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Original contribution

Pathologic significance of immunoglobulin G4–positive plasma cells in extrahepatic cholangiocarcinoma☆

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Summary Immunoglobulin G4–related sclerosing cholangitis is histologically characterized by the infiltration of immunoglobulin G4–positive plasma cells and sclerosing change. Moreover, several cases of carcinoma accompanied by immunoglobulin G4–positive cells in tissue and increased serum immunoglobulin G4 levels have been reported, but the association between cancer-associated immunity and an immunoglobulin G4 reaction is still unclear. In this study, we examined the infiltration of immunoglobulin G4–positive cells in extrahepatic cholangiocarcinoma and the pathologic significance of the immunoglobulin G4 reaction found in cancer tissues in terms of the evasion of immune surveillance by regulatory T cells. Immunohistochemistry for immunoglobulin G4, forkhead box P3, CD4, and CD8 was performed using 68 surgical specimens from patients with extrahepatic cholangiocarcinoma, and positive cells were investigated, particularly within and around cancerous tissues. Consequently, although immunoglobulin G4+ cells were few (average, <10 cells/high-power field) in most cases, 10 or more and 50 or more cells were found in 37% and 6% of cases, respectively. Immunoglobulin G4+ cells were predominantly found in the invasive front of carcinoma tissue. In the cases with 10 or more immunoglobulin G4+ cells, forkhead box P3+ regulatory T cells were also distinguishable, and a positive correlation was found between the forkhead box P3+/CD4+ ratio and immunoglobulin G4+ cell count, but few CD8+ cells invaded cancer cells (<10 cells). In conclusion, extrahepatic cholangiocarcinomas are often accompanied by the significant infiltration of immunoglobulin G4+ cells, and the immunoglobulin G4 reaction showed a positive and negative correlation with forkhead box P3+ and CD8+ cells, respectively, suggesting the evasion of immune surveillance associated with CD8+ cytotoxic T cells via the regulatory function of forkhead box P3+ regulatory T cells.

1. Introduction

Immunoglobulin G4 (IgG4) is a minor immunoglobulin subtype composing 3% to 6% of all the immunoglobulin G circulating in adults [1] but important for the formation of IgG4-related diseases that feature elevated serum IgG4 levels and abundant infiltration with IgG4-positive plasma cells in affected organs [1-3]. IgG4-related diseases incorporate various IgG4-associated inflammatory disorders including autoimmune pancreatitis (AIP), sclerosing cholangitis, sialoadenitis, retroperitoneal fibrosis, inflammatory abdominal aortic aneurysm, intestinal pneumonia, interstitial nephritis, lymphadenopathy, and inflammatory pseudotumor [3-11]. Recently, the number of cases of IgG4-related diseases has increased with the growing recognition of this

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disease entity, and clinicopathologic characteristics for a differential diagnosis have been clarified. However, the pathologic significance of increased serum IgG4 levels and marked infiltration of IgG4-positive plasma cells in target organs is still unknown.

IgG4-related diseases have varied clinical symptoms that may include features similar to malignant tumors. Because most IgG4-related diseases involving AIP are resolved by corticosteroid treatment, the diagnosis, particularly differentiation from malignant tumors, is very important [12-14]. Although using an upper normal limit for serum IgG4 of 135 mg/dL, Hamano et al [1] reported a diagnostic 95% sensitivity and 97% specificity (versus pancreatic cancer) for AIP; pathologic examination is necessary to differentiate IgG4-related diseases from tumors in any organs. Moreover, several investigators have recently reported on patients with pancreatic cancer accompanying elevated serum IgG4 levels, and some cases are speculated to arise from AIP [15-17]. Raina et al [18] reported that as many as 7% of patients with pancreatic cancer have serum IgG4 levels greater than 135 mg/dL and concluded that in patients with pancreatic mass lesions and suspicion of cancer, an IgG4 level measuring between 135 and 200 mg/dL should be interpreted cautiously and not accepted as diagnostic of AIP without further evaluation. Some kind of association between tumor immunity and IgG4 reactions has been assumed, but detailed information is not available.

The participation of CD4+CD25+ forkhead box P3 (Foxp3)+ regulatory T cells (Treg) and T helper 2–type helper T cells in the pathogenesis of the IgG4 reaction in IgG4-related diseases has been proposed [19]. Treg cells play a role in the progression of various malignant tumors, particularly in controlling the immune response against pancreatic ductal carcinoma from the premalignant stage to established cancer [20]. A high prevalence of Treg cells, moreover, seems to indicate a poor prognosis [20].

In this study, we retrospectively evaluated IgG4-positive plasma cells in extrahepatic cholangiocarcinomas including common bile duct cancers, gallbladder cancers, and cancers of the papilla of Vater and investigated the significance of the IgG4 reaction in cholangiocarcinoma from the point of view of tumor immune escape mediated by Treg cells.

2. Materials and methods

2.1. Patients and tissue preparations

Formalin-fixed and paraffin-embedded sections of 68 surgically resected specimens from 39 patients with gallbladder cancer, 21 patients with common bile duct cancer, and 8 patients with cancer of the papilla of Vater (average age, 74 years; male/female, 38/30) treated from 1998 to 2009 were obtained from the registry of liver diseases in the Department of Pathology, Kanazawa University School of Medicine. In 30 cases, follow-up data were also obtained in the present study. Each cholangiocarcinoma was classified histologically as well- (including papillary), moderately, or poorly differentiated, based on the predominant histologic grade. Special histologic types such as adenosquamous carcinoma and mucinous carcinoma were not included in the present study. Four-micrometer-thick serial sections were prepared from each formalin-fixed, paraffin-embedded block. One was stained with hematoxylin and eosin (H&E), and the others were used for immunohistochemistry.

2.2. Immunohistochemistry

The deparaffinized and rehydrated sections were microwaved in EDTA buffer for IgG4 and Foxp3, in buffer at pH 9 for CD4, and in citrate buffer for CD8 and vimentin for 20 minutes in a microwave oven. After the blocking of endogenous peroxidase, these sections were incubated at 4°C overnight with antibodies against IgG4 (mouse monoclonal, diluted 1:200; Southern Biotech, Birmingham, AL), Foxp3 (mouse monoclonal; 10 μg/mL; Abcam, Cambridge, United Kingdom), CD4 (mouse monoclonal, undiluted; Nichirei, Tokyo, Japan), CD8 (mouse monoclonal, diluted 1:20; Dako, Tokyo, Japan), neutrophil elastase (mouse monoclonal, diluted 1:100; Dako), vimentin (mouse monoclonal, diluted 1:600; Dako), and CD34 (mouse monoclonal, diluted 1:200; Beckman Coulter, Tokyo, Japan) and then at room temperature for 1 hour with antimouse immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple Staining Kit; Nichirei). After a benzidine reaction, sections were counterstained lightly with hematoxylin. No positive staining was obtained when the primary monoclonal antibody was replaced with an isotype-matched, nonimmunized immunoglobulin as a negative control of the staining procedures.

2.3. Histologic examination

In addition to histologic observation by H&E staining, the distribution of the immunopositive cells was examined. In a primary survey, we examined all tumorous area in each specimen and, for counting IgG4+, Foxp3+, CD4+, or CD8+ mononuclear cells, selected 3 representative areas containing IgG4+ plasma cells and expressed results as the mean number of each immunopositive cell in high-power fields (HPFs). For semiquantitative evaluation of the IgG4 staining, the cases with 10 or more and less 10 IgG4+ cells per HPF on average were evaluated as IgG4-positive and IgG4-negative cases, respectively. The ratio of Foxp3+ to CD4+ cells was calculated for the 3 selected areas in each case, and the average ratio (Foxp3/CD4) was compared between IgG4-rich and IgG4-poor cases because the absolute number of Foxp3+ cells was prominently affected by the number of infiltrating mononuclear cells. Moreover, the average number of CD8+ cytotoxic T cells (CTLs) within carcinoma cells was evaluated to estimate

the extent of the host immune response to the cancer. Finally, to confirm whether the IgG4 reaction is due to cancer-associated immunity, the area with IgG4-positive cells was evaluated in terms of neutrophilic infiltration, fibrosis, and granulation. For neutrophilic infiltration, neutrophil elastase–positive cells were counted in the same area as IgG4-positive cells. For fibrosis and granulation, the immunoreactivity of vimentin and CD34, respectively, was used, and the degree of change was semiquantitatively graded as follows: 0, absent; 1+, mild; 2+, intermediate; 3+, severe.

2.4. Statistical analysis

Data were analyzed using the Spearmann correlation coefficient test. Survival curves to evaluate the association between prognosis and IgG4 reactions were calculated by the Kaplan-Meier method, and analyses were conducted with the log-rank test. $P < .05$ was considered to be statistically significant.

3. Results

3.1. Detection and distribution of IgG4+ cells in extrahepatic cholangiocarcinoma

Immunohistochemistry revealed IgG4+ plasma cells to be scattered within and around cancerous nests in most cases (Fig. 1). Particularly around nests and in the invasive area facing a noncancerous biliary wall and surrounding fibroadipose tissue, these positive cells were prominent with some intermingling of other inflammatory cells (Fig. 2). Moreover, 1 characteristic of IgG4-related diseases, the perineural infiltration of IgG4+ cells, was commonly seen in extrahepatic cholangiocarcinomas. In contrast, desmoplastic change and vascular invasion by cancer cells are usually seen in extrahepatic cholangiocarcinomas, but other features of IgG4-related diseases, obliterative phlebitis caused by IgG4+ cells and storiform-type fibrosis, are rare.

A quantitative evaluation of IgG4+ cells revealed that 25 (37%), 19 (28%), and 4 (6%) of 68 cholangiocarcinoma patients had 10 or more, 20 or more, and 50 or more IgG4+ cells per HPF, respectively. There was no correlation between the density of IgG4+ cells and any clinicopathologic factor including age, sex, anatomical location (common bile ducts, gallbladder, and the papilla of Vater), or the histologic differentiation (well, moderate, and poor) of extrahepatic cholangiocarcinomas.

3.2. Association between IgG4 reactions and Foxp3+ Treg cells

The association between IgG4+ and Foxp3+ cells was evaluated in each case. Foxp3+ Treg cells were scattered in most cases with a marked IgG4 reaction (Fig. 3). The relation between the IgG4 reaction and Foxp3+ Treg cells is shown in Fig. 4. In IgG4-rich cases (≥10 IgG4+ cells/HPF), the ratio of Foxp3+/CD4+ cells correlated closely with the IgG4+ cell count, although in IgG4-poor cases (<10 IgG4+ cells/HPF), the correlation varied.

