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

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Notch1-Hes1 signaling axis in the tumorigenesis of biliary neuroendocrine tumors

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ABSTRACT

Aims: Biliary neuroendocrine tumors (NETs) are rare and mostly exist as a component of mixed adenoneuroendocrine carcinomas (MANECs). Although the NET component in biliary MANECs is generally more malignant and clinically more important to the prognosis than the ordinary adenocarcinomatous component, the histogenesis of biliary NET has not been clarified. In this study, the role of the Notch1-Hes1 signaling axis in the histogenesis of biliary NETs was examined. **Methods:** Immunohistochemistry for Notch1, its ligand Jagged1, and Hes1 was performed using surgical specimens from 11 patients with biliary MANEC. Moreover, after the knock-down of Notch1 mRNA expression by the siRNA technique in a cholangiocarcinoma cell line, the expression of chromogranin A (a neuroendocrine marker) and Ascl1 (a neuroendocrine-inducing molecule inhibited by activated Hes1) was examined by quantitative PCR. **Results:** Histological examination revealed that the adenocarcinomatous components were predominately located at the luminal surface of the MANEC and the majority of stromal invasion involved NET components. Ordinary adenocarcinomas and non-neoplastic biliary epithelium constantly expressed Notch1, Jagged1, and Hes1, but the expression of Notch1 and Hes1 was decreased or absent in NET components, suggesting interference with the Notch1-Hes1 signaling axis in biliary NET. Moreover, in the cholangiocarcinoma cell line in which the expression of Notch1 mRNA was knocked down, the mRNA expression of Ascl1 and chromogranin A was increased. **Conclusion:** The Notch1-Hes1 signaling axis suppresses neuroendocrine differentiation and maintains tubular/acinar features in adenocarcinoma and non-neoplastic epithelium in the biliary tree. Moreover, a disruption of this signaling axis may be associated with the tumorigenesis of NETs in biliary MANEC.

Key Words: biliary neuroendocrine tumors, mixed adenoneuroendocrine carcinoma, Notch1, Hes1

INTRODUCTION

Neuroendocrine tumors (NETs) including carcinoid tumors are commonly found in several organs including the pancreas and gastrointestinal tract. In contrast, most tumors originating from the intrahepatic and extrahepatic biliary trees are ordinary adenocarcinomas, irrespective of their etiology. Physiologically, a few enterochromaffin-like neuroendocrine cells exist in the biliary tree, particularly in large bile ducts and peribiliary glands of the hepatic hilus,[1] but cases of pure NET in hepatobiliary organs are very rare. Most biliary NETs exist as a component of mixed adenoneuroendocrine carcinomas (MANECs, WHO classification, 2010).[2] MANECs are found in hepatic hilar cholangiocarcinomas with hepatolithiasis, gallbladder cancers, and extrahepatic cholangiocarcinomas and show a characteristic histology.[3] Moreover, since the NET component of biliary MANEC defines the prognosis, it is important to identify it and consider indications for adjunctive therapy such as somatostatin analogues.

Notch signaling allows the establishment of patterns of gene expression and differentiation, and regulates cell fate in multiple developmental programs. The Notch signaling pathway has an important role in the development of intrahepatic bile ducts via postnatal bile duct growth and remodeling.[4] Moreover, in the developing endoderm, Notch signaling inhibits endocrine differentiation via the activation of a repressor, hairy and enhancer or spilt 1 (Hes1, a Notch effector), for the expression of a basic helix-loop-helix (bHLH) transcription factor, achaete-scute complex homolog-like 1 (Ascl1)/MASH1 which leads to the neuroendocrine phenotype.[5-8] Therefore, the Notch1-Hes1 signaling axis and Ascl1 play key opposing roles and a lack of Notch1 signaling has been demonstrated to cause the regression of Hes1 activation leading to the activation of Ascl1 and neuroendocrine differentiation in gastrointestinal carcinoids.[7]

To date, several cases of biliary NET, mostly MANEC, have been reported,[9-13] but the histogenesis of NETs is not well studied in the biliary tree area. In this study, we first examined the

expression of a panel of developmental transcription factors concerned with the Notch1-Hes1 signaling axis in biliary NET using biliary MANEC cases. Impaired Notch1-Hes1 signaling is demonstrated to play a role in the histogenesis of biliary NET.

