obtained before diagnosis or treatment

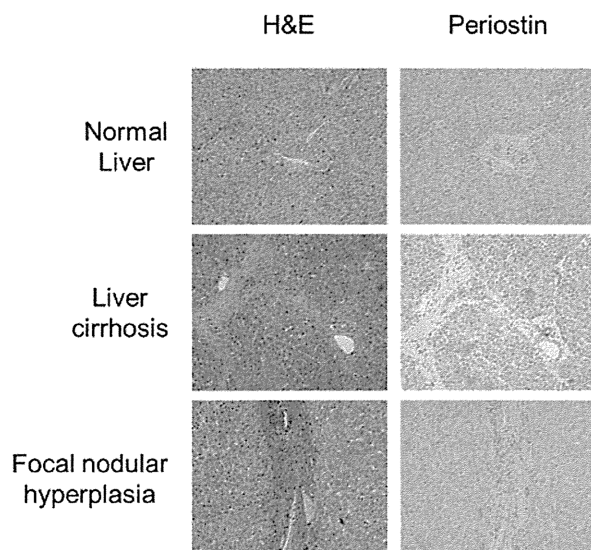

Figure 1. Immunostaining for periostin in non-malignant liver tissue. Sections from normal liver, liver cirrhosis, and focal nodular hyperplasia were immunostained with anti-periostin antibodies. Expression of periostin was visualized by 3,3'-diaminobenzidine (brown). Original magnification, x400.

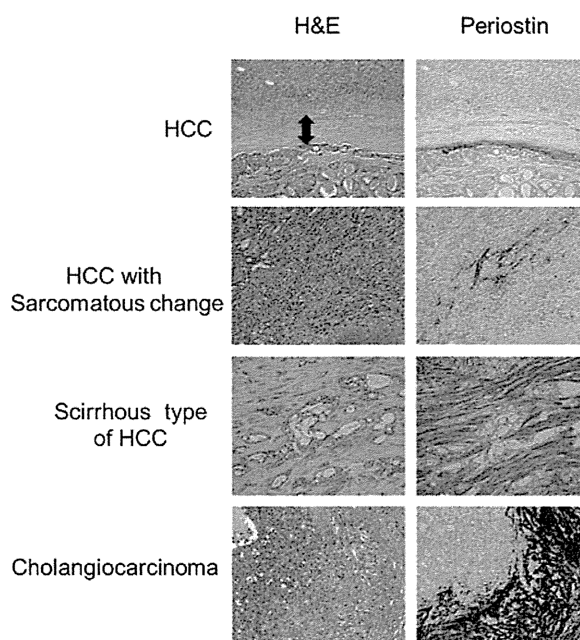

Figure 2. Immunostaining for periostin in hepatic malignant tissue. Sections from hepatocellular carcinoma (HCC), HCC with sarcomatous change, scirrhous type of HCC, and cholangiocarcinoma were immunostained with anti-periostin antibodies. Expression of periostin was visualized by 3,3'-diaminobenzidine (brown). Original magnification, x400. Arrow in left upper panel indicates capsule of HCC.

of hepatic malignancies and then stored at -80˚C until ELISA assay. We newly established a human periostin ELISA assay. Two rat anti-human periostin mAbs (clone no. SS16A and SS17B) were used to establish a sandwich ELISA assay. The SS16A mAb (2 μg/ml) was incubated overnight at 25˚C on ELISA plates (Loose MaxiSorp® Nunc-Immuno® Modules, Thermo Fisher Scientific, Rochester, NY). The ELISA plates were blocked by blocking buffer (0.5% casein, in TBS, pH 8.0) overnight at 4˚C and then washed three times with washing buffer (0.05% Tween-20 in PBS). The ELISA plates were incubated with diluted samples (1/6,000) or recombinant periostin standards for 18 h at 25˚C, followed by washing five times. Biotin-labeled SS17B mAb (5 μg/ml) was added followed by incubation for 90 min at 25˚C. After washing five times, diluted peroxidase-labeled streptavidin (1/15,000) (Strerospecific Detection Technologies) was added to the plates, which were then incubated for 1 h at 25˚C. After the ELISA plates were washed 5 times, reaction solution (0.8 mM 3,3',5,5'-Tetramethylbenzidine, 2.5 mM H_2O_2) was added, followed by incubation for 10 min at 25˚C and then the reaction was stopped by adding the stop solution (0.7 N HCl). The values were calculated by subtracting the absorbance at 550 nm (secondary wavelength) from the absorbance at 450 nm (primary wavelength) measured by a microplate reader (Bio-Rad Laboratories, Tokyo, Japan). Periostin concentrations in the serum were calculated simultaneously using the recombinant periostin proteins. We performed the ELISA assay on duplicated samples.

Statistical analysis. All continuous data were expressed as median and the 25th to the 75th percentile of the interquartile range.

The Kruskal-Wallis test was used to determine significance of intergroup differences of the serum periostin levels. In case of statistical significance, multiple pairwise comparisons were conducted with the Mann-Whitney U test with Bonferroni correction.

To test if serum periostin level can distinguish CCA group from other groups, bivariate logistic regression analysis was performed with serum periostin level as a covariate and each group and other group (dummy variables) as dependent variables. Receiver operating characteristic (ROC) analysis was used to determine the cut-off value of serum periostin levels for distinguishing CCA patients from others as previously described (28). The significance for the cut-off value of serum periostin was evaluated by sensitivity, specificity, accuracy, positive predictive value (PPV), negative predictive value (NPV), and likelihood ratio (LR). Finally, among hepatic malignant neoplasms (n=45) serum levels of tumor markers including AFP, CEA, CA19-9 and periostin for distinguishing CCA from other malignancies were reevaluated with ROC analysis.

