LABORATORY INVESTIGATION

USCAP UNITED STATES AND CANADIAN ACADEMY OF PATHOLOGY
Creating a Better Pathologist

Research Article

Efficient Establishment of Bile-Derived Organoids From Patients With Biliary Cancer

Keisuke Kinoshita[a,b], Yoshiyuki Tsukamoto[a,*], Yuka Hirashita[a,b], Takafumi Fuchino[a,b], Shusaku Kurogi[a], Tomohisa Uchida[a], Chisato Nakada[a], Takashi Matsumoto[c], Kazuhisa Okamoto[b], Mitsuteru Motomura[d], Satoshi Fukuchi[e], Ryota Sagami[f], Takayuki Nagai[g], Yasuhiko Gotoh[h], Kensuke Fukuda[b], Ryo Ogawa[b], Kazuhiro Mizukami[b], Tadayoshi Okimoto[b], Masaaki Kodama[b], Kazunari Murakami[b], Masatsugu Moriyama[a], Naoki Hijiya[a,*]

[a] Department of Molecular Pathology, Faculty of Medicine, Oita University, Oita, Japan; [b] Department of Gastroenterology, Faculty of Medicine, Oita University, Oita, Japan; [c] Department of Environmental and Preventive Medicine, Faculty of Medicine, Oita University, Oita, Japan; [d] Department of Gastroenterology, Oita Red Cross Hospital, Oita, Japan; [e] Department of Gastroenterology, Oita City Medical Association Almeida Memorial Hospital, Oita, Japan; [f] Department of Gastroenterology, Oita San-ai Medical Center, Oita, Japan; [g] Department of Gastroenterology, Oita Kouseiren Tsurumi Hospital, Beppu, Japan; [h] Department of Gastroenterology, Shinbeppu Hospital, Beppu, Japan

ARTICLE INFO

ABSTRACT

Patient-derived tumor organoids have considerable potential as an in vitro diagnostic tool for drug susceptibility testing. In the present study, we investigated whether bile collected for diagnostic purposes could be a potential source for the establishment of biliary cancer organoids. Among 68 cases of biliary cancer, we successfully generated 60 bile-derived organoids (BDOs) from individual patients. Consistent with previous reports that described biliary cancer organoids from surgical tissues, the BDOs showed diverse morphologies such as simple cysts, multiloculated cysts, thick capsulated cysts, and solid masses. They also harbored mutations in *KRAS* and *TP53* at frequencies of 15% and 55%, respectively. To enrich the cancer organoids by removing contaminated noncancerous components of BDOs, we attempted to verify the effectiveness of 3 different procedures, including repeat passage, xenografting, and selection with an MDM2 inhibitor for *TP53* mutation−harboring BDOs. By monitoring the sequence and expression of mutated *TP53*, we found that all these procedures successfully enriched the cancer organoids. Our data suggest that BDOs can be established with minimal invasiveness from almost all patients with biliary cancers, including inoperable cases. Thus, despite some limitations with respect to the characterization of BDOs and methods for the enrichment of cancer cell−derived organoids, our data suggest that BDOs have potential applications in personalized medicine.

Introduction

Advances in cancer genomics and drug development have accelerated the development of molecularly targeted therapies for certain types of tumors, including melanoma, lung cancer, and a

subset of lymphomas. On the other hand, any clinical benefit from molecularly targeted therapy is still limited to tumors such as esophageal, gastric, and biliary cancers despite extensive data on their molecular profiles. For these cancers, chemotherapy with cytotoxic drugs remains the main treatment option. Still, the therapeutic effect is unsatisfactory, mostly because of a lack of methods for the prediction of the effects of cytotoxic drugs based on genomic or transcriptomic information. Therefore, novel predictive methods are needed for more effective use of cytotoxic drugs.

Biliary cancer has a poor prognosis, with an estimated annual mortality of 174,000 worldwide.[1] Although surgical resection is the only treatment that can offer any survival benefit, biliary cancer is initially asymptomatic and can become highly invasive and metastatic at advanced stages, making curative resection difficult. Consequently, the majority of biliary cancers become inoperable, and these patients require chemotherapy, leading to a higher demand for more effective individualized treatment. Recently, clinical trials of IDH1 and FGFR inhibitors for intrahepatic cholangiocarcinoma have shown that patients with an *IDH1* mutation and the *FGFR2* fusion gene have better prognoses, respectively.[2-4] However, such patients represent only a fraction of patients with biliary cancer, and most of the frequent driver mutations in biliary cancer, such as *KRAS* and *TP53* mutations, are untargetable.[5-7] Furthermore, driver mutations are detected in a site-specific manner in biliary cancers that arise from the biliary tract epithelium. Therefore, in parallel with the development of molecularly targeted therapy, novel diagnostic methods and drugs would be highly desirable.

The development of 3-dimensional culture systems has enabled us to culture patient-derived tumor cells as organoids (patient-derived organoids [PDOs]) under physiologically and pathologically more relevant conditions than would be the case for conventional cancer cell lines.[8,9] Many efforts have been made to apply PDOs for individualized diagnosis in various types of solid tumors, including esophageal, gastric, colorectal, liver, pancreatic, and biliary cancers.[10-18] Saito et al[18,19] reported the establishment of PDOs using surgically resected biliary cancer tissues. However, the establishment of PDOs without surgically resected tissues would be more beneficial for most patients with biliary cancer because most of such cancers are inoperable. In this study, we reasoned that bile, which is routinely collected for cytology using endoscopic retrograde cholangiopancreatography (ERCP), could be used as a material for the establishment of PDOs from individual patients with biliary cancers. For this purpose, we attempted to establish and characterize bile-derived organoids (BDOs) from 68 patients, including 28 inoperable cases. Furthermore, we proposed methods for obtaining BDOs with a high proportion of cancer cells.

Materials and Methods

Establishment of Bile-Derived Organoids From Patients With Biliary Cancer

This study was approved by the Ethics Committee of Oita University Hospital (approval number: 1808). All patients provided written informed consent. In this study, all patients were diagnosed as having biliary cancer using computed tomography imaging and underwent ERCP for therapeutic and/or diagnostic purposes, with biliary cytology. We obtained bile samples using ERCP from patients with biliary cancer at Oita University Hospital and its associated hospitals. For the establishment of organoids, 5 to 20 mL of bile samples was stored at 4 °C and processed for organoid culture within 1 hour after collection. They were washed with a cold wash medium (Dulbecco modified Eagle medium [Thermo Fisher Scientific] supplemented with 1% fetal bovine serum and 1% penicillin/streptomycin), filtered through a 70-μm EASYstrainer (Greiner Bio-One), and centrifuged at 300 g for 5 minutes. The cell pellets were then washed twice by resuspending in a wash medium and centrifuged at 300 g for 5 minutes. The thoroughly washed cell pellets were suspended in Matrigel (#356231; Corning) and seeded in 12-well suspension plates (Greiner Bio-One). Once the Matrigel had solidified, the mixtures of cells and Matrigel was overlaid with an organoid culture medium composed of advanced Dulbecco modified Eagle medium/ F12 (Thermo Fisher Scientific) supplemented with 1% penicillin/ streptomycin, 1% glutamax, 10mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 1% N2 supplement, 2% B27 supplement (all from Thermo Fisher Scientific), 1.25mM N-acetylcysteine, 10nM gastrin, 10mM nicotinamide (all from Sigma-Aldrich), 0.2% primocin (InvivoGen), 50-ng/mL EGF (PeproTech), 10% Rspo-1−conditioned medium (Trevigen), 5μM A83-01 (Tocris Bioscience), 10μM forskolin (Tocris Bioscience), and 10μM Y-27632 (Sigma-Aldrich). The components of the organoid culture medium were chosen by referring to a previous study by Saito et al.[19] The medium was changed every 3 to 4 days. For passages, organoids were collected in new 15-mL tubes and digested with 2 mL of TrypLE (Thermo Fisher Scientific) at 37 °C for up to 5 minutes. They were then suspended in 10 mL of the wash medium and centrifuged at 300 g for 5 minutes at 4 °C. After removal of the supernatant, the pellet was reseeded as described above at a passaging ratio of 1:2 to 1:3, depending on the respective growth rates. Successful establishment of BDOs was defined as those cultured for >1 month and passaged twice or more. Information about the patients and the resulting organoids is summarized in Supplementary Table S1. After completing all experiments in this study, the BDOs were stocked at passages 2 to 9.

