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**Page: [O11-25]**

*Original research article*

# Establishment of cholangiocarcinoma organoids from long-term frozen tissues

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## ABSTRACT

Patient-derived tumor organoids (PDOs) are cultures of tumor cells that can be derived from individual patients, which recapitulate morphological and genetic features of the original tumor, making PDOs a very valuable tool for drug testing, biomarker and mechanism of disease research. However, the ability to make organoids consistent and in large numbers poses a technical challenges to overcome. Currently, cholangiocarcinoma (CCA) PDO is a scarce resource, because of the low number of fresh tissue available to research institutes, impeding the research to find a cure for this disease. Our work described establishments of CCA-PDOs from long-term frozen CCA tissues, and characterizations of the CCA-PDOs. We also performed drug testing on the CCA-PDOs using several drugs indicated in the standard regimens for CCA treatment. We were successful at establishing CCA-PDOs from 3-month-old, and 3-year-old frozen tissues, and found that these CCA-PDOs reserve the expression of pertinent proteins histologically specific to CCA. The CCA-PDO generated from the long-term frozen tissues also displayed qualities such as, organoid shape, protein expression, and drug response profiles indistinguishable from that of the CCA-PDO prepared from non-frozen fresh tissue. They can also be expanded and stored. Therefore, we conclude that the long-term

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preserved CCA tissue is capable to revive and preserve the characteristics of the original tissue, and suitable for precision oncology research. This procedure would allow the gathering of specimens from remote locations and enables establishment of reliable larger CCA-PDO collections.

**Keywords:** patient-derived organoid, cholangiocarcinoma, organoid collection, tissue archive, drug screening

## 1. Introduction

Cholangiocarcinoma (CCA) is one of the worst prognostic gastrointestinal cancers. Despite its low incidence worldwide, CCA is among the leading cancer deaths in low-income areas of the world. [1] The only potentially curative treatment for patients with CCA is the radical surgical resection of the lesion combined with lymphadenectomy. Unfortunately, the surgical resection is feasible in only about 20-30% of patients, and recurrence after surgery is frequent; thus, the prospects of long-term survival after resection are poor. [2, 3] For those patients who are not eligible for the surgical treatment, the treatment options are usually limited to systemic therapies. However, only approximately 25-35% of the patients benefit from systemic therapy, and most of these cases develop recurrent cancer within 6 months. [4, 5]

The low treatment efficiency of CCA therapy is due to the heterogeneity of the cancer and the lack of deep understanding of the disease mechanism. Hence, a better CCA model that recapitulates the complex physiological characteristics of the disease is necessary as a study tool for novel cancer drug screening.

For the past decade, patient-derived organoids (PDOs) has been at the central stage as a crucial tool for precision oncology research, especially in the biomarker discovery and pharmacology study. The PDOs have shown to be a better model for screening compared with other patients' avatar models such as patient-derived cells (monolayer cell culture) and patient-derived xenografts. PDOs have 3-dimensional self-organizing properties with cell-to-matrix and cell-to-cell interactions which mimic those in the body. Hence, they retain polarity, histopathological, and molecular characteristics of the original tissues. [6, 7]

A difficulty in the study of rare cancers is the limited number of cases. Thus, it is difficult to perform a perspective cohort clinical study for CCA in humans. CCA-PDOs may be used as a tool for narrowing down the drug candidates for further clinical study. Nevertheless, because CCA cases are scarce, and often found in underserved area, surgical specimens of CCA cannot be processed immediately on-site and ought to be preserved for transportation to the research center. Therefore, a collection of archival specimens that can be stored long-term is essential for CCA research.

Almost all organoid research has established organoids from fresh specimens to ensure highly efficient organoid formation. Only few studies have explored the use of frozen tissues for organoid establishment. [8-10] These studies showed that PDOs may be cultured from fresh-frozen and snap-frozen cancer (breast) and normal (endometrium, colon, ileum, duodenum, and stomach) tissues. In one study, the authors demonstrated that the breast cancer organoids cultured from fresh-frozen tissues showed similar responses in chemotherapeutic drugs as the matched organoids cultured from fresh tissues. [10] These data have encouraged researchers that it is plausible to establish CCA-PDOs from archival tissue.

To fully benefit from our archival CCA specimens, here we evaluated the procedures for establishing CCA-PDOs from fresh-frozen CCA tissues with long-term storage (duration of 3 months and 3 years).

We also established the counterpart organoids from fresh CCA tissue in 1 case and performed immunohistochemical (IHC) staining of CCA markers, stem cell markers, proliferative marker, and polarity markers on established organoids and their matched tissue specimen. Then, we screened 5 standard chemotherapeutic drugs and 2 CDK4/ 6 inhibitors based on our previous studies and compared responses between the organoids cultured from fresh and their counterpart from frozen tissues.

## 2. Materials and Methods

### Tissue specimens

Cancer tissues were collected from cholangiocarcinoma patients undergoing bile duct surgery or biopsy at the Faculty of Medicine, Siriraj Hospital, and The National Cancer Institute. The protocol was approved by the Institutional Review Board for Human Research (protocol # SI494/2019 and NCI006/ 2020). The tissues were transported to the laboratories within 1 hour. Upon arriving, the specimens were washed at least 5 times with 10 ml of phosphate buffered saline containing 1,000 U/ml penicillin/streptomycin. The tissues were then cut with surgical blades into smaller pieces, approximately 3x3x3 $mm^3$ in size, and the cut tissues were added into cryo-vials containing 500 µl of freeze medium (90% FBS [Biochrome AG, Berlin, Germany] with 10% DMSO [Sigma-Aldrich, St. Louis, USA]), not more than 5 pieces per tube. The vials were stored in a cryo-preservative box at -80ºC for 24 h, and then transferred to liquid nitrogen for longer storage. For sample CCA-PDO-NCI001, part of the fresh tissue was subjected for digestion and organoid formation.

