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Developing models of cholangiocarcinoma to close the translational gap in cancer research

Citation for published version:
Waddell, SH & Boulter, L 2021, 'Developing models of cholangiocarcinoma to close the translational gap in cancer research', *Expert opinion on investigational drugs*, pp. 1-12.
https://doi.org/10.1080/13543784.2021.1882993

Digital Object Identifier (DOI):
10.1080/13543784.2021.1882993

Link:
Link to publication record in Edinburgh Research Explorer

Document Version:
Peer reviewed version

Published In:
Expert opinion on investigational drugs

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Download date: 25. Apr. 2022

Abstract:

Introduction: Cholangiocarcinoma (CCA) is an aggressive primary liver malignancy with abysmal prognosis and increasing global incidence. Individuals afflicted with CCA often remain asymptomatic until late stages of disease, resulting in very limited possibilities for therapeutic intervention. The emergence of a large number of preclinical models both *in vitro* and *in vivo* in recent years has expanded the tool kit for researchers in CCA, though how these tools can be best applied to understand CCA biology and accelerate drug development is not clear.

Areas covered: Here, we review the literature on animal and organoid models of CCA (available through PubMed between September 2020 and January 2021) and the role they have played in investigating therapeutics for CCA. We then discuss the potential these systems have for screening therapeutics to improve CCA patient outcome.

Expert Opinion: The expansion of CCA models has led to a diverse and exciting tool kit for pre-clinical researchers. With this, however, scientists need to consider which tools are best-suited to answer the pre-clinical question, and it is likely that only a combination of advanced *in vitro* cell systems and *in vivo* testing will be necessary to accelerate translational medicine in cholangiocarcinoma.

Key Words: Cholangiocarcinoma, Organoid, GEMM, Therapeutic, Liver

Article Highlights section

1.A range of human and rodent cell lines are available for CCA research, however whether these are useful in accelerating translational research remains questionable.

2. Cell of origin is hotly contested in CCA and it is perfectly possible that different CCAs can arise from hepatocytes and cholangiocytes.

3. The best *in vivo* tools should reflect the disease history of CCA, where cancer forms on the background of repair.

4. CCA genetics should inform the generation of new models of CCA. This is now achievable using CRISPR-Cas9.

5. More complex *in vitro* tools will need developing to recapitulate the tumour stromal interactions found in CCA.

Introduction: Cholangiocarcinoma (CCA) is an aggressive primary liver malignancy associated with abysmal prognosis and rising global incidence [1–3]. CCA typically develops from biliary epithelial cells (cholangiocytes) and can be divided into intrahepatic (iCCA), distal cholangiocarcinoma (dCCA) or extrahepatic (eCCA) cholangiocarcinoma depending on whether the tumours arise from the intrahepatic, hilar or extrahepatic biliary tree (Figure 1A). CCAs are often asymptomatic in individuals and thus present at advanced stages of disease. As a result, only ~30% of patients are suitable for surgical resection, the only potentially curative therapy for CCA [1,4,5]. Surgical resection is however accompanied with a ~65% chance of recurrence. Where surgery is contraindicated, patients are administered standard of care treatment, which do not substantially improve prognosis [6]. In recent years, precision medicine approaches have been developed to target CCA with particular molecular alterations and whilst effective in some patients, the presence of one of these targetable-mutations in a patient does not necessary predict therapeutic response [7,8]. We have summarised current therapies for CCA in table 1. Because of ineffective and limited treatment options, CCA patients have a 90% mortality rate within 12 months following diagnosis and a five year survival of only 1-in-20 [9]. The combination of poor treatment options, increasing

incidence and recent improvements in pathological classification, have identified CCA as a cancer of significant impact and of severely unmet clinical need.

CCA has lagged behind other cancers as far as understanding its cellular and genetic composition. CCA can occur spontaneously, however patients with underlying disease, such as those with a diagnosis of primary sclerosing cholangitis (PSC) [10] or with chronic liver fluke infection, have a substantially increased risk of developing CCA [3] (Figure 1B). In this context, chronic disease results in iterative injury and regeneration that requires the infiltration of a number of immune and stromal cell types [11,12]. These cells that are necessary for establishing the regenerative microenvironment following ductular injury are also considered key components of the tumour microenvironment, which contribute to CCA growth by providing pro-growth signals and limiting the ability of current therapeutics to act [13]. Whilst we have a broad understanding of the cell types in CCA [14], recent years have seen an expansion in our understanding of its genetic landscape, which has been investigated more thoroughly and we now appreciate that CCA is genetically heterogeneous[15–17]. Whilst a number of exome and whole genome studies has identified a consensus group of driver mutations, these canonical mutations *in KRAS, TP53, IDH1/2, PBRM1* and *BAP1* occur in fewer than 25% of patients. In addition to these core putative driver mutations, there are a large number of genes in CCA that are mutated at low levels [16]. The contributions of these infrequently mutated genes to CCA biology, growth and therapeutic susceptibility is yet to be determined.

Given there are a number of potentially targetable therapeutic alterations in CCA, such as the FGFR2-fusions, IDH1^{R132C} neomorphic mutation and KRAS^{G12} gain of function mutation [18–20], efforts have been made to treat CCA patients with compounds, which have been

developed for other cancer types that carry these mutations. Examples of molecular targets for therapies being investigated in clinical trials include IDH1/2, receptor tyrosine kinases (RTKs; e.g. FGFR, EGFR, PDGFR), MEK and mTOR [6]. Despite there being various candidates, little success has been achieved in drug development following clinical trials. This paucity of available therapeutics is largely due to CCA heterogeneity at the histological, genetic and molecular levels [21–23]. Numerous overactive signalling pathways have been described in CCA [24]. These include developmental pathways, such as WNT [25] and Notch pathways [26,27], and pro-inflammatory cytokine signalling pathways, including IL-6-STAT3 signalling [28,29] .

The genetic and cellular complexity of human CCA makes it particularly difficult to rationalise new and emerging therapies for its treatment. On the background of the genetic and cellular complexity of human disease, a number of systems have been developed in an attempt to better model CCA growth. Whilst cancer modelling and therapeutic testing have historically relied on animal models, recent years have seen the expansion of biobanks of CCA-patient derived organoids, which show incredible promise in the development of precision medicine approaches for the treatment of CCA. An ideal therapeutic model should be as close to the human condition as possible and incorporate aspects of CCA genetics, transcriptomic and cellular composition (Figure 1C). In this review, we will summarise the models available to researchers in cholangiocarcinoma and where possible comment on how closely these animals models map the pathology of human disease.

