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REVIEW

Development and Characterization of Human Primary Cholangiocarcinoma Cell Lines

Abdulkadir Isidan,[*] Ali Yenigun,[*†] Daiki Soma,[*‡] Eric Aksu,[*] Kevin Lopez,[*] Yujin Park,[*] Arthur Cross-Najafi,[*] Ping Li,[*] Debjyoti Kundu,[§¶] Michael G. House,[‖] Sanjukta Chakraborty,[**] Shannon Glaser,[**] Lindsey Kennedy,[§¶] Heather Francis,[§¶] Wenjun Zhang,[*] Gianfranco Alpini,[§¶] and Burcin Ekser[*]

From the Transplant Division,[*] the Department of Surgery,[‖] and the Division of Gastroenterology and Hepatology,[§] Department of Medicine, Indiana University School of Medicine, Indianapolis, Indiana; the Department of General Surgery,[†] Yeditepe University Faculty of Medicine, Istanbul, Turkey; the Division of Transplantation & Hepatobiliary Surgery,[‡] Department of Surgery, University of Florida College of Medicine, Gainesville, Florida; the Division of Research,[¶] Richard L. Roudebush VA Medical Center, Indianapolis, Indiana; and the Department of Medical Physiology,[**] Texas A&M University College of Medicine, Bryan, Texas

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Address correspondence to
Burcin Ekser, M.D., Ph.D.,
Transplant Division, Department of Surgery, Indiana University School of Medicine, 550 University Blvd., Room 4601, Indianapolis, IN 46202.
E-mail: bekser@iupui.edu.

Cholangiocarcinoma (CCA) is the second most common primary liver tumor and is associated with late diagnosis, limited treatment options, and a 5-year survival rate of around 30%. CCA cell lines were first established in 1971, and since then, only 70 to 80 CCA cell lines have been established. These cell lines have been essential in basic and translational research to understand and identify novel mechanistic pathways, biomarkers, and disease-specific genes. Each CCA cell line has unique characteristics, reflecting a specific genotype, sex-related properties, and patient-related signatures, making them scientifically and commercially valuable. CCA cell lines are crucial in the use of novel technologies, such as three-dimensional organoid models, which help to model the tumor microenvironment and cell-to-cell crosstalk between tumor-neighboring cells. This review highlights crucial information on CCA cell lines, including: i) type of CCA (eg, intra- or extrahepatic), ii) isolation source (eg, primary tumor or xenograft), iii) chemical digestion method (eg, trypsin or collagenase), iv) cell-sorting method (colony isolation or removal of fibroblasts), v) maintenance-medium choice (eg, RPMI or Dulbecco's modified Eagle's medium), vi) cell morphology (eg, spindle or polygonal shape), and vii) doubling time of cells. *(Am J Pathol 2022, 192: 1200–1217; https://doi.org/10.1016/j.ajpath.2022.05.007)*

Cholangiocarcinoma (CCA) is a heterogeneous group of malignancies originating from the biliary tree.[1] CCA accounts for approximately 3% of all malignancies of the gastrointestinal system and is the second most common primary hepatic tumor, after hepatocellular carcinoma.[2,3] The prevalence of CCA varies broadly by region; while the prevalence is 1.6 per 100,000 people in the United States, it could be as high as 85 to 90 per 100,000 people in northeastern Thailand, where infection with *Opisthorchis viverrini*, a CCA risk factor, is endemic.[4] Several additional risk factors for cholangiocarcinogenesis have been identified, such as *Clonorchis sinensis* infection, bile duct cyst, primary sclerosing cholangitis, hepatolithiasis, cholelithiasis, and inflammatory bowel disease.[5] Despite the development of state-of-the-art therapies, the 5-year survival rate of CCA is still under 30%.[6,7]

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CCAs are commonly classified, according to anatomic location along the biliary tree, as extrahepatic or intrahepatic (EHCCA or IHCCA).[8] EHCCAs are subcategorized as perihilar or distal.[8] CCAs can also be subclassified based on macroscopic growth pattern (mass forming, periductal infiltrating, intraductal, or mixed), cell of origin (cholangiocytes, goblet cells, hepatic stem cells, or biliary tree stem/progenitor cells), and/or microscopic features (adeno, squamous, adenosquamous, mucinous, undifferentiated, or sarcomatous).[9,10]

Although the importance of organ-specific or cancer-specific microenvironment has been recently highlighted due to cell-to-cell crosstalk, two-dimensional cancer cell lines are one of the best sources for investigating the respective cancer type.[11,12] CCA cell lines have been used for almost 50 years to: i) better understand CCA properties, ii) investigate treatment options, iii) model the disease *in vitro*, and iv) generate *in vivo* xenograft models.[13–16] RPMI 7451 is the first known CCA cell line isolated by George Eugene Moore and his team.[17] Dr. Moore, an oncologist, surgeon, accomplished scientist, and director of the RPMI, pioneered many cancer studies, established several additional cancer cell lines, and formulated RPMI 1640, a widely used cell-growth medium.[18–22]

There is currently a growing interest in CCA-related basic and translational research, due to poor outcomes with the currently available treatment options. For recently developed novel experimental models [eg, three-dimensional (3D) organoids] and treatments, CCA cell lines have been used for the identification of novel mechanistic pathways, biomarkers, and disease-specific genes. Therefore, previously isolated human primary CCA cell lines, their isolation methods, and known essential characteristics are summarized herein to help researchers in their future studies.

