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REVIEW ARTICLE

RNA N^6-methyladenosine modification in solid tumors: new therapeutic frontiers

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Abstract

Epigenetic mRNA modification is an evolving field. N^6-methyladenosine (m^6A) is the most frequent internal transcriptional modification in eukaryotic messenger RNAs (mRNAs). This review will discuss the functions of the m^6A mRNA machinery, including its "writers" that are components of the methyltransferase complex, its "readers" and its "erasers" (specifically FTO and ALKBH5) in cancer. The writers deposit the m^6A and include METTL3, METTL14, WTAP, VIRMA, and RBM15. M^6A methylation is removed by the m6A demethylases (FTO and ALKBH5). Lastly, the most diverse members are the readers that can contribute to mRNA splicing, stability, translation, and nuclear export. Many of these functions continue to be elucidated. The dysregulation of this machinery in various malignancies and the associated impact on tumorigenesis and drug response will be discussed herein with a focus on solid tumors. It is clear that, by contributing to either mRNA stability or translation, there are downstream targets that are impacted, contributing to cancer progression and the self-renewal ability of cancer stem cells.

Introduction

In the past decade, there have been progressive studies demonstrating that mRNA modification occurs to impact RNA stability and translation, thus impacting the control of gene expression. The most common form of >170 RNA nucleotide modifications is N^6-methyladenosine (m^6A) [1, 2]. This modification is reversible [3, 4] and has been found to impact >7000 mRNAs in mammalian cell individual transcriptomes [5, 6]. There is additional data demonstrating that m^6A modification in mRNAs or non-coding RNAs impact RNA translation and transcript fate/functions. These are critical for many physiologic processes, including the DNA damage response, tissue development (hematopoiesis and neurogenesis), circadian rhythm regulation, sex differentiation, microRNA processes, RNA–protein exchanges, and carcinogenesis [5–17].

Aberrant cell growth in tumorigenesis has historically been defined by abnormalities in cell division and gene expression as dictated by abnormalities in genetic and epigenetic changes. These abnormalities can be a function of genetic changes (e.g., gene mutations, deletions, amplification, or chromosomal translocations) and/or epigenetic changes, such as DNA or histone modification. In the past 10 years, RNA epitranscriptomics or gene regulation at the RNA level has gained more interest as an additional layer of influence in the development of malignancies. Of the various RNA modifications, m^6A has been identified as a reversible RNA modification similar to the well-described histone and DNA modifications that are also reversible. With the development of high-throughput m^6A sequencing techniques, there have been identified thousands of mRNA and non-coding RNA transcripts that are associated with m^6A modifications with an additional enrichment in the 3' untranslated regions in close proximity to the stop codons of mRNAs [5, 6].

In the framework of mRNA m^6A modification, there are methyltransferases and demethylases and in between there are proteins identified as "readers." The readers can promote decay or enhance RNA stability, promote translation, and impact splicing and nuclear export of various target mRNAs [10–12, 18–22]. Therefore, the type of reader protein that