Understanding cholangiocarcinoma using Xenograft and Orthotopic transplantation models: A mainstay of cancer research, xenograft models have been utilised extensively and since their initial development have changed little (summarised in Figure 2). The essential

principle for most xenograft experiments is that cancer cells, including cells from CCA, can be isolated and injected (normally with a biodegradable extracellular scaffold, such as matrigel) into the flank of immunocompromised mice [30] or can be implanted directly into the liver [31]. Following transplantation of human cells beneath the skin, tumours form below the skin of these xenografted animals and largely consist of epithelial cancer cells, with some recruitment of vasculature, some immune cells and fibroblasts (mainly derived from the dermis). In a number of CCA studies, xenografts of human CCA cell lines have been used to test and develop therapeutic approaches aimed at inhibiting the growth of CCA cells, including the inhibition of NOTCH1 [32], YAP [33], cyclin-dependent kinases [34] and TWEAK/Fn14 [35]. While tumour growth in xenograft models occur in the absence of a functional immune system, grafts can be derived from human CCA cell lines (rather than from other species, as used in syngeneic models) thus increasing their clinical relevance. In this regard, xenograft models do reflect some of the genetic components of the cancer of origin and CCA cell lines that are well characterised at the genetic level i.e. the mutations they contain have been described. There are substantial limitations to using xenografted tumour cells as a method for therapeutic testing, though.

Tumour heterogeneity among patients makes modelling CCA exceptionally difficult, and the same issue occurs with CCA cell lines. Cell lines are normally derived from a single site from one patient, and therefore only represent the genetic complexity of that sample or biopsy core. Initial studies of CCA *in vitro* utilised immortalised cell lines, such as the human line HChol-Y1 and rat line BDE1-Neu [36–38]. While being easy to manipulate, cell lines are simple tools that do not represent cellular physiology (normal nor diseased) *in vivo* due to a lack of *bona fide* extracellular interactions and being homogeneous in cell-type. To overcome these limitations, cancer studies have moved to animal models and more sophisticated cell culture

systems, namely organoid cultures, to address whether new therapies show promise in reducing CCA growth.

Organoid Models of CCA: Organoids are three-dimensional (3D) cell cultures derived from embryonic, neonatal or adult stem/progenitor cells that mimic and maintain tissue architecture *in vitro* [39–41]. To adopt tissue structures in 3D, cells in organoid cultures are suspended in an artificial, extracellular matrix (ECM)-like hydrogel that provides homogeneous stiffness and physical cues, and are cultured in medium supplemented with a cell-type-specific mix of growth factors [40–42]. Together, these signals instruct spatial organisation and cell differentiation to yield organotypic cultures more representative of *in vivo* physiology than 2D monolayer cultures. As a result, the use of organoid cultures has significantly increased within the last decade as they prove to be powerful and reproducible tools to study development/organogenesis, disease pathobiology and drug discovery [40,43,44] (Figure 3). Organoid cultures have been described for several human organs, including brain, eye, stomach, intestines, kidneys, pancreas and liver [40,41].

Human and mouse liver organoids have been derived from both pluripotent stem cell cultures and isolated tissue (adult and embryonic) to generate hepatocellular, cholangiocellular and mixed hepatobiliary organoids [45–51]. Cholangiocellular organoids assemble as hollow spheres and express cholangiocyte molecular markers (*KRT19, SOX9* and *HNF1B* [45,47]), as well as markers for apical-basal polarity (*CFTR* [47,48,51] and primary cilia [47,52]) and cholangiocyte function (ALP and GGT [51]). Therefore, akin to cholangiocytes *in vivo,* cholangiocellular organoids have made for effective tools to model mechanisms of disease in the biliary tract. Examples include: (i) Notch inhibition and dysfunction hindered proper morphogenesis of organoid cultures, thus modelling Alagille syndrome *in vitro* [53,54]; and

(ii) both hepato- and cholangio-cellular organoids have recently shown susceptibility to SARS-CoV-2 infection, which subsequently proved to be cytotoxic and is thus in accordance with the clinical observation that >50% of infected patients present liver damage [55,56]. Additionally, diseased cholangiocellular organoids have been derived from the bile of patients with primary sclerosing cholangitis (PSC) [57], and from surgically resected tumour tissue of patients with primary liver cancers [58–60]. *L. Broutier et al* showed CCA patient-derived organoids (PDOs) express matching molecular profiles to their tissue-of-origin [58]. These PDOs consequently allowed for: (i) the identification of novel, potential prognostic biomarkers in CCA – *C1QBP* and *STMN1* expression correlated with poor survival; and (ii) determination of chemo-sensitivity in one CCA PDO to ERK1/2 inhibitor treatment *in vitro* and in PDOs grafted to mice. In another study, *Saito et al.* used CCA PDOs to characterise gene expression and correlate it to sensitivity to the epidermal growth factor receptor (EGFR) inhibitor, elortinib [59]. They subsequently discovered that CCA PDOs expressing the poor prognosis markers *KLK6* and *CPB2* showed chemo-sensitivity and –resistance, respectively, to elortinib treatment. Whilst the number of CCA PDOs generated in each study was low (n=3 in each), they demonstrate the potential role for PDOs in facilitating and developing personalised therapies for CCA patients.

Incorporation of drug screening phases using PDOs to develop personalised medicine strategies is the biggest clinical advantage to organoid cultures. While toxicity studies in CCA PDOs remain few and parallelisms between PDO- and patient-tolerance to drugs are yet to be made, an encouraging study using rectal cancer PDOs showed that cultures could predict patient response to neo-adjuvant chemoradiation [61]: *Yao et al.* demonstrated that PDOs displaying sensitivity to at least one of irradiation, 5-Fu or CPT-11 treatments indicated that the corresponding patients would respond well to such treatments in combination.

Acknowledging the genetic and molecular heterogeneity of CCs, therapeutic stratification is an astute approach to develop succeeding medicines to target biliary malignancies. As previously mentioned, the only current curative therapy to CCA is tumour resection. While this comes with limited success, it would give source to tumour tissue to which PDOs could be derived from and subjected to drug screening. Compounds that prove to be successful in the inhibition of cell survival in CCA PDOs could be delivered to the patient and potentially increase post-surgery survival rate.