Human Primary CCA Cell Lines

The first CCA cell line was established about a half-century ago, in 1971 in the United States[17]; more than half of currently available CCA cell lines were generated in Japan between 1975 and 1990. To date, researchers from Italy, Germany, China, South Korea, and Thailand have reported on the isolation of CCA cell lines and related studies. In the past 2 decades, researchers have reported the isolation of multiple CCA cell lines in Thailand more than in any other country, possibly due to the high prevalence of the disease in that geographic region.[4]

Each CCA cell line is unique in reflecting a specific genotype, sex-related properties, and patient-related signatures, making them especially valuable for scientific and commercial applications. It would undoubtedly be helpful to have an international and collective cell bank that includes cell-identification analysis from each study to appropriately define the CCA properties and prevent any misidentification of the existing cell lines.[23] For instance, the ETK-1 CCA cell line was retracted in 2004 after a short tandem repeat polymorphism analysis revealed that the ETK-1 and SSP-25 cell lines were identical.[24,25] However, later, it was understood that SSP-25 should have been retracted instead of ETK-1 because the SSP-25 and RBE cell lines were isolated from the same patient, but their short tandem repeat profiling results did not match (*https://cell.brc.riken.jp/en/rcb/rbessp-25_announce*, last accessed May 5, 2022). As a result, the ETK-1 cells have been distributed under the name of SSP-25 since 2004, but it is unclear when that cell line was initially misidentified. It is also unclear what happened to the SSP-25 cell line. Similarly, the M156, M213, and M214 cell lines were believed to have been isolated from three separate patients; however, results from recent short tandem repeat profiling showed that they were isolated from the same patient.[26]

Cross-contamination of cell lines is a long-standing problem that has resulted in scientific errors[27] and may initiate inaccurate data chains. Various levels of the cell-culturing process have been blamed for cross-contamination, including: i) accidental inoculation, ii) mislabeling, iii) confusion in freeze-thawing, iv) working with more than one cell line in a biosafety cabinet at the same time, and v) contamination of the stock bottle of media with cells.[28,29] Additionally, cancer cells may carry a greater risk for cross-contamination due to their greater capacity for proliferation. Even a minimal amount of inoculation of cancer cell lines could suppress other cell lines in the culture and take their place over time. Therefore, the preservation and maintenance of the cell line is as important and challenging as is the establishment of one.

In a different case, CHGS was misidentified as a CCA cell line in 2015 by Zach et al[30] and listed in the Cellosaurus database (accession number CVCL_M272; *https://web.expasy.org/cellosaurus*, last accessed May 5, 2022). However, the first mention of CHGS, in 1988 by Katoh et al,[31] indicates that CHGS is a CCA tissue line passaged in mice, and that cancer cells have not been isolated from this tissue line. Similarly, the CC-CL-1 cell line was studied as a CCA cell line in a research study; however, none of the references cited in the study contain information regarding the CC-CL-1 cell line, generating doubts about its credibility.[32]

In addition to primary CCA cell lines, derivative CCA cell lines have been established for drug resistance studies. QBC939/ADM is doxorubicin resistant; HuCCT1-G100, YSCCC-G100, RTFK-1, KKU-M139/GEM, KKU-213B/GEM, MT-CHC01R1.5, and SNU-1196/GR are gemcitabine resistant; and KKU-M055/46 and KKU-213B/246 are 5-fluorouracil–resistant CCA cell lines.[16,33–37] The KKU-213L5 cell line is a derivative of the KKU-213A cell line, with high metastatic activity.[38]

Table 1 Human Primary Cholangiocarcinoma Cell Lines Established between 1971 and 2000

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
1	1971	USA	RPMI 7451	CCA	—	—
2	1975	Japan	H-1 or H1	CCA	—	—
3	1981	Japan	OZ	IHCCA	Ascites	No digestion
4	1984	Germany	EGI-1	CCA	Primary tumor	—
5	1985	Japan	HChol-Y1	CCA	Primary tumor	No digestion
6	1985	Germany	SK-ChA-1 or WITT	EHCCA	Ascites	No digestion
7	1987	Japan	KMCH-1	IHCCA + HCC	Primary tumor	0.5 U/mg type IV collagenase in PBS, 37°C, 30 minutes
8	1988	Japan	HuH-28	IHCCA	Primary tumor (frozen-thawed)	500 U/mL trypsin
9	1989	Japan	HuCC-T1	IHCCA	Ascites	No digestion
10	1989	Japan	MEC	CCA	Pleural effusion	No digestion
11	1990	USA	PCI:SG231	IHCCA	Primary tumor	Type II collagenase, 37°C, 3 hours
12	1991	Thailand	HuCCA-1	IHCCA	Primary tumor	No digestion

(*table continues*)

A/A, antibiotic + antimycotic; CCA, cholangiocarcinoma; CMRL, Connaught Medical Research Laboratories medium; DMEM, Dulbecco's modified Eagle's medium; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; IHCCA, intrahepatic cholangiocarcinoma; MEM, minimal essential medium; P/S, penicillin/streptomycin.

