Dysregulated Epigenetic Modifications in the Pathogenesis of NAFLD-HCC

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Abstract

The pathogenesis of NAFLD is multi-faceted and mechanisms underlying the progression from simple steatosis to NASH have not been fully deciphered. The emerging field of epigenetics, an inheritable phenomenon capable of changing gene expression without altering DNA sequence, unveils a new perspective on the development of NAFLD and subsequent progression to HCC. In fact, numerous studies have highlighted the potential involvement of unhealthy daily habits such as physical inactivity and over-nutrition in the onset and development of NAFLD through epigenetic mechanisms. This chapter will discuss several epigenetic modulations including DNA methylation, histone modifications, functions of non-coding RNAs as well as RNA methylation implicated in the pathogenesis of NAFLD-HCC. On the basis of currently wealthy knowledge of DNA epigenetics, the rapidly growing field of RNA epigenetics will certainly drive forward a new avenue of research direction shedding light on the advancement of better diagnostics, prognostics and therapeutics in the coming era of precision medicine.

7.1 Introduction

As mentioned in previous sections, non-alcoholic fatty liver disease (NAFLD) is defined as the pathological deposition of triglycerides in hepatocytes due to causes other than excessive alcohol consumption. Non-alcoholic steatohepatitis (NASH), the more severe disease entity of NAFLD, represents the most common liver disease in the Western world and has the capacity to progress to cirrhosis and hepatocellular carcinoma (HCC) [1]. Compared to the high prevalence of NAFLD (20–30%) in Western countries, the prevalence in Asian countries is estimated to be around 5–20% [2]. As with other causes of liver disease, only a minor proportion of patients with NASH progress to advanced fibrosis, cirrhosis and/or HCC [3].

The pathogenesis of NAFLD is multi-faceted and mechanisms underlying the progression from simple steatosis to NASH have not been fully deciphered. According to the double-hit theory attempting to explain the development of NAFLD, the first hit is the accumulation of triglycerides in hepatocytes, accompanied by a sec-

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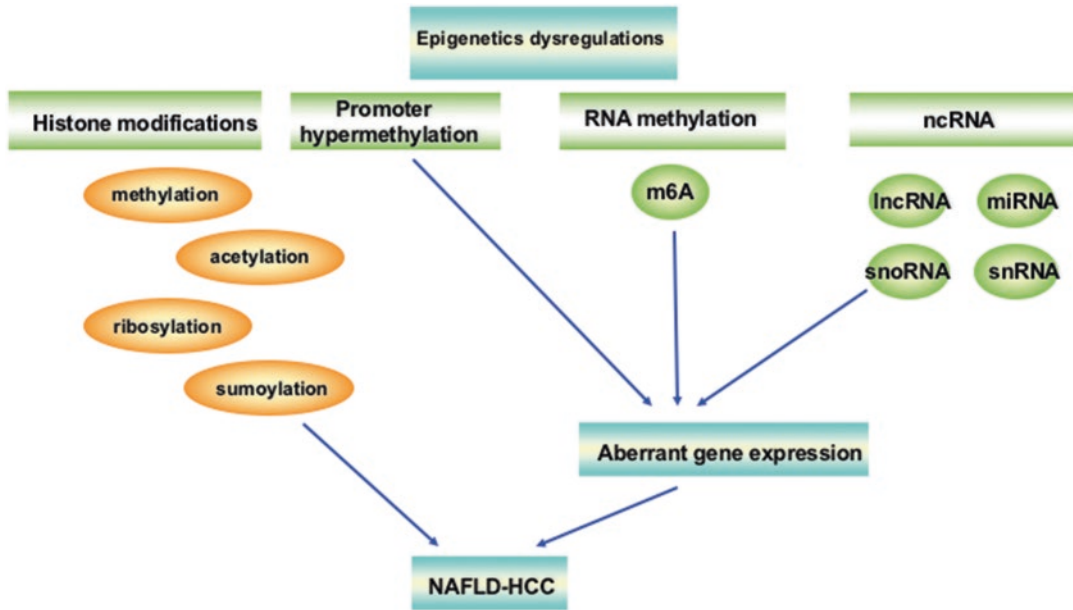
Fig. 7.1 Dysregulated epigenetic modulations including DNA methylation, histone modifications, functions of non-coding RNAs as well as RNA methylation contribute to the pathogenesis of NAFLD-HCC. *m⁶A* N⁶-methyladenosine, *lncRNA* long non-coding RNA, *snRNA* small nuclear RNA, *snoRNA* small nucleolar RNA

ond hit describing inflammatory cytokine interplay, mitochondrial dysfunction and oxidative stress causing hepatocellular injury, inflammation and fibrosis [4, 5]. Recent studies devised a new model describing multiple parallel hits in the progression of NAFLD. NAFLD pathogenesis is now commonly described as the excessive deposition of fat in hepatocytes, followed by increase in intracellular fat vacuoles, induction of endoplasmic reticulum and oxidative stress eventually leading to apoptosis of hepatocytes [6].

