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N^6-methyladenosine RNA methylation: From regulatory mechanisms to potential clinical applications

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Epitranscriptomics has emerged as another level of epigenetic regulation similar to DNA and histone modifications. N^6-methyladenosine (m^{6}A) is one of the most prevalent and abundant posttranscriptional modifications, widely distributed in many biological species. The level of N^6-methyladenosine RNA methylation is dynamically and reversibly regulated by distinct effectors including methyltransferases, demethylases, histone modification and metabolites. In addition, N^6-methyladenosine RNA methylation is involved in multiple RNA metabolism pathways, such as splicing, localization, translation efficiency, stability and degradation, ultimately affecting various pathological processes, especially the oncogenic and tumor-suppressing activities. Recent studies also reveal that N^6-methyladenosine modification exerts the function in immune cells and tumor immunity. In this review, we mainly focus on the regulatory mechanisms of N^6-methyladenosine RNA methylation, the techniques for detecting N^6-methyladenosine methylation, the role of N^6-methyladenosine modification in cancer and other diseases, and the potential clinical applications.

KEYWORDS

RNA methylation, N^6-methyladenosine, regulatory mechanisms, cancer, tumor therapy

1 Introduction

With the development of epigenetics, epitranscriptomics has emerged as another level of epigenetic regulation and has recently become a research hotspot. The epitranscriptome refers to the relevant functional changes of the transcriptome without any alteration of the RNA sequence. Conceptually, the epitranscriptome covers all the chemical modifications of RNA dynamically regulated by the removal and addition of various chemical groups in cells (Saletore et al., 2012). To date, over 170 RNA chemical modifications have been identified, including N^6-methyladenosine (m^{6}A), N^1-methyladenosine (m^{1}A), 5-hydroxymethylcytosine (hm^{5}C), 5-methylcytidine (m^{5}C), ribose 2′-O-methylation (Nm), 1-methylguanine (m^{1}G), 6-methylguanine (m^{6}G),

7-methylguanine (m^7G), N^4-acetylcytidine (ac^4C) and pseudouridine (w) (Wiener and Schwartz, 2021), but most of their functions are largely unknown. Among them, 72 variants of methyl group modifications are conjugated at distinct positions in RNA bases. Since the first discovery of m^6A RNA methylation in 1974, it has been identified as one of the most prevalent and abundant posttranscriptional modifications, widely distributed in many biological species, such as mammals (Desrosiers et al., 1974; Liu et al., 2022), plants (Yue et al., 2019; Yu Q. et al., 2021), zebrafish (Zhao et al., 2017), insects (Yang et al., 2021), yeast (Yadav and Rajasekharan, 2018), bacteria (Deng et al., 2015) and viruses (Bayoumi et al., 2020), accounting for approximately 50% of total methylated ribonucleotides in total RNA content (Wei et al., 1975). It is estimated that more than 7,000 mRNAs with m^6A modification are distributed in mammalian cells, with a frequency of 0.1–0.6% of adenosines (Ke et al., 2015). In addition, m^6A deposition also exists in other types of RNA, including rRNA, tRNA, small nuclear RNA (snRNA), small nucleolar RNA (snoRNA), long noncoding RNA (lncRNA), microRNA (miRNA), and circular RNA (circRNA) (Pendleton et al., 2017; van Tran et al., 2019; Dai et al., 2020).

In 2012, several decades after the first discovery of m^6A RNA methylation, utilizing m^6A -specific antibodies, two groups independently conducted fragmented RNA immunoprecipitation and subsequent high-throughput RNA deep sequencing (termed "MeRIP-seq" or "m^6A-seq") to map m^6A throughout the transcriptome in humans and mice (Dominissini et al., 2012; Meyer et al., 2012). The results first revealed that m^6A modification was widely distributed in mRNA, additionally, m^6A modification mainly occurred in the common motif RRACH (R = G or A, H = A, C or U), but only 1–5% of these sites were methylated in cellular RNA. Notably, most m^6A peaks were evolutionarily conserved between the human and mouse transcriptomes. More surprisingly, m^6A modification on the RRACH motif was preferentially enriched in 3′-untranslated regions (3′-UTRs) and near stop codons of coding sequences (CDS) (Meyer et al., 2012), indicating that the RRACH motif is not sufficient for the determination of m^6A modification. The m^6A levels vary in distinct cell contexts and are involved in multiple RNA metabolism pathways, such as splicing, localization, translation efficiency, stability and degradation (Kasowitz et al., 2018; Liu et al., 2020; He and He, 2021), ultimately affecting various physiological and pathological processes.

m^6A RNA methylation is dynamic and reversible and is also tightly regulated by three types of proteins, methyltransferases ("writers"), demethylases ("erasers") and m^6A binding proteins ("readers"). The m^6A methylase complex was first purified in the 1990s (Bokar et al., 1994). METTL3 is identified as a predominant component and contains a catalytically active subunit. Another methyltransferase, METTL14, is essential for structural stability to facilitate the catalysis of m^6A methylation. The larger methyltransferase holocomplex is composed of WTAP, HAKAI, RBM15, RBM15B, VIRMA and ZC3H13 and is approximately 1,000 kDa in size (Oerum et al., 2021). FTO was the first m^6A demethylase identified in 2011, followed by ALKBH5 (Niu et al., 2013). These two enzymes can remove m^6A methylation from RNA, posing a novel research field of regulation for epitranscriptomics. The functions of m^6A RNA methylation are mediated by different m^6A "readers" that selectively recognize m^6A in a direct or indirect manner and conduct distinct functions (Shi et al., 2019). The m^6A binding proteins include the YTH family, the heterogeneous nuclear ribonuclease (HNRNP) family and FMRP (Figure 1).