Xenograft Model Using Bile-Derived Organoids From Patients With Biliary Cancer

Experiments that used animals were approved by the animal ethics committee at Oita University (approval number: 220802). For xenograft models, a total of 5×10^5 to 10×10^5 BDOs were suspended in a mixture of 200 μL of an organoid medium and 50 μL of Matrigel and injected subcutaneously into the flank of male NOD scid gamma mice aged 6 to 8 weeks old (Charles River Laboratories Japan Inc). After 3 to 6 months, the mice were sacrificed, and the tumors were excised and used for further establishment of xenograft tumor−derived organoids (XDOs) and for immunohistochemistry.

Establishment of Xenograft Tumor−Derived Organoids

XDOs were established as described previously by our group, with some modifications.[10,14] Xenograft tumors were minced into 0.5-mm^3 fragments using scalpels and washed 3 times with a cold wash medium. The tumor pieces were then incubated in a digestive solution containing 0.125-mg/mL collagenase XI (Sigma-Aldrich) and 0.125-mg/mL dispase II (Thermo Fisher Scientific) in the wash medium at 37 °C for 30 minutes with shaking. After the remaining tumor fragments had settled under gravity, the resulting supernatant was transferred to a new 15-mL tube and

centrifuged at 300 g for 5 minutes at 4 °C. The collected tumor cells were embedded in a 1:2 mixture of the wash medium and Matrigel and placed as approximately 20-μL droplets in multiwell plates. An organoid culture medium consisting of the above-mentioned components was then added. The medium was changed every 3 to 4 days, and the culture was passaged as described above.

Preparation of Formalin-Fixed, Paraffin-Embedded Blocks

For the preparation of formalin-fixed, paraffin-embedded (FFPE) blocks from mouse xenografts, tissues were fixed overnight with 10% formalin (Wako FUJIFILM, Osaka, Japan) and embedded in paraffin (Pathoprep 580, Wako FUJIFILM) according to standard histologic procedures. For FFPE blocks from BDOs or XDOs, organoids were embedded in paraffin as described previously.[20] Briefly, organoids were fixed in 4% paraformaldehyde (Wako FUJIFILM) for 1 hour and embedded in gels composed of 2% Agar (INA AGAR BA-30, INA Food Industry, Ina, Japan) and 2.5% gelatin (Merck, Darmstadt, Germany). The organoid-embedded gels were then embedded in paraffin according to standard histologic procedures.

Immunohistochemistry

Immunohistochemistry was performed as described previously.[14] Briefly, paraffin sections were cut from the FFPE blocks at a thickness of 2 μm, deparaffinized with xylene, and rehydrated with ethanol according to standard protocols. For antigen retrieval, the sections were immersed in 10mM sodium citrate buffer (pH 6.0) (Iatron), autoclaved at 120 °C for 10 minutes, and cooled to room temperature (RT). Then, the sections were treated with 3% (vol/vol) hydrogen peroxide (H_2O_2) for 5 minutes at RT to inactivate endogenous peroxidase, blocked with 10% (vol/vol) rabbit serum (Nichirei) for 30 minutes at RT, and incubated overnight at 4 °C with antibodies against CK7 (Clone OV-TL 12/30, 1:100, M7018; DAKO), Hep Par 1 (Clone OCH1E5, 1:200, sc-58693; Santa Cruz Biotechnology), Ki67 (Clone MIB-1, 1:200, M7240; DAKO), and p53 (DO-7, 1:800; Leica Biosystems) diluted with DAKO Antibody Diluent (DAKO). The sections were then washed 3 times for 5 minutes with 1× phosphate-buffered saline (PBS) and incubated with biotinylated rabbit antimouse immunoglobulin (Ig)G, IgA, and IgM antibodies (Nichirei) for 30 minutes. After washing 3 times for 5 minutes with 1× PBS, the sections were incubated with a solution of avidin-conjugated Western horseradish peroxidase (Vectastain Elite ABC kit; Vector Laboratories Inc) for 20 minutes and again washed 3 times with 1× PBS for 5 minutes. Peroxidase activity was detected using an H_2O_2/diaminobenzidine substrate solution, and the sections were counterstained with hematoxylin before dehydration and mounting.

Genomic DNA Extraction and Mutation Analysis of KRAS and TP53 Genes

Genomic DNA was extracted from BDOs and XDOs using the High Pure PCR Template Preparation Kit (Roche) in accordance with the manufacturer's instructions. TaKaRa Ex Tag (Takara Bio) and the BigDye Terminator, version 3.1, cycle sequencing kit (Applied Biosystem) were used for PCR and sequencing, respectively, in accordance with the manufacturers' instructions.

Organoid Viability Assay Employing Nutlin-3a Treatment

The organoid viability assay was performed as described previously, with slight modifications.[10,14] Before the drug sensitivity assay, organoids were collected, counted, centrifuged, and resuspended in an organoid medium containing 10% Matrigel and Nutlin-3a (Med Chem Express) at concentrations of 0, 0.1, 1, and 10 μM. Small organoids at a density of 1000 organoids per 100 μL of the medium were then plated in each well of a 96-well repellent plate (Greiner Bio-One) and cultured at 37 °C for 6 days. After treatment, the Cell Titer-Glo 3D Cell Viability Assay Kit (Promega) was used to determine the viability of the organoids in accordance with the manufacturer's instructions.

Statistical Analysis

The 2-sided Student t test was used to analyze the differences in experiments using Nutlin-3a and immunohistochemistry. Data were reported as mean values ± SD of triplicate determinations. Differences at $P <.05$ were considered to be statistically significant.

Results

Establishment of Bile-Derived Organoids From Patients With Biliary Cancer

We attempted to establish BDOs from 2 patients without caner and 68 patients with biliary cancer who underwent ERCP (Fig. 1A). According to our definition of the success of the establishment of BDOs (see Materials and Methods), 2 of the 2 (100%) and 60 of the 68 (88.2%) BDOs from patients without cancer and those with biliary cancer, respectively, were generated successfully. All 8 failures were due to bacterial contamination associated with concurrent cholangitis. Of the 60 BDOs successfully established from patients with biliary cancer, 5 were from intrahepatic cholangiocarcinoma, 19 were from hilar cholangiocarcinoma, 30 were from extrahepatic cholangiocarcinoma, 1 was from gallbladder cancer, and 5 were from carcinoma of the papilla of Vater (Supplementary Table S1).

