Abstract

Cholangiocarcinoma (CCA) is a highly aggressive biliary tree malignancy with intrahepatic and extra-hepatic subtypes that differ in molecular pathogeneses, epidemiology, clinical manifestations, treatment, and prognosis. The overall prognosis and patient survival remains poor because of lack of early diagnosis and effective treatments. Preclinical in vivo studies have become increasingly paramount as they are helpful not only for the study of the fundamental molecular mechanisms of CCA but also for developing novel and effective therapeutic approaches of this fatal cancer. Recent advancements in cell and molecular biology have made it possible to mimic the pathogenicity of human CCA in chemical-mechanical, infection-induced inflammatory, implantation, and genetically engineered animal models. This review is intended to help investigators understand the particular strengths and weaknesses of the currently used in vivo animal models of human CCA and their related modeling techniques to aid in the selection of the one that is the best for their research needs.


Introduction

Cholangiocarcinoma (CCA) comprises a heterogeneous group of biliary tree malignancies. The overall incidence and mortality of CCA have been increasing, and the overall 5-year survival rate of all stages and subtypes is estimated as 7–20%. CCA can be intrahepatic (iCCA), perihilar CCA, or distal dCCA. The latter two are described as extra-hepatic cholangiocarcinoma (eCCA), and account for up to 90% of CCA cases. Combined hepatocellular carcinoma (cHCC) includes both HCC and ICCA. The anatomical subtypes have different molecular and clinical characteristics. The effectiveness of targeted therapy and immunotherapy has not been demonstrated in CCA, and the poor prognosis of CCA stems from a lack of understanding of the molecular pathogenesis of its diverse subtypes and the lack of effective treatment.

Recent discovery of genetic alterations related to CCA by next-generation sequencing (NGS) is a great leap forward. For example, the tumor protein p53 gene (TP53), Kirsten rat sarcoma virus oncogene (Kras), recombinant human mothers against decapentaplegic homolog 4 (SMAD4) and BRCA-associated protein 1 gene have been identified in nearly 40% of CCA cases. Moreover, distinct molecular mutation spectra are present in different anatomical subtypes, such as fibrous growth factor receptor (FGFR) gene fusion, mutations in isocitrate dehydrogenase 1 (IDH1) and the BRCA-associated protein 1 gene are more common in ICCA. Kras and E74 like ETS transcription factor 3 have increased mutation frequencies in eCCA, whereas alterations of epidermal growth factor receptor mutation, erb-b2 receptor tyrosine kinase 2 amplification, and phosphatase and tensin homolog (PTEN) deletion are more common in gallbladder cancer.

Although NGS has broadened our knowledge of abnormal molecular alterations in CCA, the functional consequences of these putative driver alterations have not yet been fully interpreted and translated into effective clinical management in vivo. Suitable animal models not only help in mechanistic exploration of CCA development and progression but also provide a good platform to explore new strategies for early clinical diagnosis and precision treatment of this disease. Herein, we review several current techniques and examples of CCA induction in animal models and provide insights into the advantages and limitations of these in vivo tools. Readers are also encouraged to refer to several previous review articles. Compared with previous reviews, we provide better coverage of the different aspects involved in carcinogenic mechanisms and the models used for the study of CCA. We also provide more educational background knowledge before the introduction of each specific model and its related techniques to facilitate understanding for introductory scholars. In addition, more detailed information in particular the subtypes of CCA (e.g., iCCA, eCCA, or a mixture with HCC) that can be tracked while describing each specific model is included in this review.
Li M, et al: Model selection for cholangiocarcinoma study

Chemical-mechanical and infection-induced inflammatory models

Chemical-mechanical models

Chemical carcinogens produce genotoxic effects by destroying DNA structural integrity, damaging cell membranes, and inducing inflammatory reactions, thus promoting the formation and development of CCA.\(^\text{16}\) The commonly used carcinogens are furan, thioacetamide (TAA), diethylnitrosamine (DEN), and their combined models with bile duct ligation (Table 1).

Furan-induced models

Furan is metabolized into reactive substances in the liver, and the long-term effects of these intermediates in reaction with hepatic macromolecular proteins may lead to a dose-dependent increase of liver tumors, including CCA.\(^\text{16}\) Maronpot et al.\(^\text{18}\) investigated the consequences of furan exposure in Fischer 344 rats, and found that continuous gavage with low concentrations of furan (2, 4, or 8 mg/kg body weight) for 2 years resulted in the formation of CCA in 86–100% of the rats. Short-term exposure to high concentrations of furan (30 mg/kg body weight) for 3 months eventually led to the evolution of biliary fibrosis to CCA in all the rats. Further mechanistic study has demonstrated that intrahepatic cholangiocarcinogenesis-related cellular changes, such as cholangiofibrosis and intestinal metaplasia, were induced after treatment with high concentrations of furan for 2 to 3 weeks.\(^\text{19}\) Notably, long-term sustained furan exposure disrupted the microenvironment that stimulates hepatocyte differentiation and induces irreversible bile duct lesions at high concentrations\(^\text{20}\) or non-neoplastic bile duct lesions at lower concentrations (<2 mg/kg body weight).\(^\text{21}\)