### Tissue digestion and organoid establishment

For organoid formation from fresh tissues, the tissues were further minced into a paste with surgical blades on a glass Petri-dish and transferred into a clean 15- ml conical tube. The tissue paste was washed with 12 ml of Wash Medium ( Advanced DMEM/ F12 containing 1 x Glutamax™, 10 mM HEPES, and 1 x Antibiotic-Antimycotic™, all from ThermoFisher Scientific, Waltham, MA, USA), and collected by centrifugation at 4ºC, 450xg for 5 min. After removing the supernatant, the pasted tissue was resuspended and incubated with 5 ml of Tissue Digestion Medium ( Wash Medium containing 2 mg/ml collagenase D ( Sigma-Aldrich) at 37ºC for 30 min. After 30 min, 7 ml of Wash Medium was added to the suspension and the undigested tissues were filtered out with Cell Strainer with pore size 100 µm, and the cells were collected by centrifugation. The cell pellet was resuspended with 70% Matrigel (Corning, NY, USA), added as droplets into 24- well cell culture plate, and the plate was incubated at 37ºC for 30 min. When the gel solidified, 500 µl of CCA organoid culture medium following the study by Nuciforo[11] (Advanced DMEM/F12, 1× B-27 Supplement [Thermo Fisher Scientific], 1× N-2 Supplement [ThermoFisher Scientific], 10 mM Nicotinamide [Sigma-Aldrich]. N-acetyl-L-cysteine [Sigma-Aldrich], 10 nM Gastrin I [R&D Systems, Minneapolis, USA], 10 µM Forskolin [ Stemcell Technologies, Vancouver, Canada]. 5 µM A83-01 [Stemcell Technologies], 10 ng/ml human recombinant EGF [Stemcell Technologies], 100 ng/ml human recombinant FGF10 [Stemcell Technologies], 25 ng/ml human recombinant HGF [ Peprotech, NJ, USA] , 10% R- spondin1 conditioned medium, 30% Wnt3a conditioned medium) was added to each well and the cells were cultured until organoids were formed. For PDOs from frozen tissues, after thawing, the tissues were washed with 10 ml of Wash Medium, and were subjected to digestion and organoid culture as stated earlier.

### Organoid expansion

Once the organoids grew larger than 150 µm in diameter, the Matrigel containing organoids was washed out of the cell culture plate and transferred into a clean 15-ml conical tube, and Wash Medium was added to reach 12 ml. The organoids were collected by centrifugation at 4ºC, 450xg for 5 min and the supernatant was discarded. The cell pellet was then resuspended with 1 ml of TrypLE™

Express (ThermoFisher Scientific), and the organoids were sheared into clumps of 5-10 cells by pipetting. The enzymatic reaction was stopped by diluting TrypLE™ Express with 11 ml of Wash Medium. The digested organoids were collected by centrifugation, and recultured as previously described into a 6-well cell culture plate.

**Immunohistochemical (IHC) and immunofluorescence (IF) staining**

The frozen tissues were thawed and washed with PBS to remove freeze medium, and then they were sent to a pathology laboratory for formalin fixation and paraffin embedding following the standard FFPE protocol. For analysis, when the PDOs grew to 100 μm in diameter, they were collected and washed with the Wash Medium as described previously. The organoid pellet was resuspended with 60% Matrigel and 50 μl of the organoid-gel suspension was added into a 96-well ultra-low attachment u-shape plate (Corning) and incubated at 37°C for 30 min for the gel to solidify. The CCA PDO culture medium was added and the PDOs were cultured for 3 days. For FFPE, the PDOs were fixed with 4% paraformaldehyde for 15 min at room temperature, and washed with PBS 3 times. The fixed organoids were then encased in 100 μl of 1% low-melting agarose gel (Sigma-Aldrich) and kept at 4°C for at least 1 h. The organoid-containing agarose gel was removed from the plate and wrapped with filtered paper, and was sent to a pathology laboratory for standard formalin fixation and paraffin embedding.

For IHC staining, the tissue and organoid blocks were sent to a pathology laboratory for staining for 7 protein markers (CK7, clone OV-TL12/30 (Cell Marque, Rocklin, USA), CK19, A53-B/A2.26 (Cell Marque), Ki67, MIB1 (BioGenex, Fremont, USA), OCT4, MRQ-10 (Cell Marque), SOX2, SP76 (Cell Marque), and αSMA, 1A4 (Cell Marque) following standard IHC staining protocol. For IF staining, the tissues and PDOs were subjected to staining of the 2 polarity markers E-cadherin, clone M168 (Abcam, Cambridge, UK) and β-catenin, E247 (Abcam) and the epithelial marker Pan-cytokeratin, AE1/AE3 (ThermoFisher Scientific, Waltham, USA), and the nucleus staining with DAPI (ThermoFisher Scientific) was performed at the final staining step. The tissue and organoid blocks were cut into 4 μm slices and put on charged glass slides (Matsunami Glass Industry, Kishiwada, Japan). The tissues were deparaffinized by incubating the slides in xylene (Sigma-Aldrich) solution for 3 min, and this step was repeated until the paraffin was removed. Then, the tissues and cells were hydrated by incubating the deparaffinized tissues and cells with 100% ethanol then, 95%, 70%, 50% and 30% consecutively for 3 min at each concentration. After rinsing with PBS, the slides were placed in 1XDAKO Target Retrieval Solution (Agilent, Santa Clara, USA) and boiled at 100°C for 20 min in a water bath for antigen retrieval. When the boiled slides had cooled down to room temperature, they were incubated with Odyssey™ Blocking Buffer (LICOR Bioscience, Lincoln, USA) for 30 min at room temperature and with all 3 primary-conjugated antibodies for 12 h at 4°C. After incubation with the primary antibodies, the slides were washed and stained with 2 μg/ml DAPI for 15 min. All stained slides were imaged with the objective lens at 40x magnification. The staining results were compared between the organoids derived from fresh and frozen tissues, and their original tissue.

