OPTIMIZATIONS OF PATIENT-DERIVED CHOLANGIOCARCINOMA EXPLANT CULTURE FOR 3D ORGANOIDS FORMATIONS

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ABSTRACT

The three-dimensional (3D) organoid technology has become more popular among researchers. Organoids are the small structure of tissues and organs that mimic the 3D architectures and functions of a specific organ. These 3D constructs represent near-physiological *in vitro* models and support many biomedical applications, including cancer research. Cholangiocarcinoma (CCA) is a malignant tumor of bile duct epithelium which highly endemic to the northeastern part of Thailand. CCA cell lines were established successfully as 2D cultures; however, some aspects require drug evaluation on multi-phenotypic cell types. Currently, the development of precise organoids for assessing drug response of CCA *in vitro* remains a challenge. Thus, the tissue-engineering approach might help produce CCA organoids with multi-phenotypic cells in a controllable manner. Therefore, this study aims to establish patient-derived CCA organoids using a re-assembly scheme from pre-characterized cells. From the results, explant culture was established under *in vitro* condition with cells grew out of CCA explants in a 2D plane. Single cells were further isolated and initially characterized with anti-cytokeratin 19 and anti-α-SMA antibodies to define the CCA cell and the cancer-associated fibroblast (CAF) populations. Further characterization of cells will be required, and drug responses of self-assembled organoids (CCA-CAF) will be elucidated in future.

Keywords: Three-Dimensional (3D) Organoid, Cholangiocarcinoma, Tissue-Engineering, Epithelial Cells (CCA), Fibroblasts (CAF)

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INTRODUCTIONS

Cholangiocarcinoma (CCA) is a malignant tumor of bile duct epithelium, the second most common cancer worldwide and highly endemic in Thailand's northeastern part. Patients usually present at the doctor in an advanced stage with a poor prognosis, which leads to high mortality. Nowadays, treatment includes surgery, radiotherapy, and chemotherapy. The pre-clinical models of cancer can be divided into two main models, the *in vitro* model and the *in vivo* or animal models, including two-dimensional (2D) culture, three-dimensional (3D) culture, and patient-derived tumor xenografts (PDTXs). There have been investigations of pre-clinical models to confirm drug-response predictions (Xu et al., 2018). Two-dimensional (2D) culture has several advantages for biomedical research because it can expand in monolayer with a homogenous cell populates ion, easy to maintain, and divide at several passage numbers. However, 2D culture does not mimic the physiological conditions in some aspects and may lack genetic heterogeneity in the case of cell line (Sachs & Clevers, 2014). Three-dimensional (3D) or organoid technology has become more popular among researchers. Organoids are the small structure of tissues and organs that mimic the architectures and functions of a specific organ (Mandrycky et al., 2017). These 3D constructs represent near-physiological conditions and support many biomedical applications, including cancer research. Organoids can be expanded in the long term and provide more genetic heterogeneity. Organoids can be generated from either the induced-pluripotent stem cell (iPSCs) by genetic engineering or the patient-derived tissue through biopsy samples (Drost & Clevers, 2018). Organoids can be established from explant cultures in small amounts of human tissue or organ and develop as an *in vitro* model. However, the organoid system can fulfill the research gap in some aspects because it can be expanded to several passages and observed at both endpoint and real-time. As a consequence, this study aims to optimize and control all those concerns by using a tissue engineering approach with the optimized optimal conditions for establishing patient-derived CCA organoids using multi-phenotypic cells isolated from CCA tissues via a tissue-engineering approach and proceeded to explant cultures to establish primary cells.

MATERIAL AND METHODS

Human Cholangiocarcinoma tissues

CCA tissues were obtained from cholangiocarcinoma cancer patients through resection (1-2 cm^3) at Srinagarind Hospital, Khon Kaen University. All specimens were collected and process through Cholangiocarcinoma Research Institute (CARI), Khon Kaen University, by qualified surgeons and pathologists indicating the location of the primary tumor, tumor grading, history, tumor invasion, and surgical procedure in each patient. The fresh tissue samples were maintained in Hank's Balanced Salt Solution (HBSS) contained antibiotics. The protocol of collection and study was approved by Ethic Committee for Human Research, Khon Kaen University (HE611214).

