Journal of the National Cancer Center 4 (2024) 195–202

Contents lists available at ScienceDirect

Journal of the National Cancer Center

journal homepage: www.elsevier.com/locate/jncc

Review

Primary liver cancer organoids and their application to research and therapy

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ARTICLE INFO

Keywords:
Primary liver cancer
Organoids
Drug screening
Tumorigenesis

ABSTRACT

Primary liver cancer is a leading cause of death worldwide. To create advanced treatments for primary liver cancer, studies have utilized models such as 2D cell culture and *in vivo* animal models. Recent developments in cancer organoids have created the possibility for 3D *in vitro* cultures that recapitulates the cancer cell structure and operation as well as the tumor microenvironment (TME). However, before organoids can be directly translated to clinical use, tissue processing and culture medium must be standardized with unified protocols to decrease variability in results. Herein, we present the wide variety of published methodologies used to derive liver cancer organoids from patient tumor tissues. Additionally, we summarize validation methodologies for organoids in terms of marker expression levels with immunohistochemistry as well as the presence of mutations and variants through RNA-sequencing. Primary liver cancer organoids have exciting applications allowing for faster drug testing at a larger scale. Primary liver cancer organoids also assisit in uncovering new mechanisms. Through the coculture of different immune cells and cancer organoids, organoids are now better able to recapitulate the liver cancer TME. In addition, it further aids in the investigation of drug development and drug resistance. Lastly, we posit that the usage of liver cancer organoids in animal models provides researchers a methodology to overcome the current limitations of culture systems.

1. Introduction

Cancer ranks as the second most common cause of death across all age groups as well as the primary cause of death for individuals under 85 years old in the world.[1] For age groups older than 15 years old, liver cancer and other gastrointestinal (GI) malignancies are in the top 5 cancer incidences in the world. The estimated incidence of liver cancer cases in the United States are 53,980 in 2024 with the incidence of liver cancer continuing to increase by ~2% per year in adults younger than 50 years.[1] The majority of the primary liver cancer cases are hepatocellular carcinoma (HCC) and biliary tract cancer which includes cholangiocarcinoma (CCA) and gallbladder cancer (GBC).[2] While the five-year survival rate increased for primary liver cancers and the cancer mortality rate decreased in the last 70 years, liver cancer incidence rates increased steadily annually in the last five years.

Recent breakthroughs in cancer treatment with immunotherapy have greatly improved overall survival of patients with cancers including HCC[3,4] and CCA.[5] However, treatment outcomes are still suboptimal considering the objective response rate is ~30%. There is a highly unmet need to explore innovative therapeutic strategies grounded in tumor biology including the tumor microenvironment (TME) and tumor-immune interaction. Multiple models have been employed to dissect the TME and investigate efficiency of liver cancer treatments.

Research models for many years included *in vitro* 2D cancer cell cultures and animal models. *In vitro* 2D culture systems contain a highly homogenous cell population under controlled culture conditions. However, reproducible results derived from *in vitro* 2D culture systems present challenges to recapitulate tumor heterogeneity, cell-to-cell interactions, and various biological processes within the TME. Thus, it eventually leads to poor translation into clinical use. There are multiple animal models to study different variations and disease presentations of liver cancers including genetically engineered mouse models. In addition, there are methods to create backgrounds of diseased liver such as non-alcoholic fatty liver disease (NAFLD), viral hepatitis infection, fibrosis, and many more. These mouse models do not completely recapitulate the TME and immune system interactions in addition to being time-consuming and costly. Recently there has been an attempt to overcome the limitations of *in vitro* 2D cell culture and animal models in combination with the goal to recapitulate tumor heterogeneity with the development of cancer organoids.[6]

The contemporary term “organoid” describes cells growing in a controlled 3D setting *in vitro* which leads to the creation of small clusters

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https://doi.org/10.1016/j.jncc.2024.06.002
Received 4 March 2024; Received in revised form 2 May 2024; Accepted 13 June 2024

