Animal models for hepatocellular carcinoma arising from alcoholic and metabolic liver diseases

Ken Liu¹23, Jinbiao Chen²23, Geoffrey W. McCaughan¹23

¹A.W. Morrow Gastroenterology and Liver Centre, Royal Prince Alfred Hospital, Sydney, NSW 2050, Australia.
²Sydney Medical School, University of Sydney, Sydney, NSW 2050, Australia.
³Liver Injury and Cancer Program, Centenary Institute, Sydney, NSW 2050, Australia.

Correspondence to: Dr. Geoffrey W. McCaughan, MBBS PhD FRACP FAHMS, Head of Liver Injury and Cancer Program, Centenary Research Institute, A.W. Morrow Professor of Medicine, Director A.W. Morrow Gastroenterology and Liver Centre, Director Australian Liver Transplant Unit, Royal Prince Alfred Hospital, University of Sydney, Sydney, NSW 2050, Australia. E-mail: g.mccaughan@centenary.usyd.edu.au

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Abstract

Hepatocellular carcinoma (HCC) is a major and increasing cause of clinical and economic burden worldwide. Now that there are effective therapies to control or eradicate viral aetiologies, the landscape of HCC is changing with alcoholic and metabolic liver diseases becoming major catalysts. The pathogenesis of HCC is complex and incompletely understood, hampering improvements in therapy. Animal models are essential tools for advancing study on the cellular and molecular processes in HCC and for screening potential novel therapies. Many models of hepatocarcinogenesis have been established using various methods including genetic engineering, chemotoxic agents and dietary manipulation to direct implantation of tumour cells. However, none of these can accurately replicate all features found in human diseases. In this review, we provide an overview of different mouse models of HCC with a particular focus on cancer arising from alcoholic liver disease, non-alcoholic fatty liver disease and hereditary haemochromatosis. We also highlight their strengths and limitations and provide perspectives for future study.

Keywords: Hepatocellular carcinoma, animal models, mouse models, non-alcoholic fatty liver disease, alcohol, haemochromatosis
INTRODUCTION

Hepatocellular carcinoma (HCC) is the most common type of primary liver cancer and ranks as the fifth most common incident cancer and the fourth most common cause of cancer-related death worldwide. Major causes for HCC include chronic liver disease such as infection with hepatitis B virus (HBV) or hepatitis C virus (HCV), alcoholic liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD). Over 80% of the world’s HCCs are found in less developed countries due to the influence of chronic HBV infection; however, the incidence and mortality are largely decreasing in these regions due to immunisation and antiviral therapy. Instead, the burden of HCC is increasing in Western or developed countries due to the rise of NAFLD-associated HCC. Indeed, NAFLD has either already become or is on the verge of becoming the leading cause of HCC in most Western countries. Alarming, even in non-Western countries where viral hepatitis-related HCC predominates, the proportion of patients with HCC due to NAFLD is increasing at an exponential rate. Moreover, with no effective pharmacologic agents to date, the burden of NAFLD is expected to rise further in the future.

The epidemiology of HCC in the context of ALD is poorly captured with heterogeneous geographic distribution. However, current data show alcohol accounts for 21% of HCC cases globally, making it the third leading cause (behind HBV and HCV) and the leading cause in many regions. The age-specific incidence rates for ALD-related HCC are also increasing.

Alongside NAFLD, hereditary haemochromatosis (HH) is another metabolic liver disease impacted by HCC which deserves special mention. HCC accounts for up to 28%–45% of deaths in HH patients and the relative risk of HCC development in those with cirrhosis is greater than 200. HCC has also been described in HH patients without cirrhosis. Furthermore, iron has been implicated as a cofactor for HCC development in other liver diseases such as NAFLD.

Therefore, with continuing improvements in global HBV vaccination coverage and effective therapies to control HBV and eradicate HCV, alcohol and metabolic liver diseases will take their place as the major contributors of hepatocarcinogenesis in the coming decades.

WHY DO WE NEED ANIMAL MODELS?

The biology of HCC is complex and incompletely understood with no single dominant molecular pathology. However, therapeutic approaches for primary intervention over the past ten years have resulted in numerous negative randomised controlled trials. The current approved therapies for advanced disease prolong survival by only 2–3 months. Thus, new targets for therapies are urgently needed.

Unlike other cancers, HCC can be diagnosed by imaging criteria alone and few patients (<30%) are eligible for curative surgical resection or liver transplantation. This has limited the availability of human HCC tissue samples for study. Indeed, the large number of human studies that have classified human HCC at the molecular level have almost exclusively used tissue from relatively early HCC obtained at hepatic resection or transplantation. Thus, animal models of more advanced HCC have proved to be crucial for investigating the genetic alterations, signalling pathways and microenvironment interactions involved in hepatocarcinogenesis. Importantly, they also allow for the evaluation of potential novel treatment paradigms and drugs in preclinical trials.

Although many animal models of HCC exist, this review focuses on mouse (Mus musculus) models, which are considered some of the best animal models for studying HCC owing to their compact size, short lifespan, breeding capacity and physiologic and genetic similarities to human biology. After a brief overview of HCC mouse models, the review concentrates on mouse models for HCC arising from ALD, NAFLD and HH.
MOUSE MODELS FOR HCC (GENERAL APPROACHES)

Hepatocarcinogenesis can be achieved through several different strategies either alone or in combination [Figure 1].