MATERIALS and METHODS

Patients and tissue preparations

Surgically resected hepatobiliary specimens from a total of 11 patients with biliary MANECs were retrieved from the surgical files of our laboratories and affiliated hospitals. Six patients had gallbladder cancer with cholecystolithiasis, 2 had common bile duct cancer, and 3 had hepatic hilar cholangiocarcinoma accompanying hepatolithiasis. Nine of the cases were used in another study for the clinicopathological characterization of biliary MANEC.[3] All tissue specimens containing tumorous lesions were immediately fixed in 10% neutral-buffered formalin and embedded in paraffin. Several 4μm-thick sections were prepared from each paraffin-embedded block and either routinely stained for histologic evaluation or processed for immunohistochemical analysis.

Definition of MANEC

According to the 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 signaling axis in the tumorigenesis of biliary NET. The deparaffinized sections were microwaved in citrate buffer (pH6) for 20min for antigen retrieval prior to staining. Following endogenous peroxidase blocking and incubation in normal goat or rabbit serum (1:10; Vector Lab, Burlingame, CA) for 20 min, these sections were incubated at 4°C overnight with primary antibodies against human chromogranin A (mouse IgG, 0.5μg/ml, Dako, Tokyo, Japan), synaptophysin (mouse IgG, 5μg/ml, Dako), Jagged 1 (Goat IgG, 0.5μg/ml, Santa Cruz, Santa Cruz, CA), Notch 1 (mouse IgG, 5μg/ml, OriGene, Rochkville, MD), or HES1 (Rabbit IgG, 5μg/ml, United States Biological, Swampscott, MA), and then at room temperature for 1 hour with goat anti-mouse or anti-rabbit immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Envision, Dako) or rabbit anti-goat immunoglobulins conjugated to a peroxidase-labeled dextran polymer (Simple staining kit, Nichirei, Tokyo, Japan). After a benzidine reaction, sections were weakly counterstained with hematoxylin. No positive staining was obtained when the primary antibodies were replaced with an isotype-matched, non-immunized immunoglobulin as a negative control of the staining procedures. The degree of expression of Jagged1 and Notch1 in the adenocarcinoma and NET components was semi-quantitatively evaluated as <20%, 20-80%, or >80%.

Cell culture and transfection of short interfering RNAs (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 siRNAs against mRNA of Notch 1 (siRNA 1, ACGAAGAACAGAAGCACAAAGGCGG and siRNA 2,

UCGCAUUGACCAUUCAAACUGGUGG) 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 was analyzed by real-time PCR as described below.

Isolation of RNA and polymerase chain reaction (PCR)

HuCCT1 was collected from the flasks with a cell scraper for determination of the baseline mRNA expression of Notch1, Jagged1, Hes1, Ascl1, and chromogranin A by 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 reverse transcription (RT), 1μg 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 1min and extension at 72°C for 2min, 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'-CCAGCAACTGCTGACATCAA-3', Hes1 forward, 5'-TCTGAGCCAGCTGAAAACAC-3', and reverse, 5'-GGTACTTCCCCAGCACACTT-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

(GAPDH, internal positive control), forward, 5'-GGCCTCCAAGGAGTAAGACC-3', and reverse, 5'-AGGGGTCTACATGGCAACTG-3'.

Statistical analysis

Data were analyzed using Wilcoxon signed-ranks test. $p<0.1$ 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 tumor 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 (Fig.1). These NET components were classified as NET G2 or G3 according to the WHO classification (2010)[2,14]: the tumor cells had a round- or oval-shaped nucleus and "salt-and-pepper" pattern of nuclear chromatin, general characteristics of neuroendocrine cells (Fig.1).