In this review, we will address the regulatory mechanisms of m^6A RNA methylation, the techniques for detecting m^6A methylation, the role of m^6A modification in cancer as well as the potential clinical applications.

2 Regulation of N^6-methyladenosine RNA modification

m^6A RNA methylation is functionally important and tightly modulated by several molecular mechanisms in eukaryotes. As described above, catalytic enzymes, methyltransferases and demethylases dynamically and reversibly direct the addition and removal of m^6A RNA methylation, and are termed "m^6A writers" and "m^6A erasers", respectively. Some binding proteins ("m^6A readers") recognize and function by decoding m^6A methylation as well as recruiting downstream functional protein complexes to mediate biological activities. Histone modification also guides m^6A deposition in stop codons of CDSs and 3′-UTRs. The dynamics of m^6A regulation can be achieved by some transcription factors that recruit the m^6A methyltransferase complex to specific RNA loci in distinct cellular contexts. In addition, nutrition and metabolites can reverse and modulate m^6A methylation patterns (Figure 2).

2.1 RNA N^6-methyladenosine machinery

m^6A methylation is catalysed by a multicomponent m^6A methyltransferase complex that is composed of two predominant proteins, methyltransferase-like 3 (METTL3) and methyltransferase-like 14 (METTL14), and their cofactors WTAP, HAKAI, RBM15, RBM15B, VIRMA and ZC3H13(Knuckles et al., 2018; Yue et al., 2018; Bawankar et al., 2021). Although both METTL3 and METTL14 have methyltransferase domains, only METTL3 contains a catalytically active subunit, which requires S-adenosylmethionine (SAM) as a substrate to mediate catalytic activity. The SAM binding pocket is distributed on one side of the central ß-sheet and is enclosed by the catalytic site loop (Wang et al., 2016). METTL14 is associated with the stabilization of the conformation between METTL3 and the RNA

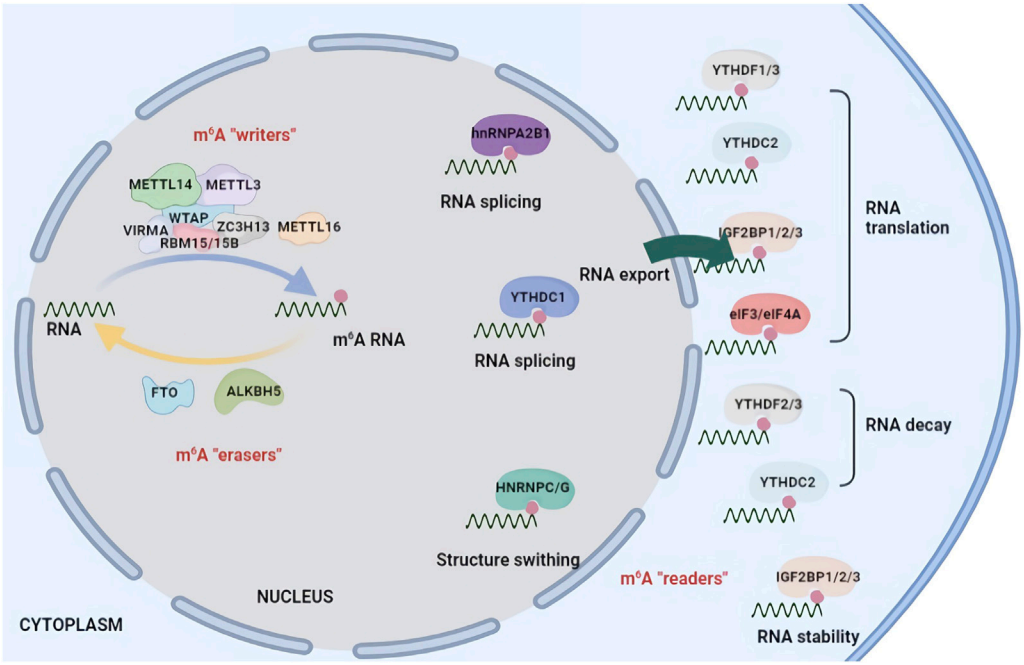


FIGURE 1
Chemical basis and molecular functions of m^{6}A machinery. m^{6}A methylation is catalysed by a multicomponent m^{6}A methyltransferase complex ("m^{6}A writers") that is composed of two predominant proteins, METTL3 and METTL14, as well as their cofactors WTAP, HAKAI, RBM15, RBM15B, VIRMA and ZC3H13. m^{6}A methylation can be removed by demethylases ("m^{6}A erasers") including FTO and ALKBH5. m^{6}A modification affects RNA fate by recruiting m^{6}A-binding proteins ("m^{6}A readers") such as YTHDF1/2/3, YTHDC1/2, IGF2BP1/2/3 and HNRNPC/A2B1. m^{6}A methylation is involved in RNA splicing, localization, translation efficiency, stability and degradation.