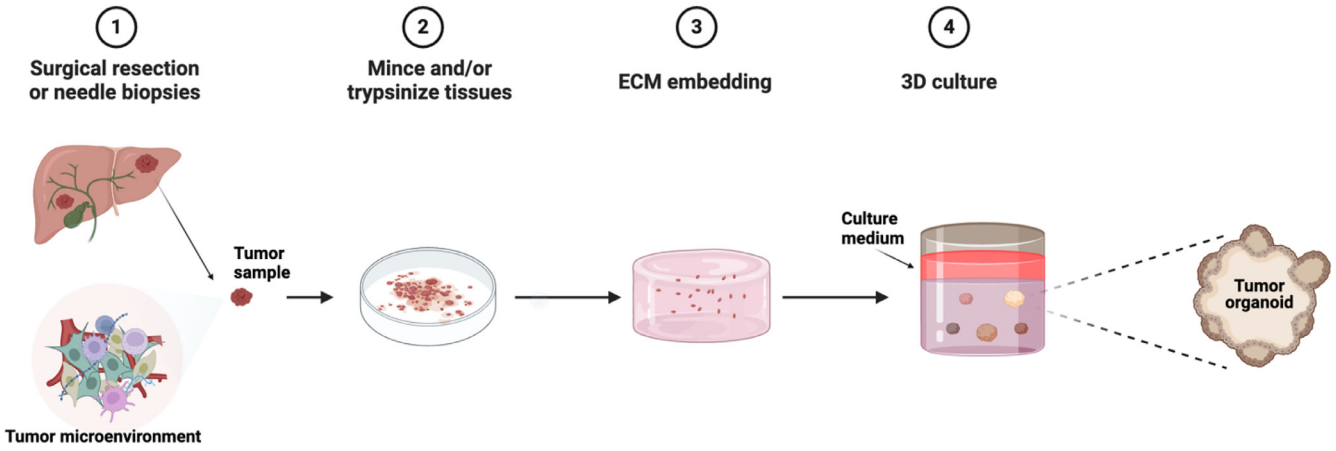

Fig. 1. Schematic diagram of patient-derived liver organoid generation *in vitro*. ECM, extracellular matrix.

of cells that autonomously organize and mature into functional cell varieties. The first successful liver organoid model was reported in 1985, where rat hepatic cells were cultured in a 3D matrix.[7] However, it took another 20 years before human liver organoids were first reported in 2008 when human liver stem cells were used to create 3D structures.[8] Subsequently, human liver cancer organoids were reported in 2017.[2] The research has witnessed significant advancements since then in terms of characteristics and application of liver cancer organoids. Organoids attempt to recapitulate the structure and operations of an organ or TME *in vivo*. With recapitulation of the tumor complexity, the value of the liver cancer organoids grows to capture the heterogeneity of the patient population and provide valuable insights into drug screening and developments. In addition, the value of liver cancer organoids allowed for easy manipulation when comparing to animal studies, providing flexibility of the 2D cell culture system but also recaitualting the 3D structure and function.[9] The advent of organoid technology has introduced an unprecedented approach for modeling human cancers *in vitro*.[10] In this review, we provide an overview of the latest advancements in liver cancer organoid and discuss the potential of application and future direction.

2. Origin

Tumor tissues from primary sites,[11] metastasis,[12] peripheral circulation,[13] and liquid effusions[14,15] have all been used to generate tumor organoids. As direct processing of fresh tissues for generating organoids may not always be possible, frozen primary tumor tissue samples have generated tumor organoids.[16] However, in primary liver cancer organoid generation, not all of the abovementioned sources of tissues have been attempted (Fig. 1). Among them, surgically resected primary liver cancer tissues have been used to successfully establish organoids that partially preserve the histological characteristics, tumorigenic and metastatic properties, and genomic and transcriptomic landscapes of the parental tumor tissues even after long-term expansion in vitro.[2,17–21] Diagnostic needle biopsies of primary liver cancer patients have also been reported to be used to generate organoids.[22]

After samples are collected, two commonly strategies have been used to process the samples before encapsulation in the 3D matrix and continuous culture (Fig. 1): (1) through single cell dissociation of tissue samples or (2) mechanical and enzymatic mincing of tissue samples into few millimeter size fragments. Furthermore, various steps were taken to avoid non-cancerous cells from being included in the organoid culture. These steps include (1) sorting tumor cells by flow cytometry or by magnetic beads before being encapsulated into matrix or (2) manual microscopic screening based on morphological differences between normal organoids and tumor organoids,[23] and (3) then adding components in the medium to suppress normal organoid formation.

3. Culture system

In order to support *in vitro* tumor organoid formation, *in vitro* culture systems have to recapitulate *in vivo* conditions. The culture system (Fig. 1) mainly consist of extracellular matrix (ECM), certain cytokines and small molecules, and basic growth medium. Faithful restoration of liver cancer-specific ECM is important to generate tumor organoid for cancer research. Matrigel and basement membrane are two commonly used materials to mimic ECM in 3D models to generate liver cancer organoids (Table 1). Following liver cancer tissue/cell isolation, tissue/cells are typically seeded into either Matrigel[17,18,20,21] or basement membrane extract[2,22] to support 3D tumor organoid formation.

