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Animal models for hepatocellular carcinoma☆

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ABSTRACT

Hepatocellular carcinoma (HCC) represents ~90% of all cases of primary liver cancer and occurs predominantly in patients with underlying chronic liver disease and cirrhosis. Establishing appropriate animal models for HCC is required for basic and translational studies, especially the models that can recapitulate one of the human disease settings. Current animal models can be categorized as chemically-induced, genetically-engineered, xenograft, or a combination of these with each other or with a metabolic insult. A single approach to resemble human HCC in animals is not sufficient. Combining pathogenic insults in animal models may more realistically recapitulate the multiple etiologic agents occurring in humans. Combining chemical injury with metabolic disorder or alcohol consumption in mice reduces the time taken to hepatocarcinogenesis. Genetically-engineering weak activation of HCC-promoting pathways combined with disease-specific injury models will possibly mimic the pathophysiology of human HCC in distinct clinical settings.

1. Introduction

Liver cancer is the second leading cause of cancer death worldwide, according to a report from World Health Organization in early 2018. Among 8.8 million cancer deaths in 2015, liver cancer accounts for 788,000 deaths (~9%). Hepatocellular carcinoma (HCC) represents ~90% of cases of primary liver cancer and occurs predominantly in patients with underlying chronic liver disease and cirrhosis [1].

Few therapeutic options exist for patients with HCC. For some years, sorafenib, a multi-kinase inhibitor with anti-proliferative and anti-angiogenic effects, is the only medical therapeutic option for advanced and unresectable HCC. Although this treatment increases mean overall survival by three months [2], only a proportion of patients benefit from this therapy and most patients suffer from considerable side effects. In 2017, the US Food and Drug Administration approved nivolumab as an anti-programmed death-1 (PD-1) monoclonal antibody immunotherapy to treat HCC in patients who have been previously treated with sorafenib [3]. The evaluation of its clinical benefit is ongoing. There is an urgent and unmet need to develop new therapies. A major issue in developing new HCC therapies is that clinical trials are predominantly of drugs that are effective for other cancers, rather than developed specifically for HCC. This approach has not improved survival in HCC above that achieved by sorafenib. Successes are largely limited to improved tolerability with non-inferiority [4].

Establishing appropriate animal models for HCC is required for basic and translational studies, especially models that can recapitulate human disease settings. A wide range of animal models are currently available, targeting HCC pathogenesis from different angles. Here we review animal models of HCC and provoke thinking on how to "humanize" the models and use these models for pre-clinical testing of cancer therapy. Some aspects are discussed in our previous review [5].

1.1. Pathogenesis of human hepatocellular carcinoma

Most patients with HCC have a clinical history of chronic liver diseases. The structure and function of the liver undergo pathological changes in response to insults and injury, resulting in inflammation, fibrosis, necrosis and cirrhosis [6]. In humans, cirrhosis is the major clinical risk factor for liver cancer [7]. Cirrhosis often develops in the setting of non-alcoholic fatty liver disease (NAFLD), or non-alcoholic

Abbreviations: CCl_4, carbon tetrachloride; DEN, diethylnitrosamine; FXR, farnesoid X receptor; GEM, genetically-engineered mouse; GNMT, glycine *N*-methyltransferase; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HFD, high fat diet; HT, hydrodynamics-based transfection; NAFLD, non-alcoholic fatty liver disease; NASH, non-alcoholic steatohepatitis; NMOR, N-nitrosomorpholine; PB, phenobarbital; PD-1, programmed death-1; PREX2, phosphatidylinositol-3,4,5-Trisphosphate Dependent Rac Exchange Factor 2; PTEN, phosphatase and tensin homolog; TAA, thioacetamide; Tet, tetracycline

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steatohepatitis (NASH), as well as viral, alcoholic or autoimmune hepatitis. NAFLD and NASH are closely linked with the metabolic syndrome, such as obesity, type 2 diabetes and dyslipidaemia. Notably, obesity is an independent risk factor for the development of HCC [8]. Half of all cases of HCC are associated with chronic hepatitis B virus (HBV) infection, with a further 25% associated with chronic hepatitis C virus (HCV) infection [9]. Other risk factors for developing HCC include alcoholic liver disease and aflatoxin-contaminated food [9]. Thus, HCC has complex aetiology, with many confounding factors affecting disease course and patient prognosis. Recent comprehensive transcriptomics has shown that human HCC has several distinct subtypes [10]. Perhaps each human HCC subtype will prove to be best reflected by a particular animal model [11]. This question needs exploration by applying transcriptomics and proteomics to each animal model for comparisons with human HCC. The integrated and multi-platform analysis of human HCC genotype [10] provides a useful tool for such comparisons. This approach has potential to confer greater clarity upon the field of HCC animal models and their applicability to human cancers.

2. HCC models in animal

Animal models are well-established tools used to understand disease pathogenesis, identify therapeutic targets and screen for effective drugs. Due to the complex aetiology and tumour heterogeneity, to develop animal models of HCC that are analogous to human disease settings is a challenge. Animal models currently available can be categorized as follows: (a) chemically-induced models, (b) genetically-engineered mouse (GEM) models, and (c) engrafted models (Fig. 1). A selection of representative models for HCC research with emphasis on the most recent findings will be discussed in this review (Table 1).

