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Establishment of a Novel Cell Line from Intraductal Papillary Neoplasm of the Bile Duct

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Abstract. *Background: Intraductal papillary neoplasm of the bile duct (IPNB) is a novel and increasingly-occurring disease. Its characteristics remain uncertain because of the lack of an in vitro culture system. We established and characterized a novel cell line from a human IPNB. Materials and Methods: We obtained tumor tissue from a surgical specimen from a patient with IPNB. Cells were primary co-cultured with mouse stromal cells in serum-free medium. Tumor characteristics were compared among the primary IPNB, established cell line, and xenograft. Results: We successfully established an IPNB cell line. We temporary termed this cell line Kobe Bile Duct Cancer (KBDC)-11. Xenograft formed a tumor which had ductal structures and mucus production as the primary tumor did. Overexpression of p53, MUC staining pattern, and CD133 expression were common among the primary IPNB, KBDC-11, and the xenograft. Conclusion: This novel cell line established from IPNB exhibited the same features as IPNB and might contribute to studies of IPNB and its process of malignant transformation.*

Intraductal papillary neoplasm of the bile duct (IPNB) is a novel emerging disease entity of cholangiocarcinoma characterized by mucin production and papillary growth. In recent years, there has been an increasing number of studies of IPNB. In general, patients with IPNB have a more favorable prognosis than patients with cholangiocarcinoma (1-4). However, the prognosis is not favorable for all patients with IPNB; high expression or positivity for MUC1 or

MUC5AC staining is a poor prognostic factor (5-7). In addition, molecular and pathological studies strongly suggest that carcinogenesis of IPNB is different from that of conventional cholangiocarcinoma (3, 4, 8, 9). We previously described differences in characteristics of IPNB and conventional intra-hepatic cholangiocarcinoma with regard to the expression pattern of CD133 (4).

IPNB has often been considered to be the biliary counterpart of intra-ductal papillary mucinous neoplasm of the pancreas (IPMN) (3, 8, 9). However, there are several differences between IPNB and IPMN. Firstly, the pancreatobiliary type is predominant in IPNB, while the intestinal type is predominant in IPMN (3). Secondly, there are more malignant tumors among IPNB than among IPMN (6, 10). Finally, IPMN is characterized by macroscopic mucin production, while only one- to two-thirds of IPNB is accompanied by macroscopic mucin production (3, 11), although most IPNBs are histologically-positive for mucin production.

Despite the growing knowledge of IPNB, detailed characteristics of this entity remain uncertain, and which biomolecular processes play crucial roles in its malignant transformation have not yet been fully-evaluated. This is probably because recent studies of IPNB were based on data from clinical samples. We have successfully established a novel cell line from a human IPNB and present it herein.

Materials and Methods

Patient. A 69-year-old man was diagnosed with cancer of the pancreatic head and perihilar cholangiocarcinoma. Endoscopic retrograde cholangiopancreatography showed a filling defect on the left hepatic duct (Figure 1a) and magnetic resonance imaging revealed that a tumor existed in the left hepatic duct (Figure 1b). Sub-total stomach-preserving pancreaticoduodenectomy and left hepatic lobectomy were performed. There was a tumor on the left hepatic duct of the resected specimen (Figure 1c). This surgical specimen was fixed with 10% phosphate-buffered formalin and embedded in paraffin. Pathology indicated an intraductal papillary carcinoma of the bile duct (the International Union Against Cancer

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(UICC) TNM classification stage I; T1 N0 M0) and a moderately-differentiated tubular adenocarcinoma (UICC stage IIB; T3 N1 M0) of the pancreas.

Cell culture. PA6 cells (a gift from Dr. Nishikawa, RIKEN, Kobe, Japan) were maintained in α -minimum essential medium (α -MEM; Invitrogen, Carlsbad, CA, USA) containing 10% fetal calf serum (FCS) and 50 U/ml of penicillin and 50 μ g/ml of streptomycin (PenStrep) (Invitrogen) at 37°C in a humidified atmosphere containing 5% CO₂.