substrate (Zhou H. et al., 2021). The METTL3-METTL14 complex is formed in the cytoplasm and is located in the nucleus, and it induces m^{6}A methylation (Scholler et al., 2018). Both *in vitro* methylation assays and CLIP combined with photoactivatable ribonucleoside-enhanced crosslinking (PAR-CLIP) suggested that the METTL3-METTL14 complex efficiently catalyses m^{6}A methylation on the GGACU or GGAC motifs of RNAs, consistent with the RRACH motif of a previous study (Liu et al., 2014). The depletion of *Mettl3* and/or *Mettl14* could greatly reduce the peak numbers and the enrichment of m^{6}A in the global transcriptome (Vu et al., 2017; Weng et al., 2018). Except for these two modulators, other m^{6}A writers lack methyltransferase activity. WTAP guides METTL3 and METTL14 into nuclear speckles to efficiently methylate target RNAs (Ping et al., 2014). RBM15 and RBM15B have been confirmed to interact with WTAP by coimmunoprecipitation and bind to specific RNA regions that are adjacent to the DRACH sequence, suggesting that RBM15 and RBM15B can recruit the METTL3-WTAP complex and direct these methyltransferases to DRACH consensus sequence sites for m^{6}A modification (Patil et al., 2016; Shi et al., 2019). RBM15 and RBM15B targets induce X-chromosome inactivation and gene silencing by binding to lncRNA XIST, and VIRMA prefers to mediate alternative polyadenylation and mRNA methylation near the 3′-UTRs and stop codon regions (Yue et al., 2018; Zhu et al., 2021). ZC3H13 complexes with WTAP or other cofactors to regulate nuclear m^{6}A RNA methylation (Wen et al., 2018). Recently, METTL16, as an independent RNA methyltransferase, was shown to catalyse the m^{6}A methylation of U6 spliceosomal RNA (snRNA) and U6-like hairpins of *Mat2a* mRNA (Shima et al., 2017). ZCCHC4, a new m^{6}A methyltransferase, was found to specifically recognize the AAC motif associated with rRNA methylation (Ma et al., 2019).

After deposition, m^{6}A methylation is reversible and can be removed by demethylases ("m^{6}A erasers"). FTO belongs to the nonheme Fe(II)- and α-KG-dependent dioxygenase AlkB family. FTO was the first enzyme reported to modulate m^{6}A demethylation in 2011. In addition, m^{6}Am RNA, m^{1}A RNA, m^{3}T single-stranded DNA and m^{3}U single-stranded RNA modifications can be demethylated by FTO (Wei et al., 2018). Mauer et al. found that the catalytic activity of FTO towards m^{6}Am was approximately 10 times greater than that towards m^{6}A (Mauer et al., 2017). It has been reported that snRNA and snoRNA are targets of FTO (Mauer et al., 2019). Another m^{6}A demethylase, ALKBH5, is a member of the ALKB family and seems to be specific for m^{6}A RNA methylation (Yu F. et al., 2021).

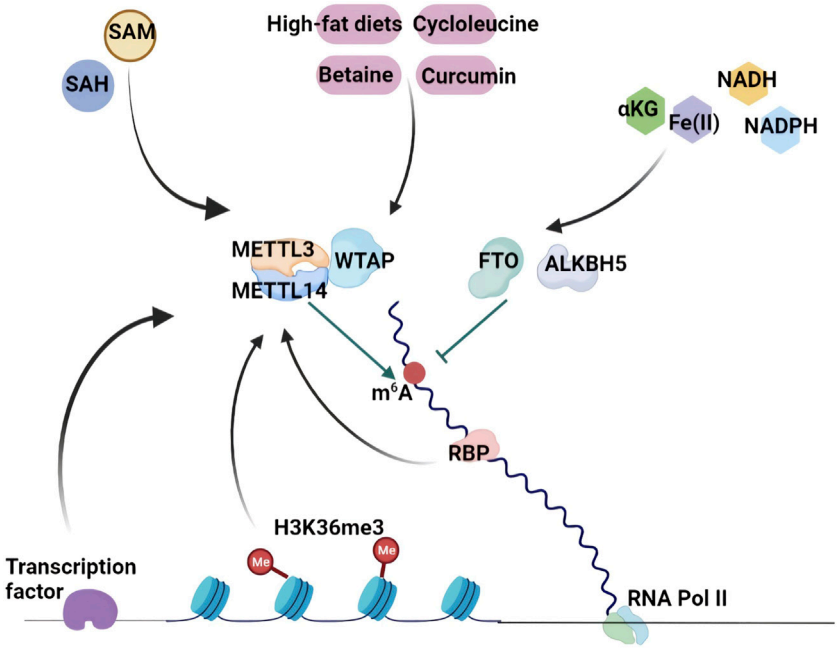


FIGURE 2
The biogenesis and regulatory mechanisms of RNA m^{6}A Methylation. Methyltransferases and demethylases dynamically and reversibly direct the addition and removal of m^{6}A RNA methylation. H3K36me3 guides m^{6}A deposition in stop codons of CDSs and 3′-UTRs. The dynamics of m^{6}A regulation can be achieved by some transcription factors that recruit the m^{6}A methyltransferase complex to specific RNA loci in distinct cellular contexts. In addition, m^{6}A methylation patterns are modulated by nutrition and metabolites including SAM, SAH, cycloleucine, betaine, curcumin, high-fat diets, αKG, NADH and NADPH.

m^{6}A modification affects RNA fate by recruiting m^{6}A-binding proteins (m^{6}A 'readers') such as YTH domain-containing proteins, insulin-like growth factor 2 mRNA-binding proteins IGF2BP1-3 and the heterogeneous nuclear ribonuclease (HNRNP) family (Zhao et al., 2020). In mammals, YTH domain-containing proteins contain five members: YTHDC1, YTHDC2, YTHDF1, YTHDF2, and YTHDF3. YTHDC1 play roles in alternative splicing events, nuclear export of RNAs into the cytoplasm and mRNA decay (Xiao et al., 2016). YTHDC2 promotes target mRNA translation (Mao et al., 2019). YTHDF1 interacts with the translation initiation factors eIF3 and eIF4A3 to enhance the translation efficiency of m^{6}A-modified mRNAs (Liu et al., 2020; Cai et al., 2022). YTHDF2 and YTHDF3 are involved in the degradation of target mRNAs associated with p-bodies, and the depletion of *Ythdf2* and *Ythdf3* causes a considerable increase in m^{6}A mRNA abundance in cells. hnRNPA2B1, hnRNPC and hnRNPG are related to mRNA splicing (Bi et al., 2019). In contrast, IGF2BP1/2/3, FMRP, and PRRC2A are essential for the stabilization of m^{6}A-modified transcripts in a m^{6}A-dependent manner (Huang et al., 2018; Edens et al., 2019; Wu et al., 2019).