All analyses were performed using SPSS statistical software (version 12.0J; SPSS, Inc., Chicago, IL) and $P<0.05$ was considered statistically significant.

Results

Immunohistochemistry for periostin. Although slight expression of periostin was seen in bile duct cells, no expression of periostin was noted in either hepatocytes or fibrous stroma in normal liver and liver cirrhosis tissues (Fig. 1). In focal nodular hyperplasia tissue, a benign liver disease, expression of periostin was not seen in either hepatocytes or fibrous scars (Fig. 1).

In HCC tissue, while no expression of periostin was seen in cancer cells themselves, weak periostin expression was localized in capsule contacting to cancer cells (Fig. 2). Similarly,

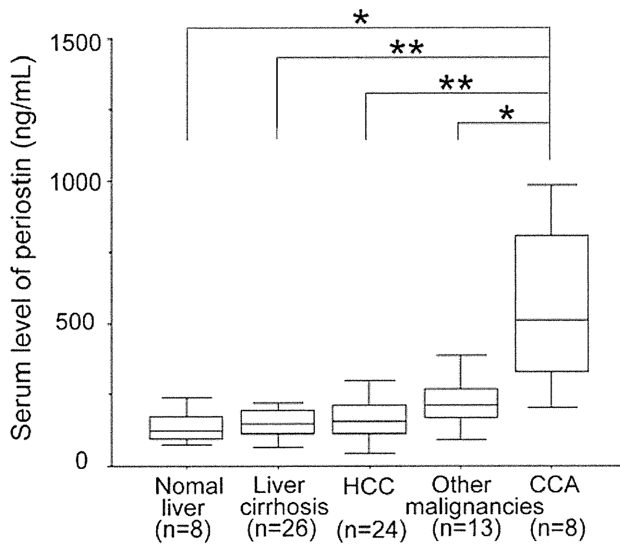

Figure 3. Box plots show serum level of periostin in patients with normal liver and various hepatic malignancies. Serum levels of periostin were measured by newly established a human periostin ELISA assay. The vertical bars indicate the range and the horizontal boundaries of the boxes represent the first and third quartiles. Statistical comparisons among multiple groups were performed by Mann-Whitney U test with Bonferroni correction. *P<0.05 and **P<0.01.

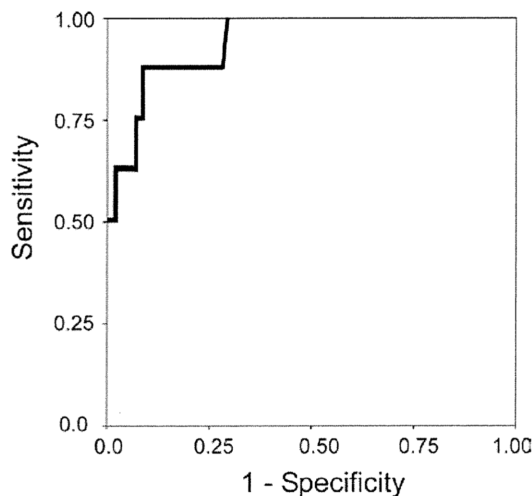

Figure 4. Graph shows receiver operating characteristic (ROC) curves for serum periostin level to distinguish CCA group from other groups. The area under the ROC curve was 0.94. The optimal cut-off value between patients with or without CCA is 302 ng/ml based on the Youden-index.

expression of periostin was detected in a portion of stroma, but not in cancer cells of sarcoma and angiomyolipoma tissues (Fig. 2). Moderate periostin expression was seen in stroma of scirrhous type of HCC, and strong periostin expression was noted in fibrous stroma of CCA tissues (Fig. 2).

Serum periostin levels. With serum periostin levels Kruskal Wallis test showed statistically significant difference (Table I, P<0.001), and multiple pairwaise comparisons revealed that serum periostin level in the CCA group was significantly higher than that in normal liver, liver cirrhosis, HCC, or other malignancies groups (Fig. 3).

Following these results we analyzed the bivariate logistic regression analysis of serum periostin levels to diagnose CCA. A significant association was seen between serum periostin level and CCA group [odds ratio, 1.014; 95% confidence interval (CI), 1.006-1.023; P=0.001].

ROC analyses and diagnostic performances for distinguishing CCA from others. We conducted receiver operating characteristic (ROC) analysis to determine the cut-off value of periostin for distinguishing CCA group from other groups. The area under the ROC curve (AUC) showed statistically significant value [0.94 (95% CI, 0.85-1.00), P=0.001] (Fig. 4). The optimal cut-off value of serum periostin levels was 302 ng/ml based on the Youden index, and respective diagnostic performances for distinguishing CCA from others were shown in Table II: sensitivity, 0.88 (95% CI, 0.47-0.99); specificity, 0.92 (95% CI, 0.83-0.96); accuracy, 0.91 (95% CI, 0.83-0.96); PPV, 0.54 (95% CI, 0.25-0.81); NPV, 0.98 (95% CI, 0.92-0.99); positive-LR, 10.4 (95% CI, 4.8-13.4); and negative-LR, 0.13 (95% CI, 0.03-0.49).

In all of hepatic malignant neoplasms (n=45), the AUC of serum periostin levels for distinguishing CCA from other hepatic malignancies was greater than that of other tumor markers, including AFP, CEA, or CA19-9 (Table III).