3.3. Association between IgG4 reactions and CD8+ CTLs

CD8+ CTLs were scattered to various degree in each case irrespective of whether they were within or around cancer nests. As a marker of immune activity against cancers, CTLs

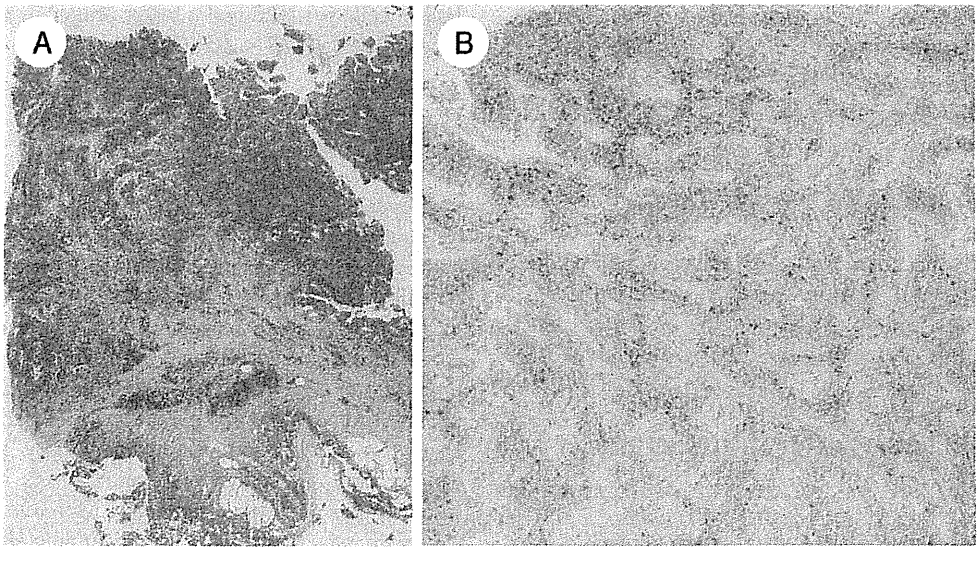

Fig. 1 Common bile duct cancer. A, Papillary adenocarcinoma. Inflammatory cells are prominent (H&E staining, original magnification, ×20). B, Immunohistochemistry for IgG4. Numerous IgG4+ cells are present in the inflamed stroma (original magnification, ×100).

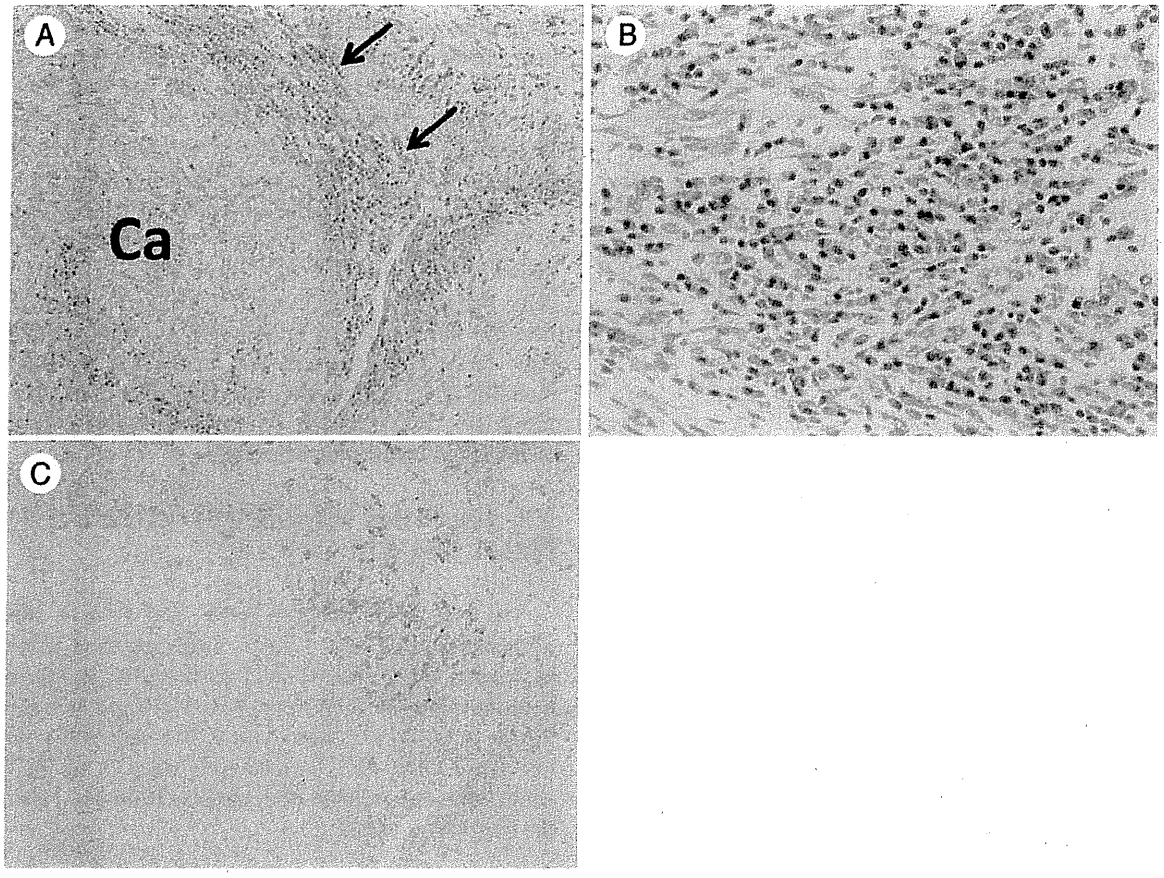

Fig. 2 Common bile duct cancer. A, A marginal zone (arrows) of poorly differentiated adenocarcinoma (Ca). Many inflammatory cells are present (H&E staining, original magnification ×100). B, Higher magnification of the marginal zone. Inflammatory cells are mostly composed of plasma cells (H&E staining, original magnification ×400). C, Immunohistochemistry for IgG4. Many positive cells are scattered in the marginal zone (original magnification ×100).

invaded cancerous nests resembling intraepithelial lymphocytes, which are found in nonneoplastic biliary epithelial layers of biliary diseases such as primary biliary cirrhosis (Fig. 5) [21]. Consequently, patients with many CD8+ CTLs showed scant IgG4 reactions (IgG4-poor cases), and all IgG4-rich cases had few CD8+ CTLs (<10 cells/HPF) (Fig. 6).

3.4. Histologic conditions for IgG4 reactions

As shown in Fig. 7, there was no correlation between the numbers of neutrophil elastase–positive neutrophils and IgG4-posiitve cells. Moreover, the degree of fibrosis and granulation did not correlate with the IgG4-positive cell count either.

3.5. Association between IgG4 reactions and patients' survival

After the surgical resection of extrahepatic cholangiocarcinomas, of the 30 patients with available outcome data, 21 died of recurrence of the cancer. The overall survival curve for the 30 patients, obtained using the Kaplan-Meier estimator, is shown in Fig. 7. The patients with 20 or more IgG4+ cells per HPF had a better prognosis than those with 20 or more cells ($P < .05$) (Fig. 8).

4. Discussion

Elevated serum IgG4 levels and the infiltration of organs by numerous IgG4+ plasma cells are clinicopathologic hallmarks of IgG4-related diseases. Moreover, obliterative phlebitis, storiform-type sclerosing fibrosis, and sometimes mass forming–type sclerosing fibrosis are also characteristic of this disease category. It is clinically and pathologically important to distinguish IgG4-related diseases from tumors of affected organs. In particular, because desmoplastic change is a common feature of biliary and pancreatic cancers, IgG4-related diseases and cancers in these organs show similar radiologic behaviors. Moreover, patients with pancreatic adenocarcinoma accompanying an IgG4 reaction and/or elevated serum IgG4 levels [16,18,22,23] and with pancreatic and biliary cancers arising from IgG4-related

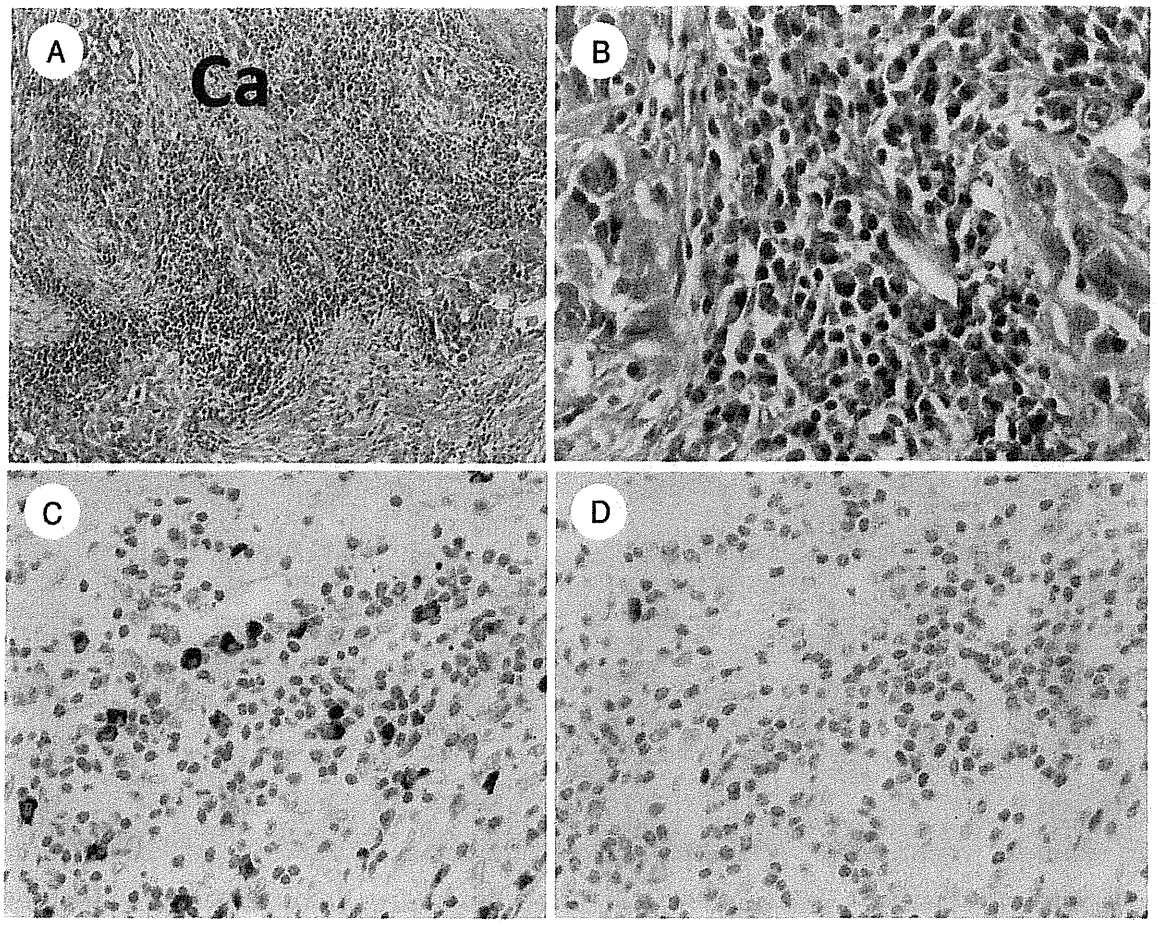

Fig. 3 Gallbladder cancer. A, An invasive area of poorly differentiated adenocarcinoma (Ca). Numerous inflammatory cells are found (H&E staining, original magnification ×100). B, Higher magnification of the invasive area. Many plasma cells are evident (H&E staining, original magnification ×400). C, Immunohistochemistry for IgG4. Several IgG4-positive cells have accumulated (original magnification ×400). D, Immunohistochemistry for Foxp3. Positive cells are scattered and slightly accumulated here and there (original magnification ×400).

