

Review

The emerging roles of m⁶A modification in liver carcinogenesis

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Abstract

The ‘epitranscriptome’, a collective term for chemical modifications that influence the structure, metabolism, and functions of RNA, has recently emerged as vitally important for the regulation of gene expression. N⁶-methyladenosine (m⁶A), the most prevalent mammalian mRNA internal modification, has been demonstrated to have a pivotal role in almost all vital bioprocesses, such as stem cell self-renewal and differentiation, heat shock or DNA damage response, tissue development, and maternal-to-zygotic transition. Hepatocellular carcinoma (HCC) is prevalent worldwide with high morbidity and mortality because of late diagnosis at an advanced stage and lack of effective treatment strategies. Epigenetic modifications including DNA methylation and histone modification have been demonstrated to be crucial for liver carcinogenesis. However, the role and underlying molecular mechanism of m⁶A in liver carcinogenesis are mostly unknown. In this review, we summarize recent advances in the m⁶A region and how these new findings remodel our understanding of m⁶A regulation of gene expression. We also describe the influence of m⁶A modification on liver carcinoma and lipid metabolism to instigate further investigations of the role of m⁶A in liver biological diseases and its potential application in the development of therapeutic strategies.

Key words: Epitranscriptome; N⁶-methyladenosine; Hepatocellular Carcinoma (HCC)

Introduction

To date, more than 170 types of RNA modifications have been identified, including 5′ cap modification, poly(A) tail, pseudouridine (Ψ), N¹-methyladenosine (m¹A) and N^{6,2′}-O-dimethyladenosine (m⁶A_m), and N⁶-methyladenosine (m⁶A) [1]. Among these modifications, m⁶A is the most abundant internal RNA modification in eukaryotic cells that widely occurs in mRNA [2] and non-coding RNAs (ncRNAs) [3-11]. Numerous studies have provided evidence that m⁶A is involved in fundamental physiological and pathological RNA metabolic processes, including splicing, nuclear export, stability, translation, and decay [12-16]. These studies advanced our understanding of m⁶A function and regulatory mechanisms in various human diseases.

m⁶A is highly conserved among eukaryotic species ranging from yeast [17,18], plants [19], and

drosophila viruses [20,21] to mammals [22,23]. However, the m⁶A field did not progress for several decades until the availability of MeRIP-seq or m⁶A-seq techniques [24,25]. Consensus motif analyses revealed that m⁶A sites depicted as “DRACH” motif (R = G or A; H = A, C, or U; where A is converted to m⁶A) in the transcriptome is not random but occurs in coding sequences (CDS), 3′-untranslated regions (3′-UTRs), and especially in the region around the stop codon [24,25]. Despite the prevalence of DRACH sequences in the transcriptome, only 1–5% of these sites are methylated *in vivo*, indicating that the DRACH motif itself is not sufficient to determine m⁶A deposition.

m⁶A modification is induced by m⁶A methyltransferase complex (composed of METTL3/METTL14/METTL16, WTAP, KIAA1429, ZC3H13, HAKAI, and RBM15/15B) [26-28], removed by m⁶A

demethylases (FTO and ALKBH5) [29,30], and recognized by RNA binding proteins, including YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3, hnRNPs [14,16,31-39]. m⁶A modification of U6 snRNA and structural RNA, 18 S rRNA and 28 S rRNA, the 5' cap m⁶A_m, and U2 snRNA internal m⁶A_m are methylated by METTL16 [10,11,40], METTL5 [41], ZCCHC4 [9,42] and PCIF1 [43,44], respectively. Recently, METTL4 was shown to be a U2 snRNA internal m⁶A_m methyltransferase [45].

It has been demonstrated that m⁶A modification influences gene expression and is involved in a variety of human cancers including hepatocellular carcinoma (HCC) [46]. In terms of high morbidity and mortality, HCC ranks the second highest cancer [47,48]. Multiple factors can trigger or worsen the pathology of HCC, including chronic hepatitis B and C viral infection, chronic alcohol consumption, obesity, metabolic disorders, and non-alcoholic fatty liver disease (NAFLD) [49]. Surgical resection is the only curative therapy and sorafenib is the only approved drug for the treatment of HCC. The survival rate is up to 70% in patients with a tumor size of less than 2 cm [50]. Therefore, early diagnosis is essential for HCC treatment. Numerous studies have demonstrated an important role of epigenetic modifications including DNA and histone modification in regulating HCC progression [51]. There is increasing evidence that total m⁶A levels and abnormal expression of m⁶A regulators are altered and associated with HCC clinical prognosis [52,53]. Furthermore, the m⁶A modification regulators play an oncogenic or tumor suppressor role in HCC by influencing the expression of special genes [54-56]. Therefore, in this review, we focused on recent progress in m⁶A detection, the function and the mechanism of m⁶A regulators in lipid metabolism, hepatic virus infection, and liver carcinogenesis, and it may provide a potential novel therapeutic and prognosis targets for HCC.

Development of m⁶A detection approaches

The global levels of m⁶A in total RNA can be detected by liquid chromatography coupled to tandem mass spectrometry (HPLC-MS/MS), dot-blot analysis [29], and m⁶A Methylation Quantification Kit [12,29]. The most commonly used approach for detecting transcriptome-wide m⁶A levels is MeRIP-seq [24] or m⁶A-seq [25] that can detect m⁶A sites in a 100-200 nt resolution. Then the SCARLET method was developed to qualify the m⁶A levels of individual modification sites [57] but it is not feasible for high-throughput applications. Subsequently, MeRIP-seq and m⁶A-seq methods were improved with an added

ultraviolet crosslinking step to identify m⁶A sites at the single-nucleotide resolution termed PA-m⁶A-seq (photo-crosslinking-assisted m⁶A sequencing strategy) [58,59] or m⁶A-CLIP-seq (UV cross-linked m⁶A antibody-bound RNA sample) [60,61]. Then, MAZTER-seq and m⁶A-REF-seq, were developed that relied on MazF RNase or ChpBK RNase by two independent groups [62,63]. However, MATER-seq or m⁶A-REF-seq sensitive to m⁶A sites occurs only in the ACA motif context. DART-seq, which rely on the cytidine deaminase APOBEC1 refused with YTH domain contain protein was used to induce C to U deamination at sites adjacent to m⁶A residues [64]. However, the quality of DART-seq relies on transfection efficiency, and its application is limited. DAPT-seq also loses some m⁶A sites and has higher false-positive and false-negative rates. Recently, an FTO-assisted m⁶A selective chemical labeling method (termed m⁶A-SEAL) was developed [65]. However, the resolution of m⁶A-SEAL is about 200 nt which needs to be improved in the future. In m⁶A-label-seq method, Se-allyl-l-selenohomocysteine substituted the methyl group with allyl on SAM [66]. Subsequently, cellular RNAs were modified with N⁶-allyl adenosine (a⁶A) at the presumed m⁶A-generating adenosine sites and will be further pinpointed by iodination-induced misincorporation at the opposite side in the complementary DNA during reverse transcription. The rapid development of m⁶A detection method will greatly accelerate the discovery and validation of m⁶A modification sites in human genome species, which will further facilitate the development of new biomarkers for cancer prognosis and classification.