2.1. Chemically induced models

Humans are inevitably exposed to toxic chemical compounds and the liver is the primary target due to its essential role in detoxification. There are several well-established chemically induced substances that induce liver injury and are therefore used as promoters in HCC models. Two types of carcinogenic compounds, genotoxic and promoting compounds, have been used in making HCC models [5]. These chemicals act predominantly to cause hepatocyte death, steatosis or cholestasis, followed by inflammation and fibrosis.

2.1.1. Diethylnitrosamine (DEN)

DEN is the most extensively used genotoxic agent for chemically induced HCC [12]. Mice mainly develop liver tumours, but can also develop gastrointestinal, skin, respiratory and hematopoietic tumours [13]. DEN induced carcinogenic activity is caused by (i) alkylating DNA structures thus causing DNA damage and subsequent cell degeneration and (ii) by inducing reactive oxygen species through the activation of the cytochrome P450 in hepatocytes [14]. Cytochrome P450 increases and reaches its peak activity between the 7th and 15th day of age and then decreases [15]. Thus DEN is commonly administered between 12 and 15 days of age by a single intraperitoneal injection (5–25 μg/g body weight in B6C3F1 mice) [16]. If the mice are older than 2 weeks, subsequent to DEN administration, a tumour promoter is required, which enhances hepatocarcinogenesis. This can be achieved by adding the following promoters: 2-AAF [17], phenobarbital (PB) [18], N-nitrosomorpholine (NMOR) [19], and carbon tetrachloride (CCl_4) [20]. Overall, DEN-induced tumorigenesis varies with age, mouse strain, sex and dose [16]. It usually takes at least a year to manifest and represent human HCC relatively effectively by itself. Combined chemical insults accelerate tumour progression.

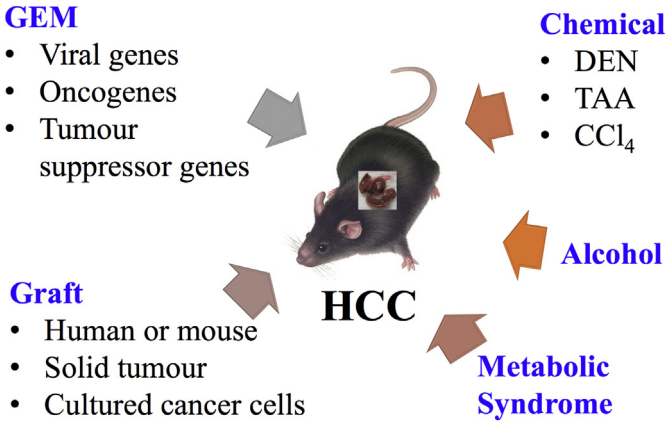

Fig. 1. Animal models of hepatocellular carcinoma (HCC) include chemical-induced models, genetically engineered mouse (GEM) model and graft models. Multiple liver insults including alcohol and metabolic syndrome synergize to accelerate experimental HCC.

2.1.2. Carbon tetrachloride (CCl_4)

CCl_4 is a potent hepatotoxin and has been used extensively to produce liver fibrosis in rats and mice, and recently in monkeys [21]. CCl_4 acts in two ways: it directly increases oxidative damage in hepatocytes and impairs the cell membrane integrity; it causes an inflammatory response by Kupffer cells and stellate cells through the production of cytokines, chemokines and other pro-inflammatory molecules [22–24]. This in turn attracts and activates monocytes, neutrophils and lymphocytes that further contribute to liver necrosis and inflammation [25]. CCl_4 is typically administered at 0.5 to 2 mL/kg body weight (diluted in paraffin oil or corn oil) via intraperitoneal injection twice weekly, leading to robust and reproducible fibrosis in 4–6 weeks. CCl_4 induced liver fibrosis can be completely resolved within several weeks after withdrawal of CCl_4 treatment [26]. The degree of susceptibility to CCl_4 - mediated liver damage varies among species, strains and administration schedules. A large number of rat models have been reported to develop both liver cirrhosis and HCC using CCl_4. In mice, some strains develop well-differentiated HCC with no evidence of cirrhosis, some show incomplete cirrhosis with portal fibrosis, bridging fibrosis, and regenerative nodules, and some show only limited portal fibrosis and bridging fibrosis with no regenerative nodules [27]. Combined DEN and CCl_4 is commonly used to reliably induce HCC [28–30].