The emerging field of epigenetics, an inheritable phenomenon capable of changing gene expression without altering DNA sequence, unveils a new perspective on the pathogenesis of NAFLD. In fact, numerous studies have highlighted the potential involvement of unhealthy daily habits such as physical inactivity and overnutrition in the onset and development of NAFLD through epigenetic mechanisms [7, 8]. This chapter will discuss several epigenetic modulations including DNA methylation, histone modifications, functions of non-coding RNAs as well as RNA methylation implicated in the pathogenesis

of NAFLD-HCC that might serve as novel diagnostic, prognostic and therapeutic options (Fig. 7.1).

7.2   DNA Methylation

The best-known and most intensively studied modification is methylation of cytosine in DNA with a methyl group. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) that transfer a methyl group from S-adenosyl-L-methionine (SAM) to cytosine with guanine as the next nucleotide known as CpG dincleotides, the clustering of which being commonly referred to as CpG islands. Majority of CpG islands are located at the promoter regions of genes and hypermethylation of CpG islands causes gene silencing [9]. On the other hand, the ten-eleven translocation methylcytosine dioxygense (TET) family of enzymes converts the modified DNA base 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC) and this modification has been proposed as the initial step of active demethylation in mammals [10, 11]. Given

the central role of DNA methylation in the regulation of gene expression, it comes with no surprise that perturbations to the homeostatic methylation level, due largely to environmental factors, contribute to aberrant gene expressions and trigger various pathological conditions.

7.2.1 DNA Methylation in Fibrosis and Progression of NASH

S-adenosyl-L-methionine (SAM) is the unique methyl donor for DNA methylation and dietary sources include folate, methionine, betaine and choline [12]. Methyl donor deficient diets have been associated with reduced DNA methylation and disturbed lipid metabolism. For example, folate deficiency has been shown to induce hepatic triglyceride accumulation and alter the expression of genes involved in fatty acid synthesis [13]. Likewise, deficiencies in methionine and choline have been correlated with reduced lipoprotein secretion and increased hepatic triglyceride generation accompanied by NAFLD development [14, 15]. Intriguingly, Tryndyak et al. [16] demonstrated *in vivo* that low SAM concentration altered the expressions of a series of genes involved in DNA repair, lipid and glucose metabolisms and hepatic fibrosis. This was consistent with a recent observation reporting a significant decrease in serum betaine levels in NASH patients as compared to those with non-alcoholic fatty liver [17], implicating proper dietary intake and maintenance of homeostatic SAM levels are critical for a harmonious hepatic lipid metabolism.

Liver fibrosis is defined by the excessive accumulation of extracellular matrix and scar formation in the context of chronic liver damage [18]. Activation and trans-differentiation of hepatic stellate cell (HSC) in response to various stimuli such as inflammation, from vitamin A storing pericyte to profibrogenic myofibroblastic phenotype, play a key role in the pathogenesis of liver fibrosis [19]. It has been demonstrated that transforming growth factor- β1 (TGF- β1), an inflammatory cytokine secreted by different types of hepatic cells, represented the main fibrogenic

cytokine behind HSC activation [20]. Although the underlying molecular mechanisms driving fibrogenesis await further investigations, DNMTs have recently been implicated in the process. In humans there are three DNMT isoforms: DNMT1, DNMT3a and DNMT3b. While DNMT1 recognizes a hemi-methylated site on a new DNA strand during cell division and regenerates the bi-methylated state thereby safeguarding the faithful propagation of methylation patterns in daughter cells, DNMT3a and DNMT3b are central to the regulation of *de novo* methylation in the absence of cell division [21]. In a mouse model, Pogribny et al. [22] documented a hepatic epigenetic phenotype predetermined individual susceptible to hepatic steatosis in association with altered expressions of DNMT1 and DNMT3a in the liver. DNMT3a has also been shown to enhance HSC activation and liver fibrogenesis via methylation and down-regulation of the GTPase Septin-9 [23]. TET enzymes, responsible for catalyzing the stepwise oxidation of methyl groups on DNA leading eventually to the restoration of the unmodified cytosine residue, have been found to fine-tune the *PPARGC1A* transcriptional program in liver. Next-generation sequencing further revealed genetic diversity at *TET* loci was associated with altered 5-hmC levels that might be accountable for the pathogenesis of NAFLD [24]. A recent study elucidating the relationship between methylome and transcriptome in patients with non-alcoholic fatty liver disease revealed differentially methylated genes might distinguish patients with advanced NASH from those with simple steatosis [25]. In the landmark piece of work, 69,247 differentially methylated CpG sites (76% hypomethylated; 24% hypermethylated) were observed in patients with advanced NASH as compared to those with simple steatosis [25]. Aberrant methylation signatures of a plethora of genes have been suggested to predict the progression from NAFLD to NASH. For instance, peroxisome proliferator-activated receptor α (PPAR-α) exhibited significantly higher DNA methylation level in severe NAFLD patients than in mild counterparts [26].