**Anticancer drug screening on CCA organoids**

The organoids were collected and washed with Wash Medium as stated previously. The PDO pellet was resuspended and incubated with 1 ml of TrypLE™ Express at 37°C for 5 min, and the organoids were further broken down into single cells by gentle pipetting. The enzymatic reaction was stopped by diluting with 11 ml of Wash Medium. The large undigested organoids were filtered out with Cell Strainer (Sigma-Aldrich) with pore size 100 μm, and the

single-cell suspension was centrifuged to collect the cells. The cell pellet was resuspended with 1 ml of CCA organoid culture medium, and total cells were counted using a hemocytometer. The number of cells plated was 1000 cells in 25 µl culture medium containing 5% Matrigel per well of ultra-low attachment 384-well plate and the cells were incubated for 3 days at 37°C to let the organoids form. Anticancer drugs (obtained from Seleckchem, TX, USA) ranging from 100-0.00001 µM final concentrations (10-fold dilutions) were added to each well in 3 replicates, and incubated for 3-5 days depending on the drug. After incubation was complete, cell viability was measured using the ATPlite™ Luminescence Assay System (PerkinElmer, MA, USA) following the manufacturer's instruction. The percentage of cell viability was analyzed and plotted as a dose-response curve. Area under the curve (AUC) and half-maximum inhibitory effect (IC50) of each drug were also calculated and compared between organoids derived from fresh and frozen tissues, and between samples.

## 3. Results

### Establishment of CCA PDOs from long-term frozen tissues

Tissues from patients diagnosed with cholangiocarcinoma according to the 8th edition of the American Joint Committee on Cancer (AJCC) and Union for International Cancer Control (UICC) Manual were collected.

Four specimens with various storage durations were used for PDO establishment in our study. In parallel to the frozen specimens, we also used a fresh specimen of NCI-001. Clinical characteristics of the specimens are shown in Table 1.

PDO preparation was performed following the protocol by Nuciforo[11] with some modification in that the tissue desegregation was performed using physical force instead of enzyme digestion (Fig. 1A). PDOs were formed from all of the specimens (4 out of 4). However, only 2 CCA-PDOs (50%), CCA-PDO-Si003 and CCA-PDO-NCI001, were able to continue growing after 3 passages (expandable) (Table 2). One tumor (CCA-PDO-Si003) was intrahepatic cholangiocarcinoma (iCCA) from a 71-year old female with T2N1Mx tumor.

Another tumor (CCA-PDO-NCI001) was iCCA from a 60-year old male with T1aNxMx tumor (Table 1). The PDOs continued to grow with consistent rates until passage 5. At this point, we considered them as well-established PDOs.

Both PDOs established from fresh and frozen specimens of CCA-PDO-NCI001 showed a very similar cystic shape appearance, while the CCA-PDO-Si003 showed as a mixture of cystic and compact shape PDOs (Fig. 1B).

### The established CCA PDOs from both fresh and fresh-frozen tumors retained the histology and protein markers expressed in the tumor tissues

To study the effect of long-term storage, we compared the CCA-PDO-NCI001 prepared from fresh and from frozen tissues. We found that PDOs prepared from fresh and frozen NCI001 tissue were histologically indistinguishable (Fig. 2). In addition, both expandable PDOs CCA-PDO-NCI001 (fresh and frozen tissues) showed histologic features of moderately differentiation and similar immunophenotypic profile with the matched primary tumor NCI001 (Fig. 2).

Both NCI001 PDOs (fresh and frozen) were positive for cytokeratin 7 (CK-7), and cytokeratin 19 (CK-19), while negative for cytokeratin 20 (CK-20), consistent with the protein expression pattern of CCA[12] (Fig. 2). They appeared to be negative for α-smooth muscle actin (α-SMA), a putative marker of hepatic stellate cells and for liver fibrosis. We also found a comparable Ki67 signal in the specimen tested (Fig. 2), confirming that the PDOs were proliferative and expandable. Since CCA has been shown to comprise a stem cell-like subpopulation[13] we explored the expression of SOX2, and OCT4, protein markers known to associate with stem cell

activation. We found negative SOX2, and OCT4 signals from the PDOs, which was identical to the results from the NCI001 tissue (Fig. 2), suggesting that CCA specimens comprise a very minute stem cell population.

**Table 1**. Demographics and pathological information of specimens used in this study.

| Samples ID | Storage time before organoid establishment (months/days) | Gender | Age at diagnosis | TNM stage (Anatomical stage) | Cell type | Histologic subtype |
|---|---|---|---|---|---|---|
| CCA-PDO-Si002 | 33.1/991 | Male | 59 | T3NxM0 (IIIA-B) | cHCC-CC | Poorly differentiate |
| CCA-PDO-Si003 | 32.3/969 | Female | 71 | T2N1Mx (IIIB) | Intra-hepatic | Moderately-differentiate |
| CCA-PDO-Si005 | 13.8/414 | Female | 53 | T4NxM0 (IIIB) | Intra-hepatic | Moderately-differentiate |
| CCA-PDO-NCI001 | 3.7/111 | Male | 60 | T1aNxMx (IA) | Intra-hepatic | Moderately-differentiate |