Explant culture

Primary CCA tissues from patients were collected according to 7.1.1 and remained in Hank's Balanced Salt Solution (HBSS) containing antibiotic-antimycotic solution overnight at 4°C. On the next day, the tissues were sliced into smaller and thin pieces, approximately 1x1x1 mm with surgical blades. After that, the explants were transferred to T25 tissue culture flasks using forceps. All pieces were cultured as explants at humidified 37 °C and 5% CO_2 to observe the cell outgrowth around the explants. In this study, the explants were cultured in 3 different cell culture media, including Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, USA), Ham’s F-12 Nutrient Medium (F-12 Nutrient Medium; Thermo Scientific, USA), and our own formulated “X Medium”.

CCA organoid culture platforms (Scaffold-free system)

The scaffold-free culture of CCA organoids was performed by seeding the cells on ultra-low attachment (ULA) 96-well round-bottomed plates supplemented with cell culture medium. Then the plates were incubated in the CO_2 incubator at 37°C, 5% CO_2 .

Hematoxylin and eosin staining (H&E)

Organoids are fixed in formaldehyde and subjected to histological section. Samples were dehydrated and embedded in paraffin. After that, the samples were cut with a microtome giving a 4 µm-thicked sections which were attached to charged slides. Then, the slides with sample sections were rehydrated and rinsed with distilled water before going through the staining process using hematoxylin and eosin. Finally, the slides were added with one drop of mounting solution and placed with the coverslips.

Immunofluorescent staining

Immunofluorescence (IF) is a method to detect antigens in cellular contents using specific antibodies. In the identification of epithelial cells (CCA) and fibroblasts (CAF), the primary cells ($2x10^5$ cells/300µl) were plated in the chamber slide and incubated at 37°C in a humidified 5% CO_2 atmosphere. After 24 h of incubation, cells on the slide were fixed in 4% paraformaldehyde for 30 min, washed with phosphate buffer saline (PBS), and blocked with 1% w/v BSA with 0.25% v/v triton-x in PBS for 1 h at room temperature, followed by incubation with primary specific antibodies (anti-α-SMA and anti-cytokeratin-19 antibodies) for 1 h at room temperature and at 4°C overnight in a moisture chamber. After incubation, the slides were washed with PBS 2 times, 5 min each. Then, the samples were incubated with secondary antibodies conjugated with Alexa Fluor® 488 or Alexa Fluor® 594 (Invitrogen, USA) at room temperature and washed twice with PBS. The samples' nucleic acid were stained with hoechst 33342 (Invitrogen, USA), 10 min at room temperature, and the slides were mounted. Finally, the samples were visualized using a wide-field fluorescent microscope EVOS M5000 (Thermo Fischer Scientific, Massachusetts, USA) equipped with standard filters including DAPI (357/447 nm), GFP (490/525 nm), and Texas Red (585/628 nm).

Statistical Analyses

Statistical analyses were performed using GraphPad Prism 9.5.0 (GraphPad Inc., San Diego, CA, USA). The statistical analysis will be performed using One-way ANOVA. The results are considered to be significant at $P < 0.05$, where Tukey's multiple comparison tests were performed to define which mean amongst a set of means differs from the rest

RESULTS

Establishment of primary cells from patient-derived CCA tissues and choosing the cell culture medium