Dynamic regulation of m⁶A

As showed in **Figure 1**, m⁶A modification can be induced by m⁶A methyltransferase complex (MTC, also known as m⁶A 'writers') [67,68], which is composed of the core catalytic subunit METTL3/METTL14 [67] and regulatory proteins, including WTAP, VIRMA, ZC3H13, HAKAI, RBM15/RBM15B [28,69]. Unlike METTL3 complex that preferentially methylates single-stranded RNAs (ssRNAs), METTL16 functions alone and selectively methylates structured RNAs in the UACAGAGAA sequence [40]. Recently, METTL5 is reported to catalyze m⁶A modification in 18 S rRNA at position A₁₈₃₂, and ZCCHC4 induces m⁶A sites in 28 S rRNA at position A₄₂₂₀, and METTL5 is stabilizes by TRMT112 [9,41,42]. The cap structure, 7-methylguanosine (m⁷G), is linked to the first transcribed nucleotide (X) that is methylated at the ribose O2 position. If the first nucleotide is adenosine, it can be further methylated on the N⁶ position, termed m⁶A_m. m⁶A_m modification adjacent to the m⁷G cap is a reversible modification

catalyzed by PCIF1 (CAPAM, cap-specific adenosine N⁶-methyltransferase) [43,44,70,71]. METTL4 has recently been identified as a novel internal m⁶A_m

methyltransferase responsible for N⁶-methylation of m⁶A_{m30} on U2 snRNA with a preference for the AAG motif [45].