Genetically engineered mouse models

The most prevalent genetic mutations in human HCC are in the promoter region of TERT (60%), TP53 (20%-30%), CTNNB1 (15%-25%), ARID1A (10%-16%) and AXIN1, while genes commonly mutated in other solid tumours such as EGFR, PIK3CA or KRAS are rarely mutated in HCC (< 5%) [20]. Various genetically engineered mouse (GEM) models have been created to reproduce these molecular features of human HCC. These models which result in activation of oncogenes or inactivation of tumour suppressor genes can be achieved via several different mechanisms including microinjection of recombinant DNA into the pronucleus of an embryo, lentiviral transduction in embryonic stem cells, homologous recombination in stem cells, conditional mutagenesis (e.g., Cre/loxP recombination system), knockdown using RNA interference and more recently genome editing with programmable endonucleases (e.g., CRISPR/Cas9 system). Liver-specific GEM models have also been created using the latter two techniques, for example with Albumin-Cre and hydrodynamic injection of plasmids, respectively [21]. Genetic modifications can also be used to produce mouse phenotypes that represent specific aetiologies of human metabolic liver diseases such as obese mice (e.g., ob/ob, db/db and foz/foz) to study NAFLD-related HCC or HFE knockout mice to study HCC in the setting of HH [22,23]. However, the use of GEM models alone cannot recapitulate human disease. Firstly, there is no single dominant molecular pathology underlying all HCCs but rather several pathways involved [24]. Sequencing of cancer genomes had revealed that a typical cancer initiating cell accumulates at least 2-8 driver mutations [25]. However, GEM models are generally limited to one specific driver mutation [21,26], hence restricting models to study only specific genes or pathways in hepatocarcinogenesis. Secondly, GEM models typically lack chronic liver injury and fibrosis and HCCs develop in almost normal livers (with the notable exception of MDR2 knockout mice). Despite this, these models have a role in providing evidence that powerful causation effects can be seen particularly following genetic ablation of key tumour suppressor genes or over amplification of oncogenic proteins. Examples of the former include liver-specific knockout of p53 (AlfpCre+Trp53Δ2-10/Δ2-10 mice), PTEN (AlbCrePtenflx/flx) or both [27-29]. Conversely, overexpression of oncogenes such as MYC and E2F1 alone or synergistically in combination can also drive hepatocarcinogenesis [30,31]. Recently, Ruiz de Galarreta et al. [32] were able to generate liver tumours with both MYC overexpression and TP53 depletion by hydrodynamic tail-vein injections of a transposon vector expressing MYC and a CRISPR/Cas9 vector expressing a single-guide RNA targeting Trp53 into C57BL/6
mice. Another method is the use of stem cell transduction, which involves retroviral infection of hepatic progenitor cells isolated from foetal livers of mice to introduce oncogenes or target tumour suppressors into a healthy liver\textsuperscript{[21]}. Manipulation of key genetic pathways in combination with liver injury models described below has now been advocated to achieve a more realistic representation of human HCC\textsuperscript{[26]}.

**Chemically- or diet-induced models**

Several chemotoxins can induce hepatocarcinogenesis by causing direct DNA damage (genotoxic) or promoting clonal expansion of preneoplastic cells (non-genotoxic). Di-ethyl-nitrosamine (DEN) is the most widely used genotoxic drug for chemically-induced HCC. Once bioactivated by cytochrome P450, DEN becomes an alkylating agent leading to the formation of mutagenic DNA adducts while also generating reactive oxygen species (ROS) which damage DNA, overall resulting in hepatocyte death. Similar to what occurs in humans, subsequent cycles of necrosis and regeneration in the mouse liver promote mutations, neoplastic transformation and eventual HCC development\textsuperscript{[33]}. Indeed, DEN tumours have consistently exhibited high mutation rates\textsuperscript{[34]}. DEN is most effective at inducing HCC when injected intraperitoneally into young male mice (less than two weeks old) when hepatocytes are still proliferating. Commonly used non-genotoxic carcinogens include carbon tetrachloride (CCl\textsubscript{4}) and thioacetamide (TAA). These agents act as tumour promoters by damaging cellular structures, increasing the risk of genetic error and stimulating cell malignant transformation by affecting proliferation, differentiation and apoptosis processes\textsuperscript{[24]}. CCl\textsubscript{4} is a potent hepatotoxin which causes centrilobular liver damage by the production of ROS and peroxidative degradation of phospholipids in plasma, lysosomal and mitochondrial membranes. Prolonged exposure (via oral, intraperitoneal or inhaled routes) leads to liver inflammation, fibrosis, cirrhosis and HCC development. TAA is another centrilobular hepatotoxin which can be administered via intraperitoneal injections or adding it to drinking water. It is bioactivated by mixed-function monooxygenases leading to its S-oxide and highly reactive S,S-dioxide, which modifies amine-lipids and proteins to initiate cellular necrosis\textsuperscript{[24]}. The carcinogenic effects of all chemically-induced HCC models vary with age, mouse strain and sex. Ethanol feeding models are discussed in the ALD-associated HCC models section.

Diet-based models are most commonly used to study fatty liver diseases, particularly NAFLD and less so ALD. Mice are usually fed ad libitum with one of the following diets: high-fat diet (HFD), high-fat high-cholesterol (HFHC), methionine and choline-deficient diet (MCD), choline-deficient high-fat diet (CD-HFD), choline-deficient L-amino acid-defined (CDAA) diet or a Western diet (WD). Although these models can reliably produce steatosis, inflammation and even fibrosis, not many of them will result in HCC development after a prolonged period\textsuperscript{[22]}. Furthermore, not all models reliably reproduce the accompanying metabolic features of the disease such as obesity and insulin resistance\textsuperscript{[21]}. For example, a major drawback of the MCD model is that mice exhibit the opposite of the human metabolic syndrome with weight loss, no insulin resistance and low serum glucose, triglyceride and cholesterol. The specifics of these diet-based models and their combinations are further discussed in the NAFLD-associated HCC models section.