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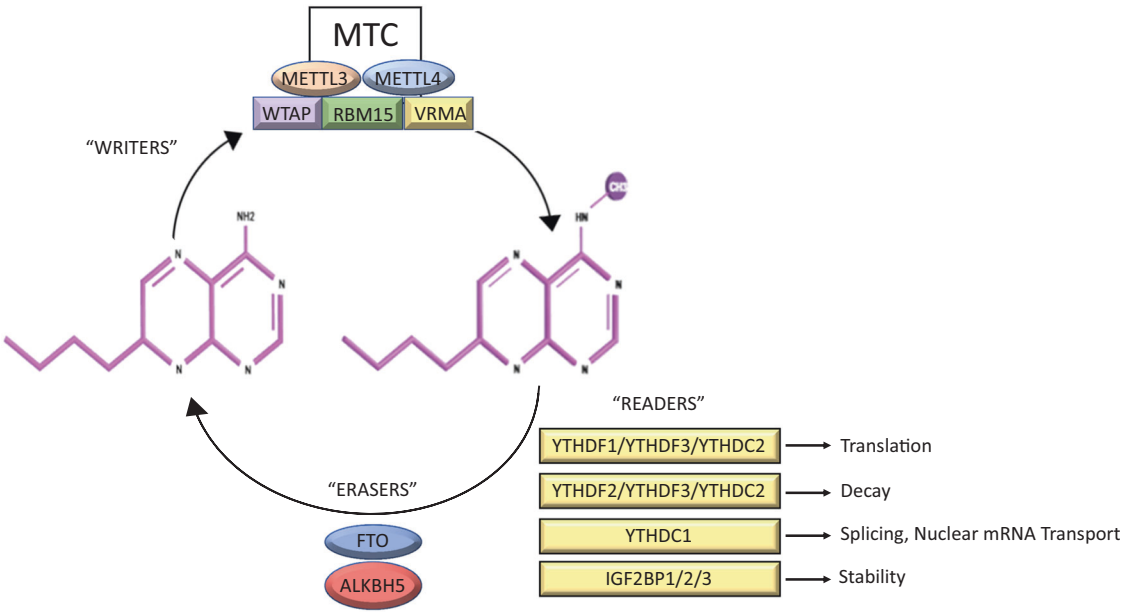

Fig. 1 m^{6}A modification machinery summary. The MTC is the m^{6}A methyltransferase complex. The MTC is composed of WTAP, VIRMA, METL3, METTL14, and RM15. The MTC serves as methylase or "writer." FTO and ALKBH5 are demethylases or "erasers." The "readers" have a variety of functions, including translation (YTHDF1, YTHDF3, YTHDC2), decay (YTHDF2, YTHDF3, YTHDC2), splicing, nuclear export (YTHDC1), and stability (IGF2BP1/2/3).

recognizes the m^{6}A modification of a given target mRNA can impact the stability of the target mRNA and can affect RNA translation, splicing, or nuclear transport (Fig. 1). This level of regulation—with the concept of mRNA "writers" (methyltransferases), "readers," and "erasers" (demethylases)—is still a field in its infancy as it pertains to dysregulation in solid tumors.

m^{6}A modification machinery

"Writers" such as methyltransferase-like 3 and 14 (METTL3 and METTL14, respectively) and their respective cofactors RBM15, Wilms tumor1-associated protein (WTAP), RBM15, and VIRMA (KIAA1429) make up the m^{6}A methyltransferase complex (MTC). This grouping of proteins functions as the m^{6}A writer and catalyzes the m^{6}A modification [8, 23–27]. Our recent studies have further characterized how m^{6}A is specifically deposited in the transcriptome. Huang et al. demonstrated that histone H3 trimethylation at Lys36 (H3K36me3), a marker for transcription elongation, guides m^{6}A deposition co-transcriptionally [28]. The mechanism is that H3K36me3 is recognized and bound directly by METTL14, which as noted above is a crucial part of the MTC and thus facilitates binding of the m^{6}A MTC to RNA polymerase II, thus delivering the m^{6}A MTC to actively transcribing RNAs to deposit m^{6}A co-transcriptionally. This work uncovers another layer of gene expression regulation involving the communication between histone modification and RNA methylation [28]. Weng et al. from our group demonstrated that METTL14 is highly expressed in normal hematopoietic stem/progenitor cells and acute myeloid leukemia (AML) cells carrying t(11q23), t(15;17), or t(8, 21) and is down-regulated during normal myeloid differentiation [29]. Inhibiting METTL14 induces terminal myeloid differentiation in AML cells and inhibits AML cell survival and growth. The pro-oncogenic role of METTL14 in AML is by regulating its mRNA targets (e.g., *MYB* and *MYC*) via m^{6}A modification [29].

Recognized demethylases or "erasers" are FTO (fat mass- and obesity-associated gene) and ALKBH5 [4, 9, 30]. These proteins function to remove the m^{6}A modification from mRNA and create a counter balance to the "writers."