Table 1. Commonly used chemical-mechanical models

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Dose</th>
<th>Route</th>
<th>Strain</th>
<th>Latency</th>
<th>Related to human CCA</th>
<th>Tumor type</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Furan</td>
<td>15–60 mg/kg bwt</td>
<td>Gavage Fischer 344 rats</td>
<td>16 months</td>
<td>Developed intestinal epithelial metaplasia and bile duct fibrosis confined to the caudate and right hepatic lobes, eventually progressing to CCA</td>
<td>iCCA 19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>TAA</td>
<td>300 mg/L Water</td>
<td>Sprague-Dawley rats</td>
<td>16–22 weeks</td>
<td>Developed multifocal bile duct hyperplasia with marked intestinal epithelial metaplasia, and then all of these rats developed invasive intestinal-type CCA with intense expression of CK19, similar to multistep progression of human CCA</td>
<td>iCCA 23</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN-BDL</td>
<td>20 mg/kg ip Syrian hamsters</td>
<td>40 weeks</td>
<td>Developed cholangiofibrosis, mucous cystadenoma, and CCA, accompanied by sequential bile duct obstruction and dilatation, formation of large cysts and necrosis and regeneration of the BECs, but without acute proliferative cholangitic lesions and epithelial hyperplasia of second order ducts</td>
<td>CCA 31</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DEN-LMBDL-DEN</td>
<td>100 mg/kg ip and 25 mg/kg oral gavage BALB/C mice</td>
<td>28 weeks</td>
<td>Developed liver injury, chronic cholestasis, fibrosis and cirrhosis, and CCA with physiopathological features of human CCA progression</td>
<td>CCA 33</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

BDL, bile duct ligation; bwt, body weight; CCA, cholangiocarcinoma; DEN, diethylnitrosamine; DMN, dimethylnitrosamine; HCC, hepatocellular carcinoma; LMBDL, left and median bile duct ligation; iCCA, intrahepatic cholangiocarcinoma; ip, intraperitoneal injection; TAA, thioacetamide.

TAA models

TAA is metabolized in the liver to highly reactive sulfur dioxide, which covalently binds to cellular macromolecules to produce hepatotoxicity and induce the development of CCA. In 1984, Praet et al.\(^\text{22}\) developed the first TAA-induced CCA model by feeding TAA-containing food to Lewis rats. Subsequently, in a study of Sprague-Dawley rats fed drinking water containing 300 mg/L TAA, 50% of the rats developed multifocal bile duct hyperplasia with marked intestinal epithelial metaplasia after only 9 weeks, and all the rats developed invasive iCCA within 16–22 weeks.\(^\text{23}\) However, the model did not show systemic metastatic foci or cause death in rats at the end of the 6-month study. In contrast, severe proliferation of bile ducts and CCA with stromal desmo-
plasia, as seen in humans, were detected histologically in Wistar rats.24 Recently, TAA-induced iCCA rat models were used to investigate the immunogenicity and efficacy of DNA cancer vaccines targeting cytotoxic T-lymphocyte antigen 4 blockade and programmed death-ligand 1.25

Combined TAA-DEN models

TAA was found to significantly potentiate the carcinogenic effects of DEN-mediated tumorigenesis in the context of precancerous lesions. The oncogenicity mainly resulted from DEN-induced DNA alkylation damage.26,27 However, this combined TAA-DEN model has a low incidence of CCA accompanied by a high incidence of HCC, which limits the study of the iCCA subtype.

Combined models of cholestasis and carcinogens

Chronic biliary diseases such as primary sclerosing cholangitis, hepatitis biliary stones, and choledochal cysts are associated with cholestasis, and the involvement of those diseases in the development of CCA is now recognized.28 Surgical procedures, such as common bile duct ligation, mimic above pathological changes.29 Various CCA models have been developed by combining the widely used chemical carcinogens DEN or dimethylnitrosamine with bile duct ligation,30–33 and the models effectively characterize the multistep pathological evolution of human CCA from cystic hyperplasia to atypical hyperplasia and to CCA. However, bile duct ligation is relatively demanding for the operator and vulnerable to anesthetic and surgical risks.33

Infection-induced inflammatory models

Liver fluke infection induces chronic inflammation of the bile ducts and is an important risk factor for CCA formation. Oral administration of Opisthorchis viverrini metacercariae combined with dimethylnitrosamine or N-dimethylaminonitrosamine induces cholangiocarcinogenesis in hamsters in vivo.34–37 Combined induction with infection and nitrosamines leads to liver injury, increased inflammation-mediated DNA fragmentation, mitochondrial apoptosis, and structural disruption, which in turn leads to tumor progression.38 Studies addressing that type of etiology will improve our knowledge of the prevention of CCA disease. Thus, the development of CCA models following infection could be of importance, especially in the Far East, in which infections with liver flukes is a public health problem. However, the latency period of such models varies.

In summary, chemical-mechanical and infection-induced models effectively mimic the continuum of pathological changes in human liver tumor initiation and progression stages caused by environmental factors and provide useful preclinical platforms to study the etiology and chemoprevention of CCA. However, such models often lead to a simultaneous development liver cancer and other systemic tumors.27 In addition, the associated genetic changes are unknown.

Implantation models

General considerations

Implantation of established human or rodent cancer cells or tissues into a host animal can generate CCA in a relatively short period of time. Modeling is influenced by various factors, such as the biological characteristics and tumorigenicity of implanted tissue or cells, the volume of cells or tissue block, the implantation route, the site and procedure, and the genetic background and immune status of the host.