**Implantation models**

Human or murine HCC cell lines can be injected into recipient mice to form orthotopic tumours (intrahepatic, intraspelic or intraportal injection) in the liver or heterotopic tumours (subcutaneous injection) typically in the flank. The main advantages of implantation models are their quick time to develop visible tumours (weeks to months in spontaneous models) that are easy to measure (especially subcutaneous heterotopic tumours) and reproducible - making them popular models for drug screening. This is counterbalanced by disadvantages such as considerable differences between cell lines necessitating multiple cell lines to be tested, the lack of tumour-liver microenvironment interactions in heterotopic models and the need for surgical expertise for orthotopic models\textsuperscript{[21]}. Implantation of human cells (xenograft models) requires immunocompromised mice to prevent rejection of these foreign cells while murine cells can be implanted into immunocompetent mice (syngeneic/allograft models). Mouse tumour cell lines harbour mutations that are neutral or not relevant in human cancer making xenograft models more
genetically applicable to human disease\textsuperscript{[35]}. Patient-derived xenograft (PDX) models in which cells from a specific patient with HCC are transplanted into immunocompromised mice have been established\textsuperscript{[36]}. PDXs faithfully recapitulate histologic, genomic and biological characteristics of the primary tumour and have been shown to predict drug response in HCC patients. However, this model is limited by engraftment failure rates of up to 60%, long time to engraftment (several months) and high cost, which make it unsuitable for large-scale drug screening\textsuperscript{[36,37]}. Furthermore, the major drawback of xenograft models (PDX or otherwise) is the lack of a tumoural immune response, which has become increasingly important as we enter the era of immunotherapies for HCC. Attempts at overcoming this with double humanised mouse models which express human hepatocytes and haematopoietic stem cells (and hence human immune cells) are technically intensive, expensive and not yet widely adopted\textsuperscript{[21]}. Finally, xenograft of human HCC models which develop metastases (more readily than GEM models) have been established, providing the opportunity to study late-stage disease\textsuperscript{[38]}. Replicability in human disease

Human HCCs are highly complex and heterogeneous and thus cannot be adequately represented by any single mouse model. For example, gene expression profiles of tumours from the commonly-used DEN model was previously shown to be most similar to a subgroup of human HCC with poorer survival\textsuperscript{[39,40]}. Correspondingly, many poor prognostic markers in human HCC are also highly expressed in DEN-induced tumours, e.g., alpha-foetoprotein (AFP). However, the DEN model lacks other hallmarks of human HCC, particularly fibrosis in the surrounding microenvironment\textsuperscript{[41]}. In a more recent integrative genomic analysis of four separate mouse models and 987 human HCC samples, DEN tumours were found to be histologically hard to classify and least similar to human disease while Stelic Animal Model (STAM) tumours (discussed further in the NAFLD-associated HCC models section) were most molecularly similar to human HCC, especially high-grade, proliferative tumours with poor prognosis\textsuperscript{[39]}. The authors further argued that DEN models should be avoided since they are dominated by mutational mechanisms not seen in human HCC. In contrast to DEN-induced and STAM tumours, MDR2 knockout tumours are most similar to human HCCs associated with better survival\textsuperscript{[42]}. However, the MDR2 knockout model produces a phenotype resembling humans with primary sclerosing cholangitis or primary biliary cholangitis rather than chronic “hepatitis” diseases caused by alcohol excess and HBV or HCV infection\textsuperscript{[43]}. Very recently, experimental hepatocyte-specific activation of $\beta$-catenin also resulted the development of a phenotype that resembled the low proliferative subclass of human HCC\textsuperscript{[32]}. Interestingly these tumours also had few intratumoral immune cells and were resistant to immune checkpoint inhibitor therapy. Therefore, it is clear that different models (and their combinations) are required to simulate specific subgroups of human HCC. Furthermore, drugs with known anti-tumour activity against human HCC do not demonstrate activity in some animal models and vice versa. Indeed, the current Food and Drug Administration-approved drugs for treating advanced HCC such as sorafenib and anti-programmed cell death receptor 1 (PD1) antibodies were trialled based on success in other cancers (advanced renal cell carcinoma and melanoma, respectively) rather than positive results in HCC animal models per se.