2.2 Histone modification guides *N*^{6}-methyladenosine deposition

Histone modification has an effect on m^{6}A deposition. H3 lysine 36 trimethylation (H3K36me3) is a classical transcription activator that shows a similar m^{6}A distribution. H3K36me3 chromatin immunoprecipitation (ChIP)-seq analysis indicated that approximately 70% of H3K36me3 sites overlapped with m^{6}A peaks, suggesting a close connection between H3K36me3 and m^{6}A modification (Huang et al., 2019) (Figure 2). SETD2 and KDM4A are H3K36me3 methyltransferase and H3K36me3 demethylase, respectively (Mar et al., 2017). More surprisingly, changes in H3K36me3 levels by utilizing dCas9-SETD2 or dCas9-KDM4A can significantly alter m^{6}A abundance in human and mouse transcriptomes, revealing that H3K36me3 is a modulator of m^{6}A deposition. Of note, most H3K36me3-dependent m^{6}A sites are targeted by METTL3, METTL14 and WTAP, demonstrating the association between H3K36me3 and m^{6}A modification. In addition, METTL14 can directly bind to H3K36me3, which leads to the recruitment of other m^{6}A methyltransferases to activate RNA Pol II and controls m^{6}A methylation on mRNA (Huang et al., 2019; Zhou X. L. et al., 2021). Therefore, the

relationship between H3K36me3 and m^{6}A provides a new way to enrich multiple aspects of gene expression regulation.

2.3 Transcription factors affect *N*^{6}-methyladenosine deposition

Transcription factors also recruit m^{6}A methyltransferases to regulate RNA modification in specific cell contexts. Zinc-finger protein 217 (ZFP217) is a transcriptional activator of some key pluripotency genes that are essential for maintaining self-renewal in mESCs. METTL3 can be bound and sequestered by ZFP217, preventing the formation of the m^{6}A methyltransferase complex and m^{6}A methylation on ZFP217 target transcripts (Aguilo et al., 2015). In contrast, the transcription factors SMAD2 and SMAD3 preferentially recruit the METTL3-METTL14-WTAP methyltransferase complex to their target transcripts and increase the m^{6}A modification of target transcripts (Bertero et al., 2018). Different functions of m^{6}A deposition on the target transcripts determine the distinct roles of ZFP217 and SMAD2/3 in ESCs. Another study reported that METTL3 could interact with the CAATT-box binding protein CEBPZ on target transcripts and mediate m^{6}A modification to promote the translation of target mRNAs that maintain the leukaemic state in acute myeloid leukaemia (AML) cells (Barbieri et al., 2017). Frequently, these transcription factors manipulate m^{6}A deposition on a subset of target transcripts in specific cellular contexts to implement dynamic regulation of gene expression (Figure 2).

2.4 Nutritional metabolism and metabolites regulate *N*^{6}-methyladenosine deposition

Evidence has shown that nutritional challenge and metabolites play crucial roles in the manipulation of m^{6}A deposition. Cycloleucine is a competitive inhibitor of methionine adenosyltransferase that decreases m^{6}A RNA methylation levels by reducing SAM concentrations (Kang et al., 2018). Betaine, as a methyl donor for SAM synthesis, prompts m^{6}A methylation by suppressing FTO expression in the adipose tissues of high-fat diet-fed mice, which increases the expression of the mitochondrial protein PGC-1α to improve metabolic disorder (Zhou et al., 2015). It has been reported that curcumin enhances m^{6}A modification by decreasing ALKBH5 and increasing METTL3 and METTL14 expression in the livers of piglets (Ding et al., 2016). Undoubtedly, high-fat diets affect m^{6}A modification in various models and tissues (Tung et al., 2010; Wu et al., 2020) (Figure 2).

SAM, a common methyl donor, is involved in most cellular methylation processes. The change in cellular SAM concentration affects DNA and histone methylation as well as RNA methylation (Duncan et al., 2013). METTL3 requires SAM as a substrate to mediate catalytic activity and m^{6}A writing. Interestingly, the SAM binding affinity of METTL3 is regulated by substrate RNA availability. S-adenosyl homocysteine (SAH) is the metabolite of SAM during the methylation reaction that can strongly inhibit METTL3 methyltransferase activity (Li F. et al., 2016; Selberg et al., 2019). Demethylases, FTO and ALKBH5 are 2-oxoglutarate (αKG)- and Fe(II) dependent. The FTO and ALKBH5 mutants of the αKG-Fe(II) oxygenase domain lost the catalytic activities of m^{6}A demethylation (Feng et al., 2014; Zhang X. et al., 2019). Recently, NADH and NADPH were identified as the direct binding partners of FTO by using a florescence quenching assay. Both NADH and NADPH could enhance FTO demethylase activity, indicating that reducing NADPH and NADH may attenuate demethylation reactions. Conversely, the induction of NADPH by glucose injection or a high-fat diet suppressed m^{6}A modification (Figure 2). In contrast, the depletion of G6P dehydrogenase (G6PD) or NAD kinase (NADK) enhanced cellular m^{6}A abundance, which was reversed by NADPH supplementation (Wang L. et al., 2020).

