World Journal of Gastroenterology

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World J Gastroenterol 2018 May 14; 24(18): 1989-1994

DOI: 10.3748/wjg.v24.i18.1989

ISSN 1007-9327 (print) ISSN 2219-2840 (online)

MINIREVIEWS

Mouse models for investigating the underlying mechanisms of nonalcoholic steatohepatitis-derived hepatocellular carcinoma

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Author contributions: Takakura K and Oikawa T wrote the manuscript; Tomita Y, Mizuno Y, Nakano M and Saeki C critically appraised the manuscript; Torisu Y and Saruta M formatted and edited the final manuscript.

Conflict-of-interest statement: All of the authors declare no potential conflicts of interest relevant to this article.

Manuscript source: Invited manuscript

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Received: March 29, 2018
Peer-review started: April 4, 2018
First decision: April 27, 2018
Revised: May 1, 2018
Accepted: May 6, 2018
Article in press: May 6, 2018
Published online: May 14, 2018

Abstract

As the incidence of hepatocellular carcinoma (HCC) caused by infection with the hepatotropic viruses hepatitis B and hepatitis C decreases, greater attention has become focused on HCC caused by nonalcoholic steatohepatitis (NASH), an advanced form of nonalcoholic fatty liver disease which has shown increasing prevalence in correspondence with the overall increase in metabolic syndrome over the recent decades. Several clinical population studies have shown a positive relationship between NASH and HCC, while also providing initial insights into the underlying mechanisms of HCC development from NASH. Research into the pathological progression of NASH to HCC has advanced by use of several beneficial rodent models. In this review, we summarize the established mouse models for preclinical research of NASH-associated HCC and discuss the underlying hepatic mechanisms of NASH-related tumorigenesis identified to date that could lead to new targets for treatment and prevention.

Key words: Hepatocellular carcinoma; Nonalcoholic steatohepatitis; Nonalcoholic fatty liver disease

Core tip: This review provides a brief overview of the molecular mechanisms underlying progression

to hepatocellular carcinoma from nonalcoholic steatohepatitis that have been identified to date using the array of mouse models currently available and popular in the experimental field.

Takakura K, Oikawa T, Tomita Y, Mizuno Y, Nakano M, Saeki C, Torisu Y, Saruta M. Mouse models for investigating the underlying mechanisms of nonalcoholic steatohepatitis-derived hepatocellular carcinoma. *World J Gastroenterol* 2018; 24(18): 1989-1994 Available from: URL: http://www.wjgnet.com/1007-9327/full/v24/i18/1989.htm DOI: http://dx.doi.org/10.3748/wjg.v24.i18.1989

INTRODUCTION

As Western diet and problems with food satiation have spread across the globe in recent years, there has been a concomitant increase in patients with nonalcoholic fatty liver disease (NAFLD) and its progressive form of nonalcoholic steatohepatitis (NASH). This increase is the result of prevailing metabolic syndrome, including obesity, diabetes and hyperlipidemia[1-4]. The distinctive characteristic of NAFLD is its diversity of conditions, from simple fatty accumulation in the liver to hepatic injury and inflammation with or without fibrosis[2,5-7]. The sequential progression to NASH puts the sufferer at risk for irreversible liver cirrhosis and hepatocellular carcinoma (HCC)[4,7], causing the patient to require more medical attention due to the increased morbidity and mortality[8]. Indeed, HCC is a leading indication for liver transplantation, especially in developed countries[9,10].

Compared with the long history of both clinical and laboratory investigations to elucidate the molecular pathogenesis of HCC derived from chronic hepatotropic virus infections, particularly with hepatitis B virus and hepatitis C virus, and from alcoholic liver disease, the pathologic mechanisms of NASH-associated HCC (NASH-HCC) remain largely uninvestigated and unknown. The public health threat associated with the increasing incidence of NASH-HCC[11], however, highlights the urgent need to gain a more comprehensive and detailed understanding of the mechanisms which mediate NASH-HCC progression. Several experimental mouse models exist for such studies[12-15] and should be continuously applied to preclinical investigations into the pathogenic pathways of NASH-HCC to advance the subsequent development of methods to manage the modern increasing clinical trend.