To confirm whether the BDOs established from the patients with biliary cancer indeed contained cancer cells, they were subjected to downstream investigations, including examination of organoid morphology, sequencing of KRAS and TP53, and the use of a xenograft model (Fig. 1A). First, we observed the morphologic characteristics of each BDO using stereomicroscopy. Both of the 2 BDOs from the patients without biliary cancer showed a regular cystic shape with a thin cyst wall (Supplementary Fig. S1). In contrast, the BDOs from the patients with biliary cancer exhibited a diverse range of morphology, such as a regular cystic shape (Fig. 1Bi), a solid mass (Fig. 1Bii), a multicystic form (Fig. 1Biii), and an irregular cyst with a markedly thick cyst wall (Fig. 1Biv). Subsequent histologic analysis revealed that the BDOs from the patients without biliary cancer formed a cyst composed of a single layer of biliary duct epithelium, without nuclear atypia (Fig. 1Ci). On the other hand, the BDOs from the patients with biliary cancer presented not only histologic features similar to those of BDOs from the patients without cancer but also a solid growth pattern (Fig. 1Cii), cribriform pattern (Fig. 1Ciii), and cystic structure with multilayered cells (Fig. 1Civ). Interestingly, these latter organoids harbored both structural and cellular atypia, such as loss of cell polarity as well as nonuniform nuclear size and shape. The BDOs were positive for the cholangiocyte marker CK7 and negative for the hepatocyte marker
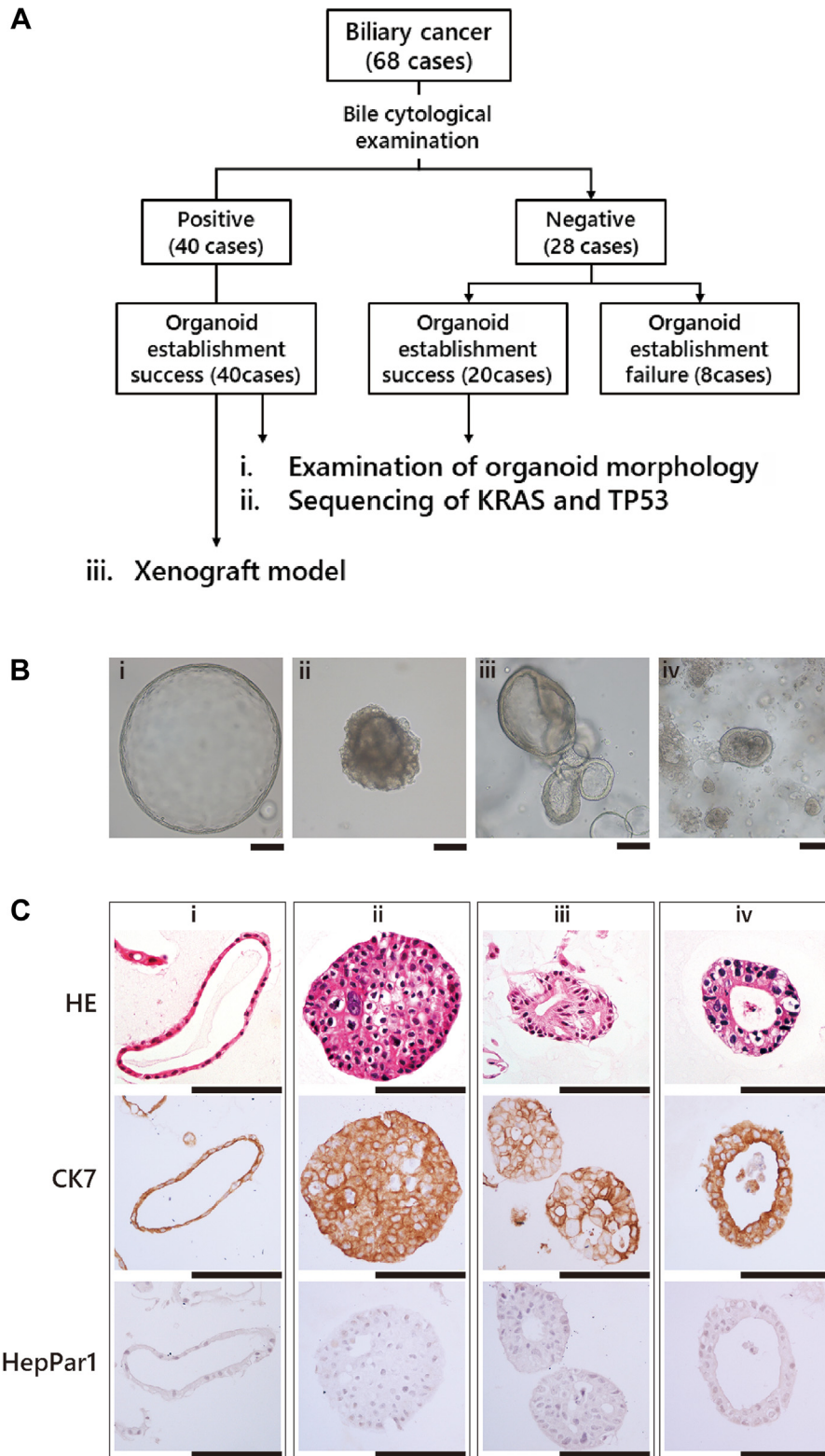
Figure 1.
Establishment of bile-derived organoids (BDOs) from patients with biliary cancer. (A) Study overview. The success rate for the establishment of BDOs from patients with biliary cancer was 88.2% (60 of 68 bile samples). Every BDO was analyzed using morphologic examination and sequencing of the cancer-related genes *KRAS* and *TP53*. In addition, in vivo tumorigenicity was investigated using 40 BDOs derived from biliary cancers diagnosed as positive using bile cytologic examination. (B) Microscopic images of BDOs from patients with biliary cancer exhibited diverse morphologic features such as (i) a regular cystic shape, (ii) a solid mass, (iii) a multicystic form, and (iv) an irregular cyst with a markedly thick cyst wall. (Bi-iv) Representative images of C (cystic), S (Solid), MC (multicystic form) and TC (irregular cyst with marked thick cyst wall), respectively, in Supplementary Table S1. (C) Histologic analysis of hematoxylin and eosin−stained slice sections of BDOs. The BDOs from patients without biliary cancer formed (i) a cyst composed of a single layer of biliary duct epithelium, without displaying nuclear atypia. The BDOs from patients with biliary cancer showed (ii) a solid growth pattern, (iii) a cribriform pattern, and (iv) a cystic structure with multilayered cells composed of both structural and cellular atypia. The BDOs were positive for the cholangiocyte marker CK7 and negative for the hepatocyte marker Hep Par 1. (B, C) Scale bar = 100 μm. HE, hematoxylin and eosin.

Hep Par 1, confirming that they were derived from cholangiocytes (Fig. 1C). These findings strongly suggested that the BDOs from the patients with biliary cancer contained organoids derived from both normal bile duct epithelium and bile duct cancer cells with heterogenous states of differentiation.

Second, we investigated the mutation status of the cancer-related genes KRAS and TP53. Among the 40 BDOs derived from bile cytology−positive patients (Fig. 1A), KRAS and TP53 mutations were detected in 6 (15%) and 22 (55%) patients, respectively, consistent with data from previous studies in which these mutations were detected in 10% to 20% and 40% to 60% of patients with biliary cancer, respectively.[21,22] In contrast, in 20 BDOs from bile cytology−negative patients, these mutations were detected in 0 (0%) and 2 (10.0%) patients, respectively. As expected, both of the 2 BDOs from the patients without cancer harbored no mutations in these genes.

Third, to investigate their tumorigenicity, 40 BDOs from bile cytology−positive patients were xenografted subcutaneously into immunodeficient mice. Sixteen (40%) of them formed obvious subcutaneous tumors 2 to 6 months after xeno-transplantation. Macroscopically, these tumors appeared as a solid mass with a granular surface and bleeding (Fig. 2A), multicystic form filled with serous fluid (Fig. 2B), or white solid mass with a smooth surface (Fig. 2C, D). Microscopic observation revealed that xenografted tumors from BDOs consisted of papillary (Fig. 2E) and tubular (Fig. 2F) structured epithelia with abundant stroma. These epithelial cells had a hyperchromatic nucleus, with a high nucleus-to-cytoplasm ratio, and exhibited atypical mitosis, indicating oncogenic transformation. Interestingly, the histologic features of the xenograft tumors derived from BDOs were quite similar to those of corresponding surgically resected tumors in each case (Fig. 2E, F). Together with the data on the frequencies of mutations in KRAS and TP53, these results suggested that a substantial proportion of BDOs from the patients with biliary cancer in this study indeed contained tumor cells and that some of the BDOs retained tumorigenicity in vivo.

Enrichment of Cancer Cell−Derived Organoids in Bile-Derived Organoids

Although our results suggested the presence of cancer cells in our BDOs, we were unable to exclude the possibility of contamination with normal biliary epithelial cells. Because such contamination hampers the application of BDOs as preclinical tumor models, enrichment of cancer cells in each BDO is required. Previous reports have also pointed out contamination with normal cells during the establishment of tumor organoids from various types of solid cancers, including biliary cancer.[19,23-27] In these previous studies, normal cell−derived organoids were distinguished from cancer cell−derived organoids based on their morphologic features such as cystic balloon-like morphology for normal organoids and solid morphology for cancer organoids. Indeed, the BDOs derived from the patients without cancer in our present study had a regular cystic shape with a thin cyst wall (Supplementary Fig. S1). However, we also found that some cystic-dominant BDOs harbored cancer-related mutations in TP53 and/or KRAS genes (B5 and B23 in Supplementary Table S1 and Supplementary Fig. S2), suggesting the presence of cancer cells. Furthermore, even cystic BDOs without mutations in either TP53 or KRAS (B22 and B41 in Supplementary Table S1 and Supplementary Fig. S2) exhibited in vivo tumorigenicity. These results suggested that a cystic structure is not definitive evidence

of normal biliary epithelial cell−derived origin in our BDOs. Therefore, we attempted strategies other than morphology-based selection to enrich cancer cell−derived organoids.

First, we found that a mutant allele in TP53 was accumulated in some BDOs during serial passage (Fig. 3). For example, B25 showed the oncogenic CGG>TGG mutation of TP53 (R248W) at a negligible level in the culture obtained after the first passage but at significant and dominant levels after the second and third passages, respectively (Fig. 3A). Similarly, B32 also showed increased levels of the oncogenic TP53 mutation in the first passaged culture compared with the primary culture (Fig. 3B). These results suggest that a subset of BDOs derived from patients with biliary cancer is contaminated at outset by normal biliary epithelial cell−derived organoids but that the proportion of biliary cancer cell−derived organoids increases with passaging, whereas that of normal biliary epithelial cell−derived organoids decreases. In fact, under our culture conditions, we found that the growth rate of noncancerous BDOs decreased at approximately third to fifth passages (data not shown). These observations support the idea that cancer cell−derived organoids can be enriched by serial passages in a subset of BDOs.

