Technology in Cancer Research & Treatment
Volume 22: 1-6

DOI: 10.1177/15330338231154090
journals.sagepub.com/home/tct
SAGE

Updates on Organoid Model for the Study of Liver Cancer

Korri E. El-Khobar, PhD[1] and Caecilia H. C. Sukowati, PhD[1,2]

Abstract

Liver cancer remains one of the most common cancers worldwide with limited therapy options. The main risk factors for hepatocellular carcinoma (HCC), the most common form of liver cancer, include chronic infection with hepatitis B or hepatitis C viruses, alcohol abuse, and metabolic disease. Current systemic therapies for advanced HCCs have greatly improved in the last decade, but there is still a need to develop more targeted drug therapy for HCCs. The development of liver organoids, a self-organising and self-renewal three-dimensional cell culture model, has greatly improved cancer research, including liver cancer. The generation of liver organoids provides a physiologically relevant model to study cancer drug screening and development, personalized medicine, liver disease modeling, and liver regeneration. However, the advent of organoid development also comes with few shortcomings that must be overcome, including the high cost of the model, the availability of origin tissues, and the need for multilineage liver organoids to replicate the true cellular heterogeneity of the liver. Despite all the limitations, liver organoids provide a reliable *in vitro* model for translational applications to develop more effective HCC therapy and to understand the underlying pathogenic mechanism in various liver diseases.

Keywords

liver cancer, HCC, liver organoid, cancer research, 3D culture

Received: September 5, 2022; Revised: January 1, 2023; Accepted: January 8, 2023.

Liver cancer is estimated to be the sixth most common cancer and the fourth leading cause of cancer death worldwide, with around 841 000 new cases and 782 000 deaths annually.[1] It is the most common cancer in countries with lower human development index (HDI), particularly in several countries in Northern and Western Africa and Eastern and South-Eastern Asia.[1] Hepatocellular carcinoma (HCC) is the most common form of primary liver cancers (PLCs), and accounts for around 90% of the cases.[2] The incidence and the mortality rates of HCC are 2 to 3 times higher among men in most countries.[1] Most HCC cases occur in patients with underlying liver disease, as the results of hepatitis B or hepatitis C virus (HBV or HCV) infection or alcohol abuse.[3] Additionally, the increase in non-alcoholic fatty liver disease (NAFLD) and resulting non-alcoholic steatohepatitis, particularly in the Western countries, together with the high rates of metabolic syndrome, adult obesity, and diabetes, have now become the growing cause of cirrhosis and HCC.[3–5] The varied etiology of HCC is reflected in the molecular heterogeneity of HCC,[5] either within an individual (intratumoral heterogeneity) or within patients (intertumoral heterogeneity).

HCC is typically diagnosed using imaging modalities such as ultrasonography (US), dynamic computed tomography or dynamic magnetic resonance imaging.[6] The combination of US method with a tumor marker detection such as alpha-fetoprotein can be used for annual HCC surveillance, especially in high-risk patients.[5,6] HCC treatment is currently determined based on the tumor stages using the Barcelona Clinic Liver Cancer (BCLC) staging system. Generally, patients with HCC in their early stage of cancer, with preserved liver function, are eligible for liver resection or transplantation. For those ineligibles for surgery, a local ablation is recommended. A transarterial therapy such as the transarterial chemoembolization is

[1] Eijkman Research Centre for Molecular Biology, Research Organization for HealthNational Research and Innovation Agency, Cibinong, Indonesia
[2] Fondazione Italiana Fegato ONLUS, AREA Science Park, Trieste, Italy