Expression of Notch signaling molecules

Immunohistochemistry revealed that Jagged1 was constantly expressed in the cytoplasmic regions of non-neoplastic biliary epithelial cells in normal bile ducts and gallbladder (Fig.2). Although the expression of Jagged1 varied in tumor cells of the adenocarcinoma and NET, semiquantitative analysis revealed no significant difference between these components in any cases except one (Fig.2). Notch1 was constantly expressed with the nuclear pattern in non-neoplastic biliary epithelial cells (Fig.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 (Fig.3). Hes1 expression was found in the cytoplasm and/or nucleus of positive cells (Fig.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 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 (Fig.4).

Expression of Notch signaling 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 (Fig.5). Ascl1 and chromogranin A were faintly found (Fig.5). The procedure using both siRNA1 and siRNA2 against the Notch1 gene interfered with the expression approximately 20% (Fig.6). In contrast, the expression of Ascl1 and chromogranin A was significantly upregulated, compared to no siRNA (transfectant only) (Fig.6). These findings suggest that Notch 1 expression could negatively regulate the expression of chromogranin A.

DISCUSSION

Several cases of biliary NET including carcinoid tumors 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 tumor 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 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 signaling allows the establishment of patterns of gene expression and differentiation, and regulates cell fate in multiple developmental programs. The four mammalian Notch receptors (Notch1 to 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 neighboring cells. In intrahepatic bile ducts, the Notch signaling 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 signaling to maintain epithelial homeostasis in the tubular/acinar epithelium.

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

overexpression of an active form of Notch1 leads to the up-regulation of Hes1, silencing of Ascl1, and the down-regulation of neuroendocrine markers: the regression of the repressor Hes1 followed by a lack of Notch signaling has been demonstrated in the histogenesis of NET.[7] Ascl1 is highly expressed in neuroendocrine tumors.[20] However, this histogenesis of NET is not common in any NETs. Wang et al.,[19] reported that gastroenteropancreatic NETs were heterogeneous with regard to the Notch1-HES1 signaling 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 signaling pathways of gastroenteropancreatic NETs. Moreover, in the developing endoderm, disruption of Notch-Hes1 signaling results in precocious endocrine differentiation in the pancreas. Because pancreatico-biliary systems embryologically share the common genesis from the foregut,[21] the Notch-Hes1 signaling 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 that HuCCT1 is 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 signaling 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 signaling is important to inhibit neuroendocrine differentiation in the adenocarcinoma phenotype of cholangiocarcinoma and an its impairment is closely associated with the tumorigenesis of biliary NETs.

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 signaling 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 degression of Notch expression in biliary NET is unknown. In prostate carcinoma, neuroendocrine differentiation has been associated with tumor 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 signaling axis suppresses neuroendocrine differentiation to maintain tubular/acinar features in biliary adenocarcinoma and normal epithelium.
- A disruption of Notch1-Hes1 signaling axis may be associated with the tumorigenesis of biliary neuroendocrine tumors in mixed adenoneuroendocrine carcinomas.
- Further studies are needed to understand the mechanism behind the modulation of Notch1 and to devise ways to improve the prognosis for mixed adenoneuroendocrine carcinomas.

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FIGURE LEGENDS

Fig.1 Gallbladder cancer with neuroendocrine tumor (NET) G3 (WHO classification, 2010). Although the luminal surface of the tumor is an ordinary tubular adenocarcinoma (A, arrow), the invasive component has a different histology showing a solid component (A, *). Tumors in the solid component consist of uniform-sized and large tumor 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), x40; (B), x400.

Fig.2 Comparative analysis of Jagged1 expression between adenocarcinomas and neuroendocrine tumors (NETs) in MANEC. 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). Semi-quantitative 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) and (B), x40; (B), x200.

Fig.3 Comparative analysis of Notch1 expression between adenocarcinomas and neuroendocrine tumors (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. Semi-quantitative evaluation revealed that Notch1 expression in NET is decreased in 8 of 11 cases and less than 20% in all cases

except one. There was a statistical significant in the expression of Notch 1 between adenocarcinoma and NET (Wilcoxon signed-ranks test, $p<0.1$, T value=2, confidence interval 5 to 31). Immunohistochemistry for Notch1 (A-C). Original magnification, (A), x40; (B) and (C), x200.

