Recent insights into the pathogenesis and therapeutic targets of chronic liver diseases

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INTRODUCTION

Fatty liver diseases, including non-alcoholic fatty liver disease (NAFLD) and alcoholic-associated liver disease (ALD), have become major health concerns worldwide, affecting millions of people.1 2 NAFLD is the most common liver disease in developed countries and is associated with obesity, insulin resistance and metabolic syndrome. ALD is caused by excessive alcohol consumption and is a leading cause of liver-related mortality. Both NAFLD and ALD can progress to liver fibrosis, cirrhosis and ultimately hepatocellular carcinoma (HCC), a primary liver cancer with a poor prognosis.3 4

Over the past decade, significant progress has been made in understanding the pathogenesis of fatty liver diseases and liver cancer. This progress has led to the identification of novel therapeutic targets for the prevention and treatment of these diseases. In the fifth Chinese American Liver Society/Society of Chinese Bioscientists in America Hepatology Division Annual Symposium, which was held virtually on 21–22 October 2022, focused on the topics related to ALD, NAFLD and liver cancer. Here, we briefly highlight the presentations that focus on the current progress in basic and translational research in ALD, NAFLD and liver cancer. The roles of non-coding RNA, autophagy, extrahepatic signalling, macrophages, etc in liver diseases are deliberated, and the application of single-cell RNA sequencing in the study of liver disease is also discussed.

ABSTRACT

Viral hepatitis, alcohol-associated liver disease (ALD) and non-alcoholic fatty liver disease (NAFLD) are the three major causes of chronic liver diseases, which account for approximately 2 million deaths per year worldwide. The current direct-acting antiviral drugs and vaccinations have effectively reduced and ameliorated viral hepatitis infection, but there are still no effective drug treatments for ALD, NAFLD and liver cancer due to the poor understanding of their pathogenesis. To better understand the pathogenesis, the fifth Chinese American Liver Society/Society of Chinese Bioscientists in America Hepatology Division Annual Symposium, which was held virtually on 21–22 October 2022, focused on the topics related to ALD, NAFLD and liver cancer. Here, we briefly highlight the presentations that focus on the current progress in basic and translational research in ALD, NAFLD and liver cancer. The roles of non-coding RNA, autophagy, extrahepatic signalling, macrophages, etc in liver diseases are deliberated, and the application of single-cell RNA sequencing in the study of liver disease is also discussed.

Alcohol-associated liver disease

Alcohol abuse causes a wide spectrum of liver diseases ranging from steatosis, hepatitis, cirrhosis, to HCC.5 6 In 2019, alcohol accounted for an estimated 25% of cirrhosis deaths6 7 and 19% of liver cancer deaths globally.8 The per-capita alcohol consumption has been increasing from 1990 to 2017 worldwide and is predicted to increase further by 2030.9 Therefore, the burden of ALD might increase in parallel. In the liver, alcohol is primarily oxidised to acetaldehyde by alcohol dehydrogenase (ADH) and the cytochrome P450 enzymes, particularly CYP2E1. The oxidation of alcohol leads to the production of reactive oxygen species, which contributes to liver injury. Excessive acetaldehyde also contributes to tissue injury by reacting with various proteins to form acetaldehyde-protein adducts. Through aldehyde dehydrogenase (ALDH)2 in the mitochondria, acetaldehyde is metabolised to acetate, which is exported into peripheral systems and can be metabolised in other tissues to acetyl-CoA, the substrate for lipid biosynthesis.10 In response to alcohol intoxication, hepatocytes undergo various forms of cell death,11 which triggers the inflammatory cascade involving distinct immune cells.12 Furthermore, alcohol contributes to gut dysbiosis and disruption of gut barrier integrity, subsequently inducing translocation of bacteria and their metabolites into system circulation, leading to liver injury.13 14 Currently, therapies for patients with ALD involve behavioural interventions, reducing inflammation and liver transplantation. Although emerging therapies are
undergoing evaluation, available effective and safe treatments are still very few for patients with ALD. In this symposium, targeting alcohol metabolism, autophagy, and extrahepatic signalling were highlighted as novel potential treatment strategies for ALD.