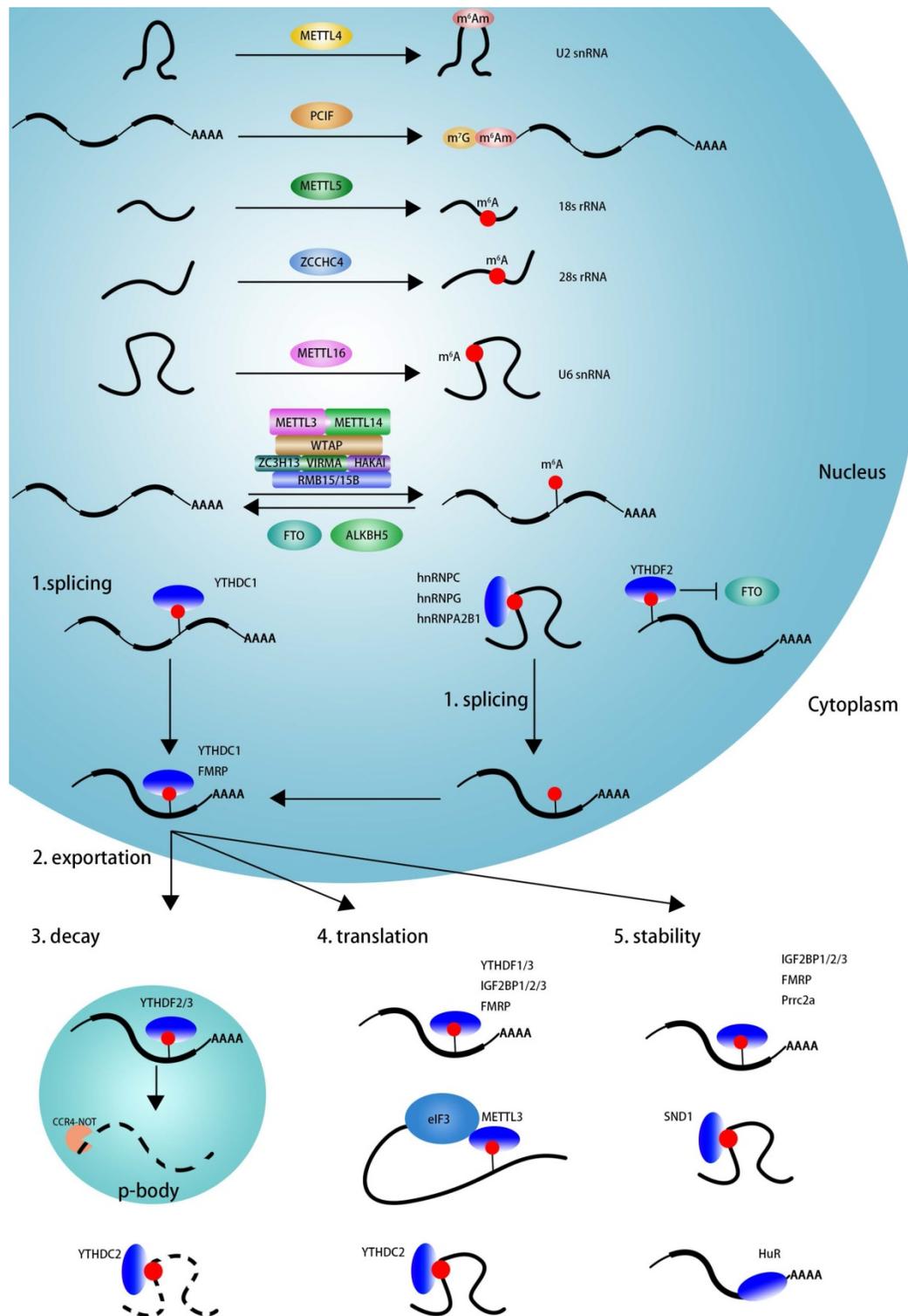


Figure 1. The dynamic regulation and function of m⁶A and m⁶A_m. The m⁶A modification is methylated by the methyltransferase complex (MTC, also known as m⁶A ‘writer’), composed of the core catalytic subunit METTL3-METTL14 and the regulator proteins WTAP, VIRMA, ZC3H13, RBM15/RBM15B and so on. METTL16 alone can methylated m⁶A deposition in structural RNA and snRNA. METTL5 is responsible for m⁶A deposition in 18 S rRNA and facilitate ZCCHC4 as 28 S rRNA methylase. METTL4 mediate internal m⁶Am modification in U2 snRNA. m⁶A modification can be eliminated by m⁶A ‘eraser’ (FTO and ALKBH5). The function of m⁶A is mediated by m⁶A ‘reader’, which affects RNA splicing, export, decay, stabilization and translation. YTHDC1 and hnRNPs (hnRNPA2B1, hnRNPC and hnRNPG) facilitate RNAs alternative splicing. The nuclear export of m⁶A containing RNAs is mediated by YTHDC1 and FMRP. The nuclear YTHDF2 binds with m⁶A in 5’UTR prevent FTO demethylation and promote cap-independent translation. The decay of m⁶A modified RNAs is mediated by YTHDF2/YTHDF3 and YTHDC2. The translation is facilitated by YTHDF1/YTHDF3, IGF2BP1/2/3, FMRP, METTL3 and YTHDC2. IGF2BP1/2/3, FMRP, PRRC2a and SND1 facilitate to keeping methylated RNAs stability.

The m⁶A mark may be removed by RNA demethylases (also known as m⁶A 'erasers'), including FTO and ALKBH5 [12,29]. The identification of FTO suggested that m⁶A modification is dynamic and reversible [29]. Further studies reveal that FTO can remove m⁶A and m⁶A_m in mRNA and U6 snRNA, and m¹A in tRNA [70,72]. ALKBH5 seems to specific demethylated m⁶A modification in RNA [12].

m⁶A is involved in numerous RNA processes, including splicing, export, decay, stability, and translation and exerts its functions mainly by recruiting m⁶A-binding proteins (also known as m⁶A 'readers'). As displayed in **Figure 1**, YTHDC1, hnRNPC, hnRNPG, and hnRNPA2B1 may regulate mRNA splicing by recognizing m⁶A sites on pre-mRNA in a direct or indirect (also known as "m⁶A switch") manner [14,33,38,73]. YTHDC1 and FMRP could mediate nuclear export of m⁶A modified RNAs into cytoplasm [74-76]. The nuclear YTHDF2 binds m⁶A sites in 5'UTR and prevents demethylation by FTO and thereby promotes cap-independent translation [77]. In the cytoplasm, YTHDF2, YTHDF3, YTHDC2 could mediate m⁶A deposited transcripts degradation in p-bodies, whereas IGF2BP1/2/3, FMRP, Prrc2a, SND1, and HuR play an important role in maintaining m⁶A-containing transcripts stabilization [35,78-84]. YTHDF1, YTHDF3, YTHDC2, IGF2BP1/2/3, FMRP, and cytoplasm METTL3 promotes m⁶A deposited mRNA translation [31,32,35,36,85-89]. In summary, m⁶A influence RNA every aspect of RNA metabolism depending on the functions of various readers.

Site specificity of m⁶A deposition

Why the distribution of m⁶A sites on DRACH motif is not random but occurs in CDS and 3'-UTR regions, especially around the stop codon, despite the high prevalence of DRACH [25]? Yue *et al.* demonstrated that VIRMA can interact with WTAP-HAKAI-ZC3H13 to recruit METTL3/METTL14 and guide region-selective methylation in 3'UTR and near stop codon [28]. FTO preferentially targets pre-mRNAs in intronic regions and regulates alternative splicing and 3'end processing [64]. Huang *et al.* described that Histone H3 trimethylation at Lys36 (H3K36me3) can guide m⁶A modification globally [90]. H3K36me3 can be recognized and bound directly by METTL14, and then facilitates the binding of MTC to adjacent RNA polymerase II, regulating m⁶A deposition in actively transcribed nascent RNAs. This study revealed a cross-talk between histone modification and m⁶A deposition. Several studies indicated that transcription factors can also influence m⁶A deposition. For instance, ZFP217 interacts with

several epigenetic regulators and modulates m⁶A deposition on their transcripts by sequestering the enzyme METTL3 [13]. The CAATT-box binding protein CEBPZ recruits METTL3 to chromatin and promotes m⁶A modification within the coding region of the associated mRNA transcripts [91]. The interaction of SMAD2/3 with METTL3/METTL14/WTAP complex mediates m⁶A modification in a subset of transcripts involved in early cell fate decisions [92]. By using the CRISPR-Cas9 technology, the m⁶A modification site can be edited by fusing dCas9 (dead Cas9) with single-chain methyltransferase domains derived from METTL3 and METTL14 or full-length of m⁶A demethylase (ALKBH5 or FTO) [88]. Programmable m⁶A editing can be realized by this approach providing a mechanistic understanding of epitranscriptome.

Aberrant m⁶A regulation in liver disease

Lipid metabolism

HCC is the second most frequent cancer in the world with high morbidity and mortality [49]. Viral hepatitis, alcohol abuse, and nonalcoholic steatohepatitis (NASH) progress to HCC. Therefore, the prevention of HCC is focused on averting virus infection (hepatitis B and C viruses), inflammation, and obesity [93]. NAFLD is a risk factor predisposing HCC formation and is associated with metabolic syndromes, including diabetes and obesity. Transcriptome-wide m⁶A profile has revealed that m⁶A-containing gene exhibit a high overlap in human HepG2 cell lines and mouse livers. Moreover, m⁶A-modified regions in liver tissues are enriched of single nucleotide polymorphisms (SNPs) related to lipid traits [94]. Functional enrichment analysis of m⁶A-modified genes in porcine liver at three developmental stages are involved in regulating growth, development, metabolic process and protein catabolic processes [95]. The diverse pattern of m⁶A-modification on genes in different stage had metabolic functions required for or specific to this stage, which indicating that the liver may be exposed to different stimuli during development. High-fat diet (HFD) induced m⁶A modification enriched in gene associated with lipid-associated processes, including cellular lipid metabolism, fatty acid metabolism, response to fatty acids and TG metabolism [96]. Xie *et al.* reveals that elevated METTL3 expression in Type 2 diabetes (T2D) patients inhibits hepatic insulin sensitivity via N⁶-methylation of Fasn (fatty acid synthase) mRNA, and subsequently promotes fatty acid metabolism [97]. Approximately one-tenth of liver genes have been identified as rhythmic, however only one-fifth of those genes are driven by de novo