Fig.4 Comparative analysis of Hes1 expression between adenocarcinomas and neuroendocrine tumors (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 significant in the expression of Hes1 between adenocarcinoma and NET (Wilcoxon signed-ranks test, $p<0.1$, T value=5, confidence interval 8 to 37). Immunohistochemistry for HES1 (A-C). Original magnification, (A), x40; (B), x400; (C), x200.

Fig.5 Detection of Notch1, Jagged1, Hes1, Ascl1, and chromogranin A in cultured human intrahepatic biliary epithelial cells (HIBEC), human cholangiocarcinoma cells (HuCCT1), and human breast cancer cells (MCF1, positive control) by RT-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).

Fig.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 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).

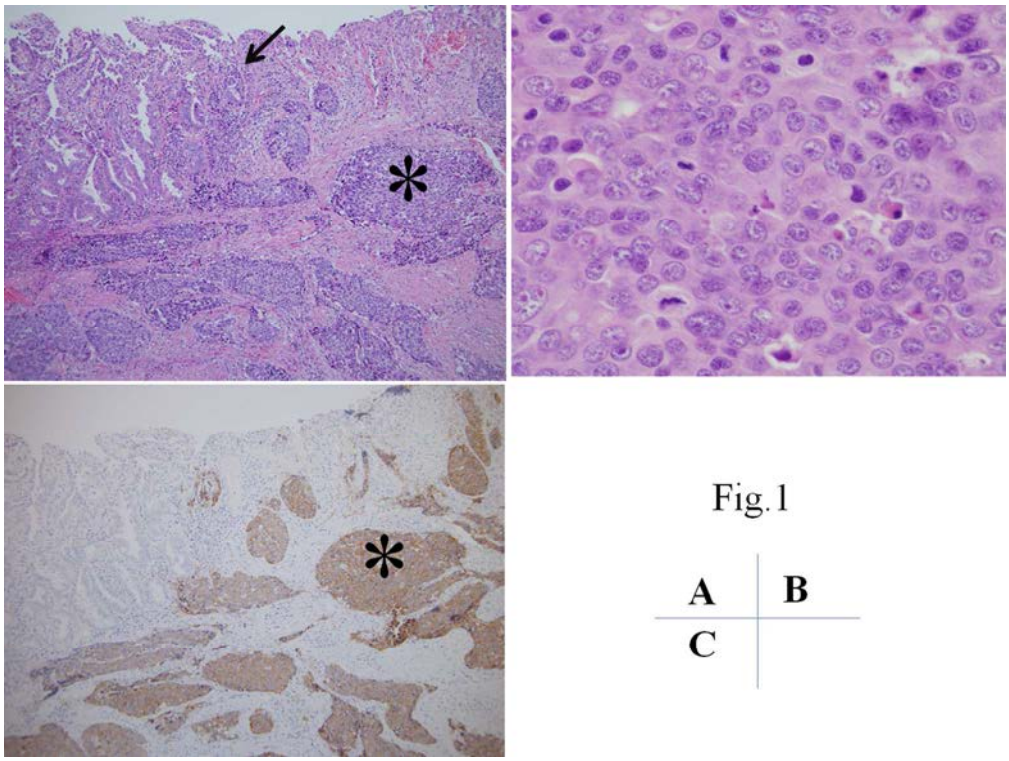

Fig. 1

A B
C

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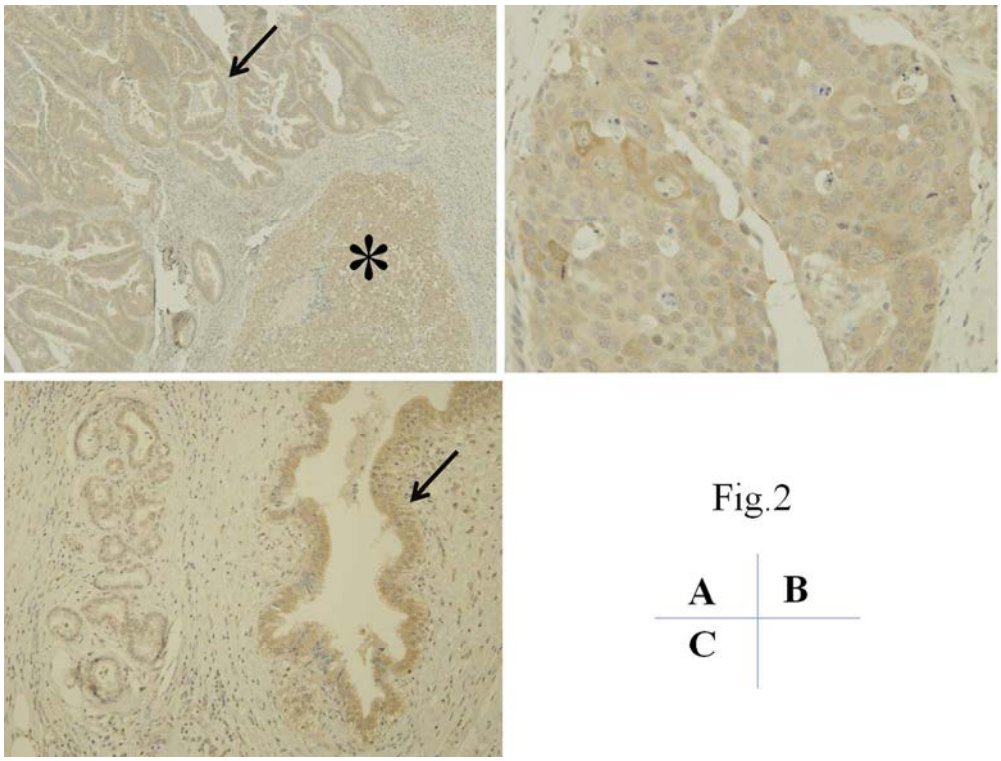

Fig.2

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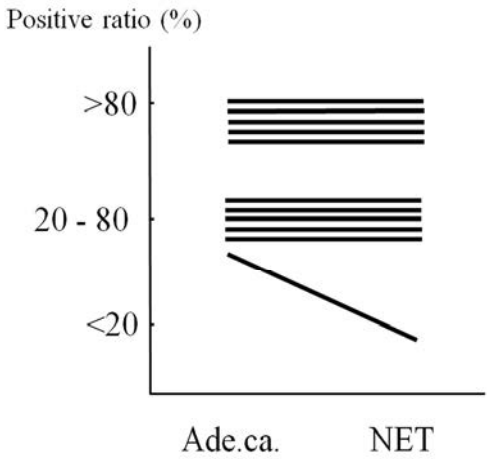