7.2.2 DNA Methylation in the Progression of HCC

Disruption of DNA methylation has long been recognized as one of the key hallmarks of all cancer types [27]. Typical lesions in cancer include loci-specific *de novo* hypermethylation at promoter regions of tumour suppressor genes (TSGs) resulting in transcriptional repression of downstream TSGs. Among the plethora of studies reporting changes in DNA methylation pattern in HCC, Villanueva et al. [28] conducted a comprehensive study profiling the DNA methylation landscape in a cohort of 304 patients with HCC treated with surgical resection. Methylome profiling covering 96% of known CpG islands and 485,000 CpG dinucleotides was performed and a methylation signature generated based on 36 methylation probes accurately predicted survival in patients with HCC. While HCC tissue samples displayed general hypomethylation in the intergenic and body regions as compared with normal liver, hypermethylated probes were mainly located in promoter regions [28]. The authors further demonstrated aberrant methylation in established and candidate epidrivers of disease including well-known tumour suppressors such as Ras association domain family member 1 (*RASSF1*), adenomatous polyposis coli (*APC*), insulin-like growth factor 2 (*IGF2*) and *NOTCH3*, supporting the pivotal role of deregulated DNA methylation in HCC development [28].

The functional relevance of aberrant DNA methylation has been tested in numerous tumour suppressor genes. For instance, sphingomyelin phosphodiesterase 3 (*SMPD3*) and heavy polypeptide (*NEFH*) overexpression could inhibit tumour cell proliferation, whereas stable knockdown of the two enhanced cell migration and invasion *in vitro* and *in vitro* [29]. Noteworthy, persistent Hepatits B virus (HBV) infection has been shown to stimulate the upregulation of DNMTs, leading to hypermethylation and inactivation of p16 and the subsequent progression of HCC [30]. A recent intriguing study uncovered a role of hypoxia in the process of tumour development. Thienpont et al. [31] observed a direct inhibition of the activity of TET enzymes in a series of cancer cell lines (including HCC) and mouse cells in response to a hypoxic environment. The reduction in activity increased hypermethylation at gene promoters resulting in aberrant gene expressions in various signaling pathways and conferring a selective advantage to cancer cells [31]. Taken together, deregulated DNA methylation will continue to be a hot research area as a more thorough understanding of the underlying mechanisms is crucial to formulating novel prognostic markers and therapeutic targets.

7.3 Histone Modifications

Condensation of 2 m of DNA into a human nucleus is achieved through interaction between DNA and specialized histone proteins to form tightly packed chromatin. The basic level of chromatin packing is known as the nucleosome with each core particle comprising of 147 bp of double stranded DNA wrapped around a complex of eight histone proteins (two copies each of H2A, H2B, H3 and H4). The structure is commonly referred to as "beads on string" with linker DNA being the string and the nucleosome core particle representing the beads. In order to allow chromosomal processes such as gene transcription to occur, the chromatin must be packed lightly (euchromatin) or tightly (heterochromatin) in a finely orchestrated fashion. Indeed, each of the core histones harbours an unstructured N-terminal amino acid tail extension that can be subject to a plethora of posttranslational modifications including acetylation, methylation, phosphorylation, ubiquitination, ribosylation and sumoylation which constitute a crucial determinant of chromatin compactness and accessibility [32].

7.3.1 Histone Acetylation in NAFLD-HCC

Among various types of posttranslational modifications, acetylation of lysine residues at the N-terminus of histone tails has been most extensively investigated [33]. While histone acetylation is catalyzed by histone acetyltransferases (HATs), histone deacetylation is mediated by histone

deacetylases (HDACs) [34]. Perturbations to the balance between HAT and HDAC have been reported to alter gene expression profiles in NAFLD [35].