Second, we hypothesized that tumors formed by transplantation of BDOs are composed primarily of cancer cells even though their matched BDOs are contaminated with organoids derived from normal cells, ie, transplantation of BDOs into mice makes it possible to enrich cancer cell−derived organoids in BDOs. To test this hypothesis, we re-established organoids from xenografts (XDOs) and compared their morphologic and pathologic features with those of corresponding BDOs. Most of the XDOs showed more solid and compact morphology than the corresponding BDOs (Fig. 4A and Supplementary Fig. S3). Furthermore, the percentages of both Ki67- and TP53-positive cells increased XDOs from B35 and B41 (Fig. 4B, C and Supplementary Fig. S4). These results suggested that transplantation enriched the proportion of cancer organoids with solid morphology and tumorigenicity.

Finally, we focused on the TP53 mutation, which is detected most frequently in biliary cancers, and determined whether Nutlin-3a, an inhibitor of MDM2, could enrich only cancer organoids harboring this TP53 mutation. As shown in Figure 5A, treatment with Nutlin-3a at a concentration of 1 μM had no suppressive effects on cell proliferation in B41 XDOs harboring the TP53 mutation (R273H). On the other hand, the treatment markedly reduced the viability of normal cell−derived organoids containing wild-type TP53 (Fig. 5A). Based on this finding, we addressed whether TP53-mutated cancer cells could be enriched by Nutlin-3a treatment in BDOs contaminated with normal epithelial cells. After treatment with Nutlin-3a, guanine at position 818 of the wild-type TP53 sequence in B41 BDOs was replaced by adenine, resulting in R273H-mutant TP53 (Fig. 5B). This enrichment of TP53-mutated cells by Nutlin-3a treatment was further confirmed using immunohistochemistry, which showed accumulation of TP53 in almost all cells (Fig. 5C, D). As another example of this enrichment, a minor fraction of TP53-mutant cancer cells (R273C) in B35 BDOs was successfully enriched by Nutlin-3a treatment, as demonstrated using sequencing and immunohistochemical analyses (Supplementary Fig. S5). These results suggested that the addition of Nutlin-3a can efficiently enrich cancer cell organoids derived from patients with TP53 mutation−positive biliary cancer. Interestingly, in both the B41 and B35 BDOs, the patterns of nucleotide replacement in the TP53 sequence were consistent between the 2 enrichment procedures, ie, the xenograft model and Nutlin-3a treatment (Fig. 5B and Supplementary Fig. S5, left panel), suggesting that the TP53
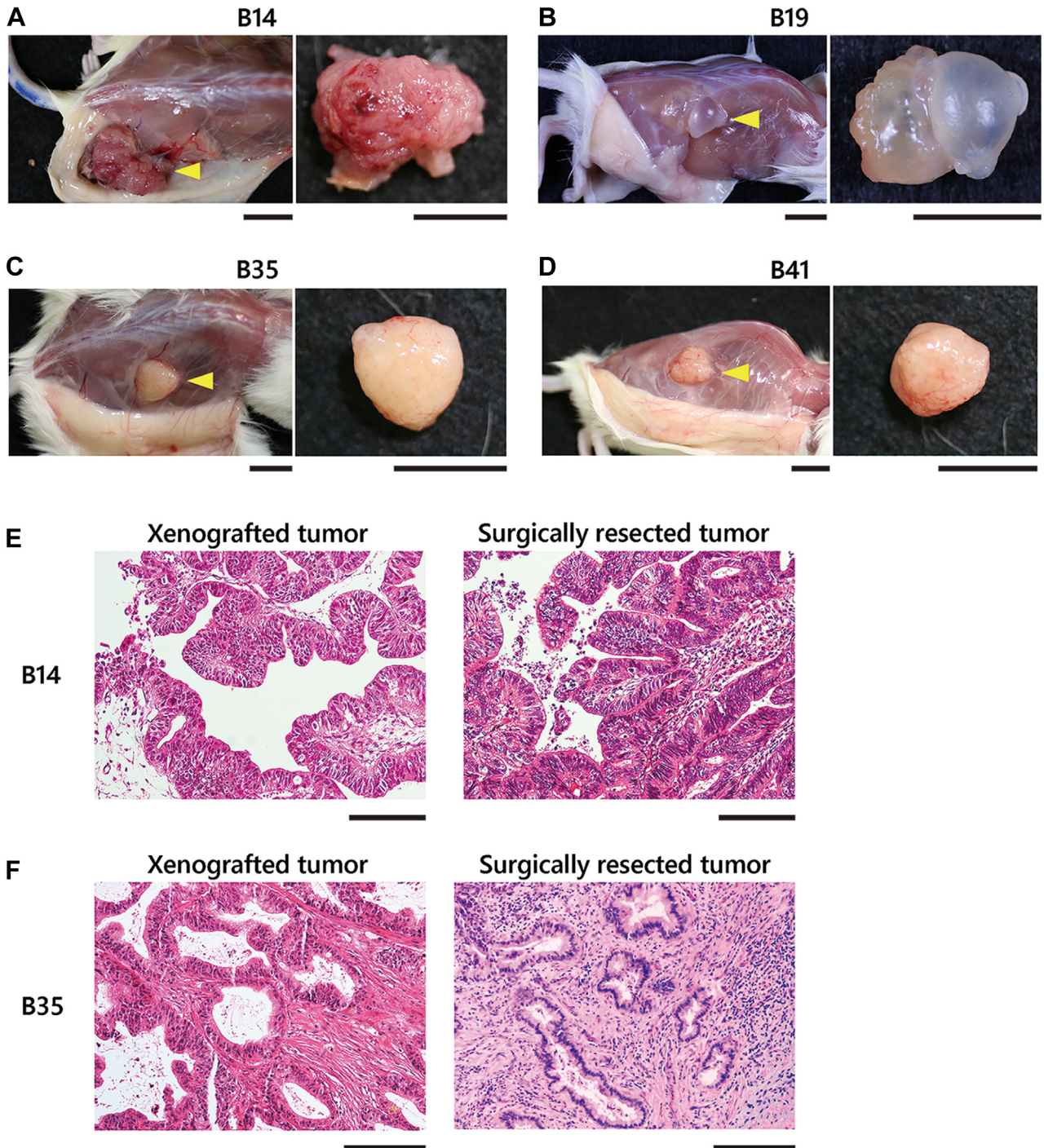
Figure 2.
Xenograft model of biliary cancer involving bile-derived organoids. (A-D) Macroscopic images of xenograft tumors showed (A) a solid mass with a granular surface and bleeding, (B) the formation of multicysts filled with serous fluid, and (C, D) a white solid mass with a smooth surface. Yellow arrowheads indicate subcutaneous tumors. Scale bar = 1 cm. (E, F) Pathologic features of xenografted tumors (E) B14 and (F) B35, similar to those of the corresponding surgically resected tumors. Scale bar = 100 μm.

mutation had not been artificially introduced during our enrichment methods but derived from minor fractions of cancer cells in these BDOs.

Together, these results suggest that cancer cell−derived organoids in BDOs can be enriched using the following 3 methods: repeat passages, re-establishment of organoids from xenografts, and addition of Nutlin-3a (Fig. 6).

