Definition of MANEC

According to WHO classification (2010), at least 30% of the main lesion has to be made up of either an adenocarcinoma or NET.[2 14] Moreover, the NET component must be positive for neuroendocrine markers such as chromogranin A and synaptophysin.

Immunohistochemistry

Using representative cancerous sections involving NET from each case, the immunohistochemical staining of chromogranin A and synaptophysin was performed to confirm the presence of NETs. Moreover, Notch1, Jagged1 and Hes1 were also immunostained to examine the Notch1-Hes1 signalling axis in the tumourigenesis of biliary NET. The deparaffinised sections were microwaved in citrate buffer (pH6) for 20 min for antigen retrieval prior to staining. Following endogenous peroxidase blocking and incubation in normal goat or rabbit serum (1:10; Vector Lab, Burlingame, California, USA) for 20 min, these sections were incubated at 4°C overnight with primary antibodies against human chromogranin A (mouse IgG, 0.5 μg/ml, Dako, Tokyo, Japan), synaptophysin (mouse IgG, 5 μg/ml, Dako), Jagged 1 (Goat IgG, 0.5 μg/ml, Santa Cruz, California, USA), Notch 1 (mouse IgG, 5 μg/ml, OriGene, Rockville, Maryland, USA) or HES1 (Rabbit IgG, 5 μg/ml, United States Biological, Swampscott, Massachusetts, USA), and then at room temperature for 1 h with goat antimouse or antirabbit immunoglobulins conjugated to a peroxidase-labelled dextran polymer (Envision, Dako) or rabbit antigoat immunoglobulins conjugated to a peroxidase-labelled dextran polymer (Simple staining kit, Nichirei, Tokyo, Japan). After a benzidine reaction, sections were weakly counterstained with haematoxylin. No positive staining was obtained when the primary antibodies were replaced with an isotype-matched, non-immunised immunoglobulin as a negative control of the staining procedures. The degree of expression of Jagged1 and Notch1 in the adenocarcinoma and NET components was semiquantitatively evaluated as <20%, 20%–80% or >80%.

Cell culture and transfection of siRNA

A commercially available cell line derived from human cholangiocarcinoma, HuCCTl, was obtained from Health Science Research Resources Bank (Osaka, Japan). This cell line was cultured in culture flasks with a standard medium (RPMI 1640, Life Technologies, Tokyo, Japan) supplemented with 10% fetal calf serum at 37°C in a water-saturated atmosphere of 95% air and 5% CO_2. Two kinds of short interfering RNAs (siRNAs) against mRNA of Notch 1 (siRNA 1, ACGAAGAACAGAAGC ACAAAGGCGG and siRNA 2, UCGCAUUGACCAUUCAAA CUGGUGG) were purchased from Life Technologies. HuCCT1 was transfected the day after plating at 30%–40% confluence using Lipofectamine (Life Technologies), according to the manufacturer's recommendation. After 48 h, cells were lysed for RNA isolation. Notch 1 and Ascl1 transcript levels were analysed by real-time PCR as described below.

Isolation of RNA and PCR

HuCCT1 was collected from the flasks with a cell scraper for the determination of the baseline mRNA expression of Notch1, Jagged1, Hes1, Ascl1 and chromogranin A by reverse transcription (RT)-PCR. Cultured normal human intrahepatic biliary epithelial cell lines established in our laboratory[15–17] and the breast cancer cell line MCF1 (Health Science Research Resources Bank) were used as positive controls. Briefly, total RNA was isolated from each sample with the RNeasy Total RNA System (QIAGEN, Hilden, German) and treated with RNase-Free DNaseI. For RT, 1 μg of total RNA, M-MLV RTase (ReverTra Ace, Toyobo, Tokyo, Japan) and oligo-dT primers were used. PCR amplification was performed using DNA polymerase (Takara EX Taq, Takara, Tokyo, Japan) and specific primers for human mRNA sequences. Following 40 cycles of annealing for 1 min and extension at 72°C for 2 min, PCR products were subjected to agarose gel electrophoresis. In addition, real-time quantitative PCR was performed for measurements of Notch1, Ascl1 and chromogranin A mRNAs according to a standard protocol using the Brilliant II SYBR Green QPCR Reagents and Mx300P QPCR system (Stratagene Japan, Tokyo, Japan) and relative gene expression was calculated using the comparative cycle threshold method. Specific primers were as follows: Notch1 forward, 5′-AGCATCACCTGCCTGTTAGG-3′, and reverse, 5′-TGGCATACACACTCCGAGAA-3′, Jagged1 forward, 5′-CTTTGCAGCTCAGAACCACA-3′, and reverse, 5′-CCAGC AACTGCTGACATCAA-3′, Hes1 forward, 5′-TCTGAGCCAGC TGAAAACAC-3′, and reverse, 5′-GGTACTTCCCCAGCACA CTT-3′, Ascl1 forward, 5′-TCGCACAACCTGCATCTTTA-3′, and reverse, 5′-CCGTTTTCTGAAAGCCATGT-3′, chromogranin A forward, 5′-CGCGCCTTGTCTCCTACTC-3′, and reverse, 5′-AGGAAAGAGCCCAGAACAGAT-3′, and glyceraldehyde 3 phosphate dehydrogenase (internal positive control), forward, 5′-GGCCTCCAAGGAGTAAGACC-3′, and reverse, 5′-AGGGGTCTACATGGCAACTG-3′.

Statistical analysis

Data were analysed using Wilcoxon signed-ranks test. p Value<0.1 was considered statistically significant.

RESULTS

Pathology of biliary MANECs

The presence of neuroendocrine components was confirmed by the expression of chromogranin A and/or synaptophysin using immunohistochemistry and in all MANEC cases selected in this study, at least 30% of the main tumour had to be made up of either component. Characteristically, the adenocarcinomatous component was located at the luminal surface and the majority of the stromal invasion including vascular invasion involved the NET component (figure 1). These NET components were classified as NET G2 or G3 according to WHO classification (2010):[2 14] the tumour cells had a round- or oval-shaped nucleus and 'salt-and-pepper' pattern of nuclear chromatin, general characteristics of neuroendocrine cells (figure 1).