7.3.1.1 Histone Acetyltransferases (HATs)

Histone acetyltransferases (HATs) acetylate conserved amino acid residues on histone proteins by transferring an acetyl group from acetyl-CoA to form ε-*N*-acetyllysine enabling enhanced gene expression. HATs can be divided into different classes depending on their subcellular localization [36]. Type A HATs are mainly located in the nucleus including Gcn5-related *N*-acetyltransferases (GNATs), p300/CBP and TAF$_{II}$250, whereas type B HATs function predominantly in the cytoplasm [36]. In particular, p300/CBP has been shown to be involved in NF-κB dependent inflammatory pathways [37]. Inhibition of hepatic p300 was further suggested to be beneficial for treating hepatic steatosis in obesity and type 2 diabetes [38]. On the contrary, a recent report demonstrated p300/CBP-associated factor inhibited the growth of HCC cells by promoting autophagy, suggesting restoration of the specific HAT might prove to be a novel therapeutic strategy of HCC treatment [39].

7.3.1.2 Histone Deacetylases (HDACs)

Histone deacetylases (HDACs) remove acetyl groups from ε-*N*-acetyl lysine residues on histone, a process that is essential for tight wrapping between histones and DNA, as well as subsequent inhibition of gene transcription. HDAC superfamily is sub-divided into four classes: I, II, III (also referred to as Sirtuins or SIRTs) and IV on the basis of varying structure, enzymatic function and subcellular localization. Not surprisingly, dysregulations of HDACs have been implicated in the progression of NAFLD. Disruption of the circadian clock by HDAC3, a member of class I HDACs, resulted in perturbation to hepatic lipid metabolism and obesity [40]. Another member HDAC6 has been documented to function as a tumour suppressor in HCC and suppression of which by induction of miR-221 accompanied by activation of down-

stream oncogenic pathways contributed to liver tumorigenesis [41].

Silent information regulator 2 proteins (Sirtuins or SIRTs) belong to class III HDACs. Seven members have been identified in human so far (SIRT1-7) with different subcellular localizations. While some are present predominantly in the nucleus, others display cytoplasmic (SIRT1,2) and mitochondrial (SIRT3,4,5) localizations [42]. Research on mammals has been focused on SIRT1, which acts as a potent protector from a wide array of pathological conditions such as diabetes, liver steatosis and various types of cancer [43]. Although overexpression of SIRT1 appeared to offer protection against DNA damage and metabolic derangement induced by high fat diet [44], recent studies highlighted up-regulation of SIRT1 facilitated HCC metastasis and self-renewal of liver cancer stem cells [45, 46]. Similarly, SIRT2 overexpression has also been demonstrated in HCC promoting epithelial-mesenchymal transition and an aggressive phenotype [47]. Another member of the SIRT family of HDACs, SIRT3, represents the primary mitochondrial deacetylase that is indispensable for the maintenance of mitochondrial integrity and metabolism during oxidative stress [48]. In a mouse model fed a high fat diet, Hirschey et al. observed down-regulation of SIRT3 and mice lacking SIRT3 exhibited exacerbated obesity, insulin resistance, hyperlipidemia and steatohepatitis supporting a role of SIRT3 in safeguarding metabolic homeostasis [49]. Studies looking into the potential roles of other SIRT members in the development of liver diseases are expanding.

7.3.2   Histone Methylation in NAFLD-HCC

Though less well studied as compared to DNA methylation, histone methylation can be associated with transcriptional activation or repression. Histone methyltransferases mediate the transfer of methyl groups from S-adenosyl-L-methionine (SAM) to lysine or arginine residues of H3 or H4 histones. Common sites of methylation that have been reported to be involved in gene activation

include H3K4, H3K48 and H3K79, whereas H3K9 and H3K27 are associated with gene inactivation [50]. Recent investigations demonstrated participation of histone methyltransferases in the development of diseases. For instance, Fei et al. recently reported the H3K9 methyltransferase SETDB1 was overexpressed in HCC and regulated tumour cell growth via di-methylation of p53 [51].

7.3.3 Histone Ribosylation in NAFLD-HCC

Adenosine diphosphate (ADP)-ribosylation refers to the addition of one or more ADP-ribose moieties from nicotinamide adenine dinucleotide (NAD) to acceptor proteins. The reaction is a reversible posttranslational modification catalyzed by two classes of enzymes: mono-ADP-ribosyltransferases and poly (ADP-ribose) polymerase (PARP) [52]. PARP is involved in a broad range of cellular functions including gene regulation, DNA damage repair, cell signaling as well as apoptosis [53, 54]. As with other types of modifications, aberrant PARP expression has been documented in various types of cancer including HCC. Poly-ADP-ribosylation and PARP expression were found to be significantly upregulated in human HCC when compared to adjacent non-tumour tissues [55]. Since then the potential of PARP as a therapeutic target for cancer has been intensively studied. In combination with DHMEQ (a novel inhibitor of NF-κB), the PARP inhibitor Olaparib has recently been shown to exert synergistic anti-tumour effects on HCC cells [56].