transcription, suggesting that both transcriptional and epigenetic mechanisms underlie the mammalian circadian clock [98]. At the core of the mammalian circadian clock gene regulatory network, the bHLH-PAS transcriptional activators BMAL1, CLOCK and NPAS2 activate the Period (Per1, Per2) and Cryptochrome (Cry1, Cry2) genes whose transcripts and proteins slowly accumulate during the daytime [98]. Some circadian clock-dependent pathways involved in regulating both anabolism and catabolism, such as lipid metabolism, glucose metabolism and bile acid synthesis [99]. Recently, a research showed that m⁶A modification involved in regulating this process, liver-specific deletion of Bmal1 (Bmal1^{-/-}) results in high levels of ROS and elevated oxidative damage, and subsequently ROS significantly elevates METTL3 expression, which finally suppresses PPAR α (the nuclear receptor peroxisome proliferator-activator α) expression in an m⁶A-YTHDF2-dependent manner, which will influence the lipid metabolism [100]. Recently, it has been reported that m⁶A modification enriched in and enhanced the expression of lipogenic genes in leptin receptor-deficient *db/db* mice, however, YTHDC2 could attenuate lipid metabolic disorder and hepatic TG accumulation by binding and decreasing the stability of lipogenic gene including sterol regulatory element-binding protein 1c (Srebp-1c), fatty acid synthase (Fasn), stearoyl-CoA desaturase 1 (Scd1) and acetyl-CoA carboxylase 1 (Acc1) [101]. m⁶A modification enhances the stability of LINC00958, and subsequently, elevated LINC00958 sponges miR-3619-5p to upregulate hepatoma-derived growth factor (HDGF) expression, thereby facilitating HCC lipogenesis and progression [102]. FTO is known to be tightly associated with increased body mass and obesity in humans [103-105]. FTO is reported to be positively related to obesity and T2D, and FTO levels were significantly increased in NAFLD group [106,107]. These results reveal that m⁶A modification is involved in regulating lipogenesis and NAFLD, which is a major risk of HCC.

Virus infection

Driving factors in hepatocyte transformation and HCC development are chronic inflammation, epigenetic modifications, early neoangiogenesis, senescence, DNA damage, and telomerase reactivation, chromosomal instability [108]. Liver cancer usually develops only after decades of HCV infection, and often accompanied by cirrhosis. As a positive-strand RNA virus, most if not all events related to HCV replication are restricted to the cytoplasm. Therefore, HCV infection induced longstanding hepatic inflammation with associated

oxidative and the potential DNA damage in the presence of cirrhosis is thus likely to contribute to the development of carcinoma [109,110]. HBV contributes to HCC progression through direct and indirect mechanisms. HBV-related HCC, cirrhosis is absent in up to one-third of patients. It has been shown that viral infection can affect m⁶A modification and thus alter m⁶A in special transcripts and influence viral infection [111]. m⁶A modification in the 5' epsilon stem-loop of pgRNA, which mediates HBV life cycle, is necessary for efficient reverse transcription of pgRNA, whereas m⁶A sites in the 3' epsilon stem-loop lead to destabilization of HBV transcripts [112]. This study reveals that m⁶A modification of HBV RNA exerts dual regulatory functions. m⁶A modification negatively regulates HCV life cycle and the production of infectious HCV particles, and m⁶A-binding YTHDF proteins relocalize to lipid droplets, sites of HCV viral assembly, and suppress this stage of viral infection [113]. Recently, it has been demonstrated that HBV infection increases METTL3 and subsequently upregulates m⁶A modification and degradation of PTEN, which consequently inhibits IRF-3 nuclear import and activation of PI3K/AKT pathway to facilitate HCC progression by affecting innate immunity [114]. Taken together, these studies indicated that m⁶A modification plays a significant role in broader HBV/HCV virus infection, and uncover regulatory strategies to inhibit replication by these established and emerging viral pathogens to prevent HCC development.

Chromosome Instability

This missegregation of genomic material is defined as Chromosome instability that is associated with poor prognosis of cancer patients including HCC [115,116]. HBV can facilitate HCC progression by inducing both chromosome instability by integrating its DNA into the host genome and mutagenesis of various cancer-related genes [117]. Furthermore, HBx could directly induce chromosomal instability by affecting the mitotic checkpoints through binding and inactivating p53 and DDB1 [118,119]. Not only virus DNA, altered cellular signaling pathways could also influence chromosome instability signatures. Weiler and colleagues showed that YAP and its binding partner TEAD4 could induce chromosome instability by interacting with FOXM1 [120]. After the inhibition of YAP, about four-fifth of CIN25 and three-fifth of CIN25 were reduced. Although it hasn't been clarified yet whether m⁶A modification is involved in affecting HCC progression by influencing chromosome instability. Several researchers have shown that YAP mRNA was m⁶A modified in HCC [121], colorectal cancer [122], NSCLC [123], and so on.

Those results hint that m⁶A modification may be implicated in HCC development through chromosome instability by influencing hepatic virus infection and some genes which need further investigation.

Neovascularization

Angiogenesis is essential for HCC progression and metastasis. Vasculogenic mimicry (VM) formed by aggressive tumor cells has been found in various types of cancer, including ovarian cancer, colorectal cancer, melanoma and HCC, and associated with poor prognosis in patients [124-126]. Pathologic angiogenesis is critical for the growth and malignant dissemination of solid tumors by providing oxygen, nutrients, and routes for metastasis [127]. Several studies demonstrated that m⁶A modification could also influence HCC progression through regulating angiogenesis. Qiao *et al.* showed that silencing METTL3 suppresses VM formation through inhibiting YAP1 expression [124]. Intratumoral hypoxia is a common signature of human solid cancer including HCC and could regulate the expression of genes that involve in regulating angiogenesis, EMT process, self-renewal of cancer stem cells, chemotherapy- and radiation- therapy resistance extracellular matrix remodeling, cancer stem cell maintenance through hypoxia-induced factor (HIF) [128]. Zhong *et al.* demonstrated that hypoxia down-regulated YTHDF2 expression, and forced YTHDF2 expression could suppress HCC carcinogenesis, proliferation and

activation of MEK and ERK in HCC cells through directly binding the m⁶A sites on EGFR 3'UTR and subsequently promoting its degradation [129]. The activation of EGFR induced by EGF and TGF- α could induce the expression of VEGF which is a predominant stimulator of angiogenesis [130]. Those results illustrated that YTHDF2 may be involved in regulating HCC tumorigenesis by regulating angiogenesis through EGFR/VEGF signaling pathway. Hou *et al.* showed YTHDF2 expression was decreased in hypoxia and tumor tissues, and HIF-2 α antagonist (PT2385) could restore YTHDF2 expression and inhibit HCC progression [131]. Mechanistically, YTHDF2 mediated the decay of m⁶A-modified interleukin 11 (IL11) and serpin family E member 2 (SERPINE2) mRNAs, which were involved in regulating the inflammation-mediated malignancy and disruption of vascular normalization. These studies showed that m⁶A modification might regulate HCC development by modulating neovascularization.