Table 1 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
—	Eagle's MEM + 10% FBS + nonessential amino acids + 60 pg/mL gentamicin	Tightly adherent, monolayer, polygonal shaped cells	—	17,39,40
—	—	—	—	40,41
Colony isolation by trypsin and EDTA-soaked filter; other cells removed by enzymatic and mechanical treatments	Williams' E + 10% newborn calf serum + P/S	Large nucleus with 1 to 2 nucleoli; dark cytoplasm; high nucleus/cytoplasm ratio; pavement-like proliferation. Abundant production of gel-like substance	48 hours	42
—	MEM + 10% FBS + 2× MEM amino acids (essential and nonessential) + 4 mmol/L L-glutamine + 1 mmol/L sodium pyruvate	Monolayer, adherent, polymorphic cells	45—50 hours	43
Fibroblasts spontaneously disappeared in 2 months	Ham's F12	Uniform, monolayer cells; including abundant granules; prominent round nucleus with multiple nucleoli	52 hours	44
Light trypsin treatment	CMRL + 15% + P/S	Adherent, spindle- to polygonal shaped, polymorphic cells; proliferating as single adherent cells or small clusters	48 hours	45,46
Colony isolation by trypsin	DMEM + 20% FBS + 35 μmol/L sodium bicarbonate + P/S	Large, round nucleus with multiple nucleoli; abundant clear cytoplasm; pavement-like proliferation; microvilli on the luminal surfaces	39 hours	47
—	RPMI 1640 + 20% FBS + %0.2 lactalbumin hydrolysate	Spindle- to polygonal shaped cells	80 hours	48
IS-RPMI medium used to eliminate fibroblasts	RPMI 1640 + 0.2% lactalbumin hydrolysate	Polygonal to spindle-shaped, abundant and clear cytoplasm, proliferation in pavement arrangement	74 hours	49,50
—	RPMI 1640 + 20% FBS	Polymorphic, epithelial-like cells; high nucleus/cytoplasm ratio	40 hours	51
—	α MEM (Earle's salts) with nucleosides + 10% FBS + 60 pg/mL gentamicin	Tightly adherent, monolayer, polygonal shaped cells; nonadherent cell clusters that formed three-dimensional tubular structures resembling bile ducts	—	40
Fibroblasts gradually decreased with each passage and disappeared in a month	Ham's F12 + P/S	Monolayer, adherent, polygonal shaped epithelial cells with occasionally multiple, round to oval nuclei; granule-filled cytoplasms; piling up of cells and occasional gland-like appearances	55 hours	52

Table 1 Human Primary Cholangiocarcinoma Cell Lines Established between 1971 and 2000

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
13	1991	Japan	KMBC	EHCCA	Xenograft	Collagenase 150—250 U/mg, 37°C, 90—110 minutes
14	1992	USA	CC-LP-1	IHCCA	Primary tumor	0.05% (w/v) collagenase type IV, 0.002% (w/v) DNase type 1, 90 minutes
15	1992		CC-SW-1			
16	1992	Japan	KMC-1	IHCCA	Xenograft	0.5 mg/nL type IV collagenase in PBS, 37°C, 60—80 minutes
17	1993	China	QBC939	EHCCA	Primary tumor	Type I collagenase, DNase I, plasminase
18	1994	Japan	TK	EHCCA	Ascites	No digestion
19	1995	Japan	OCUCh-LM1	EHCCA	Primary tumor	No digestion
20	1995	Japan	TFK-1	EHCCA	Primary tumor	1000 U/mL dispase, 37°C, 30 minutes
21	1996	Japan	KMCH-2	IHCCA + HCC	Primary tumor	150—250 U/mg collagenase in PBS, 37°C, 90—110 minutes
22	1997	Japan	ETK-1	IHCCA	Ascites	No digestion

(*table continues*)

A/A, antibiotic + antimycotic; CCA, cholangiocarcinoma; CMRL, Connaught Medical Research Laboratories medium; DMEM, Dulbecco's modified Eagle's medium; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; IHCCA, intrahepatic cholangiocarcinoma; MEM, minimal essential medium; P/S, penicillin/streptomycin.