2.1.3. Thioacetamide (TAA)

TAA is a well-established model of liver fibrosis in rodents. Like CCl_4, TAA administration was originally used to model fibrosis and cirrhosis in mice and rats [31]. The molecular mechanisms behind its hepatotoxicity are not completely understood, possibly through oxidation processes by a mixed-function oxidase system [32]. TAA can be administered either by intraperitoneal injections ranging from 100 to 200 mg/kg body weight 3 times a week for 4–8 weeks [33,34] or via drinking water (200 μg/L) for 6–18 weeks [35,36]. Intraperitoneal administration leads to hepatic centrilobular necrosis, elevated transaminase activity and robust liver fibrosis in about 6 weeks [37]. Long-term TAA treatment can cause biliary dysplasia and cholangiocarcinoma. Oral TAA administration does not lead to significant elevation of transaminases in mice [35], thus resulting in a milder health insult to experimental animals that possibly makes it a better candidate for multiple insult HCC models. TAA in conjunction with another hepatocarcinogen such as DEN in a two-stage model is commonly used to induce HCC [38,39].

2.1.4. Phenobarbital (PB)

Human relevance of tumour promotion in rodents is sometimes controversially discussed as, for example, in the case of the antiepileptic drug PB. Although PB causes tumours in rodent liver following chronic administration, epidemiological data from epilepsy patients treated with phenobarbital do not show a specific role of PB in human liver

Table 1
HCC models in rodents.

Model	Time of HCC development	Strain species	Metastasis / = not mentioned	Property	Reference
Chemical and risk factor					
DEN + 2-AAF	14 weeks	Wistar rat	/	Early hepatic pre-neoplastic event; model for HCC prevention	17
DEN + PB	40 weeks	Bcl-3^{Hep} mouse	/	Late stage, inflammation-driven HCC	18
DEN + PB	14 weeks	Wistar rat	/	Early hepatic pre-neoplastic event; model for HCC prevention	42
DEN + NMOR	16 weeks	Fischer rat	/	HCC model with altered metabolites	19
DEN + CCl_4	9 months	$CCR10^{-/-}$ mouse	/	Inflammation-driven hepatocarcinogenesis	28
DEN + CCl_4	3–6 months	$Wnt^{-/-}$ (hepatocyte-specific)	/	Chemical injury in GEM for hepatic injury, fibrosis and carcinogenesis	29
DEN + CCl_4	20 weeks	TIMP-$1^{-/-}$ mouse	/	Fibrosis-driven hepatic carcinogenesis	30
DEN + TAA	25 weeks	Wistar rat	/	Cirrhosis-associated hepatocarcinogenesis	39
DEN + alcohol	16 weeks	C57BL/6	/	Alcohol promotion of DEN-induced hepatocarcinogenesis	86
HFD	32–52 weeks	B6/129	/	HFD induced NASH–HCC progression	81
Choline-deficient, L-amino acid-defined HFD	36–60 weeks	C57BL/6 J	/	Specialised HFD that causes NASH	82
DEN + choline-deficient, L-amino acid-defined HFD	20–24 weeks	C57BL/6 J	/	Specialised HFD induced NASH accelerated to HCC by DEN	83
CCl_4 + HFD	24 weeks	C57BL/6 J	/	CCl_4 exacerbates HFD induced tumour development	84
DEN + TAA + HFD	24 weeks	C57BL/6	/	HFD accelerates DEN and TAA-induced hepatocarcinogenesis	85
DEN + CCl_4 + Alcohol	5 months	BALB/c	/	HCC model with steatohepatitis, fibrosis and alcohol-induced hepatotoxicity	20
GEM ± risk factor					
HBV-derived (HBx)	> 15 months	ATX mouse	Yes	Susceptible to HCC	45
HBx + DEN	8 months	ATX mouse	/	HBx sensitises DEN-induced carcinogenesis	49
HBx-FXR knockout	15 months	ATX-$FXR^{-/-}$	/	Spontaneous HCC model	50
HBx-β-catenin conditional depletion	8–9 months	ATX-$Ctnnb1^{-/-}$ (hepatocyte-specific)	/	β-catenin(+) hepatic progenitor cells for HCC formation, which is potentiated by HBx.	53
GMNT knockout	8–16 months	$GMNT^{-/-}$ mouse	/	Spontaneous development of steatosis, fibrosis, and HCC	55,58
Mdr2 (Abcb4) knockout	12 months	$Mdr2^{-/-}$ mouse	/	Model for beta-catenin-negative subgroup of human HCCs characterized by low nuclear cyclin D1 levels	63
Mdr2 (Abcb4) knockout	7 months	BALB/c.$Mdr2^{-/-}$	/	Model for fibrosis, and cirrhosis-driven HCC	64
Ras + c-Myc	2 months	C57BL/6	/	Combination of oncogenic mutations using hydrodynamics-based transfection	68,69
Ras + p53	1 month		/		
c-Myc + p53	7 months		/		
Engrafted model					
PLC/PRF/5 cells	7 days	Athymic mouse (NU/NU)	/	Subcutaneously injected; tumour size up to 10 mm^3	73
MH-134 cells	N/A	C3H mouse	/	Subcutaneously injected; tumour size up to 20 mm^3 in > 80% of mice	74
HCa-1 cells	N/A	C3H/HeN mouse	/	Intramuscularly into thigh; tumour to 8 mm mean diameter	75
Hepa1–6 cells	5 weeks	C57BL/6	/	Injected into the left liver lobe	76
Rat hepatoma N1-S1 cells	14 days	Sprague-Dawley rat	/	Under the liver capsule; medial aspect of left lobe	77

Table 2
Suggested criteria for the ideal animal model of HCC.