Isolation of human IPNB. The tumor tissue was minced manually using a knife and centrifuged at 300 $\times$ g for 5 min at room temperature. Precipitated cells were washed with 10 ml of phosphate-buffered saline (PBS) three times and suspended with 10 ml of serum-free Stem medium (DS Pharma Biomedical, Osaka, Japan) that contained 0.1 μ M 2-mercaptoethanol and PenStrep. Cells suspended in serum-free Stem medium were seeded on the confluent mouse stromal cell line PA6 in a 12-well plate and incubated at 37°C in a humidified atmosphere containing 5% CO₂.

After eight weeks, colonies were sub-cultured on confluent PA6 in a 12-well plate with serum-free medium. The medium was changed every three to four days. Cells were thereafter termed Kobe Bile Duct Cancer (KBDC)-11 cells.

Transplantation of KBDC cells into immunocompromised mice. To test tumorigenicity, 10-week-old male nude mice (22g) (BALB/cAjl-nu/nu) (Oriental Yeast, Tokyo, Japan) were used. All nude mice were housed and used under the approved protocols (approval number: P121203) in accordance with the Kobe University guidelines for the care and use of laboratory animals, and efforts were made to minimize suffering and the number of animals used. Nude mice were anesthetized with tribromoethanol. After a small incision was made in the left back, a mixture of KBDC cells with PA6 (2 $\times$ 10⁵ cells) in 100 μ l of Matrigel (BD Bioscience, San Jose, CA, USA) was injected subcutaneously. After eight weeks, the mice were sacrificed and tissue samples were fixed in 10% phosphate-buffered formalin overnight and embedded in paraffin.

Immunohistochemistry. KBDC cells were embedded with PA6 in Histogel™ (Thermo Scientific Richard-Allan Scientific, Kalamazoo, MI, USA) according to the manufacturer's protocol. Sample embedded in Histogel was fixed in 10% buffered formalin and routinely processed for analysis.

Immunohistochemical detection of MUC1-core, MUC2, MUC5AC, p53 and CD133 protein was performed using mouse anti-human MUC1 monoclonal antibody (mAb) (clone Ma552, 1:100 dilution in Dako REALAntibody Diluent; Novocastra Laboratories Ltd., Newcastle upon Tyne, UK), mouse anti-human MUC2 mAb (clone Ccp58, 1:100 dilution; Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA), mouse anti-human MUC5AC mAb (clone CLH2, 1:300 dilution; Chemicon International, Inc., Temecula, CA, USA), mouse anti-human p53 protein mAb (clone DO-7, 1:50 dilution; Dako, Carpinteria, CA, USA), and mouse anti-human CD133 mAb (clone AC133/1, 1:50 dilution; Miltenyi Biotec, Bergisch Gladbach, Germany), respectively. Incubation with the respective primary antibodies was performed overnight at 4°C. Immunodetection was performed using a Envision plus system (Dako, Carpinteria, CA, USA) with 3,3-diaminobenzidine (DAB)/H₂O₂ as the chromogen. Immunostained sections were then counterstained with hematoxylin and coverslipped for microscopic assessment.