Discussion

In this study we demonstrated that pathologically-determined periostin expression was stronger in the fibrous stroma of CCA compared with other hepatic malignancies. In addition, serum periostin level was significantly elevated in patients with CCA and enabled distinction between CCA and other hepatic malignancies.

Periostin occurs in fibrous stroma and plays significant roles in the development, promotion, and progression of various cancers (29). Recently, Riener *et al* and Utispan *et al* reported that periostin expression is seen in the stromal fibroblasts of CCA tissue (16,17). In good accordance with previous reports, high expression of periostin was also noted in the stroma of CCA tissue in this study.

Although the reason and the impact of this up-regulation of periostin in the fibrous stroma of CCA remain unclear, transforming growth factor β (TGF-β) seems to be a key molecule because the expression of periostin is regulated by TGF-β (30). TGF-β is reported to be involved in the cholangiocarcinogenesis (31), growth (32), invasion (33), and epithelial-mesenchymal transition of CCA (34). Thus, increased expression of periostin may be caused by TGF-β and have an impact of the malignant potential of CCA.

In the present study, we also revealed that no periostin expression was seen in normal liver, liver cirrhosis, or focal nodular hyperplasia. Furthermore, we observed that low to moderate expression of periostin was seen in the capsule of HCC tissue and in fibrous stroma of HCC with sarcomatous change. Thus, high expression of periostin was unique to the stroma of CCA. Since periostin is a secretory protein (11,22), these findings let us hypothesize that serum periostin level may enable distinction between CCA and other hepatic malignancies.

Table II. Diagnostic performance of serum periostin levels for distinguishing cholangiocarcinoma from others.

Cut-off value	TP	TN	FN	FP	Sensitivity (95% CI)	Specificity (95% CI)	Accuracy (95% CI)	PPV (95% CI)	NPV (95% CI)	LR+ (95% CI)	LR- (95% CI)
302 ng/ml	7	65	1	6	0.88 (0.47-0.99)	0.92 (0.83-0.96)	0.91 (0.83-0.96)	0.54 (0.25-0.81)	0.98 (0.92-0.99)	10.4 (4.8-13.4)	0.13 (0.03-0.49)

TP, true positive; TN, true negative; FN, false negative; FP, false positive; CI, confidence interval; PPV, positive predictive value; NPV, negative predictive value; LR+, positive likelihood ratio; LR-, negative likelihood ratio.

Table III. Areas under receiver operating characteristic curves for distinguishing cholangiocarcinoma from other hepatic malignancies.

Serum level of each tumor marker	AUC	95% CI	P-value
AFP	0.760	0.620-0.903	0.021
CEA	0.746	0.568-0.923	0.029
CA19-9	0.631	0.361-0.901	0.244
Periostin	0.936	0.849-1.000	<0.001

AUC, area under receiver operating characteristic curve; CI, confidene interval; AFP, α fetoprotein; CEA, carcinoembryonic antigen; CA19-9, carbohydrate antigen 19-9. Each AUC was measured when finding was positive as follows: differentiating cholangiocarcinoma from other hepatic malignancies was positive when each serum level of CEA, CA19-9, or Periostin was higher than any cut-off values and when serum level of AFP was lower than those.

Serum levels of periostin are evaluated in patients with non-small cell lung carcinoma and breast cancer (12,13). Although serum levels of periostin are associated with disease progression, no studies have compared levels between healthy subjects and cancer patients (12,13). Sasaki *et al* measured serum periostin level in patients with thymoma and reported that there are large overlapping ranges between serum levels of periostin in thymoma patients and controls (35). Therefore, we developed a monoclonal antibody against periostin and established new ELISA for measurement of serum periostin level. Using the new ELISA using monoclonal antibody, we first demonstrated that serum periostin level in patient with CCA was significantly elevated compared to those in patients with normal liver, liver cirrhosis, HCC, and other hepatic malignancies.

We also examine the potential use of serum periostin level as a serodiagnostic marker for CCA. In bivariate logistic regression analysis, serum periostin level was only associated with CCA group. These findings suggest that serum periostin can distinguish CCA group from other groups. In ROC analysis, AUC of serum periostin level showed more than 0.9, indicating a potential of high diagnostic accuracy (28). Moreover, ROC analyses among hepatic malignancies revealed that periostin showed the widest AUC compared to other tumor markers, such as AFP, CEA, and CA19-9. Finally, we examined diagnostic performances of serum periostin level for CCA and found the optimal cut-off value of 302 ng/ml showed acceptable sensitivity, specificity, accuracy, PPV, and NPV. In addition, positive-LR and negative-LR showed more than 10 and between 0.1-0.2, respectively, indicating a potential of clinical practical use (36). Although CA19-9, CEA, and CA-125 are currently the most widely used serum tumor markers for CCA, the sensitivity and specificity of these tumor markers are low (18,37), which consisted with our results. While serum levels of interleukin-6, trypsinogen-2, mucin 5AC, and soluble fragment of cytokeratin 19 are also reported as potential tumor makers, their clinical roles have not yet been elucidated (18,37). These previous reports and our own findings suggest that serum periostin is a novel serodiagnostic marker for CCA.

There were some potential limitations to our study. The study was retrospective and involved a relatively small number of patients. Similarly the cut-off value of serum periostin level was based on small number of patients. A prospective study with a substantially large sample from multiple centers is needed to further validate our findings.

In conclusion, we showed that periostin expression was higher in the fibrous stroma of CCA compared to other hepatic malignancies. Furthermore, serum periostin level was significantly elevated in patients with CCA, thereby, enabling distinction between CCA and other hepatic malignancies.