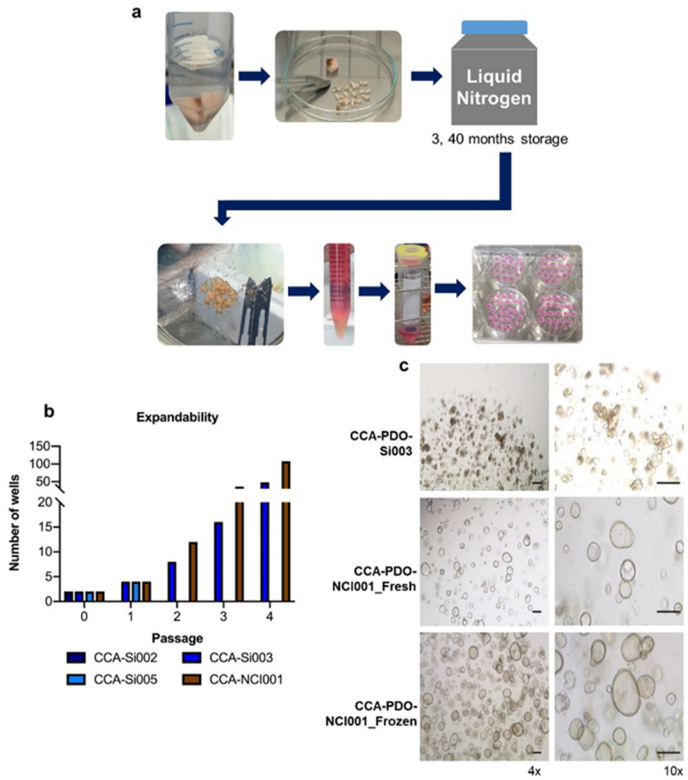


**Fig. 1.** ( a) Process of tissue preparation for organoid establishment. (b) Expandability of each established organoid from archival specimens. Morphology of the successfully expanded organoids from archival tissues ( CCA- PDO-Si003 and CCA- PDO- NCI001_ Frozen) and fresh tissues (CCA-NCI- 001_Fresh) . Scale bar = 100 µm ( objective 4x ( left) and 10x (right) magnification.

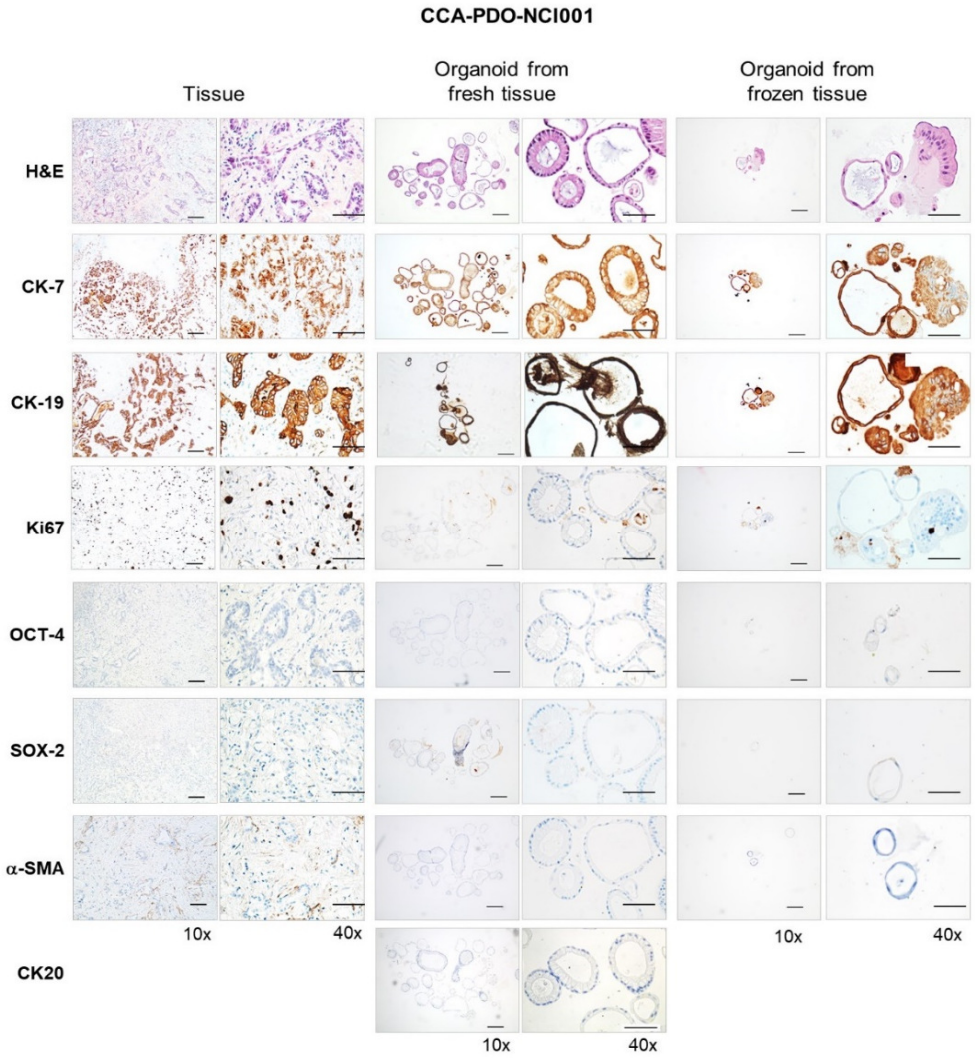


**Fig. 2.** H&E and IHC staining of 6 protein markers in tumors and their matched organoids established from fresh and frozen tissues of sample CCA-PDO-NCI001at objective lens 10x magnification (scale bar = 100 µm) and objective lens 40x magnification (scale bar = 50 µm).

