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# **Novel Mechanism of Nonalcoholic Lipid Accumulation Promoting Malignant Transformation of Hepatocytes**

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Additional information is available at the end of the chapter

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## **Abstract**

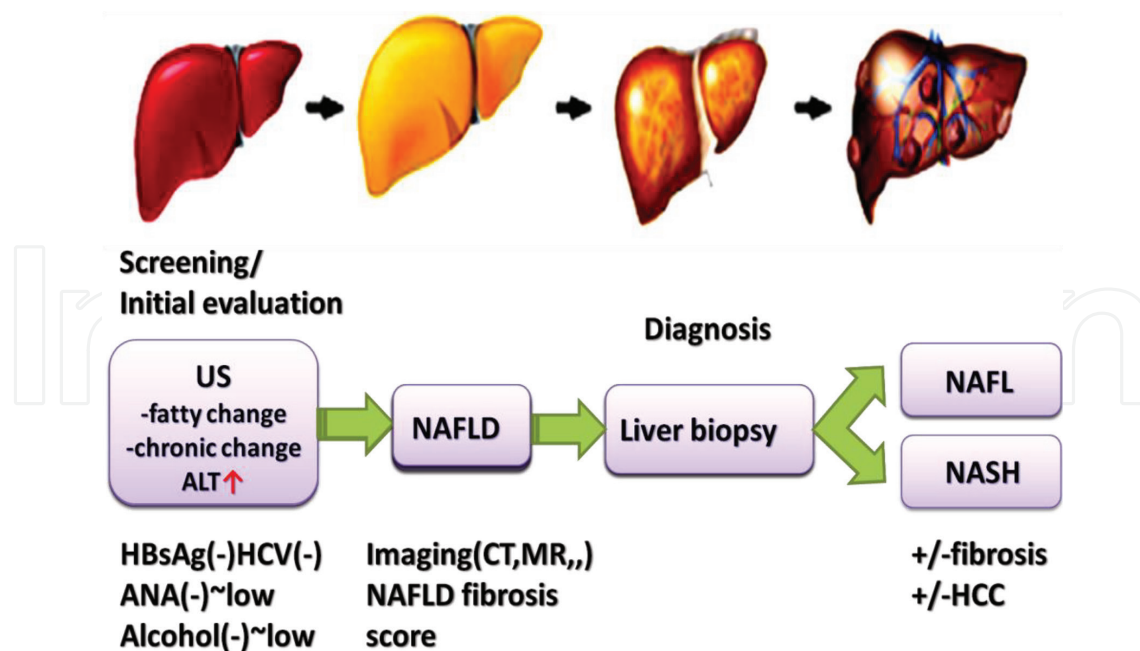
The incidence of hepatocellular carcinoma (HCC) is steadily increasing in worldwide, which has been a public concern significantly associated with diabetes and non-alcoholic fatty liver disease (NAFLD) is an emerging risk factor with increasing prevalence nowadays, with gradually instead of HBV and HCV, aflatoxin, or alcohol liver disease as major etiological factors. The deeply worrisome aspects of these high risk factors are their large spread in population. Systemic and genetic mechanisms involved in malignant transformation of liver cells as well as useful biomarkers at early stage of HCC are being investigated. However, the exact mechanisms from NAFLD to HCC still remain to be explored. In this paper, some advances of liver lipid accumulation were summarized on the relationship between NAFLD and hepatocytes malignant transformation.

**Keywords:** nonalcoholic fatty liver disease, hepatocellular carcinoma, metabolism

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## **1. Introduction**

Hepatocellular carcinoma (HCC) is one of the fifth most common malignant tumors, the third most frequent cause of cancer mortality worldwide [1, 2], and ranks the second in China among all malignancies with its mortality almost equal to its morbidity, especially in the inshore area of the Yangtze River [3, 4]. The principal treatment of HCC patient is surgical resection or liver transplantation, depending on whether the patient is a suitable transplant candidate [5, 6]. However, in most HCC patients with diagnosis at early stage is very difficult, thereby excluding the patients from definitive surgical resection. Sorafenib, the most commonly used systemic therapy, has shown to only minimally impact on patient survival



**Figure 1.** NAFLD progression and clinical diagnosis. ALT: alanine aminotransferase, NAFLD: non-alcoholic fatty liver disease, NAFL: nonalcoholic fatty liver, NASH: non-alcoholic steatohepatitis, HCC: hepatocellular carcinoma, HBsAg: hepatitis B surface antigen, HCV: hepatitis C virus, ANA: antinuclear antibody.

by several months. Besides, neither chemotherapy nor radiotherapy are generally effective. Due to the poor prognosis of HCC patients, the early diagnosis and effective therapy of HCC are needed with several being in development, either in preclinical or clinical studies [7, 8].