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Case Report

Development of intrahepatic cholangiocarcinoma after a 14-year follow-up of a patient with primary sclerosing cholangitis and ulcerative colitis

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Intrahepatic cholangiocarcinoma (ICC) is one of the life-threatening complications of primary sclerosing cholangitis (PSC). However, the incidence of ICC in Japanese PSC patients is low, and the association between the development of ICC and morbidity duration of PSC is largely unknown. Here, we describe a case of ICC that developed after a long-term follow-up of a patient with PSC and ulcerative colitis (UC). At the age of 10 years, the patient was first diagnosed with UC and its remission was achieved with systemic steroid therapy. Since then, he was routinely followed-up. At the age of 19 years, laboratory tests showed abnormalities in liver function parameters, and the patient was diagnosed with PSC. Although treatment with ursodeoxycholic acid improved the abnormalities in serum levels of biliary enzymes and no PSC-related symptoms were seen for 13 years, calculous cholecystitis frequently occurred in the patient since the age of 32 years. He developed ICC, which expressed some hepatic progenitor cell markers such as CD133, neural cell adhesion molecule, keratin 7, and keratin 19 at the age of 33 years. ICC was treated by curative partial hepatectomy and adjuvant chemotherapy with gemcitabine. Eight months later, however, the patient developed multiple metastases in the abdominal lymph nodes and lungs, and died 21 months after the onset of ICC. Here, we report a case of ICC that developed after a 14-year follow-up of a patient with PSC and UC.

Key words: cholangiocarcinoma, Japanese, long-term follow-up, primary sclerosing cholangitis, ulcerative colitis.

INTRODUCTION

PRIMARY SCLEROSING CHOLANGITIS (PSC) is a cholestatic liver disease characterized by multiple fibrotic strictures of the intra- and extrahepatic biliary tree.[1–3] Although PSC is generally slow progressive, it is refractory to therapy, and frequently results in advanced liver cirrhosis.[1–3] Intrahepatic cholangiocarcinoma (ICC) is the most feared complication of PSC and the occurrence of ICC leads to a poor prognosis for PSC patients.[4,5]

The prevalence of ICC in patients with PSC is reported to be approximately 7–15% in the USA.[2] While, a Japanese national survey disclosed the prevalence of ICC to be only 3.6% in PSC patients (14/391).[5] The survey also revealed two clinical characteristics for PSC-associated ICC in Japan. First, the average follow-up period between PSC and ICC diagnoses is relatively short (average period, 2.6 ± 3.5 years).[5] Second, PSC patients with inflammatory bowel disease (IBD) are less likely to develop ICC.[5] However, PSC-associated ICC is a rare disorder in Japan, and limited information is available about the clinical characteristics of this devastating disorder. Here, we report a case of ICC, which developed

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after a 14-year follow-up of PSC and ulcerative colitis (UC).

CASE REPORT

A 10-YEAR-OLD Japanese male visited Kurume University Hospital because of abdominal pain and hematochezia. The patient was diagnosed with UC by colonic biopsy. Since the patient had an allergic reaction to 5-aminosalicylic acid, remission of UC was achieved by systemic steroid therapy. After withdrawal of steroid therapy, exacerbation of UC occurred, and he was administered a continuous 5 mg/day of prednisolone (Fig. 1).

At the age of 19 years, laboratory tests of the patient showed abnormalities in liver function parameters with elevated serum levels of biliary enzymes, including alkaline phosphatase (ALP) (Fig. 1). Multiple strictures and dilatation of the intrahepatic bile ducts were seen on endoscopic retrograde cholangiography (ERC). His liver biopsy showed concentric periductal fibrosis (Fig. 2). Thus, the patient was diagnosed with PSC associated with UC. After treatment with 600 mg/day of ursodeoxycholic acid (UDCA), his serum levels of biliary enzymes were decreased (Fig. 1).

At the age of 25 years, the patient complained of abdominal pain and hematochezia. Colonoscopy revealed diffuse mucosal inflammation and ulcerations