7.3.4 Histone Sumoylation in NAFLD-HCC

Sumoylation describes the covalent attachment of small ubiquitin-related modifier (SUMO) proteins to acceptor proteins. Four SUMO family members, SUMO-1 to SUMO-4, have been identified so far. Though SUMO-1 exhibits 18% sequence identity with ubiquitin and the two share similar three-dimensional structures, sumoylated proteins are not designated for degradation [57]. Indeed, sumoylation is commonly involved in various cellular processes including intracellular trafficking, transcriptional regulation, response to oxidative stress and cell cycle progression [58]. Sumoylation is also a dynamic process catalyzed by SUMO-specific activating (E1), conjugating (E2) and ligating (E3) enzymes [59] and can be reversed by the family of SUMO-specific proteases (SENPs) [60]. In addition to mediating transcriptional repression through recruitment of histone deacetylases and heterochromatin protein 1, sumoylation has been implicated in tumorigenesis [61]. Recently, upregulation of one of the SUMO-specific proteases, SENP5, has been observed in HCC to promote tumorigenesis *in vitro* and *in vivo* via de-sumoylation and regulation of DNA damage response [62]. SUMO1 has also been demonstrated to possess oncogenic properties in HCC by promoting p65 nuclear translocation and regulating NF-κB activity [63].

7.4 Non-coding RNAs (ncRNAs)

Non-coding RNAs (ncRNAs) constitute a significant proportion of the transcribed genome that is not destined to be translated into proteins. ncRNAs comprise highly abundant RNAs including transfer RNAs (tRNAs), ribosomal RNAs (rRNAs), microRNAs (miRNAs), small nuclear RNAs (snRNAs), small nucleolar RNAs (snoR-NAs), extracellular RNAs (exRNAs) and long ncRNAs (lncRNAs) [64]. The plethora of ncRNAs play crucial roles in a broad spectrum of biological processes while dysregulations of which contribute to the development of various diseases.

7.4.1 miRNAs

7.4.1.1 Definition

Ever since the discovery of *lin*-4 in the nematode *Caenorhabditis elegans* (*C.elegans*) in 1993, members of the novel class of small non-coding

single strand regulatory RNAs, the microRNA (miRNAs) family, have been expanding drawing the attention of research focus [65]. miRNAs are each comprised of approximately 22 nucleotides and are found in a diverse array of organisms ranging from prokaryotes, eukaryotes to viruses. miRNAs can be either encoded by specific genes or located in the introns or exons of protein-coding genes and expressed as a by-product [65]. They play crucial roles in a wide spectrum of cellular and physiological functions, including cell proliferation, cell death, metabolism, haematopoiesis, and chromatin modification by modulating the expression of target genes [66].

7.4.1.2 Biogenesis and Mechanism of Action

Biogenesis of miRNAs in vertebrate initiates with the generation of a long primary miRNA (pri-miRNA) which is transcribed mostly by RNA polymerases type II (Pol-II). Each pri-miRNA is then processed into a hairpin-shaped precursor miRNA (pre-miRNA) of approximately 60–70 nucleotides by Drosha-like RNase III endonucleases. The pre-miRNA is subsequently transported out of nucleus into cytoplasm by Exportin-5 and Ran-GTP, and is then cleaved by Dicer-like RNase III endonuclease to form the mature miRNA duplex. Afterwards, one strand is usually incorporated into the RNA-induced silencing complex (RISC) whereas the other strand is degraded [67]. Regulation of gene expression is mediated through the canonical base pairing of miRNA seed sequence and the complementary sequence of target mRNAs followed by silencing or degradation of target mRNAs [68]. It has been reported an average miRNA has approximately 100 target sites, indicating that miRNAs are capable of regulating a large fraction of protein-coding genes [69]. Given the importance of miRNAs in the regulation of a wide array of cellular functions, it comes with no surprise that deregulating the function of miRNAs could lead to the development of multiple pathological conditions including cancer. Indeed, dysregulated miRNAs have been documented in various cancer types including chronic lymphocytic leukemia [70], breast cancer [71], lung can-

cer [72], colorectal cancer [73], prostate cancer [74] and ovarian cancer [75].