Targeting alcohol metabolism in ALD

Targeting ALDH2 in ALD has been intensively investigated. Using the ALDH2 deficient mice, a previous study discovered that ALDH2 deficiency resulted in higher levels of acetaldehyde and malondialdehyde-actetdehyde adduct, which ameliorated hepatic steatosis and liver injury in Gao-binge murine model. Given that acetaldehyde inhibited aerobic glycolysis-mediated pathways to interfere with glucose metabolism and subsequent activation in T cells, ALDH2 deficiency could attenuate ethanol-feeding-exacerbated T-cell-mediated hepatitis. However, in mice with ethanol feeding and carbon tetrachloride administration, more inflammation and fibrosis were observed in ALDH2 deficient mice, probably due to increased alcohol-induced gut leakiness. Furthermore, ALDH2 deficiency was associated with an increased risk of HCC development in patients with cirrhosis who consumed excessive alcohol and in mice with liver fibrosis and ethanol feeding. Other studies demonstrated that either activating ALDH2 by Alda-1, which reversed alcohol-reduced hepatic ALDH2 activity by mitochondria-targeted ubiquinone, or delivering ALDH2-incorporated nanocapsules reversed hepatic steatosis and hepatocyte death through accelerating acetaldehyde clearance in mice with ethanol feeding. In the current symposium, Lu et al (H Lu, B Gao, unpublished data, 2022) observed different localisation patterns of ALDH2 in healthy human and murine livers. ALDH2 was localised in pericentral hepatocytes in murine livers, while in human livers, ALDH2 was expressed throughout hepatocytes in all zonations. In patients with severe alcoholic hepatitis (SAH), RNA-sequencing and immunostaining data indicated a substantial decrease in ALDH2 expression, probably due to the loss of hepatocytes. Lu et al (H Lu, B Gao, unpublished data, 2022) also demonstrated that SAH patients with more steatosis expressed ADH1 in periportal cells and hepatocyte nuclei, which was not observed in SAH patients with minimal steatosis, suggesting distinct histopathological phenotypes existed in SAH patients. However, ADH1 deficiency did not significantly affect ALD in mice due to the compensation of ADH3. Characterisation of ethanol metabolising enzymes and further investigation of their roles would be greatly beneficial to identify SAH types in patients and develop personalised therapies for individuals with ALD.

Targeting autophagy in ALD

Autophagy protects against both acute and chronic ethanol-induced hepatotoxicity in mice through lowering hepatic lipid load and removing dysfunctional/damaged mitochondria and accumulated fatty acids. However, autophagy in the liver is differentially modulated by acute and chronic alcohol consumption. Acute alcohol exposure increases the essential ATG (eg, ATG5, ATG7) expressions and autophagic flux through activating FoxO3 in primary murine hepatocytes. The induction of autophagy by ethanol is further confirmed in primary human hepatocytes. Additionally, enhanced hepatic autophagy on acute ethanol exposure is associated with a higher nuclear transcription factor EB (TfEB) level, which positively regulates lysosomal biogenesis. On the contrary, chronic ethanol exposure inhibits the induction of hepatic autophagy with a lower nuclear TFEB level. Although chronic ethanol feeding increases hepatic autophagosome biogenesis in mice, the insufficient lysosomal number and function caused by chronic ethanol administration lead to incomplete autophagy.

The role of autophagy in aged mice with ALD is discussed. Qian et al reported that the deficiency of the autophagy receptor protein SQSTM1/p62 in aged mice developed metabolic syndrome and exacerbated liver injury after chronic plus binge ethanol treatment. Williams et al (SN Williams, X Ma, W Liu, W Ding, unpublished data, 2022) implicated that impaired TFF-mediated autophagy was involved in aging-exacerbated alcohol-induced hepatic inflammation, oxidative stress and steatosis. They suggested that enhancing autophagy could be a potential treatment for ALD in aged patients. One of the critical protective effects of autophagy against ALD is to remove alcohol-induced mitochondrial damage. A study presented by Ma et al showed that alcohol consumption impaired mitochondrial fission via decreased TFF-mediated autophagy in human alcoholic hepatitis and experimental ALD. Liver-specific DNML1 knockout mice had increased accumulation of megamitochondria and decreased mitophagy, which was associated with more severe liver damage. Newly formed megamitochondria may serve as an adaptive response to regenerate hepatic NAD+ for alcohol metabolism, and such adaptive response became maladaptive under prolonged alcohol consumption conditions due to the impaired removal of dysfunctional megamitochondria via mitophagy.

Liu et al (G Liu, X-M Yin, unpublished data, 2022) introduced a new form of autophagy, chaperone-mediated autophagy (CMA), which occurred in the liver with macroautophagy deficiency. The study described that CMA was enhanced in ATG7-deficient mice. NRF2 inhibition could further enhance CMA activity, thereby rescuing liver dysfunction caused by macroautophagy deficiency. The comparison of benefits between macro-autophagy and CMA has not been well investigated. However, manipulating CMA by targeting NRF2 might be an alternative option for patients with ALD with defective macroautophagy.