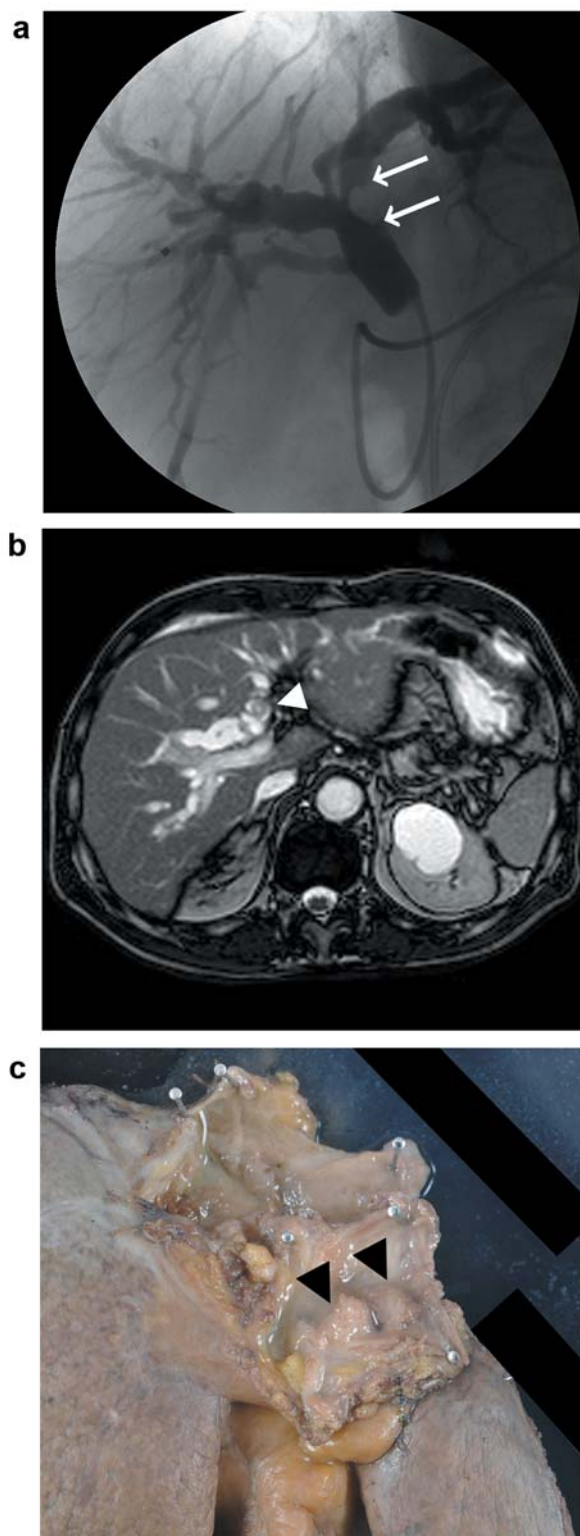


Figure 1. Preoperative imaging studies showed a filling defect of the perihilar bile duct on endoscopic retrograde cholangiopancreatography (arrows) (a) and tumor that occupied the perihilar bile duct on magnetic resonance imaging (arrowhead) (b). c: Surgical specimen of papillary tumor in the perihilar bile duct.