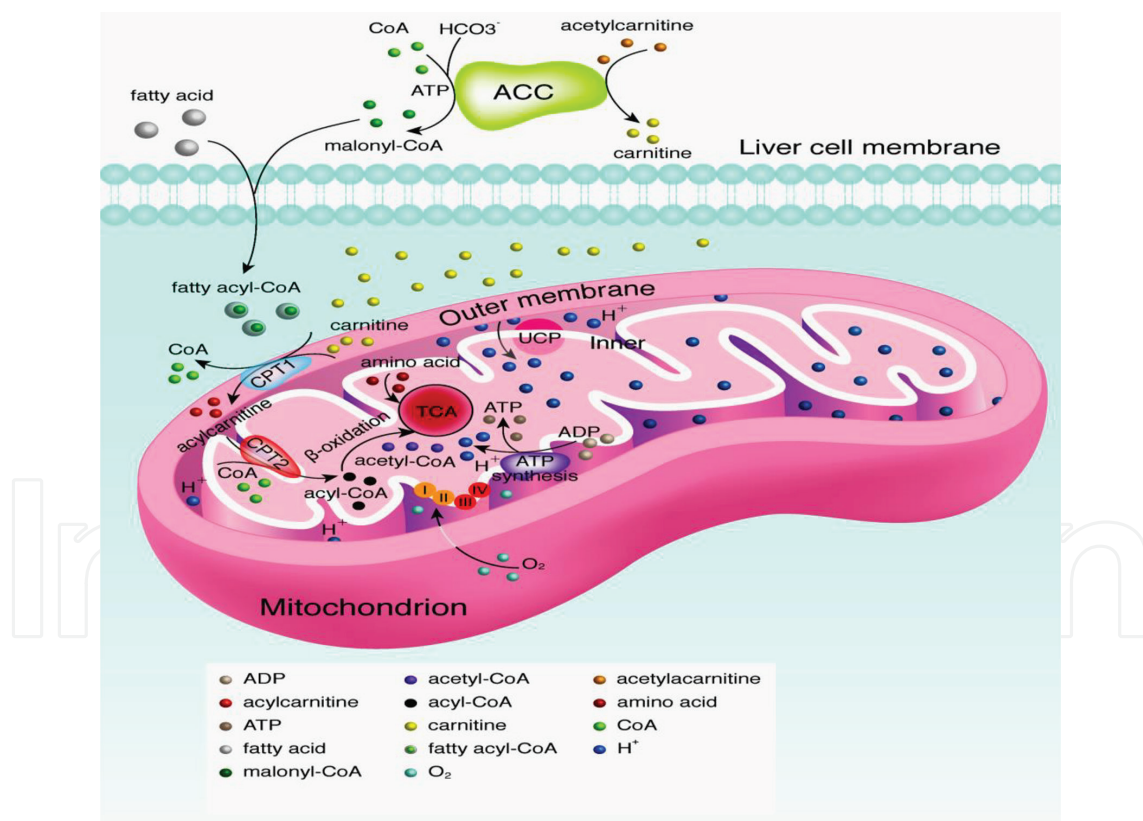
The development of HCC is a complex multi-step process involved multiple genes. Major risk factors of HCC include hepatitis B virus (HBV) or hepatitis C virus (HCV) infection, alcoholic or non-alcoholic fatty liver disease, nitrosamines, aflatoxin, and other harmful substances [9–12]. Chronic persistent infection of nonalcoholic is still the main pathological factor of inducing cirrhosis and HCC. However, with the changes of people's dietary structure and lifestyle, the incidence of fatty liver disease (FLD) also rose sharply [13–15]. A median prevalence of alcoholic fatty liver disease (AFLD) and nonalcoholic fatty liver disease (NAFLD) is 4.5 and 15.0%, respectively [16, 17]. It is worrying that if no interference is conducted in the treatment, nonalcoholic steatohepatitis (NASH) or alcoholic hepatitis can also be developed for liver fibrosis, cirrhosis and liver cancer (**Figure 1**), and its exact mechanism is worth exploring. However, the underlying molecular mechanisms that lead to malignant transformation of infected liver cells still remain to be explored. Most of HCC patients died quickly because of the rapid tumor growth, and surgical operation or liver transplantation still is the only effective treatment for HCC [18, 19]. This article summarizes new advances on relationship between NAFLD and hepatocytes malignant transformation.

## 2. Mitochondria and fatty $\beta$ -oxidation

### 2.1. Mitochondria

Liver is one of the most important organs in human for maintaining energy supply and lipid metabolism [20, 21]. The peroxisomal compartment in hepatocytes hosts several essential

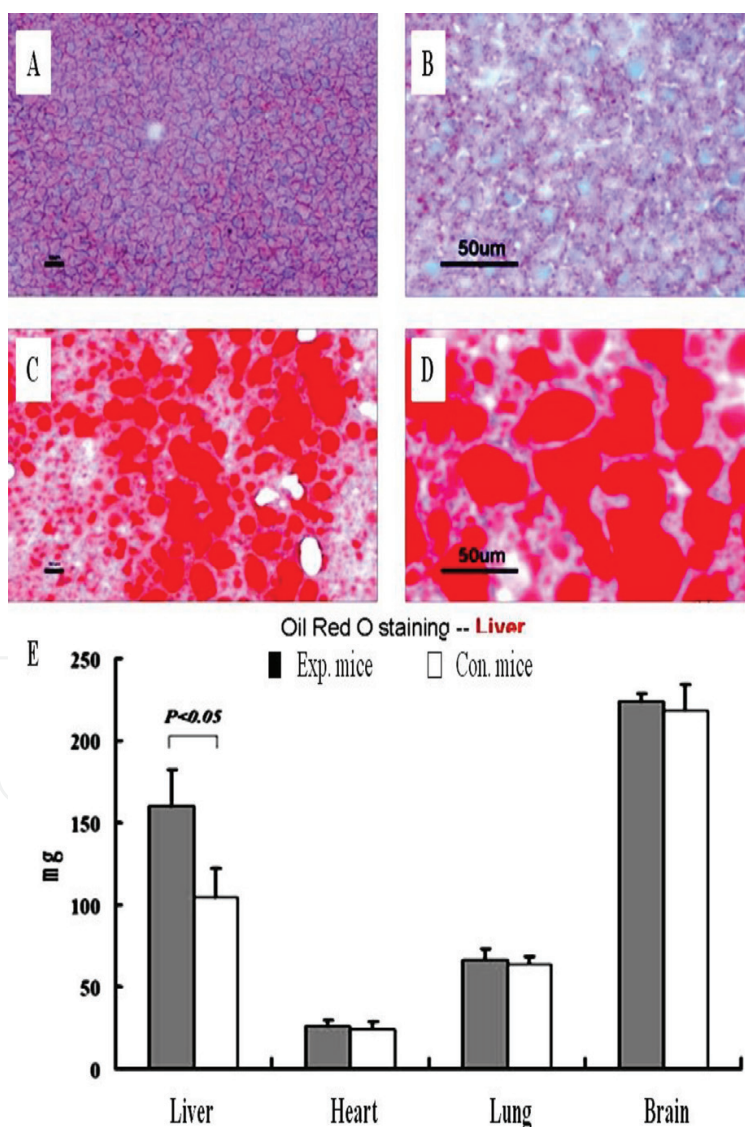
metabolic conversions. Upon nutrient deprivation, cells metabolize fatty acids (FAs) in mitochondria to supply energy. FAs mobilization depends on triacylglycerol lipolysis, whereas autophagy feeds the lipid droplet pool for continued fueling of mitochondria. Proteome imbalance of mitochondrial electron transport chain in brown adipocytes leads to metabolic benefits [22]. Lipid metabolism are defective in peroxisomal disorders that are either caused by failure to import the enzymes such as carnitine palmitoyltransferase (CPT) in the organelle or by mutations in the enzymes or in transporters needed to transfer the substrates across the peroxisomal membrane (**Figure 2**). Hepatocytes specific differences have been confirmed in mitochondrial DNA maintenance and expression [23]. Hepatic pathology is one of the cardinal features in disorders of peroxisome biogenesis and peroxisomal  $\beta$ -oxidation, although it rarely determines the clinical fate. Besides of the morphological changes, the impact of peroxisome malfunctions on other cellular compartments includes thermal instability of carnitine palmitoyltransferase II (CPT-II) variants in mitochondria and endoplasmic reticulum (ER) [24–26]. Proteomics analysis revealed numerous enzymes expression involved with the electron transport system, the tricarboxylic acid cycle, as well as lipid and amino acid metabolism in response to anoxia exposure [27].