The culture system for organoids is specific: first there is the basic medium, then the culture medium is made by adding other ingredients into the basic medium. Commonly used basic medium contains advanced DMEM/F12, antibiotics penicillin/streptomycin, HEPES (N-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid) buffer, essential amino acid for cell growth such as L-glutamine or L-glutamine substitute glutamax, and chemically defined serum-free N2 and B27 supplements to suppress cell differentiation and promote cell growth. In the culture medium, various soluble factors are used to recapitulate *in vivo* conditions. Commonly elements added to culture medium include N-Acetylcysteine, nicotinamide, dexamethasone, gastrin, and multiple growth factors (epidermal growth factor, fibroblast growth factor, hepatocyte growth factor). Conditioned medium is medium containing biologically active growth factors, such as Wnt-3A and RSPO1, derived from engineered cell lines. Occasionally, conditioned medium are added to the culture medium instead of the growth factors individually. Various small molecular drugs are also included in culture medium to activate or inhibit signaling pathways. For example, AB301 for TGF-ß pathway inhibition, Forskolin for Rho pathway inhibition, Y27632 for Rock pathway inhibition, and Noggin for BMP-4 and BMP-7 pathway inhibition.[24]

4. Validation

After the establishment of primary liver cancer organoids, it is critical to evaluate whether cultured organoids recapitulate key aspects of primary liver cancers. Charecterization of primary liver cancer-derived organoids at the proteomic level (*e.g.*, immunohistochemistry [IHC] and immunofluorescence) enables the assessment of the potential presence, pattern, and quantification of specific markers levels in comparison with the original cancer tissue. The most significant HCC markers are alpha-fetoprotein (AFP) and HepPar1b.[2] Additional IHC markers include heat shock proteins 70 (HSP70),[25] glypican-3 (GPC3),[26] and glutamine synthetase (GS). [27] The transmembrane glycoprotein epithelial cell adhesion molecule (EpCAM) is a marker characterizing CCA-derived

Table 1
Summary of previously reported studied of primary liver cancer organoids.

Study		Broutier L, Nat Med 2017	Neal JT, Cell 2018	Nuciforo S, Cell Rep, 2018	Li L, JCI Insight 2019	Saito Y, Cell Rep 2019	Wang S, J Exp Clin Cancer Res 2020	Maier CF, Int J Mol Sci 2021	Yuan B, Clin Transl Med 2022
Disease		HCC (n = 3), CCA (n = 3), CHC (n = 2)	AC (n = 2), CCA (n = 1)	HCC (n = 8), CCA (n = 3)	HCC (n = 2), CCA (n = 3)	CCA (n = 6),GBC (n = 5), BDC (n = 4)	HCC (n = 4)	CCA (n = 25)	GBC (n = 41)
Sample sources		Surgical resection	Surgical resection	Needle biopsy	Surgical resection	Surgical resection	Surgical resection	Surgical resection	Surgical resection
Digestion solution		collagenase-accutase	None	Collagenase IV, DNase	Collagenase, DNase	Dispase II, Collagenase XI	Liberase, DNase	Dispase II, Collagenase I	Collagenase IV
ECM		BME	Collagen	BME2	Matrigel	Matrigel	Matrigel	Matrigel	Matrigel
Basal medium	Advanced DMEM/F12	√	√	√	√	√	√	√	√
	HEPES	√	√	Not mentioned	√	√	√		√
	Antibiotics	√ (Pen/strep)	√(Pen/strep)	Not mentioned	√ (primocin)	√(Pen/strep)	Not mentioned	√ (primocin)	√(Pen/strep, primocin)
	Glutamax	√	√	Not mentioned	√	√	√		√
Additive reagents	B27	√	√	√	√	√	√	√	√
	N2	√		√	√	√	√		√
	NAC	√	√	√	√	√	√	√	√
	nicotinamide	√	√	√	√	√	√	√	√
	ITS							√	
	gastrin	√		√	√	√	√	√	√
	EGF	√	√	√	√	√	√	√	√
	FGF10	√		√	√		√	√	√
	HGF	√		√	√		√		√
	IGF								√
	forskolin	√		√	√	√	√	√	√
	A8301	√	√	√	√	√	√		√
	Y27632	√			√	√	√	√	√
	SB-202,190		√						
	Dex	√					√		√
	PGE2								√
	Noggin				√			√	√
	Noggin CM		√						
	R-spondin							√	√
	Rspo-1 CM	√	√	√	√	√			
	Wnt3a								√
	Wnt3a CM	√	√	√	√			√	
Validation		IHC (HepPar1, EpCAM), IF (AFP, EpCAM), RNAseq, WES	IF (KRT7), exome PCR sequencing	IHC (AFP, GPC3, GS, HSP70, KRT7 and KRT19), WES, RNAseq	IHC (KRT7, mucicarmine), IF (EpCAM, KRT19, LGR5, SOX9, HepPar1, AFP), RNAseq, WES	IHC (KRT7, mucicarmine), RNAseq, WES	IHC (AFP, GPC3, EpCAM, CD44)	IHC (KRT7, mucicarmine, TP53), IF (KRT7, KRT19, EpCAM, TP53), RNAseq	IHC (KRT7), RNAseq, WES
Yield (%)		7/8 (87.5)	2/3 (66.7)	10/38 (26.3)	5/5 (100)	6/18 (33.3)	4/4 (100)	Not available	5/41 (12.2)