SPECIFIC MOUSE MODELS FOR ALCOHOLIC AND METABOLIC LIVER DISEASE-ASSOCIATED HCC

ALD-associated HCC models

In general, mice and other species (except the golden hamster) dislike alcohol and avoid ingestion when it is offered \textit{ad libitum}\textsuperscript{[21,44]}. Therefore, ALD mouse models are established by one of three ways: (1) replacing the food and water source with a liquid diet in which 5% ethanol accounts for 36% of total calories (Lieber-DeCarli model); (2) binge feeding mice with ethanol via gavage in addition to chronic ingestion [National Institute of Alcohol Abuse and Alcoholism (NIAAA) model]; or (3) intragastric ethanol infusion via a surgically inserted infusion pump (Tsukamoto-French model) [Table 1].
| Mouse model* | Strain | Genetic modification | Diet manipulation | Chemotoxin | HCC development | Comments |
|--------------|--------|----------------------|-------------------|------------|-----------------|----------|
| **Alcohol HCC models** | | | | | | |
| Alcohol + DEN | C57BL/6 male | - | 4% Lieber-DeCarli alcohol diet from Week 9 | DEN 75 mg/kg i.p. weekly (Weeks 4-6), then 100 mg/kg weekly (Weeks 7-9) | Hepatic lesions with elevated AFP and cellular proliferation | Hepatic hyperplasia described rather than histological HCC. Alcohol hepatitis and fibrosis in non-tumour tissue. |
| Ambade et al. | | | | | | |
| Alcohol + DEN | B6C3 male and female | - | 10/20% (v/v) ethanol drinking water on alternate days at Week 16 or 40 for 8 weeks | DEN 1 mg/kg i.p. single dose 21-25 days old | No tumours at 24 weeks. At 48 weeks, tumours displayed in: Males 93.7%, Females 35.7% | Ethanol significantly increased tumour burden in male but not female DEN-treated mice. Liver injury and fibrosis in non-tumour tissue. |
| Brandon-Warner et al. | | | | | | |
| Alcohol + DEN + CCl₄ | BALB/c male | - | 9% ethanol drinking water from Week 9 | DEN 100 mg/kg i.p. single dose at Week 7. DEN 50 mg/kg gavage single dose at Week 9. CCl₄ gavage 5 mL/kg twice weekly at Week 7, increased to 8 mL/kg at Week 10. | 100% moderate to well differentiated tumours at Day 150 | 7.5% mortality rate. Hepatitis at Day 60, fibrosis at Day 90, 100% cirrhosis at Day 120 in non-tumour tissue. |
| Xin et al. | | | | | | |
| **NASH HCC models** | | | | | | |
| HFD | C57BL/6 male and female | - | HFD from Week 4 | 1/40 (2.5%) displayed tumour at 12 months | WG, IR and steatosis but no inflammation or fibrosis. HCC not described in other single diets, e.g., MCD, CD and WD. |
| Wolf et al. | | | | | | |
| CD-HFD | C57BL/6 male and female | - | CD-HFD from Week 4 | 19/75 (25%) displayed tumours at 12 months | Similar rate of tumours in male and female mice. WG, IR, steatosis, inflammation and fibrosis present. |
| Wolf et al. | | | | | | |
| CDA | C57BL/6J male | - | CDA from Weeks 6-8 | 30% displayed tumours at 6 months. 35% displayed tumours at 9 months. 89% displayed tumours at 32-52 weeks | WG, IR, steatosis, inflammation and fibrosis present. |
| De Minicis et al. | | | | | | |
| DIAMOND | B6/129 male | - | WD + high-fructose-glucose water from Weeks 8-12 | | 100% had well-differentiated tumour foci, 40% had poorly differentiated tumours. |
| Asgharpour et al. | | | | | | |
| foz/foz mouse + DEN | NOD.B10 male | foz/foz (A1ms1 mutant) | DEN 10 mg/kg i.p. single dose on Days 12-15 | 100% displayed tumours at 6 months (2 had lung metastases) | WG, IR and steatosis and inflammation present. |
| Arfianti et al. | | | | | | |
| HFD + DEN | C57BL/6J male and female | - | HFD from Week 6 | DEN 25 mg/kg i.p. single dose at Week 2 | At 9 months, tumours displayed in: Males 100%, Females 80% | WG, IR and steatosis present. |
| Park et al. | | | | | | |
| CD-HFD + DEN | C57BL/6J male | - | CD-HFD from Week 3 | DEN 25 mg/kg i.p. single dose at Week 3 | 20% displayed tumours at 16 weeks. 100% displayed tumours at 20 weeks | Mean size 2.9 mm at 24 weeks. |
| Kishida et al. | | | | | | |

Table 1. Examples of mouse models for hepatocellular carcinoma associated with alcoholic and metabolic liver diseases.
| Model | Strain | Treatment | Tumour Development |
|-------|--------|-----------|--------------------|
| HFHC + DEN | C57BL/6J male | HFD or HFHC from 6 weeks | DEN 25 mg/kg i.p. single dose at Week 3 | At 8 months, tumours displayed in: HFD 90%, HFHC 100% |
| Liang et al. | | | | Increased size and multiplicity in HFHC-fed mice compared with HFD-fed mice |
| CDAA + CCl₄ | C57BL/6J male | CDAA from Weeks 6-8 | CCl₄ 0.2 ×L/g i.p. weekly from Weeks 6-8 | 30% displayed tumours at 6 months, 100% displayed tumours at 9 months |
| De Minicis et al. | | | | Median diameter of tumours = 9 mm |
| HFD + DEN + CCl₄ | C57BL/6J male | HFD from Week 4 | DEN 25 mg/kg i.p. single dose at Day 14 TAA 300 mg/L in drinking water from Week 4 | 83% displayed tumours at 24 weeks |
| Henderson et al. | | | | Weight loss, steatosis, inflammation and fibrosis present |
| STAM | C57BL/6 male and female | HFD from Week 4 | STZ 200 ×g s.c. at Day 2 | 100% displayed tumours at 20 weeks. No female mice developed tumours |
| Fuji et al. | | | | WG, diabetes (no IR), steatosis, inflammation and fibrosis present |
| PTEN knockout | C57BL/6J male and female | Hepatocyte-specific PTEN knockout: AlbCre/PTEN<sup>fox/fox</sup> | - | At 74-78 weeks, tumours displayed in: Males 66%, Females 30% |
| Horie et al. | | | | Insulin hypersensitivity, steatosis and inflammation but no fibrosis present |
| Leptin deficiency (Ob) + DEN | C57BL/6J male | Leptin deficient genetically obese (Ob) | - | DEN 25 mg/kg i.p. single dose at Week 2 | 100% displayed tumours at 9 months |
| Park et al. | | | | WG, IR and steatosis present. |
| MUP-uPA | C57BL/6J male | MUP-uPA transgene | HFD from Week 6 | - | 78.6% displayed had tumours > 2 mm, and 35.7% displayed tumours > 10 mm at 40 weeks |
| Nakagawa et al. | | | | WG, IR, steatosis, inflammation and fibrosis present |
| Haemochromatosis HCC models | | | | |
| β2-microglobulin knockout | C57BL/6J male and female | β2-microglobulin knockout | Added 10 mg ferrous sulphate/kg normal chow from 8-15 months for 6-8 months | 4/13 (30.7%) displayed tumours at 1-2 years | Iron overload seen in the liver with sinusoidal fibrosis |
| Rothenberg et al. | | | | |
| FBXLS knockout | C57BL/6J male and female | Hepatocyte-specific FBXLS knockout: AlbCre/Fbxl5<sup>fox/fox</sup> mice | DEN 25 mg/kg i.p. single dose at Day 15 | At 36 weeks, tumours displayed in: Males 95%, Females 75% |
| Muto et al. | | | | Iron overload seen in the liver |

