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Review Article

Selecting an Appropriate Experimental Animal Model for Cholangiocarcinoma Research

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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.

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Introduction

Cholangiocarcinoma (CCA) comprises a heterogeneous group of biliary tree malignancies. The overall incidence and mortality of CCA have been increasing,[1] and the overall 5-year survival rate of all stages and subtypes is estimated as 7–20%.[2] 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.[3,4] The effectiveness of targeted therapy and immunotherapy has not been demonstrated in CCA,[5] 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.[6] 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.[7–9]

Although NGS has broadened our knowledge of abnormal molecular alterations in CCA,[10] 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 precise 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.[11–15] 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.

Keywords: Cholangiocarcinoma; Heterogeneity; Animal model; Genetically engineered model; Cancer cell of origin.

Abbreviations: Alb, albumin; CCA, cholangiocarcinoma; CHCC, combined hepatocellular carcinoma; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidine; DEN, diethylnitrosamine; ECCA, extra-hepatic cholangiocarcinoma; FGFR, fibrous growth factor receptor; GEMs, genetically engineered models; HFD, high-fat diet; ICCA, intrahepatic cholangiocarcinoma; IDH, isocitrate dehydrogenase; Kras, Kirsten rat sarcoma virus oncogene; NGS, next-generation sequencing; NICD, Notch 1 intracellular domain; PDXs, patient-derived xenografts; PTEN, phosphatase and tensin homolog; SMAD4, recombinant human mothers against decapentaplegic homolog 4; TAA, thioacetamide; TAM, tamoxifen; TGF-β, transforming growth factor beta; TP53, tumor protein p53; YAP, Yes-associated protein.

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Table 1. Commonly used chemical-mechanical models

		Dose	Route	Strain	Latency	Related to human CCA	Tumor type	Ref.
Chemicals	Furan	15–60 mg/kg bwt	Gavage	Fischer 344 rats	16 months	Developed intestinal epithelial metaplasia and bile duct fibrosis confined to the caudate and right hepatic lobes, eventually progressing to CCA	iCCA	19
	TAA	300 mg/L	Water	Sprague-Dawley rats	16–22 weeks	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	iCCA	23
Chemical-mechanical	TAA-BDL	0.05%	Water	Wistar rats	30 weeks	Developed histologically invasive intestinal and mucin-producing CCA with positive expression of CK-7 and Claudin-4	CCA	30
	DMN-BDL	20 mg/kg	ip	Syrian hamsters	40 weeks	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	CCA	31
	DEN-LMBDL-DEN	100 mg/kg ip and 25 mg/kg oral gavage	ip and oral gavage	BALB/C mice	28 weeks	Developed liver injury, chronic cholestasis, fibrosis and cirrhosis, and CCA with physiopathological features of human CCA progression	CCA	33

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.

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.[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.[17] Maronpot *et al*.[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.[19] Notably, long-term sustained furan exposure disrupted the microenvironment that stimulates hepatocyte differentiation and induces irreversible bile duct lesions at high concentrations[20] or non-neoplastic bile duct lesions at lower concentrations (<2 mg/kg body weight).[21]

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*.[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.[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-indued 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, hepatobiliary 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 tumorgenicity 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 (1×10^6 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 tumorgenicity of the implanted tissues or cells affected the modeling and the biological characteristics of CCA. For example, poorly invasive and tumorigenic BDEsp cells (4×10^6 cells) and highly tumorigenic BDEneu cells (4×10^6 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 vismodegib[44] 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 drugs[47] and mechanistic studies of either tumor progression[48] or stemness modulation[49] of iCCA. However, orthotopic CCA-PDX models are usually technically challenging 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 anticancer efficacy of chemotherapeutic agents in autochthonous environments.

Ectopic xenograft models: Ectopic transplantation generally involves subcutaneous 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*.[51] 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 microRNAs[53,54] 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 tumors. However, xenograft models do not reflect tumorigenesis,[45] immunodeficient hosts are not suitable for tumor immunology studies,[59] and phase III clinical trials of antitumor drugs screened based on the results of cell line-derived xenograft models often fail.[60] One reason is that the models do not fully encompass the heterogeneity of CAA. Highly transplantable iCCA and eCCA cell lines with disease heterogeneity have been established from a PDX model, which may be a promising platform for individualized anticancer drug screening.[61] Moreover, fresh human tumor tissue is not easily accessible. To overcome that, some studies have generated CCA models with metastasis biopsies[62] or secondary engraftment of cryopreserved tissues[63] obtained from CCA patients.