An altered m⁶A modification in HCC

m⁶A modification and m⁶A regulators have been shown to be dysregulated in liver carcinoma, and m⁶A expression influences liver progression through proliferation, metastasis, inflammation, and vascularization (as showed in **Figure 2**). METTL3 facilitates migration, invasion and EMT of cancer cells by promoting Snail (a key transcription factor of EMT) expression through m⁶A-YTHDF1 pathway [56].

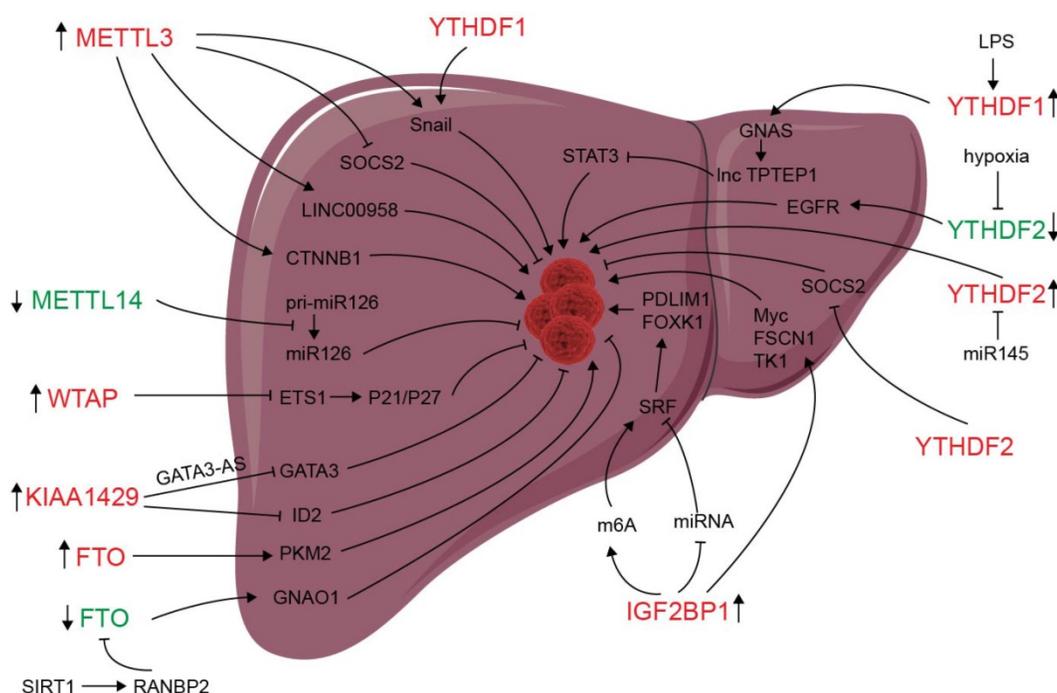


Figure 2. Deregulation of m⁶A modifiers in liver cancers. Modifiers in red indicate an oncogenic role, modifiers in green indicate a tumor-suppressive role.

SUMOylation of METTL3 was positively associated with high metastasis potential of HCC via controlling Snail mRNA homeostasis in HCC [132]. Chen *et al.* showed that METTL3 promotes liver cancer progression by suppressing SOCS2 (suppressor of cytokine signaling 2) via m⁶A-YTHDF2-mediated degradation [55]. SOCS2 has been shown to inhibit proliferation, migration, and stemness in various cancers, including oral squamous carcinoma, leukemia, and HCC [54]. METTL3-mediated m⁶A modification upregulates LINC00958 expression, and subsequently enhances hepatoma-derived growth factor (HDGF) expression by sponging miR-3619-5p, and consequently promotes HCC lipogenesis and progression [102]. METTL3 promoting HCC development by interacting with DGCR8 and promoting the maturation of miR-873-5p in an m⁶A-dependent manner, thereby inhibiting SMG1 expression [133]. METTL3 upregulation promotes hepatoblastoma (HB) progression through enhancing CTNBN1 mRNA stability and subsequently activating Wnt/ β -catenin pathway [134]. WTAP suppresses ETS proto-oncogene 1 (ETS1) in an m⁶A-HuR-dependent manner, which further reverses the inhibition of p21/p27 axis to promote G2/M phase of HCC cells, and consequently promotes HCC progression [83]. KIAA1429 upregulation facilitates migration and invasion of HCC by repressing ID2 mRNA in an m⁶A-dependent manner [135]. lncRNA-GATA3-AS facilitates KIAA1429 induces m⁶A modification and degradation of GATA binding protein 3 (GATA3) pre-mRNA in an HuR-dependent manner, and consequently promotes HCC growth and metastasis [136]. Circ_KIAA1429 (has_circ_0084922) was upregulated and accelerate HCC development by facilitating HCC migration, invasion, and EMT process through enhancing Zeb1 mRNA stability in an m⁶A-YTHDF3 dependent manner [137]. However, METTL14 is downregulated in HCC, especially metastasis cancer, can interact with the microprocessor protein DGCR8 and promote pri-miR126 maturation in an m⁶A-dependent manner, and consequently suppresses HCC metastasis [3]. In summary, those results reveal that m⁶A “writers” complex involved in regulating HCC progression. However, the function of METTL14 and other m⁶A “writers” is apparently controversial. The reasons for the above conflicting findings might be associated with the heterogeneity of clinical samples. Further investigations are needed to settle these contradictory findings and clarify the role of different m⁶A “writer” in the progression of HCC.