However, despite their clinical potential, PDOs present challenges. Firstly, to develop PDO cultures from resected tumour tissue would be time-consuming and to subject cultures to compound screens would be expensive. Secondly, although organoid cultures are more similar to *in vivo* physiology than monolayer cultures, they're not wholly representative: the cell-hydrogel interface in organoid cultures is homogenous and thus unlikely to completely represent mechanical forces and microenvironment stiffness *in vivo* [42]; and additionally, they do not include other cell types that make up the tumour microenvironment (TME). The TME is a dynamic, plastic, multi-cell-type composite that supports tumour growth, with evidence suggesting it confers drug resistance: myeloid cells (M2 macrophages) have been reported to contribute to chemo-resistance in the liver [62]. Elegant PDO-TME co-cultures have recently been described for certain cancers [63,64]: Neal et al. propagated primary tumour tissues as complex PDO cultures containing tumour-residing macrophages and lymphocytes, showing the potential of PDOs to investigate personalised immunotherapies [64]. This approach is yet to be described for CCA, and thus drug studies in current CCA PDOs lacking the presence of an immune stroma will likely not infer therapeutic effectiveness *in situ*. Finally, to establish PDO cultures, the tissue of origin must be void of normal, non-tumour cells; healthy normal cells that contaminate PDO cultures have been reported to outcompete

cancer cells, which has been attributed to optimised culture conditions for the former and genetic instability in the latter [58,65]. Once established, PDO cultures must also maintain expandability and post-cryopreservation restorability [66]. This, in combination with limited access to tissue for their derivation, makes the careful process of generating PDO cultures onerous. Nonetheless, we contend that PDOs would play a key role in improving the clinical outcome for CCA patients through accelerating drug screening and contributing to medicinal stratification.

Despite the increasing complexity of organoid systems [67] and the hope that epithelial organoids could be combined with specific extracellular scaffolds or scaffolds that more closely recapitulate the tumour cell niche [68,69] with stromal and immune cells [64], CCA organoids currently fail to recapitulate the complexity of CCA. Testing therapeutics that do not target the epithelium, but rather attempt to limit the development of the tumour microenvironment have, as such, been lacking in these systems. While organoid models that represent both the tumour epithelium and tumour microenvironment are developed, *in vivo* models of CCA are well-suited to study therapeutic effectiveness in this bipartite system of disease.

Animal models of cholangiocarcinoma.

Initially, animal models of CCA relied on the administration of chemotoxic compounds that damaged the liver epithelia. Models of CCA *in vivo* have since drastically expanded in diversity with the development of several, sophisticated transgenic models that allow for the study of patient-relevant genetics. Here, we will discuss both chemotoxic and genetic models of CCA, highlighting the benefits and limitations to both in studying therapeutic testing.

Chemically induced models of cholangiocarcinoma: Classical models of CCA have involved the chronic administration of carcinogens to rats and mice to injure the bile duct and induce a state of chronic repair and regeneration. These models rely on spontaneous mutagenesis of CCA oncogenes on the background of disease to perpetuate tumour formation. Examples of chemotoxic reagents include diethylnitrosamine (DEN), dimethylnitrosamine (DMN), furan and thioacetamide (TAA).

DEN/DMN administration and bile duct ligation

Diethylnitrosamine (DEN) and dimethylnitrosamine (DMN) are chemotoxic compounds that are hydroxylated by CYPE21 and other cytochrome P450 isozymes in the liver [70]. Bioactive derivatives of DEN metabolism result in DNA adducts and subsequently CCA in mice at 28 weeks [71]. Contrastingly, however, DMN consumption alone is insufficient to drive CCA but can be combined with *Opisthorchis viverrini* (liver fluke) infection or bile duct ligation (BDL) to accelerate tumorigenesis [72]. Similarly, BDL can be used with DEN administration to accelerate CCA [71]. BDL is a long-standing, reproducible surgical procedure to induce obstructive choleostasis in rats and mice [73]. BDL drives biliary proliferation, fibrosis and inflammation – all features of pre-malignant disease – but does not drive tumorigenesis alone [71,74]. DEN, DMN and BDL models of liver injury reconstitute a fibro-inflammatory environment, allowing investigation into pre-malignant processes and the effects of therapeutic intervention at early stages of disease. Inflammatory states represent a substantial risk factor in the development of CCA and as such developing *in vivo* models that reflect the combination of genetic insult on the background of disease are likely to more closely recapitulate human disease within the experimental setting. Using these combinatorial models could benefit patients in the clinic with liver diseases that increases

their risk to developing CCA (Primary Sclerosing Cholangitis, for example), and potentially give rise to preventative medicines that can prevent or reduce the transition from a chronic pre-malignant disease into CCA. However, as CCA is typically diagnosed at advanced stages of disease, preventative medicine may be unavailing for most cases in the clinic, therefore questioning the benefits of these models in therapeutic intervention.

Furan administration in rats

Furan is a volatile, heterocyclic organic compound that, similar to DMN/DEN, is processed by cytochrome P450 enzymes, forming *cis*-2-butene-1,4-dial that interacts with DNA to form adducts [75]. Administration of furan at high concentrations (15-60 mg/kg/per day) in rats for 2-3 weeks has been shown to drive biliary hyperplasia and the development of cholangiofibrotic lesions [76]. A longer time course of 15 months of furan administration at a lower dose (8 mg/kg/per day) gave rise to advanced pathologies with the development of CCA and cases of metastasis to the pancreas and ovary, among other tissues [77]. Furan-induced CCA in rats is a relevant model of cholangiocarginogenesis given human exposure to furan from foods, agricultural products and pharmaceuticals [75]. Additionally, associated pathological features, such as intestinal metaplasia and gene expression profiles, mimic aspects of the human disease [78], thereby allowing the researcher to identify relevant therapeutic targets that would inhibit such processes. However, this model is time-consuming and largely constrained to use in rats only.