Abbreviations: AC, ampullary adenocarcinoma; AFP, alpha fetoprotein; BME, basement membrane extract; CCA, cholangiocarcinoma; CM, conditional medium; Dex, dexamethasone; ECM, extracellular matrix; EGF, epithelial growth factor; FGF, fibroblast growth factor; GBC, gallbladder cancer; HCC, hepatocellular carcinoma; HGF, hepatocyte growth factor; GPC3, Glypican 3; GS, glutamine synthetase; HSP70, heat shock protein 70; IGF, insulin-like growth factor; IHC, immunohistochemistry; IF, immunofluorescence; ITS, insulin-transferrin-selenium; KRT, keratin; MAPK, mitogen-activated protein kinase; NAC, N-acetyl-L-cysteine; Pen/strep, penicillin/streptomycin; PGE2, Prostaglandin E2; RNASeq, RNA-sequencing; WES, whole-exome sequencing.

organoids that occurs at an early stage of the neoplastic transformation of CCA cells.[28] Cytokeratin (KRT) 7 and 19 (KRT19) have been studied in primary liver cancer though intrahepatic CCA (iCCA) tend to express higher KRT7 and KRT19 when compared to HCC.[29,30] Thus, these two molecules are useful histochemical markers for the differential diagnosis of HCC and iCCA.[30]

Similarly, primary liver cancer-derived organoids are expected to share gene expression profiles with the originating parent tumors tissues. In this regard, primary liver cancer-derived organoids could be tested with whole exome sequencing (WES) or RNA sequencing which would show concordance of mutations and copy number variations between derived cancer organoids and parent liver cancer,[31,32] as well as genomic-wide transcriptomics.[2] It has been shown that nearly 92% of global variants in tested HCC samples were preserved in the derived HCC organoids.[2] Nevertheless, single-cell RNA sequencing occasionally was used to demonstrate the recapitulation of transcriptional features and intratumoral and intertumoral heterogeneity of derived liver cancer organoids at single cell resolution.[20]

Taken together, the expression profiles and mutation landscapes similarities between parental liver cancer tissues and tumor organoids offers us an ideal insight into liver cancer tumorigenesis and progression as well as facilitating clinical decision-making.

5. Application

5.1. Model of tumorigenesis

Organoids derived from healthy liver tissues were successfully used to model the development of liver cancer through the gain or loss of function of specific genes.[33–35]

5.1.1. Organoids modeling various disease backgrounds with variation in methods

Liver organoids have allowed study of various conditions ranging from viral infections to tumor progression. Recent improvements in culture techniques made it possible to create ever-more complex and cutting-edge culture systems, such as vascularized liver organoids. Additionally, these improvements have allowed research of host- hepatitis B virus (HBV)/ hepatitis C virus (HCV) interactions *in vitro*, a key factor in the development of primary liver cancers.[36–38] With the emergent of organoid-on-a-chip, another etiology of primary liver cancer, NAFLD, was also studied.[39] Using primary liver cancer organoids as *in vitro* model to study liver cancer tumorigenesis has been established. Using samples from liver cancer needle biopsies, organoids can be generated from different major etiologies and HCC stages to explore unknown etiology-based tumor biology.[22,40] Emerging technologies such as artificial intelligence (AI) are coming into light with algorithms that are capable of quickly classifying the heterogeneity of organoids to increase efficiency in data analysis.[41–43]