Types of implantation

Allograft models

Allograft models involve the reimplantation of cells or tissues from animal into other inbred animal that have immune activity of the same strain and genetic background. Rizvi et al.,39 injected seven different C57BL/6 mouse CCA cell lines (1×106 cells) into the lateral medial lobe of the liver of the same strain of mice. All mice formed tumors histologically and morphologically similar to human CCA after 4 weeks, with positive expression of the bile duct cell markers CK-7, CK-19, and SOX9, formation of hyperplastic connective tissue and malignant glands. The tumorigenicity of the implanted tissues or cells affected the modeling and the biological characteristics of CCA. For example, poorly invasive and tumorigenic BDEsp cells (4×105 cells) and highly tumorigenic BDEneu cells (4×106 cells) from the same immortalized rat BDE1 bile duct cell lines were inoculated into the bile ducts of the same strain of Fischer 344 rats. After 21–26 days the rats transplanted with BDEsp cells formed only nonmetastatic ICCa without biliary obstruction, whereas those transplanted with BDEneu cells exhibited biliary obstruction, extensive abdominal metastasis, and weight loss.40 The above two models mimicked early versus late disease progression and metastasis of human iCCA, respectively.

Allografts can be used in immunocompetent hosts, facilitating the evaluation of the therapeutic response to antitumor drugs in vivo and have profound impacts on tumor immunology research and immunotherapeutic agent development. In a syngeneic transplantation model, cancer-associated fibroblasts in the tumor microenvironment have been identified as a potential antitumor target.41 Moreover, the antitumor activity in vivo of imatinib mesylate,42 sorafenib,43 and vismodegib44 was confirmed in several syngeneic orthotopic transplantation models. However, it is difficult to fully mimic the complex biological and molecular heterogeneity of human CCA.45

Xenograft models

Xenotransplantation involves the implantation of tumor cell lines or tissues into immunodeficient hosts of different species. Currently, the commonly used models include cell line-derived and patient-derived xenografts (PDXs). The first ectopic xenograft model was established by injecting cell line xenografts derived from intrahepatic metastatic human CCA tumor tissue subcutaneously into the flanks of nude mice. The histological characteristics were maintained after seven consecutive cell passages.46

Orthotopic xenograft models: Orthotopic transplantation involves the surgical implantation of CCA cells or tissue into the bile duct or liver. Micro-CT, MRI, ultrasound, and other methods can be used to evaluate tumor size and metastasis. Several orthotopic CCA xenograft models have been established for efficacy assessment of antitumor drugs47 and mechanistic studies of either tumor progression48 or stemness modulation49 of iCCA. However, orthotopic CCA-PDX models are usually too time-consuming to establish and require expensive and laborious longitudinal imaging to monitor tumor growth and therapeutic response. Recently, an orthotopic iCCA-PDX model has been
developed using ultrasound-guided intrahepatic injection and rapid and easy monitoring by minimally-invasive high-frequency ultrasound and bioluminescence imaging.50 Such an iCCA model provides a favorable experimental tool to test the antitumor efficacy of chemotherapeutic agents in autochthonous environments.

Ectopic xenograft models: Ectopic transplantation generally involves scubcutaneous injection of cells or tissue directly into the flanks of mice, which facilitates direct observation of tumor growth and size. In 2016, Cavalloni et al. established the first iCCA-PDX model and a subsequent iCCA-PDX model endogenously expressing the FGFR2-CCDC6 fusion protein.52 In addition, various ectopic transplantation models have been used to identify the regulatory mechanisms of CCA biological behavior, such as abnormal upstream and downstream regulation of microRNAs53,54 and long noncoding RNAs,55,56 or activation of autophagy,57,58 which provide potential therapeutic targets for antitumor drug development.

In general, xenograft models are the most important tool for preclinical drug screening and efficacy assessment because of their short latency, ease of operation, and ability to mimic many of the genetic and epigenetic abnormalities of human cancers. However, xenograft models do not reflect tumor immunology studies,59 and phase III clinical trials of antitumor drugs screened based on the results of cell line-based xenograft models often fail.60 One reason is that the models do not fully encompass the heterogeneity of CCA, and regulates cell growth through the transforming growth factor β (TGF-β) signaling pathway.71 Aberrant SMAD4 expression has been found in various digestive malignancies.72 In 2006, Xu et al.73 crossed mice carrying PTEN conditional allele loss (PTEN−/−) and/or SMAD4 conditional allele loss (SMAD4−/−) with mice carrying Alb-Cre recombinase. The findings showed that of the different genotypes, only Alb-Cre−/−; SMAD4−/−; PTEN−/− mice formed invasive CCA histologically similar to human iCCA at 4–7 months of age, and all died before 10 months of age. Mice with the SMAD4−/−; PTEN−/− genotype did not develop tumors. In contrast, homozygous deletion of PTEN alone resulted in HCC in 66.7% (8/12) of mice at 19 months of age.74 Given the similar genetic backgrounds of mice and the gene-specific recombination system used by these two research teams, it is reasonable to assume that the model used by Xu et al.73 might be a chCCA/iCCA model if the survival time of Alb-Cre−/−; SMAD4−/−; PTEN−/− mice is long enough. Xu et al.73 confirmed that cholangiocarcinogenesis involved the activation of AKT, mTOR, ERK, and CyclinD1, as well as the inactivation of FOXO1. However, the model was established in the absence of chronic liver injury and inflammation, and there was no distant metastasis. Notably, the model was accompanied by the formation of salivary gland tumors, which may be tied to nonspecific expression of the Alb promoter.