Corresponding Author:
Korri E. El-Khobar, Eijkman Research Centre for Molecular Biology, Research Organization for Health, National Research and Innovation, Soekarno Science and Technology Area, Jl. Raya Jakarta-Bogor Km 46, Cibinong, West Java 16911, Indonesia.
Email: korri.elvanita.el.khobar@brin.go.id

recommended for patients in the intermediate stage with unresectable tumors. Those in the advanced stage, with portal invasion or extrahepatic tumor spread, can receive systemic therapies including tyrosine kinase inhibitors (TKIs) and immune checkpoint inhibitors (ICIs).[2,3,6] Sorafenib is the first TKI (antivascular endothelial growth factor receptor [VEGFR]) drug available for HCC treatment,[2,6] however, recently, new TKIs and ICIs drugs have also been used for HCC treatment. A new study had shown that a combination regiment of atezolizumab (anti-programmed death ligand 1 (PD-L1) antibody) and bevacizumab (anti-VEGF antibody) resulted in overall improved survival for advanced HCC compared to the sorafenib standard of care.[7] Based on this recent study, atezolizumab and bevacizumab have now become the first-line therapies for advanced HCC, except in patients with contraindications for VEGF inhibitors or immunotherapy where sorafenib or lenvatinib is recommended instead.[2,8]

The clinical management of HCC has improved greatly in the past decade,[3] however, some issues remain to be solved. As HCC prognosis depends greatly at the stage when the tumor is detected, an early HCC detection is important to improve overall patients' survival.[6] However, in low HDI countries where HCC is most common, this is not always feasible. Screening and diagnosis rate for HBV and HCV infection in Asia-Pacific region are usually low, resulting in significant proportions of patients with chronic hepatitis B and hepatitis C left undiagnosed and receiving no treatment and care,[9] increasing their risks for developing HCC. Access to healthcare facilities for liver disease diagnosis, treatment, and care, as well as liver transplantation facilities are other limiting factors for HCC management in these countries.[9] Despite liver transplantation being the only HCC treatment which offers a real chance of cure for both HCC and the underlying cirrhosis,[5,6] the availability of liver grafts, the long waiting time for liver transplants, the high cost of the procedure, and the possibility of tumor recurrence remains the limiting factors for this procedure.[6,9] In addition, there is still a proportion of patients with advanced HCC who does not respond well to current systemic therapies and therefore has lower overall survival rate. This may be attributed to the high intranodule and internodule HCC heterogeneity and heterogeneity in the tumor evolution.[5] Since there is still limited information on the mechanisms of HCC initiation, disease progression, and drug resistance, more studies are needed in reliable preclinical models (*in vitro* and *in vivo*) which aim to understand the mechanism of HCC pathogenesis as the foundation for development of targeted clinical therapies with minimal toxic effects and cost.[2,3]

The conventional *in vitro* 2-dimensional (2D) cell culture system has been used as the platform model to study cellular and molecular mechanisms and to study drug mechanisms, toxicity, and efficacy.[10,11] The technique is simple and cost-efficient but lacks the structural, functional, and physiological features of *in vivo* cells,[11] due to the cell monolayers growth condition and the absence of extracellular matrix components, as well as the cell-to-cell and cell-to-matrix interactions.[10,12] Cancer cell lines are usually derived from primary patient materials that have been extensively adapted and selected for *in vitro* 2D culture conditions. As such the derived cell lines may have undergone substantial genetic changes and may no longer reflect the genetic heterogeneity of the original tumors.[13] Primary human hepatocytes (PHHs) grown in 2D culture is the current gold standard for assessing drug metabolism and hepatotoxicity *in vitro*. However, *ex vivo* PHHs tend to rapidly dedifferentiate and quickly lose its hepatic functionality.[14] The use of 3-dimensional (3D) culture system has overcome some of the limitations of 2D culture, especially in maintaining the cell polarity as well as the cell-to-cell and cell-to-environment interactions.[11] The 3D *in vitro* model represents a more realistic preservation of the *in vivo* conditions, processes, and microenvironment where the tumor develops.[15] A recently developed 3D organoids culture model has allowed for the development of a more physiological human healthy tissue and cancer models.[13] As such, 3D platforms may bridge the gap between 2D cultures and animal models, enabling for a more relevant preclinical model for cancer drug discovery and testing.[10,15]