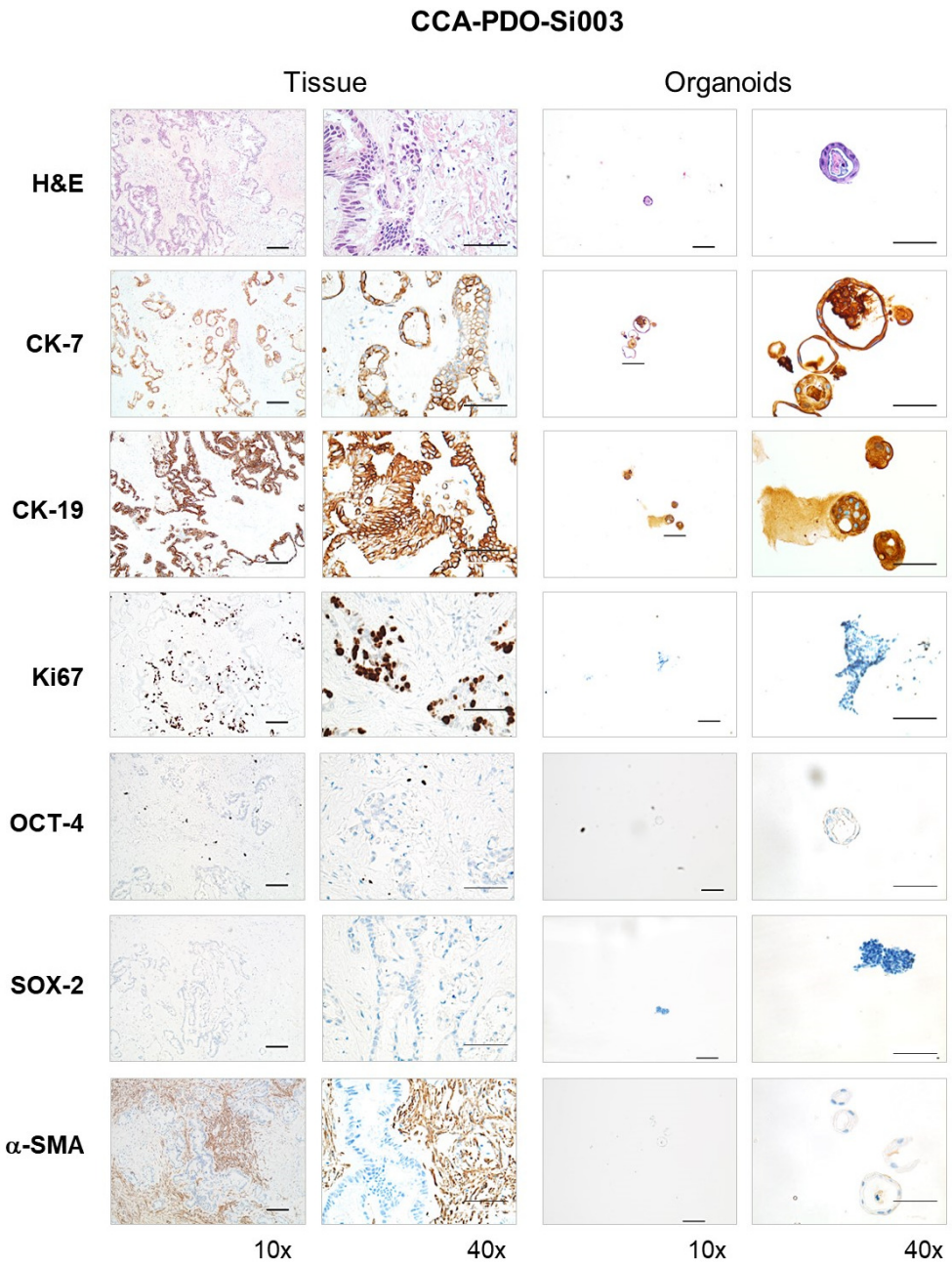


**Fig. 3**. H&E staining and IHC staining of 6 protein markers in tumors and their matched organoids established sample CCA-PDO-Si003 at objective lens 10x magnification (scale bar = 100 µm) and objective lens 40x magnification (scale bar = 50 µm).

**Table 2.** Characteristics of the CCA-PDOs.

| Samples ID | Tissue status used for organoid culture | Organoid formation at passage 0 | Organoid expansion over 3 passages | Organoid morphology | Current passage | Explanation |
|---|---|---|---|---|---|---|
| CCA-PDO-Si002 | Fresh-frozen | Yes | No | Cystic | 1 | Organoid failed to expand due to the low number of organoids formed |
| CCA-PDO-Si003 | Fresh-frozen | Yes | Yes | Compact and Cystic (mostly compact) | 5 | Time to each passage: 14 days |
| CCA-PDO-Si005 | Fresh-frozen | Yes | No | Compact | 0 | Organoid failed to expand due to the low number of organoids formed |
| CCA-PDO-NCI001 | Fresh | Yes | Yes | Cystic | 9 | Time to each passage: 10 days |
| | Fresh-frozen | Yes | Yes | Cystic | 11 | Time to each passage: 10 days |

We also performed the same analyses on the expandable PDOs prepared from the 3-year old frozen Si003 tissue (CCA-PDO-Si003). We found that the PDOs showed the histologic features of moderately differentiated CCA and shared the same immunophenotypic profile with primary tumor (Fig. 3). We also found that the CCA-PDO-Si003 were positive for CK-7, CK-19, and Ki67, and was negative for α-SMA, SOX2, and OCT4 expressions (Fig. 3) which was indistinguishable from the matched tissue (Fig. 3). Therefore, we conclude that viable and expandable PDOs could be established from the long-term frozen tissue, i.e. in our case, frozen tissues up to more than 3 years (969 days), and that the PDOs showed a protein expression profile well matched to that of the original tissues from the patients. Characteristics of the CCA-PDOs are summarized in Table 2.