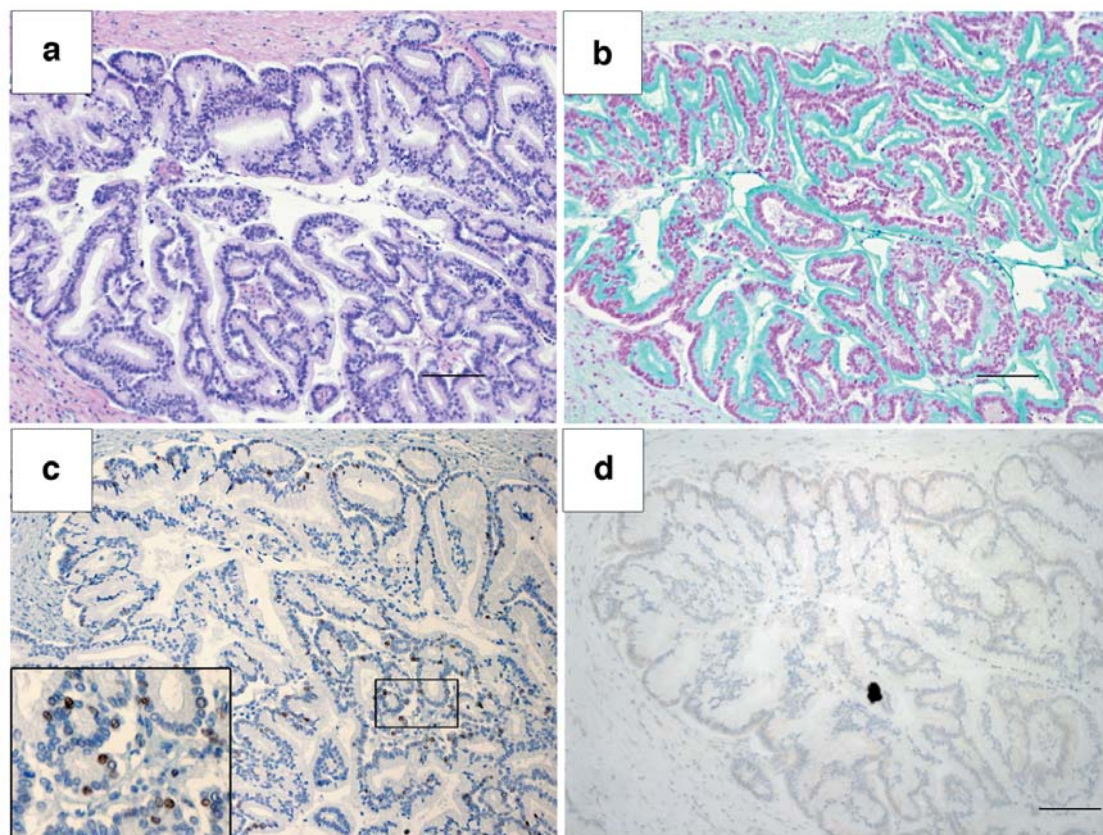


Figure 2. Histopathological features of the primary tumor. a: Hematoxylin and eosin staining; b: Alcian blue staining; c: p53 staining; and d: CD133 staining (bar=100 μ m, $\times$ 40).

Ethical considerations. The study protocol was approved by the Institutional Ethics Committee and adhered to the principles outlined in the Guideline for Good Clinical Practice (January 1997) and Declaration of Helsinki (1996). The patient provided written informed consent was for the use of surgical tissue for this research.

Results

Primary tumor. Histological features of the primary IPNB tumor are shown in Figure 2. Prominent papillary growth was revealed by hematoxylin-eosin staining (Figure 2a) and gross mucin production by Alcian blue staining (Figure 2b). These cells have mucin in their columnar cells with low cellular atypism. These findings are compatible with the histological features of the pancreatobiliary type of IPNB.

Establishment of tumor cell line. Five days after the primary culture, some colonies were formed when we cultured the KBDC cells on PA6 cells in serum-free medium, whereas no colony was formed in either serum-free medium or in α -MEM containing 10% FCS without PA6 (data not shown). The colonies of KBDC-11 were passaged 23 times over a 1-year period.

KBDC-11 in vitro. Microscopy showed that spheric colonies were dominant in serum-free medium (Figure 3a). It took more than two weeks for a colony to form from a single cell. When we exchanged serum-free medium for DMEM containing 10% FCS, sphere colonies of KBDC-11 changed to planar colonies. KBDC-11 had mucin production and was positive for p53 staining and negative for CD133, similar to the primary tumor (Figure 3b).

Tumor formation by KBDC cells. Eight weeks after implantation of KBDC-11 in mice, a subcutaneous tumor grew at the site of injection (Figure 3a). However, we did not detect any metastatic tumors. The xenograft exhibited ductal formation (Figure 3b). Xenograft also showed mucin production and was positive for p53 staining and negative for CD133 similarly to the primary tumor (Figure 3b).

Characteristics of mucin production of KBDC 11 cell graft. To investigate tumor characteristics, we examined expression of MUC1, MUC2, and MUC5AC (Figure 4). The primary tumor was positive for MUC1 and MUC5AC and negative for MUC2. The xenograft had a similar pattern to the primary tumor.