5.1.2. Primary liver cancer subtypes and the transcriptomic landscape

Moreover, primary liver cancer organoids help us investigate deeper into origins and subtypes of primary liver cancer. It has been noted that there is cell-fate conversion from hepatocytes to cholangiocytes during liver injury, indicating the high plasticity features and the fate of transdifferentiation between the two main primary liver cancer subtypes: HCC and iCCA.[44] Indeed, iCCA organoids cultured with differentiation medium or tissue specific liver ECM significantly induced an intrahepatic phenotype.[45,46] This phenotype inlcudes upregulation of hepatic markers (albumin, CYP3A4 and HNF4A), increased the bile acid production, suppressed Wnt signaling pathway including downstream protein DNMT1/3B[45,46] These changes which indicates that iCCA could evolve towards hepatic direction after transdifferentiation induction.[45,46] Conversely, HCC could also gain cholangiocellular-like phenotypes during cancer progression.[46] In addition, primary liver cancer organoid can be used to study tumor-initiation from cancer stem cells and explore novel therapeutic cancer stem cell target.[47]

Notably, genomic and transcriptomic alterations vary between patients even in the same zone iof the same type of primary liver cancer tissue.[48,49] Using a homogenous patient population, we can study liver cancer organoids from the same zone of different tissue samples to study intertumoral heterogeneity. On the other hand, liver cancer organoids generated from a specific portion are not representative when considering intratumor spatial heterogeneity.[50] Samples of different zones from the same patient can cultured into liver organoids to study intramural heterogeneity. These models could assist in the studies of epigenetic and/or genetic changes which may underlie drug resistance.[40]

5.1.3. The tumor microenvironment

Liver cancer tumor microenvironment is a complex ecosystem that can assist in tumor initiation, development, and metastasis through the interaction between tumor cells, stromal cells, immune cells, ECM proteins and the plethora of chemokines/cytokines. Thus, simulation of the composition and behavior of cells in the TME is critical to portray the entire landscape of primary liver cancer. The coculture of HCC organoids with endothelial cells and cancer-associated fibroblasts (CAFs) enabled the exploration of the interactions between stromal and HCC cells.[51,52]

The coculture of endothelial cells with HCC cells can mimic the vasculature of HCC and model angiogenesis. From these vascularized HCC organoids, it was noted that endothelial cells could activate tumor necrosis factor (TNF) signaling pathway and polarize macrophages in the TME.[53,54] These results indicate endothelial cells play a critical role on the generation of the inflammatory HCC TME.

Currently, CAFs are widely recognized to facilitate liver tumor progression through directly affecting tumor cells via paracrine signaling, exosome transfer and physical contact as well as indirectly impacting tumor progression by communication with stromal cells.[55,56] By co-culturing CAFs with HCC and CCA organoids, they not only confirmed the critical role of CAFs on liver cancer growth and drug resistance but also revealed the feedback regulation of CAF phenotypes by liver cancer paracrine signaling.[52] Furthermore, notable experiments were conducted where HCC organoids included induced pluripotent stem cell-derived endothelial cells and mesenchymal cells and then orthotopically implanted into the livers of immune-deficient mice with various diseases.[57] These experiments demonstrated that TME in fibrotic conditions could promote tumor amplification.[57] These findings suggest that certain microenvironmental stimuli contribute to the development of primary liver cancer.

Interactions of immune cells with liver cancer cells is one of the active fields in the research of TME of primary liver cancer. A recent study has shown tumor organoids derived from biopsied bile duct ampullary adenocarcinoma preserved fibroblast stroma and a set of diverse immune cells including T, B, natural killer, natural killer T cells, and macrophages. Importantly, these tumor organoids faithfully recapitulated the T cell receptor (TCR) repertoire of parental tumor tissue and immune checkpoint blockade in vitro.[19] This result provides an unprecedent model to study the complex interaction of immune cells with liver cancer cells *in vitro* and to explore novel therapeutic strategies. It has been demonstrated that coating the surface of T cells with a flexible DNA network as a protective scaffold potentiates tumor killing activities as evidenced by increased antitumoral cytokines and apoptotic cells.[58] In addition, when cocultured with autologous HBVs+ HCC organoid, T cells with HBV surface protein-specific chimeric antigen receptors exhibited stronger anti-tumor behaviors than normal which suggests a therapeutic option in the future.[59]

In summary, primary liver cancer organoids offer us a reasonable model to explore primary liver cancer progression and evolution.