Discussion

In this study, we successfully established BDOs from patients with biliary cancer. Tissue-derived organoids (TDOs) from a surgically resected specimen of biliary cancer have already been reported.[19] In comparison with conventional TDOs, these BDOs have several advantages. First, the success rate of BDO establishment is
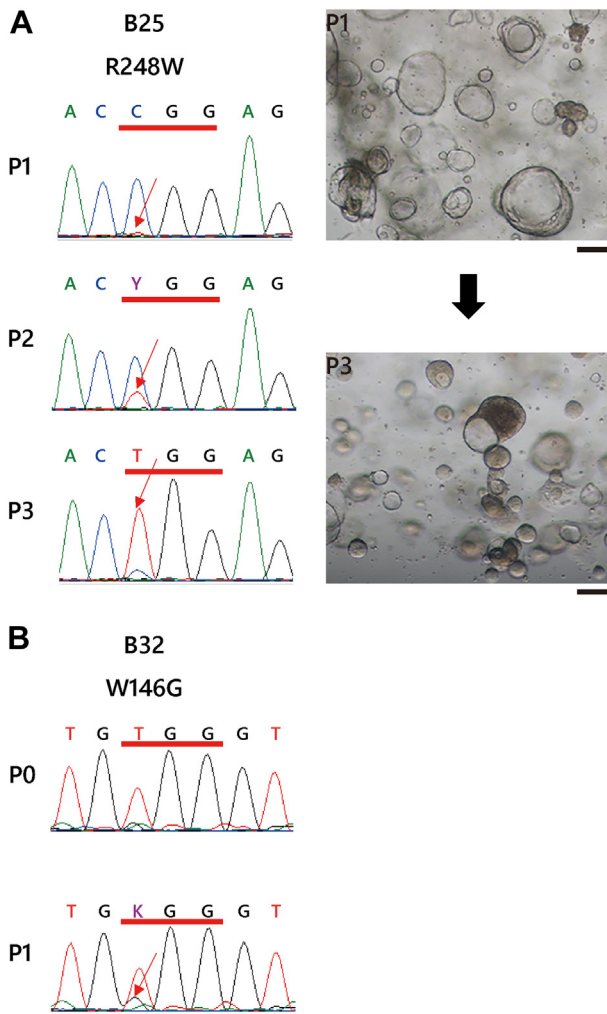
Figure 3.
Sequence analysis of *TP53* gene mutations at each passage. (A) The level of the oncogenic CGG>TGG mutation of *TP53* (R248W) increased during passage. Microscopic images of B25 in the third passaged culture (lower right panel) showed fewer cystic organoids and more solid and compact organoids compared with those in the first passaged culture (upper right panel). Scale bar = 100 μm. (B) B32 also showed increased levels of the oncogenic TGG>GGG mutation of *TP53* (W146G) in the first passaged culture compared with those in the primary culture. Red arrows and red underbars indicate the mutation sites and the associated changes in codons, respectively.

higher than that of TDO establishment (88% vs 33%, respectively).[19] This difference can be explained in terms of the quality and purity of resected tissues. Resected tissues may be damaged by long-term ischemic stress during surgical operations, and such specimens contain abundant stromal components that disturb the proliferation of cancer cells in 3-dimensional cultures.[19] As a source of BDOs, we used fresh bile in sufficient volumes immediately after collection. This predominantly contains epithelial cells and few nonepithelial components other than blood cells. Second, BDOs can be established from almost all patients with biliary cancers. In contrast to TDOs, which can be established only from patients undergoing surgery, BDOs can be established from any patients who undergo ERCP. Currently, bile is routinely collected from patients in whom biliary cancers are clinically suspected because bile cytology is essential for definitive diagnosis of biliary cancers. In addition, personalized medicine based on genetic analysis of individual cancer tissues is currently being promoted. In this context, the possibility of establishing cancer organoids from patients with inoperable cancers, for whom

therapeutic options other than surgery are limited, would be significantly beneficial. Third, the procedures for the establishment of BDOs have extremely low invasiveness for patients because they involve the use of bile remaining after sampling for cytological examination or drainage. Fourth, it would be possible to establish a series of BDOs that represents the whole course of tumor progression from individual patients. For example, by collecting bile and establishing BDOs before and after treatment, it would be possible to compare the characteristics of BDOs before treatment with those of BDOs that acquire resistance to treatment. This would be useful for devising therapeutic strategies appropriate to the disease stage in individual patients and for helping to clarify the molecular mechanisms that underlie resistance to therapy, assisting the development of novel therapeutic strategies for targeting cancer resistance.

Furthermore, we proposed methods for avoiding contamination with noncancerous organoids, which has been a major issue while establishing PDOs from various solid cancers.[19,23-27] The fundamental reason for such contamination is that the cancer tissues used for the establishment of PDOs usually contain normal epithelial cells and cancer cells. Moreover, efficient expansion of normal epithelium−derived cells is unavoidable during the establishment of organoids because the main components of the organoid medium are optimized to maintain adult stem cells[8,28] by supplying their niche factors such as Wnt, R-spondin, and EGF. To minimize such contamination and establish PDOs with high tumor cell purity, previous studies employed approaches suited to specific cancer types.[13,16,29-33] For the establishment of PDOs from gastric cancer, noncancer and cancer organoids have been morphologically characterized and sorted based on manual selection.[13] To establish PDOs from colorectal and pancreatic cancers, culture media lacking a single niche factor or a combination of factors have been used to enrich organoids with niche-independent growth facilitated by cancer type-specific mutations, such as *APC* and *KRAS* mutations in colorectal and pancreatic cancers, respectively.[30-33] Initially, we attempted to sort cancer organoids from BDOs based on morphologic features because possible contamination with normal epithelium−derived organoids was observed in BDOs. However, even among BDOs dominated by cystic organoids, which were typically derived from normal epithelium,[19,34-36] some harbored *TP53* and/or *KRAS* oncogenic mutations, and others showed in vivo tumorigenicity, suggesting that cystic morphology cannot be regarded as a definitive feature of organoids derived from normal cells. As an alternative to morphology-based sorting, we proposed the following 3 methods for enriching cancer organoids in BDOs by monitoring the proportion of oncogenic mutations in *TP53* as an indicator of enrichment: repeat passages, re-establishment of XDOs, and modification of culture conditions (Fig. 6). Among these 3 methods, the most reliable method for enrichment might be the establishment of XDOs. The XDOs established in this study, a total of 9 lines, could be potentially useful and valuable models of high-grade biliary cancers because some of them were derived from highly malignant and inoperable cancers. However, this method is time consuming and not practicable for individualized diagnosis through BDO drug response feedback. Furthermore, this method cannot be used for BDOs that are not transplantable into immunodeficient mice. The enrichment method involving repeated passages might also have only limited application because it is not possible to grasp accurately the degree to which cancer organoids are enriched by each passage. Compared with the 2 abovementioned methods, modification of the organoid medium according to the oncogenic mutation status of individual BDOs would be effective for the enrichment of cancer organoids and
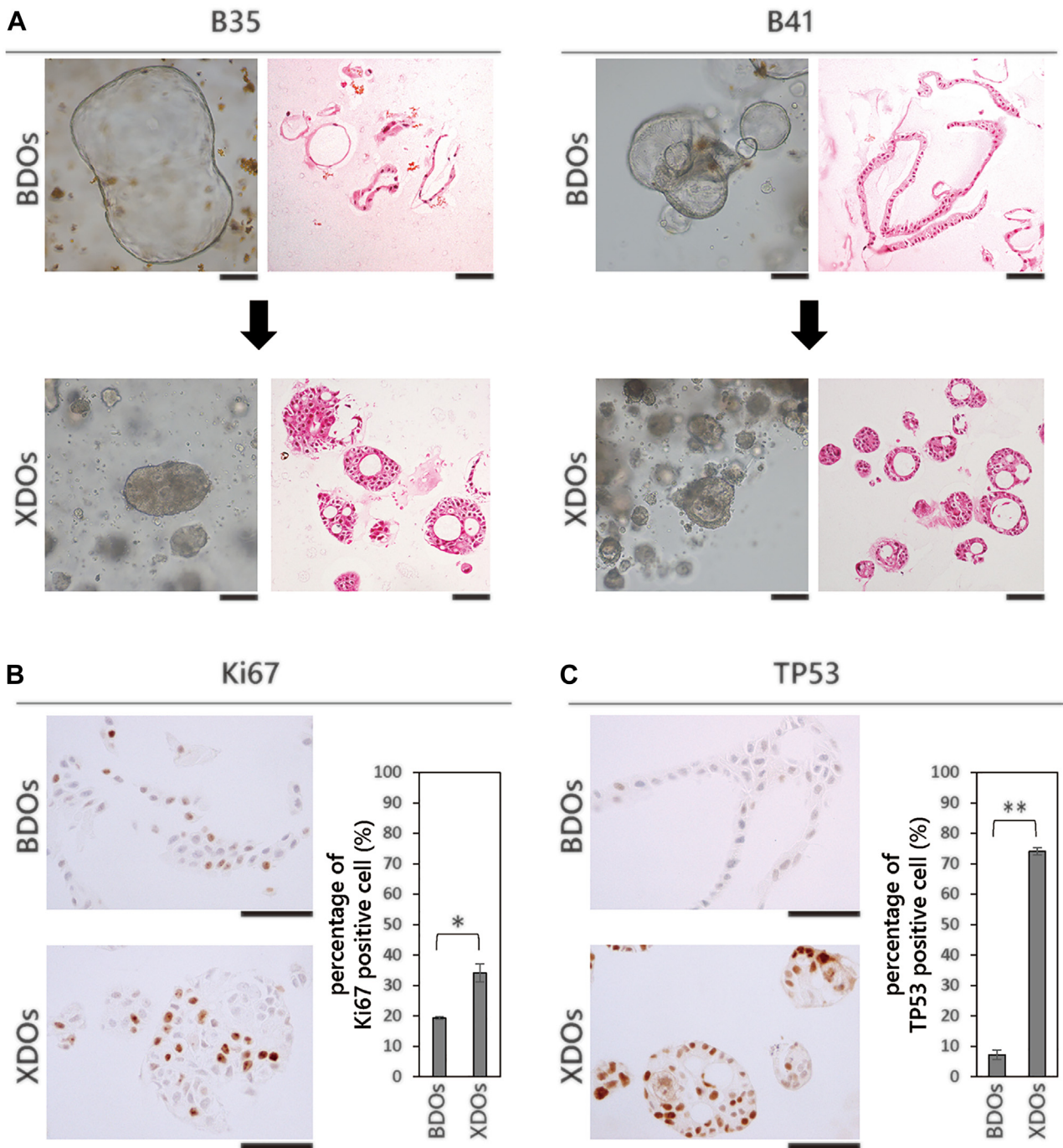
Figure 4.
Establishment of xenograft-derived organoids (XDOs). (A) Microscopic images of bile-derived organoids (BDOs) and XDOs for B35 (left) and B41 (right). XDOs showed a more solid and compact morphology than the corresponding pretransplant BDOs. Scale bar = 100 μm. (B, C) Immunohistochemistry of BDOs and XDOs for B41. XDOs show increased (B) Ki67-positive and (C) TP53-positive cells as compared with BDOs. The percentages of (B) Ki67-positive and (C) TP53-positive cells are shown as mean values ± SD of triplicates. The differences were analyzed statistically using the Student *t* test (n = 3). *P < .01 and **P < .001. Scale bar = 50 μm. As another example, the immunohistochemical analysis of BDOs and XDOs for B35 is shown in Supplementary Fig. S4. BDOs, bile-derived organoid; XDOs, xenograft-derived organoid.