Genetically engineered models

Genetically engineered models (GEMs) induce CCA by overexpression, deletion, or mutation of genes related to carcinogenesis through transgenes or gene transduction. GEMs can be used to explore the causes and molecular mechanisms of cholangiocarcinogenesis, progression, and metastasis at the level of specific genes, to identify biomarkers for prognosis, and to preclinically assess the therapeutic response to targeted drugs.64,65 More importantly, GEM-based tumors are generated de novo in immunocompetent animals and are more representative of human tumorigenesis.

Conditional GEMs

Recently, genomic complexity has been partially revealed by high-throughput sequencing, and the deletion of tumor suppressor genes such as TP53, SMAD4, and PTEN, or the activation of actionable oncogenes, like KrasG12D, have been found in CCA.7 More importantly, those genetic driver mutations can be functionally mimicked by a site-specific Cre recombinase (Cre)-loxP system in specific tissues or cells without affecting normal gene expression in other tissues or cells.66 Cre activity can be induced by liver-specific albumin (Alb) promoter. Such recombinase activity is low at birth and gradually increases because of the gradual loss of a floxed target gene in the liver lineage, reaching its maximum activity at 4–6 weeks of age.67 In contrast, a modified Cre-ERT recombinase system68 can realize tissue- and time-specific manipulation of Cre recombinase activity by controlling the administration time of exogenous tamoxifen (TAM). Alb-Cre is expressed in both cholangiocytes and hepatocytes, and Alb-Cre driven GEM models often induce a mixture of iCCA and HCC. In addition to Alb, other promoters including Ah and SOX9, have also been used to mediate the activation of Cre recombinase. Here, we summarize the conditional gene expression and/or deletion models based on the commonly used Cre-loxP system in Table 2.

Liver-specific PTEN-SMAD4 knockout models

Various alterations abrogate the antagonistic effect of PTEN on the PI3K/AKT/mTOR pathway, leading to biliary tract malignancies.68 Although the frequency of PTEN variation in CCA was found to be only 0.6–11% through NGS,7 human clinical specimens have shown that PTEN expression is lost or downregulated in CCA tissues compared with paracancerous tissues.70 SMAD4 is one of the most common tumor suppressor genes in CCA, and regulates cell growth through the transforming growth factor β (TGF-β) signaling pathway.71 Aberrant SMAD4 expression has been found in various digestive malignancies.72 KrasG12D; FGFR2-CCDC6 mice developed using ultrasound-guided intrahepatic injection into the flanks of mice, which facilitates direct observation of tumor behavior, such as abnormal upstream and downstream regulation of microRNAs53,54 and long noncoding RNAs,55,56 or activation of autophagy,57,58 which provide potential therapeutic targets for antitumor drug development.

In general, xenograft models are the most important tool for preclinical drug screening and efficacy assessment because of their short latency, ease of operation, and ability to mimic many of the genetic and epigenetic abnormalities of human cancers. However, xenograft models do not reflect tumor immunology studies,59 and phase III clinical trials of antitumor drugs screened based on the results of cell line-based xenograft models often fail.60 One reason is that the models do not fully encompass the heterogeneity of CCA, and regulates cell growth through the transforming growth factor β (TGF-β) signaling pathway.71 Aberrant SMAD4 expression has been found in various digestive malignancies.72 In 2006, Xu et al.73 crossed mice carrying PTEN conditional allele loss (PTEN−/−) and/or SMAD4 conditional allele loss (SMAD4−/−) with mice carrying Alb-Cre recombinase. The findings showed that of the different genotypes, only Alb-Cre−/−; SMAD4−/−; PTEN−/− mice formed invasive CCA histologically similar to human iCCA at 4–7 months of age, and all died before 10 months of age. Mice with the SMAD4−/−; PTEN−/− genotype did not develop tumors. In contrast, homozygous deletion of PTEN alone resulted in HCC in 66.7% (8/12) of mice at 19 months of age.74 Given the similar genetic backgrounds of mice and the gene-specific recombination system used by these two research teams, it is reasonable to assume that the model used by Xu et al.73 might be a chCCA/iCCA model if the survival time of Alb-Cre−/−; SMAD4−/−; PTEN−/− mice is long enough. Xu et al.73 confirmed that cholangiocarcinogenesis involved the activation of AKT, mTOR, ERK, and CyclinD1, as well as the inactivation of FOXO1. However, the model was established in the absence of chronic liver injury and inflammation, and there was no distant metastasis. Notably, the model was accompanied by the formation of salivary gland tumors, which may be tied to nonspecific expression of the Alb promoter.