In this study, the explants were cultured in 3 different cell culture media, including Dulbecco's Modified Eagle Medium (DMEM; Thermo Scientific, USA), Ham's F-12 Nutrient Medium (F-12 Nutrient Medium; Thermo Scientific, USA), and the Advanced DMEM with supplements. The results are shown in Table 2 and figure 1. In DMEM medium, 1 out of 5 cases presented cells proliferating out of the explants with fibroblast-like characteristics, which took 21 days to grow from the explant. In HAM's F12 medium, 1 out of 5 cases exhibited cell proliferating out of explants with only fibroblast-like cells. However, Advanced DMEM showed cells proliferating out of explants in 5 cases with epithelial cells, fibroblasts, or mixed populations. Hence, Advanced DMEM was chosen for culturing cells in the subsequent experiment. All tissue cases were further cultured with Advanced DMEM supplemented with 2.5% FBS referred to the results primary cells isolated from patients' tissues were stained to confirm the characteristics of the cell via the immunofluorescence (IF) method with specific primary antibodies, including anti-cytokeratin-19 (for CCA cells) and anti-α-SMA (for CAFs) and then added with secondary antibody Alexa Fluor® 488 and 594 (Invitrogen). Case numbers

J074, K092 and CR64-79 show mixed populations of CCA cells and CAFs, whereas L134 and L197 showed only CAF population **(Figure 1)**

Table 1 Cost per litre of cell culture medium*

DMEM		**HAM's F12**		**Advanced DMEM**	
Chemicals	**Cost per litre (THB)**	**Chemicals**	**Cost per litre (THB)**	**Chemicals**	**Cost per litre (THB)**
DMEM powder	278.20	HAM's F12 powder	267.50	Advanced DMEM basal medium, bottle, sterile, 500 mL	5,778.00
$NaHCO_3$	18.89	$NaHCO_3$	18.89	-	-
Sterile water for injection, 1L	65	Sterile water for injection, 1L	65	-	-
1N HCL (pH Adjustment)	61.13	1N HCL (pH Adjustment)	61.13	-	-
0.22 µm filter	347.75	0.22 µm filter	347.75	-	-
Antibiotic	186.18	Antibiotic	186.18	Antibiotic	186.18
FBS	1,560.06	FBS	1,560.06	FBS	390.02
-	-	-	-	Nicotinamide	0.052
-	-	-	-	2-Phospho-L-ascorbic acid	0.003
-	-	-	-	HEPES	331.70
-	-	-	-	Dexamethasone	0.001
-	-	-	-	Glutamax	159.43
-	-	-	-	Recombinant human EGF	406.60
Total	**2,517.21**	**Total**	**2,449.96**	**Total**	**7,251.98**

* Cost per litre of cell culture medium was calculated based on products sold by Thailand's distributors in 2022. The prices are VAT include

Table 2 Time course of cell expansion in different medium condition

Cases / **Media**	**DMEM medium**	**HAM's F12 medium**	**Advance DMEM medium**
J074	No cell*	No cell*	9 days (Mixed cell)
K092	No cell*	No cell*	10 days (Mixed cell)
L134	No cell*	No cell*	14 days (Epithelial-like)
L197	21 days (Fibroblast-like)	21 days (Fibroblast-like)	15 days (Fibroblast-like)
CR64-79	No cell*	No cell*	15 days (Mixed cell)