Currently, therapies for ALD that specifically target autophagy are not available. A previous preclinical study indicated that enhancing autophagy using carbamazepine or rapamycin decreased steatosis and liver injury in mice with chronic ethanol exposure.
Guo, W Zhong, L Hao, H Dong, X Sun, R Yue, Z Zhou, unpublished data, 2022) reported the activation of mTORC1 by free fatty acid (FFA) in the livers of mice with chronic ethanol feeding and patients with SAH. The activation of mTORC1 induces endoplasmic reticulum (ER) stress, reduces LAMP2 protein levels and impairs autophagic flux. The inhibitor of mTORC1 rapamycin restores LAMP2 expression and improves autophagic flux.37 38 However, Song et al (Q Song, Z Song, unpublished data, 2022) demonstrated that the protective effect of mTORC1 inhibition was independent of autophagy, as inhibiting autophagy failed to abrogate mTORC1 inhibition-induced protection. A more recent study showed that alcohol deceased tuberous sclerosis complex 1 (TSC1) resulting in persistent activation of mTORC1 in human alcoholic hepatitis livers and Gao-binge ALD mouse model. Interestingly, loss of cholangiocyte but not hepatocyte TSC1 led to more severe liver damage including ductular reaction and cell death in Gao-binge alcohol-fed mice.39 Although targeting mTORC1 might be a promising therapeutic treatment for ALD, whether and/or how targets specific cell types in the liver needs to be further determined.

Targeting extrahepatic signaling in ALD
Alcohol modulates the gut-liver axis in multiple aspects, including gut microbiome, intestinal barrier, and bacterial products and metabolites. Li et al (F Li, W Feng, unpublished data, 2022) extracted faecal extracellular vesicles (EVs) from patients with alcohol-associated hepatitis (AH) and healthy controls. These faecal EVs were administered to mice fed with ethanol. The data showed that faecal EVs from AH patients, but not from healthy controls, exacerbated hepatic steatosis and injury. This was due to a dramatic decrease in butyrate-producing bacteria and a significant increase in murein-lipoprotein, one of the most abundant membrane proteins in Gram-negative bacteria. Recent metagenomic analyses of the intestinal microbiome of patients with chronic alcohol abuse and mice with ethanol feeding have revealed that alcohol reduces bacteria diversity and phyla shift towards a greater abundance of Proteobacteria (a major phylum of Gram-negative bacteria) and a lower abundance of Bacteroidetes and Firmicutes (include the main butyrate-producing bacteria).40-42 Li et al’s finding (F Li, W Feng, unpublished data, 2022) is consistent with previous studies and provides new evidence supporting that the microbiome might be involved in ALD pathogenesis. It has also been reported that faecal microbiota transplantation (FMT) from alcohol-resistant donor mice restores gut homeostasis and prevents alcohol-induced hepatic steatosis, inflammation and injury in alcohol-sensitive recipients.43 Recently, a phase 1 clinical trial is underway to transplant faecal microbiota from a donor enriched in Lachnospiraceae and Ruminococcaceae (both belonging to Firmicutes) to patients with cirrhosis with alcohol use disorder (AUD). The result of this trial showed that FMT was safe and increased bacterial diversity, the levels of butyrate/isobutyrate and further reduced AUD-related serious adverse events over 6 months.44 This trial demonstrated the feasibility of FMT in the management of ALD. The study by Li et al (F Li, W Feng, unpublished data, 2022) proposed a novel method to manipulate microbiota EVs to potentially treat patients with ALD.

Alcohol-induced gut microbiota alternation also results in the disruption of bile acid signalling. In patients with ALD, intestinal microbiota abnormalities induce an over-representation in intestinal bacteria with chlorelglycine hydrolase and disrupt farnesoid X receptor (FXR) activation in enterocytes, leading to reduced fibroblast growth factor (FGF)15 (FGF19 in mice).45 Enhancing bile acid/FXR/FGF15 signalling has been reported to improve ALD in mice.46 Notably, activation of the FXR pathway by FXR agonist or supplement of exogenous FGF19 in mice stimulates hepatic FGF21 expression and secretion, which could reverse metabolic syndrome.47 48 FGF21 has been reported to be increased in circulation and livers in both humans and mice with alcohol exposure.49-52 FGF21-deficient mice exhibited exacerbated ALD and increased mortality with impaired lipolysis, increased lipogenesis and inflammation.51 52 Recombinant FGF21 suppresses alcohol-induced hepatic steatosis and injury.53 Mechanistically, SIRT1 is involved in FGF21-mediated protection against ALD.52 55 Li et al (G Li, M Zang, unpublished data, 2022) reported a novel mechanism involving serine-arginine protein kinase (SRPK)2-mediated lipo- genic pre-mRNA process, which is inhibited by FGF21 for combating ALD pathology. FGF21 also exerts extrahepatic functions. It has been acknowledged that FGF21 could reduce the preference for alcohol in both mice and non-human primates through a projection-specific subpopulation of KLB-expressing neurons in the basolateral amygdala.54 55 Increased systemic FGF21 during alcohol exposure could also promote adipose lipolysis to reverse metabolic syndrome in ALD.49 In the current symposium, Chen et al56 discovered elevated FGF21 expression in the epididymal white adipose tissue in CRAMP-deficient mice feeding with high-fat diet (HFD) plus alcohol, contributing to the protection from steatosis and injury through the adiponectin-mediated adipose-liver axis.