Expression of notch signalling molecules

Immunohistochemistry revealed that Jagged1 was constantly expressed in the cytoplasmic regions of non-neoplastic biliary epithelial cells in normal bile ducts and gallbladder (figure 2). Although the expression of Jagged1 varied in tumour cells of the adenocarcinoma and NET, semiquantitative analysis revealed no significant difference between these components in any cases except one (figure 2). Notch1 was constantly expressed with the nuclear pattern in non-neoplastic biliary epithelial cells (figure 3). In the carcinoma cells composing the ordinary adenocarcinomatous component in biliary MANECs, the expression of Notch1 varied, but in the NET component, all cases except one showed <20% positivity (figure 3). Hes1 expression was found in the cytoplasm and/or nucleus of positive cells (figure 4). Because Hes1 is a transcription factor, its nuclear expression indicates the activated form and was evaluated as positivity in this study. Consequently, normal biliary epithelial

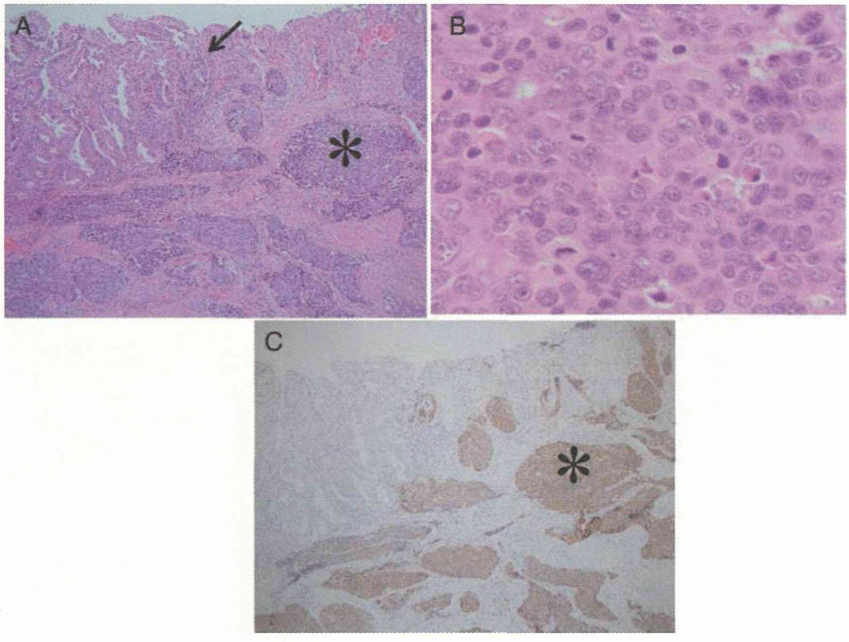

Figure 1 Gallbladder cancer with neuroendocrine tumour G3 (WHO classification, 2010). Although the luminal surface of the tumour is an ordinary tubular adenocarcinoma (A, arrow), the invasive component has a different histology showing a solid component (A, *). Tumours in the solid component consist of uniform-sized and large tumour cells resembling large cell neuroendocrine carcinoma of the lung (B) and are diffusely positive for chromogranin A (C). (A) and (B), H&E staining. (C) Immunohistochemistry for chromogranin A. Original magnification, (A) and (C), ×40; (B), ×400.

cells and also carcinoma cells of the adenocarcinoma constantly showed positivity. In contrast to the ordinary adenocarcinomatous component, in carcinoma cells of the NET component, nuclear-type Hes1 expression was lacking in all cases except one (figure 4).

Expression of Notch signalling molecules and interference of Notch 1 in cultured cholangiocarcinoma cells

The expression of Notch1, Jagged1 and Hes1 mRNAs was constitutively detected in a cultured cholangiocarcinoma cell line, HuCCT1, and normal human intrahepatic biliary epithelial cells by the RT-PCR analysis (figure 5). Ascl1 and chromogranin A were faintly found (figure 5). The procedure using both siRNA1 and siRNA2 against the Notch1 gene interfered with the expression approximately 20% (figure 6). In contrast, the expression of Ascl1 and chromogranin A was significantly upregulated, compared with no siRNA (transfectant only) (figure 6). These findings suggest that Notch 1 expression could negatively regulate the expression of chromogranin A.

DISCUSSION

Several cases of biliary NET including carcinoid tumours have been reported, but most involve biliary NETs arising from or accompanying ordinary adenocarcinomas (MANEC), mostly in patients with gallbladder cancer and extrahepatic cholangiocarcinoma. In cases of biliary MANEC, the adenocarcinomatous component was located at the luminal surface of the main tumour and the majority of the stromal invasion including vascular invasion and lymph node metastasis involved the NET component. Therefore, we have suggested that the NET component in biliary MANEC defines the prognosis.[3] Although biliary MANEC has a very characteristic histology and it is important