The "readers" are a functionally more heterogeneous family of proteins with more diverse functionality. The YT521-B homology (YTH) domain family including YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3 are considered direct readers [10–12, 18–20]. YTHDF2, YTHDF3, and YTHDC2 promote degradation of their target mRNAs. YTHDF1, YTHDF3, and YTHDC2 promote translation, while YTHDC1 influences splicing and targets mRNA exportation. Our group has reported that insulin-like growth factor 2 mRNA-binding proteins (IGF2BPs; including IGF2BP 1/2/3) are a distinct family of m^{6}A readers [22]. These target many mRNA transcripts via identifying the consensus GG(m^{6}A)C sequence [22]. In contrast to the mRNA-decay-promoting function of YTHDF2, IGF2BPs promote the stability and storage of

their target mRNA (e.g., *MYC*) and also promote their translation in an m^6A-dependent manner and therefore impact gene expression [22]. Additional readers include eukaryotic initiation factor 3 (eIF3) and cytoplasmic METTL3 [13, 31].

Recent work has intimated that antitumor immunity may be in part controlled through mRNA m^6A methylation and the "reader" YTHDF1 in dendritic cells [32]. In the context of antitumor immunity, tumor regression often correlates to the neoantigen burden. Han et al. show that durable neoantigen-specific immunity is regulated by m^6A methylation via the reader YTHDF1. They demonstrate that *Ythdf1*-deficient mice showed an amplified antigen-specific CD8+ T cell antitumor response. More specifically, the loss of YTHDF1 in classical dendritic cells amplified the cross-presentation of tumor antigens and the cross-priming of CD8+ T cells in vivo. Transcripts encoding lysosomal proteases are marked by m^6A and identified by YTHDF1. The binding of YTHDF1 to these specific transcripts amplifies the translation of lysosomal cathepsins in dendritic cells and inhibition of the cathepsin increases cross-presentation of wild-type dendritic cells. In addition, the therapeutic efficacy of programmed death ligand 1 checkpoint blockade is much more effective in *Ythdf1*−/− mice, implying that YTHDF1 is a potential target that can amplify anticancer immunotherapy [32].

Aberrations in the m^6A mRNA modification machinery have recently been associated with several malignancies (Table 1), including leukemia, glioblastoma, breast cancer, hepatocellular cancer (HCC), cervical cancer, lung cancer, and gastric cancer (GC) [29, 33–36]. There is also emerging data that targeting various aspects of this system will lead to novel therapies. The aim of this review is to highlight recent developments on the role of the m^6A mRNA modification machinery as it pertains to the development, propagation, and treatment of solid tumors.

The oncogenic "ERASER" FTO: a role in liquid and solid tumors

Approximately 10 years ago, single-nucleotide polymorphisms (SNPs) in FTO were found to be strongly associated with obesity and body mass index (BMI) in humans as determined by genome-wide association studies [37, 38]. More recently, as the m^6A mRNA modification proteins have been characterized, there has been more interest in FTO as a demethylase or "eraser." FTO catalyzes the demethylation of 3-methyl-thymine in single-stranded DNA with Fe(II) and 2-oxoglutarate producing carbon dioxide, formaldehyde, and succinate [3]. Based on its jelly-roll motif protein folding structure, FTO functions with high affinity to m^6A in mRNA whereby it functions as an efficient demethylase [4].

The direct causality of FTO and higher BMI or obesity has not been definitively elucidated. However, it is generally believed to be associated with a greater intake of calories perhaps secondary to FTO expression in the hypothalamus [39, 40]. As obesity is associated with several cancers, there are studies that correlate the connection between FTO and some obesity-associated cancers [41, 42]. One mechanism whereby the FTO gene is regulated is by DNA methylation. Hypomethylation of specific CpG sites in the FTO gene leads to increased FTO expression and this correlates with the presence of type 2 diabetes mellitus and some cancers [43].