3 Approaches for detecting *N*^{6}-methyladenosine RNA methylation

Several techniques have been developed for detecting m^{6}A RNA methylation (Table 1). Although immuno-northern blot and m^{6}A dot blot facilitate easier and faster observation of global m^{6}A levels, the disadvantages are obvious with lower sensitivity and are semiquantitative accuracy (Nagarajan et al., 2019). High-performance liquid chromatography–mass spectrometry (HPLC–MS/MS) is used for quantifying m^{6}A levels with high sensitivity; however, this approach cannot provide details about RNA sequence and localization information (Thuring et al., 2017). Site-specific cleavage and radioactive labelling followed by ligation-assisted extraction and thin-layer chromatography (SCARLET) is suitable for stoichiometric quantification, but it is very tedious and is only used to validate the known m^{6}A changes at a given site (Liu and Pan, 2016). Above all, these techniques are not appropriate for widespread identification and localization of modified sites; subsequently, high-throughput sequencing methods have emerged and have been rapidly developed. m^{6}A-specific antibody-based high-throughput sequencing strategies are widely used for the identification of m6A, which include m^{6}A-Seq, MeRIPSeq, PA-m^{6}A-Seq, m^{6}A-CLIP and m^{6}A individual-nucleotide resolution cross-linking and immunoprecipitation (miCLIP) (Dominissini et al., 2012; Meyer et al., 2012; Chen et al., 2015; Ke et al., 2015; Linder et al., 2015). MeRIP-seq was first identified in 2012, allowing for m^{6}A analysis with 100- to 200-nucleotide resolution. In terms of MeRIP-seq, mRNA was fragmented into 100-nucleotide lengths

TABLE 1 Approaches for the detection of m^{6}A RNA methylation.

Approaches	Principle	Advantages	Limitations	References
m^{6}A dot blot	Antibody based immunoblot	Easy, fast	Low sensitive, semi-quantitative	Nagarajan et al. (2019)
HPLC-MS/MS	Mass spectrum	High sensitive, quantitative	Lack of RNA sequence and localization	Thuring et al. (2017)
SCARLET	Thin-layer chromatography	Quantitative	Complicated, low-throughput	Liu and Pan, (2016)
m^{6}A-Seq	Antibody based sequencing	High-throughput	100–200 nucleotide resolution	Dominissini et al. (2012)
MeRIPSeq	Antibody based sequencing	High-throughput	100–200 nucleotide resolution, antibody specificity	Meyer et al. (2012)
miCLIP	Antibody based sequencing	High-throughput, single site	Low cross-linking efficiency, antibody specificity	Linder et al. (2015)
MAZTER-Seq	Endoribonuclease based sequencing	High-throughput, single site	Preference of the enzyme	Garcia-Campos et al. (2019)
m^{6}A-REF-Seq	Endoribonuclease based sequencing	High-throughput, single site	Preference of the enzyme	Zhang Z. et al. (2019)
m^{6}A-SEAL	Chemical labeling sequencing	High-throughput, single site	Chemical labeling efficiency	Wang Y. et al. (2020)
m^{6}A-label-seq	Chemical labeling sequencing	High-throughput, single site	Chemical labeling efficiency	Shu et al. (2020)
m^{6}A-SAC-seq	Chemical labeling sequencing	High-throughput, single site	Chemical labeling efficiency	Hu et al. (2022)

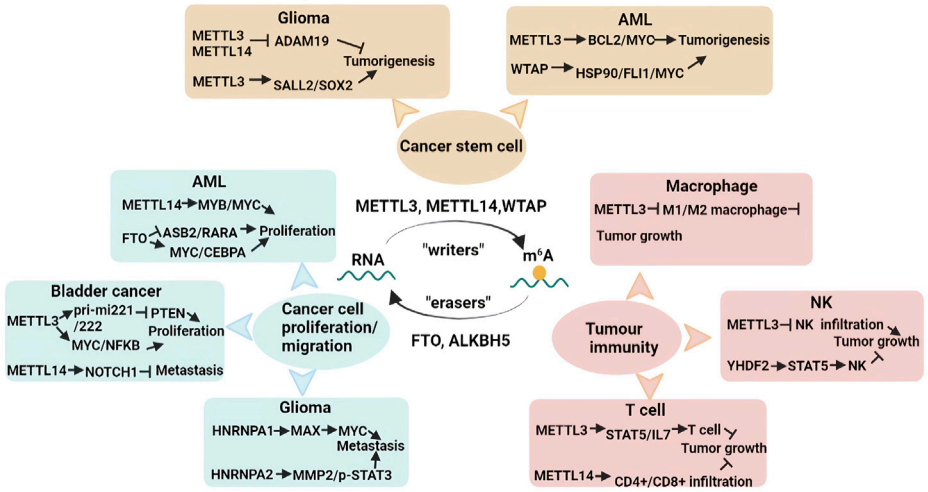


FIGURE 3
Diverse functions of m^{6}A RNA methylation in cancers. m^{6}A RNA methylation plays a crucial role in tumorigenesis and cancer progression. m^{6}A RNA methylation is reversible and is also tightly regulated by "writers" and "readers". m^{6}A RNA methylation contributes to mediating CSC pluripotency, cancer proliferation, tumour metastasis, and tumor immunity. More details are shown in a graphical form.