Since its identification as the demethylase of m⁶A, FTO has been reported to play an important role in many cancers including liver cancer [138-141]. Li *et*

al. reported a correlation between up-regulated FTO in liver cancer and poor prognosis. It was also shown that FTO mediates demethylation of PKM2 mRNA and promotes its translation to promote HCC progression [142]. Besides its oncogenic function, FTO also suppresses HCC progression. Liu *et al.* reported that SIRT1 activate RANBP2 that mediates FTO SUMOylation and degradation, and subsequently leads to m⁶A deposition in HCC tumor suppressor guanine nucleotide-binding protein G (o) subunit alpha (GNAO1) and inhibits its mRNA expression, and consequently results in liver cancer progression [139]. Recently, a study reveals that FTO plays a protective role in HCC development [143]. Hepatocyte-specific depletion of FTO not only impacts HCC initiation phase (increased tumor numbers) but also influences HCC development (increased numbers of larger tumors) by inhibiting Cul4a translation [143]. Intrahepatic cholangiocarcinoma (ICC) is the second most common form of primary liver cancer. Rong *et al.* demonstrated that loss of FTO in ICC related to poor prognosis, mechanistically, FTO suppresses the anchorage-independent growth and mobility of ICC cells through demethylation and impairing oncogene TEAD2 mRNA stability [140]. These conflicting functions of FTO in HCC and ICC might be associated to heterogeneity of clinical samples and the context-specific m⁶A roles between HCC and ICC. ALKBH5 inhibits HCC cells proliferation and invasion by suppressing IGF2BP1-mediated LY6/PLAUR Domain Containing 1 (LYPD1) RNA stability [144].

The oncogenic or tumor suppressor role of m⁶A readers may be associated with their effect on transcripts processing. Several studies have indicated that chronic inflammation is involved in HCC progression [145]. Innate immune cell types, including macrophages, innate lymphoid cells, NK cells, Dendritic cells (DCs), and mucosal-associated invariant T cells (MALTs) are enriched in liver and play essential roles in liver homeostasis [146]. Traditionally, macrophages can be classified as M1 (pro-inflammatory), M2 (anti-inflammatory), or M_{reg} (immunosuppressive), and the balance of M1-M2 polarization involved in hepatic inflammation and repair [147]. It has been demonstrated that METTL3 facilitates M1 macrophage polarization via the methylation of STAT1 mRNA [148]. Ding *et al.* found that LPS used to stimulate the inflammatory response in HCC cells enhances GNAS expression in an m⁶A-YTHDF1-dependent manner [149]. Elevated G-protein alpha-subunit (GNAS) expression can promote inflammation-related HCC progression through promoting STAT3 activation by inhibiting lncRNA TPTEP1 interaction with STAT3 (signal

transducer and activator of transcription 3). YTHDF2 promotes the liver cancer stem cell phenotype and cancer metastasis by promoting OCT4 expression [150]. Hou and colleagues described that hypoxia-inducible factor-2 α (HIF-2 α) inhibits YTHDF2 expression, resulting in elevated expression of m⁶A-containing interleukin 11 (IL11) and serpin family E member 2 (SERPINE2) mRNAs, which further leads to inflammation and vascular abnormalities in HCC [151]. YTHDF2, downregulated by hypoxia, can suppress HCC progression through binding m⁶A sites of EGFR 3'UTR and inducing its degradation, and consequently suppresses cell proliferation, tumor growth, and activation of MEK and ERK in HCC cells [129]. However, another research reported that YTHDF2 upregulated and exerts as an oncogene in HCC and can be inhibited by miR-145 [152]. ICF2BP1/2/3 protein were identified as m⁶A-binding proteins and plays an oncogenic role in HeLa (cervical cancer) and HepG2 (liver cancer) cells via enhancing the stability of MYC, FSCN1, and TK1 mRNAs [35]. IGF2BP1 promotes SRF expression in an m⁶A- and miRNA- dependent manner and subsequently promotes SRF downstream target gene including PDLIM7 and FOXK1 translation, consequently PDLIM7 and FOXK1 promote tumor cell growth and enhance cell invasion, and indicate a poor overall survival probability in liver, ovarian and lung cancer [153].

These studies illustrate a complicated role of m⁶A modification and m⁶A regulators in liver carcinogenesis. m⁶A modification and m⁶A regulators involve in regulating cancer cell proliferation, migration, invasion, immunity, vascular abnormalization, and EMT process. However, some of the studies have reported conflicting results on the expression and function of various m⁶A regulators. Most of MTCs upregulated and promote HCC progression, however, METTL14 is decreased and could suppressed liver cancer cell metastasis. The role of FTO and YTHDF2 are double-edged sword. When they down-regulated in carcinoma tissues, they may play a tumor suppressive role. However, if they up-regulated, they may be an oncogenic factor. The expression of FTO and YTHDF2 can be influenced by tumor heterogeneity, hypoxia, and post-translational modification. All the discrepant results of the above studies uncover a complicated role of m⁶A modification and m⁶A regulators in human HCC. Further investigation and effort will be required to reconcile these paradoxical results. Altered expression, function, and mechanism of m⁶A regulators in liver carcinoma are listed in **Table 1**.

Prognosis and therapeutic potency of targeting altered m⁶A regulators

Since m⁶A modification levels and m⁶A regulators play essential roles in regulating the progression of liver disease, targeting abnormal expressed m⁶A regulators may serve as a biomarker and potential therapeutic targets. Several studies showed that liver carcinogenesis is related to the altered expression of m⁶A regulators [3,129,135]. Bioinformation analysis in several studies reveal that METTL3 and YTHDF1 are both significantly upregulated and are associated with poor prognosis in HCC patients [53,154,155]. METTL3, VIRMA, and hnRNPC showed higher expression in tumor samples, while METTL14, ZC3H13, FTO, ALKBH5, YTHDF2, YTHDC1, and YTHDC2 showed higher expression in normal tissue samples [53,152,156,157]. High expression level of METTL14 is associated with a better prognosis in HCC patients [156]. YTHDF2 expression is closely associated with HCC malignance, and can be inhibited by miR-145 [152]. YTHDF2, YTHDF1, METTL3, KIAA1429, and ZC3H13 could be a prognostic signature of malignant HCC patients [52,157-159]. The expression of two genes (hnRNPA2B1 and RBM15) are closely related to the TNM stage and metastasis risk and can act as a prognostic indicator for HBV-related HCC patients in the early TNM stage [160]. In conclusion, METTL3, YTHDF1 are elevated and METTL14 is down-regulated in HCC and can be prognosis biomarker of HCC.

m⁶A levels in circulating tumor cells (CTCs) can be detected by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS). The authors showed that m⁶A modification levels in CTCs was significantly elevated compared to the whole blood cells and m⁶A levels in CTCs might be a non-invasive approach for cancer diagnosis [161]. Therefore, the altered expression of m⁶A regulators may be a biomarker for prognostic prediction in HCC patients.