Recapitulate human HCC aetiology
Genetic alterations that are similar to human HCC
Reflect the stages of disease progression in human HCC
Simulate the human tumour microenvironment, including tumour-parenchymal cell response and immune system response
Represent human HCC regarding primary tumour and metastases, including intravascular invasion
Reasonable time frame and affordable manipulations
Minimise health and safety risks for experimenters
Recapitulate various disease severities seen in recruits for human clinical trials

cancer risk [40]. In rodents, the effects of PB on liver cancer are also controversial. HCC was induced in Wistar rats using DEN for initiation and PB as a promoting agent [41]. The pro-inflammatory cytokine interleukin-6, tumour markers, angiogenesis markers, lipid peroxidation and nitric oxide were significantly increased in DEN/PB-induced rats [41]. However, Braeuning et al. have demonstrated that chronic PB treatment of mice exerts a dual role in liver tumour formation by promoting the growth of hepatocellular adenoma but inhibiting the growth of HCC [42]. The mechanisms by which PB causes tumour promotion or inhibition are not fully understood.

2.2. Genetically engineered mouse (GEM) models

During HCC pathogenesis, pre-neoplastic/neoplastic cells accumulate genetic mutations, thus enhancing the tumorigenic potential and ultimately becoming malignant and metastatic HCC. An exome sequencing analysis of liver tumours in HCC patients has revealed mutations centred on aberrant telomerase reverse transcriptase (*TERT*) activation, Wnt/β-catenin signalling pathway (*CTNNB1*), and aflatoxin B1 and HBV infection (*Tp53*) [43]. Other pathways altered are PI3K/AKT/mTOR/MAPK signalling, epigenetic and chromatin regulation, and cell cycle control [1]. GEM models for HCC with alterations in candidate oncogenes, tumour suppressor genes, growth factors, or tumour microenvironment have been summarized [25,44]. Data from transgenic mouse models suggest that viral hepatitis genes could have a primary role in initiating or promoting liver carcinogenesis whilst non-viral genes including oncogenes and growth factors could increase the chance of tumour development. However, genetic profiles of human and mouse HCC are likely variable and different. Certain genetic mouse models closely reproduce the gene expression patterns of HCC in humans, while others do not [11]. Comparison of gene expression profiles of human and mouse HCC could effectively identify appropriate GEM to study certain subtypes of human HCC. Here we consider recent research on GEM models.

2.2.1. Hepatitis virus transgenic mice

Hepatitis B virus X protein (HBx) is an HBV protein that has multiple cellular functions. Kim et al. placed the HBx regulatory gene directly into the germline of mice [45]. Transgenic animals harbouring this viral gene began with multifocal areas of altered hepatocytes, followed by the appearance of adenomas, and proceeding to the development of carcinomas. HBx has been shown to induce oxidative stress in liver cells [46], trigger inflammation [47], and regulate fatty acid oxidation to promote HCC survival during metabolic stress [48]. HBx expression contributes to the development of DEN-mediated carcinogenesis by promoting the proliferation of altered hepatocytes [49]. Recent work has found that ablation of a nuclear receptor, called farnesoid X receptor (FXR), enhances the carcinogenic activity of HBx in mice [50]. Epidemiological and animal studies have previously shown that males are more susceptible to HCC, it has been suggested that the stimulatory effect of androgens and the protective effect of oestrogens may play a mechanistic role. However, this study reports that the incidence of tumour and pre-neoplastic lesions in female $FXR^{-/-}$ HBx mice are significantly greater than in their male counterparts [50]. It is generally accepted that male animals are used for HCC. This highlights a need to consider the sex and strain of the animals used in these experiments.

2.2.2. Oncogenic transgenic mice

In a study of 109 patients with HCC, 14.6% harbored activating mutations in β-catenin gene *CTNNB1* mutation [51]. Since global genetic knockout of β-catenin results in embryonic lethality, $Ctnnb1^{flx/flx}$ mice were crossed with Alb-Cre mice to generate the conditional depletion of *Ctnnb1* in hepatocytes. Loss of β-catenin impairs the ability of liver to counteract chemical DEN-induced oxidative stress [52]. When *Alb-Cre*;$Ctnnb1^{flx/flx}$ mice were further crossed with HBx transgenic mice (*HBx*;$Ctnnb1^{flx/flx}$), the rate of liver tumour development was much faster (8–9 month of age compared to 20 month of age) and the number of tumours was increased [53]. This indicates that these combined insults synergise and accelerate tumour development.