TAA administration in rats

The most common chemical model of cholangiocarcinoma is the administration of thioacetamide (TAA) which has been used to model liver cancer since the middle of the last century [79]. TAA is a potent hepatotoxin that can cause hepatocellular carcinoma,

hepatocellular adenoma and CCA in rats when consumed [80,81]. It is believed that liver uptake of TAA results in its oxidation to form bioactive, mutagenic metabolites that likely react with the amine groups of proteins and phospholipids [82]. TAA metabolism induces an inflammatory response driving hepatic fibrosis, cirrhosis and ductular reactions, and has been shown to recapitulate the multi-step progression of human CCA [72,78,83]. As such, TAA is an excellent model of CCA and recapitulates many of the cellular processes that precede the formation of a tumour *in situ*. Furthermore, the histopathological appearance of TAA-induced cholangiocarcinoma is similar to that found in human patients; however, what the genetic changes are in TAA-driven cholangiocarcinoma and whether they recapitulate the alterations found in human disease is not clear. A further limitation of the TAA model is that it is technically challenging to use this model to understand the fundamental processes that promote CCA formation and growth. Whilst transgenic rats have been generated and this has been made more efficient by the discovery of CRISPR-Cas9 [84], there are a limited number of genetically modified rat strains that allow for transgenic modulation of signalling pathways or cell types. As such, in the TAA rat model, intervention is largely limited to pharmacological dosing of experimental compounds; however, due to the size of the rat as an experimental tool, even this approach can be limiting and requires quantities of therapeutics beyond what is normally feasible within the basic research setting.

To this end, the mouse has become the animal of choice for developing models of CCA, as it offers a high level of genetic plasticity with an enormous range of constitutive and conditional mutant mouse lines that can be crossed into mouse models of CCA to better understand the biology of this disease. Within the CCA field, there are two major molecular processes to generate transgenic mice in order to study CCA biology: 1. Classical genetically engineered

mouse models (GEMMs) and 2. The introduction of genetic material using a hydrodynamic injection.

Considerations of the Cell of Origin: In the development of animal models to investigate CCA, the origin of the tumour is contested. Histopathologically, CCA is located closely to the biliary tract in patient disease and therefore the assumption is that in humans, CCA tumours arise from the bile duct [85]. However, experiments in mice have demonstrated that epithelial cells in the liver (hepatocytes and cholangiocytes) have high levels of lineage plasticity and can transdifferentiate between these two lineages [86,87]. A number of mouse models of CCA (including the hydrodynamic models) rely on this lineage plasticity and the transdifferentiation of hepatocytes into a cholangiocyte-like cell prior to oncogenic transformation [88]. Along with these hepatocyte-based models, there are also classical GEMMs that utilise the deletion of oncogenes specifically within the bile duct, which we will discuss further in this review. Whilst cell of origin is likely important in the genetics and pathophysiology of CCA, further work will be necessary to determine whether CCA does indeed arise from hepatocytes in patients. However, the tools that have been developed will allow us in the meantime to address the fundamentals of CCA.

Genetically engineered mouse models (GEMMs) of cholangiocarcinoma: Classical GEMM models of cancer utilise *Cre* recombinase to recombine *loxP* sites that flank the DNA of tumour suppressors (so called *floxed mice*). The result of this is that the gene is deleted and tumour suppression is lost. Moreover, this system has been adapted to overexpress oncogenic proteins, such as KRAS^{G12D} in a *Cre* dependent manner [89]. In *Cre/loxP*-based systems, lineage specificity is achieved by expressing *Cre* recombinase under a lineage specific promotor and temporal control is conferred by fusing *Cre* to the Tamoxifen-responsive

Oestrogen receptor (ER^T). These *CreER^T* transgenic lines are silent until the animal is dosed with tamoxifen, at which point the ER is uncoupled from the *Cre*, which can then access DNA and recombine *loxP* sites.

Transgenic animals that develop CCA have used two principal *Cre* strategies. The first recombine in the fetal stages of life, such as in the *Alfp-Cre* mouse where *Cre* is under the control of the *Alb* promotor and *Afp* regulatory elements [90]. This approach deletes tumour suppressors or activates oncogenes in hepatoblasts (embryonic epithelial precursors) before they become specified into cholangiocytes or hepatocytes[90]. The benefit of this approach is that it is highly penetrant and the tumours that form following this approach tend to develop rapidly and early in life[91]. Using this developmental-biology approach, a number of signalling pathways including Notch [91]and YAP (via *Nf2*-deletion) [92] have been altered *in vivo* and are sufficient to generate CCA in adult mice. A substantial limitation of this approach, however, is that genetic recombination of floxed alleles in development means that they are propagated throughout the liver. This has the potential to initiate tumorigenesis from a number of different epithelial cell types, therefore making the cell of origin difficult to ascertain and often producing tumours that have aspects of both CCA and hepatocellular carcinoma. Furthermore, juvenile CCA is incredibly rare (with an incidence of 0.0036 per 100,000) [93]; as such, these approaches fail to recapitulate many important aspects of CCA, a predominantly adult disease. Despite these caveats however, conditional activation of oncogenes or deletion of tumour suppressors do generate murine CCA models with a high level of penetrance and have a moderate level of flexibility; it is, in these animals, completely possible to swap different floxed alleles and therefore represent different CCA genetic profiles. This strategy is animal intensive and does normally require multiple generations of breeding and crossing to get the correct mutant allele combination. However, once the model

is generated, these GEMMs are good candidate for testing therapies, though do not always recapitulate the underlying disease seen in the clinic.

The second GEMM strategy for developing mouse models of cholangiocarcinoma is by using conditional *Cre* alleles that recombine in the adult bile duct following tamoxifen administration. *Keratin-19-CreER*^{T}[94], *Sox9-CreER*^{T}[95] and *Hnf1b-CreER*^{T}[96] have all been used to recombine floxed alleles in the adult bile duct. The benefits of this approach is that oncogenic transformation of cholangiocytes can occur in the adult and in a timed manner. However, as with many CreER^{T} lines, the level of *Cre*-mediated recombination can be limiting, with only a proportion of cholangiocytes being recombined.