Non-alcoholic fatty liver disease
The burden of NAFLD has been continually increasing worldwide in past years. The estimated global incidence of NAFLD is 46.9 cases per 1000 persons, and the overall prevalence of NAFLD is estimated to be 32.4%.56-58 During 2016-2030, non-alcoholic steatohepatitis (NASH) prevalence is predicted to increase by 15%-56% in different countries and regions, while advanced liver diseases and mortality will at least double due to the increasing ageing population.50 Currently, NAFLD-related HCC accounts for 10%-38% of the HCC burden in different Western countries and regions, and NAFLD-related HCC will dramatically increase over time by ranging from 47% to 130% by 2030.50 61
NAFLD is a multifactorial and progressive disease that starts with the stage of simple steatosis, characterised by triglyceride accumulation through de novo lipogenesis in hepatocytes. Increased de novo lipogenesis resulting from continuous fructose uptake, saturated fatty acids accumulation or cholesterol accumulation in the ER, etc induce cellular stress (lipotoxicity) in hepatocytes, leading to the activation of hepatic immune cells including macrophages. Fructose also increases intestinal permeability, further enhancing liver inflammation by the gut-liver axis. The cellular stress pathways ultimately contribute to fibrosis development to progress NAFLD to NASH. The exacerbation of this deleterious cycle potentially promotes the transition to cirrhosis and HCC.62–64 Currently, there are numerous ongoing clinical trials targeting lipogenesis, insulin resistance, inflammation, bile acid signalling; however, none of them has been approved to treat NAFLD.65–67 In this symposium, targeting non-coding RNA, bile acid signalling, and macrophages were discussed as novel potential treatments for NAFLD.

**Targeting non-coding RNA in NAFLD**

MicroRNAs (miRNAs) are a class of small non-coding RNAs (approximately 18–22 nucleotides in length), which are highly conserved. They have epigenetic functions and could target other RNAs, especially mRNA, by promoting mRNA degradation or repressing protein translation.68,69 Previous studies have identified a plethora of miRNAs as therapeutic targeting for NAFLD. Among them, miR-34a, miR-122 and miR-192 are the best candidates as biomarkers for NAFLD diagnosis and staging, and may serve as therapeutic targets for NAFLD.68–72 In a meta-analysis study, miR-34a showed the best diagnostic accuracy for discriminating NASH from NAFLD.72 In mice with miR-34a deficiency or overexpression in hepatocytes, or treated with miR-34a inhibitor, miR-34a has been demonstrated to promote NASH induced by a diet high in fat, cholesterol and fructose (HFCF).73 Mechanistically, miR-34a directly targets SIRT1, HNF4α, ATG4B and the small GTPase RAB8B, which are involved in lipid metabolism, cholesterol synthesis, fatty acid oxidation and lipophagy.73–77 In the liver, miR-34a expression is controlled by FXR/SHP/p53 signalling.78 Ahamed et al (F Ahamed, Y Zhang, unpublished data, 2022) reported an increase of miR-34a in SHP-deficient macrophages in vitro and in the livers of myeloid cell-specific SHP knockout mice, which were more susceptible to HFCF-induced NASH progression with exacerbated monocyte infiltration and liver fibrosis but without affecting hepatocyte steatosis. Ahamed et al (F Ahamed, Y Zhang, unpublished data, 2022) also demonstrated that PPARγ was the direct target of miR-34a in macrophages, and its downregulation by miR-34a blocked the anti-inflammatory macrophage differentiation, subsequently leading to aggravated NASH. This study revealed a novel role of miR-34a in macrophages in NASH progression in addition to its prosteatotic role in hepatocytes and provided substantial evidence to support the therapeutic application of miR-34a inhibition in NAFLD.

Long non-coding RNAs (lncRNAs) are RNA transcripts with more than 200 nucleotides. Generally, lncRNA could physically interact with DNA, RNA, and proteins either through nucleotide base pairing or forming structural domains by RNA folding, contributing to regulating gene expression at epigenetic, transcriptional and post-transcriptional levels.80,81 A plethora of abnormal expressions of lncRNAs have been reported and associated with metabolic diseases, including NAFLD.80,81 In this symposium, lncRNAs H19 and Gm19619 were highlighted to be involved in metabolic regulation.