Initial studies identified some FTO gene SNPs that were associated with the risk of certain cancers, including endometrial cancer and pancreatic cancer [44–46]. In breast cancer, SNPs in intron 1 of FTO including rs8047395, rs9939609, and rs7206790 have been identified as having important associations with development of this disease [47]. The precise mechanisms whereby these malignancy risk-associated SNPs in FTO remain to be elucidated.

Our group demonstrated that FTO facilitates oncogenesis in AML [48]. FTO targets genes such as *ASB2* and *RARA* as a demethylase. FTO is overexpressed in certain subtypes of AML and promotes leukemogenesis and prevents all-*trans*-retinoic acid-induced leukemia cell differentiation. Thus FTO functions as an oncogene in this disease by inhibiting mRNA targets such as *ASB2* and *RARA* by reducing their m^6A levels and stability [48]. This work revealed a previously unidentified method of gene regulation in carcinogenesis and highlights the significance of the FTO gene and m^6A mRNA demethylation in cancer.

Table 1 Described m^6A member machinery in cancer.

Component	m6A role	Role	Cancer studied	References
FTO	Eraser	Oncogenic	AML, GBM	[48, 52]
ALKBH5	Eraser	Oncogenic	GBM, breast, pancreas	[53, 66, 75]
METTL3	Writer	Oncogenic, tumor suppressor	AML, GBM, HCC, pancreas, breast	[52, 67, 74, 77]
METTL14	Writer	Tumor suppressor	GBM, HCC, endometrial	[36, 52, 73]
IGF3BP1/2/3	Writer	Oncogenic	GBM	[22]
YTHDF1	Reader	Oncogenic	Colorectal cancer, melanoma	[32]

Su et al. from our group has also demonstrated that high levels of FTO sensitize leukemic cells to the oncometabolite R-2-hydroxyglutarate (R-2HG) [34]. R-2HG is produced to relatively high levels by mutant isocitrate dehydrogenase1/2, which is found in 10–20% of AML patients [49]. R-2HG exerts antitumor activity via inhibition of leukemia cell proliferation/viability and induction of cell-cycle arrest and apoptosis. R-2HG inhibits FTO (demethylase) activity and therefore increases m^6A mRNA modification. This in turn decreases the stability of MYC/CEBPA transcripts and thus suppresses relevant downstream pro-tumor pathways [34]. These mechanistic findings have been limited in solid tumors; however, there is emerging data of FTO and other members of the m^6A mRNA modification machinery and the implications of tumorigenesis. Most recently, Huang et al. developed an effective FTO-specific inhibitor, namely, FB23–2, and showed that targeting FTO by small-molecule inhibitors such as FB23–2 can significantly inhibit AML cell viability/growth, promote apoptosis and inhibit AML progression in vivo [50]. Thus these studies provide proof-of-concept evidence suggesting that FTO is a druggable target and targeting FTO by effective inhibitors holds great therapeutic potential to treat FTO-overexpressing AML [34, 50].

In solid tumors such as melanoma, a recent report found that FTO regulates melanoma tumorigenecity and response to anti-programmed death 1 (anti-PD-1) blockade. FTO knockdown increased the m6A methylation of protumorigenic genes, including PD-1, C-X-C chemokine motif receptor 4, and SOX10 that lead to increased RNA decay via the reader YTHDF2. In addition, FTO knockdown sensitized melanoma to interferon gamma and subsequently to anti-PD-1 treatment in mice. This highlights the role of targeting FTO in melanoma to improve sensitivity to checkpoint inhibitors [51].

Glioblastoma

Glioblastoma is the most common and aggressive primary malignant brain tumor, and even with surgical resection, recurrence is common [52]. Cui et al. found that RNA m^6A RNA methylation regulates the self-renewal and tumorigenesis of glioblastoma stem cells (GSCs) by the regulation of mRNA m^6A enrichment and expression [53]. In glioblastoma, similar to in leukemia, R-2HG displays antitumor effects by inhibition of proliferation/survival in FTO-overexpressing cancer cells and targeting of the FTO/m^6A/MYC/CEBPA signaling [34]. These pathways are important in cell proliferation and survival. This work demonstrated why it would be reasonable to target FTO in both glioblastoma and AML as noted above.