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 $Kras^{G12D}$ 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 system[68] 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.[69] 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 beta (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 ($PTEN^{L}$) and/or *SMAD4* conditional allele loss ($SMAD4^{L}$) with mice carrying Alb-Cre recombinase. The findings showed that of the different genotypes, only $Alb\text{-}Cre^{+}$; $SMAD4^{L/L}$; $PTEN^{L/L}$ 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 $SMAD4^{L/L}$ alone 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 cHCC/iCCA model if the survival time of $Alb\text{-}Cre^{+}$; $SMAD4^{L/L}$; $PTEN^{L/L}$ 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 promotor.

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 $Kras^{G12D}$ expression and *PTEN* homozygous deletion was induced intrahepatic cholangiocarcinogenesis.[77] Further investigation showed that in the presence of $LSL\text{-}Kras^{G12D}$, 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 $Alb\text{-}Cre^{+}$; $LSL\text{-}Kras^{G12D}$; $PTEN^{L/L}$ mice (AKPP) developed only iCCA; $Alb\text{-}Cre^{+}$; $LSL\text{-}Kras^{G12D}$; $PTEN^{L/+}$ mice (AKP) developed iCCA and HCC; while $Alb\text{-}Cre^{+}$; $LSL\text{-}Kras^{G12D}$; $PTEN^{+/+}$ 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

Genes targeted	Cre	Chemical induction	Latency	Tumor type	IHC	Comments (advantages/A; disadvantages/D)	Ref.
SMAD4$^{L/L}$; PTEN$^{L/L}$	Alb-Cre	–	4–7 months	iCCA	CK-19^{+}; Mucicarmine^{+}; Mucin 5AC^{+}; Hep Par1^{-}	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	73
LSL-Kras$^{V12/-}$; PTEN$^{L/L}$	AhCre^{ERT}	BNF/TAM	NA	GBC; iCCA	CK-19^{+}	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$^{L/L}$ 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	76
LSL-Kras^{G12D}; PTEN$^{L/L}$	Alb-Cre	–	7 weeks of age	iCCA	α-SMA^{+}; Mucicarmine^{+}; CK-19^{+}; Pan-CK^{+}; Hep Par1^{-}	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	77
	Alb-Cre^{ERT2}	Administered TAM at P10	2 months	iCCA	Mucin^{+}; CK-19^{+}; Pan-CK^{+}; Hep Par1^{-}	A: Developed exclusive iCCA; iCCA originated from the cholangiocytes; D: The type of cell in which Cre-mediated recombination occurs varies with age	77
	SOX9-Cre^{ERT2}	TAM	12 weeks	iCCA, eCCA and pancreatic cancer	CK-19^{+}	A: Short latency; developed pancreatic cancer, iCCA and eCCA using Cre-loxP under the control of SOX9 promotor, indicating that SOX9 had a potential role in hepatopancreatic ductal system and the intrapancreatic and intrahepatic ductal networks; provided a mouse model for study hepatopancreatic ductal carcinomas; D: Mixed liver cancer and pancreatic cancer, not exclusive iCCA; AKPP animals succumbed rapidly to ill-health, with a marked survival deficit	78
LSL-Kras^{G12D}; TP53$^{L/L}$	Alb-Cre	–	9 weeks	iCCA and HCC and cHCC-iCCA	Pan-CK^{+}; AFP^{+}	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	81
	AAV8-TBG-Cre	DDC diet	12–66 weeks	iCCA and HCC and cHCC-iCCA	Pan-CK^{+}, CK-19^{+}; Hnf 4α^{-}in iCCA; Hep Par1^{+} in HCC	A: Full penetrance; tumor developed in the liver injury of ductular reaction, fibrosis, and inflammation similar to human liver cancer; D: Not exclusive iCCA	83
	SOX9-Cre^{ERT2}	TAM; DDC diet	30 weeks (average) postinjection	iCCA and HCC and cHCC-iCCA	Pan-CK^{+}; CK-19^{+}; Hnf 4α^{-}in iCCA	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	83
LSL-IDH2^{R172K}; LSL-Kras^{G12D}	Alb-Cre	–	33-58 weeks	iCCA	CK-19^{+}; Hep Par1^{-}	A: Developed multifocal iCCA with peritoneal metastasis and splenic invasion; full penetrance; D: Long latency	85

(continued)