In NASH patients, H19 expression increases in the liver.82 On FFA treatment and HFD feeding, H19 expression is induced in hepatocytes in vitro and in vivo, respectively.83,84 It is reported that miR-130/PPARγ, MLX-interacting protein-like (MLXIPL) and PI3K/mTOR pathways mediate H19-promoted lipogenesis in NAFLD.83,84 Additionally, using murine models of H19 deficiency and overexpression, a previous study has demonstrated that H19 interacted with PTBP1, an RNA-binding protein, to promote its association with SREBP-1c mRNA and protein, contributing to the enhanced nuclear transcripational activity of SREBP-1c, which further augmented lipid accumulation.85 Yang et al (Z Yang, J Ma, N Huda, Y Jiang, K Perez, S Liangpunsakul, unpublished data, 2022) confirmed H19 and PTBP1 interaction and found that H19 inhibited betaine-homocysteine methyltransferase expression by interfering with its mRNA stability through PTBP1, thus promoting hepatic steatosis. All of the above provide substantial evidence that targeting H19-mediated lipogenesis could be a potential therapeutic treatment for NAFLD.

Fang et al86 determined the biological function of Gm19619, which has never been investigated. Hepatic Gm19619 is highly upregulated by diet-induced obesity and overnight fasting in mice but is dramatically repressed by vertical sleeve gastrectomy, which is one of the most common and effective surgeries for sustained weight loss, remission of diabetes, and other comorbidities, including NAFLD. Mechanistically, Gm19619 binds to the promoter region of FOXO1 to activate G6PC/PCK1-dependent gluconeogenesis. Gm19619 also binds to the upstream region of leptin receptor (LEPR) to inhibit its expression and signalling to facilitate lipid accumulation. Knockdown of Gm19619 in HFD-fed mice significantly improves glucose tolerance, insulin resistance and lipid metabolism, suggesting Gm19619 could be a new potential lncRNA target for treating NAFLD.

**Targeting bile acid signalling in NAFLD**

Primary bile acids are synthesised by cholesterol 7α-hydroxylase (CYP7A1) and steroid 27-hydroxylase (CYP27A1). Subsequently, they are secreted into the intestine through the bile ducts after conjugating with taurine and glycine to promote emulsifying dietary lipids. The primary bile acids form secondary bile acids with the aid of intestinal...
microbiota. Most of the bile acids in the intestine return to the liver through the portal vein and the homoeostasis is maintained through the enterohepatic circulation of bile acids and FXR-dependent negative feedback loops.

The disruption of bile acid homoeostasis is associated with NAFLD occurrence and progression. To target disturbed bile acid homoeostasis, Pan et al (X Pan, Y Zhang, unpublished data, 2022) and Eppler et al (N Eppler, Y Zhang, unpublished data, 2022) manipulated hepatocyte KLF10 and HuR, respectively, to treat NASH in mice fed with an HFCF diet. Hepatocyte-specific KLF10 or HuR deficiency alters bile acid content probably through dysregulation of bile acid synthesis and transport, thus contributing to NAFLD progression. Their studies suggested that targeting dysregulated bile acid signalling through augmenting KLF10 and/or HuR in hepatocytes might be an attractive strategy for treating NAFLD. In addition to manipulating hepatic targets, Jones et al (E Jones, Y Zhang, unpublished data, 2022) provided an alternative way to restore bile acid homoeostasis by taking advantage of enterohepatic circulation of bile acid signalling. Jones et al (E Jones, Y Zhang, unpublished data, 2022) discovered that SHP knockout in the intestinal epithelium impaired intestinal lipid absorption due to changes in intestinal structure and subsequent increased faecal excretion of bile acids, ultimately protecting mice against NAFLD development induced by the Western diet or HFCF diet.

Given that FXR plays an indispensable role in the bile acid homoeostasis, targeting FXR has been intensively investigated in different metabolic scenarios. FXR-deficient mice have been reported to exhibit hepatic steatosis. FXR activation by non-bile acid agonist GSK3234 or obeticholic acid improves hepatic steatosis by reducing lipid absorption and lipogenesis. It is shown that deleting FXR only in hepatocytes or intestinal epithelium is insufficient to alter hepatic steatosis in mice fed with Western diet. However, an earlier study reported that intestinal FXR promotes hepatic steatosis by regulating ceramide synthesis in NAFLD induced by HFD.

To unravel the specific roles of hepatocyte FXR and intestinal FXR in NASH development, Henry et al (ZR Henry, GL Guo, unpublished data, 2022) compared different parameters evaluating NASH among FXR knockout, hepatocyte-specific FXR knockout, intestinal-specific FXR knockout, and WT mice in both males and females using a NASH ‘Fast Food’ diet (Western diet plus milk fat, cholesterol and sucrose). Henry et al (ZR Henry, GL Guo, unpublished data, 2022) found that intestinal FXR did not show significant benefits in protecting mice against NASH, and deficiency of hepatocyte FXR is associated with more severe liver injury and NASH development in females. These findings suggest that FXR activation in specific cell types needs to be considered in treating NAFLD.