Models combining liver-specific PTEN deletion with Kras activation

Kras mutation has been found in 16.7% of iCCA cases.75 In 2013, Marsh et al.76 achieved PTEN deletion with Kras activation in both gallbladder epithelial cells and the intrahepatic bile duct system in adult mice with an Ah promoter-driven Cre-loxP system. It was found that PTEN deletion alone without Kras activation was sufficient to cause slow transformation of normal bile duct epithelium into low-grade malignancies, while dual mutations further shortened the latency of tumorigenesis and transformed tumors into more invasive phenotypes. Based on Cre activities mediated by Alb and TAM administration, or SOX9 promoter, mice with specific liver KrasG12D expression and PTEN homozygous deletion was induced intrahepatic cholangiocarcinogenesis.77 Further investigation showed that in the presence of LSL-KrasG12D, the type of PTEN gene deletion (homozygous or heterozygous) determined the fate of liver tumors with regard to formation from biliary or hepatocyte lineages because immunohistochemical staining revealed that Alb-Cre−/−; LSL-KrasG12D; PTEN−/− mice (AKPP) developed only iCCA; Alb-Cre−/−; LSL-KrasG12D; PTEN−/− mice (AKP) developed only iCCA and HCC; while Alb-Cre−/−; LSL-KrasG12D; PTEN−/+ mice (AK) developed only HCC. Notably, the spatiotemporal specificity of TAM-induced recom-
Table 2. Conditional genetically engineered models mediated by Cre-LoxP recombinase system

<table>
<thead>
<tr>
<th>Genes targeted</th>
<th>Cre</th>
<th>Chemical induction</th>
<th>Latency</th>
<th>Tumor type</th>
<th>IHC</th>
<th>Comments (advantages/A; disadvantages/D)</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMAD4&lt;sup&gt;L/L&lt;/sup&gt;; PTEN&lt;sup&gt;L/L&lt;/sup&gt;</td>
<td>Alb-Cre</td>
<td>–</td>
<td>4–7 months</td>
<td>iCCA</td>
<td>CK-19&lt;sup&gt;+&lt;/sup&gt;; Mucicarmine&lt;sup&gt;+&lt;/sup&gt;; Mucin 5AC&lt;sup&gt;+&lt;/sup&gt;; Hep Par1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>A: The formation of iCCA follows multistep progression of histopathological changes; 100% penetrance; progressed into invasive iCCA; histologically is similar to human iCCA; D: All mice died at about 10 months of age before potential HCC formation; No metastasis; Salivary gland tumor developed</td>
<td>73</td>
</tr>
<tr>
<td>LSL-Kras&lt;sup&gt;G12D&lt;/sup&gt;; PTEN&lt;sup&gt;L/L&lt;/sup&gt;</td>
<td>AhCre&lt;sup&gt;ERT&lt;/sup&gt;</td>
<td>BNF/TAM</td>
<td>NA</td>
<td>GBC; iCCA</td>
<td>CK-19&lt;sup&gt;+&lt;/sup&gt;</td>
<td>A: Short latency; widespread papillary neoplasia of BECs formed; D: Dual mutant mice did not survive long enough to develop the types of lesions seen in PTEN&lt;sup&gt;L/L&lt;/sup&gt; mice; developed extensive noninvasive papillary neoplasms in the intrahepatic biliary system and invasive moderately differentiated adenocarcinomas of gall bladder with stromal desmoplasia without specific phenotype</td>
<td>76</td>
</tr>
<tr>
<td>LSL-Kras&lt;sup&gt;G12D&lt;/sup&gt;; PTEN&lt;sup&gt;L/L&lt;/sup&gt;</td>
<td>Alb-Cre</td>
<td>–</td>
<td>7 weeks of age</td>
<td>iCCA</td>
<td>α-SMA&lt;sup&gt;+&lt;/sup&gt;; Mucicarmine&lt;sup&gt;+&lt;/sup&gt;; CK-19&lt;sup&gt;+&lt;/sup&gt;; Pan-CK&lt;sup&gt;+&lt;/sup&gt;; Hep Par1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>A: All AKPP mice demonstrated abdominal distension accompanied by jaundice and weight loss, which recapitulates well those frequently observed in human iCCA; D: Short median survival</td>
<td>77</td>
</tr>
<tr>
<td>SOX9-Cre&lt;sup&gt;ERT2&lt;/sup&gt;</td>
<td>Tam</td>
<td>Administered TAM at P10</td>
<td>2 months</td>
<td>iCCA</td>
<td>Mucin&lt;sup&gt;+&lt;/sup&gt;; CK-19&lt;sup&gt;+&lt;/sup&gt;; Pan-CK&lt;sup&gt;+&lt;/sup&gt;; Hep Par1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>A: Developed exclusive iCCA; iCCA originated from the cholangiocytes; D: The type of cell in which Cre-mediated recombination occurs varies with age</td>
<td>77</td>
</tr>
<tr>
<td>LSL-Kras&lt;sup&gt;G12D&lt;/sup&gt;; TP53&lt;sup&gt;L/L&lt;/sup&gt;</td>
<td>Alb-Cre</td>
<td>–</td>
<td>9 weeks</td>
<td>iCCA and HCC and cHCC-iCCA</td>
<td>Pan-CK&lt;sup&gt;+&lt;/sup&gt;; AFP&lt;sup&gt;+&lt;/sup&gt;</td>
<td>A: Widespread local and distant metastasis; multistage progression; two of the most common gene mutations in human iCCA were involved; tumors arise from the malignant progression of precursor lesions in the bile ducts; D: A certain proportion of HCC is present, which may limit its application in iCCA research</td>
<td>81</td>
</tr>
<tr>
<td>AAV8-TBG-Cre</td>
<td>DDC diet</td>
<td>12–66 weeks</td>
<td>iCCA and HCC and chHCC-iCCA</td>
<td>Pan-CK&lt;sup&gt;+&lt;/sup&gt;; CK-19&lt;sup&gt;+&lt;/sup&gt;; Hnf 4α in iCCA; Hnf 4α in HCC</td>
<td>A: Full penetrance; tumor developed in the liver injury of ductular reaction, fibrosis, and inflammation similar to human liver cancer; D: Not exclusive iCCA</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>SOX9-Cre&lt;sup&gt;ERT2&lt;/sup&gt;</td>
<td>TAM; DDC diet</td>
<td>30 weeks (average postinjection)</td>
<td>iCCA and HCC and chHCC-iCCA</td>
<td>Pan-CK&lt;sup&gt;+&lt;/sup&gt;; CK-19&lt;sup&gt;+&lt;/sup&gt;; Hnf 4α in iCCA</td>
<td>A: Full penetrance; tumor developed in the liver injury, fibrosis, and inflammation; adjacent liver to iCCA showed biliary intraepithelial neoplasia similar to precursor of human iCCA; D: Not exclusive iCCA</td>
<td>83</td>
<td></td>
</tr>
<tr>
<td>LSL-IDH2&lt;sup&gt;R172K&lt;/sup&gt;; LSL-Kras&lt;sup&gt;G12D&lt;/sup&gt;</td>
<td>Alb-Cre</td>
<td>–</td>
<td>33-58 weeks</td>
<td>iCCA</td>
<td>CK-19&lt;sup&gt;+&lt;/sup&gt;; Hep Par1&lt;sup&gt;−&lt;/sup&gt;</td>
<td>A: Developed multifocal iCCA with peritoneal metastasis and splenic invasion; full penetrance; D: Long latency</td>
<td>85</td>
</tr>
</tbody>
</table>