Similar to the question of which transgenic *Cre* is best, there is little consensus as to which combination of floxed alleles gives rise to CCA tumours in the mouse that closely resemble patient disease. Early studies demonstrated that the deletion of *Smad4* and *Pten* throughout the liver (using an *Alb-Cre*, which recombines floxed alleles in the embryo) could lead to CCA formation[97]. However, *Pten* deletion alone is also sufficient to drive substantial hepatomegaly without cancer formation in these mice and, as such, this model has not been widely taken up by the field. More recently, a number of oncogenes have been deleted throughout the liver, including *Trp53* and overexpression of Notch intracellular domain (NICD), which results in rapid CCA formation. However, these deletions rely almost completely on hepatocyte to cholangiocytes transdifferentiation[91]. Within the CCA modelling field, cancer that has been generated through the deletion of oncogenes in the biliary epithelium appears to have gained more traction; a number of models have been developed that rely on various combinations of *Trp53* deletion with either concurrent deletion of *Pten* or expression of the oncogene *KRas*^{G12D}[98]. The ultimate effect of both of

these pathways is to activate AKT and MAPK signalling thereby driving oncogenic transformation. What is interesting in many of these models is that recombination of the oncogenes alone is either insufficient to promote tumour formation or induce tumours that are sporadic and take a long time to develop[98,99]. To overcome this, a number of studies have combined GEMMs with injury models such as the TAA model (discussed above) or treatment with 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) supplemented diet, a model that results in ductular occlusion and compensartory ductular proliferation. Both of these models result in the iterative injury and repair of the biliary epithelium and trigger the generation of a complex stromal and immune microenvironment that promotes cholangiocyte proliferation. In this context, GEMMs develop CCA from the bile duct, on the background of chronic disease and regeneration and appear to develop a stroma that recapitulates those seen in human – superficially, at least. The combination of a GEMM and injury model increasing the incidence of tumour formation also indicates that mutation alone (in the bile duct) could be insufficient for tumour initiation in many cases and that signals from the stromal cells in the cancer could be essential for tumour initiation and growth, however this needs to be formally assessed. The relevance of classical GEMM models to drug development is contested across the tumour spectrum and whilst they often represent the human pathology well, they are by their nature very stereotyped as to reduce experimental variability; because of this, fields often become dominated by a small handful of models that represent a subset of human disease. Work in intestinal cancer has demonstrated that by broadening the range of available GEMMs, so that they represent the genetic and histopathological variation seen in human disease, improves applicability to therapeutic testing and developing precision medicines (reviewed here: [100]). GEMMs to generate CCA do not currently represent the range of phenotypes seen in patients, though with an

increased understanding of CCA genetics and cellular heterogeneity expanding the GEMM repertoire will invariability improve drug discovery.

Transposon-mediated integration of DNA in mice:

The murine tail vein is as an effective entry route for systemic delivery of DNA, RNA and proteins [101]. Initial experiments performing rapid injections of DNA plasmids encoding β-galactosidase and luciferase reporter genes showed high levels of corresponding protein expression specifically in the liver, with smaller degrees of expression in the kidneys, spleen, lungs and heart [102,103]. The high speed introduction of solution into the vascular punctures its endothelium and results in leakage [101,104]. Hepatocytes are particularly targeted by the hydrodynamic tail vein injection due to their close proximity to capillary endothelial cells; the pressurised endothelium permeabilises the membranes of neighbouring hepatocytes, which subsequently allows uptake of the contents of the solution introduced.

This gene delivery system has proven advantageous for studying liver cancers. A number of studies have utilised this technique for the incorporation of *naked* DNA plasmids into the hepatocyte genome (reviewed in [88]). Hepatocellular carcinoma (HCC) can be easily recapitulated through sleeping beauty (SB)-mediated integration of a number of different transposon plasmids: example combinations include *MET* and constitutively active *CTNNB1* (*ΔN90-β-Catenin*), or constitutively active *AKT* (*myr-AKT)* and *ΔN90-β-Catenin,* or *myr-AKT* alone. To drive cholangiocarcinogenesis via hydrodynamic tail vein injection, a DNA plasmid encoding a gene that will ultimately result in hepatocyte-to-cholangiocyte transdifferentiation is required, such as *NOTCH1* intracellular domain (*NICD1*) [105,106]. *NICD1* can be injected alone or with *myr-AKT* to drive cholangiocarcinogenesis in the mouse (CCA develops within 20 weeks of injection of the former, whereas tumours can be observed

macroscopically from 3.5 weeks post-injection of the latter combination) [105]. Alternatively, a DNA plasmid combination of *myr-AKT* with *YAP*^{S127A} also models CCA [107]. However, in this scenario, hepatocyte transdifferentiation is also dependent on activating Notch signalling downstream. Both plasmid combinations result in cytokeratin 19 (KRT19)-positive, well-differentiated CCA tumours.

Modelling CCA with hydrodynamic tail vein injections generates tumours quickly while under an intact immune surveillance (Figure 4, top panel). Additionally, the researcher can define the genetic landscape of tumours (to some extent). One major limitation of the hydrodynamic tail vein model is that all tumours are derived from hepatocytes, and as such, this tool is wrapped in the debate discussing cell-of-origin of CCA as mentioned above. To generate CCA from hepatocytes, the hydrodynamic tail vein model must include one DNA plasmid that drives hepatocyte-to-cholangiocyte transdifferentiation, and this therefore shapes the molecular profile of the resulting CCA tumours. However, other oncogenes of interest and reporter genes can easily be integrated to form a cocktail of *naked* DNA for injection. This method thus allows assessment of the contribution individual genes make to tumour initiation, development, shaping the tumour microenvironment (TME) and responding to therapeutics. The amenability of the hydrodynamic tail-vein injection model in combination with *in vivo* therapeutic experiments makes it a valuable tool to study genomic-drug interactions.

A recent development of the hydrodynamic tail vein model is the inclusion of CRISPR-Cas9 into this system. The CRISPR-Cas9 system has been generated to be incredibly modular and it is now relatively trivial to clone a gRNA to target Cas9 to a particular gene or genes in order to generate a mutation at that site (Figure 4, lower panel). *Werber et al* initially described the

shuttling of the CRISPR-Cas9 machinery into the SB-transposon system in order to generate gRNA libraries of common liver cancer mutations that could be screened *in vivo* [108]. Since then, a number of studies have used a similar approach to delete single candidate genes or a number of genes in the liver in order to assess which loss of function mutations are critical for oncogenic transformation. The repertoire of Cas9-genome editors is increasing and are the next steps in understanding how CCA could initiate from endogenous gain of function mutations directly in the liver, using a range of base-editing tools or homology directed repair *in vivo*, rather than relying on the overexpression of oncogenes.