**Single cell RNA sequencing and targeting macrophages in NAFLD**

Macrophages are the key players in human NAFLD, and an increase in periportal macrophages was shown as an early hallmark. The perportal CCR2+ inflammatory monocyte-derived macrophages in NASH patients are associated with disease severity and fibrosis. The liver resident macrophages, Kupffer cells (KCs), are reported to release proinflammatory mediators to promote steatohepatitis as early players. It has been reported that either reducing monocyte-derived macrophage recruitment or depleting KCs at the initiation phase attenuates hepatic inflammation and the severity of NAFLD. The mechanisms underlying the role of macrophages in NAFLD have been reported to be mediated by damage-associated molecular patterns released from dying hepatocytes, pathogen-associated molecular patterns, inflammasome, dysbiosis and fatty acid metabolism.

Different groups in the current symposium evaluated four macrophage-derived targets for treating NAFLD. Yang et al demonstrated that myeloid Myd88 knockout or a Myd88 inhibitor (LM8) protected mice against HFD feeding mice through attenuated saturated fatty acid-mediated inflammatory activation in macrophages. Guo et al (X Guo, C Wu, unpublished data) demonstrated that myeloid stimulator of interferon genes (STING) was positively associated with the degrees of hepatic inflammation in NAFLD patients. Deletion of STING in myeloid cells alleviated hepatic steatosis and inflammation in mice fed with HFD. Ahamed et al (F Ahamed, Y Zhang, unpublished data, 2022) demonstrated that myeloid SHP knockout mice were more susceptible to HFCF-induced NASH by exacerbating monocyte infiltration and liver fibrosis without affecting hepatic steatosis. Mechanically, SHP promoted anti-inflammatory macrophage polarisation by regulating miR-34a/PPAR signalling. Wei et al (X Wei, H Wang, unpublished data) discovered that β-arrestin (Arrb)2 expression in monocytes is positively correlated with liver inflammation in NAFLD patients. Arrb2 in macrophages promotes ubiquitination of IRG1, leading to reduced itaconate, enhanced SDH activation, and subsequently reduced oxidative phosphorylation (OXPHOS), elevated mitochondrial ROS release, and HIF1α/IL-1β signalling, ultimately contributing to NASH development. All of these studies revealed potential strategies to target macrophages for treating NAFLD.

Recently, single cell RNA sequencing (scRNAseq) and single nucleus RNA sequencing (snRNAseq) have shed new light on the heterogeneity of hepatocytes, hepatic stellate cells (HSCs), liver sinusoid endothelial cells (LSECs), and immune cells in human and murine NAFLD. The keynote speaker Dr. Jiandie Lin comprehensively elaborated on the emerging roles of macrophages in NASH and NASH-associated liver cancer. Notably, his group previously identified a TREM2+CD9+ population of hepatic macrophages, named NASH-associated macrophages (NAM), using scRNAseq. In mice with dietary NASH, embryonic...
KCs undergo death, and monocyte-derived KCs emerge and replenish the hepatic macrophage pool. The NASH diet induces LXR-mediated reprogramming in hepatic macrophages to induce partial loss of KC identity and increase expression of TREM2 and CD9.115 In the FFA-enriched environment, TREM2 in hepatic macrophages represses the initial progression of NAFLD by restraining the release of miR-106b-5p-containing exosomes that targets MFN2 and contributes to mitochondrial fragmentation in hepatocytes.114 Another study reported that TREM2+macrophages were located at the sites of hepatocellular damage, inflammation, and fibrosis in steatotic livers in mice fed with a high-fat high-cholesterol (HFC) diet. And haematopoietic TREM2 deletion augmented NASH development through impaired lipid handling.115 Although emerging data suggest a protective function of TREM2+macrophages in NASH pathogenesis, the induction of TREM2+ macrophages might predispose NASH to progress to liver cancer in the presence of CD8+T exhaustion.108 Therefore, the strategy of using TREM2+NAM for treating NAFLD needs further evaluation.