Table 2. *(continued)*

Genes targeted	Cre	Chemical induction	Latency	Tumor type	IHC	Comments (advantages/A; disadvantages/D)	Ref.
LSL-$Kras^{G12D}$; $TGFBR2^{L/L}$; $CDH1^{L/L}$	$K19Cre^{ERT}$	TAM; IL-33	20 days	eCCA	CK-7^+; CK-19^+; α-SMA^+	A: Formed a model of biliary injury-related eCCA from EHBD; suggested PBGs as the cellular origin of eCCA; eCCA spread laterally along the biliary tree, and cancer cells were highly malignant and metastasized to the regional lymph nodes; periductal infiltrating growth and lymph node metastasis are characteristics of human eCCA; D: All KTC-$K19Cre^{ERT}$mice died within 4 weeks after TAM administration	89
$TP53^{L/L}$	–	CCL4	29 weeks	iCCA	CK-19^+	A: The initiation and progression of iCCA in the setting of bile duct proliferation and associated fibrosis; developed lymph node metastasis; shares feature prominent in the human disease, including the presence of intrahepatic fibrosis, increased inflammation and a molecular profile; D: Long latency; penetrance is not high (54%)	93
	$Alb\text{-}Cre^{ERT}$	TAM; TAA;	26 weeks	CCA	NA	A: CCA arises following chronic inflammation caused by injury with TAA; developed multifocal invasive CCA; high penetrance (80%); D: Lack of detailed CCA subtype and phenotype identification results	93

AFP, alpha-fetoprotein; AKPP mice, $Alb\text{-}Cre^+$; $LSL\text{-}Kras^{G12D}$; $PTEN^{L/L}$ mice; BECs, biliary epithelial cells; BNF/TAM, β-naphthoflavone and tamoxifen; CCL4, carbon tetrachloride; CDH1, a gene encoding E-cadherin molecule; cHCC-iCCA, combined hepatocellular carcinoma and intrahepatic cholangiocarcinoma; CK, cytokeratin; CK-19, cytokeratin 19; DDC, 3,5-diethoxycarbonyl-1,4-dihydrocollidin; EHBD, extra-hepatic bile duct; $K19Cre^{ERT}$, Cre^{ERT} induced by TAM under the control of CK-19 promoter; NA, not available; Pan-CK, pan-cytokeratin; PBG, peribiliary gland; P10, postnatal day 10; SOX9: SRY-related high mobility group box transcription factor 9.

binase activity may be correlated with specific tumor type, as evidenced by the fact that TAM administration at 10 days of age induced Cre recombinase activity in hepatocytes and biliary cells, culminating in the intrahepatic cholangiocarcinogenesis, whereas administration on day 56 mediated genetic recombination only in hepatocytes, culminating in the formation of HCC and hepatocyte dysplasia, which also supports the conclusion that hepatocytes can serve as the cellular origin of iCCA. Based on the results obtained in the above models, Lin *et al*.[78] crossed mice expressing the $Kras^{G12D}$ allele and/or the *PTEN* allele with mice expressing Cre recombinase under the control of the *SOX9* gene and screened for $SOX9^+$; Cre^{ERT2+}; $LSL\text{-}Kras^{G12D}$; $PTEN^{L/L}$ (S^+KPP) mice. The model achieved *PTEN* deletion and *Kras* activation in the intrahepatic and extra-hepatic biliary epithelium and the pancreatic ductal epithelium, which eventually formed iCCA, eCCA, and pancreatic cancer, providing a platform for studying hepatopancreatic ductal carcinoma. Notably, $SOX9^+$ cells with deletion of the *PTEN* gene alone already have the potential to form HCC and iCCA using the same inducible Sox9-Cre^{ERT}-based approach,[79] indicating that loss of *PTEN* alone is sufficient to drive the transformation of $SOX9^+$ cells in the liver. In addition, a pancreatic and duodenal homeobox 1 promoter-driven Cre recombination system was used to mediate the knockout of *PTEN* or activation of $PIK3ca^{H1047R}$, a mutant of PI3K. Both were found to produce conditional GEMs of eCCA,[80] which faithfully recapitulates human eCCA and provides a novel platform for genome-wide mutagenesis screening.