Organoid is defined as a self-organising 3D structure of cells that displays organ-specific functionalities and capable of self-renewal and self-organisation.[11,16] Different from spheroid 3D culture that are grown in scaffold-free environment, organoid culture needs to be embedded into a 3D scaffolding matrix such as Matrigel and Cultrex, most often supplemented with growth factors and cytokines.[11,17] Once formed, organoids can be expanded in the long term, can be genetically modified and cryopreserved, and remain genetically and phenotypically stable.[13] Organoids are not immortalized, and as such are far more suitable model to study the oncogenic factors that drive cancer initiation and progression.[18] The first method of generating organoid culture from adult stem cells (ASCs) was initiated in 2009 by Sato et al. that established 3D epithelial organoids from mouse intestinal leucine-rich repeat containing G protein-coupled receptor 5 stem cells.[19] This protocol formed the basis of generating other organoid culture protocol from different mouse and human epithelia including liver, colon, pancreas, and other organs that all retain their original tissue functions.[13,20] Nowadays, human liver organoids are mainly formed from patient-derived tumor tissues,[21] human embryonic stem cells (ESCs),[22] and induced pluripotent stem cells (iPSCs) (Figure 1).[14,23]

Liver organoids are ideal tools for personalized HCC treatment and drug development. Using samples from liver cancer needle biopsy, it is possible to generate organoids from all different HCC disease stages and from major etiologies to develop targeted HCC therapies since the tumor organoids preserve all the morphology and genetic heterogeneity of the originating tumors.[24] Organoids generated from healthy and tumorous tissue samples of patients can be used to profile epigenetic and/or genetic changes that may cause drug resistance, enabling for specific and tailored treatment regimens for patients.[13] These approaches also allow for the identification of disease-driving mutations that occurs during tumorigenesis, and for drug screening purposes by specifically targeting the tumor cells.[11,13] In addition, self-renewing liver organoids can also