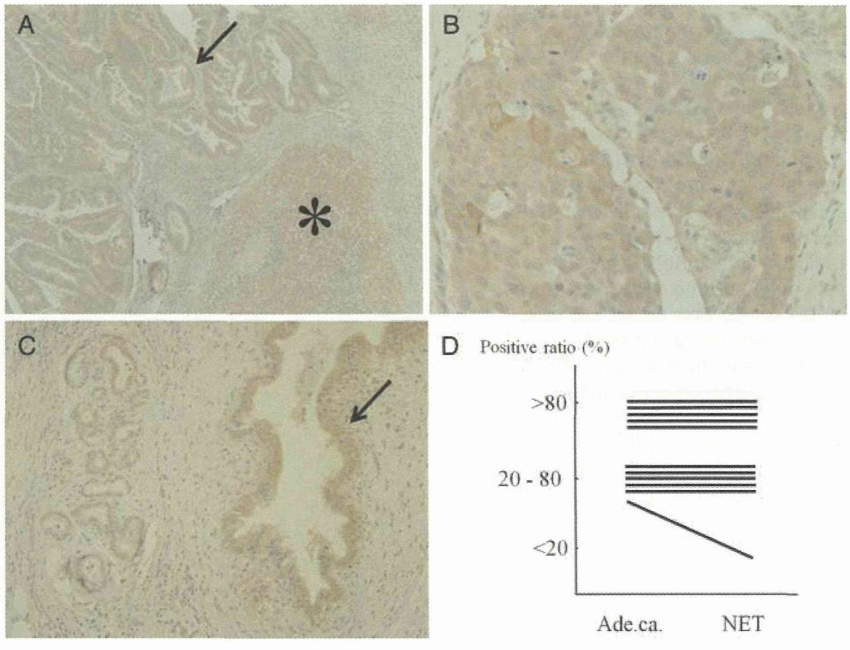

Figure 2 Comparative analysis of Jagged1 expression between adenocarcinomas and neuroendocrine tumours (NETs) in mixed adenoneuroendocrine carcinomas. Cytoplasmic expression is diffusely found in both adenocarcinomas (A, arrow) and NETs (A, * and B). Non-neoplastic biliary epithelial cells lining the intrahepatic large bile duct are also positive (C). Semiquantitative evaluation revealed that the expression varied, but there was no significant difference between the adenocarcinoma (Ade.ca.) and NET except in one case (D). Immunohistochemistry for Jagged1 (A–C). Original magnification, (A), ×40; (B), ×400; (C), ×100.

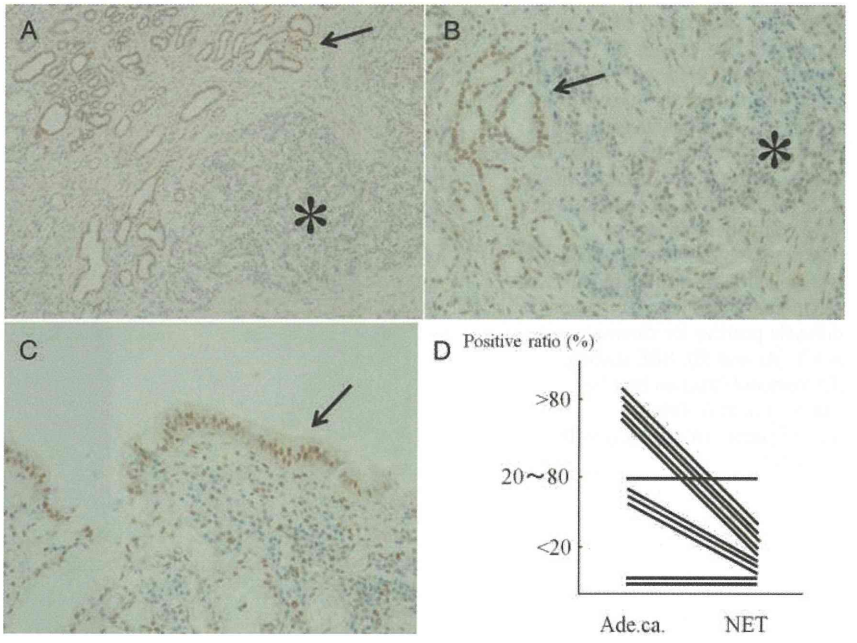

Figure 3 Comparative analysis of Notch1 expression between adenocarcinomas and neuroendocrine tumours (NETs). Nuclear expression is found in the adenocarcinomas (A and B, arrows), but rarely in NETs (A and B, *). Normal biliary epithelial cells lining the gallbladder are also constantly positive for Notch1 with a nuclear pattern (C). Semiquantitative evaluation revealed that Notch1 expression in NET is decreased in eight of 11 cases and less than 20% in all cases except one. There was a statistical significance in the expression of Notch 1 between adenocarcinoma and NET (Wilcoxon signed-ranks test, $p<0.1$, T value=2, CI 5 to 31) (D). Immunohistochemistry for Notch1 (A–C). Original magnification, (A), ×40; (B) and (C), ×200.

to identify and consider indications for adjunctive therapy, our understanding of how the neuroendocrine phenotype in biliary NETs is regulated is limited. The histogenesis or origins of biliary NET including MANEC found in biliary trees may be (1) the ectopic pancreas or adrenal gland, (2) metaplastic enterochromaffin-like neuroendocrine cells caused by chronic inflammation and (3) the transformation of adenocarcinoma cells. In the present study, the third hypothesis was verified using biliary MANEC cases.

Notch signalling allows the establishment of patterns of gene expression and differentiation, and regulates cell fate in multiple developmental programmes. The four mammalian Notch receptors (Notch1–Notch4) are single-pass, heterodimeric transmembrane proteins that serve as receptors for Notch ligands, Delta-like (Dll-1, Dll-3 and Dll-4) and Jagged (Jagged1 and Jagged2) expressed on neighbouring cells. In intrahepatic bile ducts, the Notch signalling pathway has an important role during development and a mutation of Jagged1 has been discovered in patients with Alagille syndrome.[18] The present study revealed that non-neoplastic biliary epithelial cells covering intrahepatic and extrahepatic bile ducts including gallbladder constantly expressed Notch1 and Jagged1 and the expression of Notch1 showed a nuclear pattern suggesting translocation into the nucleus from the cytoplasm after the activation of Notch1. Moreover, ordinary adenocarcinomas also expressed Notch1 in the nucleus accompanying Jagged1 expression. This suggests that adenocarcinomas as well as normal biliary epithelial cells were regulated by Notch1 signalling to maintain epithelial homeostasis in the tubular/acinar epithelium.

After its activation by Notch ligands expressed on neighbouring cells, Notch1 translocates to the nucleus and then transactivates target genes including Hes1. In gastrointestinal carcinoids,