disease [16,24,25] have been reported, although a cause-and-effect relationship between the IgG4 reaction and cancers has not been demonstrated. Therefore, the presence of IgG4+ cells is not a histologic hallmark of IgG4-related diseases, and the IgG4 reaction is speculated to occur nonspecifically in carcinoma tissues [26]. In this study, we retrospectively examined IgG4 reactions in cases of extrahepatic cholangiocarcinoma including common bile duct cancers, gallbladder cancer, and cancers of the papilla of Vater. Consequently, 10 or more IgG4+ cells per HPF were observed in 37% of cases, and the cases with marked infiltration (≥50 IgG4-positive cells/HPF) and resembling IgG4-related diseases made up 6% of the total. As the pathologic diagnostic criteria of IgG4-related disease, the essential number of IgG4+ cells varied from 5 to 50 per HPF depending on the affected organs, but in IgG4-related sclerosing cholangitis, 10 or more IgG4+ cells per HPF are proposed according to the HISORt criteria published for AIP [27,28]. In our cases, therefore, several cancers accompanying remarkable infiltration of IgG4-positive cells (≥50 cells/HPF) were speculated to originate from the preceding IgG4-related disease, but follow-up data before discovery of the cancers are needed to demonstrate this. Because cases of pancreatic and biliary cancers arising

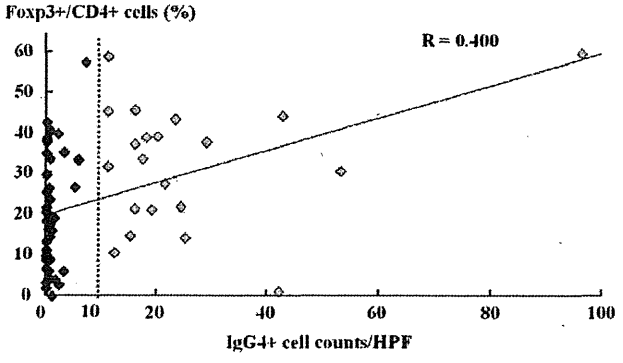

Fig. 4 Relationship between IgG4+ cells and Treg cells in extrahepatic cholangiocarcinoma. In cases with 10 or more per HPF IgG4+ cells (IgG4-rich cases), there is a good correlation between IgG4+ and the ratio of Foxp3+/CD4+ cells ($R = 0.400$).

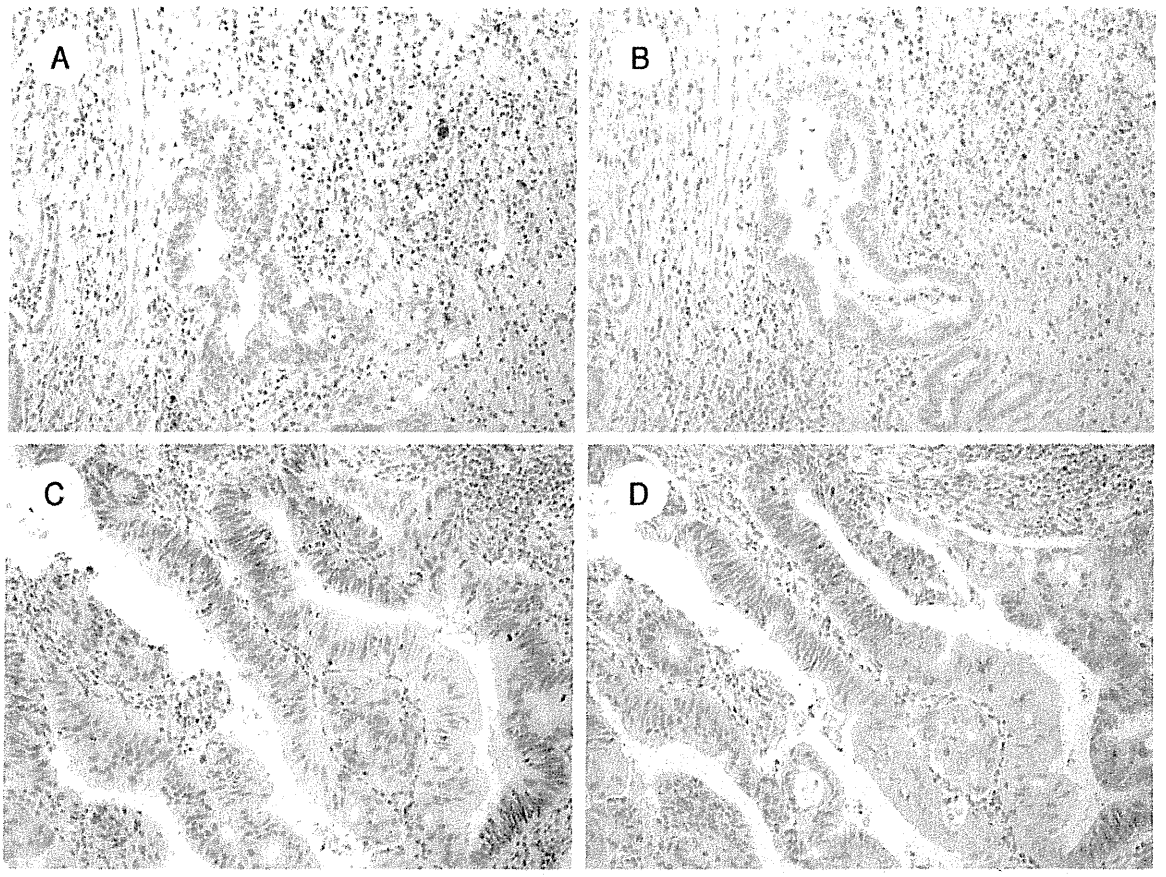

Fig. 5 Comparison of CD8+ cytotoxic T cells in IgG4-rich (A and B) and IgG4-poor (C and D) cases of extrahepatic cholangiocarcinoma. Immunohistochemistry for IgG4 (A and C) and CD8 (B and D). In IgG4-rich cases, CD8+ as well as IgG4+ cells are found around adenocarcinoma tissue, but no CD8+ cells are present in cancer cells lining the adenocarcinoma. In contrast, many CD8+ cells invade cancer cells lining the adenocarcinoma in IgG4-poor cases (original magnification ×200).

from IgG4-related disease were reported [16,24,25], IgG4-related sclerosing cholangitis is thought to be an important preceding disease in the carcinogenesis of cholangiocarcinoma. Irrespective of whether the patients have

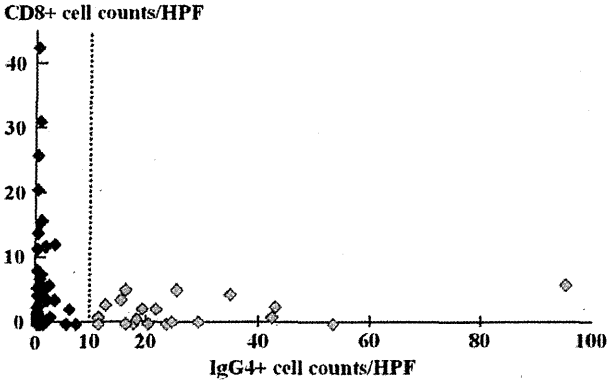

Fig. 6 Relationship between IgG4+ and CD8+ cytotoxic T cells in extrahepatic cholangiocarcinoma. In all cases with 10 or more per HPF IgG4+ cells (IgG4-rich cases), the number of CD8+ cells is less than 10.

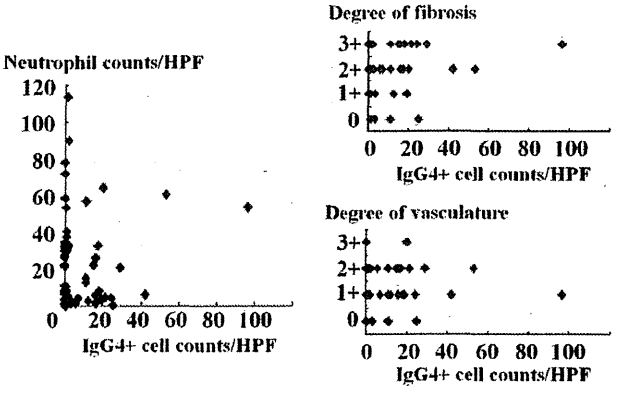

Fig. 7 Correlation of IgG4 reactions with neutrophil infiltration, fibrosis, and granulation in cholangiocarcinoma. The degree of neutrophil infiltration was evaluated from the neutrophil elastase–positive cell counts. The degree of fibrosis and granulation was semiquantitatively graded as follows: 0, absent; 1+, mild; 2+, intermediate; 3+, severe, based on the vimentin-positive area and CD34-positive neovasculature, respectively. Consequently, no correlation was found between the degree of neutrophil infiltration, fibrosis, or granulation and IgG4-positive cells.