feasible for personalized medicine because the method actively eliminates organoids derived from normal cells. Although we focused only on the most frequent oncogenic mutation in biliary cancer, *TP53*, and added Nutlin-3a to the organoid medium to eliminate normal cell−derived organoids, we could expand our medium-based enrichment methods to other BDOs by identifying other oncogenic mutations or signaling pathways that render cancer cells independent of niche factors.

Despite potential contamination by normal organoids, BDOs are valuable as materials for individualized diagnosis involving gene panel testing, for which cancer tissues admixed with normal cells are generally used. Recently, Kamp et al[37] reported that biliary brush cytology samples could be used for gene panel testing. However, in terms of the following 2 points, BDOs may be more useful for predicting drug efficacy in comparison with the direct use of bile for gene panel testing. First, the amount of cancer
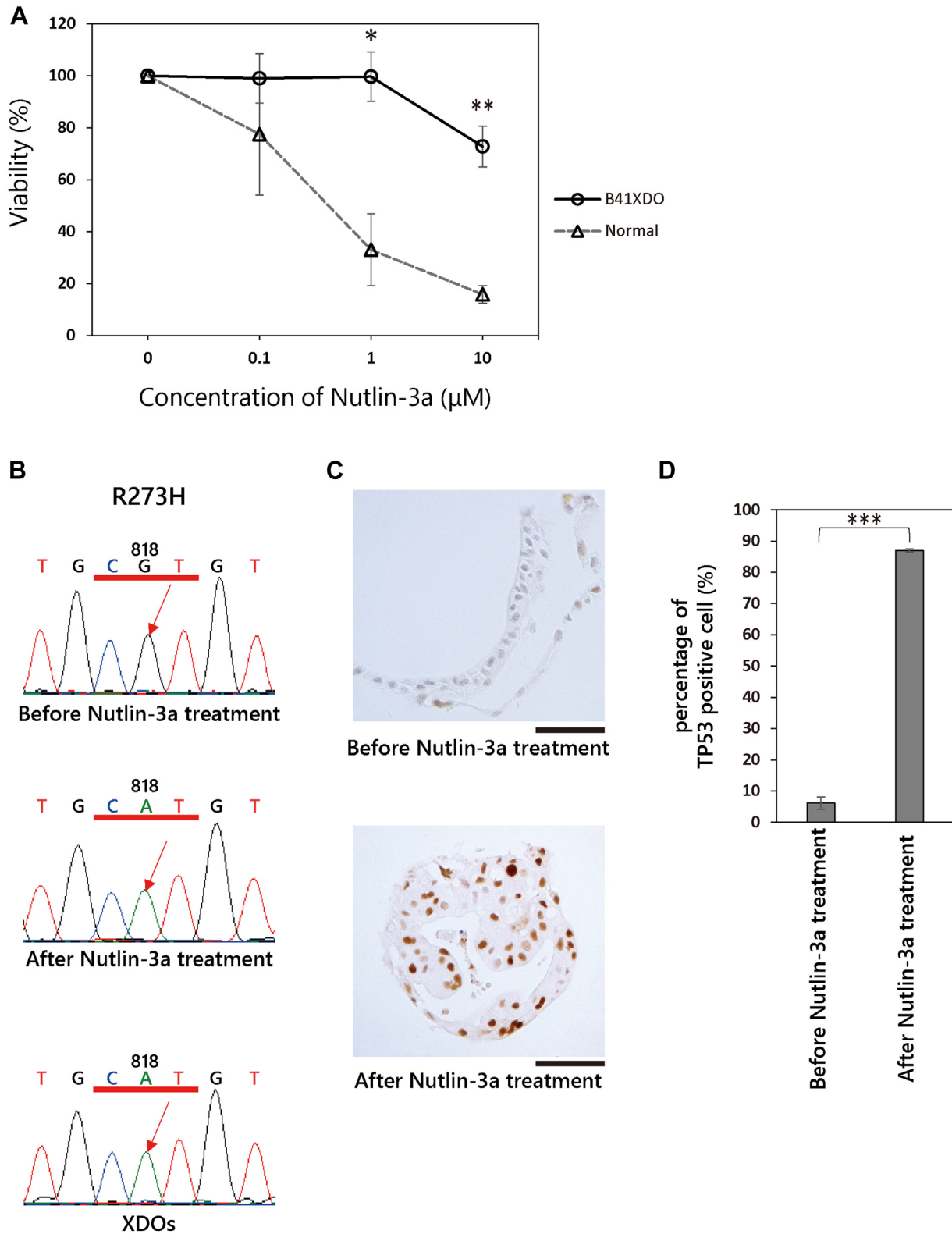
Figure 5.
Enrichment of *TP53*-mutated cancer cells in bile-derived organoids (BDOs) using Nutlin-3a treatment. (A) Effects of Nutlin-3a on cell viability in organoids with mutant *TP53* (B41 xenograft-derived organoid [XDO]) or without mutant *TP53* (normal). The cell viabilities at each point are shown as mean values ± SD of triplicates. The differences were analyzed statistically using the Student *t* test (n = 3). (B) Genomic sequences around R273 in B41 BDOs before and after Nutlin-3a treatment and B41 XDOs. The frequency of the oncogenic CGT>CAT mutation in *TP53* (R273H) is clearly shown to be increased in B41 BDOs after Nutlin-3a treatment, similarly to that in B41 XDOs. Red arrows and red underbars indicate the mutation sites and the associated changes in codons, respectively. (C) Immunohistochemistry of TP53 in B41 BDOs before and after Nutlin-3a treatment. Scale bar = 50 μm. (D) The percentages of TP53-positive cells are shown as mean values ± SD of triplicates. The differences were analyzed statistically using the Student *t* test (n = 3). *P < .01, **P < .001, and ***P < .0001. XDO, xenograft-derived organoid.
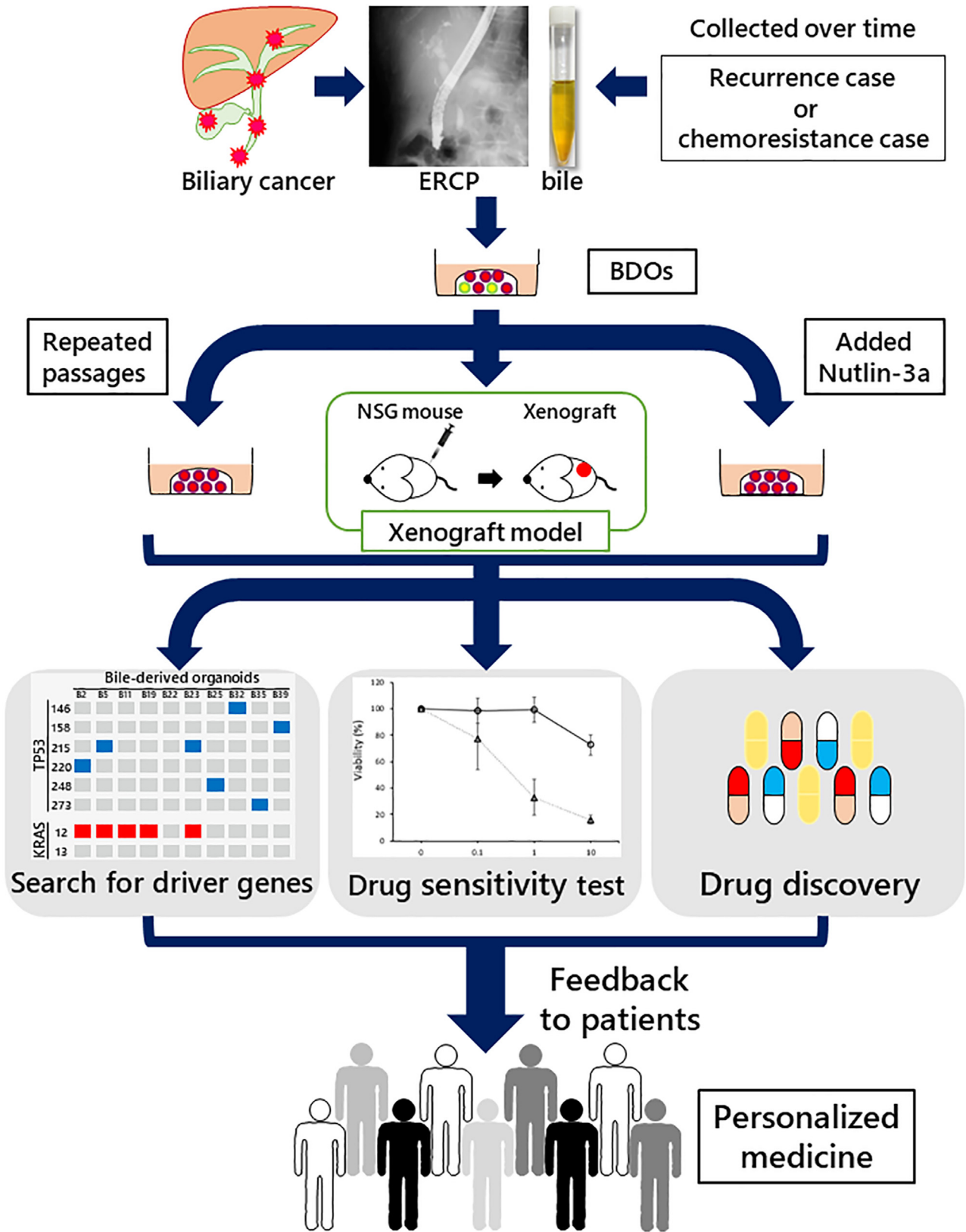
Figure 6.
The future outlook for personalized medicine using bile-derived organoids (BDOs). For increasing the content of cancer cells in BDOs derived from patients with biliary cancer, it would seem feasible to increase the number of passages, production of xenografts, and addition of Nutlin-3a to enrich cancer cell organoids while eliminating contamination with normal biliary epithelial cells as much as possible. Furthermore, BDOs could be used as a material for genetic panel testing and drug sensitivity testing, allowing personalized feedback to individual patients as well as contributing to drug discovery as a biobank for biliary cancers, including many inoperable cases. ERCP, endoscopic retrograde cholangiopancreatography; NSG, NOD scid gamma.