*List of models presented are not intended to be exhaustive but to give representative examples of different combinations of methods to achieve hepatocarcinogenesis in alcoholic and metabolic liver diseases. AFP: alpha-fetoprotein; CCl₄: carbon tetrachloride; CD: choline-deficient; CD-HFD: choline-deficient high-fat diet; CDAA: choline-deficient L-amino acid-defined; DEN: di-ethyl-nitrosamine; HCC: hepatocellular carcinoma; HFD: high-fat diet; i.p.: intraperitoneal; IR: insulin resistance; MCD: methionine and choline-deficient diet; NASH: non-alcoholic steatohepatitis; s.c.: subcutaneous; STAM: stelic animal model; STZ: streptozotocin; TAA: thioacetamide; WD: Western diet; WG: weight gain.
Aside from their natural aversion to alcohol, mice metabolise alcohol five times faster than humans\(^{43}\). As a result, the aforementioned ALD mouse models tend to exhibit less liver injury than seen in human disease\(^{21}\). The Lieber-DeCarli model induces mild steatosis with little to no inflammation or fibrosis. The technically demanding Tsukamoto-French model produces severe steatosis but only mild inflammation and mild fibrosis. Although chronic or binge ethanol feeding regimens cause minor liver changes by themselves, their combination in the NIAAA model synergistically induces more severe steatosis and inflammation, with only mild chicken-wire fibrosis\(^{46}\).

Many have studied liver injury patterns of ALD mouse models; however, few have examined hepatocarcinogenesis specifically. As described above, the mild severity of liver inflammation and fibrosis induced by standalone mouse models of ALD means HCCs do not develop spontaneously. Therefore, a “second-hit” usually consisting of a chemical hepatotoxin is required for progression of ALD to cirrhosis and/or HCC. Indeed, of the hepatotoxins, DEN-induced C57BL/6 tumours have recently been matched to most resemble alcohol-induced HCC both morphologically and by comparative genomic hybridisation in a study comparing five different HCC models with human data\(^{47}\). Ambade et al.\(^{48}\) established a model of alcohol-driven HCC in adult C57BL/6 male mice. The four-week-old mice were administered six doses of DEN (or saline) intraperitoneally (75 mg/kg weekly for three weeks and then 100 mg/kg weekly for three weeks) followed by the Lieber-DeCarli diet (or calorie-matched control diet) for seven weeks before sacrifice at 15 weeks. Compared to mice fed with a control diet, alcohol-fed mice had greater liver inflammation (raised alanine aminotransferase) and fibrosis. The alcohol-fed group also exhibited numerous liver nodules of hepatic hyperplasia associated with increased AFP expression and cellular proliferation, which the authors thought represented signs of early hepatocarcinogenesis. There were no hyperplastic nodules seen in the alcohol-fed saline-injected group or the control-fed DEN-injected group, thus confirming the need for a second stressor to initiate hepatocarcinogenesis. Early precancerous lesions were also described in another model using the combination of DEN and alcohol diet\(^{49}\). In this study, male C57BL/6 mice were injected intraperitoneally with DEN (25 mg/kg) at two weeks of age and then fed with the Lieber-DeCarli diet at eight weeks of age for 21 days. Over half of DEN-injected alcohol-fed mice developed precancerous basophilic foci compared to none in the DEN-injected control diet group. Interestingly, dietary luteolin (a flavonoid with anti-cancer properties) co-administration completely abrogated the development of precancerous lesions potentially by restoring sirtuin 1 activity and increasing downstream proliferator-activated receptor gamma coactivator 1 alpha protein expression. In a longer model, Brandon-Warner et al.\(^{50}\) studied DEN-injected alcohol-fed B6C3 mice for 48 weeks and observed tumours in 94% and 36% of males and females, respectively. While chronic ethanol feeding exacerbated tumour formation in DEN-injected males, fewer and smaller tumours were observed in females exposed to ethanol compared to DEN-injected control-fed mice of respective sexes. Further analysis of liver mRNA revealed elevated SMAD3 in male compared to female mice in response to liver injury from DEN and alcohol, suggesting that increased TGF\(\beta\)-SMAD3 signalling may enhance HCC promotion. Indeed, gender disparity (males > females) in liver cancer both in humans and in DEN-injected mice is well-recognised and may be related to sex differences in MyD88-dependent IL-6 production mediated by the protective effect of oestrogen\(^{51}\).

The combination of alcohol and CCl\(_4\) has also been experimented, although predominantly in rats. Weekly injections of CCl\(_4\) and alcohol administration through drinking water led to HCC after 104 weeks in mice\(^{52}\). The impact of chemical carcinogens on HCC formation appears to be additive. Recently, Xin et al.\(^{53}\) combined DEN (100 mg/kg intraperitoneal and 50 mg/kg gavage once each), CCl\(_4\) twice weekly and 9% alcohol as drinking water together in adult (seven-week-old) BALB/c mice. Multifocal HCC was noted only five months (150 days) after DEN injection. Tumours were moderate to highly differentiated and secreted AFP, resembling human HCC. Furthermore, there was no evidence of toxicity in this model as these mice survived until sacrifice.
NAFLD-associated HCC models

Many models have been developed to represent NAFLD and non-alcoholic steatohepatitis (NASH), although, as aforementioned, not all of them exhibit features of metabolic syndrome. This is particularly important in NAFLD-related HCC since the presence of obesity and/or diabetes are themselves independent risk factors for the development of cancer.\(^{[54]}\)