**CCA PDOs showed the apical-in formation**

We also performed immunofluorescent staining of polarity markers (E-cadherin and β-catenin) and cancer markers (pan-Cytokeratin) on tissues and organoids to evaluate the cell organization in the organoids compared with their tissues of origin.

The immunofluorescent staining results showed orderly organized cell-cell structures of the pan-cytokeratin positive CCA PDOs (Fig. 4). The results showed positive staining of the adherent junction (basolateral) membrane–associated protein E-cadherin and β-catenin on cells of the PDOs, indicating the "apical-in" formation of the PDOs, which is similar to the pattern of the patient tissues, both in the cases of CCA-PDO-NCI001 and CCA-PDO-Si003.[14] We also found that the organoids prepared from the long-term preserved CCA tissue contain a comparable cell component with those prepared from the fresh specimen (compare CCA-PDO-NCI001_Fresh vs CCA-PDO-NCI001_Frozen).

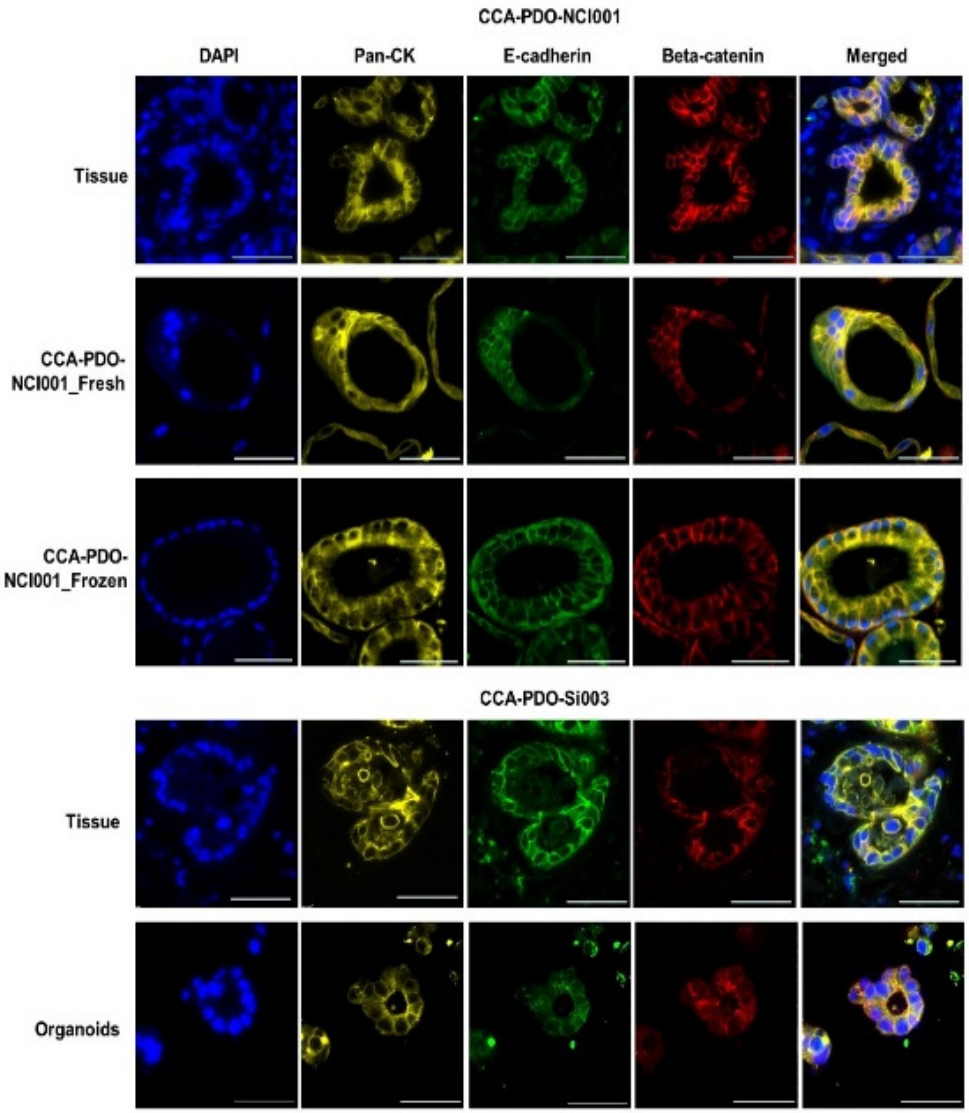


**Fig. 4.** Immunofluorescent staining of polarity markers in tumors and their matched organoids (scale bar = 100 µm).

**Response to chemotherapeutic and targeted treatments of the CCA PDOs**

The CCA- PDO- Si003, and CCA-PDO-NCI001 (prepared from both fresh and frozen tissue) were treated with 5 standard chemotherapeutic drugs and 2 targeted drugs of interest (palbociclib and abemaciclib both are CDK4/6 inhibitors). We first compared drug response results between CCA- PDO-NCI001 established from fresh *vs* frozen tissue, in order to investigate whether PDOs established from archival specimen reflect the drug response profiles from those established from fresh specimens (originally from the same tissue). We found that the drug response results in PDOs established from the frozen tissue were consistent with the results from the PDOs from the fresh tissue in all drugs tested, indicated by comparable area under the curves (AUCs) of the drugs tested (Fig. 5A, B).