Table 1 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
Fibroblasts scraped away and completely disappeared after several passage	DMEM + 5% FBS + 12 mmol/L sodium bicarbonate + P/S	Polymorphic epithelial-like cells; one or more large, irregular, round to oval nuclei with a few prominent nucleoli; relatively poor, round to polygonal cytoplasm; pavement-like proliferation; tubular formation	30 hours	53,54
Centrifugation on double-layer (75%/100% v/v) Ficoll-Hypaque gradients; fibroblasts removed by differential trypsinization	DMEM + 15% FBS + 2 mmol/L L-glutamine + antibiotics	Cobblestone-like proliferation of monolayers; stratification of cells in some areas	180 hours 72 hours	55
Tumor cells suppressed the fibroblasts by the time	DMEM + 10% or 5% FBS	Monolayer, pavement-like proliferation; clear cytoplasm, oval-shaped nuclei; tubular formation; some cells with mucin in the cytoplasm	54 hours	56
Fibroblasts gradually decreased by passaging	RPMI 1640 + 10% FBS	Monolayer, polymorphic, adherent cells; round or oval nuclei; high nucleus/cytoplasm ratio	24 hours	57,58
Colony isolation using 0.33% agar	RPMI 1640 + 15% FBS + 2 mmol/L glutamine + 1 mmol/L sodium pyruvate	Monolayer, adherent proliferation; forming gland-like structures; lobated or dark, large nuclei	29 hours	59
Fibroblasts gradually decreased and disappeared in 1 month	DMEM + 10% FBS + 2 mmol/L L-glutamine + 0.5 mmol/L sodium pyruvate + P/S	Monolayer, pavement-like proliferation; clear cytoplasm and oval nuclei	31 hours	60
Fibroblasts removed by mechanical scraping and differential attachment selection with trypsin	RPMI 1640 + 10% FBS + P/S	Polygonal epithelial monolayers; pavement-like proliferation	37 hours	61
Fibroblasts removed by mechanical scraping	DMEM + 5% FBS + 12 mmol/L sodium bicarbonate + P/S	Monomorphic polygonal cells; large round to oval nuclei with a few prominent nucleoli; pavement-like proliferation	44 hours at 20th passage; 32 hours at 55th passage	62
Limiting dilution	RPMI 1640 + 10% FBS + 10 mmol/L HEPES + 2 mmol/L L-glutamine + 0.1 mmol/L nonessential amino acids + 1 mmol/L sodium pyruvate + 0.005 mmol/L β-mercaptoethanol	Small polygonal cells; round to oval nuclei and prominent nucleoli; uniform, monolayer with a pavement-like proliferation	71 hours	41

Table 1 Human Primary Cholangiocarcinoma Cell Lines Established between 1971 and 2000

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
23	1997	Japan	ICBD-1	EHCCA	Primary tumor	No digestion
24	1997	Japan	SSP-25	IHCCA	Primary tumor	No digestion
25			RBE			
26	1998	Japan	TBCN-1	EHCCA	Primary tumor	—
27	1999	Thailand	KKU-213A	IHCCA	Primary tumor	0.25% trypsin-EDTA, 37°C, 1 hour
28			KKU-213B			
29			KKU-213C			
30	1999	Japan	YSCCC	CCA	—	—
31	2000	Japan	HBDC	EHCCA	Ascites	No digestion

(*table continues*)

A/A, antibiotic + antimycotic; CCA, cholangiocarcinoma; CMRL, Connaught Medical Research Laboratories medium; DMEM, Dulbecco's modified Eagle's medium; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; HCC, hepatocellular carcinoma; IHCCA, intrahepatic cholangiocarcinoma; MEM, minimal essential medium; P/S, penicillin/streptomycin.

Tables 1 and 2 summarize the CCA cell lines available between 1971 and 2000, and between 2001 and 2021, respectively, from the English and non-English published literature. Tables 1 and 2 include: i) the type of CCA (eg, intra- or extrahepatic), ii) isolation source (eg, primary tumor or xenograft), iii) tissue-digestion method (eg, trypsin or collagenase), iv) cell-sorting and -purification methods (colony isolation or removal of fibroblasts), v) maintenance-medium choice (eg, RPMI or DMEM), vi) cell morphology (eg, spindle or polygonal shape), and vii) doubling time of cells.

Overview of Isolation Methods

The general principles of the CCA cell—isolation protocol are summarized in Figure 1. CCA cell isolation requires either a solid sample (eg, primary tumor or xenograft) or a liquid sample (eg, ascites or pleural effusion). Samples can come from autopsy, surgery, paracentesis, or animal harvesting. Samples can be transferred into Ham's F12 culture medium at 4°C.[52] It is better to process the samples as soon as possible to avoid ischemia-related problems in cells. If the CCA

Table 1 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
Fibroblasts gradually decreased and disappeared in 1 month	RPMI 1640 + 10% FBS + 0.5 mmol/L sodium bicarbonate + 2 mmol/L L-glutamine + P/S	Large round to oval nuclei with a few nucleoli; relatively poor, polygonal to oval cytoplasm; pavement-like proliferation	31.5 hours	63
Fibroblasts gradually disappeared, limiting dilution	RPMI 1640 + 10% FBS	Spindle-shaped cells	64 hours	64
		Monolayer of polygonal cells, pavement-like proliferation	45 hours	
—	RPMI 1640 + 10% FBS	—	54 hours	65
—	DMEM + 10% FBS + A/A	Small, spindle-shaped cells	23 hours	26
		Irregular polygonal cells; high nucleus to cytoplasmic ratio; patch-like structure	24.5 hours	
		Irregular polygonal cells; high nucleus to cytoplasmic ratio	25.6 hours	
—	RPMI 1640 + 10% FBS	Lymphocyte-like	—	66, *https://cellbank.brc.riken.jp/cell_bank/CellInfo/?cellNo=RCB1549&lang=*, last accessed May 5, 2022
Centrifugation on triple-layer (75%/100%/25) Ficoll-Hypaque gradients; colony isolation using porcelain cloning rings	Williams' E + 10% FBS + 2 ng/mL HGF + 2 mmol/L L-glutamine + 6 mmol/L glucose + 0.5 mmol/L sodium bicarbonate + 100 ng/mL kanamycin + 10 ng/mL fungizone	Polygonal or spindle-shaped polymorphic cells; occasional large vacuoles in the cytoplasm; forming small clusters or clumps; one or more large, irregular, round or oval nuclei with a few prominent nucleoli; pavement-like proliferation	32 hours	67