(continued)
The most common gene mutations in CCA are Kras and TP53.ym In 2012, O’Dell et al. established Alb-Cre<sup>+</sup>; Kras<sup>G12D</sup>, TP53<sup>fl/fl</sup> CCA models. Liver tumors formed as early as 9 weeks of age and were histopathologically confirmed to be 66% iCCA, 17% mixed HCC/iCCA, and 17% HCC. Most of the mice had symptoms of bloody ascites and tumor necrosis. In addition, 75% of the tumors invaded adjacent organs or developed distant metastases. It was also found that TP53 gene deletion alone was not sufficient to cause liver lesions even over a sufficiently long time period. However, when combined with Kras<sup>G12D</sup> activation, both heterozygous and homozygous TP53 mutations accelerated tumorigenesis and metastasis. Of note, a certain proportion of HCC was present in this model. To identify mechanisms driving precancerous lesions and subsequent progression toward invasive tumors that faithfully recapitulate human iCCA, a model that combined Kras<sup>G12D</sup> and TP53<sup>fl/fl</sup> lines was used. Cre recombinase activity driven by the SOX9 promoter eventually led to the transformation of SOX9+ cells in the liver. In addition, 75% of the tumors invaded adjacent organs or developed distant metastases. It was also found that TP53 gene deletion alone was not sufficient to cause liver lesions even over a sufficiently long time period. However, when combined with Kras<sup>G12D</sup> activation, both heterozygous and homozygous TP53 mutations accelerated tumorigenesis and metastasis. Of note, a certain proportion of HCC was present in this model. To identify mechanisms driving precancerous lesions and subsequent progression toward invasive tumors that faithfully recapitulate human iCCA, a model that combined Kras<sup>G12D</sup> and TP53<sup>fl/fl</sup> lines was used. Cre recombinase activity driven by the SOX9 promoter eventually led to the transformation of SOX9+ cells in the liver. In addition, 75% of the tumors invaded adjacent organs or developed distant metastases. It was also found that TP53 gene deletion alone was not sufficient to cause liver lesions even over a sufficiently long time period. However, when combined with Kras<sup>G12D</sup> activation, both heterozygous and homozygous TP53 mutations accelerated tumorigenesis and metastasis. Of note, a certain proportion of HCC was present in this model. To identify mechanisms driving precancerous lesions and subsequent progression toward invasive tumors that faithfully recapitulate human iCCA, a model that combined Kras<sup>G12D</sup> and TP53<sup>fl/fl</sup> lines was used. Cre recombinase activity driven by the SOX9 promoter eventually led to the transformation of SOX9+ cells in the liver. In addition, 75% of the tumors invaded adjacent organs or developed distant metastases. It was also found that TP53 gene deletion alone was not sufficient to cause liver lesions even over a sufficiently long time period. However, when combined with Kras<sup>G12D</sup> activation, both heterozygous and homozygous TP53 mutations accelerated tumorigenesis and metastasis.
Li M, et al: Model selection for cholangiocarcinoma study

iCCA lesions at 33–58 weeks of age with peritoneal metastases and splenic invasion, whereas mice with KrasG12D activation alone formed single HCC nodules. Mechanistically, mutant IDH inhibited the differentiation of hepatic progenitor cells in the liver after hepatocyte nuclear factor 4a inactivation, thus promoting iCCA formation. Because of the high mutation rate of IDH in tumors and the relatively mature clinical studies of IDH inhibitors, the model is of great significance for the direct evaluation of therapeutic response to anti-iCCA agents. However, the model has a relatively long incubation period.