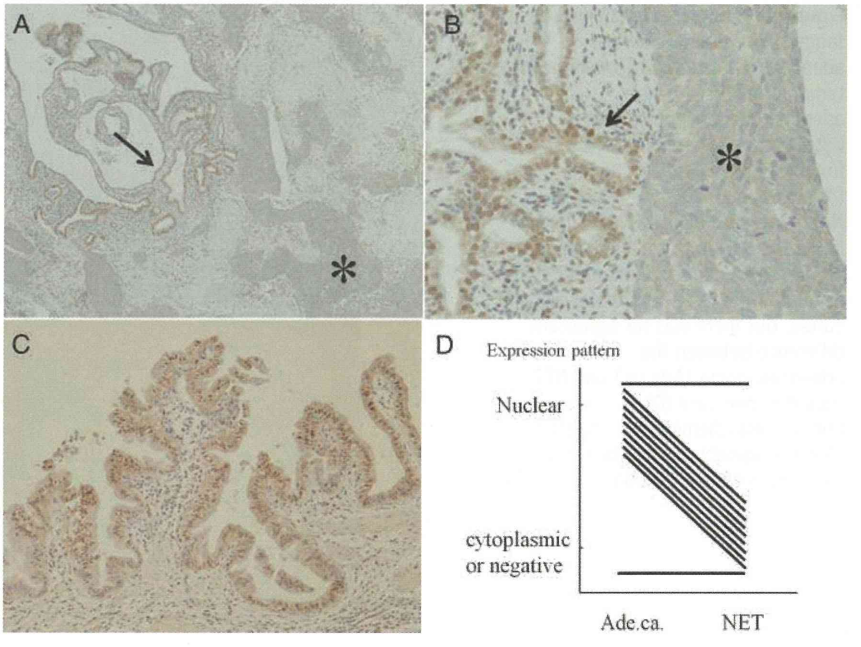

Figure 4 Comparative analysis of Hes1 expression between adenocarcinomas and neuroendocrine tumours (NETs). Nuclear expression is found in the adenocarcinomas (A and B, arrows), but not NETs (A and B, *). Normal biliary epithelial cells lining the gallbladder also constantly express Hes1 in a nuclear pattern (C). Evaluation of the Hes1 expression pattern revealed no nuclear expression in NET components except one case, though there was a statistical significance in the expression of Hes1 between adenocarcinoma and NET (Wilcoxon signed-ranks test, $p<0.1$, T value=5, CI 8 to 37) (D). Immunohistochemistry for HES1 (A–C). Original magnification, (A), ×40; (B), ×400; (C), ×200.

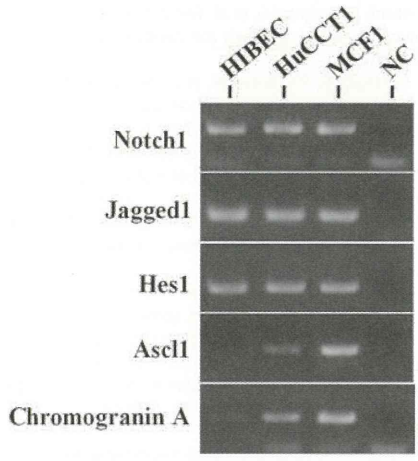

Figure 5 Detection of Notch1, Jagged1, Hes1, Ascl1, and chromogranin A in cultured human intrahepatic biliary epithelial cells, human cholangiocarcinoma cells (HuCCT1), and human breast cancer cells (MCF1, positive control) by reverse transcription-PCR analysis. All cultured cell lines express the mRNAs of Notch1, Jagged1 and Hes1. Although Ascl1 and chromogranin A were detected in MCF1, they were only faintly detected in HuCCT1. 'NC' was a negative control (distilled water instead of the reverse transcriptase for reverse transcription).

the overexpression of an active form of Notch1 leads to the upregulation of Hes1, silencing of Ascl1 and the downregulation of neuroendocrine markers: the regression of the repressor Hes1 followed by a lack of Notch signalling has been demonstrated in the histogenesis of NET.[7] Ascl1 is highly expressed in NETs.[19] However, this histogenesis of NET is not common in any NETs. Wang *et al*[20] reported that gastroenteropancreatic NETs were heterogeneous with regard to the Notch1-HES1 signalling pathway; Notch1 and Hes1 were preferentially expressed in rectal NETs and a subset of pancreatic NETs, but uniformly negative in ileal NETs, indicating the heterogeneity in signalling pathways of gastroenteropancreatic NETs. Moreover, in the developing endoderm, disruption of Notch-Hes1 signalling results in precocious endocrine differentiation in the pancreas. Because pancreatico-biliary systems embryologically share the common genesis from the foregut,[21] the Notch-Hes1 signalling axis is important in maintaining tubular/acinar function by preventing the differentiation into neuroendocrine cells during biliary development. In the present study, we used cultured human cholangiocarcinoma HuCCT1 cells, though it is unknown if HuCCT1 originated from intrahepatic or extrahepatic cholangiocarcinoma. Moreover, as a positive control, we used human intrahepatic biliary epithelial cells established in our laboratory[17] because we could not prepare cultured biliary epithelial cells from extrahepatic bile ducts. However, these cultured cells constantly expressed Hes1 as well as Notch1 and Jagged1, suggesting that the Notch-Hes1 signalling axis could prevent neuroendocrine differentiation to maintain biliary homeostasis. Moreover, this study using surgical specimens revealed that ordinary adenocarcinoma components constantly expressed Notch1, Jagged1 and Hes1, but the expression of Notch1 and Hes1 was mostly lacking in biliary NET components. This finding suggests that Notch-Hes1 signalling is important to inhibit neuroendocrine differentiation in the adenocarcinoma phenotype of cholangiocarcinoma and its impairment is closely associated with the tumourigenesis of biliary NETs.

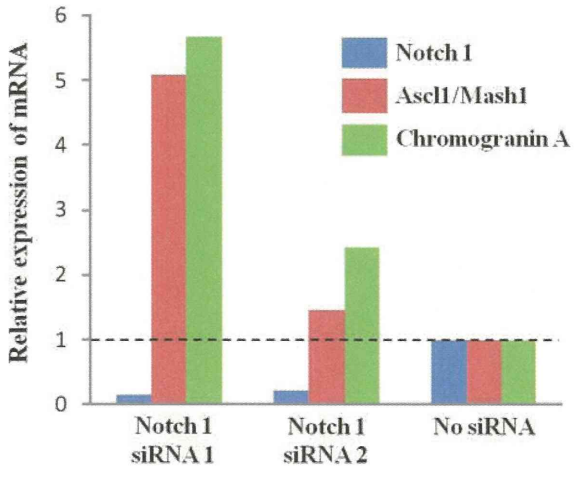

Figure 6 Quantitative analysis of mRNAs of Notch1, Ascl1 and chromogranin A in a cultured cholangiocarcinoma cell line, HuCCT1. The knock-down of Notch1 mRNA expression by approximately 20% using the short interfering RNA (siRNA) technique increased the expression of Ascl1 and chromogranin A significantly. siRNA1 and siRNA2 interfered with different sites of the Notch1 gene, but had similar effects. Results are shown as a percentage compared with no siRNA (control).