Here, we summarize the established mouse models for preclinical research of NASH-HCC progression (Table 1) and discuss the revealed mechanisms and the future prospective of NASH-related tumorigenesis in liver which could lead to new targets for treatment or prevention (Figure 1). Of note, we recognize the existence of other available rodent models which can also be used for assessing the mechanisms of NASH-HCC; however, we focused this review on the ones which are most representative of metabolic syndrome-associated steatohepatitis and which generate HCC unfailingly from NASH status within a certain period of time.

CONFIRMED TUMORIGENIC MECHANISMS OF CURRENT NASH-HCC MOUSE MODELS

The established mouse models for preclinical research of NASH-HCC progression are listed below (Table 1).

PTEN null mice

PTEN, a tumor suppressor gene which antagonizes the PI3K/Akt pathway, is mutated in many human cancers, including HCC, and is essential for maintaining homeostasis and preventing oncogenesis in the liver. Decreased *Pten* expression leads to increased tumor grade, advanced stage and poor prognosis. Hepatocyte-specific *Pten* null mice were generated by Horie *et al*[12], wherein steatohepatitis emerges at 10 wk old and hepatic tumors at 40-44 wk old. The liver tumors become adenomas in 100% of these mice or HCC in 66% at 74-78 wk old, due to the *Pten* deficiency (*Pten* knock-out, KO) causing lipid accumulation in hepatocytes. In general, these mice have revealed that *Pten* function is crucial for preventing tumorigenesis in liver.

Several other research groups have uncovered different mechanisms of NASH-HCC by using the *Pten* null mouse model. For example, a study of eicosapentaenoic acid (EPA; a typical dietary n-3 polyunsaturated fatty acid contained in fish oil and a reagent for upgrading lipid metabolism[16]) performed by Ishii *et al*[17] showed the effect of EPA on steatohepatitis and tumor formation in *Pten* null mice. The data confirmed that the steatotic change, accumulation of inflammatory cells and presence of ballooning hepatocytes were significantly decreased in the EPA group compared with the control group. In addition, liver adenomas developed in 63% of the control group mice, as compared with 0% of the EPA group mice, by 40 wk of age. HCC developed in 75% of the control group and 13% of the EPA group of the *Pten* KO mice at 76 wk old. In addition, MAPK and Akt, which are both downstream signaling molecules of Ras, were found to be activated in hepatocytes of the *Pten* KO mice, thereby promoting tumorigenesis[18]. Collectively, these data suggested that EPA alters fatty acid composition in liver and suppresses the development of HCC by inactivating these signaling pathways in *Pten* null mice.

In another study of the *Pten* null mice, reduction of glucose-regulated protein 78 (GRP78; a molecular chaperone elevated in several human cancers, including HCC[19,20], and which is critical for endoplasmic reticulum folding, stress signaling and PI3K/Akt activation) promoted liver steatosis and liver injury at

Table 1 Mouse models of nonalcoholic steatohepatitis-associated hepatocellular carcinoma

List	Backgrounds	Inducer of NASH/HCC	Carcinogenic duration	HCC occurrence (%)	Ref.
PTEN null mice	Genetic	Spontaneous	40 wk	66 (74-78 wk)	[12,17,18,21,22]
MC4R KO mice	Genetic	HFC diet	1 yr	100	[13,29,31]
STAM mice	DM/HL	Streptozotocin, HFC diet	20 wk	100	[14,32-36]
ALR KO mice	Genetic	Spontaneous	1 yr	60	[15]

HFC: High fat/calorie; DM: Diabetes; HL: Hyperlipidemia; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