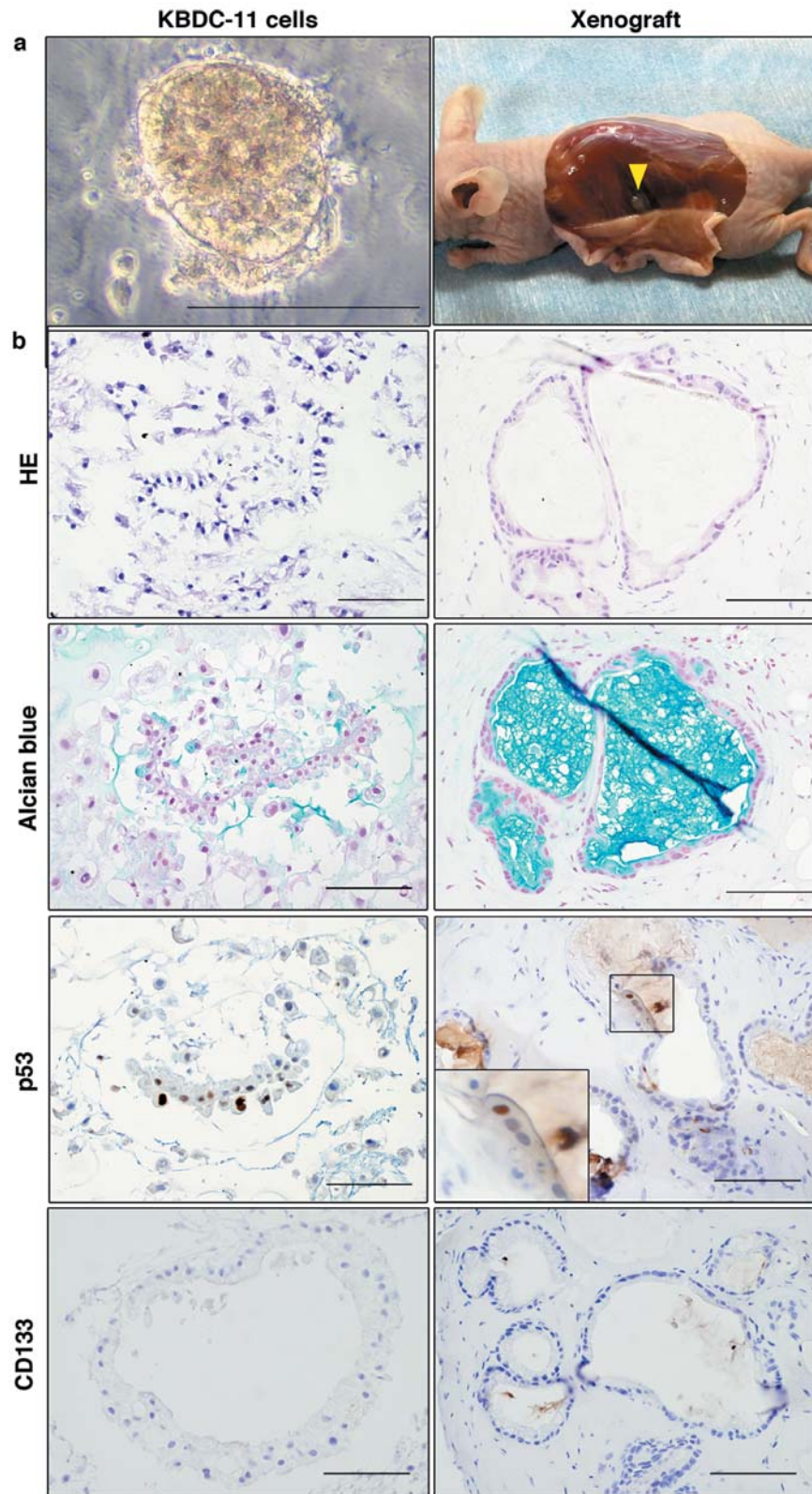


Figure 3. a: Spherical colony of KBDC-11 cells in vitro (bar=100 μ m, $\times$ 200) and tumor formation from a xenograft of KBDC-11 cells in a nude mouse (arrowhead). b: Histopathological features of a colony in vitro and of xenograft as shown by staining with hematoxylin and eosin, Alcian blue, and for p53, and CD133.