and immunoprecipitated by a m^{6}A-specific antibody with the combination of high-throughput deep sequencing. miCLIP enables the detection of m^{6}A residues at precise positions with single-nucleotide resolution. However, the number of identified m^{6}A peaks is limited due to the low cross-linking efficiency of this method. m^{6}A-specific antibody-based sequencing approaches have some obvious drawbacks. m^{6}A antibodies are not strikingly specific for m^{6}A, may also bind to m^{6}Am sites and other non-m^{6}A-specific sequences. Distinct commercial m^{6}A antibodies show differences in affinity for m^{6}A (Haussmann et al., 2016). Endoribonuclease-based techniques include antibody-free m^{6}A sequencing methods such as m^{6}A-REF-Seq and MAZTER-Seq and rely on the endoribonuclease activity of MazF (Zhang Z. et al., 2019; Garcia-Campos et al., 2019). Therefore, the motif preference of endoribonuclease determines the limitation, and these methods detect a portion of the m^{6}A sites. Recently, chemical labelling strategies have been reported, including m^{6}A-SEAL, m^{6}A-label-seq and m^{6}A-SAC-seq (Wang Y. et al., 2020; Shu et al., 2020; Hu et al., 2022). Nevertheless, improvements in the chemical labelling efficiency are needed. A specific method for m^{6}A detection in the global transcriptome should be validated by other techniques to obtain a more accurate m^{6}A landscape.

4 Role of N^6-methyladenosine RNA methylation in human diseases

m^{6}A RNA methylation is involved in multiple RNA metabolism pathways, such as splicing, localization, translation efficiency, stability and degradation, ultimately affecting various physiological and pathological processes. For instance, m^{6}A methylation has been demonstrated to regulate the haematopoietic system, the central nervous system, immunity stemness, mammalian spermatogenesis and brain development. The dysregulation of m^{6}A methylation is associated with various diseases, especially cancer (Figure 3).

4.1 N^6-methyladenosine and cancer

4.1.1 N^6-methyladenosine in cancer stem cells

Cancer stem cells (CSCs) are a type of cells possessing a stem cell-like capacity to self-renew, differentiate and survive and give rise to many types of cancers (Nassar and Blanpain, 2016). CSCs lead to the tolerance of standard therapeutics and CSCs, tumour recurrence, and distant metastasis (Walcher et al., 2020). Cui et al. found that m^{6}A methylation played a role in the tumorigenesis of glioma stem cells (GSCs). *Mettl3/14* knockdown prominently promoted GSC self-renewal and tumorigenesis by decreasing m^{6}A levels, whereas METTL3 overexpression exerted negative effects (Cui et al., 2017; Zhang et al., 2017). MeRIP-seq revealed that silencing *Mettl3/14* altered m^{6}A enrichment and that m^{6}A RNA methylation of ADAM19 regulated GSC self-renewal. Similarly, Visvanathan et al. confirmed that METTL3 was essential for glioma cell differentiation and GSC maintenance. Furthermore, METTL3 is also involved in radiosensitivity and DNA repair through the SOX2 axis in GSCs (Visvanathan et al., 2018).

Wang et al. provided the first evidence of the interplay between m^{6}A methylation and osteosarcoma stem cells (OSCs). Compared with non-OSCs, METTL14 and FTO were significantly reduced in OSCs. Meanwhile, MeRIP-seq and RNA-seq analyses of OSCs and non-OSCs revealed that differentially expressed genes containing differentially methylated m^{6}A peaks were associated with the Wnt pathway and the pluripotency of stem cells (Wang et al., 2019).

Ly P Vu and others revealed that METTL3 expression was elevated in leukaemia cells compared with normal haematopoietic cells. miCLIP analysis showed that METTL3 enhanced the m^{6}A methylation of target genes such as *Bcl2*, *Myc* and *Pten* in the human acute myeloid leukaemia (AML) MOLM-13 cell line, which promoted the mRNA translation of these genes, thereby retaining pluripotency properties and inhibiting cell differentiation. Notably, silencing of *Mettl3* in human myeloid leukemia cell lines promoted cell differentiation and cell apoptosis (Vu et al., 2017). WTAP is associated with haematopoietic stem cell (HSC) homeostasis and haematopoietic regeneration (Wang H. et al., 2018; Li et al., 2018). Recent research conducted by Bansal et al. suggested a role for m^{6}A modification in myeloid leukaemia. WTAP expression was increased in AML cells derived from patients with AML, while knockdown of *Wtap* led to the repression of cell proliferation, the activation of cell differentiation and apoptosis in a leukaemia cell line (Bansal et al., 2014) (Figure 3).

4.1.2 N^6-methyladenosine in cancer cell proliferation and migration

Numerous reports have elucidated that m^{6}A modification is involved in cancer cell proliferation and tumour metastasis in different types of cancers. Recent findings revealed that METTL14 exerted an oncogenic function by increasing the expression levels of targets such as MYB and MYC in AML, while SPI downregulated the expression level of MEETL14 (Weng et al., 2018). FTO demethylase is elevated and plays an oncogenic role in AML. It has been demonstrated that a high level of FTO prompts cell proliferation and viability, whereas it reduces cell apoptosis and global m^{6}A methylation by repressing the expression of ASB2 and RARA (Li Z. et al., 2017). Su et al. observed that R-2-hydroxyglutarate (R-2HG) attenuated FTO activity and augmented global m^{6}A modification in R-2HG-sensitive AML cells, which decreased the stability of *Cebpa*/*Myc* mRNA and the activities of relevant cell signalling pathways (Su et al., 2018).