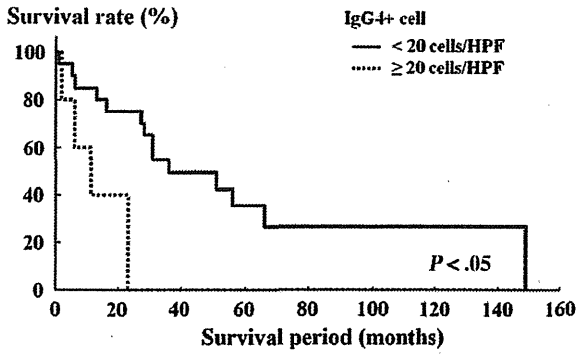

Fig. 8 Survival curve of patients with extrahepatic cholangiocarcinoma. Kaplan-Meier plots of the postoperative survival period were made for 2 groups of patients, those with less than 20 and 20 or more IgG4+-positive cells per HPF. Log-rank analysis of the postoperative survival periods indicated that the patients with less than 20 IgG4+ cells per HPF had a better prognosis ($P < .05$).

cholangiocarcinoma and a marked IgG4 reaction or cancers arising from IgG4-related sclerosing cholangitis, the presence of adenocarcinoma should be taken into account in the pathologic diagnosis of IgG4-related cholangitis, particularly using small specimens such as biopsy materials.

The present study demonstrated that an IgG4 reaction is often found to some degree in extrahepatic cholangiocarcinoma as well as IgG4-related diseases. Moreover, the perineural infiltration of IgG4+ cells, which is a feature of IgG4-related cholangitis and AIP, was prominent in IgG4-rich cholangiocarcinoma cases. In contrast, patients with IgG4-related cholangitis are generally older men (85%) [27], but this male domination was not found in the IgG4 reaction of cholangiocarcinoma. Obliterative phlebitis and storiform fibrosis are also characteristics of IgG4-related diseases but rare in cholangiocarcinoma. These histologic features of IgG4-related diseases differing from cholangiocarcinoma are not located at the superficial biliary mucosa, unfortunately suggesting that these characteristic findings are not useful for a differential diagnosis using biopsy specimens. The IgG4 reaction is not a specific immune reaction of IgG4-related diseases, but the immunopathogenesis of IgG4 reactions should be different in IgG4-related diseases and cancers. As shown in Fig. 7, no correlation between the number of IgG4-positive cells and degree of neutrophilic infiltration, fibrosis, or granulation was found, suggesting that the IgG4 reaction in cholangiocarcinoma is due to cancer-associated immunity via the different mechanisms of IgG4-related diseases characterized by fibrosis as well as IgG4 reactions. Further study is necessary to clarify the histogenesis of IgG4 reactions in cholangiocarcinoma.

IgG4 does not have the ability to activate complement, and its physiologic and pathologic significance are still unknown in healthy and IgG4-related diseased patients. In the pathogenesis of the IgG4 reaction in IgG4-related sclerosing cholangitis and pancreatitis, the participation of the T helper 2–type cytokine milieu and interleukin 10 produced by Treg cells is assumed to involve IgG4 class switching and/or the progressive proliferation/differentiation of IgG4+ plasma cells [10,19,29-31]. In the carcinogenesis of pancreatic cancer, the prevalence of Treg cells increases and that of cytotoxic CD8+ cells conversely diminishes in cancer tissues [20]. Moreover, the prevalence of Treg cells is negatively correlated with the prognosis of patients with pancreatic cancers [20]. These findings suggest that Treg cells play a role in controlling the immune response against pancreatic cancer, especially the evasion of tumor-associated immune surveillance. The present study using extrahepatic cholangiocarcinomas also demonstrated that in the IgG4-rich cases (≥10 IgG4+ cells/HPF), the number of IgG4+ cells in cancer tissue positively correlated with that of Foxp3+ Treg cells, and conversely, the number of cytotoxic CD8+ CTLs was constantly small. Therefore, extrahepatic cholangiocarcinomas as well as pancreatic cancers could cause the evasion of immune surveillance via the regulatory function of Treg cells, involving the concomitant IgG4 reaction. However, high Foxp3+/CD4+ cells ratio were also seen in many IgG4-poor cases, suggesting that the presence of Foxp3+ cells is not sufficient for the induction of IgG4 reaction. A functional analysis of infiltrating Foxp3+ Treg cells and possible other mechanisms of IgG4 reactions in cholangiocarcinoma also should be considered.

Finally, we examined the prognosis. Clinical follow-up data were available for only 30 of the 68 patients because of the retirement or transfer of primary physicians, a cessation of digestive surgery at the affiliated hospitals, and other reasons. Moreover, as more than 10 years had passed since the operation in several cases, sometimes the clinical records themselves had been destroyed. In addition to the analysis using cytotoxic CD8+ CTLs as mentioned above, survival curves obtained using the follow-up data for the 30 patients also indicated the patients with less than 20 IgG4-positive cells per HPF to have a better prognosis than those with 20 or more cells. Although the role of IgG4+ plasma cells in cancer tissue is unclear, the degree of IgG4+ cell infiltration might be a pathologic marker of extrahepatic cholangiocarcinoma.

In conclusion, this study revealed that extrahepatic cholangiocarcinoma often accompanies significant infiltration by IgG4+ cells, indicating a need to distinguish it from IgG4-related diseases, especially when using small specimens such as biopsy materials. Moreover, the IgG4 reaction in cholangiocarcinoma might be associated with the evasion of immune surveillance by CD8+ CTLs and tumor progression through Treg cells.

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Bile duct expression of pancreatic and duodenal homeobox 1 in perihilar cholangiocarcinogenesis

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Aims: Pancreatic and duodenal homeobox 1 (Pdx1) is a transcription factor that is crucial in embryogenic development and differentiation of pancreas, and its overexpression is reportedly involved in the progression of many malignancies, including pancreatic carcinoma. In this study, the role of Pdx1 was examined in cholangiocarcinogenesis.

Methods and results: Forty-three cases of human cholangiocarcinoma (CC) and 66 cases of hepatolithiasis or primary sclerosing cholangitis (PSC) with biliary intraepithelial neoplasia (BilIN) lesions and also eight fetal and 20 adult normal livers were examined immunohistochemically. Pdx1 was constantly expressed in the nuclei of fetal bile ducts, but was virtually absent in the large bile ducts of adults. By contrast, Hairy and enhancer of split 1 (Hes1), which represses pancreatic exocrine and endocrine differentiation, was expressed frequently in the adult bile ducts. Pdx1 was expressed in 67% of invasive CCs. In large bile ducts, expression of Pdx1 increased while that of Hes1 decreased during the progression of BilIN lesions to CC. Expression of Pdx1 correlated with proliferative activities in CCs. In an *in vitro* study, all three CC cell lines expressed Pdx1 mRNA and protein.

Conclusion: Up-regulation of Pdx1 is a feature of cholangiocarcinogenesis associated with chronic cholangitis. Furthermore, expression of Pdx1 in CC is related to increased proliferative activity in CCs.

Keywords: bile ducts, biliary intraepithelial neoplasia, cholangiocarcinoma, pancreatic cancer, pancreatic and duodenal homeobox 1

Abbreviations: BilIN, biliary intraepithelial neoplasia; CC, cholangiocarcinoma; Hes1, hairy and enhancer of split 1; Neurog3, neurogenin 3; Pdx1, pancreatic and duodenal homeobox 1; PSC, primary sclerosing cholangitis

Introduction

During development, the biliary system and ventral pancreas arise from a contiguous region of the endoderm, and transcription factors such as pancreatic and duodenal homeobox 1 (Pdx1) and hairy and enhancer of split 1 (Hes1) are involved in the differentiation of both the pancreas and biliary system.[1–4] Pdx1 is a transcription factor of the homeobox gene family, which is important in differentiation and development of the pancreas,[2] while Hes1 encodes the basic helix–loop–helix protein, which represses expression of neurogenin 3 (Neurog3), a promoter of pancreatic exocrine and endocrine differentiation.[3] Recently, a new approach to elucidate carcinogenesis has been reported finding possible derangement of embryonic morphogenesis with the re-activation of stem cell-related genes such as Sonic Hedgehog, Notch and pancreatic and Pdx1 during the development of tumours in of several organs.[4–6] In this context, Pdx1 may thus be involved in the malignant transformation of pancreaticobiliary system.

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Although several studies have shown overexpression of Pdx1 in a variety of malignant tumours,[5,7] including pancreatic ductal adenocarcinomas,[5,8,9] and also their precursor, pancreatic intraepithelial neoplasia (PanIN),[10] there have been no reports of Pdx1 expression in cholangiocarcinoma (CC) and its precursor, biliary intraepithelial neoplasia (BilIN).[11–13]

We hypothesize that the same factors operating in the development of the biliary system, and which may be involved in the development of PanIN and pancreatic carcinomas, are also involved in the development and progression of CC. In this study, we first surveyed the expression of Pdx1 and Hes1 in normal fetal and adult bile ducts, CCs and their precursors in the presence or absence of chronic cholangitis. We also examined the functional role of Pdx1 in cholangiocarcinogenesis using cultured CC cell lines.

Materials and methods

HUMAN TISSUE STUDIES

In this study, the hilar bile ducts and intrahepatic large bile ducts are collectively called the large bile ducts.

Case selection

Tissue specimens of the bile ducts and pancreas were obtained from our department and affiliated hospitals (Table 1). Normal adult (20 cases) and fetal (eight cases) hepatobiliary specimens were selected. CCs with hepatolithiasis (27 cases) or primary sclerosing cholangitis (PSC) (one case) or CCs unassociated with the aforementioned diseases (15 cases) were classified into well-differentiated, moderately differentiated and poorly differentiated adenocarcinoma, and rare variants (Table 1). Hepatolithiasis cases were divided into those with and without invasive CC. All CC cases in this study were of perihilar CC, including hilar CC.[14] Using the criteria of the *WHO Classification of Tumors of the Digestive System* (2010),[12,13] BilIN was classified into three grades based on the degrees of cellular and structural atypia. So-called 'carcinoma *in-situ*' was included in BilIN3. In this study, BilIN lesions composed of at least 150 epithelial cells were graded as above in each case. Consequently, a total of 104 BilIN lesions were examined consisting of BilIN1 (number of foci, 36), BilIN2 (n = 36) and BilIN3 (n = 28) from 63 cases of hepatolithiasis, and BilIN1, 2 and 3 (n = 2, 1 and 1, respectively) from three PSC cases.

As a control, 39 cases of pancreatic invasive ductal adenocarcinoma, all surgical cases, were used. In these cases, six foci of pancreatic intraepithelial neoplasm (PanIN-1b) and 12 foci of PanIN-2/3 were found in non-carcinomatous areas. Pan-IN-1, 2 and 3 were classified according to Takaori *et al.*[11]

Informed consent for research was obtained from all patients and the study was approved by the Kanazawa University Ethics committee (approval number 64).