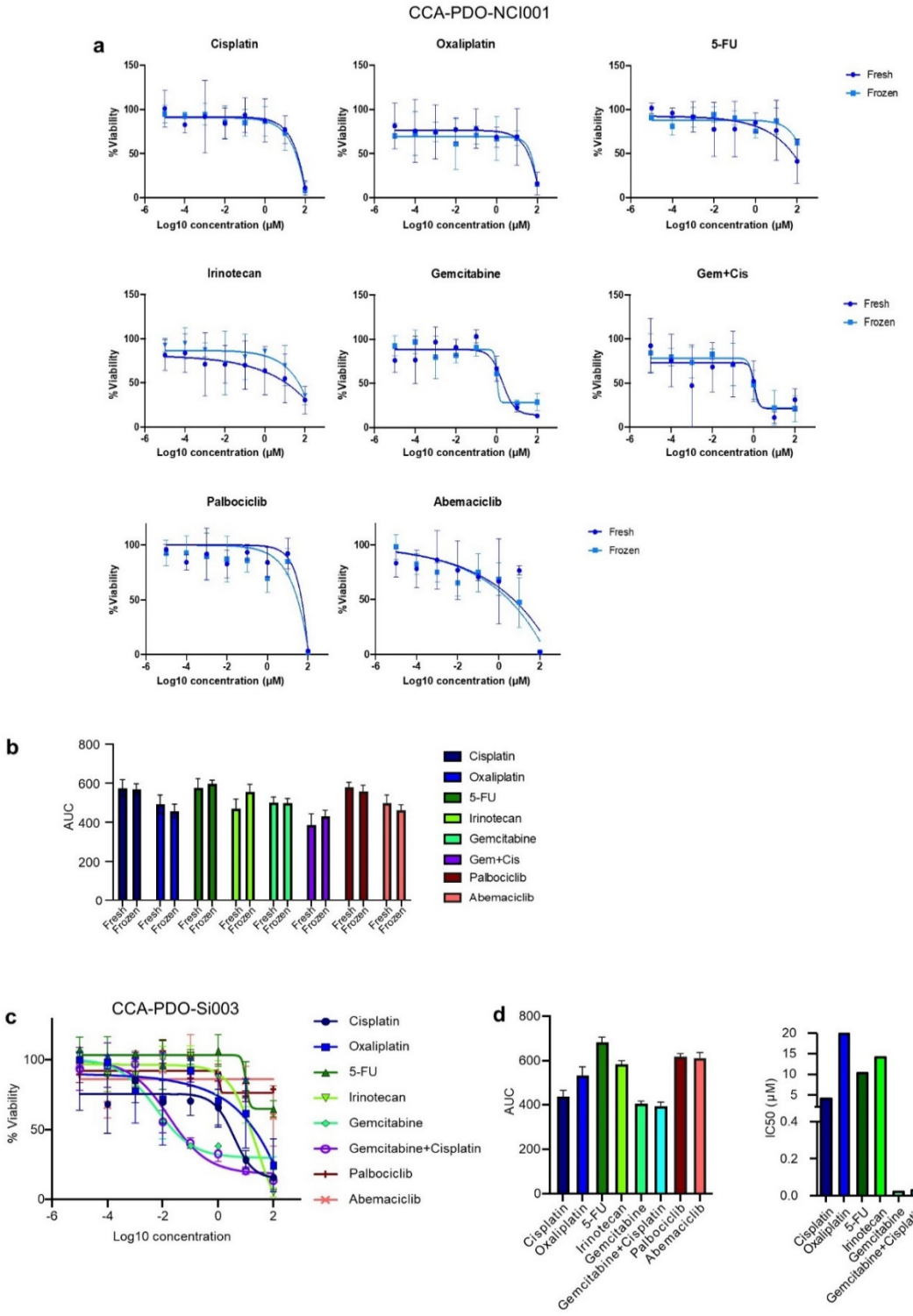
CCA-PDO-NCI001
a
Cisplatin
Oxaliplatin
5-FU
Irinotecan
Gemcitabine
Gem+Cis
Palbociclib
Abemaciclib
% Viability
Log10 concentration (μM)
Fresh
Frozen
b
AUC
Cisplatin
Oxaliplatin
5-FU
Irinotecan
Gemcitabine
Gem+Cis
Palbociclib
Abemaciclib
c
CCA-PDO-Si003
% Viability
Log10 concentration
Gemcitabine+Cisplatin
d
AUC
IC50 (μM)

**Fig. 5.** (a) Dose-response curves of standard chemotherapeutic drugs and CDK4/6 inhibitors in organoids established from fresh and frozen tissues of sample NCI-001. (b) Comparison of AUC from dose-response curves between organoids established from fresh vs frozen tissues of sample NCI-001. (c) Dose-response curves of standard chemotherapeutic drugs and CDK4/6 inhibitors of sample Si-003. (d) AUCs from the dose-response curves of each drug from sample Si-003. Note: IC50 of palbociclib and abemaciclib cannot be determined.

Patient NCI001, with incomplete resection (R1), has received gemcitabine as treatment regimen. A 6-month follow up showed that the patient is alive without evidence of recurring tumor, implicating that the disease may be controlled by gemcitabine. To this end, gemcitabine responses in CCA-PDO-NCI001 established from both fresh and frozen tissues showed that the IC50s ($IC50_{fresh}$ = 1.9 µM and $IC50_{frozen}$ = 1.03 µM) were significantly lower than the clinical maximum tolerable dose (Cmax) of gemcitabine (89 µM), suggesting that PDOs from both fresh and frozen tissues of patient CCA-PDO-NCI001 were responsive to gemcitabine treatment. Therefore, the PDO drug testing results were concordant with the clinical outcome.

Unfortunately, we were not able to analyze drug response data for the remaining drugs for patient NCI-001, since the drugs were not used in the patient.