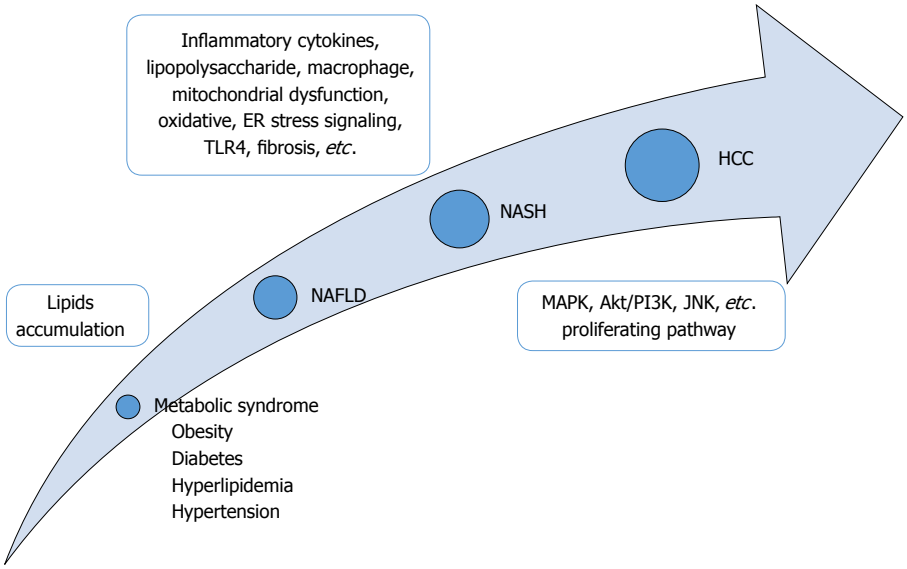

Figure 1 Developmental process of hepatocellular carcinoma *via* nonalcoholic steatohepatitis. Based on excessive lipids accumulation, several factors such as inflammatory cytokines, oxidative stress or proliferating pathways are involved in the whole process of hepatocellular carcinoma development from nonalcoholic steatohepatitis status *via* nonalcoholic fatty liver disease. NAFLD: Nonalcoholic fatty liver disease; HCC: Hepatocellular carcinoma; NASH: Nonalcoholic steatohepatitis.

3 mo of age and liver tumors at 6 mo of age[21]. These effects proceeded HCC or cholangiocarcinoma, which developed at 8-9 mo of age and was accompanied by elevation of p-JNK; in contrast, the GRP78 normal *Pten* null mice never generated tumor lesions in liver, as assessed out to 14 mo of age[21]. Collectively, these data suggested that JNK might contribute to acceleration of tumorigenesis in liver. Accordingly, these data demonstrated GPR78 as a regulator for *Pten* loss-mediated liver steatosis and tumor progression on the basis of p-JNK elevation.

In a third study of the *Pten* null nice, Miura *et al*[22] showed that liver tumors emerged after 36 wk of age, although no liver tumors were found in *Pten* normal mice until 72 wk of age. Toll-like receptor (TLR) 4 expressed on macrophages was found to contribute to the development of steatohepatitis and HCC in *Pten* KO mice. In general, gut-derived materials stimulate the immune system, including the TLRs which recognize bacterial components. TLR4, in particular, senses components of Gram-negative bacteria, including the lipopolysaccharide (LPS)[23]. In this way, TLRs affect the development of liver diseases. Moreover, macrophages are known to be a major source of proinflammatory cytokines which facilitate the progression of steatohepatitis[24,25] and Ly6C is a marker for inflammatory macrophages[26]. Hepatic macrophages isolated from the *Pten* null mice showed an increased expression of Ly6C. In addition, TLR4 signaling was shown to promote hepatic inflammation as well as subsequent liver tumor growth in the *Pten* null mice. Antibiotic treatment suppressed the tumor growth, in concert with a decreasing LPS level in the portal vein, suggesting that the gut microbiota serves as a source of TLR4 ligand(s) and that the Ly6C-positive macrophages play a role in tumor development in *Pten* null mice. Collectively, these data indicate that gut-derived LPS-induced inflammation *via* TLR4 on macrophages and TLR4-mediated inflammation result in HCC.