sample needs to be preserved, it can be freeze-thawed in 10% dimethyl sulfoxide in culture medium[48] or can be preserved in histidine-tryptophan-ketoglutarate organ-preservation solution at 4°C until tissue processing.[68]

CCA cell lines can be isolated using several methods.[44,47] Herein, the general perspective of isolation protocols is briefly summarized. All tissue processing should be performed under aseptic conditions in a biological safety cabinet with sterile or disposable surgical instruments. The first and the most essential step of CCA cell isolation from solid samples is cutting the tissue into small pieces (>1 mm in diameter).[44–47] After that, the protocol can be continued either with or without enzymatic digestion. For the protocols without enzymatic digestion, a stainless-steel mesh is helpful for releasing CCA cells.[44] Chemical digestion usually proceeds at 37°C, and the digestion time is dependent on the concentration and type of the enzyme used (Table 1). Several types of collagenases (types I, II, and IV) and trypsin have been successfully used for chemical digestion (Table 1). Plasminase to digest fibrin clumps and DNase I to lyse DNA leaked into cell suspension can be included in the dissociation protocol (Figure 1).[57]

After chemical dissociation, the cell suspension should be filtered with 70 μm of mesh and centrifuged[47]; the pellet can then be resuspended in a variety of media conditions [RPMI, Dulbecco's modified Eagle medium, minimum

Table 2 Human Primary Cholangiocarcinoma Cell Lines Established between 2001 and 2021

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
1	2001	Republic of Korea	Choi-CK	IHCCA	Primary tumor	No digestion
2			Cho-CK			
3			JCK			
4			SCK			
5	2002	Thailand	HubCCA-1	IHCCA	Intrahepatic biliary fluid	—
6	2002	Thailand	KKU-100	IHCCA	Primary tumor	0.025% trypsin-EDTA, 37°C, 1 hour
7	2002	Republic of Korea	SNU-245	EHCCA	Primary tumor	No digestion
8			SNU-1079	IHCCA		
9			SNU-1196	EHCCA		
10	2004	Japan	TKKK	IHCCA	—	—
11	2005	Thailand	KKU-M055	IHCCA	—	—
12			KKU-OCA17			
13	2005	Japan	TBCN6	EHCCA	Xenograft	0.25% trypsin in PBS, 37°C, 10 minutes
14			TGBC-47			

(*table continues*)

CCA, cholangiocarcinoma; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EGF, epidermal growth factor; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; IHCCA, intrahepatic cholangiocarcinoma; KFSM, keratinocyte serum free medium; MEM, minimal essential medium; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; ROCK, Rho-associated kinase.

Table 2 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
Differential trypsinization, scraping, or G418 treatment (100 μg/mL)	Opti-MEM + 10% FBS + 30 mmol/L sodium bicarbonate + antibiotics	Polygonal and compact cells with indistinct cell boundaries	—	75
		Polygonal and compact cells with indistinct cell boundaries		
		Polygonal morphology and some spindle-shaped cells		
		Monolayer, epithelioid or spindle-shaped cells; fewer polygonal shaped cells		
—	Ham's F12 + 20% FBS + P/S	Adherent, monolayer, polygonal to spindle-shaped cells	—	76,77
Fibroblasts removed with a cell scraper and by differential trypsinization	Ham's F12 + 20% FBS + P/S	Compact, polygonal to spindle-shaped cells; floating or clumping in a confluent monolayer; large nucleus containing two to five nucleoli and a clear cytoplasm	72 hours	77,78
Differential trypsinization used to remove fibroblasts	RPMI 1640 + 10% FBS	Monolayer, adherent cells; trabecular arrangement with acinar formation	54 hours	79
		Monolayer, adherent cells; pleomorphic appearance with multiple cytoplasmic processes; numerous cytoplasmic vacuoles in some cells; some multinucleated cells	72 hours	
		Monolayer, adherent cells; trabecular pattern consisted of spindle to polygonal shaped cells having vesicular nuclei and multiple small nucleoli	48 hours	
—	DMEM (low glucose) + 10% FBS	Adherent, monolayer, epithelial-like cells; pavement-like proliferation	—	80
—	Ham's F-12 + 10% FBS + P/S	—	—	81
—	DMEM + 10% FBS	Adherent, monolayer, polygonal cells	38 hours	82

Table 2 Human Primary Cholangiocarcinoma Cell Lines Established between 2001 and 2021