* No cells observed after 2-month culture

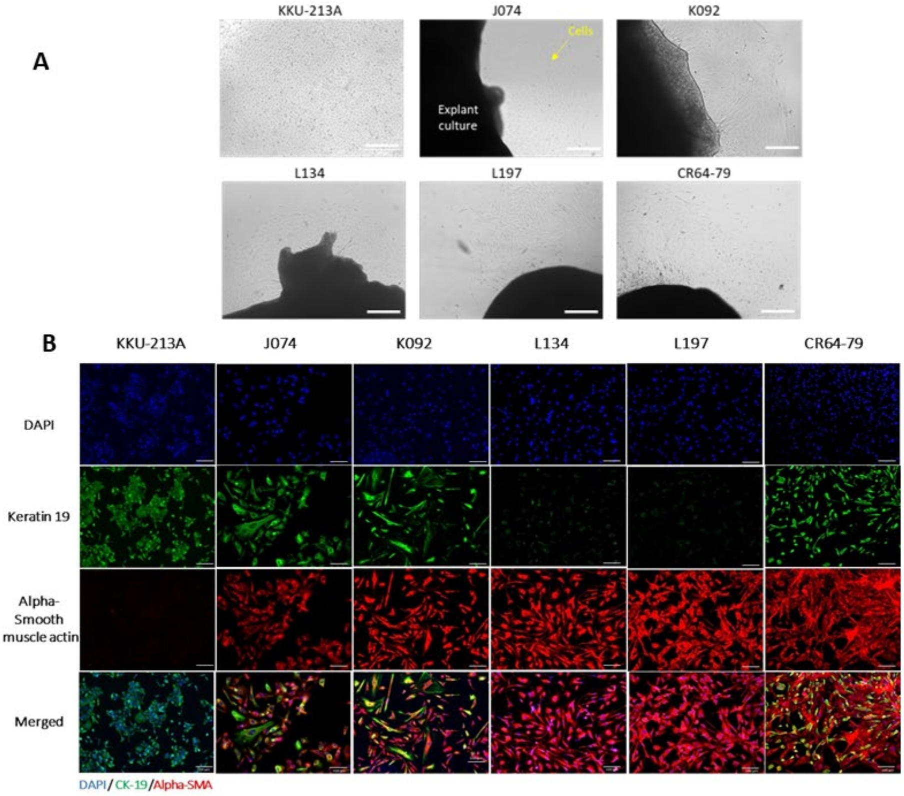
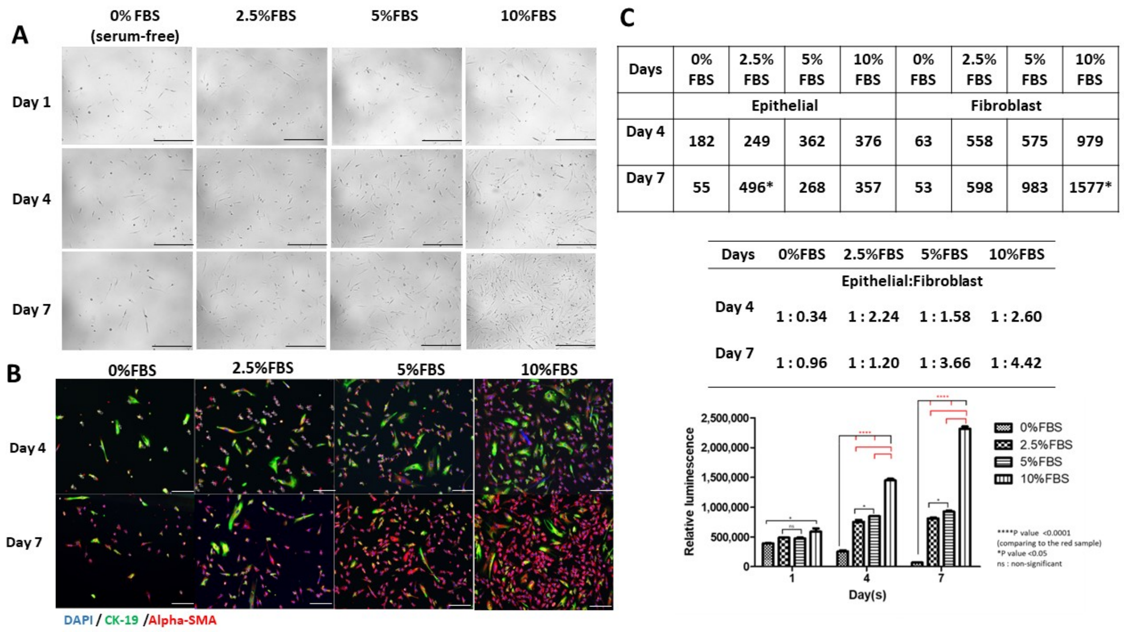


Figure 1 Workflow of our study (A)Schematic of tissue delivery for experiments are shown on the top the tissue sample was identified by a pathologist and separated for pathology diagnosis, biobanking, and leftover specimen and then tissues were dissected and cultured in T25 flasks via an explant culture scheme and The cells were grown from the explant and ready to harvest for experiments or cryostorage.(B) Pictures of primary cells expanded from explants culture the expanded cells generated into epithelial-liked cells and fibroblast-like population(C) Fluorescence images of primary cells CK-19 (green, marker for biliary cells),α-SMA (red, marker for fibroblast) and DAPI (blue, nuclear staining). Scale bar represents150 µm