Melanocortin 4 receptor KO mice

Melanocortin 4 receptor (MC4R), a seven-transmembrane G protein-coupled receptor, is involved in regulation of body weight; hence, *MC4R* gene mutation is the major monogenic origin of obesity in human[27,28]. Feeding of a high-fat diet to MC4R-deficient (MC4R-KO) mice for 20 wk and 1 year leads to NASH and multiple well-differentiated HCC formations in the liver, respectively[13]. Similar to the findings in *Pten* null mice, Konuma *et al*[29]

found that highly-purified EPA treatment of MC4R-KO mice effectively inhibited the development of liver fibrosis without affecting body weight.

According to their previous study, hepatic crown-like structures (hCLSs), a unique histological feature, were found to play a pivotal role in the progression from simple steatosis to NASH[30], with EPA markedly suppressing hCLS formation and fibrosis *via* prevention of hepatocyte injury. Thus, it was concluded that the beneficial effect of EPA involved the hCLSs. In addition, canagliflozin (CANA, a sodium glucose cotransporter 2 inhibitor and antidiabetic drug) was shown to attenuate NASH-HCC in another study[31]. Based on the evidence that CANA induces adipose expansion without promoting macrophage augmentation, inflammation or fibrosis and altered glutathione metabolism to reduce oxidative stress in adipose tissue, the authors concluded that the decreased hepatic fat accumulation upon CANA treatment suppresses hepatic inflammation, fibrosis at 20 wk and subsequent NASH-HCC at 52 wk in Western diet-fed MC4R-KO mice.

STAM mice

The STAM mouse model was generated by neonatal male C57BL/6J mice exposure to low-dose streptozotocin at 2 d after birth followed by feeding with a high-fat diet after 4 wk of age[14]. As a result, NASH developed at 8 wk and HCC at 16-20 wk. This mouse model has specific positive features, such as the average duration of HCC occurrence being within 16-20 wk of age, the number of HCC nodules being over 4 in any single mouse, the basal liver function being relatively preserved and there being no visible metastasis in the entire body[32]. Moreover, this model has the substantial benefit of its HCC development from NASH being identical to the known progression in human patients, but with the whole process being completed within a relatively short period of time.

By using the STAM model, four studies have uncovered several of the mechanisms underlying NASH-HCC. First, Lau *et al*[33] demonstrated that cancer-associated fibroblasts, which regulate liver tumor-initiating cells, are augmented in parallel with increasing human growth factor (HGF) level during fibrosis and that HGF-induced FRA1 activation is related to fibrosis-dependent HCC development. These data suggest that cancer-associated fibroblast-derived, HGF-mediated FRA1 can be a new therapeutic target for NASH-HCC. Second, Fernandes *et al*[34] showed that solithromycin, a novel macrolide antibiotic, suppressed NASH, fibrosis and NASH-HCC by modulating the gluconeogenesis pathway, in particular the components of fructose 1, 6-biphosphatase and glucose-6-phosphatase which are regulated by protein kinase C epsilon. Solithromycin improved the hepatic morphological features, such as the hepatocyte ballooning degeneration, and functions, as evidenced by reduction in NAFLD activity score along with decreased inflammation, fibrosis and HCC progression. This mechanism was ultimately suggested as a candidate factor of novel treatment of NASH-HCC.

Third, Conti *et al*[35] revealed that aberrant expression of hepatic micro (mi)RNAs, such as miR-34a-5p, miR-93-5p, miR-221-3p and miR-222-3p, indicates their mechanistic significance in NASH-HCC tumorigenesis; specifically, 10 over-expressed miRNAs were identified. It is well known that human HCC tumorigenesis is associated with extensive genomic alterations. Therefore, the authors concluded that the altered expression profile of these miRNAs could be a surrogate marker for the initiation and progression of NASH-HCC.