Zhang et al. queried The Cancer Genome Atlas (TCGA) to assess which components of the m^6A machinery were associated with poor patient prognosis [54]. They found that the "eraser" ALKBH5 predicted poor prognosis in all data sets [54]. They showed that targeting ALKBH5 impaired self-renewal and decreased proliferation and tumorigenesis in GSCs. The downstream targets of ALKBH5 in GSCs were evaluated and FOXM1, a key transcription factor important in GSCs, was identified [54]. Mechanistically, ALKBH5 was found to demethylate FOXM1 nascent transcripts leading to increased expression and this may be an avenue to therapy for glioblastoma [54].

RNA methylation in GC

GC is the third most frequent cause of cancer-related mortality and is the fifth most common cancer in the world [55, 56]. The management for early disease is surgery with or without systemic therapy. Advanced disease is managed with chemotherapy and several targeted therapies as a function of tumor characteristics. Xu et al. demonstrated that, by immunohistochemistry and tissue microarray, FTO is markedly increased in GC tissues compared to adjacent non-tumor tissues (56% vs 38%) [57]. FTO expression was significantly associated with poor differentiation and lymph node metastases and positively correlated with worse stage. High FTO expression was also significantly associated with poor prognosis. Downregulation of FTO expression inhibited the proliferation, migration, and invasion of GC cell lines in vitro [57].

Mechanistically, Zhang et al. demonstrated that reduced m^6A modification predicts malignant phenotypes and augmented Wnt/phosphoinositide 3-kinase (PI3K)-Akt signaling in GC [33]. Utilizing a proteomics-based GC cohort they had previously generated and the TCGA-GC cohort, they merged the expression of canonical m^6A writers (METTL3/METTLE14), readers (YTHDF1/YTHDF2/YTHDF3), and erasers (ALKBH5/FTO), respectively, as W, R, and E signatures to represent the m^6A modification. They stratified patients according to those signatures to decipher m^6A's associations with critical mutations, prognosis, and clinical indices. m^6A's biological function in GC was predicted by gene set enrichment analysis and validated via in vitro experiments. W and R were potential tumor-suppressive signatures and E was a potential oncogenic signature in GC. Based on W/R/E stratifications, patients with low m^6A were associated with higher mutations of specific genes (*CDH1*, *AR*, *GLI3*, *SETBP1*, *RHOA*, *MUC6*, and *TP53*) and also demonstrated worse clinical outcomes [33]. Via in vitro experiments, they demonstrated that m^6A suppression (as METTL14 knockdown) promoted cell proliferation and invasiveness via activating Wnt and PI3K-Akt signaling, while m^6A elevation (i.e., *FTO* knockdown) reversed these changes [33]. In addition, the findings implied that m^6A modification may be involved in

interferon signaling and immune responses in GC. These data imply that targeting the "erasers" such as FTO or amplifying the "writers" such as METTL14 may be therapeutic avenues to pursue as it pertains to the m^6A machinery in GC [33].

Writers and erasers in breast cancer

Breast cancer is the most prevalent malignancy in women. Although there has been great progress in this disease, the primary cause of mortality is secondary to distant metastases [58]. The population of tumor-initiating cells or breast cancer stem cells (BCSCs) have the capability of self-renewal [59]. The phenotype of these cells is characterized by the expression of several core pluripotency proteins, including Kruppel-like factor 4 (KLF4), Octamer-binding transcription factor 4 (OCT4), sex-determining region Y (SRY)-box 2 (SOX2), and NANOG [60–64]. In the context of metastatic disease, intratumor hypoxia leads to the expression of the transcription factor hypoxia-inducible factor (HIF)-1α [65]. There is recent work indicating that HIFs are necessary for the maintenance of BCSCs via transcriptional regulation of genes encoding the pluripotency-associated genes NANOG, SOX2, and KLF4 [66]. Pluripotency factors have been associated with changes in mRNA stability as dictated by m^6A mRNA methylation. Zhang et al. have reported that exposure of breast cancer cells to hypoxia induces m^6A demethylation and stabilization of NANOG mRNA, thus supporting the BCSC phenotype [67]. In addition, they showed that downregulating the expression of the *ALKBH5* (coding a demethylase or eraser) or HIF-1s (which activate *ALKBH5* gene transcription in hypoxic breast cancer cells) led to decreased NANOG expression and growth inhibition of BCSCs in vivo [67]. Further work is needed to assess whether competitive agonists of ALKBH5 may be useful as therapy that targets BCSCs.