The optimal percentage of fetal bovine serum (FBS)

FBS is one factor that is crucial when culturing primary cells established from either tissue dissociation or explant culture. In the case of mixed cell populations, a high percentage of FBS may cause the domination of fibroblasts over some other cell types. Moreover, several publications demonstrated that a cell culture medium with reduced serum formula (less than 10%) offers better growth for epithelial cells(Malm et al., 2018)·(Ryan, 1979). Therefore, different percentage of FBS in Advanced DMEM was investigated in this experiment. The results from Cell TiterGlo® (**Figure 2**) showed K092 cells proliferated higher when % serum in the media increased. However, that could not be the only factor, as the ratio of epithelial cells per fibroblast must be taken into account. Images of cells from 8 frames from 3 individual wells were counted and analyzed using ImageJ program. The ratio of each cell was calculated and shown in **Figure2C** The results revealed 10% FBS had 1:4.42 (epithelial cell: fibroblast) while 5% FBS presented 1:3.66, 2.5% FBS demonstrated 1:1.2, and 0% had 1:0.96 over 7 days. Maintaining the populations of epithelial type and fibroblast type is crucial for our experiment. Hence, a ratio close to 1:1 will maximize both populations' yield, which is necessary for the next step such as cell sorting. Therefore, 2.5% FBS was chosen as this condition yielded epithelial type: fibroblast type near 1:1 with increasing and equal cell proliferation rate over 7 days.



Days	0% FBS	2.5% FBS	5% FBS	10% FBS	0% FBS	2.5% FBS	5% FBS	10% FBS
	Epithelial				Fibroblast			
Day 4	182	249	362	376	63	558	575	979
Day 7	55	496*	268	357	53	598	983	1577*

Days	0%FBS	2.5%FBS	5%FBS	10%FBS
	Epithelial:Fibroblast			
Day 4	1 : 0.34	1 : 2.24	1 : 1.58	1 : 2.60
Day 7	1 : 0.96	1 : 1.20	1 : 3.66	1 : 4.42

Figure 2 The optimal percentage of fetal bovine serum (FBS) of primary mixed cell types case no. K092 (A)Brightfield imaging of K092 cell of %FBS, which are 0% FBS, 2.5% FBS, 5% FBS and 10% FBS from day 1 to day 7, magnification = 100X (B) Fluorescence images of K092 detection of CK-19 (green, marker for biliary cells),α-SMA (red, marker for fibroblast), and DAPI (blue, nuclear staining) to identify the number of cell populations (C) Number of total cells of K092 , Ratio of cells population of K092 and Growth rate of K092 cell via Cell Titer-Glo®.

Development of patient-derived CCA organoids

After Immunofluorescence staining validation, J074 and K092 were tested with the 3D formation. The results showed the reduction of projected area in the case of J074 but dynamically changed in K092. Both cases improved their circularity and roundness over 7 days of culture **(Figure 3)** This experiment employed the selected mixed-phenotypic cases (J074 and K092). From the original CCA tissue profile, we demonstrated that there are variations in the degree of fibrosis. In order to mimic the fibrosis condition, we designed the 3D co-culture experiment.