Electrical integration of DNA plasmids: Whilst providing high levels of experimental flexibility, the standard hydrodynamic approach produces CCA with high levels of variability and due to the nature of transposon integration, cancers form across the liver. This does not represent human disease which is normally focal or constrained to particular sites within the liver. An alternative approach to the hydrodynamic tail vein injection for delivery and expression of transgenes to model CCA is liver electroporation [109]. Gürlevik, Fleischmann-Mundt et al. introduced SB plasmids into the hepatocytes of *Trp53 floxed* mice via subcapsular injection of the large lobe of the liver, and subsequently applied electrical pulses for plasmid transduction. This system allowed for the expression of *Cre* to delete *Trp53* and the constitutive expression of oncogenic $KRas^{G12V}$ to drive the formation of a singular CCA tumour node that could be resected within 3-5 weeks post-transduction. This model is therefore useful for the generation of a focal tumour and investigation into surgical intervention of CCA, as well as post-surgical recurrence and associated metastases. The limitation of this model lies with the need for surgical involvement. However, given the devastating rates of recurrence after surgical resection of tumours in the clinic, modelling CCA with electroporation of SB transgenic plasmids proves to be an underused tool to trial therapeutic efficacy in cases of

recurrence and metastases. The work by Gürlevik, Fleischmann-Mundt et al. showed an increase in median survival following tumour resection in mice treated with gemcitabine [109]; the effectiveness of using targeted therapies based on the genetic and molecular profile of the primary tumour on recurring and distal secondary tumours would be an interesting route of investigation, and informative for taking the next steps in improving prognosis post-surgery.

Conclusion: CCA is an aggressive primary liver malignancy with abysmal prognosis and limited therapeutic intervention methods. CCA can be modelled *in vivo* using xenograft and orthotopic transplantation models, chemically-induced models and transgenic models. Whilst the tools to investigate this cancer have advanced over the last decade, many models only represent a simplified version of disease, such as organoids representing the cancerous epithelium, but not the complex stromal and a cellular environment found in disease, or GEMMs that model only a single aspect of the complex and heterogeneous genetics of CCA. Despite these limitations, *in vitro*, xenograft and transplantation models, and GEMMs continue to have a central role to play in investigating CCA pathogenesis and treatment.

Expert Opinion: Early models of cholangiocarcinoma (CCA) relied almost exclusively on the chronic administration of mutagenic compounds to experimental animals and on cell lines derived from patients. These models remain a powerful cornerstone of work on CCA, and a number of experimental medicines have been identified by treating tumour-bearing rats or human cell lines. These models are limited, however; cell lines in particular are poor predictors of drug efficacy, as they normally represent a single cancer specimen and have grown accustomed to life in a 2D culture system. More recently, focus has now moved from these tools to a suite of animal models and organoids to understand CCA biology.

Innovative animal models have been developed using classical transgenesis, in particular *Cre/LoxP* technology, but also the hydrodynamic model to overexpress candidate CCA oncogenes, or more recently, delete tumour suppressors in the liver using CRISPR-Cas9. Both types of animal model have their benefits and limitations, and whilst most of these models have a normal immune system and the tumour develops *in situ*, many of these models rely on a very limited genetic profile. For example, *Cre/LoxP* methods often require the deletion of *Trp53* (in combination with other genes such as *Pten* or expression of oncogenic *KRas*). However, *TP53* is lost in only ~30% of patient CCA, therefore whether developing therapies in these model systems is going to be appropriate to target patients with a range of mutations (and not *TP53* mutations) is not yet clear. Similarly, the hydrodynamic model is driven by a very stereotypical set of driver genes (namely expression of gain of function oncogenes such as *Notch, Yap* and *Akt*). Whether these models will predict patient response to a drug or combination of drugs has not yet been determined. That is not to say that animal models no longer have a place in CCA research. The wide uptake of CRISPR-Cas9 editing has opened up CCA research and we are now able to model the diversity of human CCA. As drug development in other cancers has undergone somewhat of a revolution following molecular stratification, we are now at those crossroads in CCA, and by integrating data from largescale human genetics datasets into our animal models, such that they more accurately reflect the human condition, we can test therapies that target either specific molecular subtypes of CCA or broad biological processes that are shared across CCA subtypes.

In addition to animal models, the advancements in organoid technologies has substantially added to the CCA researchers' arsenal and has significantly improved our ability to model this 'cancer in a dish'. These patient-led tools will inevitably improve the transition from drug development to therapy. While current organoid models have a limited cellular composition

(they exclusively contain cancer epithelial cells), these organoids can be grown from patients and propagated and expanded. Moreover, organoids can be used for medium to high throughput drug screening to identify chemical families that inhibit CCA growth. These novel compounds can then be developed further and forward-engineered to improve their pharmacological characteristics. Organoids from other tissues have been shown to reflect the biology of the primary tumour more closely than 2D cell lines and improve therapeutic selection. While Mohr et al rightfully argue that organoid cultures are limited by the absence of an intact immune system [110], we believe advancements will be made for CCA PDOs, as we have seen for other cancers [64], that allow for multi-cell-type cultures that represent patient tumours in their entirety *in vitro*, which will prove valuable for high-throughput drug screening and develop our understanding of the stromal response to therapeutics.. Over the coming years, it will be exciting to watch the expansion of CCA organoid banks that represent the genetic and phenotypic diversity of human CCA. Furthermore, expanding this platform to incorporate an extracellular matrix that more closely resembles that found in CCA, as well as the fibrogenic and immune cells that we know are important in CCA development and in drug responsiveness, will set these complex organoid systems at the front of therapeutic screening and development - not just for small molecules, but for biological agents too.

Drug development and translational medicine for treating CCA is important, however the reality is that the majority of patients are diagnosed when their cancer is advanced and often metastatic. In our opinion, this poses as the biggest challenge to developing models and therapeutics for the treatment of CCA. We need to know (and be able to model) the natural disease processes more closely to ask why certain patients with underlying disease are more prone to cancer. Further to this, we should begin to study how established CCA can move to other, distant sites and form metastasis – organoid and animal models represent the ideal

platforms to understand the biology around these processes. Employing these tools in CCA research will establish coherent therapeutic strategies to be used as a prophylactic for those patients with increased risk of CCA or whose cancer exhibits the potential to metastasise. In the coming years, as we develop a deeper understanding of the genetics of CCA we anticipate the expansion of models that reflect the complex interplay of genotype and microenvironment, including some of the common mutations and fusions in *KRAS*, *IDH* and *FGFR* which have been identified in patient sequencing studies. As we move forward, we should seek to view CCA in the round, not as simply a mutated epithelium, but as a tumour organ with an extensive immune and stromal component both of which are susceptible to therapeutic modulation and are essential for cancer cell survival.