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
15	2006	Thailand	KKU-M139	CCA	Primary tumor	—
16	2006	Thailand	RMCCA-1	IHCCA	Primary tumor	No digestion
17	2007	China	HKGZ-CC	IHCCA	Primary tumor	1200—2000 U/mL collagenase and DNase in 10 mL/g DPBS
18	2007	Japan	IHGGK	IHCCA	—	—
19	2009	Thailand	CL-2	CCA	—	—
20			CL-6			
21			CL-19			
22	2010	Japan	NCC-BD1	EHCCA	Xenograft	No digestion
23			NCC-BD2	EHCCA		
24			NCC-CC1	IHCCA		
25			NCC-CC3-1	IHCCA		
26			NCC-CC3-2	IHCCA		
27			NCC-CC4-1	IHCCA		
28	2013	China	HCCC-9810	IHCCA	—	—
29	2015	Italy	MT-CHC01	IHCCA	Xenograft	200 U/mL collagenase, 3 hours, 37°C
30	2016	USA	ICC1	IHCCA	—	Trypsin, 30 minutes, 37°C
31			ICC2			
32			ICC5			
33	2016	China	ZJU-0826	EHCCA	Primary tumor	—
34			ZJU-1125	IHCCA		
35	2017	Germany	CCC-5	EHCCA	Pleural effusion	No digestion

(*table continues*)

CCA, cholangiocarcinoma; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EGF, epidermal growth factor; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; IHCCA, intrahepatic cholangiocarcinoma; KFSM, keratinocyte serum free medium; MEM, minimal essential medium; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; ROCK, Rho-associated kinase.

Table 2 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
—	Ham's F12 or RPMI 1640 + 10% FBS + P/S	Adherent, monolayer, polygonal cells; pavement-like proliferation	17 hours	16,76
Differential trypsinization used to remove fibroblasts	Ham's F12 + 20% FBS + EGF + 250 μg/mL amphotericin + P/S	Circular to spindle shape with many processes and ornamental fringes; granulated nucleus and cytoplasm	48 hours	83
—	DMEM	Adherent, monolayer, epithelial-like cells	48 hours	84
—	RPMI 1640 + 10% FBS + P/S	Adherent, monolayer, epithelial-like cells		85, *http://www2.idac.tohoku.ac.jp/dep/ccr/TKGdate/TKGvo106/0623.html*, last accessed May 5, 2022
—	DMEM + 15% FBS + P/S	—	—	86,87
Fibroblasts removed by mechanical scraping	RPMI 1640 + 10% FBS + 2 mmol/L L-glutamine + P/S	Adherent, monolayer, epithelial-like cells; pavement-like proliferation	—	88
—	RPMI 1640 + 10% FBS + P/S	—	20 hours	89
—	Knockout/DMEM/F-12 + 10% FBS + P/S	Monolayer, adherent cells	40 hours	90
—	RPMI or DMEM/F12 + 5% FBS + P/S	—		91
Fibroblasts eliminated by differential trypsinization and differential attachment	RPMI 1640 + 10% FBS + antibiotics	Monolayer, adherent, homogeneous cells with characteristic loose pleomorphic cells and rare multinucleated cells	63 hours	92
		Monolayer, epithelial-like, adherent cells; polygonal, occasionally multinucleated	44 hours	
—	DMEM + 20% FBS: KFSM - 2:1	Monolayer, spindle- to polygonal shaped cells, pavement-like proliferation; anaplastic, multinucleated giant cells	60 hours	93

Table 2 Human Primary Cholangiocarcinoma Cell Lines Established between 2001 and 2021

Cell no.	Year	Country	Cell name	Diagnosis	Isolation source	Digestion method
36	2017	Thailand	KKU-023	IHCCA	Primary tumor	1 mg/mL collagenase, 37°C, 30–45 minutes
37			KKU-452	EHCCA		
38	2018	China	ICC-1	IHCCA	Primary tumor	No digestion
39			ICC-2			
40	2019	Thailand	KKK-D049	IHCCA	Xenograft	1000 U/mL collagenase + 0.1 mg/mL DNase I, 3 hours
41			KKK-D068			
42			KKK-D131			
43			KKK-D138			
44	2021	Italy	82.3	IHCCA	Xenograft	Collagenase 200 U/mL, 3 hours, 37°C

(*table continues*)

CCA, cholangiocarcinoma; DMEM, Dulbecco's modified Eagle's medium; DPBS, Dulbecco's phosphate-buffered saline; EGF, epidermal growth factor; EHCCA, extrahepatic cholangiocarcinoma; FBS, fetal bovine serum; IHCCA, intrahepatic cholangiocarcinoma; KFSM, keratinocyte serum free medium; MEM, minimal essential medium; PBS, phosphate-buffered saline; P/S, penicillin/streptomycin; ROCK, Rho-associated kinase.

essential medium, William's, and Ham's F12].[42–44,47,49] Stromal fibroblasts are the primary contaminating cells in CCA cell cultures.[69] After cell attachment, CCA cells can be purified using scraping of the contaminating cells, colony isolation, and/or passaging cells until all contaminating cells have been removed (Figure 1).[42,44,49]

Need for More CCA Cell Lines

Cell lines provide indispensable *in vitro* model systems for the assessment of features of cancer cells, since they are direct descendants of the primary tumors.[12] Under the right conditions, most of the phenotypic and genotypic properties of the primary tumor are preserved in cancer cell lines.[11] After >50 years of research, these features have allowed for the establishment of thousands of cell lines from various malignancies, of which only 70 to 80 belong to the CCA family (Tables 1 and 2). While each new cell line is not necessarily superior to previous lines, collectively, they have been important for the understanding of common and differing features of CCA, including: i) antigenic variations, ii) new genotypes, and iii) enzymes that sustain tumorigenic growth to drug sensitivities or resistances.