Several studies demonstrated that dysregulation of m⁶A regulators associated with drug resistance. Taketo and colleagues showed that METTL3-depleted pancreatic cancer cells showed higher sensitivity to anticancer reagents such as 5-fluorouracil, gemcitabine, cisplatin and irradiation [162]. METTL3 knockdown in NSCLC inhibits tumor growth, metastasis and DDP resistance [163]. METTL3 induces the production of p53 R273H mutant protein that results in acquired multidrug resistance in colon cancer cells [164]. Elevated METTL3 expression is implicated in glioma stem-like cell maintenance and radioresistance [165]. The upregulation of FTO

enhances chemo-radiotherapy resistance in cervical cancer [166]. Depletion of METTL3 under hypoxia significantly enhances sorafenib-resistant of HCC by decreasing the stability of FOXO3, and overexpression of FOXO3 restores m⁶A-dependent sorafenib sensitivity [167]. These results highlight the therapeutic value of targeting m⁶A modification and m⁶A regulators in drug-resistant tumors.

m⁶A regulators can be targeted by inhibitors. The nature product rhein is the first identified FTO inhibitor that exerts good inhibitory activity of FTO demethylation and led to increases of m⁶A modification levels [168,169]. However, it is not an FTO selective inhibitor, as it can also target ALKBH5 [170]. Entacapone, an FDA approved drug used to treat Parkinson's disease, has also be identified as FTO inhibitor that elicited the effects of FTO on gluconeogenesis in the liver and thermogenesis in adipose tissues in mice by acting on an FTO-FOXO1 regulatory axis [171]. Meclofenamic acid (MA), a non-steroidal, anti-inflammatory drug, is to be identified as highly selective inhibitor of FTO [30]. Most recently, Su *et al.* reported two small-molecule inhibitors of FTO that can suppress leukemia stem/initiating cell maintenance and immune evasion by

suppressing expression of immune checkpoint genes, especially LILRB4 [172]. Administration of PT2358, an antagonist HIF-2 α , restores YTHDF2 expression which attenuates tumorous inflammation and angiogenesis, thus inhibiting HCC progression [151].

The m⁶A modification in liver can be influenced by gut microbiota, which can further alter lipid metabolism and insulin signaling [173]. Lu *et al.* reported that curcumin can affect METTL3, METTL14, ALKBH5, FTO, and YTHDF2 expression and subsequently increase m⁶A modification to inhibit LPS-induced liver injury and lipid metabolism disorders in piglets through inhibiting SREBP-1c (sterol regulatory element binding proteins) and SCD-1 (stearoyl-CoA desaturase 1) expression in an m⁶A dependent manner [174]. Those studies reveal that altered m⁶A modification involved in regulating liver cancer cell metabolism, proliferation, invasion, and immunity, and is associated with clinical prognosis and progression, indicating that altered expression of m⁶A regulators may serve as potential biomarkers and therapeutic targets. However, more studies are required to determine the function of m⁶A modification on liver carcinogenesis and the potential as therapeutic targets.

Table 1. Roles of m⁶A regulators in liver carcinoma

Proteins	Cancer	Role	Function	Mechanism	Reference
METTL3	HCC	Oncogene	Regulating EMT progression	Promoting Snail expression	[56, 132]
	HCC	Oncogene	Promoting HCC cell proliferation and migration	Regulating SOCS2 expression	[55]
	HCC	Oncogene	Promoting HCC lipogenesis and progression	m ⁶ A modification enhances LINC00958 RNA stability and expression that sponged miR-3619-5p to upregulate HDGF expression	[102]
	HCC	Oncogene	Promoting the development of HCC	Promoting the maturation of miR-873-5p and thereby inhibiting SMG1 expression	[133]
	HB	Oncogene	Promoting proliferation of HB	Regulating Wnt/ β -catenin pathway activation through regulating CTNBN1 expression	[134]
METTL14	HCC	Suppressor	Inhibiting the migration and invasiveness	Regulating pre-miR-126 maturation through interacting with DGCR8	[3]
WTAP	HCC	Oncogene	WTAP modulated the G2/M phase of HCC cells	Suppressing ETS1 expression in m ⁶ A-HuR dependent manner	[83]
KIAA1429	HCC	Oncogene	Enhancing proliferation, migration, and invasion of HepG2 cells	Inhibiting ID2 expression	[135]
	HCC	Oncogene	Promoting proliferation and metastasis of HCC cells	Leading to the degradation of GATA3 pre-mRNA	[136]
FTO	HCC	Oncogene	Promoting proliferation and <i>in vivo</i> tumor growth	Promote PKM2 expression	[142]
	HCC	Suppressor	SIRT1 activates RANBP2 that further mediates FTO SUMOylation and degradation	Decreased FTO leads to hypo m ⁶ A modification of GNAO1 and reduced its RNA stability	[139]
	HCC	Suppressor	FTO exerts protective role in the initiation of HCC	Inhibiting Cul4a translation	[143]
	ICC	Suppressor	FTO suppresses the anchorage-independent growth and mobility of ICC cells	Impairing oncogene TEAD2 mRNA stability	[140]
ALKBH5	HCC	Suppressor	ALKBH5 inhibits the proliferation and invasion capabilities of HCC cells <i>in vitro</i> and <i>in vivo</i>	Reducing the stability of LYPD1 mediated by m ⁶ A reader IGF2BP1	[144]
YTHDF1	HCC	Oncogene	Regulating EMT progression	Promote METTL3 target gene snail translation	[56]
	HCC	Oncogene	Promoting LPS-induced HCC cell growth and invasion	Promoting GNAS translation	[149]
YTHDF2	HCC	Oncogene	Promoting HCC proliferation and migration	Interacting with METTL3 to regulating SOCS2 expression	[55]
	HCC	Oncogene	Promoting the liver cancer stem cell phenotype and cancer metastasis	Promoting OCT4 expression	[150]
	HCC	Suppressor	YTHDF2 silencing provokes inflammation, vascular reconstruction and metastatic progression of HCC	Mediating decay of IL11 and SERPINE2	[151]
	HCC	Suppressor	Suppressing HCC cell proliferation, growth and induces apoptosis	Destabilizing of EGFR mRNA	[129]
YTHDF3	HCC	Oncogene	Facilitating HCC migration, invasion, and EMT process	Enhancing Zeb1 mRNA stability	[137]
IGF2BP1	HCC	Oncogene	Promoting tumor cells proliferation colony formation ability and cell migration/invasion	Enhancing the stability of MYC, FSCN1, and TK1	[35]
	HCC	Oncogene	Promoting tumor cells proliferation and invasion	Enhancing SRF mRNA stability in an m ⁶ A-dependent manner	[153]