Acknowledgements: LB is funded by CRUK fellowship: C52499/A27948. All figures were generated using BioRender. We would like to thank Edmund Boulter-Comer for technical reading of the manuscript.

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Tables and Figures:

Current treatment options in cholangiocarcinoma		
Intervention	Eligibility/Inclusion	Outcome
Surgical resection	~25% of patients are eligible, the remaining have metastatic or locally advanced disease.	Potentially curative, however relapse rates following resection can be as high at 70%
Surgical resection with adjuvant treatment	A number of studies have shown lack of improvement in post-surgical relapse, however, the BILCAP trial demonstrated that capecitabine following curative resection improved relapse free survival	Capecitabine recommended as the standard of care following curative resection
Palliative Chemotherapy	Non-resectable disease	First line chemotherapy involves cisplatin and gemcitabine. Following progression on first-line therapies FOLFOX can be used as a second-line therapy to manage
Targeted therapies	The presence of a tumour with an actionable mutation. Particularly in IDH-1 or involving FGFR-translocations.	A number of IDH1, IDH2 or dual inhibitors are in trial as are a number of compounds that target active FGFR, however these have a limited effect in the majority of cases

Table 1: Summary of current treatment options in cholangiocarcinoma.

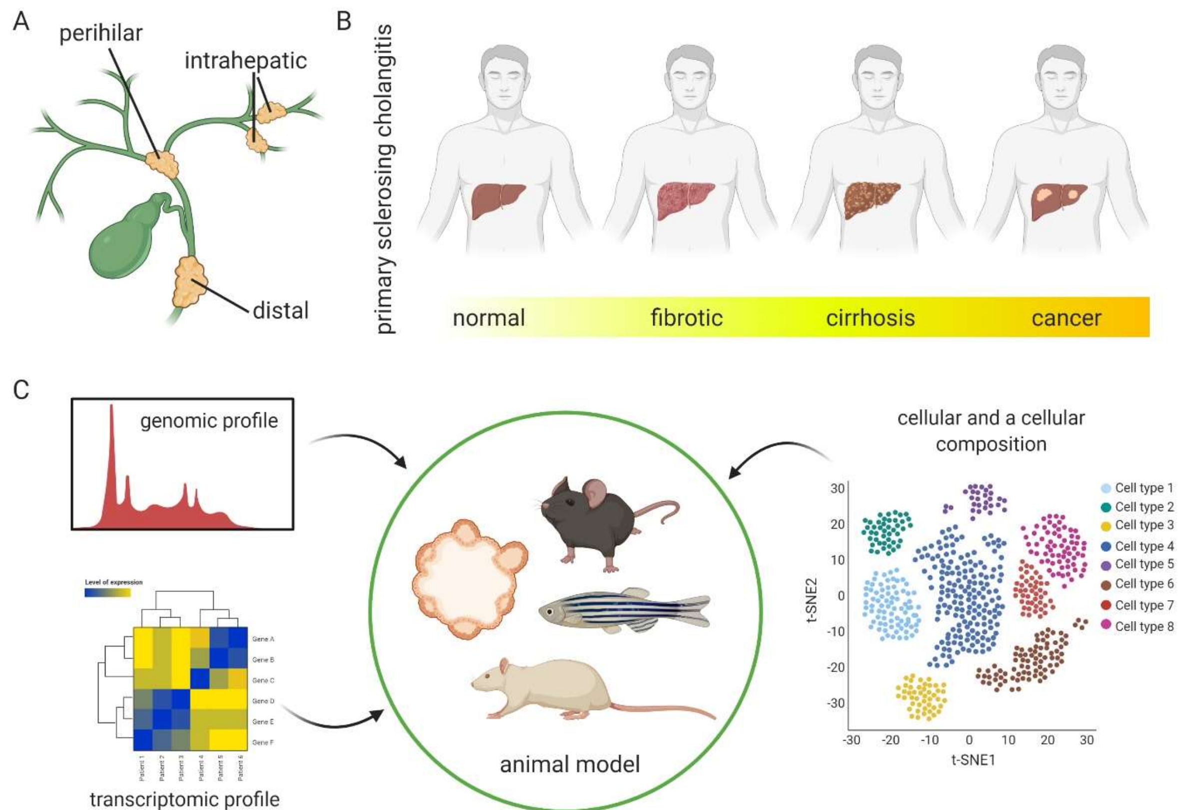


Figure 1: Cholangiocarcinoma origins, development and challenges for disease modelling: A. Cholangiocarcinoma (CCA) can form anywhere along the biliary tree from the intrahepatic ducts, through to the large ducts that leave the liver and connect it to the bowel. **B.** CCA occurs at increased rates on the background of chronic disease, with patients with PSC or infection with *Opisthorchis viverrini* having a substantially increased chance of developing CCA. **C.** Developing animal and in vitro models of CCA has been challenging for a number of reasons. Models of CCA should have a genetic and transcriptomic profile that reflects human disease and should histopathologically represent human cancer, with analogous cell types and extracellular matrices.