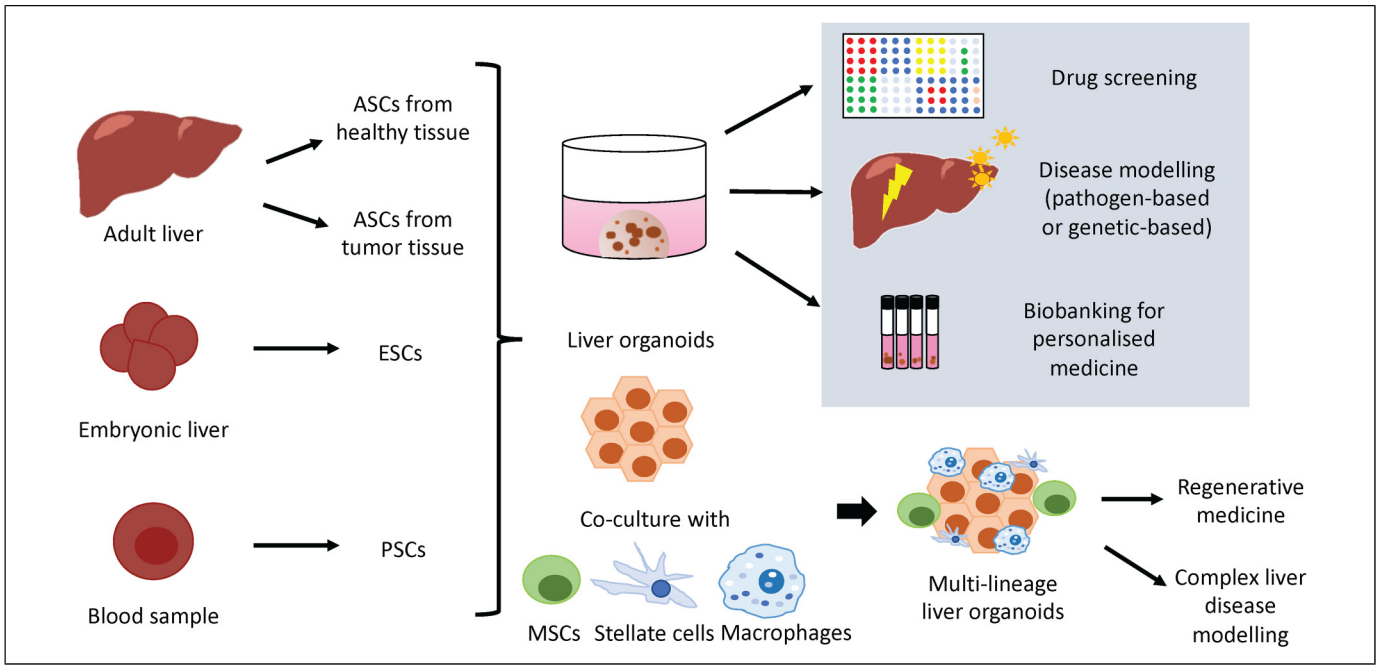

Figure 1. The liver organoid derivation origins and potential use for liver cancer research. Liver organoids can be generated from different source of human stem cells, including ASCs from healthy and tumorous adult liver tissues, ESCs from embryonic liver, and PSCs from blood samples. Organoids are form embedded in an extracellular matrix material to allow for 3-dimensional organization. Organoids typically recapitulate the phenotypic and genotypic heterogeneity of the tissue of origins. Conventional liver organoids can be used for high-throughput liver cancer drug screening and development, liver disease modeling, either induced by pathogen infection including viral hepatitis (hepatitis B or hepatitis C virus) and alcohol use or through genetic disorders resulting in abnormal liver development. Organoids can also be cryopreserved and biobanked to allow for personalized medicine approach, to estimate effective drug dose and type for patient treatment. In addition, recent co-culture development for liver organoids together with MSCs, stellate cells, and/or immune cells like macrophages may better reflect the true heterogeneity of human liver cells, allowing for generation of multilineage liver organoids that can be used to study more complex liver diseases such as inflammation-derived fibrosis and organ–organ interaction on liver cancer such as the gut-liver axis. Generation of vascularized multilineage liver organoids also open-up the potential use of organoids as an alternate for autologous liver transplantation for liver regeneration following an injury. Abbreviations: ASCs, adult stem cells; ESCs, embryonic stem cells; MSCs, mesenchymal cells; PSCs, pluripotent stem cells.

be used for high-throughput drug toxicity assay for potential new drugs on clinical trials since they retain the metabolic functionalities of the hepatocytes.[13,14,25] Large collections of patient-derived tumor and matching healthy tissue organoids that encompasses most known HCC subtypes are being generated and biobanked.[13,24] These resources would be useful for high-throughput screen of potential drugs for personalized therapy (Figure 1).[21] For instance, extracellular signal regulated kinase (ERK) inhibitors were identified as a potential therapeutic approach for PLCs treatment following drug screening on primary liver organoids.[21] A 2019 study tested a total of 129 US Food and Drug Adminstration (FDA)-approved cancer drugs utilizing 27 liver cancer organoids derived from primary HCC and cholangiocarcinoma (CCA) specimens.[26] The results showed that most of the tested drugs were ineffective, and only 9 were found pan-effective across all lines. These drugs can be categorized into 4 different classes based on their mechanism of action, including histone deacetylase inhibitors (romidepsin and panobinostat), proteaseome inhibitors (ixazomib, bortezomib, and carfilzomib), DNA topoisomerase II inhibitors (idarubicin, daunorubicin, and topotecan), and RNA synthesis inhibitors (plicamycin). Four of the pan-effective drugs in the study, panobinostat, topotecan, bortezomib, and idarubicin are currently on clinical trials for HCCs and for both HCCs and CCAs.[26]

A recent study in 2022 had generated 52 liver organoids from 153 patients with PLCs to understand the mechanism of drug resistance in PLCs.[27] The study showed that despite using only essential growth factors in the organoid culture medium, the resulting organoids presented the exact histopathological features of the original PLCs. Further, organoids that were generated from specimens with larger tumor size, with the presence of vascular invasion, and advanced BCLC stages had higher successful rate than those derived from specimens with different clinical characteristics. This may be related to the aggressive PLC tumor cell subpopulations in these specimens that allowed more growth advantages for the generated organoids. Indeed, the resulting organoids highly expressed stemness and proliferation regulating genes, including POSTN, SLC1A7, MMP12, TREM1, and CLEC5A.[27] Using a generated sorafenib-resistance organoids, the study also identified that the acquired sorafenib resistance was related to the upregulation of stemness and epithelial-mesenchymal transition regulating genes. In addition, specific targeting of the mammalian target of rapamycin (mTOR) signaling pathways, particularly phosphorylated S6 kinase, with an mTOR inhibitor