**Models combining Kras activation with TGF-βR2 and CDH1 inactivation**

FGFR2 gene fusions are seen in 13–45% of iCCA patients, and frequent abnormal changes in TGF-β family receptors have been detected in eCCA by NGS. Nakagawa et al.89 first knocked CreERT into the endogenous K19 locus to obtain K19CreERT mice with TAM administration. Effective genetic recombination was confirmed with reporter mice. Then, K19CreERT/LSL-KrasG12D/FGFR2R2/4 mice (KT-K19CreERT) were generated by crossing LSL-KrasG12D, FGFR2R2/4 and K19CreERT mice and induced with TAM. All (15/15) KTK19CreERT mice died of respiratory failure, which was probably caused by lung adenocarcinoma. CDH1 gene deletion has been shown to promote liver tumor development in mice and to lead to a series of pathological changes similar to those of primary sclerosing cholangitis in humans. These mice showed an increased ductular reaction after 7 months of a high-fat diet (HFD). HFDs cause nonalcoholic fatty liver disease, and HFD-related models are a good tool for the study of the pathogenesis of iCCA in the context of chronic liver damage. HFDs also promote the initiation and deterioration of chCC/iCCA in CDH1/L/L/KrasG12D mice. By crossing KTK19CreERT and CDH1/L/L mice with KrasG12D mice, Nakagawa et al.89 established a KT-K19CreERT mouse model characterized by CDH1/FGFR2 dual knockout and Kras activation, in which the pathological manifestations were histologically similar to human eCCA, with jaundice and lymph node metastases, but no bile duct tumors were observed with alterations in any of the aforementioned genes. However, the mice demonstrated lung adenocarcinomas, leading to lung failure or death within 4 weeks, which is not suitable for long-term experimental studies.

**Models combining TP53 deficiency with carcinogens**

A common limitation of transgenic CCA models is that the tumor initiation and formation do not involve chronic inflammation and liver injury, which limits the aggressive development of tumors. The exposure of transgenic mice to carcinogens can compensate for the lack of an inflammatory background in transgenic models. Intraportal injection of transgenic mice with TP53 deletions with CCL3, three times a week for 4 months resulted in 54% of TP53+/c mice developing iCCA, and approximately 14% (1/7) mice developing lymph node metastasis at 29 weeks of age. Using a similar approach, Guest et al.86 fed hepatotoxic TAA to biliary epithelium-specific TP53-knockout transgenic mice in an attempt to induce a tumorigenic stress response. After 26 weeks, 80% of TP53+/c mice developed multifocal, invasive CCA in the liver.

**Notch models**

Aberrant Notch activation can activate Notch 1 intracellular domain (NICD), which has been implicated in a variety of tumors. The pathophysiological role of the Notch signaling pathway has been partially elucidated in CCA GEMs. For example, Alb-Cre: NICH transgenic mice generated by crossing mice carrying a sequence encoding NICD overexpression with Cre mice activates the Notch signaling pathway, making mature hepatocytes transdifferentiate into biliary epithelial cells. Implanting liver tissue from 9-month-old transgenic mice subcutaneously into SCID mice results in the formation of iCCA after 3 weeks.87 Biliary tract malignancies are often accompanied by elevated levels of phosphorylated AKT. Cellular fate-tracing results have shown that overexpression of NICD promotes cell proliferation and to the development of iCCA originating from hepatocytes. Cirrhosis, chronic hepatitis B and C, and liver fluke infection are major risk factors for iCCA, which is often accompanied by chronic liver inflammation. In this context, iCCA has a high rate of TP53 gene mutation.99 In the basis of liver injury by TAA administration, TP53/L/L transgenic mice develop iCCA originating from biliary epithelium.100 This model mimics a common situation in human cholangiocarcinogenesis.

**Nonconditional GEMs**

Nonconditional GEMs are usually established by local injection in the liver or bile duct and transposon- or duct-specific promoter-mediated constitutive activation of oncogenes. Transposons can carry relatively large exogenous gene fragments for efficient transposition in animals and are important tools in the field of transgenic animal modeling, of which a relatively commonly used one is the Sleeping Beauty transposon. Currently, several iCCA models have been constructed based on that system.