cells in bile varies from patient to patient, and fewer tumor cells in bile could potentially make gene panel testing less accurate. In such a situation, the amplification of cancer cells by organoid culture could increase the accuracy of diagnosis. Second, BDOs could be used as functional materials to predict the efficacy of drugs by increasing the proportion of cancer cells in BDO cultures during gene panel testing, for which a period of 3 to 5 weeks is usually necessary to establish a diagnosis. For patients without any drug recommendations from gene panel testing, ie, without any targetable oncogenic mutations, BDOs could be used to predict the efficacy of cytotoxic drugs because gene panel testing is usually employed to suggest molecular targeting drugs based on oncogenic mutations. Furthermore, if patients are able to receive multiple drug recommendations from such testing, BDOs could also be used to select the most promising drug among those indicated by the drug sensitivity assay. Further studies are needed to verify the feasibility of this approach.

In the present study, we established 5 BDOs from bile obtained using ERCP for intrahepatic cholangiocarcinoma. The guidelines recommend diagnosis using liver biopsy for intrahepatic cholangiocarcinoma.[38] However, although liver biopsy has the advantage of diagnostic accuracy, it carries a risk of such serious complications. Therefore, ERCP could be an alternative option for intrahepatic cholangiocarcinoma when a patient is suspected of having a risk of biopsy-related complications; has obstructive jaundice, without evidence of a solid mass on imaging; has indications for surgery; or does not consent to liver biopsy diagnosis. In such cases, BDOs could be advantageous because bile is the only diagnostic material with a low risk. Furthermore, bile is also available in cases of intrahepatic cholangiocarcinoma with obstructive jaundice because therapeutic ERCP is frequently performed in such cases. All 5 patients with intrahepatic cholangiocarcinoma had obstructive jaundice and received ERCP treatment with their consent. From 1 of them with cytology-positive intrahepatic cholangiocarcinoma (B34), we successfully established a BDO, in which we detected the TP53 mutation as a characteristic of cancer. BDOs were also established from the other 4 patients with cytology-negative intrahepatic cholangiocarcinoma (B1, B12, B17, and B55); however, neither TP53 nor KRAS mutations were detected. Therefore, it remains unknown whether these BDOs contained cancer cells. In this study, because the number of intrahepatic cholangiocarcinoma–derived BDOs was small, further studies will be needed to validate the advantage of BDOs for patients with ERCP-treated intrahepatic cholangiocarcinoma. It might also be interesting to establish organoids from liver biopsies of intrahepatic cholangiocarcinoma to test their feasibility for clinical diagnosis and translational research.

It is unclear whether all the BDOs examined in this study had at least 1 organoid derived from bile duct cancer cells. In order to define cancer cells as those with genomic aberrations, whole-genome sequencing and array comparative genomic hybridization analysis using matched normal genomic DNA from the same patient are required. However, this was beyond the scope of the present study and was not performed. Of the 40 BDOs with positive bile cytology, 29 (72.5%; B2, B5, B11, B14, B16, B19, B20, B22, B23, B25, B28, B33, B34, B35, B36, B38, B39, B41, B49, B50, B51, B52, B54, B56, B58, B60, B61, and B62) had typical cancer characteristics, such as TP53 mutations, KRAS mutations, or in vivo tumorigenicity, strongly suggesting that these organoids contained cancer cells. Among the other 11 BDOs, 6 had typical morphologic features of cancer (multicystic organoids in B27 and B59 and irregular cysts with markedly thick walls in B40, B42, B43, and B63). However, these characteristics were less suggestive than genetic mutations or in vivo tumorigenicity. The other 5 patients (B6, B9, B24, B31, and B46) showed no TP53 mutation, KRAS mutation, or in vivo tumorigenicity. Still, they had cystic morphology similar to organoids derived from normal bile duct epithelium, suggesting that they did not contain cancer cells. However, we cannot rule out the possibility that these organoids contained cancer cells. On the other hand, the proportion of BDOs with mutations in either or both TP53 and KRAS was significantly lower in the 20 BDOs from cytology-negative cases than that in the 40 BDOs from cytology-positive cases (2/20 vs 23/40, Fisher exact test; $P = .00063$). Therefore, it is highly possible that some of the BDOs derived from cytology-negative cases might have had only organoids composed of normal epithelial cells, especially cytology-negative BDOs with cystic morphology, such as B8, B12, B13, B15, B17, B21, and B47. Overall, further studies using whole-genome sequencing and array comparative genomic hybridization analysis will be needed to clarify whether cystic organoids without either TP53 mutations or KRAS mutations, including B6, B9, B24, B31, B46, B8, B12, B13, B15, B17, B21, and B47, harbor any cancer cells.

In conclusion, we established, for the first time, BDOs, with a high success rate and reduced invasiveness, from patients with biliary cancers, including inoperable cases. BDOs could be a promising tool for translational application as a biliary cancer biobank that would include not only operable but also inoperable cases. Further characterization, such as the capability for immortalization and improvement of enrichment methods, would facilitate the application of BDOs to personalized medicine as a useful and safe model for individual patients based on their in vitro drug response.

Author Contributions

K.K., Y.T., and N.H. conceived the study and performed the formal analysis and investigation. K.K., Y.T., and N.H. prepared the original draft. Y.H., T.F., S.K., T.U., C.N., T.M., K.F., R.O., K.Mizukami, T.O., M.K., and K.Murakami reviewed and edited the manuscript. Y.T. and K.Murakami performed funding acquisition. K.O., M.Motomura, S.F., R.S., T.N., and Y.G. procured the resources. K.Murakami, M.Moriyama, and N.H. supervised the study. All authors have approved the final version of the paper.