**Figure 2.** Fatty acid oxidation and ATP production in mitochondria. The distribution of mitochondrial CPT-I or CPT-II with regulation plays important roles in fatty acid metabolism. Fatty acid (FA)  $\beta$ -oxidation requires successive carnitine acyltransferases to translocate acyl-coenzyme As (acyl-CoAs) from the cytoplasm into matrix. As initial and rate-limiting CPT-I generates acylcarnitines that traverse mitochondrial membranes via specific transporters into matrix, CPT-II produces acyl-CoAs from acylcarnitines for FA  $\beta$ -oxidation to acetyl-CoA. Then carnitine crosses the inner membrane, binds with the endogenous or exogenous acyl CoA to prevent acyl CoA accumulation causing poisoning. ACC: acetyl CoA carboxylase, CoA: coenzyme A, TCA: tricarboxylic acid cycle, UCP: uncoupling protein, I II III IV: electron transfer complex [29].

## 2.2. Carnitine level

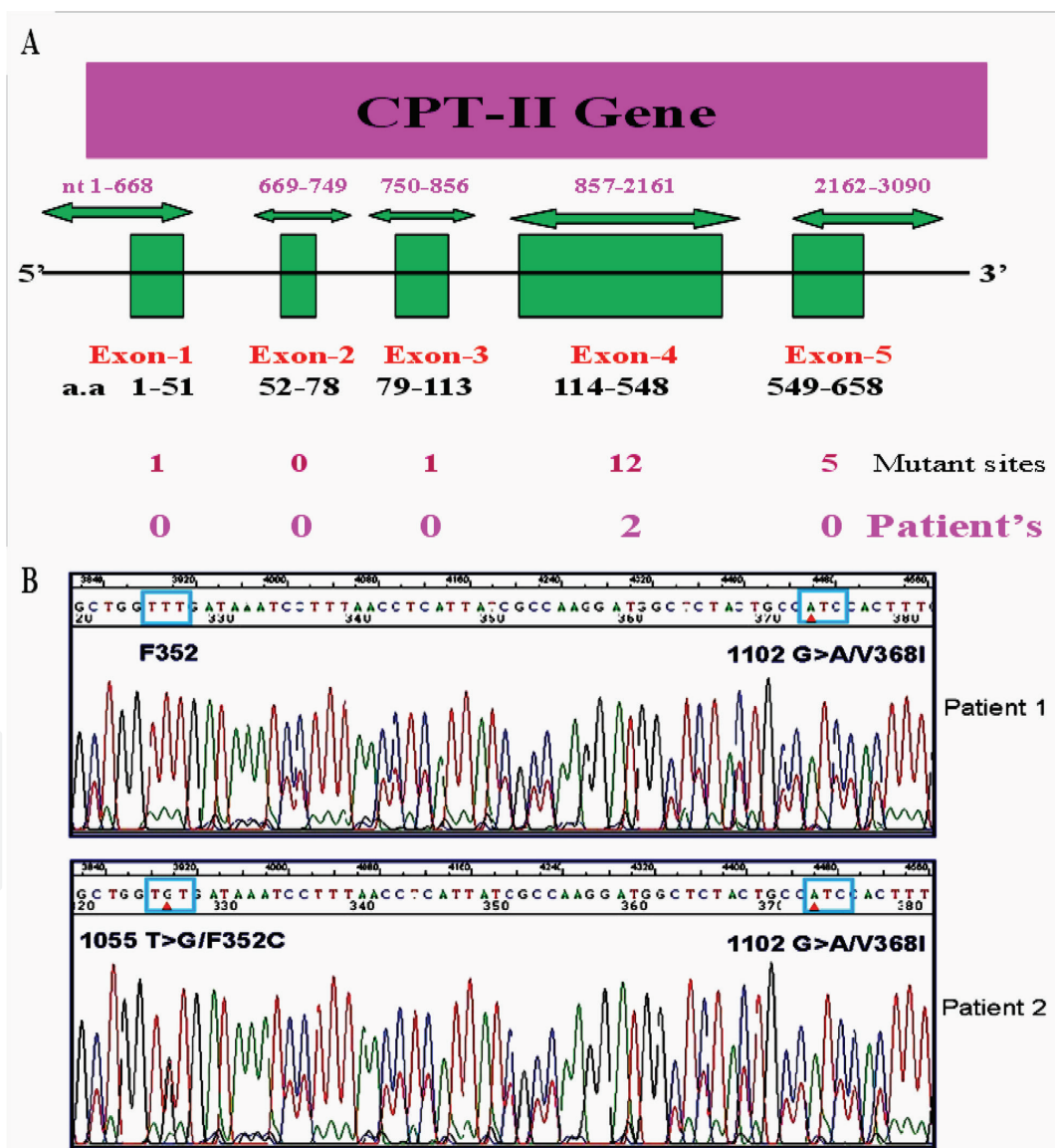
Carnitine is a physiological substance that is essential for the proper metabolism of fat and energy production that actually transports both long and medium fatty acid chains. L-carnitine attracts long and medium fatty acid chains, breaks them down, and carries them to the mitochondria of the cells where they are metabolized (burned). The L-carnitine plays important roles in the catabolism of long-chain fatty acids in the mitochondria, not only due to increased mitochondrial fatty acid oxidation reflected by increased mitochondrial biogenesis, but also to changes in plasma clearance and reduced triacylglycerol (TAG) biosynthesis [28]. The ultimate result is that you burn more fats, and in the process give your body more natural energy. In the previous study, the increasing liver weight with lipid accumulation was discovered during the course of the wild-type mice (**Figure 3**) in circulating carnitine analogues [3-(2,2,2-trimethyl hydrazinium) propionate dihydrate, THP] [29, 30].