(phenformin [RTP]), was effective in treating the sorafenib-resistance organoids.[27]

Both the 2019 and 2022 studies had demonstrated the usefulness and suitability of patient-derived liver tumor organoids for the purpose of screening novel or existing drugs for single or combination therapies, due to the maintenance of the tumor heterogeneity of the originating specimens on the generated organoids. A combinatorial CRISPR-Cas9 systematic screen of existing drugs in HCC patient-derived organoids, had identified ifenprodil, a vasodilator drug with good safety profile, as a potential adjunct drug to sorafenib for HCC treatment.[28] Another approach using a hybrid experimental-computational approach, Quadratic Phenotypic Optimization Platform (QPOP), had identified the combination of ixazomib (proteasome inhibitor) and dinaciclib (cyclin-dependent kinase inhibitor) as a potential treatment for HCC. Confirming QPOP result in HCC-derived organoids, it was found that combination of ixazomib and dinaciclib resulted in more pro-apoptotic and antiproliferative results in tumor formation compared to sorafenib, most likely through the JNK (c-Jun N-terminal kinase) signaling pathway.[29]

Despite the potential of organoid culture in developing a clinically relevant cancer treatment, the challenges of generating them include the high costs of the system as well as the generally long and difficult preparation steps.[15] ASCs-derived organoids rely heavily on fresh primary tissue biopsies, which are relatively hard to acquire and are not readily available. This prevents the use of ASCs-derived organoids for large-scale organoids-based studies.[12] As direct processing of fresh tissues for generating organoids may not always be possible, freezing the fresh tissues may be a feasible option. A 2016 study has shown that organoids can be generated from frozen primary tumor tissue samples with only minimal impact on the drug-screening results.[30] The limited access to surgically resected HCC specimens may be overcame by using HCC needle biopsies, which have been shown as a good source for generation of liver organoids.[24] To eliminate the need for primary tissue resection, the use of iPSCs, which are made from cells from blood sample collection, offers a near unlimited source for generating a large number of organoids for high throughput screening.[12,14]

Organoids typically recapitulates the phenotype and genetic make-up of the originating tissues, which allowed them to be used for disease modeling.[21] Liver organoids from healthy donor had been generated to study HBV infection and its related tumorigenesis.[31] These organoids were infected with the virus and were able to produce covalently closed circular DNA, expressed HBV RNAs and proteins, and produced infectious HBV particles. Different types of liver organoids have also been used to model for NAFLD[32] and alcoholic liver disease[22] to study the underlying pathogenesis mechanisms. Human liver organoids can also be used as models for liver embryonic development and liver regeneration. Guan *et al* have utilized human liver organoids as models for several genetic diseases including Alagille syndrome (genetic disorder causing impaired bile duct formation), tetralogy of Fallot (complex congenital heart diseases), and congenital form of hepatic fibrosis,[33,34] to identify a mutation-induced pathogenic effect of the genetic disease. Using organoids from patients with recognized disease or with confirmed genetic mutations and comparing them to healthy matched controls will allow for the development of personalized medicine for different types of genetic disorders.[32]

Organoids are typically comprised of single cells derived from the source epithelial tissues. As such, the limitations of organoids cultures are the lack of stroma/microenvironment, blood vessels, and immune cells that are important in *in vivo* systems.[10,13,15] Thus, more advanced organoids cultures are needed to incorporate additional cellular elements.[13] For the liver, improved organoids models must reflect the cell heterogeneity on the liver, not only hepatocytes and cholangiocytes but also inclusion of Kupffer cells and hepatic stellate cells,[18] that have been shown to be important for liver inflammation-induced fibrosis and cancer progression.[18,32] This limitation can be overcome by co-differentiating both epithelial and stromal lineages for pluripotent stem cell lines (PSCs) resulting in multicellular human liver organoids. Following treatment with free fatty acid, these organoids were able to recapitulate the progressive liver disease from steatosis, inflammation, and fibrosis.[32] A different approach has also successfully produced multilineage liver organoids from human PSCs that are intra-luminally polarized and were comprised mainly hepatic epithelial cells, co-differentiated with stellate-like and hepatic macrophage-like cells that can be used for liver inflammation modeling.[34] The lack of physiological complexity of organoids culture can also be tackled by performing organoids co-culture approach, where a co-culture of liver organoids with mesenchymal cells resulted in improved alcohol metabolism activity.[18,22] The tumor microenvironment (TME) plays a crucial role in tumorigenesis, as it has been identified to sustain and maintain tumor growth and metastasis.[35] Of the cells circulating in the TME, the cancer-associated fibroblasts (CAFs) have been shown to support tumor growth and invasion and promote tumor resistance.[35,36] A co-culture between PLC-derived organoids and CAFs provided a more reliable *ex vivo* liver cancer microenvironment model through direct cell-to-cell interaction between the tumor cells and CAFs.[35,36]