One reason that CCA remains difficult to cure is the lack of a precise understanding of oncogenesis.[70] The established CCA cell lines can be utilized to identify the properties of cells that can help in predicting positive or negative responses to antigen- or pathway-targeted therapies. CCA cell lines could be powerful in determining many aspects of CCA phenotypes, especially when used in a 3D microenvironment in the presence of other liver cells to study the impact of adjacent cells in the tumor milieu.[71] The 3D tumor organoid system can be used for the identification of candidate novel therapies for future clinical trials.[72]

Table 2 (*continued*)

Sorting method	Maintenance medium	Cell morphology	Doubling time	References
Fibroblasts aseptically removed with a cell scraper	Ham's F12 + 10% FBS + P/S + nonessential amino acids + 12.5 mmol/L HEPES + 50 μg/mL cefazolin + 10 μg/mL ciprofloxacin + 2.5 μg/mL amphotericin B + 5 μmol/L ROCK inhibitor	Ovoid to cuboid shape polygonal cells; seldom multinucleated; forming compact monolayer with occasional multinucleated cells	34 hours	94
		Spindle-shaped cells; seldom multinucleated; segregated and spread surround	17 hours	
—	—	—	—	73
Stromal cells sequentially removed by partial trypsinization and mechanical removal	DMEM + 10% FBS + P/S	Epithelial-like cells; high nuclear to cytoplasmic ratio; tight clustering	—	95
		Epithelial-like, polygonal and spindle-shaped cells; high nuclear to cytoplasmic ratio	—	
		Epithelial-like, polygonal and spindle-shaped cells; high nuclear to cytoplasmic ratio	—	
		Epithelial-like, polygonal and spindle-shaped cells; high nucleus-to-cytoplasm ratio	—	
	10% FBS + P/S + gemcitabine	Monolayer, adherent, epithelial-like cells	53 hours	74

In a recent study, an established IHCCA cell line was used for understanding the impact of RA190, a proteasome subunit ADRM1 inhibitor that induces cell apoptosis, in a pathway-targeted therapy model.[73] Although the outcomes of that *in vitro* study were encouraging, the study did not evaluate the roles of the tumor microenvironment and other CCA cell lines to determine whether a genotypic difference within the CCA lines may have modulated (increased or decreased) the response and sensitivity to RA190.[73] A counterexample is evidenced by the recent discovery in Italy of a novel IHCCA cell line characterized by the presence of genes encoding resistance to fluorouracil, carboplatin, and oxaliplatin—drugs actively and frequently used in CCA treatment.[74]

Future Perspectives

The establishment of new, well-defined, and well-characterized CCA cell lines is crucial in enhancing the understanding of this aggressive disease. The establishment of oncogenesis through the cancer cells provides information on their drug-sensitivity and drug-resistance mechanism, and helps to identify possible novel drug targets. The inclusion of tumor stromal cells, such as hepatic stellate cells, liver sinusoidal endothelial cells, and tumor-associated macrophages, in the CCA cell lines makes these culture models more representative of the CCA tumor microenvironment (ie, 3D tumor organoids)[71] (Figure 2). Therefore, the inclusion of multiple other cell types will help to determine the ways in which CCA cells interact with other neighboring cells and alter their genotypes, phenotypes, and transcriptomic profiles.

The establishment of well-characterized CCA cell lines with a close resemblance to primary tumors, especially in a 3D microenvironment, will provide an infinite capacity for replicability, limiting the use of small animal *in vivo* studies and making them prime materials for CCA research.