**Figure 3.** Liver lipid accumulation and liver weight tissues in mice models after carnitine analogues. A–D, the mice liver tissues with Oil red O staining: A & B, the control livers; C & D, the experimental livers; E, the alterations of different tissue weight after the experimental mice with carnitine analogues [29].

### 2.3. Carnitine palmitoyltransferase (CPT)

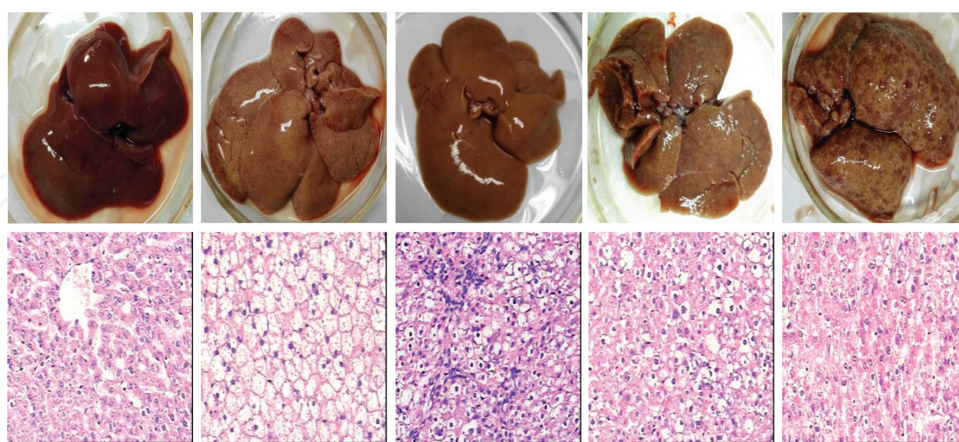
Hepatic CPT-II is a mitochondrial protein which is transported to mitochondrial inner membrane. It together with CPT-I oxidizes long-chain fatty acids in mitochondria. Defects or mutation of this gene are associated with mitochondrial long-chain fatty-acid oxidation disorders. Decreasing of its activity is a disorder of mitochondrial fatty acid oxidation with autosomal recessive mode of inheritance. The variants exert a dominant-negative effect on the homotetrameric protein of the enzyme (**Figure 4**), with reduced activities, thermal instability, fatty acid  $\beta$ -oxidation decreased to 30–59%, intracellular ATP to 48–79%, a significantly decreasing of mitochondrial membrane potential with increasing temperature at



**Figure 4.** Mutation of CPT-II gene and hepatic lipid accumulation. (A) The CPT-II gene exon 1–5. (B) The sequence fragments of CPT-II gene exon 4 were amplified on mitochondrial inner membrane. The mutation analysis of CPT-II gene exon-4 using the specific primers were designed by sequencing with 1974 nucleotides coded 658 amino acids. Compared with the original sequence from Genbank, the two substitution sites were found at 1618 (G→A) and 1858 (T→C), and code amino acids at V368I and F448L, respectively [29].

41°C, and shortening half-lives of CPT-II, and the enzyme variant proteins were polyubiquitinated and rapidly degraded by a lactacystin-sensitive proteasome pathway [24]. The very unstable CPT II variants with decreased enzymatic activities may bring mitochondrial fuel utilization below the phenotypic threshold during high fever in humans with hepatitis virus infection, and thus might be as novel potential mechanisms for NAFLD formation [31, 32].

The dynamic alterations of hepatic CPT-II expression in the mitochondrial inner membrane were investigated during the malignant transformation of hepatocytes induced by abnormal fatty accumulation. After the male Sprague-Dawley (SD) rats were fed with control, high fat (HF), and HF containing 2-fluorenylaceta-mide (2-FAA) diet, respectively. The rats were divided into control, fatty liver, degeneration, pre-cancerous, and cancerous groups according to the hematoxylin and eosin staining (H&E) of liver pathological examination, hepatic lipids accumulation were confirmed with the Oil Red O staining. Massive lipid accumulation hepatocytes were seen in rats on HF and HF containing 2-FAA diets. The lipid levels in the control group were significantly lower than those in the fatty liver, degeneration, precancerous, and cancerous groups. The serum triglyceride and total cholesterol levels in the degeneration, precancerous, and cancerous groups were 2–3 times higher than those in the control group. The serum aspartate aminotransferase and alanine aminotransferase levels (**Figure 5**) in the degeneration, precancerous, and cancerous groups were significantly higher (4–8 times) than those in the control group. The specific concentration ( $\mu\text{g}/\text{mg}$  protein) of liver CPT-II expression was significantly reduced during hepatocyte malignant transformation, as confirmed by immunohistochemistry, with the CPT-II levels significantly lower in the cancerous group than in any of other groups, indicated that low hepatic CPT-II expression might lead to abnormal lipid accumulation in hepatocytes, which should promote the malignant transformation of hepatocytes [33, 34].



**Figure 5.** Rat liver tissues and their pathological examination. Liver alterations after the rats (from left to right: upper, A, B, C, D, and E; under A1, B2, C3, D4, and E5) were sacrificed at different time according to the plan schoule. (A) A representative liver from rat with normal diet; (B) a representative liver from the rat with high-fat diet (HFD) without 2-fluorenyl acetamide (2-FAA); (C) a representative liver from the rats with HFD containing 2-FAA at early stage; (D) a representative liver at interim stage; and (E) a representative liver at later stage. The liver sections were examined with hematoxylin and eosin staining and then divided into the control (A1), fatty liver (B1), degeneration (C1), precancerous (D1), and cancerous (E1) groups; A1-E1: The original magnification of the corresponding rat liver sections was  $\times 200$  [29].