Most dietary models of NAFLD (HFD, HFHC, MCD, WD and CD diet) rarely induce HCC development alone.\(^{[22]}\) If spontaneous HCC does occur, it is time-consuming (e.g., 2.5% for C57BL/6 mice fed HFD for 12 months).\(^{[55]}\) Combination diets such as CD-HFD and CDA are shown to significantly increase rates of tumour formation, although overall rates are still low: 25% after 12 months and 35% after 9 months, respectively.\(^{[55,56]}\) Indeed, these diets can recapitulate the key features of human NASH (including fibrosis) and metabolic syndrome more so than single diets. Susceptibility to tumour formation in dietary models also appears to be strain-dependent with DBA/2J > C57BL/6 > A/J.\(^{[57,58]}\) Asgharpour et al.\(^{[59]}\) generated an isogenic strain (B6/129) derived from a cross of two common mouse strains, C57BL/6 and 129S1/SvImJ, and fed them a high-fat-high-carbohydrate diet with high-fructose-glucose water - so-called DIAMOND mice. This promising model mimicked all the physiological, metabolic, histological and transcriptomic gene signature and clinical endpoints of human NASH including HCC in 89% at 32-52 weeks. These tumours had gene signatures which strongly resembled the S1 and S2 human subclasses of HCC. Interestingly, neither C57BL/6J nor 129S1/SvImJ parent strain mice fed with the same diet developed HCC.

Combining dietary models with a hepatotoxin substantially hastens and increases HCC formation (i.e., up to 100% of male C57BL/6 mice fed CDA, HFD, CD-HFD or WD + intraperitoneal injections of DEN or CCl\(_4\), at 6-9 months) as well as tumour size. The addition of cholesterol to a HFD (HFHC) in a DEN-induced model appears to further increase tumour burden. In another model, Henderson et al.\(^{[61]}\) treated male C57BL/6 mice with DEN (25 mg/kg once at 14 days old), TAA (300 mg/L in drinking water ad libitum from four weeks old) and HFD. These agents acted synergistically to develop HCCs in 83% of mice as early as 24 weeks of age, which was significantly more than control mice or those treated with DEN and TAA only. However, combining with hepatotoxins needs to be tempered by some limitations. For example, use of CCl\(_4\) can induce liver metabolism enzymes (which may impact the use of this model for drug discovery) and also mitigate metabolic processes involved in NASH, particularly susceptibility to diet-induced obesity and insulin resistance. As mentioned above, the STAM mouse was recently shown to be the mouse model (out of four studied) that most closely resembles human HCC at a molecular level. Specifically, STAM tumours carried mutations of CTNNB1 at a rate comparable to human tumours, and (less frequently) mutations of TP53 - the most frequently altered genes in human HCC.\(^{[34]}\) In contrast, CTNNB1 and TP53 were rarely mutated in DEN-induced tumours, which instead carried Hras, Braf and APC mutations rarely seen in human cancers. The STAM combination involves first treating neonatal C57BL/6 male mice with low-dose streptozotocin (STZ) at Day 2, which induces diabetes by causing death of pancreatic B cells, resulting in lean mice with hypoinsulinaemia and hyperglycaemia, but no insulin resistance (the phenotype of type 1 diabetes). STZ is also a DNA alkylating agent (similar to DEN) with potential carcinogenic effects. When these mice are then fed with HFD, they develop weight gain, NASH by eight weeks, cirrhosis and HCC relatively quickly by 16-20 weeks. Takakura et al.\(^{[65]}\) characterised STAM tumours at 20 weeks by clinical parameters used in human liver disease (i.e., Child-Turcotte-Pugh score and dynamic contrast-enhanced computed tomography (CT) measurements of HCCs). Interestingly, the authors deduced that STAM mice had cirrhosis corresponding to Child-Turcotte-Pugh Class B (significant coagulopathy, occasional ascites, no encephalopathy and normal albumin and bilirubin) and tumours equivalent to Barcelona Clinic Liver Cancer Stage B (intermediate) or C (advanced) disease in humans. No HCCs develop when STZ is given alone, again pointing to the need for an additional stimulus. Female mice treated with the STAM regimen also fail to develop tumours, akin to the gender disparity seen in other models.
Genetically obese mice with metabolic syndrome such as ob/ob (leptin deficient) db/db (leptin-receptor deficient), and foz/foz (mutated Alms1 gene) promote tumorigenesis in the presence of a secondary insult (e.g., DEN) but do not otherwise develop HCC spontaneously\[^{41,62,67,69}\]. Furthermore, ob/ob and db/db mice fail to develop significant liver fibrosis or NASH histology without the addition of one of the dietary models above\[^{21}\]. Park \textit{et al.}\[^{62}\] utilised a dietary (HFD) and genetic (ob/ob) obesity model in combination with DEN to show that obesity (no matter how it was achieved) promoted the development of DEN-induced HCC in C57BL/6 mice by enhanced production of the pro-inflammatory cytokines IL-6 and TNF. Many other genetic models have been developed to study NAFLD-associated HCC including \textit{PTEN}\[^{39}\] knockout, \textit{PPAR}\[^{\alpha}\] knockout, \textit{AOX}\[^{21}\] knockout, \textit{KK-Ay/a} (agouti gene mutation) and \textit{MAT1A} knockout mice. While they all reliably form HCC, they fail to recapitulate NASH itself (in \textit{KK-Ay/a} mice) or its associated aspects such as obesity and metabolic syndrome (in \textit{PTEN}, \textit{PPAR}\[^{\alpha}\], \textit{AOX} and \textit{MAT1A} knockout mice)\[^{21,62,67}\]. As an example, \textit{PTEN} knockout mice (which develop tumours between 40 and 78 weeks) are hyper-responsive to insulin instead of being insulin resistant. Unsurprisingly, gene expression signatures from \textit{PTEN} knockout mice are markedly different from that of other NASH mouse models\[^{21,62,67}\].