Patient CCA Si003 has received 6 cycles of 5-FU in the adjuvant setting, which provided a 196-day disease control before a relapse. Along with all of the drugs tested in the CCA-PDO-Si003, we found that the PDO responded well to the 5-FU treatment with 5-FU IC50 = 10.22 µM, significantly lower than the drug's Cmax (426 µM)[15] (Fig. 5C). AUCs of the tested drugs are shown in Figure 5D. This result suggested that the CCA-PDO-Si03 may have a similar 5-FU response to that of the patient CCA-PDO-Si003. Interestingly, CCA-PDO-Si003 also responded to gemcitabine-based treatment ($IC50_{gemcitabineSi003}$ = 0.02 µM, $IC50_{gemcitabine+cisplatinSi003}$ = 0.03 µM) (Fig. 5D). Of note, gemcitabine Cmax = 30 µM.[16] However, we were not able to compare drug response data for the remaining drugs, since these drugs were not used in the patient.

Our previous studies have demonstrated that proliferation of cholangiocarcinoma cells and PDXs required activity of CDK4/6, and that CDK4/6 inhibitors, palbociclib and abemaciclib, decreased growth of cholangiocarcinoma cell lines that showed high expressions of cyclin D1 and intact pRB.[17] In this study, we showed the differential responses in CCA-PDO-Si003, CCA-PDO-NCI001 PDOs to the drugs. The CCA-PDO-NCI001 was responsive to 2 CDK4/6 inhibitors (palbociclib and abemaciclib), while the CCA-PDO-Si003 was consistently not responsive to both drugs ($IC50_{palbociclibNCI001}$ = 39 µM *vs* Undetermined $IC50_{palbociclibSi003}$, $IC50_{abemaciclibNCI001}$ = 4 µM *vs* Undetermined $IC50_{abemaciclibSi003}$, $AUC_{palbociclibNCI001}$ = 498 *vs* $AUC_{palbociclibSi003}$ = 619, and $AUC_{abemaciclibNCI001}$ = 464 *vs* $AUC_{abemaciclibSi003}$ = 612). These results showed that PDOs might be an excellent tool for the mechanistic study of the response to the CDK4/6 inhibition in CCA.

## 4. Discussion

To understand the tumorigenesis and improve treatment of CCA, models that closely resemble the tissue of origin are essential for the advance of the field. Patient-derived cancer organoids have taken a prominent role in pre-clinical and translational research and have been generated for most common solid tumors. Because of the requirement that the specimen for PDO should be obtained fresh, collections of rare cancer PDOs, such as cholangiocarcinoma, are harder to obtain. Cholangiocarcinoma is an immense health

burden for middle to low-income countries. In Thailand, for example, this disease is prominent in the North-East and North of the country, in areas with rural/ agricultural economies and low research capacity. The ability to preserve the CCA tissue long-term for reactivation is the central issue for a construction of the active CCA PDO archive.

Here, we have demonstrated successful cases of establishing stable in vitro culturing of CCA PDOs derived from both fresh and long-term frozen tissues. These PDOs derived from CCA closely recapitulated the histopathology of the primary tumors as shown by protein expression, and cellular polarity. The PDO prepared from frozen tissue (CCA-PDO-NCI001) demonstrated a phenotype that was indistinguishable from that of the fresh tissue, indicating that, when needed, the frozen tissue could be conveniently used for the PDO preparation.

Out of 4 cases, we established 2 PDO models (50% success). From those 2 failure cases, we found they contained very low percentages of tumor cells. Therefore, it may be possible that an insufficient number of cancer cells in the tissue contributed to the failure. However, to better gauge the rate of success, more PDO preparation from specimens with various storage time, disease condition/ staging, and genetic background are still required.

Interestingly, we showed an encouraging result that tissue stored for up to 969 days could be reactivated. Previous reports showed the longest preserved tissue of cancer was 12 months (colorectal cancer and breast cancer), [9,10] and 18 months (540 days).[18]

The PDOs we generated were used for drug testing with results that were in line with the data from clinic. However, this was only correlational observation. To precisely compare the drug responses in PDO vs in patient, a prospective cohort study is required.

PDOs could be a very powerful tool for the discovery of new targeted drugs for CCA. We tested CDK4/6 inhibitors against the CCA-PDOs. Interestingly, we found a differential sensitivity between the CCA-PDOs, i.e. CCA-PDO-Si003 was very resistant, while CCA-PDO-NCI001 was moderately responsive. These results indicated that the PDOs are good tools to study the molecular pathways, or the tumor microenvironment that influences the responsiveness to CDK4/6 inhibition.

From our results, we believe that CCA PDOs generated from long-term preserved tissues can be a powerful research tool for the clarification of molecular pathogenesis and the discovery of drugs for treatment of CCA. Until now, only a small number of organoids derived from CCA have been reported. We believe that the results from our practice will promote the construction of a CCA PDOs cohort that is consistent and large enough to effectively study the precision treatment for the disease. We hope that the experience we have shared will further broaden the application of organoid technologies especially for CCA, as samples can be obtained from patients treated at smaller rural hospitals that do not have the required research infrastructure to initiate organoid cultures themselves. Lastly, as the project is part of the E-Asia joint research program and Thailand's National Science and Technology Development Agency (NSTDA), the CCA-PDOs reported here may be used in the scientific community to advance the knowledge for CCA treatment.

## 5. Conclusion

The long-term preserved CCA tissue is capable of reviving and preserving the characteristics of the original tissue, and is suitable for precision oncology research. This procedure would allow the gathering of specimens from remote locations and enables the establishment of reliable larger CCA-PDO collections.

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## Conflicts of Interest

The authors declare no conflict of interest.

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