6. Drug screening and investigation of drug resistance mechanism

The ability of primary liver cancer organoids to finely recapitulate different aspects of the original liver cancers lays the foundation for its application in large-scale drug screening and drug discovery in primary liver cancers. It has been reported an overall sensitivity of 100%, a specificity of 93%, a positive predictive value of 88%, and a negative predictive value of 100% when using tumor organoids to predict tumor responsiveness to anticancer drugs.[12] Adjacent normal tissue in biopsy or surgically resected samples can be used to develop control healthy organoids to confirm the tumor-specific efficacy and drug toxicity of the tested drugs.[22] Biobanking of various HCC and CCA subtypes alone with matched healthy tissue organoids can be established.[22,40] These resources would be useful for high-throughput screening of potential drugs for primary liver cancer and possibly extend personalized therapeutic options.

More specifically, human primary liver cancer organoids have been used to explore various cancer drugs (Table 2).[2,17,22,60] In one of the studies, extracellular signal regulated kinase (ERK) inhibitor, SCH772984, was identified as a potential therapeutic approach for primary liver cancer after testing 6 primary liver cancer organoids with 29 anticancer compounds. Nevertheless, genotype-response correlations were identified that one primary liver cancer organoid harboring the *CTNNB1* gene mutation was resistant to porcupine inhibitor LGK974.[2] In addition, the correlation between prognosis and mutational profiles in primary liver cancer organoids was identified that biomarkers C19ORF48, UBE2S and DTYMK (for HCC) and C1QBP (for iCCA). These biomarkers were linked with poor primary liver cancer prognosis. Meanwhile, it was shown that biopsy-derived liver cancer organoids from different patients had different sensitivities to sorafenib. Interestingly, the study found that sorafenib may be a potential therapeutic option for a rare lymphoepithelioma-analogous histological type of iCCA with semi-inhibitory concentration value that was comparable to sorafenib-sensitive HCC organoid.[22] With established GBC organoids, it was found

Table 2
Drug screening using primary liver cancer organoids.

Cancer type	No. of drugs tested	Sensitive drugs and MOA	Reference
HCC (2 lines) CCA (2 lines) Combined HCC/CCA (2 lines)	29	Gemcitabine (DNA replication inhibitor) Sorafinib (Targeting PDGFR, Kit, VEGFR) AZD8931 (Targeting ERBB1/2/3) SCH772984 (ERK1/2 inhibitor) Taselisib (Pi3K inhibitor) Gemcitabine (DNA replication inhibitor) Sorafinib (Targeting PDGFR, c-KIT, VEGFR) EMD1214063 (MET inhibitor) LGK974 (Targeting porcupine) Gemcitabine (DNA replication inhibitor) Sorafinib (Targeting PDGFR, c-KIT, VEGFR) Taselisib (Pi3K inhibitor) LGK974 (Targeting porcupine)	Broutier L, Nat Med 2017
HCC (10 lines) CCA (17 lines)	129	Bortezomib(Proteasome inhibitor) Ixazomib (Proteasome inhibitor) Carfizomib (Proteasome inhibitor) Romidepsin (HDAC inhibitor) Panobinostat (HDAC inhibitor) Idarubicin (DNA replication inhibitor) Daunorubicin (DNA replication inhibitor) Topotecan (DNA replication inhibitor) Plicamycin (RNA synthesis inhibitor)	Li L, JCI Insight 2019
HCC (4 lines)	4	GANT61 (Hedgehog inhibitor)	Wang S, J Exp Clin Cancer Res 2020
GBC (5 lines)	20	CUDC-907 (Dual PI3K/HDAC inhibitor) CUDC-901 (Dual EGFR/HDAC inhibitor) Vorinostat (HDAC inhibitor) Curcumin (HDAC inhibitor) Amuvatinib (Targeting c-KIT, PDGFR and FLT3)	Yuan B, Clin Transl Med 2022
HCC (9 lines) CCA (3 lines)	1	Sorafinib (Targeting PDGFR, c-KIT, VEGFR)	Nuciforo S, Cell Rep 2018
BTC (6 lines)	339	Cabazitaxel (Antimicrotubule) Docetaxel (Antimicrotubule) Epothilone B (Antimicrotubule) Vindesine (Antimicrotubule) Vinorelbine (Antimicrotubule) Afratinib (Suppress mTORC1) Dasatinib (Tyrosine kinase inhibitors) Lapatinib (Tyrosine kinase inhibitors) Clofarabine (DNA replication inhibitor) Gemcitabine (DNA replication inhibitor) Pralatrexate (Antimetabolite) Everolimus (mTOR inhibitors) Rapamycin (mTOR inhibitors) Bortezomib (Proteasome inhibitor) Carfizomib (Proteasome inhibitor) Aclarubicin (Topoisomerase inhibitor) Mitomycin C (Antitumor antibiotic) 5-Aza-2′-deoxycytidine (DNA methylation inhibitor) Amorolfine (Antifungal) Fenticonazole (Antifungal) Cerivastatin (HMG-CoA reductase) Talipexole (Dopamine D2 receptor agonist)	Saito Y, Cell Rep 2019
HCC (2 lines)	1	Desloratadine (Histamine antagonist)	Tan XP, Signal Transduct Target Ther 2023
HCC (2 lines)	1	Ifenprodil (NMDAR inhibitor)	Xu F, Cancer Res 2021