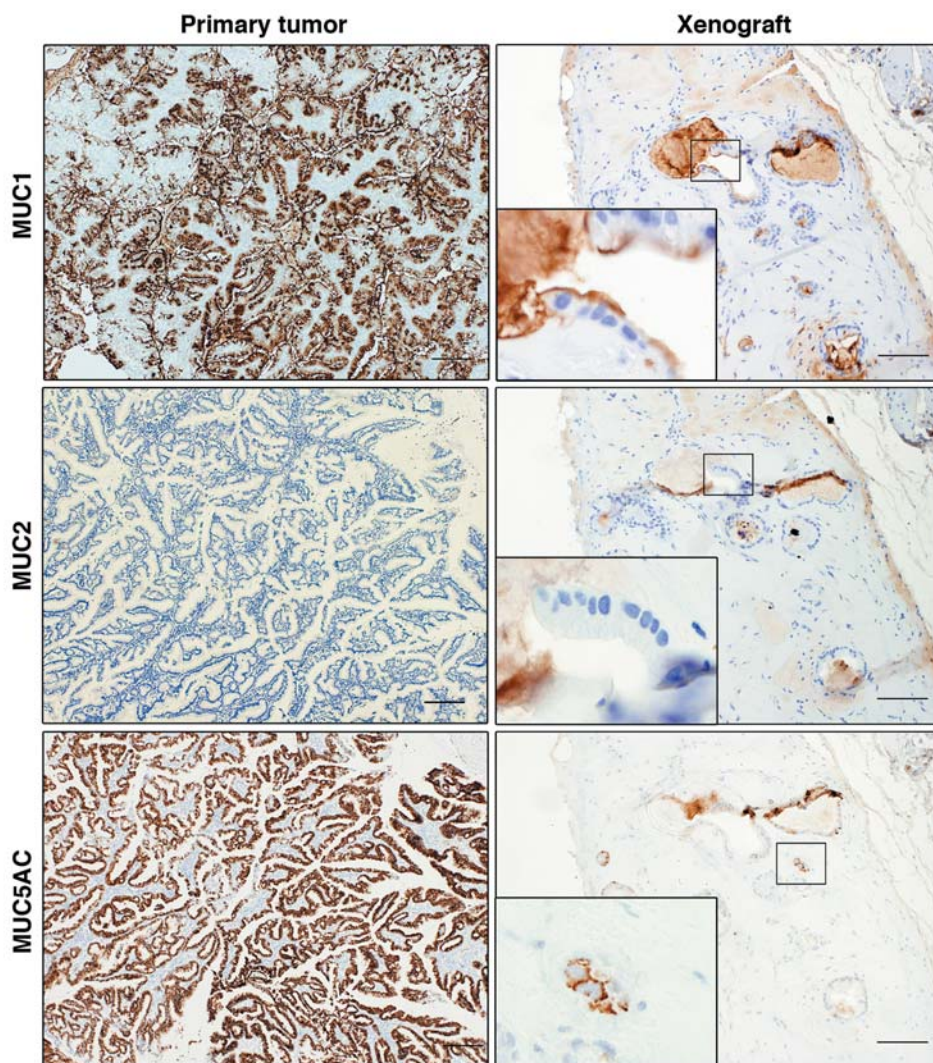


Figure 4. Staining pattern of MUC1, MUC2, and MUC5AC in primary tumor (bar=100 μ m, $\times$ 40) and KBDC-11 xenograft (bar=100 μ m, $\times$ 200).