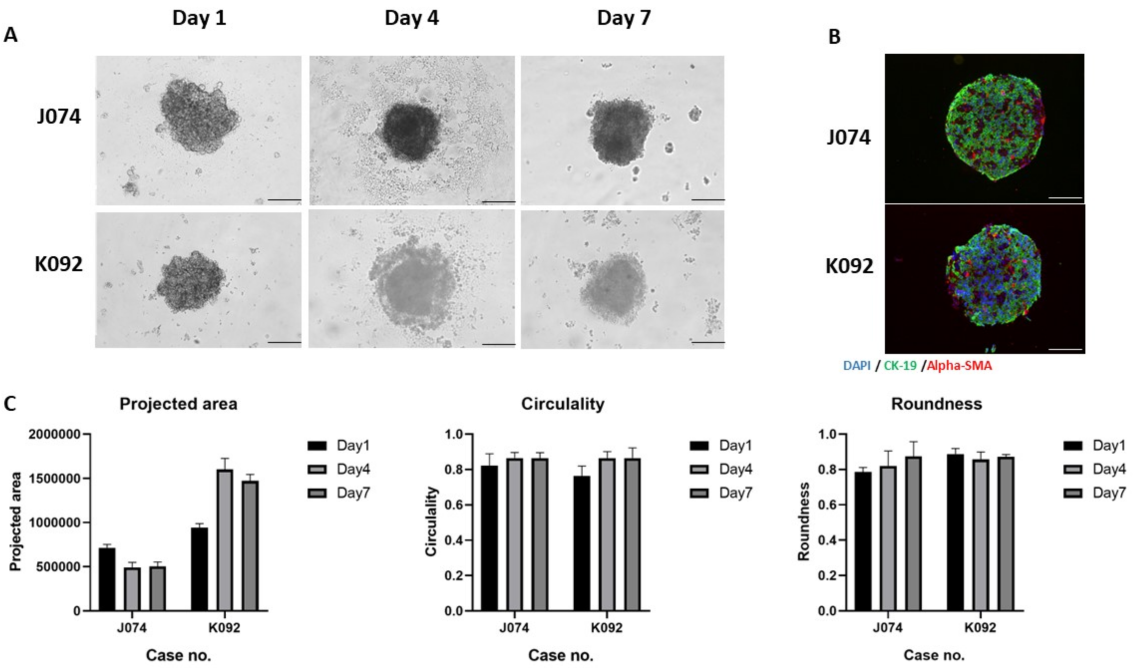


Figure 3 Representative pictures of 3D J074 and K092 from day 1-7, showing the projected area (100X magnification) and Quantification of projected area, circularity, and roundness. Fluorescence images of 3D of J074 and K092 detection of CK-19 (green, marker for biliary cells),α-SMA (red, marker for fibroblast), and DAPI (blue, nuclear staining).

DISCUSSION AND CONCLUSIONS

The pre-clinical models of cancer can be divided into two main models, the *in vitro* model and the *in vivo* or animal models, including two-dimensional (2D) culture, three-dimensional (3D) culture, and patient-derived tumor xenografts (PDTXs). There have been investigations of pre-clinical models to confirm drug-response predictions (Xu et al., 2018). The different drug responses may be due to the different microenvironments of the tumors. The tumor microenvironment is the main factor that can reduce the effectiveness of the chemotherapeutic treatment (Son et al., 2017; Wu et al., 2021). Specifically, patients present with diverse tumor phenotypes which dynamically evolve through the progression(Dagogo-Jack & Shaw, 2018). This impairs the drug mechanism and patient response against tumors with varying origins and phenotypes. Therefore, the effective development of personalized cancer therapies will depend on the capability to scientifically define and model cancer heterogeneity in the laboratory(LeSavage et al., 2022). Throughout history, the *in vitro* models were established from the primary tissues using explant culture, primary cell extraction and culture, and histoculture. When the advancement in stem cell research has taken place, there has also been a genetic-engineered model to recapitulate gene defects that leads to cancer progression. Here we focused on obtaining the primary cells through explant culture to depict the multi-phenotypic cells and expanding them *in vitro*. Explant culture is present primary culture when using tissue from patient. The tissue piece's companion offers certain benefit to migrating cells. Although the absence of cells to the extracellular matrix (ECM), which longer isolation times. The presence of other cell types cannot pose a problem for obtaining pure MSC population because it is limited to the primary culture. The tissue is composed of ECM and cellular components, both of which are active during the process of primary culture.(Hendijani, 2017) As a consequence, this study aims to optimize and control all those concerns by using a tissue engineering approach with the optimized culture system. Sato and the team demonstrated that

3D epithelial organoids could be established from laminin-rich Matrigel or growth factors, including epidermal growth factor (EGF), Noggin, Wnt, and R-spondin (Sato et al., 2011).