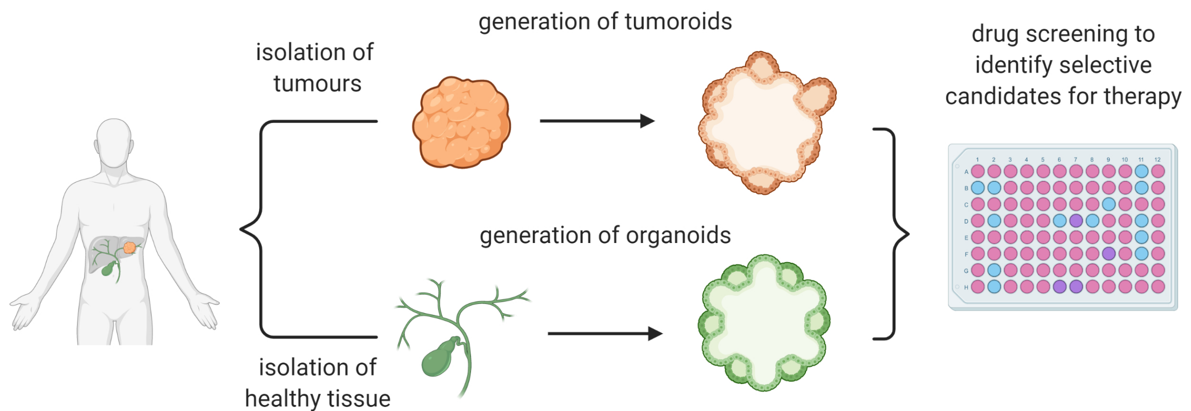


Figure 2: Developing organoid technologies for high throughput therapeutic screening: Increasingly, biopsy samples taken from patients with CCA are being used for testing experimental drugs. The benefit of this approach is that it is relatively straightforward to isolate tumour organoids (tumouroids) and isogenic, non-tumour organoids from the same patient. This strategy provides a precision medicine approach to therapeutic testing while also being able to identify potential therapeutic off-target affects.

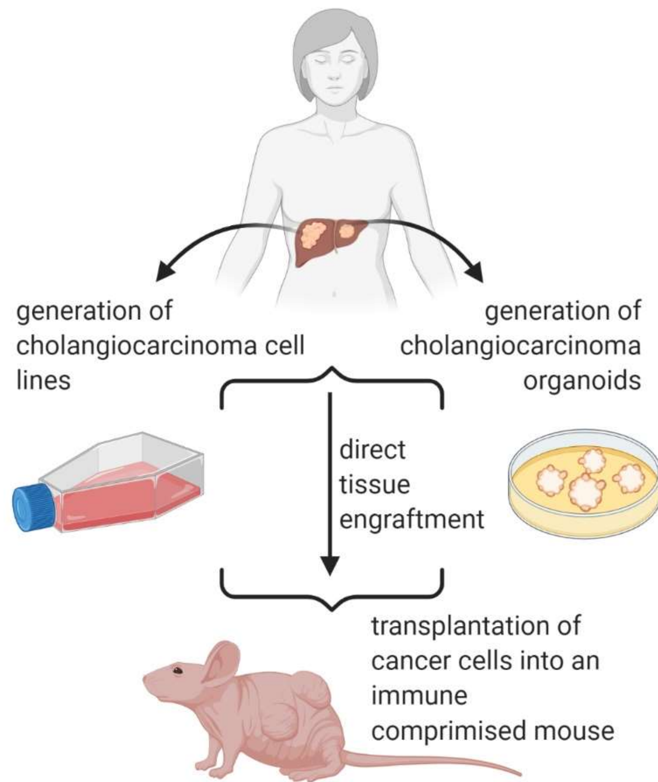


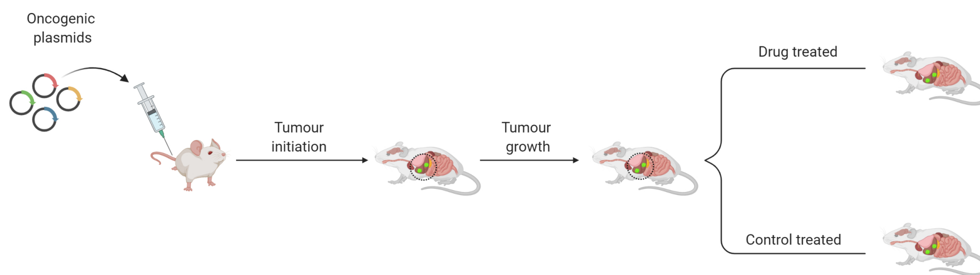
Figure 3: Developing humanised approaches for cancer therapeutics. Cell transplantation from human CCA is a very powerful approach for therapeutic testing. The development of these approaches relies on the use of immunocompromised mice, which lack all or part of an immune system. 2D-cultured, immortalised cell lines, CCA organoids or small pieces of tumour can all be transplanted into these immunocompromised mice to propagate tumours.

1. Hydrodynamic tail-vein injection of oncogenic plasmids

1. Hydrodynamic tail vein injection of oncogenic constructs

2. Transdifferentiaton of hepatocytes into cholangiocytes and oncogenic proliferation of cancer cells

3. Therapeutic intervention to determine the effectiveness of treatment on the model



2. In vivo screening of oncogenic mutations using hydrodynamic tail-vein injection and Cas9/CRISPR

1. Hydrodynamic tail vein injection of gRNA

2. Tumour development and selection of mutations that drive cholangiocarcinoma formation

3. Tissue collection and gRNA analysis

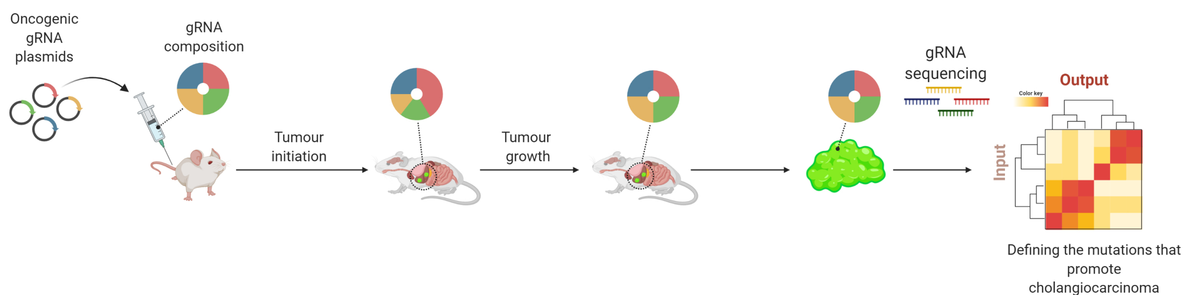


Figure 4. Developing the HTVI model to generate diverse models of cholangiocarcinoma. 1. Liver cancer can be generated by the injection of the plasmids into the tail vein under high hydrodynamic pressure. These plasmids express the cDNA of oncogenic forms of genes that cooperate to generate tumours alongside a transposase, which incorporates these oncogenes into the DNA. The composition of the plasmids determines the type of cancer that forms. **2.** The hydrodynamic tail vein injection model has further been developed to utilise CRISPR-Cas9 to edit endogenous loci and could be used for mutational screening and for developing new models of cholangiocarcinoma.