Tissue preparation

All tissue specimens were fixed in 10% neutral buffered formalin and embedded in paraffin; 4-μm-thick sections were cut from each paraffin block, and stained with haematoxylin and eosin (H&E).

Table 1. Main clinical features of materials examined in this study

Case	No. of cases	Years (mean)	Male:female	Histological grades (well/mod/poor/v)
Hepatolithiasis	63	45–81 (63)	1:1.7	
Primary sclerosing cholangitis	3	35–65 (45)	1:2	
Cholangiocarcinoma				
With HL or PSC	28	34–83 (62)	1:2	13/10/1/4
Without HL or PSC	15	51–79 (65)	1.5:1	8/4/3/0
Normal adult liver	20	70–90 (76)	1:1.4	
Normal fetus liver*	8	8–41 weeks	–	
Pancreatic ductal adenocarcinoma	39	51–88 (69)	1.2:1	12/16/7/4

Well, Well-differentiated type; mod, moderately differentiated type; poor, poorly differentiated type; v, variant type; HL, hepatolithiasis; PSC, primary sclerosing cholangitis.
*Fetus liver containing bile ducts.

IMMUNOHISTOCHEMISTRY

Immunostaining was performed using paraformaldehyde (PFA) and formalin-fixed paraffin-embedded (FFPE) tissue sections of surgically resected liver specimens of hepatolithiasis, PSC and CC. The antibodies and their sources, optimal dilution and antigen retrieval are shown in Table 2. After blocking the endogenous peroxidase and incubation in protein block solution (DakoCytomation, Tokyo, Japan), the sections were incubated overnight at 4°C with primary antibodies against Pdx1, Hes1 or Ki-67. The sections were then treated with secondary antibodies conjugated to peroxidase-labelled polymer, EnVision system (DakoCytomation). Colour development was performed using 3,3′-diaminobenzidine (DAB) and the sections were counterstained with haematoxylin. Negative controls were also included.

Semiquantitative analysis of immunostaining

Expression of Pdx1, Hes1 and Ki-67 in the nuclei was regarded as positive. For Pdx1 and Hes1, their immunoreactivity was graded semiquantitatively in each lesion, as follows: 0, no positive cells; 1+, only weakly expressed in almost all positive cells; 3+, expressed strongly in almost all positive cells; and 2+, between +1 and +3. For Ki-67, more than 300 nuclei were evaluated in each case or lesion and the Ki-67 labelling index (LI) was shown as a percentage of positive nuclei; the LI was calculated in all 43 cases of CC.

Double immunostaining for Pdx1 and Hes1 or Ki-67

This was performed using TSA Plus fluorescence (tyramide signal amplification (TSA) Plus fluorescein/TMR kits; PerkinElmer®, Yokohama, Japan). First, immunostaining for Pdx1 was performed as above using TSA Plus tetramethylrhodamine for the colour development. After blocking endogenous peroxidase, sections were then incubated overnight at 4°C with Hes1 or Ki-67 and treated with anti-rabbit or anti-mouse immunoglobulins conjugated to peroxidase-labelled polymer using the EnVision system (DakoCytomation). Colour development was performed using TSA Plus fluorescein. In three CC cases in which Pdx1-positive and negative were evaluable separately, Ki-67 LI was compared in both lesions in each case.

MICRODISSECTION AND RNA EXTRACTION

Frozen, PFA and FFPE tissues were cut into 10-μm sections on membrane slides, and normal to reactive lesions and BilIN2/3 lesions were microdissected using a laser microdissection system (Leica, Tokyo, Japan).

RNA extraction from frozen tissues

Using the RNeasy Plus® micro kit (Qiagen, Tokyo, Japan), total RNA was extracted from whole frozen sections of CC tissue (three cases) and one focus of microdissected normal biliary epithelial cells (BEC). Total RNA (1 μg) was used to synthesize single-strand complementary DNA with reverse transcriptase (ReverTra Ace; Toyobo, Osaka, Japan).

RNA extraction from PFA FFPE tissues

Two foci of reactive lesions and two foci of BilIN2/3 lesions were microdissected from PFA or FFPE sections. For these microdissected samples, the RNeasy® FFPE kit (cat. no 74404) was used for RNA extraction.

RNA amplification and cDNA purification

Total RNA extracted from microdissected samples was amplified using Ribo-SPIA technology with the WT-Ovation™ FFPE RNA amplification system V2 (cat. no. 3400-12; NuGEN Technologies, San Carlos, CA, USA). For purification of SPIA cDNA, RNeasy MinElute Spin column (Qiagen, Tokyo, Japan) was used.

Table 2. Antibodies used in this study: source and dilution

Primary antibody against	Clone (product code)	Company	Optional dilution	Antigen retrieval method	Positive control
Pdx1	Goat poly (sc-14664)	Santa Cruz (Santa Cruz, CA, USA)	1:100	Citrate buffer AC	Islet cells in human pancreas (N)
Hes1	Rabbit poly (H2034-35)	US Biological (Swampscott, MA, USA)	1:500	Citrate buffer MW	Human fibroblast (N)
Ki-67	Mouse mono (M 7240)	Dako (Tokyo, Japan)	1:200	Citrate buffer MW	Human colon cancer (N)

Poly, Polyclonal; mono, monoclonal; AC, autoclave; MW, microwave; N, nuclear; Pdx1, pancreatic and duodenal homeobox 1; Hes1, hairy and enhancer of split 1.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative real-time PCR

The sequences of the primers for PDX1 used were as follows: 5′-TTCACGAGCCAGTATGACCTTCAC-3′ (forward), 3′-GAAGACAGACCTGGGATGCACA-5′ (reverse). The PCR products were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide. Quantitative real-time PCR was performed according to a standard protocol using the SYBR Green PCR Master Mix (Toyobo, Osaka, Japan) and Mx 3005P™ (Stratagene, Japan, Tokyo, Japan). Cycling conditions were incubation starting at 95°C for 10 min, 40 cycles of 95°C for 30 s, 62°C for 1 min and 72°C for 1 min, followed by one cycle of 95°C for 1 min, 62°C for 30 s and 95°C for 30 s. mRNA levels were evaluated by taking the ratio with glyceraldehyde-3-phosphate dehydrogenase by qRT-PCR.

CELL CULTURE AND RNA EXTRACTION

All the following *in-vitro* experiments were performed at least in triplicate. Human cholangiocarcinoma cell lines (HuCCT1 and TFK1) were provided by the Health Science Research Resources Bank (Osaka, Japan), and human pancreatic cancer cell lines (PANC-1, MIA Paca2, KP4-1, PK-59) were provided by Riken BRC (Saitama, Japan). A human cholangiocarcinoma cell line (CCKS-1) was established in our laboratory. To maintain the cell lines, RPMI-1640 (Roswell Park Memorial Institute 1640; Life Technologies, Inc., Rockville, MD, USA) was used for HuCCT1, PANC-1 and PK59. Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Inc.) was used for MIA Paca2 and DMEM/F-12 (DMEM and nutrient mixture F-12, 1:1; Life Technologies, Inc.)

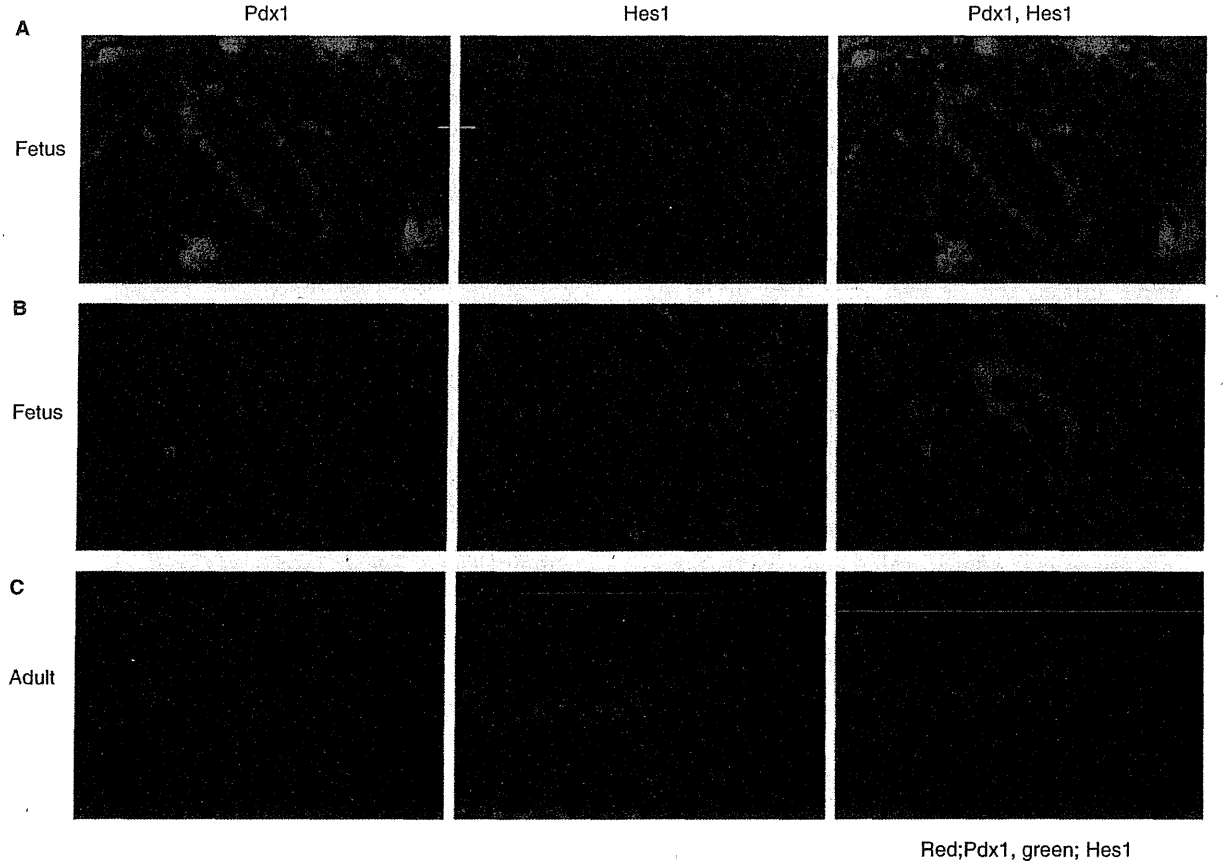

Figure 1. Expression of pancreatic and duodenal homeobox 1 (Pdx1) and hairy and enhancer of split 1 (Hes1) in normal bile ducts of fetus and adult: double staining of Pdx1 (red) and Hes1 (green). Pdx1 was strongly positive in the nuclei of lining epithelia of the fetal bile duct, while Hes1 showed a variable expression pattern; Hes1 was negative in A and variably positive in B. In the merged figure, some nuclei expressing both Pdx1 and Hes1 were yellow in B. In adult bile ducts, Pdx1 expression was less frequent than in fetus cases, while Hes1 was expressed strongly; Pdx1-negative and Hes1-positive cases (C). No yellow colour in the nuclei in C.

was used for CCKS1 and KP4-1. Each medium was supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic–antimycotic (all from Invitrogen, Carlsbad, CA, USA). All cultures were incubated in 7.5% CO_2 at 37°C. Total RNA from cultured cells was extracted using an RNA extraction kit (RNeasy mini; Qiagen) and was used to synthesize cDNA with reverse transcriptase (ReverTra Ace).