As mentioned above, we speculate that the NET component of biliary MANEC originated from adenocarcinoma cells. Therefore, we directly demonstrated that a cultured cholangiocarcinoma cell line showing adenocarcinomatous features transformed into NET. Immunohistochemistry using MANEC specimens suggested that the Notch1-Hes1 signalling axis prevented the expression of neuroendocrine features, although the expression of Jagged1 was preserved in biliary NETs. Therefore, the knock-down of Notch1 expression in a cultured cholangiocarcinoma cell line was performed by the siRNA technique. Consequently, under conditions where Notch1 expression was reduced approximately 20%, the expression of Ascl1 and chromogranin A was significantly upregulated. This finding directly demonstrated that the regression of Notch 1 expression could result in NET features, probably via Hes1. The mechanism of digression of Notch expression in biliary NET is unknown. In prostate carcinoma, neuroendocrine differentiation has been associated with tumour progression and a poor prognosis, and induced by hypoxia; hypoxia triggered a significant decrease of Notch expression, with subsequent downregulation of Hes1 and upregulation of neuroendocrine markers.[22] Biliary MANEC is also possibly associated with hypoxia in the histogenesis of the NET component.

Take home messages

- Notch1-Hes1 signalling axis suppresses neuroendocrine differentiation to maintain tubular/acinar features in biliary adenocarcinoma and normal epithelium.
- A disruption of Notch1-Hes1 signalling axis may be associated with the tumourigenesis of biliary neuroendocrine tumours in mixed adenoneuroendocrine carcinomas (MANECs).
- Further studies are needed to understand the mechanism behind the modulation of Notch1 and to devise ways to improve the prognosis for MANECs.

Contributors KH was mainly involved in the concept of this study and preparation of the manuscript. YN was the organiser. The other authors were advisors.

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Competing interests None.

Patient consent Obtained.

Ethics approval Kanazawa University Ethic Committee.

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Notch1-Hes1 signalling axis in the tumourigenesis of biliary neuroendocrine tumours

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Original article

Monocyte chemoattractant protein-1 derived from biliary innate immunity contributes to hepatic fibrogenesis

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ABSTRACT

Aims Monocyte chemoattractant protein-1 (MCP-1) is a major chemotactic factor for hepatic stellate cells (HSCs) associated with hepatic fibrosis. In this study, among several fibrogenetic factors derived from biliary epithelial cells (BECs), MCP-1 produced by the biliary innate immune system was found to be most critical in the histogenesis of hepatic fibrogenesis.

Methods Using cultured human BECs, the expression of five fibrogenetic factors including MCP-1 on stimulation with Toll-like receptor ligands, inflammatory cytokines or bile acids was examined. Moreover, in situ detection of MCP-1 and α-smooth muscle actin proteins was performed using sections from normal and diseased livers by immunohistochemistry.

Results All fibrogenetic factors were detected in BECs, but only MCP-1 expression was upregulated, by all the Toll-like receptor ligands, IL-1β, and tumour necrosis factor-alpha. Proliferating bile ductules in interface areas expressed MCP-1 in diseased livers accompanying α-smooth muscle actin-positive activated HSCs.

Conclusions Bile ductules proliferate in various hepatobiliary diseases, and its significance is still unknown. This study demonstrated that BECs in bile ductules could produce MCP-1, particularly, via biliary innate immunity, suggesting that MCP-1 derived from BECs plays an important role in the recruitment of HSCs to interface areas and the activation of HSCs resulting in the progression of periportal fibrosis.

INTRODUCTION

Hepatic fibrosis is a major feature of advanced liver diseases and defines the prognosis. Hepatic fibrosis spreads within portal tracts and also periportal areas in patients with hepatitic and cholestatic liver diseases. Although periportal fibrosis is thought to be associated with the accumulation and activation of hepatic stellate cells (HSCs), its mechanisms are not fully understood. HSCs undergo differentiation towards an activated phenotype, and this process is enhanced by soluble mediators such as platelet-derived growth factor (PDGF) and transforming growth factor-β (TGF-β), which mediate the increase in cell proliferation and extracellular matrix production.[1 2] Then, HSCs migrate into damaged areas in response to a chemokine, secreting monocyte chemotactic protein-1 (MCP-1/CCL2) that recruits monocytes and lymphocytes.[3] Moreover, MCP-1 triggers the migration of activated, but not quiescent, HSCs in a dose-dependent manner. Experiments in vitro using HSCs isolated from normal human livers revealed that MCP-1-dependent signals were not transduced by the chemokine receptor of MCP-1, CCR2, and may be mediated by alternative chemokine receptors.[3] Recently, Ramm *et al*[4] reported that hepatocyte-derived MCP-1 induced by a hydrophobic bile acid, taurocholate, could result in the recruitment of HSCs in cholestatic liver injury as the major fibrogenesis in a paediatric cholestatic liver disease such as biliary atresia. In contrast, biliary epithelial cells (BECs) may promote fibrogenesis by a number of mechanisms including the synthesis of matrix constituents and the release of mediators such as MCP-1, PDGF-BB, TGF-β, connective tissue growth factor (CTGF) and endothelin-1.[5] The role of BECs in the pathogenesis of hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis in various hepatobiliary diseases including chronic viral hepatitis (CVH) and primary biliary cirrhosis (PBC), is speculated to be important to the disease's progression, but its precise mechanism is still unknown.

Our previous study demonstrated that human BECs possess several inflammatory cytokine receptors and Toll-like receptors (TLRs) and produced cytokines and chemokines in response to inflammatory cytokines and pathogen-associated molecular patterns (PAMPs), respectively.[6–9] In particular, bile ductules located between interlobular bile ducts in portal tracts and bile canaliculi in hepatocytes are frequently increased in number under a variety of pathological conditions of the liver and take part in various immunological responses and the pathogenesis of biliary diseases.[10–12] This ductular reaction is speculated to be closely associated with hepatic fibrosis, particularly periportal fibrosis accompanying interface hepatitis, but its precise mechanism is still unknown. In this study, we examined the possibility that BECs produce MCP-1 and play a role in fibrogenesis in periportal areas via MCP-1 production.