Discussion

Currently there are a few available cell lines for cholangiocarcinoma. These cells originate from conventional bile duct carcinoma (12-16), gallbladder carcinoma (14, 17, 18), and intra-hepatic cholangiocarcinoma (12, 19, 20). To our knowledge, we are the first to establish a cell line from IPNB.

The primary tumor exhibited prominent papillary growth and gross mucin production as shown by alcian blue staining. These features are compatible with IPNB, which is the biliary counterpart of IPMN. IPNB was sub-classified into four types based on hematoxylin-eosin staining according to the classification of IPMN: gastric type, intestinal type, pancreatobiliary type, and oncocytic type (3). These four types have different MUC staining patterns (21-23). In the

present study, the primary tumor was sub-classified as the pancreatobiliary type from the viewpoint of both tumor cell type and MUC staining pattern. The xenograft of KBDC-11 cells in nude mice retained histological features similar to those of the primary tumor. These results confirmed that KBDC-11 was derived from the primary tumor of the patient with IPNB.

There are two different types of biliary carcinogenesis: one is the IPNB type and the other is the flat type associated with biliary intra-epithelial neoplasia (24). Expression of p53 in IPNB is less common than in non-papillary intra-hepatic cholangiocarcinoma (25). In addition, p53 expression was observed in the early stage of IPNB, while only the flat type of cholangiocarcinoma was observed in the invasive state (24). In contrast, p53 expression has been shown to be

associated with IPNB invasiveness (7, 9, 26). In our case, the primary tumor was histologically staged as T1 with p53 overexpression, and the KBDC-11 cells were also positive for p53, suggesting that this type of tumor has the potential to become invasive.

IPNB is distinguished from conventional biliary tumors, which is similar to the relationship between IPMN and pancreatic ductal adenocarcinoma. Comparison of IPNB with IPMN has been the topic of several reports (3, 6, 10, 11). However, reports of comparisons of IPNB and conventional biliary tumors are scant. Our previous study revealed that no CD133 expression was observed in IPNB tumor, while extrahepatic bile duct cancer expressed CD133 in 17.2% of cancer cells (4). This difference was also observed between pancreatic IPMN and pancreatic cancer (27). Our established cell line, KBDC-11, is also negative for CD133. This shows that KBDC-11 has characteristics of IPNB, as well as papillary formation and mucin production.

In addition, we cultured KBDC-11 cells on mouse mesenchymal feeder cells, PA6. KBDC-11 did not form any colonies without PA6 cells present. KBDC-11 is a feeder-dependent cell line like embryonic stem cells or induced pluripotent stem cells. Cell–cell interaction plays an important role in organ function (28) and tumor–stromal interaction plays an important role in cancer progression (29, 30). With regard to the method of the primary culture, a co-culture system was used by several cancer researchers (31, 32). However, the function of PA6 cells in the case of co-culture with carcinoma is uncertain. Further study is necessary to clarify the molecular mechanisms involved PA6 feeder cells.

Another factor in sphere formation is the use of serum-free medium. In the case of cholangiocarcinoma, tumor markers such as carbohydrate antigen 19-9 or carcinoembryonic antigen were more greatly stimulated in medium containing FBS than in inactivated serum medium (19, 20). Serum-free medium allows for the maintenance of an undifferentiated stem cell state (33). It was suggested in some reports that under serum-free conditions, new cell lines could be established that formed spherical colonies with the phenotype of cancer stem cells from gallbladder cancer (34) and ovarian cancer (35).

In summary, we have established a new human IPNB cell line (KBDC-11). KBDC-11 showed overexpression of p53, positivity for MUC1 and MUC5, and negativity for MUC2 and CD133. This cell line can contribute to the further study of various aspects of IPNB, such as its molecular biological processes, carcinogenesis, mechanism of invasive phenomenon, or biomarkers for diagnosis.

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