WESTERN BLOT ANALYSIS

Total proteins were extracted from the cells using T-PER protein extraction reagent (Pierce Chemical, Rockford, IL, USA). The protein was subjected to 10% sodium dodecyl sulphide-polyacrylamide gel electrophoresis, and then transferred electrophoretically onto a nitrocellulose membrane. The membrane was incubated with primary antibodies against PDX1 (diluted 1:100, the same antibody used for the immunohistochemistry) and actin (1:2000, AC-15, mouse monoclonal; Abcam, Cambridge, MA, USA). Protein expression was detected using an EnVision system (DakoCytomation), and DAB was used as the chromogen. Semiquantitative analysis of the results was performed using NIH J image software (National Institutes of Health, Bethesda, MD, USA). The fold difference compared with actin expression was calculated.

KNOCKDOWN OF PDX1 IN CULTURED CELLS

Transfection of small interfering RNA

Knockdown of PDX1 was performed using Accell small interfering RNA (siRNA), purchased from Thermo Scientific Dharmacon (Yokohama, Japan). The Hu-CCT1 cell line was plated 24 h ahead using standard medium. For the transfection, medium was exchanged with Accell delivery mix, containing human PDX1 Accell siRNA (1 μM) and Accell siRNA delivery media (Thermo Scientific Dharmacon). After 72 h, knockdown was confirmed using RT-PCR and Western blot, after which a proliferation assay was performed.

WST1 cell proliferation assay

The effects of knockdown of Pdx1 on cell proliferative activity were assessed using a WST1 assay (Roche Diagnostics KK, Indianapolis, IN, USA). The cells were seeded on 96-well dishes, and were incubated with standard medium for 24 h. After pre-incubation, siRNA was transfected. The WST1 reagent was then added after 72 h, and the cell proliferative activity was determined spectrometrically.

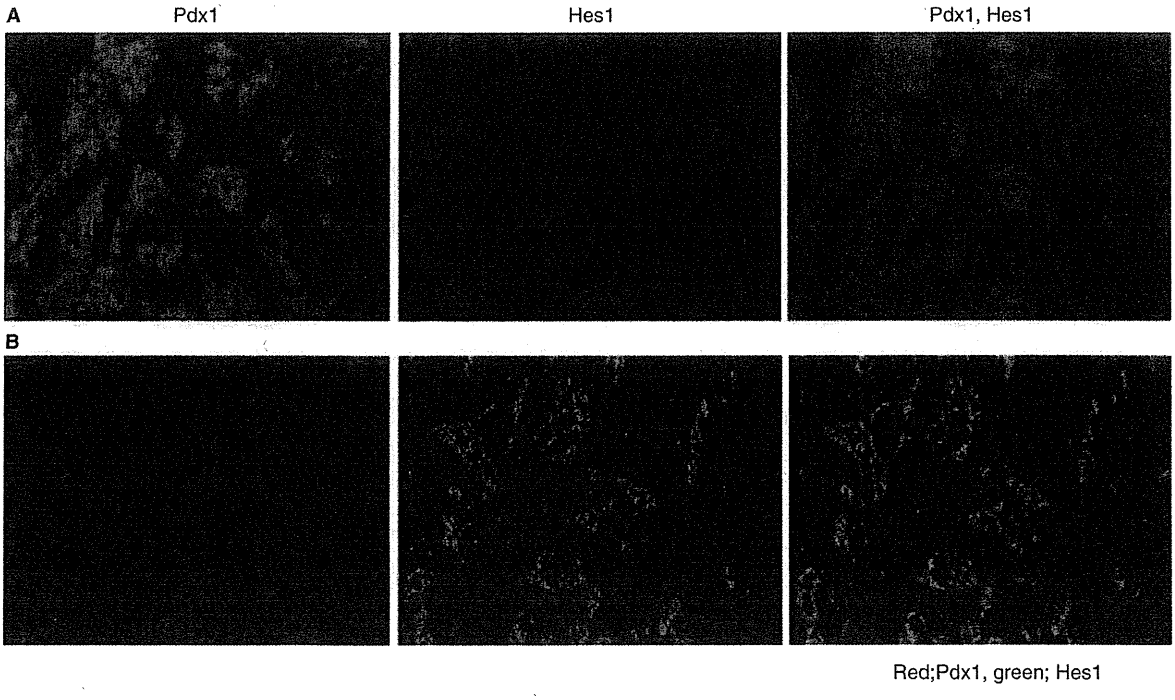

Figure 2. Expression of pancreatic and duodenal homeobox 1 (Pdx1) and hairy and enhancer of split 1 (Hes1) in cholangiocarcinoma: double staining of Pdx1 (red) and Hes1 (green). Representative cases of $Pdx1^+/Hes1^-$ (A) and $Pdx1^-/Hes1^+$ (B) are shown in this figure. The merged figure showed no yellow colour.

STATISTICAL ANALYSIS

Data from different groups were compared using one-way analysis of variance and examined with the Mann–Whitney *U*-test or Welch's *t*-test. Differences in the proportions of categorical data were tested using the chi-square test. The correlation coefficient of two factors was evaluated using Spearman's rank correlation test. The results were considered significant if the *P*-value was <0.05.

Results

HUMAN TISSUE STUDIES

Normal bile ducts

Pdx1 was expressed consistently in the nuclei of epithelia lining the bile ducts in all fetal material (Figure 1A,B). Conversely, all the 20 adult cases showed no expression (14 cases) in bile ducts or only +1 expression (six cases). No cases showed 2+/3+ expression of Pdx1 (Figure 1C). In contrast, five of eight fetal cases showed 2+/3+ Hes1 expression in the nuclei of lining epithelia, while 11 of 20 adult cases showed 2+/3+ Hes1 expression.

Cholangiocarcinoma

Nineteen cases (68%) of CC with hepatolithiasis or PSC and 10 cases (67%) of CC alone showed 2+/3+ Pdx1 expression in nuclei of the tumour cells [total, 29 cases (67%) in 43 CC cases] (Figure 2A) (Table 3A). In contrast, eight cases (29%) of CC with hepatolithiasis or PSC and seven cases (47%) of CC alone showed 2+/3+ Hes1 expression [total, 15 cases (35%) in 43 CC cases] (Figure 2B). There was no statistically significant correlation with histological grade (Table 3C,D), but there was a trend towards 2+/3+ expression of Pdx1

Table 3. Expression of pancreatic and duodenal homeobox 1 (Pdx1) (A) and hairy and enhancer of split 1 (Hes1) (B) in cholangiocarcinoma (CC) with/without hepatolithiasis or primary sclerosing cholangitis (PSC) and correlation between histology (C,D)

	CC with HL or PSC	CC without HL or PSC	Total Case
(A) Pdx1			
0	4	0	4
1+	5	5	10
2+	13	8	21
3+	6	2	8
Total (2+/3+ %)	28 (68)	15 (67)	43 (67)
(B) Hes1			
0	10	6	16
1+	10	2	12
2+	6	5	11
3+	2	2	4
Total (2+/3+ %)	28 (29)	15 (47)	43 (35)

	Histological differentiation			
	Well	Mod-poor	Variants	Total Case
(C) Pdx1				
0/1+	9	4	1	14
2+/3+	12	14	3	29
(D) Hes1				
0/1+	14	11	3	28
2+/3+	7	7	1	15

being more frequent in moderately to poorly differentiated adenocarcinoma (14 cases, 78%) in comparison with that in well-differentiated adenocarcinoma (12 cases, 57%). Such difference was not found for Hes1 expression.

Proliferative activities of CC with respect to Pdx1 expression

The Ki-67 LI was higher in Pdx1-positive CC cases [29 cases, 12.6 ± 7.1%, mean ± standard deviation (SD)] than in Pdx1-negative CC cases (14 cases, 5.2 ± 3.3%) ($P < 0.01$). In three representative cases in which Pdx1-positive and -negative lesions were evaluable separately (three foci in each), the Ki67 LI was 21.6 ± 4.9% in Pdx1-positive lesions and 2.7 ± 5.8% in negative lesions in case 1 ($P < 0.01$), 11.3 ± 2.1% and 2.1 ± 1.8% in case 2 ($P < 0.01$) and 14.0 ± 4.3% and 10.6 ± 6.8% in case 3, respectively (Figure 3A,B). The LI was higher in Pdx1-positive CC lesions (15.6 ± 5.0%, mean ± SD of three cases) than in Pdx1-negative CC lesions (5.0 ± 2.5%) ($P < 0.05$).

BilINs in PSC and hepatolithiasis without CC

Expression of Pdx1 at a level of 2+/3+ was found in 18 BilIN1 foci (47%), 25 BilIN2 foci (68%) and 22 BilIN3 foci (76%) (Figure 4A,B, Table 4A). The incidence of 2+/3+ expression increased with the progression of BilIN lesions ($P < 0.05$) (Figure 4B). In contrast, 2+/3+ expression of Hes1 decreased with progression: 26 BilIN1 foci (68%), 20 BilIN2 foci (54%) and seven BilIN3 foci (24%) ($P < 0.05$) (Figure 4B, Table 4B). The degree of Pdx1 expression was higher in the Hes1 (0/1+) group than in the Hes1 (2+/3+) group ($P < 0.01$) (Figure 4C). Double immunostaining revealed that some foci of BilINs showed simultaneous expression of Pdx1 and Hes1 in their nuclei (Figure 4D), while other BilINs showed the expression to be mutually exclusive (Figure 4A,E).