For 3D culture establishment consists of Matrigel as ECM substitutes and specific culture medium. Components in organoid culture medium mostly advanced Dulbecco's modified Eagle's medium (ADMEM)/F12 with another supplement or growth factor that have promote of organ development stimulate proliferation, migration, invasion, differentiation carcinogenesis and progression of cancers, and apoptosis such as penicillin/streptomycin, primocin, Glu-taMAX, HEPES, B27, N2, EGF, FGF10, FGF7, hepatocyte growth factor (HGF), Wnt3A, Noggin, R-spondin-1, gastrin, prostaglandin E2, nicotinamide, neuregulin 1, N-acetylcysteine and molecule inhibitor Y27632 (a Rho kinase inhibitor), A-83- 01 (a transforming growth factor-beta inhibitor), and SB202190 (a p38 inhibitor) (Xu et al., 2018)

Although this technique has been employed for decades, the challenges of maintaining live tissues and getting the primary cells remain. Therefore, we optimized the explant culture with 3 different cell culture media, namely, DMEM, Ham's F-12, and Advanced DMEM. The first two media are widely used in the laboratory with different cell types, while the Advanced DMEM is used with specific supplements for specific purposes of cell culture, including CCA organoid culture(Saito et al., 2019). The results showed Advanced DMEM has more capability to generate monolayer cells growing out of the explant structure from different CCA samples. However, the cell types varied from case to case, and most were fibroblast-like cells or mixed phenotypes (fibroblast-like and epithelial-like cells). With advanced DMEM, the time required to generate enough monolayer cells is usually around 2-3 weeks, whereas the other two media did not provide the monolayer cells or took a long time. Hence, we continued other experiments with Advanced DMEM with superior performance despite the higher cost of culture. However, Advanced DMEM showed cells proliferating out of explants with epithelial cells, fibroblasts, or mixed populations. Hence, Advanced DMEM was chosen for culturing cells in the subsequent experiment. Regarding Advanced DMEM composition, it has more types of supplements added to the medium, compared to DMEM and Ham's F-12. We have noticed that the fibroblast-like population dominated the epithelial-like population when doing several subcultures. The literature review stated that adjusting the optimal percentage of FBS may help slow down the domination of fibroblasts and maintain the epithelial population. Therefore, a varying ratio of FBS related to the cell populations was observed in the experiment. In this study, we employed case No. K092 with mixed epithelial-fibroblast phenotypes to study.

Our results showed that 5% and 10% FBS induced more overall cell growth with strong fibroblast domination. While 2.5% FBS can induce overall cell growth but maintain the ratio of epithelial: fibroblast to 1:1. Although the overall cell growth of 2.5% FBS was less than 5% and 10% FBS and may take a longer time for subculture, we continued using 2.5% FBS to maintain the ratio of multi-phenotypic cells. The epithelial-like and fibroblast-like populations of all cases were further characterized with immunofluorescent staining with specific markers to confirm the phenotype. We used cytokeratin 19 (CK19) and alpha-smooth muscle actin (α-SMA) antibodies to differentiate the population.(Massani et al., 2013) The results showed the sample got stained with 2 markers when they were mixed phenotypes and got stained only α-SMA when they had only CAF population.

Finally, the tissue-engineering approach to reassemble the multi-phenotypic cells back to the 3D structure of the organoid. This technique can precisely control cell number and population related to organoid quality control. Therefore, each patient-derived organoid must be individually evaluated with optimized chemotherapeutic agents and concentrations. Our organoid generation pipeline is useful for biomedical applications and can be further developed for a more efficient drug evaluation platform for CCA patients and This study has demonstrated efficient alternative for obtaining patient-derived organoid. However, further investigations are required to characterize the organoid that resemble the true physiological environment of the patient's tumors. Therefore, several factors are needed to examine.

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