7.4.1.3 miRNAs in the Progression from NAFLD to HCC

Recent studies have demonstrated aberrant expressions of miRNAs are involved in the acquisition of NAFLD and subsequent progression to NASH. Deregulations of some of the key regulatory miRNAs have been shown to disturb normal glucose, cholesterol and lipid metabolism leading to intra-hepatic excessive accumulation of triglycerides and fatty acids [76]. It has also been demonstrated miRNAs are frequently dysregulated in different phonotypes of NAFLD, from simple steatosis through NASH to cirrhosis and eventually HCC [77].

One of the very first miRNAs associated with lipid metabolism and homeostasis is miR-122, the most abundant miRNA in adult human liver accounting for 70% of the liver's total miRNAs [78]. Using murine models, Krutzfeldt et al. documented antagomirs targeting miR-122 resulted in reduction of plasma cholesterol levels coupled with altered expression of several genes involved in hepatic lipid biosynthesis [79]. A further study demonstrated inhibition of miR-122 significantly increased hepatic fatty acid oxidation and decreased the biosynthesis of hepatic fatty acid and cholesteral *in vivo* [80]. Although specific miRNA signatures responsible for NAFLD progression await further investigations, accumulating evidence has implicated a pivotal role of miR-122 in the process. For instance, mice lacking the gene encoding miR-122a were viable but later developed temporally controlled steatohepatitis, fibrosis and HCC [81]. Reduced expression of miR-122 has also been reported to upregulate modulators of tissue remodeling (including hypoxia-inducible factor 1 and vimentin) contributing to NASH-induced liver fibrosis [82]. A comparison of miR-122 levels in hepatocytes and primary human HCC cells revealed that miR-122 was down-regulated in HCC cells and the tumorigenic properties of cancer cells could be reversed by re-introduction of miR-122 [83]. Consistently, diminished expression of miR-122 has recently been shown to contribute to the acquisition of

sorafenib chemoresistance in HCC [84] while miR-122 restoration in human stem-like HCC cells was capable of prompting tumour dormancy via Smad-independent TGF-β pathway [85], implicating that miR-122 might serve as a potential therapeutic target in HCC.

Being one of the firstly identified oncomirs, miR-21 upregulation has been demonstrated in the liver of patients with NAFLD and hepatic miR-21 expression is positively correlated with the severity of NASH [86, 87]. Using different mouse models of NASH, Loyer et al. [88] showed miR-21 was overexpressed in hepatic biliary and inflammatory cells while inhibition of miR-21 diminished liver injury, inflammation and fibrosis via restoration of peroxisome proliferator-activated receptor alpha (PPARα). miR-21 is also involved in the pathogenesis of HCC by suppressing expression of the tumour suppressor gene phosphatase and tensin homolog (PTEN) [89]. A recent study reported that miR-21 acted downstream of the oncogenic signal transducer and activator of transcription 3 (STAT3) mediating the tumorigenic properties of HCC cells, suggesting miR-21 inhibition or suppression might prove to be a novel treatment of HCC [90].

miR-34a represents another key oncomir displaying elevated expression in patients with NAFLD and positive association with the degree of NASH [86]. It has been shown that the miR-34a/SIRT1/p53 pro-apoptotic pathway played a significant role in human NAFLD development which could be suppressed by the inhibitor ursodeoxycholic acid (UDAC) [91]. Administration of pifithrin-α p-nitro (PFT), a p53 inhibitor, was capable of attenuating steatosis and liver injury in a mouse model of NAFLD partially attributed to the resulting transcriptional suppression of miR-34a [92]. In contrast, miR-34a functions as a tumour suppressor in HCC. A small molecule modulator of miR-34a, termed Rubone, has recently been demonstrated to dramatically inhibit tumour growth in a mouse xenograft model via restoration of miR-34a expression and functioning [93].

In an attempt to identify the pattern of altered gene expression at various time points in a high fat diet-induced NAFLD mouse model, Hur et al. [94] found reduced levels of miR-451 in palmitate-exposed HepG2 cells and mouse liver tissue. *In vitro* analysis further showed miR-451 negatively regulated palmitate-induced interleukin-8 (IL-8) and tumour necrosis factor alpha (TNFα) production supporting a role of miR-451 in preventing the progression from simple steatosis to severely advanced liver disease [94]. Concomitantly, miR-451 has also been documented to function as a potential suppressor of tumour angiogenesis in HCC by targeting IL-6R-STAT3-VEGF signaling, thereby implicating a promising therapeutic role of miR-451 in HCC [95]. miR-221/222, which has been intensively studied in the carcinogenesis of breast cancer, has recently been shown to be overexpressed in human liver in a fibrosis progression-dependent manner [96]. miR-221/222 was further established to promote liver tumorigenesis in a mouse transgenic model [97]. Taken together, studies aiming at elucidating the roles of various miRNAs in the progression from NAFLD to HCC are emerging and further investigations are highly anticipated for detailed insights.