Expression of Pdx1 in invasive ductal adenocarcinoma of pancreas and its precursors

In 18 cases (47.4%) of invasive ductal adenocarcinoma of pancreas, 2+/3+ Pdx1 expression was found while

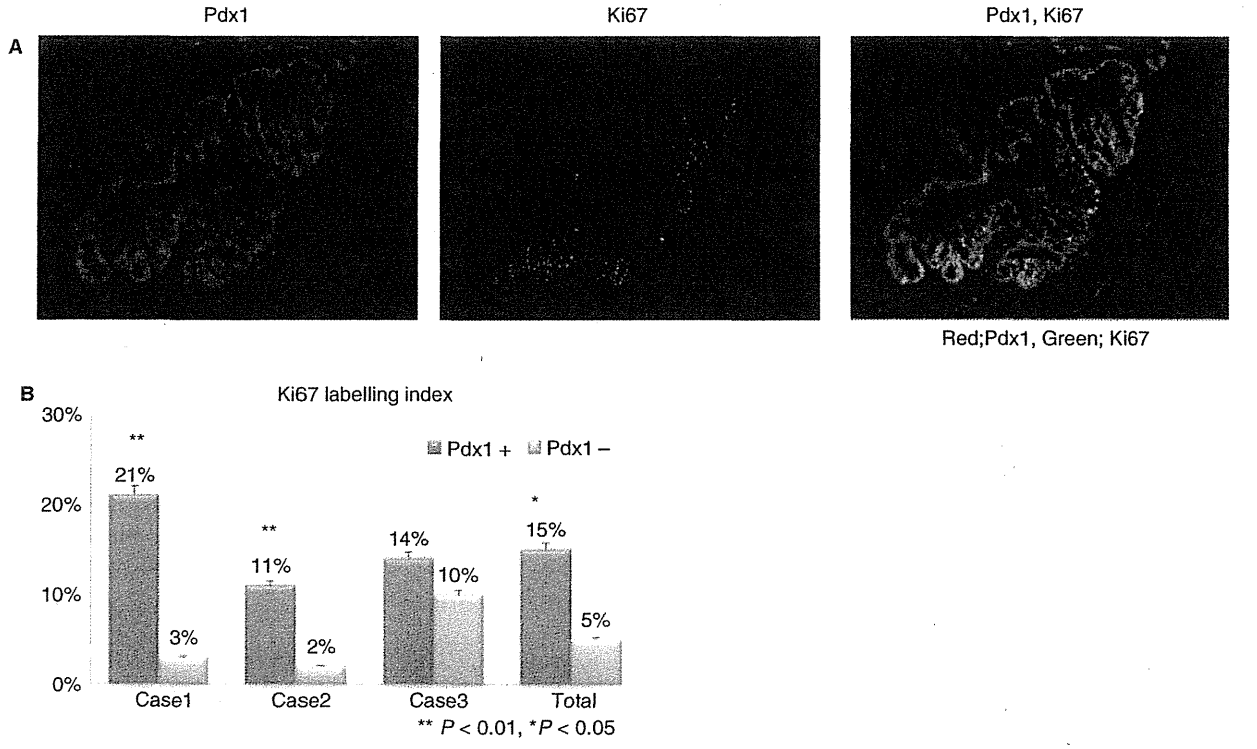

Figure 3. Ki-67 labelling index in pancreatic and duodenal homeobox 1 (Pdx1)-positive cholangiocarcinoma (CC) and Pdx1-negative CC. **A**, Double immunostaining of Pdx1 (red) and Ki67 (green) in a Pdx1-positive CC case. A majority of CC cells were strongly positive for Pdx1 (red), and about one-third of these CC cells were simultaneously positive for Ki67. The merged figure showed yellow-coloured nuclei, suggesting positivity for Pdx1 and Ki67. **B**, Ki-67 labelling index (LI) was higher in Pdx1-positive CC lesions than in negative CC lesions in cases 1, 2 and 3, respectively, and the average of Ki-67 LI in three cases was higher in Pdx1-positive CC lesions than in negative CC lesions ($P < 0.05$).

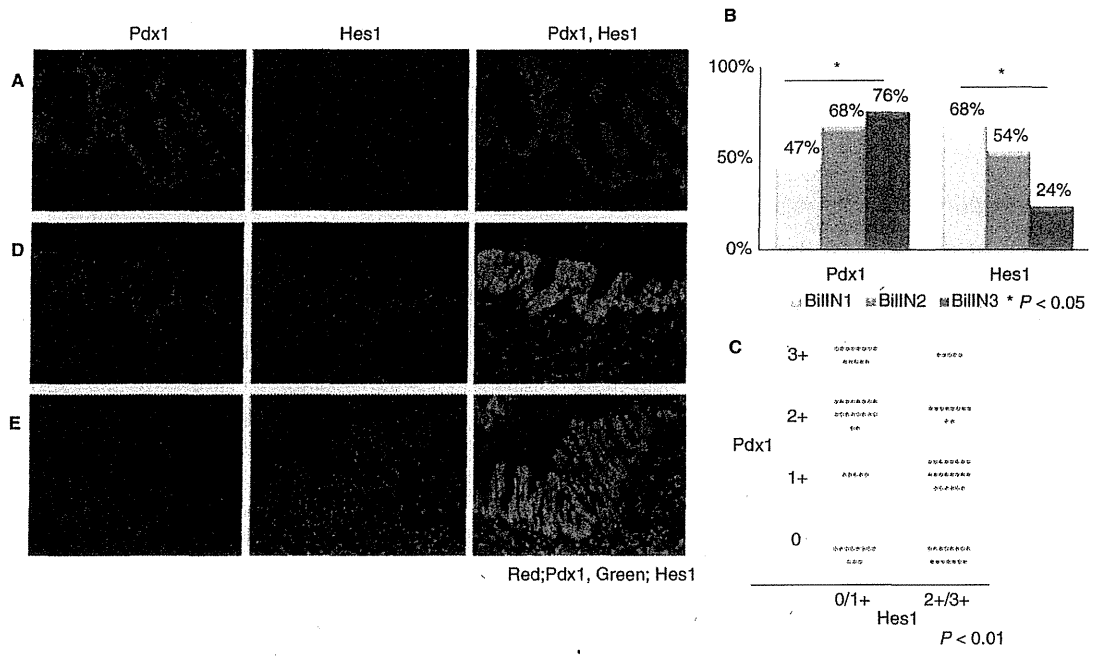

Figure 4. Expression of pancreatic and duodenal homeobox 1 (Pdx1) and hairy and enhancer of split 1 (Hes1) in biliary intraepithelial neoplasia (BilIN) lesions: double staining of Pdx1 (red) and Hes1 (green). Representative cases of $Pdx1^{+}/Hes1^{-}$ (A), $Pdx1^{+}/Hes1^{+}$ (D) and $Pdx1^{-}/Hes1^{+}$ (E) are shown. Percentages of moderate to strong (2+/3+) expression of Pdx1 and Hes1 in BilIN1, 2, 3 are shown in B. The expression of Pdx1 increased with a higher grade of BilIN ($P < 0.05$), while expression of Hes1 decreased ($P < 0.05$). Expression of Pdx1 and Hes1 in BilIN lesion appears to be reciprocal (C). In each case, the expression of Pdx1 was divided into two groups, Hes1 0/1+ and Hes1 2+/3+ groups. Significant difference was seen between these two groups ($P < 0.01$).

this level of immunoreactivity was seen in five of six PanIN-1 lesions and in all 12 PanIN-2/3 lesions. Islet cells were constantly positive for Pdx1.

Detection of Pdx1 mRNA by RT-PCR and quantitative qRT-PCR

Pdx1 mRNA was detectable in one normal BEC sample, two reactive biliary epithelia, two BilIN2/3 foci (microdissected) and three CCs (whole tissue) using RT-PCR (Figure 5). qRT-PCR revealed that the levels of Pdx1 mRNA were increased significantly in BilIN2/3 foci and CCs in comparison with those in normal BECs and reactive foci (Figure 5).

CULTURE STUDIES

Pdx1 expression

mRNA level. Pdx1 mRNA was detectable variably in cultured CC cells (HuCCT, TFK1 and CCKS1), pancreatic carcinoma cells (KP4-1, PK59, MIA Paca2 and PANC1) and four cultured non-neoplastic BECs (BEC1–4). qRT-PCR revealed that the levels of Pdx1 mRNA were significantly higher in cultured CCs and pancreatic carcinoma cells than in non-neoplastic cultured BECs (Figure 6A).

Protein. Protein extracted from cultured CC cells and pancreatic carcinoma cells and non-neoplastic BECs was examined by Western blot. Semiquantitative analysis performed using NIH J image in comparison with actin expression showed that there was a tendency towards higher expression levels of Pdx1 in carcinoma cells than in non-neoplastic BEC lines (Figure 6B).

Knockdown of Pdx1 in cultured HuCCT1 cells

Proliferative activities of cultured HuCCT1 cells increased in controls after 72 h culture, although after knocking down the expression of Pdx1 with siRNA, the proliferative activities were decreased significantly (Figure 6C). This suggests that proliferative activities of the latter were inhibited.

Discussion

The findings obtained in this study can be summarized as follows: (i) nuclear expression of Pdx1 is extensive and consistent in the lining epithelia of fetal bile ducts, but is almost absent in adult large bile ducts. In contrast, Hes1 expression is frequent in adult bile ducts. (ii) Pdx1 is detectable in about two-thirds of invasive

CCs. Cultured CC cell lines also express Pdx1 mRNA and protein. Expression of Pdx1 is related to proliferative activity in CCs, and this was supported by knocking down Pdx1 mRNA in cultured CC. (iii) Pdx1 is frequently 're-expressed' in BilIN lesions and this expression increases with the progression of BilIN, while expression of Hes1 decreases with such progression. (iv) Increased expression of Pdx1, along with decreased expression of Hes1 in the large bile ducts in hepatolithiasis and PSC, may play a role in cholangiocarcinogenesis.

Table 4. Expression of pancreatic and duodenal homeobox 1 (Pdx1) (A) and hairy and enhancer of split 1 (Hes1) (B) in biliary intraepithelial neoplasia (BilIN) lesions

	BilIN1	BilIN2	BilIN3*
(A) Pdx1			
0	10	6	5
1+	10	6	2
2+	14	17	12
3+	4	8	10
Total (2+/3+ %)	38 (47)	37 (68)	29 (76)
(B) Hes1			
0	7	12	10
1+	5	5	2
2+	17	13	3
3+	9	7	4
Total (2+/3+ %)	38 (68)	37 (54)	29 (24)

*Significant difference was seen in BilIN3 compared with BilIN1 ($P < 0.05$).