In other work, Cai et al. demonstrated that METTL3 overexpression in breast cancer drives the progression of breast cancer via inhibiting tumor-suppressor let-7g [68]. Initial observations were that the overexpression of both METTL3 and the oncoprotein mammalian hepatitis B X-interacting protein (HBXIP) were associated with breast cancer [68–70]. Mechanistically, they were able to demonstrate that HBXIP modulates *METTL3* by inhibiting miRNA let-7g, which downregulates the expression of *METTL3*. Interestingly, they found that METTL3 promoted the expression of *HBXIP* via m^6A modification essentially creating a feedback loop [70]. These findings provided new insights into the mechanism of m^6A mRNA modification in the progression of breast cancer and more work remains to be done in this field.

m^6A machinery in the liver: non-alcoholic steatohepatitis (NASH) and HCC

NASH is a rising etiology of liver failure worldwide. With the rise of obesity, the prevalence of NASH continues to increase and also correlates with the incidence of HCC in this population [71]. At present, there are no Food and Drug Administration-approved medications for the treatment of NASH. NASH is histologically characterized by hepatocyte ballooning, inflammation, focal fibrosis, and steatosis [72]. It is further described as lipotoxicity in hepatocytes. There are limited treatment options. Lim et al. demonstrate that the expression of FTO is significantly increased in the livers of NASH patients as well as in a rodent model [72]. They demonstrated that genetic silencing of FTO protects against palmitate-induced oxidative stress, mitochondrial dysfunction, endoplasmic reticulum stress, and apoptosis in vitro [72]. These results indicate that FTO overexpression may have a deleterious role in hepatocytes, and perhaps this contributes to the increased liver damage in NASH. Studies need to be done further to explore FTO targeting as a route to mitigate NASH and perhaps decrease the risk of HCC development in the future in these patients. In addition, it is not clear whether the lipotoxicity is inducing the FTO expression or FTO expression is facilitating lipotoxicity.

In liver cancer, it has been reported that the writer components function as a tumor suppressors. Ma et al. found that, in HCC, METTL14 and m^6A levels were decreased relative to normal or paratumor controls with unchanged levels of the other writers METTL3 and WTAP [36]. In addition, they found that METTL14 expression correlated with poor prognosis. METTL14 knockdown facilitated HCC metastasis and overexpression-amplified tumor invasion and metastasis via m^6A-dependent modulation of microRNA (i.e., mir-126) processing via interaction with DGCR8 [36]. In another study, Chen et al. found that METTL3 was actually overexpressed in HCC compared to normal control, with WTAP levels unchanged [73]. METTL3 overexpression in this cohort correlated with worse prognosis. Furthermore, they demonstrated that overexpression of METTL3 augmented growth of HCC both in vitro and in vivo while downregulation of METTL3 inhibited tumorigenesis and lung metastasis in vivo [73]. These findings were associated with negative regulation of *SOCS2* expression by an m^6A- and YTHDF2-dependent mechanism [73]. These data therefore imply that potential therapeutic targets in HCC development include FTO in the context of NASH and the writers METTL3 and METTL14 as they pertain to HCC growth and metastasis.