MATERIALS AND METHODS

Cultured human BECs

Two human intrahepatic BEC lines were established from explanted livers with PBC as described previously.[7] These cell lines had been confirmed to be BECs by the expression of biliary-type cytokeratins.

Stimulation

Cultured BECs were stimulated with inflammatory cytokines, PAMPs and bile acids (table 1). As an NF-κB inhibitor, isohelenin (30 μM, Calbiochem, Darmstadt, Germany) was added to the culture medium before the stimulation. We previously confirmed that BECs possess the receptors for all these cytokines and PAMPs.[6] Cell samples for the examination of mRNA as shown below were prepared 2 h after the stimulation.

RT-PCR and real-time PCR

For the evaluation of mRNAs of MCP-1, PDGF-B, CTGF, TGF-β1, endothelin-1 and glyceraldehyde 3-phosphate dehydrogenase (internal control) in cultured BECs, total RNA was isolated from BECs, and RT-PCR and real-time quantitative PCR were performed according to a standard protocol using specific primers (table 2).

ELISA

Cultured BECs were stimulated with lipopolysaccharide, Pam3CSK4, poly(I:C) and IL-1β for 24 h, and supernatants were tested for human MCP-1 by ELISA (Biosource International, Camarillo, California).

Liver tissues

A total of 26 surgical or wedge liver biopsy specimens were obtained from patients with CVH (n=8, hepatitis C virus-related, male/female=5/3, average age 58-year-old., F1/F2=4/4), PBC (n=5, all female, average age 68-year-old, Nakanuma's classification[13] Stage 2/3=4/1, Scheuer's classification Stage 1/2=2/3) and congenital hepatic fibrosis (CHF, n=5, used as a case of activated HSC-poor case[14], male/female 4/1, average age 23-year-old), and six cases with no significant histopathological change ('normal liver').

Immunohistochemistry

Deparaffinised sections were pretreated in Target Retrieval Solution (Dako, Tokyo, Japan) and incubated with a primary antibody against MCP-1 (10 μg/ml; Abcam, Tokyo, Japan) or α-smooth muscle actin (αSMA, activated HSC marker) (1 μg/ml, Dako), and then Envision-HRP (Dako) was used. No positive staining was obtained when the primary antibody was replaced with an isotype-matched, non-immunised immunoglobulin.

The simultaneous detection of combined CCR2 and αSMA, and CCR2 and CD68, was evaluated by double fluorescence immunohistochemical staining. After incubation with the antibodies for these combinations, rabbit antibody (CCR2, 1:400, Epitomics, Burlingame, California) and mouse antibodies (αSMA and CD68, Dako) were visualised by Alexa Fluor 488 and 594 (Molecular Probe, Eugene, Oregon), respectively.

Table 1 Stimulants

Stimulants	Concentration	Supplier
Cytokines		
Interleukin-1β	1000 U/ml	PerpoTech
Interleukin-6	1000 U/ml	PerpoTech
Interferon-γ	1000 U/ml	PerpoTech
Tumour necrosis factor-α	1000 U/ml	PerpoTech
Pathogen-associated molecular patterns		
Lipopolysaccharide (TLR4 ligand)	1 μg/ml	Invitrogen
Pam3CSK4 (TLR1/2 ligand)	100 ng/ml	Invitrogen
Poly(I:C) (TLR3 ligand)	25 μg/ml	Invitrogen
Bile acid		
Taurochenodeoxycholic acid	200 μM	Calbiochem
Taurocholic acid	200 μM	Calbiochem
Taurodeoxycholic acid	200 μM	Calbiochem
Tauroursodeoxycholic acid	200 μM	Calbiochem

Invitrogen, San Diego, California; PerpoTech, London.
TLR, Toll-like receptor.

Table 2 Primers used for RT-PCR and quantitative PCR

Transcript	Primers	Product size (bp)
Monocyte chemoattractant protein-1	Forward 5′-CTGAATTTTGTTTGTTGATGTGAAA-3′ Reverse 5′-GCAATTTCCCCAAGTCTCTG-3′	128
Platelet-derived growth factor-B	Forward 5′-GAGGACCTCTCAGCATAGCC-3′ Reverse 5′-GGGGTTTTCTCCAGTCTGTG-3′	135
Connective tissue rowth factor	Forward 5′-GCAGGCTAGAGAAGCAGAGC-3′ Reverse 5′-ATGTCTTCATGCTGGTGCAG-3′	153
Transforming growth factor-β1	Forward 5′-CAGAAATACAGCAACAATTCCTGG-3′ Reverse 5′-TTGCAGTGTGTTATCCCTGCTGTC-3′	186
Endothelin-1	Forward 5′-CCATGAGAAACAGCGTCAAA-3′ Reverse 5′-AGTCAGGAACCAGCAGAGGA-3′	213
Glyceraldehyde 3-phosphate dehydrogenase	Forward 5′-GCACCGTCAAGGCTGAGAAC-3′ Reverse 5′-ATGGTGGTGAAGACGCCAGT-3′	142

RESULTS

Detection of MCP-1 in cultured human BECs

RT-PCR revealed that all fibrogenic cytokines were detected in human BECs in the basal cultures (figure 1). Quantitative PCR revealed that only MCP-1 was upregulated following stimulation with all PAMPs and two cytokines (IL-1β and tumour necrosis factor-alpha (TNF-α); figure 2). Moreover, ELISA demonstrated that the concentration of MCP-1 protein in the supernatants was increased by these stimulants (figure 3). None of the bile acids upregulated the expression of MCP-1 (figure 2). The Pam3CSK4-induced upregulation of MCP-1 mRNA was significantly inhibited by pretreatment with the NF-κB inhibitor (figure 2).