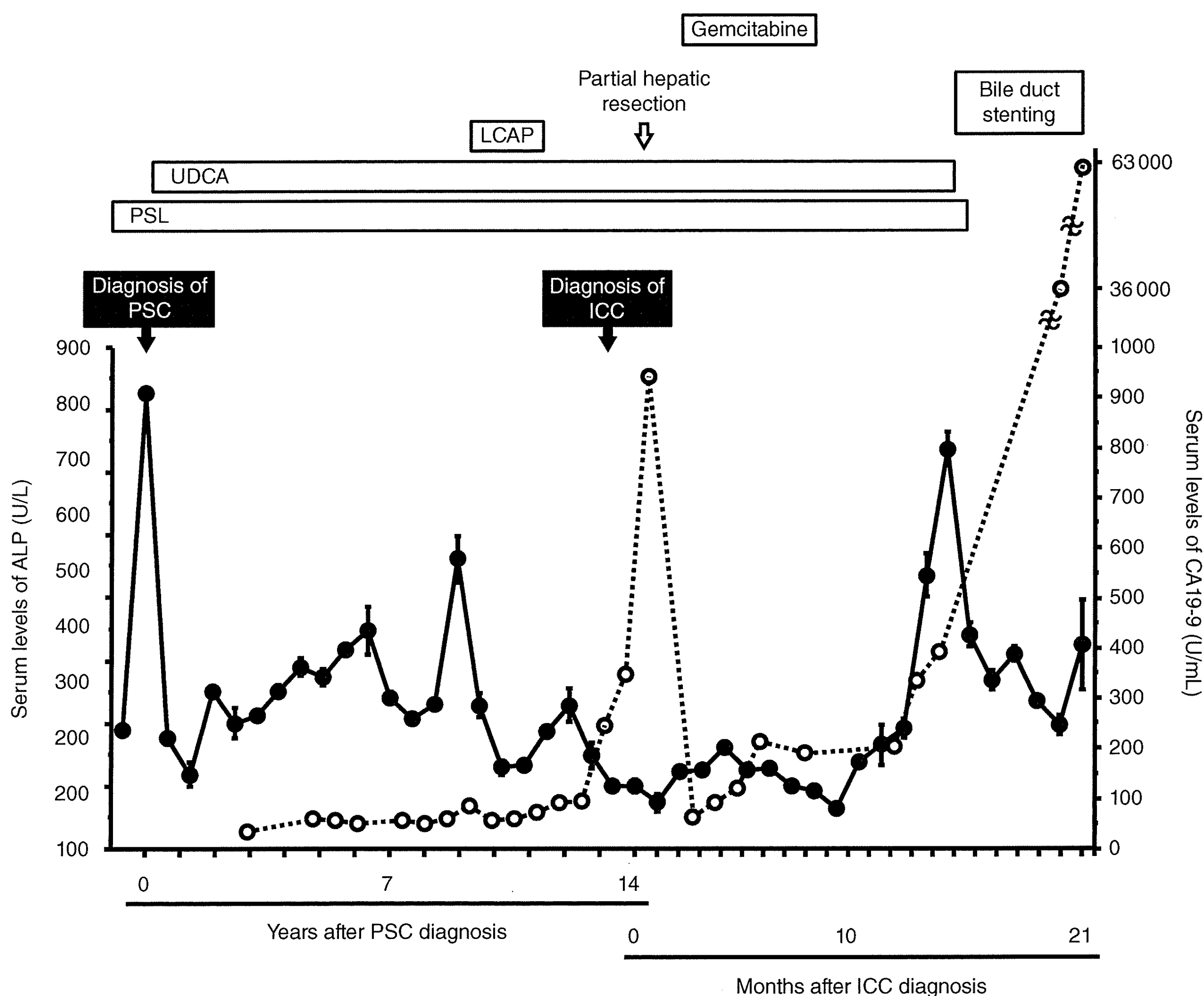

Figure 1 Clinical course and changes in serum alkaline phosphatase (ALP) and carbohydrate antigen 19-9 (CA19-9) levels. Serum levels of ALP (black circle) are shown as mean ± standard deviation (SD) of the all data measured in the indicated year or month, and serum levels of CA19-9 (white circle) are shown as absolute values. PSC, primary sclerosing cholangitis; ICC, intrahepatic cholangiocarcinoma; PSL, prednisolone; UDCA, ursodeoxycholic acid; LCAP, leukocytapheresis. (–●–): ALP; (···○···): CA19-9.

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in the descending colon. Simultaneously, laboratory tests showed elevated serum levels of biliary enzymes, including ALP and total bilirubin levels, indicating exacerbation of both UC and PSC. Leukocytapheresis therapy was initiated and marked improvement in clinical symptoms and colonoscopic findings were noted (Fig. 1). Furthermore, serum levels of biliary enzymes decreased, as previously reported.[6]

At the age of 32 years, the patient complained of abdominal pain with no hematochezia. After evaluation of biochemical and diagnostic images, the patient was diagnosed with calculous cholecystitis. His abdominal pains and biochemical abnormalities were improved by a conservative therapy of total parenteral nutrition. Although brush cytology using ERC showed suspected adenocarcinoma, ICC was not detected by computed tomography (CT) and ERC (Fig. 3). Thereafter, frequent recurrence of calculous cholecystitis was observed.

When the patient was 33 years of age, a hepatic space-occupying lesion (SOL; diameter, 35 mm) was detected by the abdominal ultrasonography (Fig. 4a). His biochemical parameters are summarized in Table 1. His serum carbohydrate antigen 19–9 (CA 19–9) level was 72.6 U/mL. On CT, contrast enhancement was not seen in the hepatic SOL (Fig. 4b). The hepatic SOL showed an accumulation of fluoro-2-deoxyglucose on positron emission tomography (Fig. 4c). On the basis of these findings, the hepatic SOL was clinically diagnosed as ICC. Since bile duct histology demonstrated no malignancy under ERC, curative partial hepatectomy was performed. The pathological diagnosis of the cancerous lesion was ICC and that of non-cancerous lesion was non-cirrhotic PSC (Inuyama classification A1, F1) (Fig. 4d–f). No infiltration of IgG4-positive cells was found in his liver specimens (Fig. 4g). We also performed immunohistochemistry for hepatic progenitor cell (HPC) markers such as CD133, neural cell adhesion molecule (NCAM), keratin 7 (K7), and K19 of the resected tissue (Fig. 5a–h). Some of ICC cells were weak immunoreactive for CD133 (Fig. 5b) and NCAM (Fig. 5d), and most of the ICC cells were strong immunoreactive for K7 and K19 (Fig. 5f,h).