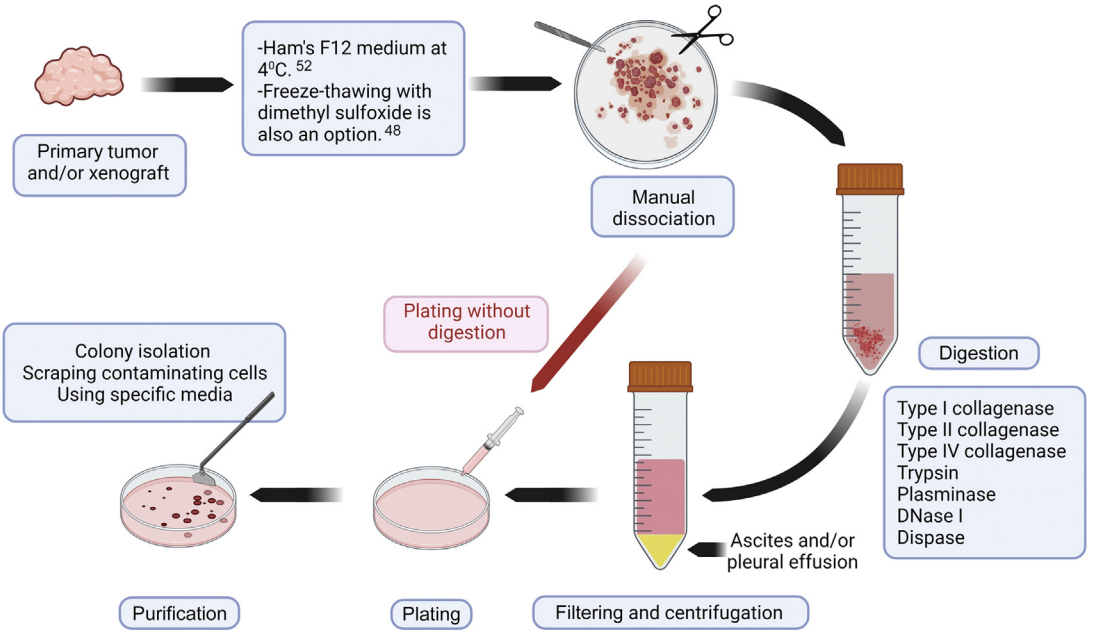

Figure 1 CCA cell isolation method. CCA cell lines can be isolated from either fresh samples (eg, HuCCA-1 cell line) or frozen-thawed samples (eg, huh-28 cell line) from primary tumors or from xenografts, kept in different mediums, after manual dissociation, plated with or without digestion.

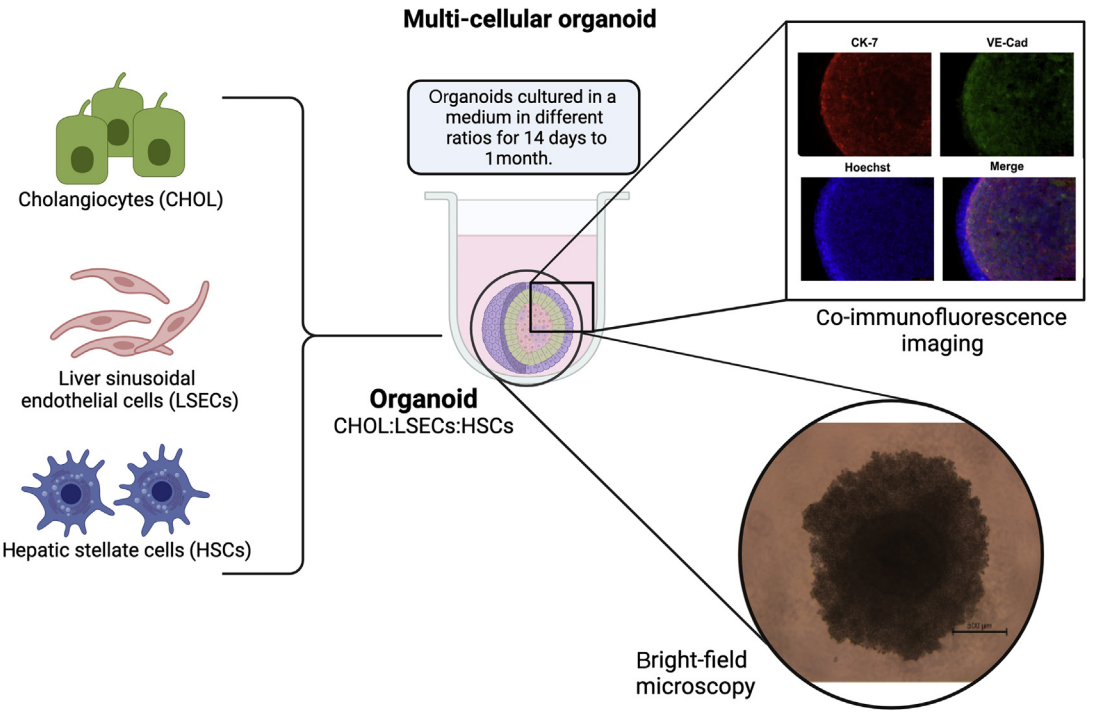

Figure 2 CCA organoids with multiple cell lines. Three-dimensional (3D) CCA organoids include multiple cell lines, such as CCA cell lines (cholangiocytes; CHOL), liver sinusoidal endothelial cells (LSECs), and hepatic stellate cells (HSCs) (representative). Other liver cells, such as hepatocytes and Kupffer cells, can also be added, if needed. These organoids can be made scaffold-free without any biomaterial (eg, Matrigel) in a low-binding plate using culture mediums and can be kept for 14 to 28 days. Evidence suggests that CCA cells express cytokeratin (CK)-7, a cholangiocyte marker, and organoids can be stained with other cellular markers (eg, vascular endothelial cadherin; VE-Cad) that are present in organoids. CCA organoids can be exposed to immune cells, as seen at the bottom right. Bright-field microscopy shows a 3D CCA organoid exposed to mast cells. Scale bar = 500 μm. Original magnification, ×10.

Author Contributions

A.I. drafted the manuscript. A.Y., D.S., and E.A. wrote and reviewed the manuscript; B.E. developed the concept and wrote and critically reviewed the manuscript. All of the authors approved the final version.

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