Fig.2D

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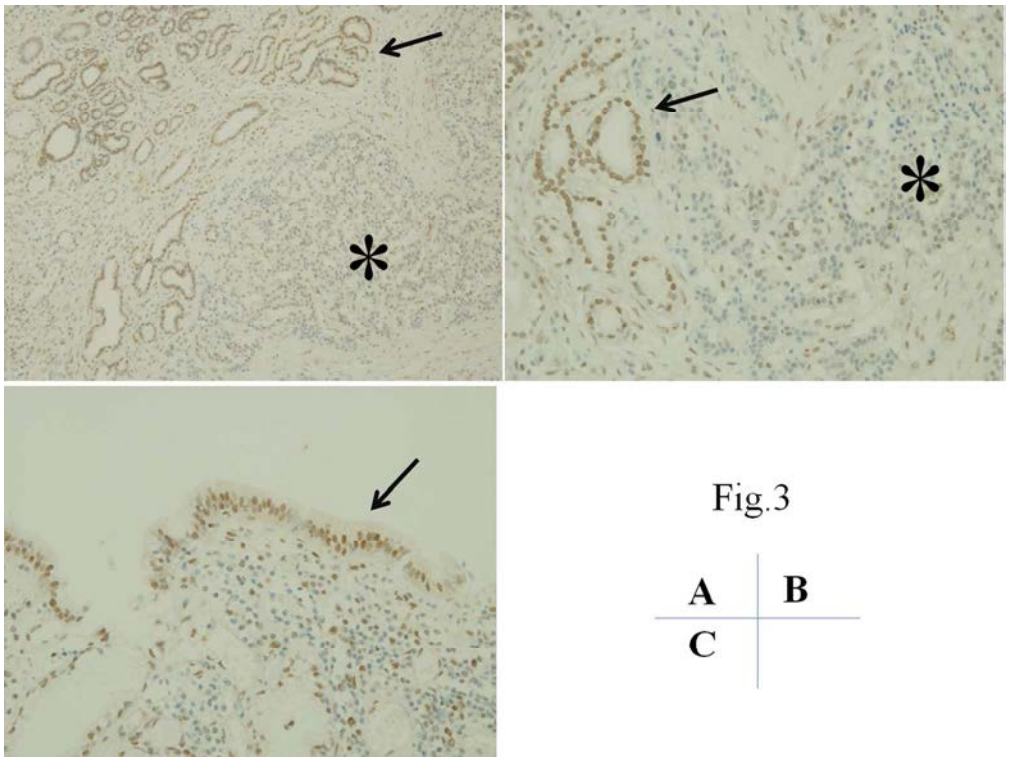

Fig.3

A B
C

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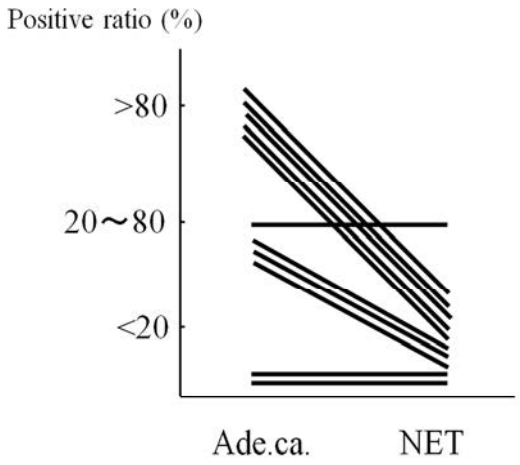

Fig.3D

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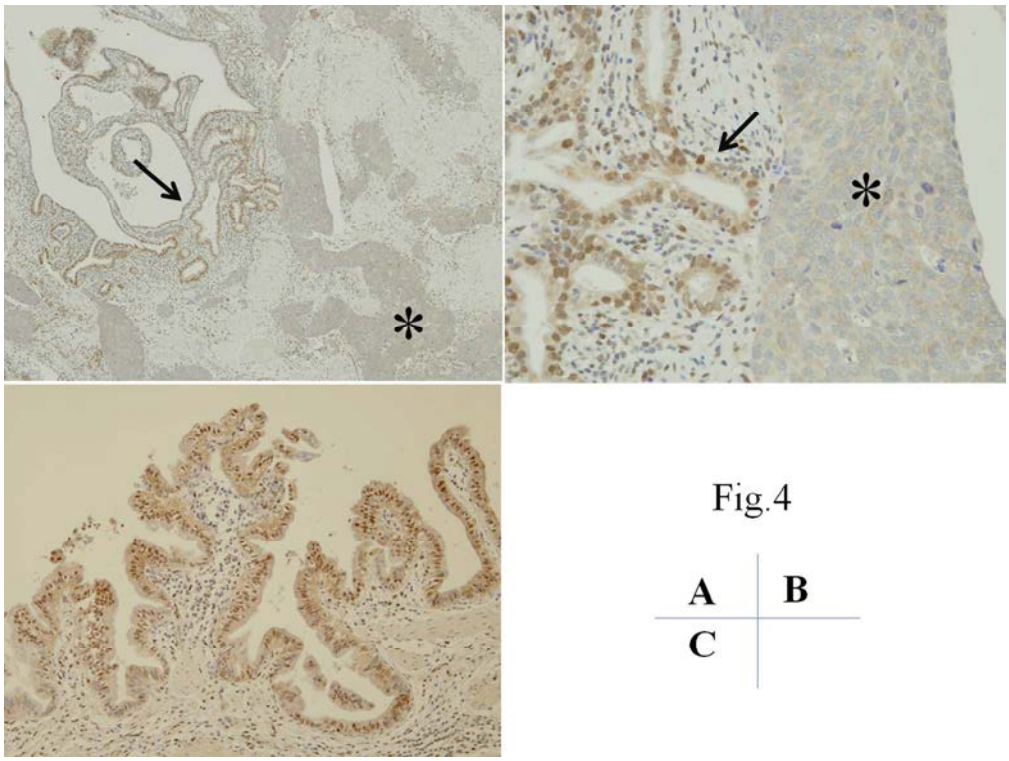