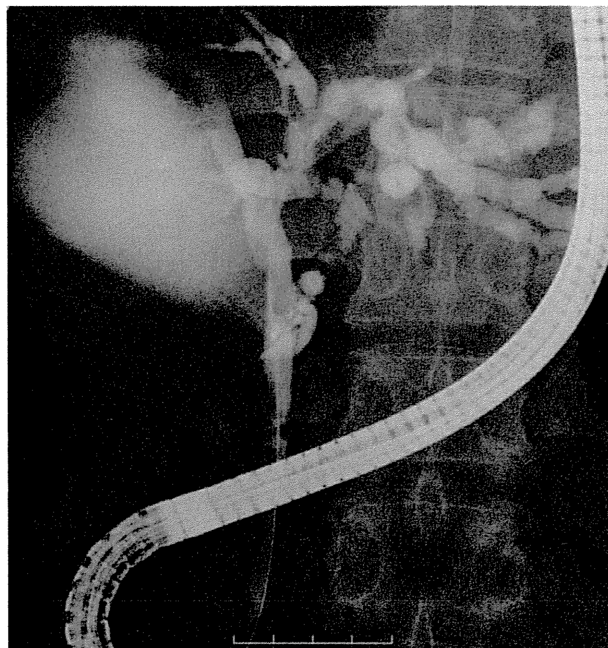

Figure 3 Endoscopic retrograde cholangiography shows multiple strictures and dilatation of the intrahepatic bile ducts. There are no findings indicating cholangiocarcinoma.

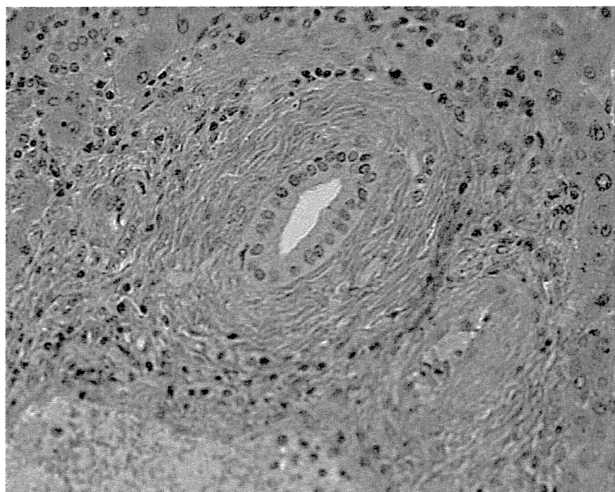

Figure 2 Histology of liver biopsy. Examination of liver biopsy specimen shows concentric periductal fibrosis, "onion-skin appearance". Original magnification × 200.

After partial hepatectomy, he was administered gemcitabine (1400 mg/day) every 2 weeks for 6 months as adjuvant chemotherapy. However, 2 months after termination of chemotherapy, he developed multiple metastases in the liver, abdominal lymph nodes, and lungs and died 21 months after the onset of ICC (Fig. 1).

DISCUSSION

THE CHARACTERISTICS OF ICC in patients with PSC are different depending on the geographical location.[2,3,5] In this report, we presented a case of ICC that developed after a 14-year follow-up of PSC and UC in Japan.

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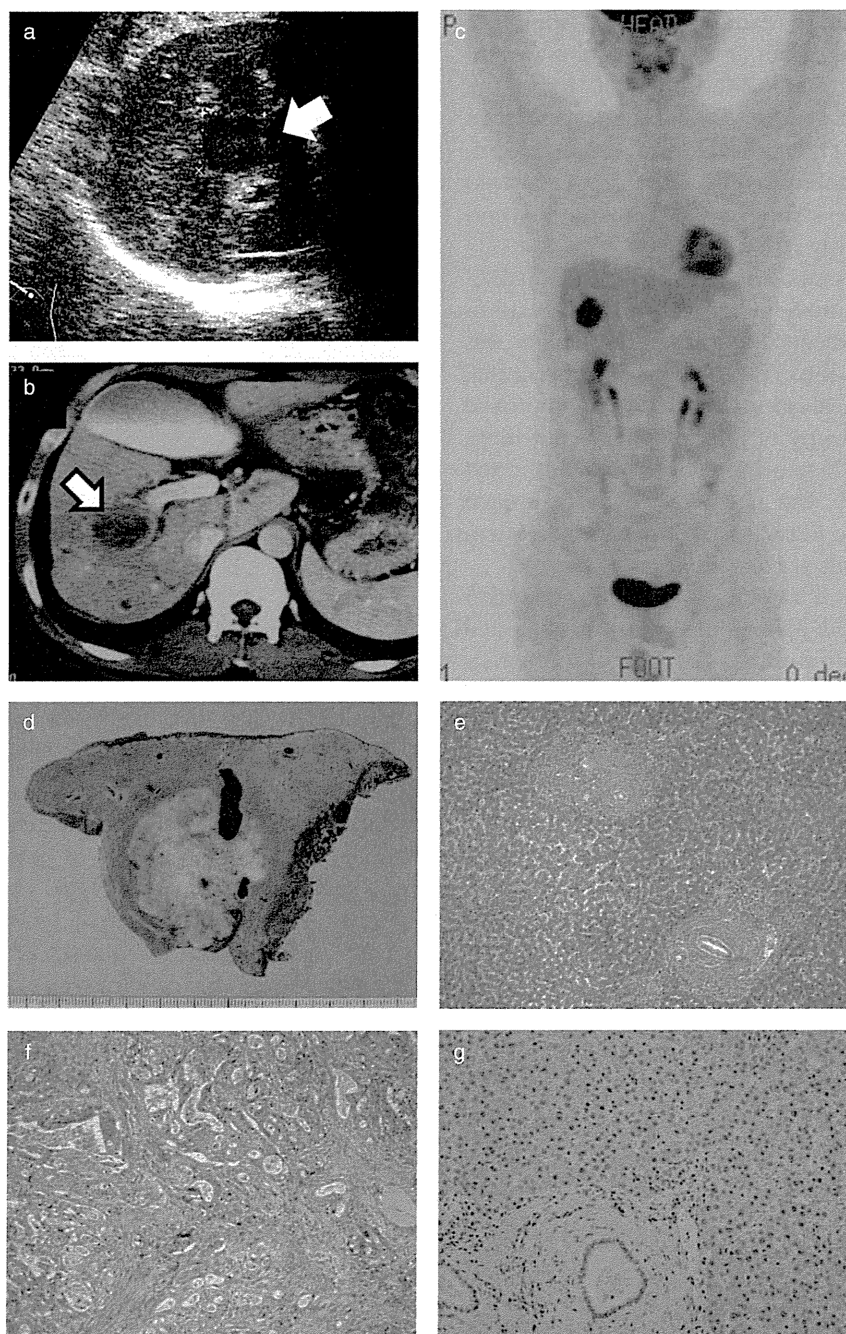