A more complex multiple organ organoids cultures can be used to study more complex liver diseases including liver fibrosis (Figure 1). It can also model for organ–organ interaction such as the gut-liver axis in liver cancer.[18] In one study, a mouse hepatobiliary tubular organoids were generated using small hepatocytes and EpCAM-positive cholangiocytes. The established connection between the hepatocytes and cholangiocytes in the organoids was confirmed through the bilirubin and bile acid transports from the hepatocytes to the biliary system.[37] In another study, a different group had established a protocol for generating hepatic, biliary, and pancreatic structures from human PSCs, by fusing 2 distinct spheroids of differentiating PSCs resulting in an inter-organ connectivity model.[38] Recently, a new microfluidic system had enabled the establishment of human iPSCs-derived liver and pancreatic islet organoids. These co-cultures liver and islet organoids exhibited tissue-specific functions and metabolically relevant signaling pathways.[39]

The development of vascularized organoids from human iPSCs demonstrated the potential use of liver organoids for regenerative medicine as an alternative for orthotopic liver transplants (Figure 1).[40] Human PSCs-derived and ESCs-derived organoids can be expanded and cultured indefinitely to produce sufficient cell numbers[23,40,41] to allow for autologous transplant liver repopulation following liver injury.[22,42] Liver cell (organoids) transplant may provide less invasive treatment in comparison to liver organ transplantation. Furthermore, a study had shown that, despite the low rates of engraftment and repopulation, the transplanted organoids managed to survive up to 2 years post-transplantation.[41] A different study had also shown that cholangiocyte organoids engraftment into human livers resulted in a repaired human biliary epithelium.[43] However, more work is still needed prior to embarking on the use of organoids for *ex vivo* hepatocyte growth. For example, finding an effective way to generate massive production of liver organoids with good reproducibility for liver grafts. Because although PSCs could be easily expanded into $>10^8$ organoids with reproducible hepatic functions,[42] the resulting organoids were still in the micrometer sizes, way smaller than the centimeter sizes of human livers.[44] Other issues include the low engraftment rate, the high costs of the technique in the clinical settings,[45] the concern of possible malignant transformation of stem cell-derived organoids, and the optimal method for organoids production.[46] In addition, the current organoids derivation protocol utilized bioengineered growth factors and extracellular matrix that may contain animal-derived materials which may cause unwanted reactions in the human host.[46]

In summary, the developments of liver organoids from human stem cells have significantly improved the availability of liver *in vitro* model for drug screening and disease modeling. Liver organoids also have the potential use for liver regeneration and as an alternative for liver organ transplantation alternative. Nevertheless, the current liver organoids derivation protocol still needs to be improved to suffice the need to develop multilineage organoids models for a more complex liver disease modeling. The initial investment for organoids model derivation and biobanking is currently higher than the cost of maintaining 2D cell culture model. However, considering the near *in vivo* model-like quality of organoids culture, the potential benefit may outweigh the initial set-up cost, since, hopefully, it may also eliminate the use of *in vivo* animal models. Overtime, significant improvements on the development techniques may reduce the cost of 3D organoids modeling, enabling better access for liver organoids study in low-income and middle-income countries where HCC is more prevalent.

Acknowledgment

CHCS received 2022 grant of the Fondazione Umberto Veronesi, Milan, Italy.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors received no financial support for the research, authorship, and/or publication of this article.

ORCID iD

Korri E. El-Khobar https://orcid.org/0000-0002-9383-931X

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