Abbreviations: BTC, biliary tract cancer; CCA, cholangiocarcinoma; EGFR, epithelial growth factor receptor; FLT3, fms-like tyrosine kinase 3; GBC, gallbladder cancer; HCC, hepatocellular carcinoma; HDAC, histone deacetylase; MOA, drug mechanism of action; mTOR, mammalian target of rapamycin; NMDAR, N-methyl-D-aspartate receptor; PDGFR, platelet-derived growth factor receptor; VEGFR, vascular endothelial growth factor receptor.

that dual PI3K/HDAC inhibitor CUDC-907 significantly inhibited the growth of GBC organoids providing a novel therapeutic option.[20]

Furthermore, larger scale of drug screening could be accomplished by multiple primary liver cancer organoids. In one study, 129 Food and Drug Administration (FDA)-approved antitumor drugs were screened with 27 liver cancer organoid models with different regions of HCC and CCA specimens. The results showed that only 9 drugs were pan-effective across all organoid lines. These drugs included histone deacetylase inhibitors (romidepsin and panobinostat), proteasome inhibitors (ixazomib, bortezomib, and carfilzomib), DNA topoisomerase II inhibitors (idarubicin, daunorubicin, and topotecan), and RNA synthesis inhibitors (plicamycin).[17] Not all positive responsive drugs have been tested clinically in HCC or CCA. In another relatively new study, a compound library consisting of 419 FDA-approved drugs were screened with different models including primary liver cancer organoids. This study demonstrated that desloratadine may be a novel anticancer drug, and the expression of N-myristoyl transferase and visinin-like protein could be a potential biomarker.[61] These results indicate that the liver cancer organoid could be used as a powerful *in-vitro* model to screen novel anticancer drugs for primary liver cancers on a large scale.

Recent advance on genetic modification technology and computational approach in the field has powered screening strategies with primary liver cancer organoids. For example, a combinatorial CRISPR-Cas9 systematic screen of existing drugs in HCC organoids identified N-methy-D-aspartate receptor antagonist ifenprodil synergized with sorafinib for HCC treatment.[62] Ifenprodil, a safe vasodilator drug, synergized with sorafenib was found to downregulate Wnt signaling pathway inducing an unfolded protein response, triggering cell cycle arrest, and reducing cancer stemness.[62] A hybrid organoid-computational approach, Quadratic Phenotypic Optimization Platform (QPOP), was applied on a pool of nine drugs comprising proteasome inhibitors, kinase inhibitors, and chemotherapy agents to improve proteasome inhibitor-based therapeutic efficacy and clinical potential.[63] The study identified the combination of proteasome inhibitor ixazomib and cyclin-dependent kinase inhibitor dinaciclib as a potential combination treatment for HCC, evidenced by enhanced pro-apoptotic and antiproliferative results.

On the other hand, key molecules responsible for reversing therapy resistance are fluctuating when considering therapy-resistance as a dynamic process with a series of genetic alterations through epigenetic regulation. Liver cancer organoids could be used to explore the evolution of genetic and epigenetic alternations, mechanisms of drug resistance, and approaches to overcome this resistance. In a study dissecting the resistance mechanism of sorafenib on HCC, it was found that CD44-positive HCC organoids were resistant to sorafenib as well as sorafenib increased CD44 levels. Thereafter, various tumor-initiating cell inhibitors, including Notch, Hippo, Hedeghog, and Wnt signaling inhibitors, were tested on CD44-positive HCC organoids. The *in vitro* and *in vivo* results showed that hedgehog signaling inhibitor (GANT61) potently suppressed HCC organoid cell viability and synergized with sorafenib to supress of cell viability and malignant properties on CD44-positive HCC organoids.[18]

Abovementioned breakthroughs have provided a favorable scientific reference for the future implementation of personalized precision medicine for patients with primary liver cancer.

7. Current limitations and future directions

The use of liver cancer organoid for drug screening and study of drug resistance to guide clinical treatment is still in the early stage. Many limitations remain to be solved and plenty of clinical studies need to be completed to confirm its reliability and safety.