Figure 4 (a) Abdominal ultrasonography shows hypoechoic a space-occupying lesion (SOL; diameter, 35 mm) in the liver (arrow). (b) The hepatic SOL shows no contrast enhancement in the early phase of computed tomography (arrow). (c) The hepatic SOL shows accumulation of fluoro-2-deoxyglucose on positron emission tomography scan. (d) Macroscopic view shows a mass with a blurred border arising in a non-cirrhotic liver. (e) Liver histology demonstrates concentric periductal fibrosis, mild inflammation, and fibrous expansion of the portal area (Inuyama classification A1, F1). Hematoxylin-eosin staining. Original magnification × 20. (f) Tumor histology shows an adenocarcinoma showing small tubular, acinar or cord-like structures with a slit-like lumen. Hematoxylin-eosin staining. Original magnification × 100. (g) No infiltration of IgG4-positive cells is seen in the liver specimens. Immunostaining for IgG4. Original magnification × 100.

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Table 1 Biochemical parameters measured at the onset of cholangiocarcinoma

Examination	Reference value	Value
Red blood cell ($\times 10^4$/mm^3)	430–570	567
Hemoglobin (g/dL)	14.0–18.0	15.7
White blood cell (/mm^3)	4000–9000	6000
Platelet ($\times 10^4$/mm^3)	13–36	29.6
Aspartate transaminase (U/L)	13–33	32
Alanine aminotransferase (U/L)	8–42	49
Lactate dehydrogenase (U/L)	119–229	161
Alkaline phosphatase (U/L)	115–359	286
γ-glutamyl transpeptidase (U/L)	10–47	101
Total protein (g/dL)	6.70–8.30	7.64
Albumin (g/dL)	4.00–5.00	3.90
Total bilirubin (mg/dL)	0.30–1.50	0.89
C-reactive protein (mg/dL)	<0.40	0.17
Total cholesterol (mg/dL)	128–220	190
Fasting blood glucose (mg/dL)	80–109	102
Hemoglobin A1c (%)	4.3–5.8	4.9
Prothrombin activity (%)	60–130	97
α-fetoprotein (ng/mL)	<8.7	6.1
Protein induced by vitamin K absence (mAU/mL)	<40	30
Carcinoembryonic antigen (ng/mL)	<5.0	3.6
Carbohydrate antigen 19-9 (U/mL)	<37.0	76.2
DuPan-2 (U/mL)	<150	63
S-pancreas-1 antigen (U/mL)	<30	25
Erastase-1 (ng/dL)	100–400	170
IgG4 (mg/dL)	4.8–105.0	39.9

The average period between PSC and ICC diagnoses are reported to be 2.3 years in Sweden.[7] Similarly, a Japanese national survey for PSC conducted in 2003 reported that a relatively short period between PSC and ICC diagnoses is a characteristic of PSC-associated ICC (average period, 2.6 ± 3.5 years).[5] However, the period between PSC and ICC diagnoses in our case was 14 years, which is more than five times longer than the average period in Japan. Although the reason for this discrepancy remains unclear, a possible reason is that the national survey was performed in hospitals specializing in gastroenterology. In the Japanese survey, 50% of ICC cases were diagnosed within a month after diagnosis of PSC, suggesting that about a half of the PSC patients were referred to medical specialists for examination of suspected ICC, and ICC might be already developed when these patients visited the hospitals. Another possible reason is UDCA administration. UDCA is known to inhibit taurocholate and taurolithocholate-induced growth of human cholangiocytes.[8] In addition, the incidence of ICC in PSC patients treated with UDCA is reported to be lower than that in PSC patients not treated with UDCA.[9] In our case, administration of UDCA from the early stage of PSC may have contributed to late onset of ICC.

Another feature of ICC in Japanese PSC patients is that the complication of IBD is a negative factor for the development of ICC. Although our patient had UC, the impact of IBD on the development of ICC is unclear. Recently, Melum *et al.* reported that the natural killer cell receptor G2D, which plays a crucial role in tumor surveillance by NK cells,[10] is involved in preventing development of ICC in patients with PSC.[11] On the other hand, steroids inhibit natural killer cell receptor G2D-mediated NK cell activity.[12] Since our patient had an allergic reaction to 5-aminosalicylic acid, the cumulative dose of steroids administration is more than 25 000 mg. Thus, IBD might have had an indirect effect on the development of ICC in our patient.

Even in transplanted patients with PSC-associated ICC, average tumor-free survival rate is only 30–35% in 3 years,[4] therefore, data on risk factors and effect of chemotherapy for ICC may provide important information on the clinical management of PSC patients. ICC is generally considered a late complication of advanced PSC-related liver cirrhosis.[13] However, histological examination in non-cancerous lesions showed mild inflammation and fibrous expansion of the portal area in our patient. Likewise, the prevalence of esophageal varices is low in PSC patients with ICC,[14] suggesting that ICC may not inevitably be a late complication of PSC; therefore, it may be necessary to be alert to the development of ICC in any stage of PSC.