Liver cancer
Liver cancer is the second most lethal tumour with a 5-year survival rate less than 20%.116 It comprises mainly two clinical subtypes, HCC and intrahepatic cholangiocarcinoma (iCCA). Chronic infection with hepatitis B or C virus and alcohol abuse are major risk factors for liver cancer. However, NAFLD, including NAFL and NASH, has been increasingly linked to the liver cancer burden in Western countries.8 Different aetiological factors may drive tumourigenesis and tumour progression through distinct molecular mechanisms, leading to a complex genomic landscape in liver cancer. While mutations in genes such as TERT, TP53, CTNNB1 and AXIN1 are relatively frequent in liver cancer, there are few driver mutations commonly linked to liver cancer.117 Treatment options for patients with liver cancer are very limited, especially for those with advanced stages of the disease. Sorafenib is the most effective systemic drug despite its inadequate efficacy.119 Encouragingly, advances in immunotherapy have recently revolutionised the landscape for the frontline treatment of liver cancer owing to the success of the combination of atezolizumab and bevacizumab in treating HCC patients.120 However, immunotherapy responses vary a lot among patients and are linked to aetiologies.121 The authors determined the role of myeloid-enriched miR-223 in regulating TME in the HCC. Using two mouse models of inflammation-associated HCC, they found miR-223 knockout mice had higher levels of infiltrated programmed cell death 1 (PD-1)+ T cells and programmed cell death ligand 1 (PD-L1)+macrophages compared with wild-type mice. Mechanistically, both in vivo and in vitro studies demonstrated that miR-223 could regulate PD-1 and PD-L1 in immune cells by shaping the TME via the HIF1α in HCC. In addition, gene delivery of miR-223 via adenovirus inhibited hypoxia-mediated PD-1/PD-L1 axis activation in both HCC models. Collectively, miR-223 may serve as a novel target for HCC due to its pivotal role in regulating TME. As the most common paediatric liver cancer, mechanisms of tumourigenesis in hepatoblastoma (HB) are not well understood. Fan et al established orthotopic liver cancer models that tumours are found to be linked to cancer.124 Ma et al evaluated mitochondria fission via DRP1 and mitophagy fusion via MFN1/2 in liver tumourigenesis by using liver-specific DRP1 knockout (L-DRP1KO), L-MFN1 KO, L-MFN2-KO, L-MFN1/MFN2 double KO (DKO) and L-DRP1/MFN1/MFN2 triple KO (TKO) mouse models. They found that all male L-DRP1 KO mice developed spontaneous liver tumours up to 15 months, while a lower incidence was observed in female L-DRP1 KO mice. In contrast, other mouse models either failed to develop liver cancer or had a much lower incidence rate. In addition, Ma et al found that alcohol-fed L-DRP1 KO mice had significantly increased liver tumour burden and size.125 This study demonstrates the role of mitochondrial fission via DRP1 in promoting liver tumourigenesis and alcohol in exacerbating liver cancer development.

Amplification of oncogenes or depletion of tumour suppressor genes is commonly observed in various cancer types. In HCC, low expression of phosphatase and tensin homolog (PTEN), a tumour suppressor gene, is very frequent. However, the loss of PTEN alone or the activation of other pathways simultaneously being required for liver cancer development is partially understood. Followed their work of loss of PTEN synergising with c-Met in promoting HCC,126 Hu et al (J Hu, N Liu, G Song, unpublished data, 2022) further explored the question by manipulating both PTEN and Yes-associated protein 1 (YAP1). They used hydrodynamic injection, Sleeping Beauty and CRISPR/Cas9 to ablate PTEN and to overexpress YAP1 in hepatocytes of wild-type male FVB/NJ mice. The authors found YAP1/sqPTEN mice developed lethal HCC within 18 weeks, whereas sqPTEN or YAPI mouse models alone failed to develop HCC. Further analysis demonstrated an immunosuppressive tumour microenvironment (TME) in YAP1/sqPTEN mice with an accumulation of regulatory T cells via activating the NF-κB/CCL20/CCR6 axis. This study suggests that loss of PTEN cooperating with YAP1 overexpression promotes HCC development by impairing antitumour immunity.

MicroRNAs have been demonstrated their critical roles in cancer. In a study performed by Fu et al,127 the authors determined the role of myeloid-enriched miR-223 in regulating TME in the HCC. Using two mouse models of inflammation-associated HCC, they found miR-223 knockout mice had higher levels of infiltrated programmed cell death 1 (PD-1)+ T cells and programmed cell death ligand 1 (PD-L1)+macrophages compared with wild-type mice. Mechanistically, both in vivo and in vitro studies demonstrated that miR-223 could regulate PD-1 and PD-L1 in immune cells by shaping the TME via the HIF1α in HCC. In addition, gene delivery of miR-223 via adenovirus inhibited hypoxia-mediated PD-1/PD-L1 axis activation in both HCC models. Collectively, miR-223 may serve as a novel target for HCC due to its pivotal role in regulating TME.
transplantation mouse models in postnatal day 5 (P5) and 60 (P60) mice (P5Tx and P60Tx models) using various HB cell lines to understand HB tumourigenesis. They found P5Tx models were more tumorigenic and metastatic compared with P60Tx models. Further analysis demonstrated that P5Tx models had significantly higher levels of CXCL1, which can mediate the migration and survival of HB cells. Collectively, Fan et al demonstrate that the neonatal liver provides a protumourigenic niche for HB development.128