Firstly, the current cancer organoid culture system still provides an incomplete understanding of the TME of liver cancer. Suboptimal *in vitro* culture conditions could contribute to the loss of cancer cell heterogeneity, tumor evolution during serial passaging, selecting for specific clones, and possibly altering/reducing clonality over time.[2,64] It has been reported that the alternation of the mutation panel is due to the adaption and expansion during culturing influencing their reliability and reducing efficiency for functional and drug discovery studies.[31,32] Moreover, current suboptimal culture condition may partially contribute to a low yield of liver cancer organoids[22,60] and favorite proliferative and aggressive cancer subsets[22] which restricts organoid practical applications. In addition, high costs of ECMs, cocktails of growth factors and small molecules as well as the time-consuming aspect of cancer organoid generation also limits future broad applications. Furthermore, *in vivo* TME systems consist of biochemical and biophysical spatiotemporal cues that we attempt to replicate using the ECM, growth factors and small molecules; however, these factors *in vivo* are highly dynamic in order to regulate tumor cell behaviors. Thus, the ideal *in vitro* liver cancer organoid culture system also needs to be highly dynamic in biochemical and biophysical properties to accommodate or control changes in liver cancer organoid structure during culture. Therefore, the future optimization of culture conditions (*e.g.* spatiotemporally controlled and standardized protocols) will be critical to enable clinical use of precision medicine.

Secondly, in addition to variability in reported liver cancer organoid culture mediums, there is no consensus on the procedure of liver cancer organoids generation, including sample harvesting, sample processing, cell culture, cryopreservation, and recovery. Large amounts of modifications have been tried but these changes have not been validated through replication. Thus, standardization of protocols to process samples will be critical to generalize findings. It is also necessary to improve the automation of the liver cancer organoid generation in order to reduce the interference of operator variability. Hence, to facilitate scalable production of high quality liver cancer organoids, unified protocols are needed. However, there is already ongoing research to modernize cell culture technologies with a combination of bioengineering techniques to construct more complicated and precise organoids, such as novel coculture systems, microcapsules,[65–68] and 3D bioprinting.[69–71] In another effort to attempt to develop standardized protocol, there could be an open database where the scientific community enter the detail methods employed for specific organoids to increase reproducibility. The organoid culture system can mimic some of the TME as the traditional animal models, however, does not provide the overall structure of vascularization and ECM completely as *in vivo* models. Therefore, to possibly address this, one may consider a combination with other coculture systems with technologies that could possible simulate natural tissue ECM.[72–74]

Thirdly, current liver cancer organoids are largely comprised of a singular liver cancer cell type and singular noncellular matrix which alters tumor cell behaviors and results in underrepresentation of the typical multi-cellular TME of liver cancers. Additionally, as the field cancer research continues to interrogate immunotherapy and stromal targets, organoids must represent these populations as well. One solution is to set up co-culture systems of liver cancer organoids with a variety of cell types, including patient-derived immune cells, CAFs, and endothelial cells. This would offer a complementary tool for modelling the dynamic interactions between cancer cells and neighboring cells in the TME.[51,52,59] In addition, creating multi-cellular liver cancer organoids in which epithelial cells interact with various stromal cells and immune cells is necessary for the disease modelling of primary liver cancer;[19] however, this approach has not been broadly adopted so far. Another approach is implantation of primary liver cancer organoids in mice with humanized immune cell populations. This approach will assist in overcoming the limitation of current culture systems which lack an immune microenvironment significantly accelerating the future study of immunotherapy of primary liver cancer organoids.[75] Although multiple aspects of the microenvironments are being recapitulated, however, vascularization continues to be a challenge in liver cancer organoids.[76] Glomerular vascularization has been shown in kidney organoids that are subjected to high fluidic shear stress.[72] Perhaps such technology can be employed in liver tumor organoids to induce vascularization.

In conclusion, primary liver cancer organoid is a promising 3D model for basic and translational research of liver cancer. Liver cancer organoids, although cultured in various ways, provides a medium to study liver cancers that is consists of both the flexibility from 2D cell culture systems and the ability to mimic *in vivo* TME. In addition, the ability of liver cancer organoids to recapitulate patient and tumor heterogeneity provides a meaningful and efficient way for therapeutic screening to provide reliable results. The combination of primary liver cancer organoid platform and new technologies will continuously advance application limitations and bring novel advancements to research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

This research was funded by the Physician-Scientist Early Investigator Program at CCR of NIH/NCI (grant number: ZIA BC 011888).

Author contributions

All authors contributed to writing the manuscript and/or designed and prepared the figures and legends.

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