### 3. Abnormal liver lipid accumulation

#### 3.1. Lipid accumulation

Lipid accumulation in liver or HCC will cause tumor-associated molecular signaling alteration including NF- $\kappa$ B (nuclear factor-kappa B), JNK (c-Jun N-terminal kinase)/activation protein-1 activation, and alterations of HCC development-related genes, respectively. For example, liver unsaturated fatty acids (UFA) inhibit the expression of phosphatase and tensin homolog (PTEN) deleted on chromosome 10 (10q23.3) via activating NF- $\kappa$ B/mTOR (mammalian target of rapamycin) complex [35]. As a tumor suppressor gene PTEN regulates the PKB/akt (serine-threonine kinase protein kinase B) pathway, and PTEN deficiency induces the proliferation of hepatocytes by inhibiting cell apoptosis and promoting HCC formation confirmed in mice models with the PTEN deficiency in resembling non-alcoholic steatohepatitis (NASH) features with developing steatosis, and inflammation damages or fibrosis in liver tissues [36].

DNA injury affects hepatic lipid metabolism. Reactive oxygen species (ROS) is an important factor in carcinogenesis. It can be induced in NAFLD patients with contiguous DNA damage by some of hepatic inflammatory cytokines or hepatitis virus infection and react with polyunsaturated fatty acids derived from hepatocyte membrane phospholipids, and subsequently results in reactive aldehydes production as lipid oxidation (LPO) byproducts, for example, 4-hydroxynonenal (4 HNE) that can react with DNA to form mutagenic exocyclic etheno-DNA adducts. Importantly, they are preferably formed in codon 249 of TP53, resulting in inactivation of tumor suppressor p53 gene, secondary growth advantage, and anti-apoptosis [37].

#### 3.2. Adipokines

Adipokine is a plethora of pro- and anti-inflammatory cytokines that secretes from adipose tissue with low-grade inflammation. Adiponectin and leptin have evolved as crucial signals in many obesity-related pathologies including (NAFLD) [38–40]. Adiponectin regulates the metabolism of blood glucose and hepatic fatty acid, and is decreased in NAFLD that might be critically involved in the pro-inflammatory state associated with obesity and related disorders, overproduction of leptin, a rather pro-inflammatory mediator, is considered of equal relevance [41, 42]. An imbalanced adipokine profile in obesity consecutively contributes to metabolic inflammation in NAFLD, which is also associated with a substantial risk for developing HCC in the non-cirrhotic stage of disease [43, 44]. Both related to liver tumorigenesis especially in preclinical models, especially in hepatic satellite cell activation with stimulating the tissue inhibitor of metalloproteinase 1 production via the JAK/STAT (Janus kinase/signal transducer and activator of transcription) pathway promoting fibrogenesis [45, 46], or angiogenesis or progression from NASH to HCC that has been confirmed in mice models [47, 48].

According to the data from animal models with HCC cell lines, adiponectin could increase JNK activation and induce cell apoptosis with AMPK alteration, which could inhibit mTOR phosphorylation, xenograft growth, tumor growth and metastasis by suppression of tumor angiogenesis in nude mice. However, lower circulating adiponectin favors tumorigenesis in NASH model [49]. Adipose-derived tumor necrosis factor is a potent activator of pro-oncogenic



pathways involving in mTOR, JNK, NF- $\kappa$ B, and extra-cellular signal-regulated kinases; Interleukin-6 (IL-6) combining with its receptors on liver or non-parenchymal cells can promote signal-transmuting receptor (gp130) complex with IL-6R activating JAK1 signaling, and STAT3 activation or phosphorylation promotes the proliferation and anti-apoptosis of cancer cells [50], indicated that adiponectin coefficient action from adipose tissues and related- cytokines affect fatty acid metabolism and hepatocyte malignant transformation via many signal molecules.

## 4. Inducing roles of related proteins

### 4.1. Sterol regulatory element-binding proteins (SREBPs)

Lipid reprogramming has been considered as a crucial characteristic in HCC initiation and progression. SREBPs are the key transcription regulators of hepatic lipogenesis, and activate hepatic steatosis at the early stage. Tat-interacting protein 30 (TIP30) is a tumor suppressor protein that has been found to be expressed in a wide variety of tumor tissues that is involved in the control of cell apoptosis, growth, metastasis, angiogenesis, DNA repair, and tumor cell metabolism. TIP30 regulates lipid metabolism in human HCC by regulating SREBP1 (sterol regulatory element-binding protein 1) through the Akt/mTOR signaling pathway [51, 52]. In human HCC tissues, SREBP1 could significantly induce lipogenesis and be associated with a poor prognosis [53].

SREBP1c gene at mRNA level was up-regulated in human HCC tissues and not in their adjacent non-cancerous or non-cancerous liver tissues. The inhibition of SREBP1 expression resulted in growth arrest and apoptosis of cancerous cells, and increased the cell proliferation ability. HBx (HBV protein X) expression induces lipid accumulation in hepatic cells mediated by the induction of SREBP1, a key regulator of lipogenic genes in the liver. HBx interacts with LXRA (liver X receptor alpha) and enhances the binding of LXRA to LXRE (LXR-response element), thereby resulting in the up-regulation of SREBP1 and fatty acid synthase, suggested that HBV infection can stimulate the SREBP1-mediated control of lipid accumulation [54].

### 4.2. Loss of tripartite motif 24 (TRIM24)

Aberrantly high expression of TRIM24 occurs in human HCC clinical samples and positively correlated with HCC tumor grade. Its knockdown inhibits proliferation and migration in HCC cells *in vitro*, with impeding of tumor growth *in vivo* [55, 56]. TRIM24 in mice is reportedly a liver-specific tumor suppressor, and appears to promote liver tumor development via AMPK signaling [57]. TRIM24 as an epigenetic co-regulator of some gene transcription that directly or indirectly inhibits mouse hepatic lipid accumulation, liver cell inflammation, liver fibrosis, and hepatocyte damage. Additionally, the global expression analyses of TRIM24<sup>-/-</sup> livers unveiled signaling pathways that closely associated with some features of NAFLD, inflammatory, cell apoptosis, and hepatocyte damage. The loss of liver TRIM24 expression could lead to the progression from patients with NAFLD to NASH or HCC in a time dependent manner [58].

### 4.3. Osteopontin (OPN)

According to accumulating data, human liver OPN is a multifunctional protein involved in some pathological alterations including hepatic immunity, hepatocytes inflammation, liver

fibrosis, and the development of HCC. Deficiency of OPN in obese mice fed with a high-fat diet reduced hepatic steatosis and inflammation, and liver cell ballooning, portal leukocyte infiltration and macrophage accumulation were attenuated. It is induced by Hedgehog signaling, may directly promote pro-fibrogenic responses in steatohepatitis, or act as a paracrine factor secreted by bile duct or natural killer T cells (NKT), and also can be as an autocrine factor promoting fibrosis in hepatic satellite cells (HSC) [59].