**Single-cell dissection of liver tumour biology**

The development of single-cell technologies provides unique opportunities to understand liver tumour biology at an unprecedented resolution.129 Accordingly, the single-cell landscape of liver tumour ecosystems has been unveiled. Remarkably, the extensive intertumour and intratumour heterogeneity (ITH) of malignant cells was uncovered. While tumour cell biodiversity is an appearance, understanding the determinants and consequences of tumour heterogeneity is more critical for developing novel therapeutic strategies.130 131 Based on single-cell transcriptomic profiling of primary HCC or iCCA tumours from 19 patients with liver cancer, Ma et al determined the level of ITH by using principal component analysis. They found that patients with a higher level of ITH had much worse survival outcomes. Additionally, high-diversity tumours can reprogram the TME via secreting more VEGF. Ma et al further investigated ITH by identifying tumour functional clones within a tumour based on single-cell transcriptomic profiling of 46 tumours from 37 patients with liver cancer. Ma et al developed a robust machine-learning-based approach to determine tumour functional clones and found that the functional clones within a tumour can serve as a predictor for patient outcomes.135

Most single-cell studies can only capture a snapshot of a tumour during its long evolutionary history due to the difficulties of obtaining longitudinal tumour biopsies. However, how tumour cells evolve under the surveillance of the immune system or on treatment is crucial to understand tumour biology. In the single-cell study performed by Ma et al,133 the authors were able to profile longitudinal samples from patients with liver cancer who were enrolled at the NIH clinical centre for immunotherapy clinical trials. They found some patients had similar tumour cell populations before and after treatment, while others had completely shifted tumour cells as well as TMEs. Further analysis demonstrated that SPP1 was a key regulator of tumour evolution. Since a small number of patients with liver cancer with longitudinal samples are available in the study, future exploration of tumour evolution in a larger cohort is a need.

As part of a tumour ecosystem, the dual roles of the TME in tumour progression cannot be neglected. Single-cell analysis has provided a cellular atlas of cell types and even cell states with a cell type in liver cancer. Well-known and newly discovered immune and stromal cells have been uncovered. Zhang et al generated a landscape of immune cells by profiling CD45+ cells from tumour and immune-relevant sites of 16 treatment-naive liver cancer patients by using full-length and 3' scRNAsq. They performed a detailed classification of immune cells and annotated 40 clusters of T cells, B cells, myeloid cells and NK cells. Specifically, they found LAMP3+ dendritic cells held the potential to migrate from tumours to lymph nodes to induce the immune response. Using a similar strategy, Xue et al performed the scRNAseq analysis of the TME from 189 human and mouse samples. They identified 12 groups of tumour-associated neutrophils (TAN) and found that the TAN populations were associated with an unfavourable prognosis of patients with liver cancer.

The continuous interactions between tumour cells and the TME represent a common phenomenon during tumour evolution. These communications may serve as a fingerprint embedded in a tumour ecosystem and further reflect intrinsic tumour biology. In a single-cell study performed by Ma et al,136 they generated single-cell transcriptomic profiles from multiple locations of a tumour, including three regions from the tumour core, one region from the tumour border, and one from the adjacent normal tissue, from patients with liver cancer who received surgical resection. The authors found that the interactions between tumour cells and the TME were very stable within each patient by evaluating the ligand-receptor interactions. However, patient-specific interactions were evident and were universally linked to clinical outcomes in several liver cancer patient cohorts. Specifically, the communications between tumour cells and tumour-associated macrophages via the ligand-receptor pairs of LGALS9-SLC1A5, and SPP1-PTGER4 were highly elevated in aggressive tumours. Further RNAseq experiments demonstrated the colocalisation of the genes associated with the pairs in space. Collectively, the study demonstrates that the communications of tumours and the TME may reflect the intrinsic tumour biology in liver cancer.

**CONCLUSION**

Fatty liver disease and HCC continue to be significant public health concerns, causing millions of deaths worldwide each year. Currently, there is still a need for effective drug treatments for ALD, NAFLD and liver cancer. The CALS/SCBA Hepatology Division Annual Symposium provided a platform for researchers to discuss the latest advances in basic and translational research related to these liver diseases. The presentations highlighted the roles of non-coding RNA, autophagy, extrahepatic signalling, macrophages, etc in advancing our understanding of the pathogenesis of these diseases. This symposium represents a critical step forward in the fight against chronic liver diseases, and it is hoped that these insights will lead to the development of more effective treatments and improved patient outcomes.
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