In bladder cancer, METTL3 interacts with DGCR8 to facilitate pri-miR221/222 maturation, which leads to a decrease in PTEN and ultimately promotes cell proliferation (Han et al., 2019). Similarly, another study showed that METTL3 was significantly elevated in bladder cancer, and *METTL3* knockdown dramatically suppressed cancer cell proliferation, cell invasion, and tumour formation through the AFF4/MYC/NF-kB axis cell signalling pathway (Cheng et al., 2019). Xie et al. revealed that METTL3 also binds to YTHDF2, which induces the degradation of target tumour suppressor mRNAs, including *Klf4* and *Setd7*, regulating the progression of bladder cancer (Xie et al., 2020). Nevertheless, METTL14 has been confirmed to be decreased in bladder cancer, and depletion of *Mettl14* accelerated cell proliferation, tumour metastasis and self-renewal by decreasing the stability of m^{6}A-modified Notch1 transcripts (Gu et al., 2019).

Evidence indicates that the hnRNPA1 expression level is elevated by EGFRvIII, leading to increased glycolytic gene expression in gliomas. Meanwhile, hnRNPA1 promotes *Max* mRNA splicing and then induces the generation of Delta Max, which promotes Myc-dependent cell transformation (Babic et al., 2013). Another study showed that silencing *hnRNPA2* represses cancerous cell viability, cell invasion, tumour metastasis and chemoresistance by decreasing the expression of MMP-2 and phospho-STAT3. Notably, hnRNPA2 has been regarded as an

oncogenic driver in gliomas (Deng et al., 2016) (Figure 3). Numerous studies have described how m^{6}A methylation contributes to cell proliferation, cell invasion, and tumour metastasis in other cancers, including breast cancer, ovarian cancer, cervical cancer, prostate cancer, lung cancer, hepatocellular carcinoma, gastric carcinoma, pancreatic cancer and colorectal cancer (Hu et al., 2019; Yang et al., 2019; Ma et al., 2020; Yang et al., 2020; Guo et al., 2021; Zhang and Xu, Forthcoming 2022).

4.1.3 *N*^{6}-methyladenosine in tumour immunity

Recent studies have revealed that m^{6}A RNA methylation induces the activation and infiltration of various immune cells into the tumour microenvironment (TME), influencing the efficacy of cancer immunotherapy. Macrophages are closely associated with tumour initiation and progression. Yin et al. elucidated that METTL3 in macrophages regulates tumour development. Silencing of *Mettl3* in macrophages facilitated tumour growth and lung metastasis. The TME was reshaped by inducing regulatory T (Treg) cells into tumour sites and promoting the infiltration of M1-and M2-like tumour-associated macrophages (Yin et al., 2021). *Mettl14* knockdown in macrophages suppressed the antitumour activity of CD8$^+$ T cells and improved tumour growth (Dong et al., 2021).

Natural killer (NK) cells play an important role in cancer immune surveillance and can directly recognize and kill cancer cells. YTHDF2 was critical for modulating NK-cell maturation, NK-cell homeostasis, IL-15-mediated survival, and antitumor activity due to the regulation of downstream target genes such as *Stat5*, *Eomes* and *Tardbp* (Ma et al., 2021). Song et al. found that METTL3 expression was decreased in tumour-infiltrating NK cells of cancer patients. In mice, they observed that depletion of *Mettl3* enhanced NK-cell responsiveness to IL-15 and promoted tumour progression and metastasis by targeting SHP-2 (Song et al., 2021).

Silencing of *Mettl3* in CD4$^+$ T cells destroyed T-cell differentiation and homeostasis by repressing the activation of IL-7-mediated STAT5/suppressor of cytokine signalling (Li H. B. et al., 2017). Yao et al. revealed that conditional depletion of *Mettl3* in CD4$^+$ T cells inhibited T follicular helper differentiation and maturation, thereby preventing the antibody response of B cells by promoting the degradation of m^{6}A-modified *Tcf7* mRNA (Yao et al., 2021). In breast cancer, the expression levels of METTL14 have a positive correlation with the infiltration of CD4$^+$ T cells, CD8$^+$ T cells, dendritic cells, macrophages and neutrophils, but they negatively correlated with Treg cells in breast cancer (Gong et al., 2020) (Figure 3).

4.2 *N*^{6}-methyladenosine and other human diseases

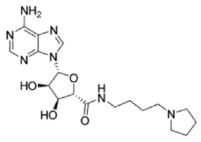
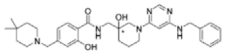
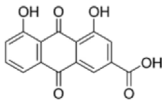
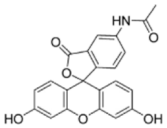
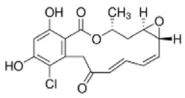
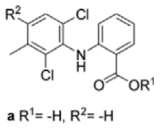
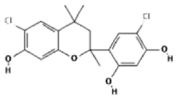
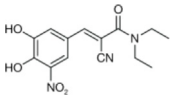
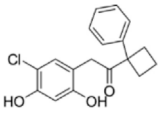
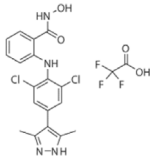
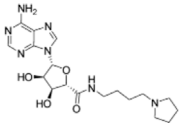
Emerging evidence has demonstrated that m^{6}A RNA methylation is closely related to other human diseases, including cardiovascular disease, metabolic syndrome, psychiatric disorders and autoinflammatory disorders. Dorn et al. (Dorn et al., 2019) showed that the global m^{6}A level of cardiomyocytes was significantly elevated in response to hypertrophic stimulation and that METTL3 played a vital role in cardiomyocyte hypertrophy. Notably, upregulated m^{6}A modification resulted in compensated cardiac hypertrophy, whereas downregulated m^{6}A levels led to eccentric cardiomyocyte remodelling and dysfunction. Overexpression of *Mettl3* increased the expression levels of mitogen-activated protein (MAP)3K6, MAP4K5, and MAPK14 in cardiomyocytes, which was positively correlated with cardiomyocyte size, revealing that METTL3 is sufficient to drive cardiomyocyte hypertrophy. However, no histopathologic changes were observed in *Mettl3*-overexpressing mice.