7.4.2 lncRNAs

7.4.2.1 Definition and Functions

Long non-coding RNAs (lncRNAs) are another class of non-protein coding transcripts longer than 200 nucleotides in length that can be further divided into three subtypes: (1) antisense lncRNAs overlapping known protein-coding regions; (2) intronic lncRNAs overlapping transcripts and (3) long intergenic RNAs encoded in the intergenic space between protein-coding areas [98]. The majority of lncRNAs display high specificity with respect to cell subtype, tissue and developmental stage. Although lncRNAs are implicated in the fine-tuning of a wide array of biological processes related to liver homeostasis and cancer including cell proliferation, differentiation and migration, in-depth mechanisms by which the majority of lncRNAs mediate their actions remain largely unknown.

LncRNAs are responsible for the regulation of basal transcription machinery, mRNA processing

and stability, protein translation and signal transduction [64]. One of the best-characterized lncRNAs functions in X chromosome inactivation in which the 17 kb transcript *Xist* recruits suppressive epigenetic factors to guarantee repression of gene expression and proper gene dosage in females [99]. Since then, research interest has been focusing on the emerging roles of lncRNAs in carcinogenesis with few reports mentioning the potential functions of lncRNAs in NAFLD. Until recently, Chen et al. [100] demonstrated the lncRNA steroid receptor RNA activator (SRA) promoted hepatic steatosis in mouse model via repressing the expression of adipose triglyceride lipase. In contrast, quite a number of studies have documented the roles of various lncRNAs in the development of HCC.

Highly upregulated in liver cancer (HULC), a 500 nt transcript discovered by cDNA microarray sequencing, is overexpressed 33-fold in HCC [101]. Using a transient silencing approach, the authors further reported HULC knockdown altered the expression of several genes related to the proliferation of HCC. HULC might also serve as a novel biomarker since it could be detected in the peripheral blood of HCC patients [101]. HOX transcript antisense intergenic RNA (HOTAIR) is a 2158 nt lncRNA displaying overexpression in HCC that is predictive of tumour recurrence in liver transplant patients [102]. Transient knockdown of HOTAIR has been shown to suppress tumorigenesis through inhibition of tumour cell growth and induction of cell cycle arrest [103]. Another recently identified lncRNA MALAT1 acts as a proto-oncogene via Wnt pathway activation and induction of the oncogenic splicing factor SRSF1 [104]. By and large, future studies using next generation sequencing will certainly shed light on the roles of more lncRNAs in hepatocarcinogenesis.

7.5 RNA Methylation

7.5.1 Introduction

The central dogma of molecular biology coined in the 50's explains the flow of genetic information in living organisms from DNA to RNA and RNA to protein. As such, messenger RNA (mRNA) represents a bridging link faithfully translating the secrets of life encoded in DNA sequences into functional proteins. However, cellular protein levels are not necessarily associated with mRNA levels, suggesting post-transcriptional mRNA modifications are crucial in the regulation of gene expression [105]. In fact, more than 100 different types of chemical modifications have so far been identified in cellular RNA, including mRNA, ribosomal RNA (rRNA), transfer RNA (tRNA), snRNA and lncRNA [106]. The most prevalent modification among all is adenosine methylation, also known as m^6A or N^6-methyladenosine.

Analysis of nucleic acid modifications and the corresponding effects on epigenetic status has been garnering heated research intention. As mentioned in previous sections, much efforts and interests have been focusing on changes in the chemistry of DNA and the actions of histone proteins as well as their subsequent modifications. It was not until the 1970s with the discovery of m^6A in a broad spectrum of eukaryotes-ranging from yeast, *Drosophila*, viruses to mammals-that investigators had realized and added a whole new RNA dimension to the field of epigenetics [107, 108]. Owing to a series of hindrances including the lack of knowledge of m^6A demethylating enzymes, the short life-span of most RNAs, the resulting idea that m^6A modifications are unalterable, coupled with technical limitations in detecting m^6A-containing mRNAs, however, the pace of RNA epigenetic research had slowed down [109]. In 2011, a new surge of interest was sparked by the discovery that the fat mass and obesity associated protein (FTO) was capable of demethylating RNA, implicating m^6A RNA modifications are highly dynamic subject to finely orchestrated regulations [110]. Elucidation of the methylated transcriptome in mammals was further achieved by technical breakthroughs such as m^6A RNA immunoprecipitation followed by high-throughput sequencing (MeRIP-Seq) [111, 112]. Since then studies aiming at deciphering novel functions of the m^6A modification and more members of the methylation/demethylation machinery have been on the rise.