Phosphorylated AKT was found to be upregulated in eCCA.101 Similar to AKT, Yes-associated protein (YAP) is a transcriptional activator associated with primary liver cancer development.102 The method used to establish AKT/YAP models was to directly inject a transposase mixture containing AKT/YAP plasmids into the bile duct of wild-type C57BL/6 mice while ligating the bile draining duct so that the targeted oncogene remained in the left lobe of the liver. The mitogen IL-33, which has the ability to promote bile duct cell proliferation, inflammation, and liver fibrosis, was continuously injected intraperitoneally for 3 days after surgery, and its effect had been confirmed in previous studies. Seventy-two percent of mice transduced with the AKT/YAP gene and treated with IL-33 developed tumors that had a morphology and phenotype similar to human CCA, accompanied by high expression of the cholangiocyte markers CK-7, CK-19, and SOX9. Only 20% of mice transduced with the AKT/YAP gene alone developed iCCA, indicating that IL-33 plays an important role in iCCA formation. However, knockout of focal adhesion kinase (FAK), a nonreceptor tyrosine kinase, in AKT/YAP mice delayed iCCA development and progression.104 Wang et al.105 used the same gene delivery technique to target liver with exogenous co-expression of myr-AKT and Fbxw7, a dominant negative form of the tumor suppressor Fbxw7, and found the development of iCCA in YAP wild-type mice within a short time of approximately 54 day. In YAP homozygous deleted mice, the tumor latency was significantly prolonged. Using the same methods, histone lysine methyltransferase G9a, and NICD have been demonstrated to be involved in cholangiocarcinogenesis.106,107 This model develops tumors quickly and can be used to study new therapeutic drugs for iCCA. However, it is technically demanding because it requires surgical ligation of the bile ducts and bile duct perfusion with drugs.
different effects on the type of tumor that develops. For example, delivery of plasmids containing Myc and mutant NRAS proto-oncogene or AKT2 via tail vein injection induces HCC, whereas transfection of the same plasmids by electroporation induces ICCA formation.\(^{108}\) That indicates that the tumor microenvironment plays an important role in the development of CCA, and gene overexpression and transcription levels of both CCA and the tumor microenvironment. Bovine protein 5 is a promoter that is actively expressed in both the stratified and pseudostratified epithelia of several organs, and is an important tool for constructing animal models of gene overexpression. A mouse model overexpressing wild-type erb-b2 receptor tyrosine kinase 2 under the control of the Bovine protein 5 promoter has been modeled, and all of these mice developed gallbladder adenocarcinoma at 4 months of age.\(^{109}\) The model recapitulates the multistep evolution of gallbladder lesions.

Recently, clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR associated protein 9 (Cas9) system, a new somatic gene editing technology, has been developed to mediate highly specific and irreversible genomic screening. An advantage of the technique is that genome-wide genome screening identifies several novel genes associated with tumorigenesis. For example, Weber et al.\(^{110}\) used the method to directly mediate multiple genetic mutations of up to 18 target gene sets in adult mouse liver somatic cells, and found that 100% of mice developed mixed HCC/CCA after 20–30 weeks. CCA mouse models have also been established either via CRISPR/Cas9-mediated knockout of NF1,\(^{111}\) or through CRISPR/Cas9 system-based KRAS-G120 activation and TP53 deletion.\(^{112}\) Notably, the latency period of ICCA in the latter was significantly shorter than that in the comparable conditional GEMs model.\(^{111}\) Dasatinib sensitivity was tested in CRISPR/Cas9-mediated human iCCA cells with IDH1 mutation.\(^{113}\)

Taken together, somatic gene integration models, especially those based on hydrodynamic injection and Sleeping Beauty transposon, are flexible, relatively easy to establish and have a short tumorigenic latency, therefore they are important tools to study gene and promoter functions. However, target gene transfection is mainly limited to the pericentral region and only lasts for a few hours to days.\(^{114}\) Hydrodynamic delivery can also cause transient liver damage. In the meantime, because mutations are present in cancers in adult human cancers and affect only a small number of cells, CRISPR/Cas9-based models are more responsive to tumorigenesis in humans.

**Conclusions**

There is no perfect animal model that meets all the needs of human CCA research. Choosing the right animal model for each experimental purpose is key. Multiple parameters such as tumor type, host immune activity, genetic alterations, and the tumor microenvironment should be considered to weigh the advantages and disadvantages when selecting a model. For example, chemical-mechanical and infection-induced inflammatory models can simulate the entire process of tumor development by changing environmental factors, but an obvious shortcoming is the poor specificity of the tumors that develop, which may include tumors of multiple systems. Implantation models are easy to establish, but the tumors grow in immunodeficient animals, which makes it difficult to truly reflect the growth of human tumors. In contrast, GEMs can simulate the initiation of CCA at the genetic and molecular level, but available models using Alb-driven Cre-IOX system usually induce ICCA or a mixture of ICCA and HCC. Moreover, the latency period is long, the technology is demanding, and it is difficult to develop a system where the transgenic products fully and accurately reflect the growth of human tumors.

With the development of targeted therapy and immunotherapy, PDX models and GEMs are playing key roles in precision medicine. Humanized PDX models have benefits in immunotherapy drug screening in malignancies, such as nasopharyngeal carcinoma\(^{115}\) and triple-negative breast cancer.\(^{116}\) They also recapitulate the interactions of cancer, the tumor microenvironment, and the immune system in humans. Efforts should be made to develop humanized PDX models of all CCA subtypes to promote the development of individualized immunotherapy in the future. Simultaneously, there has been an active search for promoters that specifically target intrahepatic or extra-hepatic bile ducts. Optimization of existing genetic recombination systems is a promising option. In addition, flexible CRISPR/Cas9 gene editing technique may be another favorable choice.

**Conflict of interest**

The authors have no conflict of interests related to this publication.

**Author contributions**

Conceived the work, designed the outline of the review and supervised all aspects of the manuscript (YY); Participated in the literature search, scrutiny and interpretation, as well as in writing and editing the manuscript (ML, XZ, WW, YS, QD, JY); Contributed to review of the data and critical revision of the review (BJ). All authors read and approved the final manuscript.

**References**


Li M, et al: Model selection for cholangiocarcinoma study
Model selection for cholangiocarcinoma study


Li M, et al: Model selection for cholangiocarcinoma study

Journal of Clinical and Translational Hepatology 2022

9


Li M, et al: Model selection for cholangiocarcinoma study

98159.