The silencing OPN gene transcription by specific shRNA could result in increasing Bax, decreasing Bcl-2/Bcl-xL and X-linked inhibitor of apoptosis protein expression, and NF- $\kappa$ B activation, and induction of mitochondria-mediated apoptosis in HCCLM3 cells [19]. There were statistically significant differences in plasma OPN levels between the HCC group and the other groups. Regarding the validity of plasma OPN was a predictor of fatty change, with 50% diagnostic accuracy, 70% sensitivity, 45% specificity, 50% positive predictive value, and 75% negative predictive value at a cutoff value of 134 ng/mL. The data indicated that plasma OPN level could be of diagnostic potential value in NAFLD [60].

## 5. Promoting role of related cells

### 5.1. Hepatic satellite cells (HSC)

Human hepatic satellite cells (hHSC) in the perisinusoidal space between sinusoids and hepatocytes are the predominant fibrogenic cells in liver tissues, and activated by liver cell injury to transdifferentiate from a quiescent state to proliferate matrix producing myofibroblasts [61–63]. The excessive production of extra-cellular matrix might result in cirrhosis occurrence. Human amphiregulin could increase the cell proliferation via EGFR, PI3K, and p38 mitogenic signaling pathways, inducing significantly up-regulation of fibrogenic biomarkers, confirmed by the mice NASH model that exhibited rapid progression of advanced fibrosis and HCC, with mimics histological, immunological and transcriptomic features of NASH, and a useful tool for preclinical drug testing [64, 65]. In addition, fatty liver as a pro-metastasis microenvironment with hHSCs could promote HCC migration and proliferation. Fasting and specific microRNAs could inhibit hHSC activation or potentiate anti-cancer activity of Sorafenib in HCC [66, 67].

### 5.2. Immune cells

Activated immune cells interact with cells in tissues by metabolic stress will migrate to liver and drive the progression from NAFLD to HCC. The dysregulation of lipid metabolism in NAFLD from mice models or human samples causes a selective loss of intrahepatic CD<sub>4</sub><sup>+</sup> but not CD<sub>8</sub><sup>+</sup> T lymphocytes, leading to accelerated hepatocarcinogenesis via cross-talk with liver cells [68, 69]. The NKT cells primarily cause steatosis in liver tissues via secreted a type II trans-membrane protein (a TNF ligand super-family member, TNFSF14), and both of CD<sub>8</sub><sup>+</sup> and NKT cells cooperatively induce liver injury by feeding choline-deficient high-fat diet [70]. CD<sub>4</sub><sup>+</sup> T lymphocytes have greater mitochondrial mass than CD<sub>8</sub><sup>+</sup> T lymphocytes and generate higher levels of mitochondrially derived reactive oxygen species (ROS) [71].

Disruption of mitochondrial function by linoleic acid, a fatty acid accumulated in NAFLD, causes more oxidative damage than other free fatty acids such as palmitic acid, and mediates

selective loss of intrahepatic CD<sub>4</sub><sup>+</sup> T lymphocytes. Hepatic immune cells recognize cell injury or pathogen invasion with intracellular or surface-expressed pattern recognition receptors, subsequently initiating signaling cascades that trigger the release of factors promoting inflammatory response during NAFLD progression, demonstrating that the transition from NASH to HCC through liver cell lymphotoxin- $\beta$  receptor (LT $\beta$ R) and NF- $\kappa$ B signaling. *In vivo* blockade of ROS reversed NAFLD-induced hepatic CD<sub>4</sub><sup>+</sup> T lymphocyte decrease and delayed NAFLD-promoted HCC [72, 73].

### 5.3. Polyploidization

Polyploidization is one of the most dramatic genomic changes with rarely reported. The physiological events occur in liver development or adult life. However, the pathological polyploidization takes place in NAFLD, a widespread metabolic disorder that maybe is a risk factor for HCC. The liver parenchyma in NAFLD models displayed the process alterations with a large ratio of highly polyploid mononuclear cells, but was not observed in normal liver parenchyma. Biopsies from NASH patients revealed the alterations in hepatocyte ploidy compared with tissue from controls. Hepatocytes from NAFLD mice revealed that progression through the S/G2 phases of the cell cycle was inefficient and associated with activation of a G2/M DNA damage checkpoint, which prevented activation of the cyclin B1/CDK1 complex. The oxidative stress promotes the highly polyploid cells, and antioxidant-treated NAFLD hepatocytes resumed normal cell division and returned to normal state of polyploidy, indicated that oxidative stress promote pathological polyploidization in NAFLD that might contribute to HCC [74].

## 6. Alterations of small molecules

### 6.1. Oxidative stress

NAFLD is characterized by excess lipids in hepatocytes, due to excessive fatty acid influx from adipose tissue, de novo hepatic lipogenesis, in addition to excessive dietary fat and carbohydrate intake [44, 19]. Serious imbalance was found between limited antioxidant defenses and excessive formation of reactive species produced by liver oxidative stress such as ROS or RNS (reactive nitrogen species). Obese persons could increase free fatty acids uptake, stimulates FA oxidation for compensating excessive liver fat storage, and accelerate  $\beta$ -oxidation leads to increased production of ROS that damage mitochondrial membrane and DNA [75, 37].

Chronic lipid overload in hepatocytes induces mitochondrial oxidative stress or hepatocytes damage leading the NAFLD developing into a more severe liver disease condition, NASH, cirrhosis or HCC. Oxidative stress may induce endoplasmic reticulum (ER) dysfunction for liver malignancy. ER plays an important role in NAFLD pathogenesis, and consecutive increasing oxidative stress, inflammation and activation of NF- $\kappa$ B and JNK signaling pathways lead to the accumulation of intracellular lipids [76]. Extra-cellular signal-regulated protein kinase (ERK) is highly expressed in HCC via PIK13 activation. Among others, copper is one of the main bio-metals required for the preponderance of the enzymes involved in physiological redox reactions, which primarily occurs during mitochondrial respiration. Antioxidant food

agents recognized to improve NAFLD and its complications have been described in the copper-related literatures [77, 78].