Challenges and Perspectives

m⁶A is the most abundant internal modification which randomly exists on almost all types of RNAs, including mRNA, lncRNA, circRNA, miRNA, rRNA and snoRNA. Numerous studies have shown that m⁶A modification plays an essential role in physiological and pathological processes including cell differentiation, meiosis, sex determination, cancer progression, circadian rhythm, neuronal function, and chromatin state. m⁶A modification is induced by MTC consisting of METTL3, METTL14, WTAP, VIRMA, ZC3H13, HAKAI and RBM15/RBM15B, in which METTL3 is the only catalytic subunit. m⁶A in structural RNAs and U6 snRNAs can be methylated by METTL16, whereas METTL5 mediates m⁶A modification of 18 S rRNA and facilitates ZCCHC4 as the 28 S rRNA modification enzyme. m⁶A_m modification is induced by PCIF1 and METTL4 mediates m⁶A_m modification in internal U2 snRNAs. m⁶A deposition is removed by eraser proteins (FTO and ALKBH5). As the first identified m⁶A demethylase, FTO can not only demethylate internal m⁶A and cap m⁶A_m but also mediates demethylation of m⁶A and m⁶A_m in snRNAs and m¹A in tRNAs.

In recent years, global m⁶A modification has been well described well in different disease contexts by using NGS. m⁶A deposition is not randomly distributed in transcripts but collectively deposited in 3'UTR and CDS regions, especially the stop codon. m⁶A deposition sites can be influenced by writers, erasers, histone modification, transcription factors, virus infection, chemical carcinogens, carRNAs, and environmental stimuli. The global m⁶A abundance can be detected by MeRIP-seq or m⁶A-seq. However, this approach has some limitations; it needs more initial RNA material; the m⁶A-antibody cross talks with m⁶A_m and it cannot accurately position on m⁶A sites. Another technique uses m⁶A-sensitive RNA-endoribonuclease, which can cleave RNA at unmethylated ACA motifs and is used to detect m⁶A distribution (m⁶A-REF-seq or MAZTER-seq). However, this method is not useful for m⁶A sites in other sequence contexts. Mapping m⁶A sites precisely is a critical step for understanding its function and regulatory pathways. In this context, DART-seq has substantially improved the transcriptome-wide m⁶A level detection and provides an efficient detection method for limiting-amounts of RNA samples.

In many disease types, global m⁶A abundance, as well as m⁶A regulators, are altered. Because of the simultaneous alteration of m⁶A writers and erasers, the global m⁶A level changes may not be informative in carcinoma. Some investigators demonstrated that increased m⁶A modification in liver carcinoma is

associated with poor prognosis. However, other groups demonstrated that global m⁶A levels decrease in HCC tissues especially metastatic tumors. Therefore, the mechanism of m⁶A regulation in liver carcinoma progression needs further investigation. Recent studies also revealed that m⁶A modification affects ncRNAs processing such as maturation, cellular location, and function. Further investigations are required to understand the molecular details of these progresses affecting liver cancer progression.

Conclusions

m⁶A modification is a new layer of post-transcription regulation of gene expression. The dysregulated expression of m⁶A regulators involved in human carcinogenesis has been demonstrated in various cancer types, including HCC. Altered m⁶A regulators expression will influence target gene expression by modulating the mRNA stability and translation. m⁶A modification also plays an essential role in mediating cancer cells response to immunotherapy, radiotherapy, and chemotherapy. However, the functional role of m⁶A in HCC are paradoxical, further studies are needed to illustrates the heterogeneity and complexity of m⁶A modification and m⁶A regulators in HCC carcinogenesis. Given the pivotal role of m⁶A modification and regulation in many cancers, targeting dysregulated m⁶A regulators may be a therapeutic option in cancer. Therefore, a better understanding of m⁶A may improve cancer therapy in the future.

Abbreviations

m⁶A: N⁶-methyladenosine; HCC: Hepatocellular Carcinoma; Ψ: pseudouridine; m¹A: N¹-methyladenosine; m⁶A_m: N⁶, 2-O-dimethyladenosine; ncRNAs: non-coding RNAs; CDS: coding sequences; 3'UTRs: 3'untranslated regions; MTC: m⁶A methylase complex; IGF2BPs: insulin-like growth factor 2 mRNA-binding proteins; NAFLD: non-alcoholic fatty liver disease; HPLC-MS/MS: liquid chromatography coupled to tandem mass spectrometry; PA-m⁶A-seq: photo-crosslinking-assisted m⁶A sequencing strategy; m⁶A-CLIP-seq: UV cross-linked m⁶A antibody-bound RNA sample; a⁶A: N⁶-allyladenosine; hm⁶A: N⁶-hydroxymethyladenosine; dm⁶A: N⁶-dithiolisitolmethyladenosine; m⁷G: 7-methylguanosine; carRNAs: chromosome-associated regulatory RNAs; FMRP: fragile X mental retardation protein; CSC: cigarette smoke condensate; PM: particulate matter; HMPV: human metapneumovirus; dCas9: dead Cas9; NASH: nonalcoholic steatohepatitis; HFD: high-fat diet; T2D: Type 2 diabetes; Fasn: fatty acid synthase; PPARα: the

nuclear receptor peroxisome proliferator-activator α ; Scd1: stearyl-CoA desaturase 1; Acc1: acetyl-CoA carboxylase 1; HDGF: hepatoma-derived growth factor; HBV: Hepatitis B virus; HB: hepatoblastoma; ETS1: ETS proto-oncogene 1; GATA3: 3' UTR of GATA binding protein 3; GNAO1: guanine nucleotide-binding protein G (o) subunit alpha; ICC: Intrahepatic Cholangiocarcinoma; DCs: dendritic cells; MALTs: mucosal-associated invariant T cells; GNAS: G-protein alpha-subunit; HIF-2 α : hypoxia-inducible factor-2 α ; IL11: Interleukin 11; SERPINE2: serpin family E member 2; CTCs: circulating tumor cells; LC-ESI-MS/MS: liquid chromatography-electrospray ionization-tandem mass spectrometry; MA: Meclofenamic acid; SREBP-1c: sterol regulatory element-binding protein 1c; SCD-1: stearyl-CoA desaturase 1.

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Authors' contributions

X.Y.P conceived and wrote the manuscript. C. H and J.L. conceived and revised the manuscript. All authors read and approved the final manuscript.

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Competing Interests

The authors have declared that no competing interest exists.

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