2.2.3. Metabolic dysfunction induced in transgenic mice

Glycine *N*-methyltransferase (GNMT) is frequently downregulated in human HCC [54]. GNMT knockout ($Gnmt^{-/-}$) mice can spontaneously develop chronic hepatitis, fatty liver, and HCC [55], through modulating key aspects of metabolic syndrome in mice via PI3K/Akt pathway [56] and/or inducing degradation of PREX2, which is a novel PTEN inhibitor [57]. Multiple tumour nodules approximately 5 mm in diameter have been found in the livers of $GNMT^{-/-}$ mice at 16 months of age. This $GNMT^{-/-}$ mouse model has since been used to search for early diagnosis biomarkers of hepatocarcinogenesis [58].

Table 3
Advantages and disadvantages of currently available animal models of HCC.

Model	Advantages	Disadvantages
Chemicals	Reflect liver's essential role in detoxification	Underlying mechanisms not fully understood
	Genotoxic, act as promoters in HCC models	Chemical agents can be metabolized differently in humans
		Average time for forming a tumour is long
GEM	Good model for studies concerning the genetic and molecular mechanism of HCC development	Single gene mutation is generally inefficient at inducing cancer
		Downstream pathway from gene mutated in mouse may be different from that in human
	Conditional and inducible gene expression systems can be liver-targeted	Embryonic lethality can occur
		Expensive and time-consuming
Engrafted	Allow studies using human tumour tissue, which carries human genetic material	Omit the entire process of hepatocarcinogenesis in the context of chronic liver injury
	Fast solution for drug screening	Immunity and tumour microenvironment are derived from mouse, not human
	Orthotopic xenograft model replicates the tumour microenvironment; appropriate for HCC metastasis investigation	Multiple cell lines have to be used in experiments because of the heterogeneity
Combined injury	Recapitulate interactions between different etiologic agents	More manipulation
	Shortened time to induce carcinogenesis	

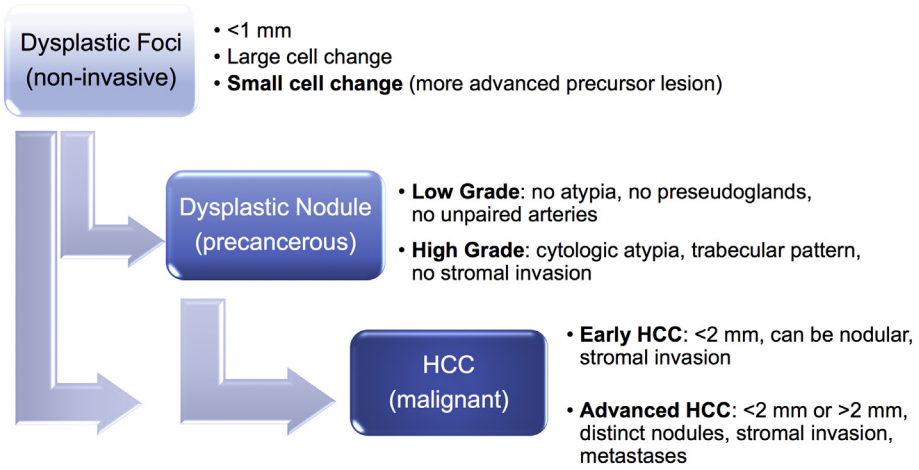

Fig. 2. The progression of precancerous lesions to hepatocellular carcinoma (HCC) in human [88].

Table 4
Histopathology of mouse liver tumour after Becker 1982 [93].

	Properties	Histopathological features
Type I	Arise in strains (C3H, B6C3F1) susceptible to spontaneous HCC	Size: 1 mm ~ 2.5 cm Tumour usually has a peripheral border (~2–8 mm thick) A gradient of alteration of glycogen and fat towards centre of the tumour
Type II	Arise in strains (C3H, B6C3F1) susceptible to spontaneous HCC	Size: 5 mm ~ > 2.5 cm Foci of trabeculation
Type III	Arise in strains with a genetic propensity of tumorigenesis that had received carcinogen	Foci of frank malignancy Cells usually present features of cytological atypia, including increased nuclear/cytoplasmic ratio, abnormalities in nucleus and chromatin structure, high mitotic rates Trabeculae with increased thickness Tumour invades into their original nodule or extend outward into the normal parenchyma
Type IV	Arise in strains with a genetic propensity to tumours or in strains that show little or no tendency towards tumorigenesis, both having received chemical carcinogens (C57BL/6)	Foci of frank malignancy in the parenchyma of liver No visible remnant of type I and II tumour

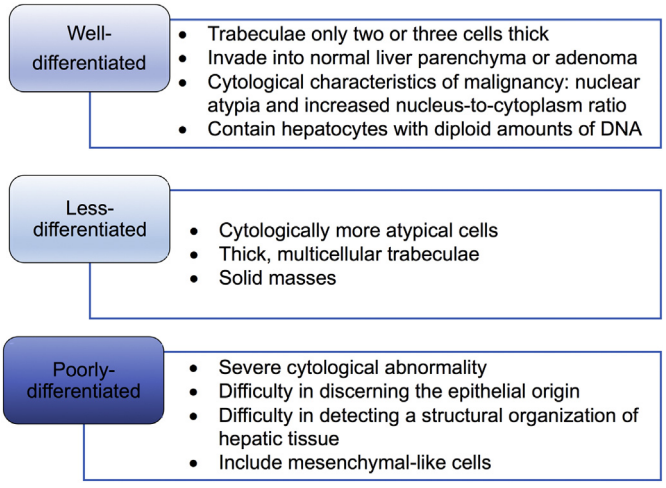

Fig. 3. The histopathology features of HCC subtypes. After Grisham 1996 [94].