The extrahepatic biliary system has a close developmental relationship with the pancreas. Experimental studies also suggest that the biliary tract shows some potential for pancreatic differentiation in mice lacking the transcription factor Hes1.[3] In this study, Pdx1 was expressed consistently and extensively and Hes1 was also expressed variably in the nuclei of lining of the fetal bile ducts, suggesting that the Pdx1-expressing fetal bile duct may have the potential for pancreatic differentiation, but achieves normal biliary organogenesis. In the adult, we found extensive expression of Hes1 and almost no expression of Pdx1 in the lining epithelia of large bile ducts, suggesting that such combined expression of Hes1 and Pdx1 may reflect an inhibition of the pancreatic differentiation program inherent in the large bile duct.

There have been several studies on Pdx1 expression in malignant tumours.[5,8,9,15] Koizume *et al.*[8] reported that 43% of pancreatic cancers express Pdx1, while Pdx1 was expressed only in islet cells in the non-neoplastic adult pancreas. Patients that had tumours that were positive for Pdx1 had a significantly poorer prognosis than those negative for Pdx1.[8] Furthermore, Liu *et al.*[5] reported that Pdx1 overexpression is related to increased cell proliferation and invasion of carcinoma cells, thus constituting an aggressive marker of pancreatic cancer. This study showed that Pdx1 was expressed extensively in 47% of invasive ductal adenocarcinoma of the pancreas, and that four cultured

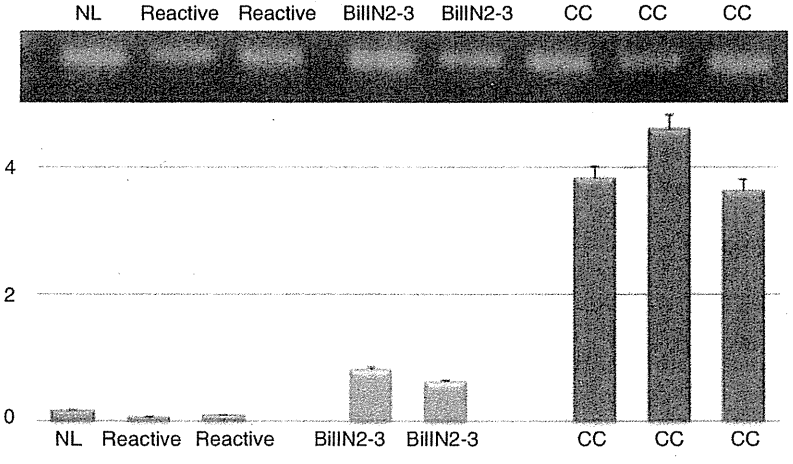

Figure 5. The levels of pancreatic and duodenal homeobox 1 (Pdx1) mRNA were significantly higher in biliary intraepithelial neoplasia (BilIN)2/3 foci and cholangiocarcinomas (CCs). mRNA expressions of Pdx1 in human normal bile duct, reactive foci, BilIN foci and CC tissue were detected by reverse transcription-polymerase chain reaction (RT-PCR). Quantitative analysis was carried out by real-time PCR using glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as the internal control for this assay. The data are shown as the mean of three sets.

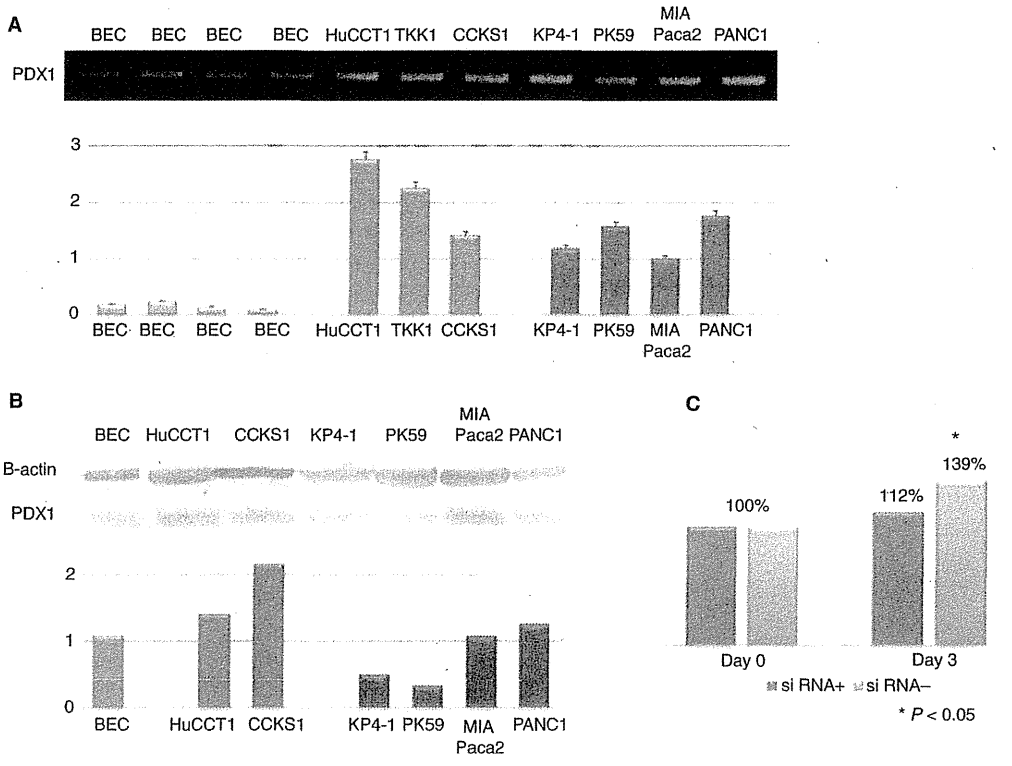

Figure 6. The levels of pancreatic and duodenal homeobox 1 (Pdx1) mRNA (A) and protein (B) in cultured cholangiocarcinoma (CC) cells were higher than in biliary epithelial cells (BEC) cells. A, mRNA expressions of Pdx1 in human BEC cells (1–4), human CC cells (HuCCT1, TFK1, CCKS1) and human pancreatic cancer cells (KP4-1, PK59, MIA Paca2, PANC1) as a reference were detected by reverse transcription-polymerase chain reaction (RT-PCR). Quantitative analysis was carried out using RT-PCR. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for this assay. B, Protein levels of Pdx1 in human BEC cells, CC cells (HuCCT1, CCKS1) and pancreatic cancer cells (KP4-1, PK59, MIA Paca2, PANC1) as a reference were analysed semi-quantitatively using NIH J image software. Beta actin was used as the internal control for this assay. C, Knockdown of Pdx1 leads to decrease of the cell proliferative activity of HuCCT1. Significant difference was seen on day 3 ($P < 0.05$). The data are shown as the mean of three sets (A) and two sets (B,C).

pancreatic carcinoma cell lines expressed increased levels of Pdx1 mRNA and protein.[16] Furthermore, it has been suggested that pancreatic carcinoma cells expressing Pdx1 may represent tumour stem cells; re-expression of Pdx1 may thus represent an alternative surface marker of pancreatic cancer stem cells.[9] In contrast, Ma *et al.* reported that Pdx1 was expressed in only 10 of 39 cases of human gastric cancer, and a negative correlation between Pdx1 and Ki-67 expression was found in gastric cancer tissues and one gastric cancer cell line. They suggested that Pdx1 functions as a putative tumour suppressor in gastric cancer.[15]

To date, there have been no studies of the expression and the role of Pdx1 in the development of human CCs. In our study, the significance of Pdx1 expression in CCs seems to be similar to that of pancreatic cancer, as described above.[1,5,8,9]

It was found in this study that Pdx1 was frequently 're-expressed' in about two-thirds of CCs, and that this expression was associated with poorer differentiation and higher proliferative activities in the tumour cells. In addition, Pdx1 was expressed frequently in BilIN lesions and this expression increased along with the progression of BilIN; in parallelthe Pdx1 mRNA level was also increased considerably in microdissected BilIN2/3 tissue. This suggests that the expression of Pdx1 may be responsible for the development of BilIN.[10] However, it remains unclear why Pdx1, a stem cell-like marker of pancreas, is re-expressed in BilIN lesions and CC during multistep cholangiocarcinogenesis. PanIN, a flat precursor lesion of pancreatic duct adenocarcinoma, resembles BilIN of the large bile duct.[1,11,13] In the adult pancreatic duct, re-expression of Pdx1 is known to be induced by its inflammation or regeneration and is also observed in early carcinogenetic stages of pancreatic carcinoma.[16,17] Recently, Park *et al.*[10] showed that Pdx1 re-expression occurred in PanIN in addition to pancreatic duct adenocarcino-

mas, and this was confirmed in this study. Recently, Pdx1-positive cells have been shown to be a possible origin of pancreatic carcinoma.[16] Thus, Pdx1 is required not only for pancreatogenesis and maintenance of pancreatic homeostasis, but also plays a key role in mediation of carcinogenesis.[5,16]

In our study, the expression patterns of Pdx1 and Hes1 were reciprocal and related closely to the progression of BilINs, suggesting that BilIN lesions of the large bile ducts with Pdx1 re-expression and decreased Hes1 expression may develop pancreatic neoplastic characteristics. The biliary tract and pancreas have several pathological in common processes, in addition to the embryonic development and differentiation.[1] In this context, the reactivation or re-expression of embryonic transcription factors such as Pdx1 could be involved in cholangiocarcinogenesis of the biliary tract, in a similar manner to that in the pancreas.

CC is one of the most intractable malignant tumours, and there have been very limited reports on effective chemotherapy using gemcitabine and cisplatin. While a few approaches to molecular-targeted therapies in CC have been reported, at present complete surgical resection of CC at its early stage is the only established curative approach.[18–20] This study focused on Pdx1 and Hes1, and these provide promising data for a novel therapeutic approach to CC. In particular, aberrantly expression of Pdx1 in CC and its precursor lesions suggest that this molecule could be a possible target. Further studies on the mechanisms of re-expression of Pdx1 in CC and the correlation between Pdx1 and other cancer-related genes involved in cholangiocarcingenesis are indicated to evaluate this possibility.

In conclusion, Pdx1 was re-expressed frequently in CCs, and its expression increased stepwise with the progression of BilIN lesions, suggesting that Pdx1 may play a role in cholangiocarcinogenesis. In addition, expression of Pdx1 in CCs was associated with poor differentiation and increased proliferative activity of CC cells. *In vitro* studies supported these human studies. Further studies on the roles of Pdx1 in CC are warranted to evaluate whether Pdx1 is indeed a candidate for molecular-targeted therapy of CC.

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