Fig.4

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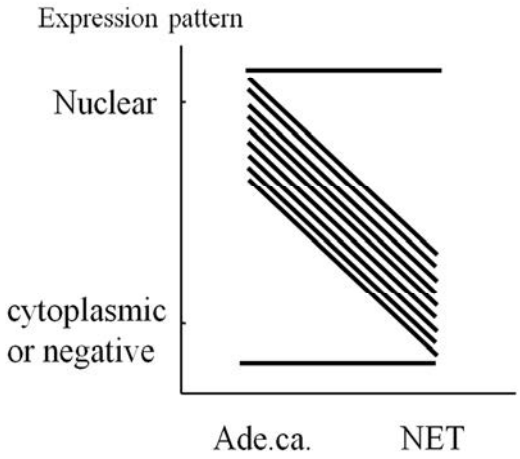

Fig.4D

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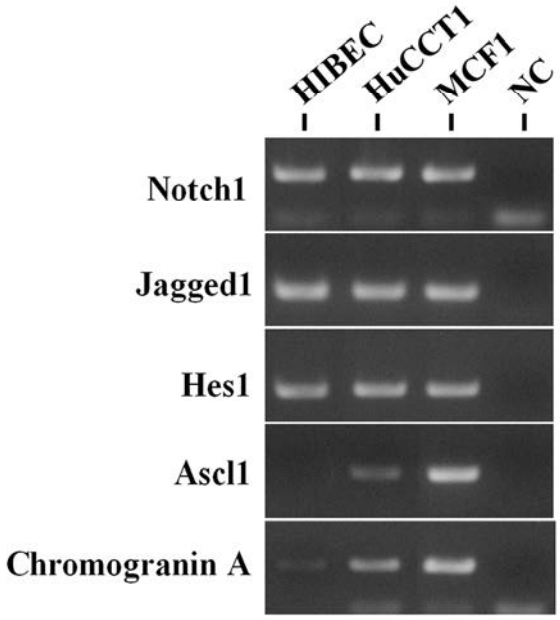

Fig.5

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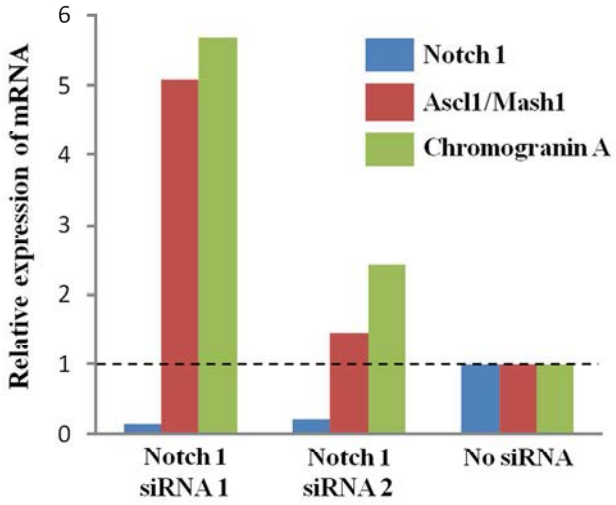

Fig.6

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