2.2.4. Inflammatory cholangitis induced in transgenic mice

ABCB4 (ATP-binding cassette, sub-family B, member 4), also known as multidrug resistance 2 (MDR2), is the transporter of phosphatidylcholine at the bile canalicular membrane of mouse hepatocytes, which has an essential role in bile formation [59]. Mutations in the human homolog MDR3 are associated with several liver diseases [60]. The Mdr2 knockout ($Mdr2^{-/-}$) mouse was first described in 1993, showing a defective secretion of phosphatidylcholine into bile [61]. $Mdr2^{-/-}$ mice as a model of non-suppurative inflammatory cholangitis and hepatocarcinogenesis was reported in 1994 [62]. Liver inflammation and hepatotoxicity induced by bile salts in $Mdr2^{-/-}$ mice leads to the development of hepatocyte dysplasia, and by 16 months of age, nearly all $Mdr2^{-/-}$ mice have liver tumours [63]. When the $Mdr2^{-/-}$ mouse was genetically backcrossed onto the fibrosis susceptible BALB/c substrain, accelerated liver fibrosis and early signs of cirrhosis occur at 12 weeks of age. Liver tumours develop as early as 7 months of age [64]. Due to its pathological properties, the $Mdr2^{-/-}$ mouse is an appropriate animal model to study sclerosing cholangitis [65,66] and consequent cirrhosis associated HCC [59].

2.2.5. Conditional and inducible gene expression systems

GEM models with proto-oncogenes or tumour suppressor genes often result in embryonic lethality and fail to mimic sporadic multistep tumorigenesis. With the advent of conditional and inducible in vivo systems, it allows for the induction of somatic mutations in a tissue specific and time-controlled manner. An inducible system allows for temporal control over genetic changes. He et al. has reviewed three widely used inducible systems: (a) tetracycline (Tet) controlled (b) tamoxifen controlled and (c) virus mediated Cre delivery [67]. Recently, a simple method for liver transgenesis was used in which naked DNA plasmids encoding a gene of interest were directly delivered into the liver via hydrodynamics-based transfection (HT) method [68,69]. This HT method uses the physical force generated by rapid injection of a large volume of DNA solution into the lateral tail vein. HT is highly specific for hepatocytes and HT models have shown increased tumour development when a combination of oncogenic mutations was