Finally, based on the confirmed finding that NASH-HCC is associated with metabolic alterations in hepatic lipid homeostasis, Pogribny *et al*[36] indicated that one of the specific features of NASH-HCC is a significant dysregulation of 1-carbon homeostasis, with decreased expression of key 1-carbon metabolism genes, especially of the S-adenosylhomocysteine hydrolase (*Ahcy*) gene, and increased expression of the S-adenosyl-L-homocysteine (*SAH*) gene. Their results suggest that the inhibition of *Ahcy* expression may be a trigger of SAH elevation and subsequent progression of NASH-HCC.

Augmenter of liver regeneration-KO mice

Augmenter of liver regeneration (ALR), a hepatic growth factor, is widely known as a pleiotropic protein. ALR is critical for mitochondrial function, lipid homeostasis and cell survival. Gandhi *et al*[15] generated a liver-specific ALR-L-KO mouse and reported that depletion of hepatic ALR caused steatosis, mitochondrial degeneration and apoptosis of hepatocytes at 2 wk of age. These effects were followed by consecutive cell death, sustained inflammation at 4 wk, fibrosis/cirrhosis at 8 wk and eventually HCC formation (in 60%) at 1 year. Thus, it was theorized that inhibition of ALR synthesis in hepatocytes could lead to mitochondrial dysfunction and cell death, resulting in consecutive NASH and HCC occurrence.

FUTURE PERSPECTIVES FOR THE STUDY OF NASH-HCC BY ANIMAL MODELS

The "two-hit" hypothesis of the underlying mechanism of NASH-HCC involves the excessive accumulation of lipids in liver as the first step, thereby promoting sensitization to LPS, oxidative stress and inflammatory cytokines, representing the second hit[37-39] (Figure 1). Recently, Tilg and Moschen[40] proposed a "multiple-hit" hypothesis, in which various factors derived from gut and adipose tissue might take place in parallel during the progression from NAFLD to NASH. However, the definitive mechanisms in the progression from simple fatty liver to NASH and HCC are still under investigation, due to the inherent complexity of the functional combination of several factors. For some time, it was believed that the lack of appropriate animal models which were able to sufficiently reflect the actual

process of human NASH-HCC progression was the main obstacle to such research[41]. In recent years, however, the situation has changed according to the development and availability of several rodent models. Each model harbors different specific characteristics, including genetic background, obesity status, diet induction, *etc*. Thus, researchers can now evaluate the mechanisms of NASH-HCC related to a specific factor/parameter by using these animal models.

According to the overall analyses of hepatocarcinogenesis in each of the mouse models discussed above, it is the STAM mice that generate HCC unfailingly and most rapidly. The considerable demerit of this mouse model, however, is the obscurity of the original gene of tumorigenesis for HCC due to lack of genetic manipulation and the inclusion of diabetes and hyperlipidemia in the background. Genetic manipulation in mouse models, such as of the PTEN-KO or ALR-KO, is a useful means by which to clarify the role of a specific gene in the molecular foundation of NASH-HCC progression; although, the sequential progression to HCC in these models has a relatively long duration and HCC occurrence is uncertain.

It is still questionable whether or not these available mouse models represent the initiating and/or progression processes of *bona fide* human NASH-HCC. Furthermore, it is noteworthy that among actual NASH patients there are individual differences in degree of fibrosis and timing of tumorigenesis in liver. At the present time, however, it is undoubted that these mouse models are essential for investigating the underlying mechanisms of NASH-HCC. Therefore, the future research targets may move forward towards gaining a more comprehensive NASH-HCC evaluation by using these mouse models.

CONCLUSION

Several mouse models have become available in recent years that support investigation into the underlying mechanisms of NASH-HCC. In response to the growing demand for better management of NASH-HCC, further inquiries are expected by researchers upon selecting an appropriate NASH mouse model according to the specific mechanisms and/or therapeutic targets of interest. After that, we hope to get some breakthrough for new treatment or prevention of NASH-HCC in the near future.

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P- Reviewer: Gonzalez-Reimers E, Namisaki T, Peltec A
S- Editor: Gong ZM **L- Editor**: A **E- Editor**: Huang Y

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ISSN 1007-9327