## 6.2. Insulin resistance

NAFLD is associated with insulin resistance (IR) leading to a resistance in the antilipolytic effect of insulin in adipose tissue with an increase of FFAs. The increase of FFAs induces mitochondrial dysfunction and lipotoxicity [79]. Liver steatosis defined as lipid accumulation in hepatocytes is very frequently found in adults and obese adolescents. Etiologically, obesity and IR or excess alcohol intake are the most frequent causes of liver steatosis. Insulin as a key hormone regulates lipogenesis and lipolysis in adipose depots. The adipose tissue becomes resistant to the antilipolytic effect of insulin and FA release is increased with lipolysis or lipid intake, promoting triglyceride synthesis with lipid accumulation occurrence in livers [80, 81].

Liver lipid accumulation causes IR by the activation of NF- $\kappa$ B pathway and leads to hyperinsulinemia to activate phosphatidylinositol-3 kinase (PI3K)/Akt signaling pathway, implicated the malignant transformation of hepatocytes or in hepatocarcinogenesis [82]. Hyperinsulinemia up-regulates insulin-like growth factor-1 that stimulates cell proliferation and inhibits cell apoptosis [83]. Insulin activates insulin receptor substrate-1 (IRS-1) with up-regulating expression in HCC [84]. The IRS-1-mediated related-signaling molecules may act as survival factors, promote liver cell proliferation via mitogen-activated protein kinase and PI3K, and protect against transforming growth factor  $\beta$ 1-induced apoptosis in HCC progression [85, 86].

## 6.3. Iron deposition

Liver is the main storage site for iron in the body because of its rich reticuloendothelial system [87]. Acquired hepatic iron overload is seen in a number of NAFLD patients. The dietary iron supplementation enhances experimental steatohepatitis induced by long-term high-fat diet feeding rats [88]. Excess liver iron may increase NASH risk and its progression to HCC [89, 90]. Abnormal iron deposition in liver is more frequent in NASH patients, in which necroinflammation may be the driving factor. Iron and the coexistence of hyperinsulinemia are risk factors for NASH development and together they may contribute to insulin resistance, disease progression and HCC. Iron reduction has been proposed as treatment for dysmetabolic iron overload syndrome and NAFLD or iron deprivation can suppress HCC growth *in vivo* and *in vitro* experiments [91].

## 6.4. Alcohol

While tobacco and alcohol are established risk factors for HCC, the most common type of primary liver cancer [92]. Chronic alcohol intake results in the induction of liver cytochrome P<sub>450</sub> 2E1 leads to generation of ROS with direct or indirect carcinogenic consequences [93]. Many genetic factors regulating alcohol metabolism could predispose in developing alcoholic pancreatitis or cirrhosis. Some studies revealed that alcohol could be metabolized by oxidative and non-oxidative. The main oxidative pathway includes alcohol dehydrogenase, aldehyde dehydrogenase, and cytochrome P<sub>450</sub> 2E1. In addition, neurocan in neuronal tissue is also expressed in liver and the common polymorphism of its gene rs2228603 is associated with HCC in alcoholic liver disease [94].

### 6.5. MicroRNA (miR)

Regulating control miRs are highly conserved, small non-coding RNAs (about 18–25 nucleotides in length) regulates transcription or translation of target genes and fatty acid metabolism. Both of miR-197 and miR-99 were associated with liver fibrosis in NASH patients. Altered miRNA expression was associated with activation of major hepatocarcinogenesis-related pathways, including the TGF- $\beta$ , Wnt/ $\beta$ -catenin, ERK1/2, mTOR, and EGF signaling. The over-expression of the miR-221-3p and miR-222-3p and oncogenic miR-106b~25 cluster was accompanied by the reduced protein levels of their targets, including E2F transcription factor 1, phosphatase and tensin homolog, and cyclin-dependent kinase inhibitor 1. miR-93-5p, miR-221-3p, and miR-222-3p have been confirmed over-expressed in HCC. Aberrant expression of miRNAs may have mechanistic significance in NASH-associated liver carcinogenesis and may serve as an indicator for the development of NASH-derived HCC [94, 95].

Some studies found that miR-122 is a key regulator of glucose and lipid metabolism in livers [96] and significantly higher circulating miR-122, miR-34a, and miR-16 expression were found in NAFLD. During the development of NAFLD patients with simple steatosis to steatohepatitis, the serological levels of miR-122 and miR-34a were positively correlated with disease severity, liver enzyme activities, fibrosis staging, active inflammation, and silencing of microRNA-122 is an early event during hepatocarcinogenesis from NASH [97], suggesting that the alteration of circulating miR-122 could be an early event from NASH to hepatocarcinogenesis.

## 7. Genetic factors

The development and progression of NAFLD are determined by environmental and genetic factors [10, 98]. The effect of genetic factors has been demonstrated by familial studies, twin studies and several cross-sectional studies. The data from the genome-wide association studies (GWAS) have shown that patatin-like phospholipase domain-containing protein 3 (PNPLA3) involved in metabolism of triglyceride on chromosome 22 is a genetic factor that promotes NASH development, and PNPLA3 gene variant I148M showed a strong relationship with the development and progression of NAFLD, NASH, and NAFLD-related HCC. Single nucleotide polymorphism (SNP, rs738409) is closely related to fatty liver involved in fibrosis progression of NAFLD. The C<G variation in SNP rs738409 also increases HCC risk in NAFLD patients [99, 100].

The whole exome sequencing finds that apolipoprotein B mutations (c.6718A>T, K2240X) represent a paradigm of rare variant influencing liver fat content and HCC risk. Besides, the Patatin-like phospholipase domain-containing 3 [the trans-membrane 6 superfamily member 2 (TM6SF2)] genes variant E167K was associated with NAFLD [101, 102]. Telomerase reverse transcriptase (TERT) mutations have been associated with hepatic steatosis. The deficiency of TERT can reduce the response to liver damage inducing the formation of steatosis and fibrosis. In conclusion, the occurrence of NAFLD-HCC seems to be influenced by common genetic variants as PNPLA3 and by rare genetic variants. Several genes have been proposed as candidate genes to be associated with NAFLD based on case–control studies [103].