One promising genetic model of NASH-driven HCC is the MUP-uPA transgenic mouse combined with HFD\[^{71}\]. MUP-uPA mice express high amounts of urokinase plasminogen activator in hepatocytes leading to hepatocyte-specific endoplasmic reticulum stress and liver damage. These mice exhibited weight gain, insulin resistance, classic signs of NASH (steatosis, inflammation, ballooning), fibrosis and, importantly, spontaneous HCC in 80% at 40 weeks via processes dependent on TNF produced by inflammatory liver macrophages\[^{71}\]. As expected, HFD-fed wild type mice developed simple steatosis and no HCC over the same period. Furthermore, transcriptomic data from MUP-uPA mice and human NASH datasets showed signalling similarities, especially in the regulation of the immune system, innate immune response and the response to cytokine gene sets\[^{67}\].

Recently, Shalapour \textit{et al.}\[^{72}\] used both MUP-uPA and STAM mice fed with HFD to make a landmark discovery that hepatocarcinogenesis in NASH was facilitated by immunosuppressive liver-resident IgA\(^+\) plasma cells, which directly inhibit anti-tumour cytotoxic CD8\(^+\) T lymphocyte activation.

Of the models mentioned above, it seems the MUP-uPA and DIAMOND mice (which require a combination of genetic modification and dietary manipulation) best replicate NASH-associated HCC. However, tumour formation in these models requires lengthy periods and there is considerable heterogeneity in their mutational landscapes which may limit utility and reliability in some settings, e.g., drug development studies\[^{67}\]. Although STAM mice can develop tumours more quickly than these models (20 weeks vs. 40 weeks), they are physiologically less similar to human NASH (lacking insulin resistance).

**HH-associated HCC models**

Hepatocarcinogenesis arising from iron accumulation is thought to be secondary to oxidative DNA damage from ROS generated by free hepatic iron. This leads to a cycle of cell death, and compensatory proliferation, which favours the accumulation of mutations in hepatocytes and ultimately malignant transformation\[^{13,73}\]. Recreating this in an animal model is difficult. The most common form of HH is caused by mutations in the \textit{HFE} gene. Although \textit{HFE} gene knockout produces the phenotype of HH in mice, spontaneous liver tumours do not develop\[^{74}\]. In a dietary model where BALB/c\(^\text{a}\) male mice were fed \textit{ad libitum} with chow supplemented with 3% carbonyl-iron, hepatic iron concentrations at 12 months were 13-fold that of normal chow-fed controls\[^{75}\]. No liver tumours developed; however, hepatocyte nuclei changes were observed (iron-containing ferritin inclusions, enlarged nucleus, increased mitotic index and abnormal mitotic figures), which may have represented preneoplastic changes. Rothenberg \textit{et al.}\[^{76}\] created a model of HH by knocking out \(\beta\)2-microglobulin (the chaperone protein for HFE) in C57BL/6 mice and reported that spontaneous HCCs developed in only a minority (31\%) of mice. Because tumour development was not predictable and time-consuming (taking up to two years), this model has not been widely used to study HH-related HCC. Recently, Muto \textit{et al.}\[^{77}\] developed a novel model of HCC induced by iron overload by deleting the iron-sensing ubiquitin ligase FBXL5 specifically in hepatocytes and
exposure to DEN. Alb-Cre/Fbxl5^flex/flox^ mice were injected with DEN (25 mg/kg) intraperitoneally at Day 15 and tumours were significantly increased in number and size compared to DEN-injected control mice at 36 weeks in both males and females. The study demonstrated FBXL5 deficiency led to a sequence of events (iron overload, oxidative stress, liver damage and regenerative proliferation), which, with the addition of DEN, gave rise to liver tumours with high mutational load. Previously, hepatocyte-specific FBXL5 deletion without the addition of DEN was shown to cause liver inflammation but not tumours. The authors went on to analyse FBXL5 mRNA expression in five different human HCC cohorts and found that low FBXL5 expression level was indeed strongly associated with poorer prognosis in human HCC. Finally, the impact of iron on hepatocarcinogenesis has also been evaluated using a xenograft model. In this study, 3–4-week-old female BALB/c athymic mice (nu/nu) were injected subcutaneously with human HCC cell lines (Hep3B or HepG2) and followed for 21 days. The authors showed that TSC24 (a potent iron chelator) suppressed tumour growth in a dose-dependent manner by reducing available iron, and triggering cell-cycle arrest and apoptosis.

THE ROLE OF THE GUT MICROBIOME

Increasingly, the role of the gut microbiome has been implicated in alcoholic and metabolic liver diseases and HCC via the gut-liver axis, which refers to bidirectional communication between the gut (and its microbiome) and the liver. In one direction, the liver secretes bile acids and antibodies into the intestine, which influences the gut microbiome composition. Reciprocally, the microbiome and its metabolites translocate the gut to reach the liver via the portal vein (the enterohepatic circulation) and regulate metabolic functions. This gut-liver axis exists in a homeostasis, which becomes disrupted in metabolic liver diseases.

Bacterial dysbiosis has been consistently demonstrated in the gut microbiomes of patients and mice with metabolic liver diseases and HCC. Mouse model studies have already revealed several mechanisms by which the gut microbiome contributes to HCC development.

Bacterial metabolism of compounds

In a model of NASH-associated HCC, Yoshimoto et al. induced HCC by treatment with a chemical carcinogen [dimethylbenz (a)anthracene] and HFD. The authors found a strong increase in Gram-positive bacteria (particularly Clostridium spp.) as well as levels of deoxycholic acid (DCA), a secondary bile acid whose production relies on metabolism of primary bile acids by bacteria such as Clostridium. Significantly, DCA was shown to promote a senescence-associated secretory phenotype in hepatic stellate cells, which leads to hepatocarcinogenesis via activation of the TLR2 pathway.