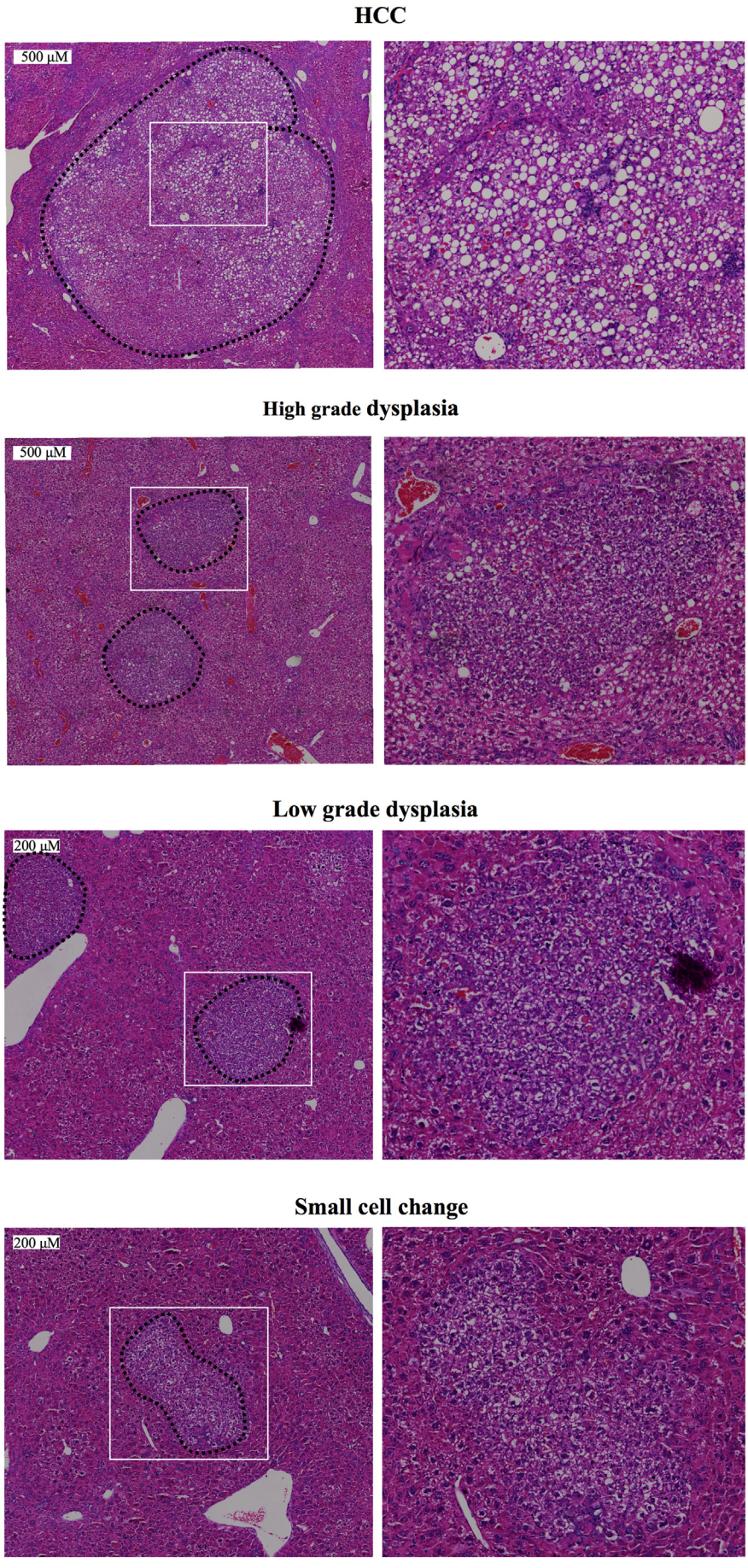

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Fig. 4. H&E stained paraffin sections of liver from mice treated with DEN at 14 days of age, and then with TAA and high fat diet at 4–24 weeks of age [85]. The lesions depicted were classified as either HCC, high grade dysplasia, low grade dysplasia or small cell change. Scale Bars = 500 μm or 200 μm. Dotted line outlines each lesion. Each boxed area is shown at greater magnification in a panel to the right.

introduced into the liver. Resulting liver tumours revealed moderately differentiated HCC (Ras and c-Myc mutation at 2 months), highly malignant and poorly differentiated HCC (Ras and p53 mutation at 1 month), and well differentiated HCC (c-Myc and p53 mutation at 7 months) [68,69]. A limitation of HT is that achieving prolonged expression of a transgene is difficult.

2.3. Engrafted HCC mouse models

In xenograft models, the tumours are formed by implantation of fragments of human solid tumours or cultured human cancer cells into a host mouse, either under the skin (ectopic) or into the organ of tumour origin (orthotopic). An advantage of xenograft is that it allows studies using human tumour tissue, which carries human genetic material; this is more representative of the properties and mutations of the human cancer. A limitation is that this model omits the entire process of hepatocarcinogenesis; when transplanted into a normal liver, it lacks the typical hepatic tumour microenvironment [70]. Immunodeficient animals are often used in order to avoid cell and tissue rejections. However, immune cells are an important component of the HCC microenvironments, so immunodeficient animals do not mimic the situation in human patients. Developing methods to "humanize" host mice might overcome this issue in future HCC studies [71,72]. An alternative is syngeneic tumour models where immortalized mouse cancer cell lines are engrafted back into the same inbred immunocompetent mouse strain. However, immunity and tumour microenvironment are derived from mouse. There can be difficulty in interpreting and predicting how a mouse immune response translates back to human.

Cell lines, of human or murine origin, used in transplantable HCC models, are reviewed elsewhere [44]. Tumour heterogeneity greatly complicates the development of molecular targeted agents for HCC. Thus, it is essential to test multiple cell lines in xenograft models, especially to screen new anticancer drugs. Investigating anti-cancer effects of combined sorafenib and capsaicin has used human HCC cell line PLC/PRF/5 in a xenograft model [73]. A study on the synergistic effect of cytokine induced killer cells with valproic acid used four human HCC cell lines for xenograft: MH-134 (a mouse HCC cell line), Huh-7 (a well-differentiated HCC cell line), SNU-475 (a poorly differentiated HCC cell line), and SNU-761 (a poorly differentiated HCC cell line) [74]. Murine and rat hepatoma cell lines used in syngeneic tumour models include HCa-1 [75], Hepa1-6 [76], and Rat N1-S1 [77]. These examples illustrate how a multiple cell lines strategy can be achieved.

2.4. Combined insults to model HCC

Animal models with genetic, anatomical, and physiological similarities to humans are desirable for elucidating the diverse mechanisms involved in the pathogenesis of HCC, to discover novel therapeutic targets and to evaluate novel therapies. Using a single approach to recapitulate human HCC in animals is not sufficient because human HCC is commonly driven by viral, chemical or other insults that may overlay a genetic predisposition. Combinations of liver - targeted insults in animal models are likely to recapitulate interactions between different etiologic agents seen in human, as the proposed "multiple parallel hit hypothesis" resulting in a shift towards NASH- and NAFLD- driven HCC development [78].