Data Availability

The data that support the findings of this study are available from the corresponding authors upon reasonable request.

Declaration of Competing Interest

The authors declare no competing interests.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of Oita University Hospital (approval number: 1808). All patients provided written informed consent.

Supplementary Material

The online version contains supplementary material available at https://doi.org/10.1016/j.jaac.2022.12.026

References

1. Ouyang G, Liu Q, Wu Y, et al. The global, regional, and national burden of gallbladder and biliary tract cancer and its attributable risk factors in 195 countries and territories, 1990 to 2017: a systematic analysis for the Global Burden of Disease Study 2017. *Cancer.* 2021;127(13):2238−2250.
2. Mosele F, Remon J, Mateo J, et al. Recommendations for the use of next-generation sequencing (NGS) for patients with metastatic cancers: a report from the ESMO Precision Medicine Working Group. *Ann Oncol.* 2020;31(11):1491−1505.
3. Abou-Alfa GK, Macarulla T, Javle MM, et al. Ivosidenib in *IDH1*-mutant, chemotherapy-refractory cholangiocarcinoma (ClarIDHy): a multicentre, randomised, double-blind, placebo-controlled, phase 3 study. *Lancet Oncol.* 2020;21(6):796−807.
4. Abou-Alfa GK, Sahai V, Hollebecque A, et al. Pemigatinib for previously treated, locally advanced or metastatic cholangiocarcinoma: a multicentre, open-label, phase 2 study. *Lancet Oncol.* 2020;21(5):671−684.
5. Nakamura H, Arai Y, Totoki Y, et al. Genomic spectra of biliary tract cancer. *Nat Genet.* 2015;47(9):1003−1010.
6. Zou S, Li J, Zhou H, et al. Mutational landscape of intrahepatic cholangiocarcinoma. *Nat Commun.* 2014;5(1):5696.
7. Simbolo M, Fassan M, Ruzzenente A, et al. Multigene mutational profiling of cholangiocarcinomas identifies actionable molecular subgroups. *Oncotarget.* 2014;5(9):2839−2852.
8. Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology.* 2011;141(5):1762−1772.
9. Sachs N, Clevers H. Organoid cultures for the analysis of cancer phenotypes. *Curr Opin Genet Dev.* 2014;24:68−73.
10. Tsukamoto Y, Kurogi S, Shibata T, et al. Enhanced phosphorylation of c-Jun by cisplatin treatment as a potential predictive biomarker for cisplatin response in combination with patient-derived tumor organoids. *Lab Invest.* 2022;102:1355−1366.
11. Kijima T, Nakagawa H, Shimonosono M, et al. Three-dimensional organoids reveal therapy resistance of esophageal and oropharyngeal squamous cell carcinoma cells. *Cell Mol Gastroenterol Hepatol.* 2019;7(1):73−91.
12. Vlachogiannis G, Hedayat S, Vatsiou A, et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science.* 2018;359(6378):920−926.
13. Yan HH, Siu HC, Law S, et al. A comprehensive human gastric cancer organoid biobank captures tumor subtype heterogeneity and enables therapeutic screening. *Cell Stem Cell.* 2018;23(6):882−897.
14. Hirashita Y, Tsukamoto Y, Kudo Y, et al. Early response in phosphorylation of ribosomal protein S6 is associated with sensitivity to trametinib in colorectal cancer cells. *Lab Invest.* 2021;101(8):1036−1047.
15. Van de Wetering M, Francies HE, Francis JM, et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell.* 2015;161(4):933−945.
16. Broutier L, Mastrogiovanni G, Verstegen MM, et al. Human primary liver cancer-derived organoid cultures for disease modeling and drug screening. *Nat Med.* 2017;23(12):1424−1435.
17. Huang L, Holtzinger A, Jagan I, et al. Ductal pancreatic cancer modeling and drug screening using human pluripotent stem cell- and patient-derived tumor organoids. *Nat Med.* 2015;21(11):1364−1371.
18. Saito Y. Establishment of an organoid bank of biliary tract and pancreatic cancers and its application for personalized therapy and future treatment. *J Gastroenterol Hepatol.* 2019;34(11):1906−1910.
19. Saito Y, Muramatsu T, Kanai Y, et al. Establishment of patient-derived organoids and drug screening for biliary tract carcinoma. *Cell Rep.* 2019;27(4):1265−1276.
20. Karakasheva TA, Kijima T, Shimonosono M, et al. Generation and characterization of patient-derived head and neck, oral, and esophageal cancer organoids. *Curr Protoc Stem Cell Biol.* 2020;53(1):e109.
21. Li M, Zhang Z, Li X, et al. Whole-exome and targeted gene sequencing of gallbladder carcinoma identifies recurrent mutations in the ErbB pathway. *Nat Genet.* 2014;46(8):872−876.
22. Silverman IM, Hollebecque A, Friboulet L, et al. Clinicogenomic analysis of *FGFR2*-rearranged cholangiocarcinoma identifies correlates of response and mechanisms of resistance to pemigatinib. *Cancer Discov.* 2021;11(2):326−339.
23. Dijkstra KK, Monkhorst K, Schipper LJ, et al. Challenges in establishing pure lung cancer organoids limit their utility for personalized medicine. *Cell Rep.* 2020;31(5), 107588.
24. Driehuis E, van Hoeck A, Moore K, et al. Pancreatic cancer organoids recapitulate disease and allow personalized drug screening. *Proc Natl Acad Sci U S A.* 2019;116(52):26580−26590. https://doi.org/10.1073/pnas.1911273116
25. Bartfeld S, Bayram T, van de Wetering M, et al. In vitro expansion of human gastric epithelial stem cells and their responses to bacterial infection. *Gastroenterology.* 2015;148(1):126−136.
26. Derouet MF, Allen J, Wilson GW, et al. Towards personalized induction therapy for esophageal adenocarcinoma: organoids derived from endoscopic biopsy recapitulate the pre-treatment tumor. *Sci Rep.* 2020;10(1), 14514.
27. Gao D, Vela I, Sboner A, et al. Organoid cultures derived from patients with advanced prostate cancer. *Cell.* 2014;159(1):176−187.
28. Xian W, Duleba M, Zhang Y, et al. The cellular origin of Barrett's esophagus and its stem cells. *Adv Exp Med Biol.* 2019;1123:55−69.
29. Boj SF, Hwang CI, Baker LA, et al. Organoid models of human and mouse ductal pancreatic cancer. *Cell.* 2015;160(1-2):324−338.
30. Crespo M, Vilar E, Tsai SY, et al. Colonic organoids derived from human induced pluripotent stem cells for modeling colorectal cancer and drug testing. *Nat Med.* 2017;23(7):878−884.
31. Fujii M, Shimokawa M, Date S, et al. A colorectal tumor organoid library demonstrates progressive loss of niche factor requirements during tumorigenesis. *Cell Stem Cell.* 2016;18(6):827−838.
32. Seino T, Kawasaki S, Shimokawa M, et al. Human pancreatic tumor organoids reveal loss of stem cell niche factor dependence during disease progression. *Cell Stem Cell.* 2018;22(3):454−467.
33. Matsuura T, Maru Y, Izumiya M, et al. Organoid-based ex vivo reconstitution of Kras-driven pancreatic ductal carcinogenesis. *Carcinogenesis.* 2020;41(4):490−501.
34. Roos FJ, Wu H, Willemse J, et al. Cholangiocyte organoids from human bile retain a local phenotype and can repopulate bile ducts in vitro. *Clin Transl Med.* 2021;11(12):e566.
35. Rimland CA, Tilson SG, Morell CM, et al. Regional differences in human biliary tissues and corresponding in vitro-derived organoids. *Hepatology.* 2021;73(1):247−267. https://doi.org/10.1002/hep.31252
36. Huch M, Gehart H, Van Boxtel R, et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell.* 2015;160(1-2):299−312.
37. Kamp EJ, Dinjens WN, van Velthuysen ML, et al. Next generation sequencing mutation analysis on biliary brush cytology for differentiation of benign and malignant strictures in primary sclerosing cholangitis. Preprint. Posted online October 14, 2022. *Gastrointest Endosc.* doi:10.1016/j.gie.2022.10.014
38. Bridgewater J, Galle PR, Khan SA, et al. Guidelines for the diagnosis and management of intrahepatic cholangiocarcinoma. *J Hepatol.* 2014;60(6):1268−1289.