Although the focus of this review is primarily on m^6A methylation of mRNA, there is developing data signifying the importance of m^6A methylation of ribosomal RNA

(rRNA). Recently, a new m^{6}A methyltransferase, ZCCHC4, which functions to target human 28S rRNA for methylation, was identified [74]. When ZCCHC4 is knocked out, there is less global translation and cell proliferation. In HCC in particular, ZCCHC4 protein is overexpressed and knockdown of this gene allows for HCC tumor regression in xenograft models.

m^{6}A mRNA methylation in endometrial cancer: an METTL14 hotspot

Sequencing studies have identified that 70% of endometrial tumors have reduced total m^{6}A mRNA methylation compared to adjacent normal cells [75]. However, the functional significance was largely unknown. The specific METTL14 (R298P) mutation occurs at the RNA-binding groove and leads to inhibiting m^{6}A mRNA methylation in tumors [75]. In addition, endometrial tumors have significantly reduced METTL3 m^{6}A methyltransferase. It appears that these abnormalities are mutually exclusive [75]. Liu et al. have found that either METTL14 mutation or reduced expression of METTL3 led to increased proliferation and tumorigenicity of endometrial cancer cells via activation of the AKT pathway [75]. Similar to several other solid and liquid tumors, they conclude that change in m^{6}A methylation is an oncogenic mechanism in endometrial cancer. A great deal needs to be studied about these mechanisms in order to develop relevant therapeutics for the treatment of endometrial cancer.

RNA modification in pancreatic cancer

Pancreatic ductal adenocarcinoma is a fatal malignancy with a 5-year survival of 9% [76]. He et al. found that *ALKBH5* was downregulated in pancreatic cancer cells, in which a long non-coding RNA, *KCNK15-AS1* is a direct target of ALKBH5 and thus is also downregulated; forced expression of *ALKBH5* or *KCNK15-AS1* could inhibit pancreatic cancer cell migration and invasion [77]. More recently, Zhang et al. reported that cigarette smoke condensate could cause hypomethylation in the *METTL3* promoter region and thereby upregulate the expression of *METTL3*, which in turn promotes the maturation process of primary microRNA-25 (miR-25) in pancreatic duct epithelial cells. The excessive miR-25-3p maturation results in the activation of the oncogenic AKT-p70S6K signaling, which promotes malignant phenotypes of pancreatic cancer cells [76]. This study revealed a previously unappreciated link between cigarette smoke, m^{6}A modification, microRNA maturation, and the pathogenesis of pancreatic cancer.

Colon cancer: METTLE3 is associated with tumor progression

The role of m^{6}A methylation in colorectal cancer (CRC) remains largely unexplored. Li et al. have found via the TCGA that *METTL3* expression correlated with poor prognosis in CRC [78]. *METTL3* knockdown led to decreased CRC cell self-renewal, stem cell frequency, and migration in vitro and inhibited growth and metastases in vivo [78]. METTLE3 was also found to target SOX2. Mechanistically, they found that, when SOX2 transcripts were methylated, they were then recognized by a specific m^{6}A reader, IGF2BP2, to prevent SOX2 mRNA degradations [78]. In addition, they found that the combination of "writer" METTL3, "reader" IGF2BP2 and "target" SOX2 correlated with better prognostic accuracy for CRC patients than each individual component [78, 79]. More work is needed to assess the efficacy of targeting components of this combination as a therapeutic strategy.

Conclusion

In summary, it is clear that the m^{6}A mRNA machinery is an important mechanism in gene regulation and expression. In nearly all malignancies studied, there appears to be a role in contributing to cancer stem cell self-renewal. Targeting the various functions of "writers," "readers," and "erasers" is a field of great interest and the oncogenic roles of the m^{6}A RNA methylation machinery needs to be further elucidated. There is a great deal to be learned by this novel epigenetic regulation at the RNA level. Development of effective and selective small-molecule compounds or other agents/tools targeting the dysregulated m^{6}A machinery components is urgently needed as these may provide more effective novel therapies for cancer treatment.

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Compliance with ethical standards

Conflict of interest JC has a patent filed based on his R-2HG/FTO work. JC is a scientific founder and the chief scientific officer of Genovel Biotech Corp. and also holds equity with this company.

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