In situ detection of MCP-1 and activated HSCs

MCP-1 was mainly expressed in bile ducts, and the majority of proliferating bile ductules predominantly expressed MCP-1 in periportal and interface areas in cases of CVH and PBC (figure 4). Moreover, αSMA-positive cells (activated HSCs) were scattered around these bile ductules (figure 4). These characteristic findings were made in CVH and PBC accompanying interface hepatitis and ductular reaction, but rare or absent in normal livers. Periportal hepatocytes were also frequently positive for

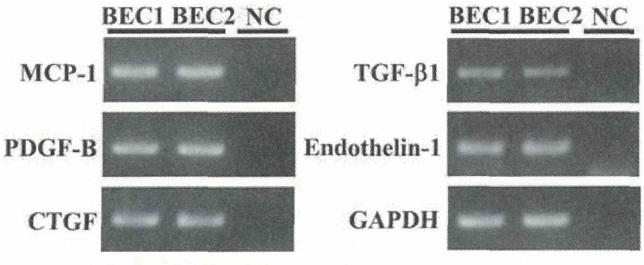

Figure 1 Detection of fibrogenic cytokines in cultured human BECs. The two human biliary epithelial cell lines (BEC1 and BEC2) express all mRNAs of fibrogenic cytokines (monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor (PDGF)-B, connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β)1, and endothelin-1 (GAPDH)) and the internal control (glyceraldehyde 3-phosphate dehydrogenase) under basal conditions. Each PCR product showed the predicted size as a single band. Negative controls (NC) were obtained by replacing reverse transcriptase with RNase- and DNase-free water.

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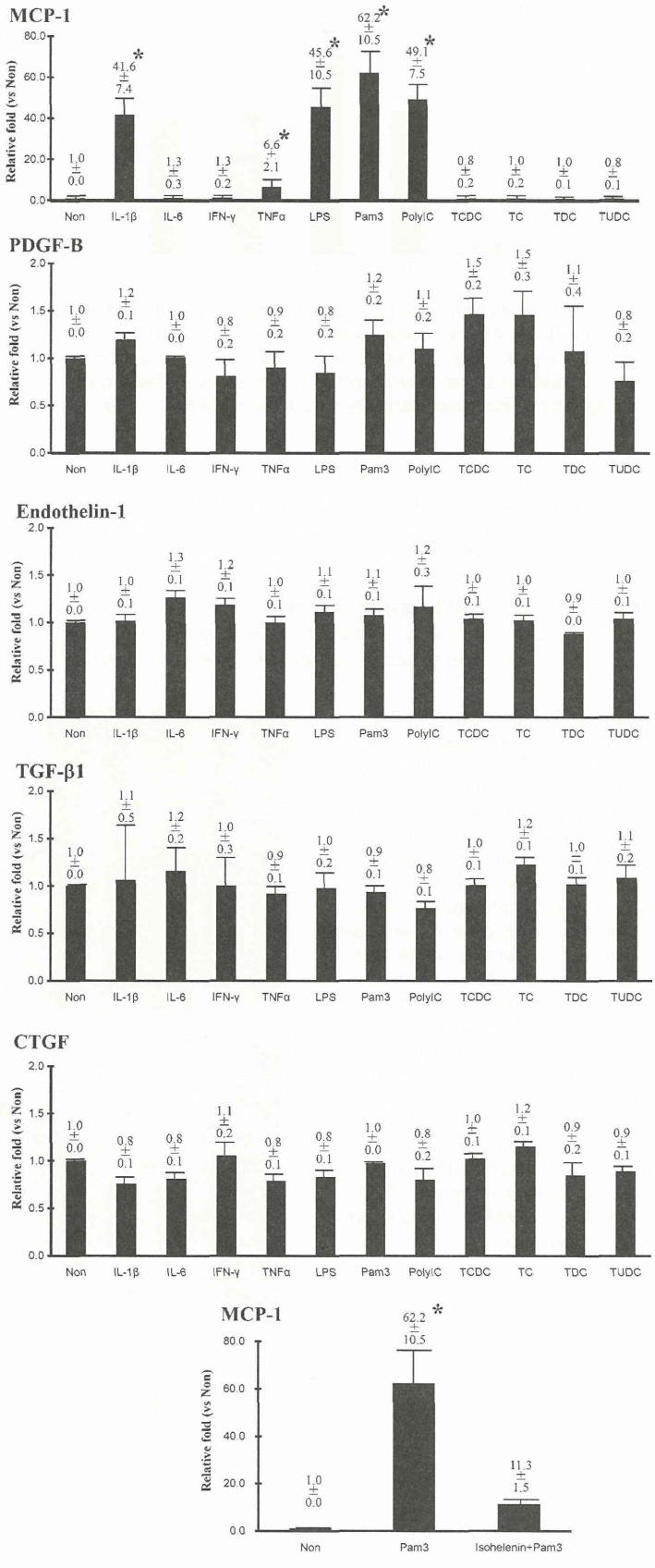

Figure 2 Quantitative PCR analysis for mRNA of fibrogenic cytokines (monocyte chemoattractant protein-1 (MCP-1), platelet-derived growth factor (PDGF)-B, connective tissue growth factor (CTGF), transforming growth factor-β (TGF-β)1, and endothelin-1). MCP-1 expression alone was upregulated by stimulation with Toll-like receptor (TLR) ligands (lipopolysaccharide (LPS), Pam3CSK4, and poly(I:C)) and cytokines (interleukin-1β (IL-1β) and tumour necrosis factor-alpha (TNF-α)). No bile acids (taurochenodeoxycholic acid (TCDC), taurocholic acid (TC), taurodeoxycholic acid (TDC) or tauroursodeoxycholic acid (TUDC)) significantly upregulated MCP-1 mRNA expression. Other fibrogenic cytokines were not affected by any stimulants. A lower figure denotes that Pam3CSK4-induced upregulation of MCP-1 mRNA expression was inhibited by pretreatment with an NF-κB inhibitor, isohelenin. Bars indicate the mean±SEM. *<0.05. INF-γ, interferon-γ.

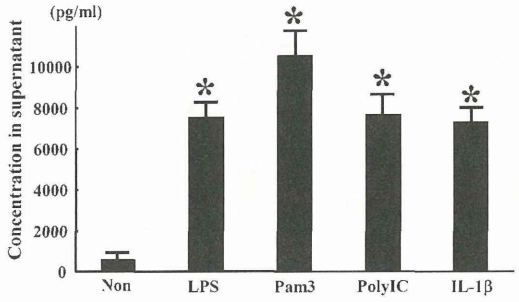

Figure 3 Measurement of monocyte chemoattractant protein-1 (MCP-1) protein by ELISA. After stimulation with lipopolysaccharide (LPS), Pam3CSK4 (Pam3), poly(I:C), and IL-1β for 24 h, the concentration of MCP-1 protein in cultured supernatants of human biliary epithelial cells was significantly increased. Bars indicate the mean±SEM. *<0.05.