## 8. Microbiota and toxic substances

### 8.1. Gut microorganisms

NAFLD has become the most common chronic liver disease worldwide and is well-accepted that gut dysbiosis is associated with NAFLD [103]. The gut-liver axis has been proposed as a key player in the pathogenesis of NAFLD, as the passage of bacteria-derived products into the portal circulation could lead to a trigger of innate immunity, which in turn leads to liver inflammation. In intestine, there are trillions of microorganisms including bacteria, archaea, yeasts and viruses collectively called intestinal ecosystem through energy harvesting and fat storage [78, 104]. The relationship between gut microbiota and NAFLD is dependent on levels of choline, bile acid, larger production of endogenous ethanol, higher prevalence of intestinal dysbiosis, higher prevalence of increased intestinal permeability, bacterial translocation, pro-inflammatory molecules, endotoxemia, and cytokines. The hepatic manifestation of the dysregulation of insulin-dependent pathways leads to IR and adipose tissue accumulation in NAFLD patients with liver injury, indicated that the gut liver-axis is the way by which the bacteria and their potential hepatotoxic products (LPS, DNA, RNA, etc.) can easily reach liver [105, 106].

The interaction between the gut epithelia and some commensal bacteria induces the rapid generation of ROS. The main goal of any therapy addressing NASH is to reverse or prevent progression to liver fibrosis/cirrhosis [78]. Recently, a new isoform of human manganese superoxide dismutase (MnSOD) has been shown to be a powerful antioxidant capable of mediating ROS dismutation, penetrating biological barriers via its uncleaved leader peptide, and reducing portal hypertension and fibrosis in rats affected by cirrhosis [107]. Primary bile acids which derived from cholesterol become secondary bile acids under the action of intestinal microbes. If the bile acids bind to G-protein-coupled cell surface receptor (TGR5), it could inhibit inflammation via suppressing NF- $\kappa$ B pathway in macrophages. Many genetic and environmental factors have been suggested to contribute to the development of obesity and NAFLD, but the exact mechanisms might be the issue of further investigations [108, 109].

### 8.2. Toxic substances

NAFLD has been implicated in some conditions such as IR, obesity, metabolic syndrome, hyperlipemia, hypertension, cardiovascular disease, and diabetes. Dietary or genetic obesity induces alterations of gut microbiota, thereby increasing the levels of deoxycholic acid, a gut bacterial metabolite known to cause DNA damage [78, 110]. Glyceraldehyde-derived advanced glycation end-products (Glycer-AGEs) are the predominant components of toxic AGEs (TAGE). More data suggested that TAGE with its receptor might change intracellular signaling, pro-inflammatory molecules gene expression, and also elicited the oxidative stress generation in liver cells including hHSCs. Circulating TAGE levels were significantly higher in NASH patients than those with simple steatosis or healthy subjects. Moreover, their TAGE levels inversely correlated with adiponectin. Increased lipid availability in livers might provide ATP and structural support for cancerous cell proliferation [111, 112].

## 9. NAFLD in hepatocarcinogenesis

Recent epidemiological studies have identified NASH, a progressive form of NAFLD, as a major risk factor for HCC. Elucidating the underlying mechanisms associated with the development of NASH-derived HCC is critical for identifying early biomarkers for the progression of the disease and for treatment and prevention [97, 113].

Liver derangements in lipid metabolism, importing FFA and manufacturing, storing, and exporting lipids could lead to NAFLD development [114]. The dysregulation of hormonal axes, mitochondrial carnitine palmitoyltransferase-II inactivity, and cytokines in NAFLD promotes a worse cycle between metabolic and inflammatory stimulus lead to malignant transformation of hepatocytes [33, 71]. The majority of NAFLD patients had steatosis about 20% present as NASH that was defined by microscopic finding, and consists of liver injury, steatosis, parenchymal and portal inflammation, and different fibrosis. Alterations of miRNA in hepatocarcinogenesis were associated with TGF- $\beta$ , Wnt/ $\beta$ -catenin, ERK1/2, mTOR, and EGF signaling pathways. Importantly, miR-93-5p, miR-221-3p, and miR-222-3p were also significantly over-expressed in human HCC. Aberrant expression of miRNAs might have mechanistic significance in NASH-associated liver carcinogenesis and serve as an indicator for the development of NASH-derived HCC [115, 116].

Hepatic lipid accumulation is accompanied by distinct patterns of perilipin expression, suggested that abnormality of hepatic lipid accumulation might promote hepatocyte malignant transformation [33]. The levels of high leptin and low adiponectin are hallmarks of obesity and involved in NAFLD and carcinogenesis [117]. Obesity-promoted HCC occurrence was dependent on increasing IL-6 and TNF levels, which resulted in liver inflammation and oncogenic STAT3 activation. The long-term chronic inflammatory in obesity plus higher IL-6 and TNF might be the risk factor for HCC [118]. The prospective studies (25,337 patients with HCC) demonstrated that both of excess body weight and obesity in males or females are related to an increased risk factor for HCC occurrence [119]. The prospective studies with longer follow-up periods should screen the malignant transformation of hepatocytes with specific biomarkers among NASH or NAFLD populations [3, 120].

## 10. Perspectives

In the past decade, the discussion of substantially NAFLD increased by hypernutrition and HCC had become a cocktail party cliché, and its impact on public health cannot be dismissed. With both relationship gradually deepening, more and more evidences have supported that NAFLD might promote the malignant transformation of hepatocytes because of liver lipid accumulation, its toxicity, endoplasmic reticulum dysfunction, IR, and abnormal fat metabolism. Although the exact mechanisms from NAFLD tumor-promoting mechanism triggered by hypernutrition remain to be explored [121], however, the patients with the excessive fat deposition feeds this tumor-promoting inflammatory flame and should be treated in time to avoid the occurrences of hepatocyte malignant transformation [7, 122].

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## Abbreviations

CPT	carnitine palmitoyltransferase
HBV	hepatitis B virus
HCV	hepatitis C virus
HSC	hepatic satellite cell
IL-6	interleukin-6
NAFLD	non-alcoholic fatty liver disease
NASH	nonalcoholic fatty hepatitis
NF- $\kappa$ B	nuclear factor kappa B
MiR	microRNA
OPN	osteopontin
HCC	hepatocellular carcinoma
PNPLA	patatin-like phospholipase domain-containing protein
ROS	reactive oxygen species
SREBP	sterol regulatory element-binding protein
TAGE	toxic advanced glycation end-products

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