7.5.2   m⁶A Modification and Regulation

Following two independent studies unequivocally demonstrating that m⁶A is a widespread phenomenon in mRNA, further investigations revealed m⁶A residues are enriched in 5′ untranslated regions (5' UTRs), around stop codons and in 3' UTRs adjacent to stop codons in mammalian mRNAs [111–113]. Bioinformatic analysis of MeRIP-Seq data identified the recognition sequence for m⁶A methylation as RRACH (where R = G/A and H = A/C/U). Occurrence of the consensus motif has been estimated at 1 in 2000 bases in human and almost 90% of all m⁶A peaks contain at least one of the motif variants [111, 112]. The dynamic regulation of m⁶A methylation is mediated by adenosine methyltransferases ("writers") and demethylases ("erasers").

Methyltransferase like 3 (METTL3) is established as the S-adenosyl-L-methionine (SAM)-binding component of a multiprotein methyltransferase complex responsible for catalyzing m⁶A mRNA methylation [114, 115]. The catalytic function of METTL3 was then confirmed by *in vitro* studies demonstrating METTL3 knockdown diminished m⁶A peaks in mRNAs from various cell lines [112, 113]. Intriguingly, localization of METTL3 in both cytoplasm and nucleus has been reported, implying m⁶A mRNA methylation could occur in both cytoplasmic and nuclear compartments [116]. As a close homologue to METTL3, METTL14 has also been shown to mediate methylation reactions and a complex formed by METTL3 and METTL14 possesses much more efficient activity than separated components [117]. As mentioned previously, the discovery of FTO as the first m⁶A mRNA demethylase ignited the conception of m⁶A as reversible modification and resurged research interest in RNA methylation. Functional investigations documented silencing of FTO increased m⁶A peaks while ectopic expression reduced m⁶A peaks [110]. AlkB Homolog 5 (ALKBH5), another member of the mRNA demethylase family, was later identified by *in vitro* and *in vivo* analyses [118].

7.5.3   Role of m⁶A Methylation in Disease

Given that m⁶A modifications have been demonstrated in many housekeeping genes influencing a wide array of biological processes including transcription splicing, nuclear RNA export, translation, energy production and cell differentiation, it comes with no surprise that dysregulation of the modification inevitably contributes to obesity, brain development abnormality and other pathological conditions [119–121]. In the field of hepatic diseases, FTO was found to be overexpressed in the livers of NASH patients. *In vitro* studies showed FTO knockdown was protective against palmitate-induced oxidative stress, mitochondrial dysfunction, ER stress and apoptosis, suggesting inhibition of FTO might serve as a treatment option for NASH [122].

The year of 2016 witnessed several inspiring studies documenting the involvement of m⁶A mRNA modifications in cancer. A hypoxic tumour microenvironment has been reported to stimulate breast cancer stem cell phenotype by increasing NANOG mRNA and protein expression via induction of HIF and ALKBH5 [123]. In lung adenocarcinoma, METTL3 promoted the growth, survival and invasion of cancer cells [124]. Recently, Ma et al. [125] demonstrated a pivotal role of METTL14 in the progression of HCC. Down-regulation of METTL14 accounted for reduced m⁶A modifications in HCC, acted as an adverse prognostic factor for disease-free survival and was significantly correlated with tumour metastasis *in vitro* and *in vivo*. The authors further showed METTL14 depletion reduced expression of the tumour suppressor miR-126 by modulating binding of the microprocessor protein DGCR8 to pri-miR-126 in an m⁶A-dependent manner [125]. Taken together, while detailed regulations and mechanisms of DNA epigenetics and histone modifications have been thoroughly studied and are already being targeted in various cancer therapies, the emerging RNA epigenetics may represent the next avenue for investigation in the pursuit for novel prognostic and treatment options.

7.6 Concluding Remarks and Future Perspectives

This chapter highlights some of the key epigenetic modulations implicated in the development of NAFLD-HCC. Further in-depth studies would undoubtedly reveal a more comprehensive picture of the role of epigenetics in the development of pathological conditions. On the basis of currently wealthy knowledge of DNA epigenetics, the rapidly growing field of RNA epigenetics will certainly drive forward a new avenue of research direction shedding light on the advancement of better diagnostics, prognostics and therapeutics in the coming era of precision medicine.

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