Leaky gut

Increased levels of lipopolysaccharide in the systemic circulation (due to increased intestinal permeability) and its interaction with TLR4 have been demonstrated to promote HCC formation in a CD-HFD-fed NASH model as well as a chemotoxin model with combination DEN and CCl_4. This process can be abrogated by gut sterilisation with oral antibiotics, especially in late-stage disease.

Immunosuppressive microenvironment

The gut microbiome also modulates tumoural adaptive immune responses. The aforementioned study by Shalapour et al. showed that manipulating the gut microbiome in mice with NASH-driven HCC either by knocking out their polyimmunoglobulin receptor (which regulates IgA transport into the gut lumen and maintains microbial homeostasis) or giving them broad-spectrum antibiotics (which reduces gut bacterial load) promoted and inhibited HCC development, respectively. Both these interventions modulate liver and circulating IgA levels and hence anti-tumour cytotoxic T cell activation, as discussed above.
The gut microbiome (and its associated HCC risk) can be transmissible between mice and, interestingly, this risk can also be transferred via the microbiome across generations to offspring of treated mothers\cite{79,85}. This opens up another avenue to induce hepatocarcinogenesis alongside GEM, hepatotoxins and dietary manipulation in future models.

Thus, as we explore the new frontier of gut microbiome, animal models will be crucial for understanding causality, pathogenesis and testing of therapeutic options targeting the microbiome (e.g., antibiotics, probiotics, synthetic bile acids and faecal microbiota transplantation). Although mouse and human gut microbiome communities are dominated by the same set of bacterial phyla, they are on the whole distinct from one another\cite{78}. Therefore, experimental findings from microbiome studies in mouse models need validation in human studies. The emerging use of a humanised gnotobiotic model (human donor stool transplanted into germ-free mice) may also improve the applicability of preclinical findings\cite{84}.

**CURRENT CHALLENGES AND FUTURE DIRECTIONS**

Although, as described above, there are many different animal models for HCC related to alcoholic and metabolic liver diseases, a single model faithfully recapitulating all features of human disease is lacking and unlikely to exist. This is partly because human HCC is genetically heterogeneous, consisting of several subtypes that are clearly different in behaviour, prognosis and response to treatment themselves. Clearly, the identification of models that represent different human HCC subsets is required. Yan et al\cite{26} argued for combining a chronic injury model (e.g., NASH, CCl\textsubscript{4} or MDR2 knockout) with alterations in oncogenes or tumour suppressor genes found in human HCC which alone are not sufficient to cause hepatocarcinogenesis (e.g., weak activation of pathways by heterozygous deletion or targeting only a small percentage of hepatocytes) to achieve a more realistic representation of human HCC. The optimal combinations for each aetiology are yet to be determined and will be an area of further research. When achieved, this would not only help improve our understanding of the pathobiology of aetiology-specific HCC but also improve our preclinical testing of new targeted treatments as we work towards personalised medicine. Humanised mouse models may be a bridge for translating findings from mouse studies to humans and presents a promising future strategy. However, several major challenges need to be overcome not the least of which is the engraftment of a humanised immune system.

The amount of time required for tumourgenesis is another obstacle, as most models take more than nine months to produce macroscopic HCCs. Furthermore, time is also needed to establish steatosis, inflammation, fibrosis and cirrhosis\cite{21}. While implantation HCC models are established within weeks, they are lacking these biologically important changes in the background liver. Indeed, human liver disease typically takes decades to progress to cirrhosis and HCC. For example, patients with NASH progress at a mean rate of only 0.09-0.14 fibrosis stages per year\cite{54}. Thus, the models most representative of human HCC may require the most time which is suboptimal for studying response to therapy.

At present, almost all mouse studies assess tumour size and number at the one time point of sacrifice; however, in clinical practice, HCC is diagnosed and monitored regularly using imaging (CT, magnetic resonance imaging and ultrasound). Although these imaging modalities give reliable measurements that correlate with tumour size at sacrifice, they are currently time-consuming and labour intensive (requiring scanners, anaesthesia and injection of intravenous contrast agents)\cite{21}. Since tumour development can be lengthy and their responses to treatment (especially new immunotherapies) are dynamic over time\cite{83}, measurement of experimental tumours on imaging will likely play an increasingly important role in the future.

Recently, three-dimensional *in vitro* cell culture systems (organoids) using cells isolated from human biopsies have been developed to study HCC. These tumour organoids (tumouroids) have been shown to
recapitulate the histological architecture, expression profile, genomic landscape and in vivo tumourigenesis of the parental tumour, even after long-term (> 1 year) expansion in culture\textsuperscript{[12]}. Furthermore, tumouroids could be established within 2-3 months after isolation. Therefore, tumouroids fulfil many of the criteria for a reliable cancer model which animal models could not and may represent a promising advancement for understanding tumour biology and drug efficacy testing in future studies of HCC. However, they currently lack the human immune and stromal microenvironment that is thought to be crucial in understanding tumour progression and response to treatment, particularly immune-based therapies.

CONCLUSION

Alcoholic and metabolic liver diseases will be major contributors to HCC burden in the future. Many aspects of human HCC development and progression remain unknown, negatively impacting therapeutic advancement. Animal models play a crucial role in improving our understanding of human HCC and developing novel therapeutic strategies. Currently, no animal model can faithfully replicate the complexity of the cancer and its background liver disease but mere aspects of it with varying degrees of technical demand. The careful combination of different animal models and use of novel technologies such as human organoids may help bridge this gap in the future. For the time being, the use of HCC mouse models needs to be tailored to specific experimental hypothesis or clinical testing.

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Acquisition, analysis and interpretation of data, drafting of the article, critical revision of the article: Liu K, Chen J, McCaughan GW
All authors have read and approved the final version.

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