2.4.1. Chemical injury with metabolic disorder

With advances in antiviral therapy, the number of patients with virus associated HCC is expected to decline. However, NAFLD- and NASH- derived HCC has been rising worldwide, with annual incidence ranging 2.4–12.8%, due to increased prevalence of metabolic syndrome [79]. High fat diet (HFD) is clearly associated with significant promotion of HCC progression [80]. HFD induces NASH and progresses to HCC after 32 weeks [81]. A dietary NASH model of choline-deficient, L-amino acid-defined HFD has found that C57BL/6 J mice can develop advanced NASH and progress towards HCC in a period of 36 weeks [82]. A similar diet of choline-deficient HFD with multiple DEN injections does not cause significant fibrosis, but induces intrahepatic tumour formation from 20 weeks, some of which are glutamine synthetase positive [83]. Combining CCl_4 and HFD for 24 weeks recapitulates the progressive stages of NASH and HCC [84]. We have found that the addition of HFD to TAA treatment in mice can generate liver fibrosis more rapidly and reliably than TAA [85]. This model mimics the human situation in which liver fibrosis often associates with overnutrition and/or diabetes. Also, mild body weight loss is likely with TAA administration [36], but less likely with the addition of HFD to TAA. This model may provide a good representation for HCC associated with NASH or with NAFLD combined with toxin. Other recent work also shows that multiple liver insults synergize to accelerate experimental HCC. The addition of HFD to DEN/TAA treatment of mice leads to an increased number of histologically confirmed HCC lesions at 24 weeks of age, along with more intrahepatic inflammation and fibrosis [85]. Such HCC models provide relatively rapid ways of examining HCC.

2.4.2. Chemical injury with alcohol

Combined use of alcohol in a conventional chemical induced mouse liver cancer model is used to represent some types of human HCC. Alcohol feeding of male mice for 16 weeks from 7 weeks after an injection of DEN has shown increased numbers of pre-cancerous foci and liver tumours [86]. This chronic alcohol consumption activates the Wnt/β-catenin signalling pathway, which increases hepatocyte proliferation thus promoting tumorigenesis following an initiating insult in the liver [86]. Treatment with alcohol, DEN and CCl_4 in mice results in inflammation, fibrosis, cirrhosis and HCC in the liver [20]. This method uses less time (5 months) for HCC development compared with the conventional chemical induced method alone [20].

2.4.3. Chemical injury with genetic mutation

Incorporation of etiological factors and mutations into animal models can result in a translational model resembling human HCC. Yan et al. proposes combining chronic injury with mutations in oncogenes or tumour suppressors commonly found in human HCC to achieve a more realistic representation of human HCC [87]. Possible approaches are using GEM with weak activation of HCC - promoting pathways, e.g. by heterozygous deletion of tumour suppressors, or by targeting a small percentage of cells. Combining these genetic modifications with disease specific injury models, such as a chemical or NASH model, may be able to mimic the pathophysiology of human HCC in distinct clinical settings [87].

No "ideal" animal model for HCC exists. An optimal animal model would facilitate the basic and translational studies of HCC (Table 2). Careful evaluation of the advantages and disadvantages of the currently available models (Table 3) is crucial when selecting an animal model.

2.5. HCC histopathology

In humans, HCC has heterogeneous histopathology and a variety of hepatocellular nodules have been identified [88,89]. Focal areas of abnormal, immature hepatocytes in the liver are commonly defined as dysplastic foci (< 1 mm) and dysplastic nodules (> 1 mm). These dysplastic foci can be subclassified as large cell dysplasia and small cell

dysplasia [88,89]. Dysplastic lesions can also be classified as either low grade or high grade dysplastic nodules [90,91]. Small cell dysplasia (small cell change), low grade dysplastic nodules, and high grade dysplastic nodules are all considered to be precursors to HCC, either directly, or indirectly in a progression through stages [91]. The presence of stromal invasion is considered to be the hallmark feature that differentiates early HCC from dysplastic lesions [88,90] (Fig. 2).

Rodent HCC shows histopathological features that are largely similar to, but also divergent from, human. Rat HCC exhibits largely trabecular growth patterns and most lesions have haemorrhage, necrosis, pigment deposition, angiectasis and/or focal fatty change [92]. Morphological characteristics of mouse liver tumours have been described [93] (Table 4). In rodents, trabecular cytology occurs in relatively low-grade malignancy, the foci associated with malignancy demonstrate high mitotic rates with nuclear atypia, and cystic and necrotic areas are often present [93]. In human and rodent, HCC demonstrates a histological/cytological spectrum from well-differentiated to poorly differentiated [94] (Fig. 3). Some examples of lesions from mouse livers are depicted (Fig. 4).

3. Conclusion

There is much to consider when choosing an animal model of HCC to represent the genetic and physiological development and microenvironment of human HCC. Each model may well mimic a particular subtype of human HCC. Nevertheless, the greatest difficulty currently is the time taken to induce HCC in animals. Given the multifactorial development of HCC in humans, combining different insults can not only accelerate hepatocarcinogenesis, but may also be more suitable for preclinical treatment or prevention studies.

Conflict of interest

The authors have nothing to disclose; COI forms completed.

Transparency document

The Transparency document associated with this article can be found, in online version.

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