Intrahepatic cholangiocarcinoma shows a variable cholangiocytic differentiation[15] and ICC with HPC phenotypes has been proposed recently.[16–19] In our case, some of the ICC cells were weak immunoreactive for CD133 and NCAM, and most of the ICC cells were strong immunoreactive for K7 and K19, suggesting that the origin of ICC is derived from HPCs. Even though curative partial hepatectomy was performed, intra- and extra-hepatic metastases and poor prognosis were seen in our case. HPC phenotype is reported to be an independent factor for worse prognosis of ICC patients.[20] Thus, poor prognosis could be related to the HPC phenotype in our case and immunohistochemistry for HPC markers may be important for selecting therapeutic strategy and predicting prognosis for ICC patients.

Concerning chemotherapy, gemcitabine was administered as adjuvant chemotherapy in our patient.

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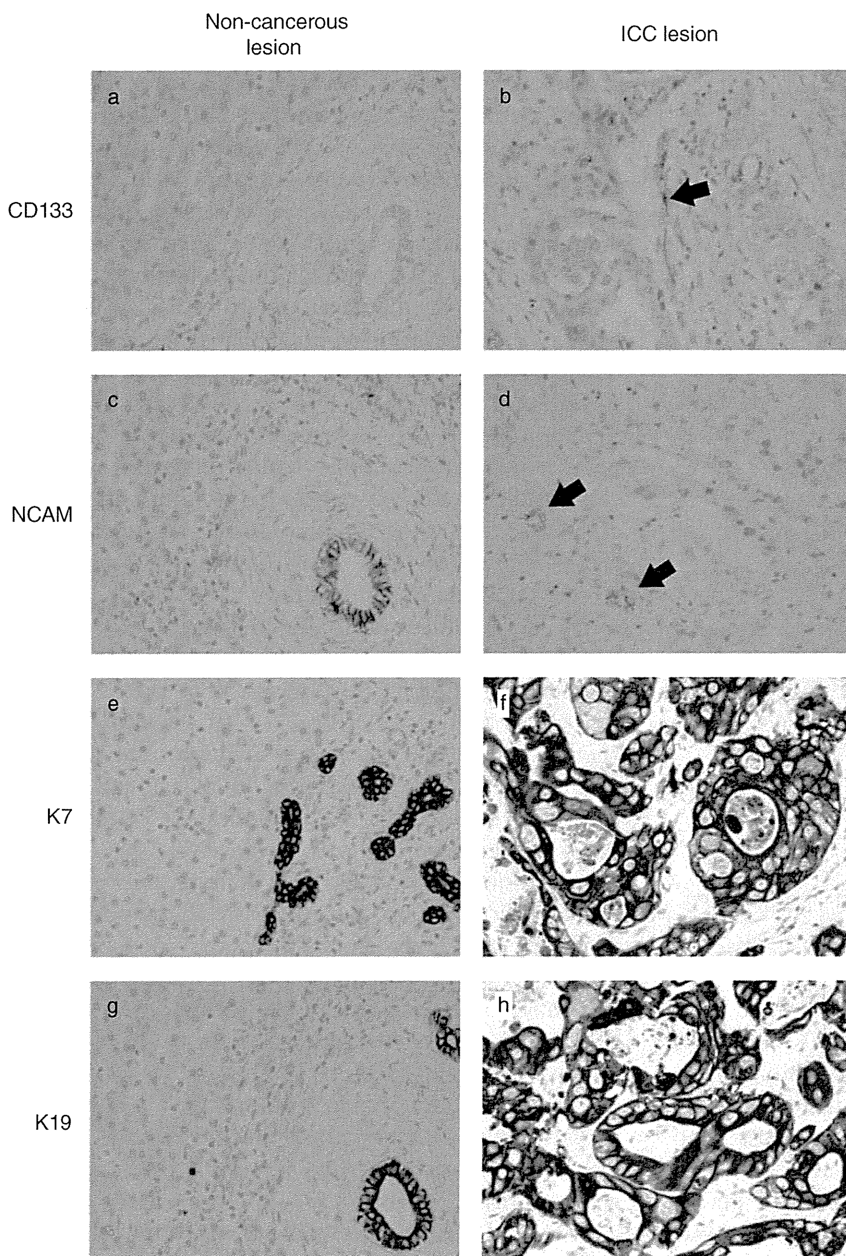

Figure 5 Immunostainig for CD133 (a and b), neural cell adhesion molecule (NCAM) (c and d), K7 (e and f), and K19 (g and h) of non-cancerous and Intrahepatic cholangiocarcinoma (ICC) lesions. Some ICC cells are weak immunoreactive for CD133 (b; arrow) and NCAM (d; arrow), and most of the ICC cells are strong immunoreactive for K7 (f) and K19 (g). Original magnification × 400. K, keratin.

However, multiple metastases occurred in the patient and he died 21 months after the onset of the ICC, suggesting that gemcitabine may not have had significant beneficial effects on the prognosis of our patient. Since there is no specific data on chemotherapy for PSC-associated ICC, it is hoped that ongoing study of molecular targeted therapy for epidermal growth factor receptors will result in a better prognosis for patients with PSC-associated ICC.[4] Thus, future studies will be focused on examining risk factors and chemotherapy for ICC in patients with PSC.

Here, we report a case of ICC that developed after a 14-year follow-up of a patient with PSC and UC in Japan. This report provides novel information on the association of development of ICC with morbidity duration of PSC and complication of IBD in Japan.

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