Models combining $Kras^{G12D}$ activation with TP53 knockout

The most common gene mutations in CCA are *Kras* and *TP53*.[6] In 2012, O'Dell *et al*.[81] established $Alb\text{-}Cre^+$; $Kras^{G12D}$; $TP53^{L/L}$ 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 $Kras^{G12D}$ 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 $Kras^{G12D}$ expression with a 3,5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) diet had the potential to mimic multistep pathological changes of chronic cholangitis, ductular hyperplasia, cystic atypical hyperplasia, and eventually iCCA.[82] The model well represented the initiation and evolution of iCCA precursor lesions. When combined $Kras^{G12D}$ activation with *TP53* deletion in liver, the mice formed mixed HCC/iCCA. Notably, the cells of tumor origin differed with different promoters. For example, Cre-mediated $Kras^{G12D}$ activation and *TP53* deletion driven by the thyroid-binding globulin promoter in mice could result in mature hepatocyte-derived cHCC/iCCA, while the same genetic alterations driven by the SOX9 promoter eventually led to HCC/iCCA of biliary lineage origin.[83]

Models combining Kras activation and IDH2 mutation

IDH 1/2 mutations have been found in approximately 20% of iCCA cases.[84] $Alb\text{-}Cre^+$; $LSL\text{-}IDH2^{R172K}$; $LSL\text{-}Kras^{G12D}$ mice formed multifocal and palpable CK-19^+, Hep $Par1^-$

iCCA lesions at 33–58 weeks of age with peritoneal metastases and splenic invasion, whereas mice with *Kras*^{G12D} activation alone formed single HCC nodules.[85] Mechanistically, mutant *IDH* inhibited the differentiation of hepatic progenitor cells in the liver after hepatocyte nuclear factor 4α inactivation, thus promoting iCCA formation. Because of the high mutation rate of *IDH* in tumors and the relatively mature clinical studies of *IDH* inhibitors,[86] 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,[87,88] and frequent abnormal changes in TGF-β family receptors have been detected in eCCA by NGS.[6] Nakagawa *et al*.[89] first knocked Cre^{ERT} into the endogenous K19 locus to obtain K19Cre^{ERT} mice with TAM administration. Effective genetic recombination was confirmed with reporter mice. Then, *K19Cre*^{ERT}; *LSL-Kras*^{G12D}; *TGFβR2*$^{L/L}$ mice (KT-K19Cre-^{ERT}) were generated by crossing *LSL-Kras*^{G12D}, *TGFβR2*$^{L/L}$ and *K19Cre*^{ERT} mice and induced with TAM. All (15/15) *KT-K19Cre*^{ERT} 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[90] and to lead to a series of pathological changes similar to those of primary sclerosing cholangitis in human. These mice showed an increased ductular reaction after 7 months of a high-fat diet (HFD).[91] HFDs cause nonalcoholic fatty liver disease,[92] 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*$^{L/L}$/*KRAS*^{G12D} mice.[91] By crossing *KT-K19Cre*^{ERT} and *CDH1*$^{L/L}$ mice with *Kras*^{G12D} mice, Nakagawa *et al*.[89] established a *KTC-K19Cre*^{ERT} mouse model characterized by *CDH1/TGFβR2* 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 developed 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. Intraperitoneal injection of transgenic mice with *TP53* deletions with CCl_4 three times a week for 4 months resulted in 54% of *TP53*$^{L/L}$ mice developing iCCA, and approximately 14% (1/7) mice developing lymph node metastasis at 29 weeks of age.[93] Using a similar approach, Guest *et al*.[94] 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*$^{L/L}$ 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.[95] The pathophysiological role of the Notch signaling pathway has been partially elucidated in CCA GEMs. For example, *Alb-Cre:: NICD* 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.[96] Implanting liver tissue from 9-month-old transgenic mice subcutaneously into SCID mice results in the formation of iCCA after 3 weeks.[97] Biliary tract malignancies are often accompanied by elevated levels of phosphorylated AKT.[69] Cellular fate-tracing results have shown that overexpression of *NICD* combined with *AKT* leads to the development of iCCA originating from hepatocytes.[98] 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.[103] 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 *Fbxw7F*, 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.

In addition to tail vein injection, electroporation can be used to introduce exogenous DNA into cells. However, different models of gene introduction have been found to have

different effects on the type of tumor that develops. For example, delivery of plasmids containing *Myc* and mutant *NRAS* proto-oncogene or *AKT1* 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 based on gene transduction modalities interact with 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 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/iCCA 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*^{G12D} 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.[112] Dasatinib sensitivity was tested in CRISPR/Cas9-mediated human iCCA cells with *IDH* 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-loxP 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 duct cells. Optimization of existing genetic recombination systems is a promising option. In addition, flexible CRISPR/Cas9 gene editing technique may be another favorable choice. Ongoing optimization of preclinical animal models through the integration of various technologies will contribute to rapid translation from bench to bedside.

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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.

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