MCP-1, but the intensity was similar to or less than that in bile ductules. In contrast, MCP-1-positive bile ductules were not found in CHF. Moreover, the expression of αSMA was limited in portal tracts, and αSMA-positive HSCs were not found in CHF.

Characterisation of CCR2-expressing cells

Fluorescence double immunostaining revealed that cells double positive for CD68 and CCR2 were scattered mostly in interface areas of CVH and PBC, but cells double positive for αSMA and CCR2 were not found in any area (figure 5).

DISCUSSION

Chronic liver injury is generally characterised by inflammation and subsequent fibrosis, and, regardless of the aetiology, a cytokine-rich environment caused by inflammation and infection is closely associated with hepatic fibrosis. HSC is considered the most important effecter cell associated with fibrogenesis in hepatic parenchyma including the interface between portal tracts and periportal hepatocytes, and the fibrous enlargement of portal tracts and fibrous extension from portal areas are closely associated with activated HSCs and their transformed version, myofibroblasts. Within portal tracts, in contrast, fibroblasts and fibrocytes are also important to the histogenesis of portal fibrosis. Several cytokines such as PDGF-B, CTGF, TGF-β, endothelin-1 and MCP-1, are reported as fibrogenetic factors in liver.[5] These cytokines could chemoattract HSCs or directly activate the production of collagen in myofibroblasts.

We have previously reported that human BECs possess receptors for inflammatory cytokines (IL-1β, IL-6, Interferon-γ and TNF-α) and an innate immune system consisting of TLRs.[6 8 9] Therefore, in this study, we examined whether BECs contribute to hepatic fibrosis using cultured human BECs. Consequently, although mRNAs of all fibrogenic cytokines were detected in

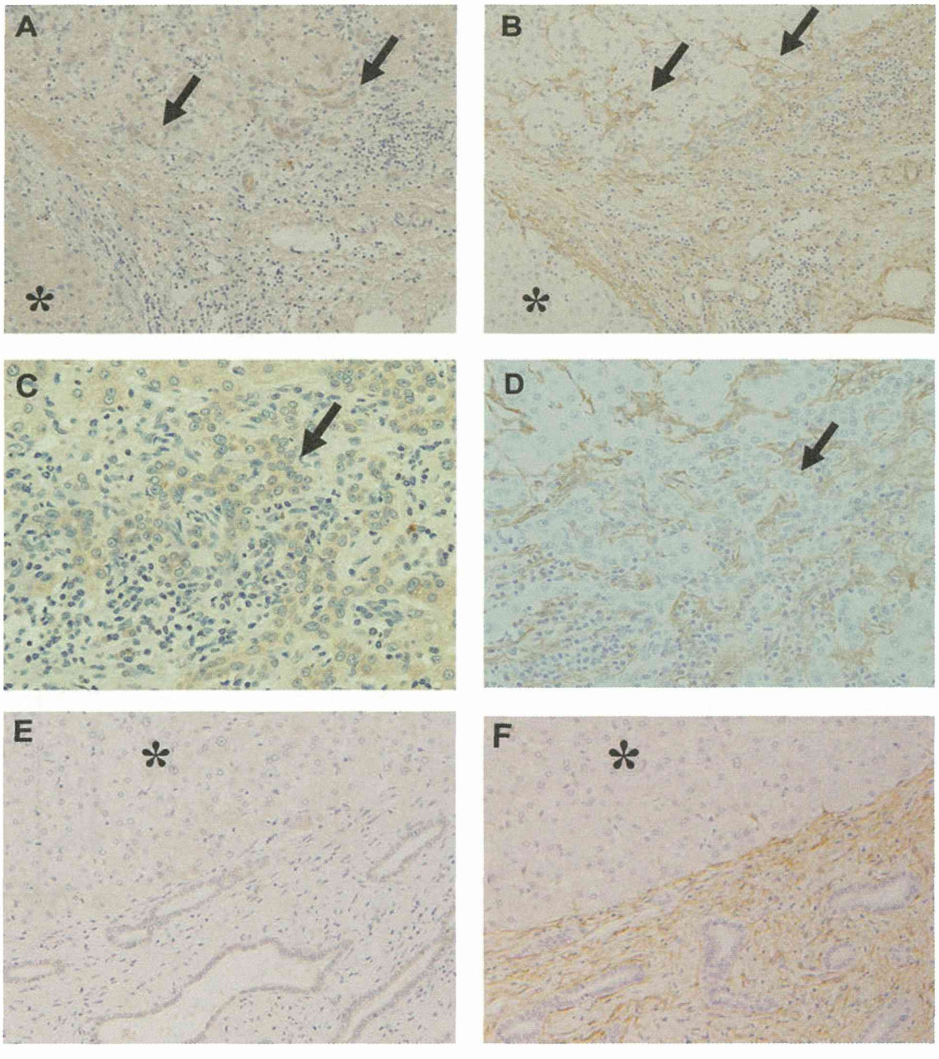

Figure 4 Immunohistochemistry for monocyte chemoattractant protein-1 (MCP-1) (A, C, E) and α-smooth muscle actin (αSMA) (B, D, F). (A, B) Hepatitis C virus-related chronic viral hepatitis. Proliferating bile ductules at the interface of periportal areas express MCP-1 (A, arrows) and αSMA-positive cells showing hepatic stellate cell (HSC) morphology are found in the same area (B, arrows). In contrast, αSMA-positive HSCs are not found in periportal parenchyma lacking bile ductules (asterisks). (C, D) Primary biliary cirrhosis. MCP-1-positive proliferating bile ductules and αSMA-positive cells are intermingled (arrows). (E, F) Congenital hepatic fibrosis. No biliary bile ducts or ductules express MCP-1, and no αSMA-positive HSCs are found in periportal areas (asterisks). The expression of αSMA is limited in portal tracts (lower right).