Zhou et al. identified that YTHDC2 was significantly repressed in nonalcoholic fatty liver disease (NAFLD) patients, and *Ythdc2*-depleted hepatocytes led to the accumulation of excessive triglycerides (TGs) by reducing the expression levels of lipogenic genes, including fatty acid synthase, sterol regulatory element-binding protein 1c, and acetyl-CoA carboxylase 1 (Zhou B. et al., 2021). Furthermore, m^{6}A sequencing was performed in human type 2 diabetes islets, and sequencing analysis showed that multiple hypomethylated transcripts were associated with insulin secretion, the insulin/IGF1 signalling pathway and cell cycle progression. *Mettl14* knockout in mouse ß-cells caused a reduction in global m^{6}A levels, giving rise to a similar islet phenotype in human T2D (De Jesus et al., 2019).

In Alzheimer's disease mouse models, the global m^{6}A level was increased in the hippocampus and the cortex compared to C57BL/6 mice, and the interaction of FTO and APOE contributed to the increase in Alzheimer's disease risk. The overall m^{6}A level was elevated in the cortex and the hippocampus of APP/PS1 (Alzheimer's disease) mice compared to C57BL/6 control mice, and FTO was found to interact with APOE, which was associated with Alzheimer's disease risk in a prospective cohort study (Keller et al., 2011; Han et al., 2020). Huang et al. found that ALKBH5/FAAH enhanced the expression of circSTAG1, which attenuated astrocyte dysfunction and depressive-like behaviours *in vitro* and *in vivo* (Huang et al., 2020).

m^{6}A methylation also has a contributory effect on autoinflammatory disorders. Luo et al. identified that the expression levels of METTL14, ALKBH5 and YTHDF2 were downregulated in peripheral blood mononuclear cells of systemic lupus erythematosus (SLE) patients (Luo et al., 2020). In addition, multivariate logistic regression analysis showed that repression of ALKBH5 and YTHDF2 was considered a risk factor for SLE. However, direct mechanistic data should be provided for the function of m^{6}A modification in SLE progression. Another study found that *Mettl14* knockdown inhibited the activation of Treg cells, which impaired the balance between Th17 and Treg cells, leading to the development of spontaneous colitis (Lu et al., 2020).

TABLE 2 Overview of the small-molecule drugs and m^{6}A-related factor inhibitors described in the text.

Drug/inhibitor	Molecular structure	m^{6}A proteins involved	Function	References
SAM mimic		METTL3	METTL3 inhibitor	Bedi et al. (2020)
UZH1a		METTL3	METTL3 inhibitor	Moroz-Omori et al. (2021)
Rhein		FTO	FTO inhibitor	Li Q. et al. (2016)
Fluorescein derivative		FTO	FTO inhibitor	Wang et al. (2015)
Radicicol		FTO	FTO inhibitor	Wang R. et al. (2018)
Meclofenamic acid (MA)	 a R^1= -H, R^2= -H	FTO	FTO inhibitor	Huang et al. (2015)
CHTB		FTO	FTO inhibitor	Qiao et al. (2016)
Entacapone		FTO	FTO inhibitor	Peng et al. (2019)
N-CDPCB		FTO	FTO inhibitor	Qiao et al. (2017)
Dac51		FTO	FTO inhibitor	Liu et al. (2021)
MV1035		ALKBH5	ALKBH5 inhibitor	Malacrida et al. (2020)

5 Potential clinical applications of N^6-methyladenosine RNA modification

Due to the major role of m^{6}A RNA modification in tumour and other disease progression, m^{6}A-associated proteins can be developed as potential therapeutic targets for tumours and other diseases (Table 2). METTL3 attenuates the sensitivity of colon cancer cells to chemotherapy of L-OHP and CPT-11 by upregulating the expression level of CBX8 in a m^{6}A-dependent manner (Zhang Y. et al., 2019). Meanwhile, *Ythdf1*-depleted colon cancer cells are more sensitive to 5-FU and L-OHP (Nishizawa et al., 2018). Another study identified that gemcitabine drove the apoptosis of pancreatic cancer cells with low METTL3 expression (Taketo et al., 2018). The combination of m^{6}A methylation and chemotherapeutic drugs contributes to resolving drug resistance in tumours. Cas13-directed methyltransferase has been used for cancer treatment, targeting m^{6}A of specific RNA loci (Wilson et al., 2020; Lo et al., 2022).

Specific inhibitors based on m^{6}A-related enzymes have been studied. Bedi et al. (Bedi et al., 2020) performed a virtual screening assay to identify potential METTL3 inhibitors from 4,000 adenosine derivatives. One compound, a SAM mimic, was found to be the first inhibitor of METTL3. However, the therapeutic value of this compound is still somewhat limited due to cell penetration issues and nonspecific targets. They also identified another METTL3 inhibitor, UZH1a, that decreased the m^{6}A/A ratios in three different cell lines, MOLM-13, HEK293T and U2OS, but the specificity of UZH1a needs further improvement (Moroz-Omori et al., 2021).

Currently, more papers are focused on FTO inhibitors. Rhein, a competitive inhibitor of FTO, binds to the catalytic domain and blocks the catalytic activity of FTO (Li Q. et al., 2016). Fluorescein derivatives, radicicol, IOX3 and MA (a nonsteroidal anti-inflammatory drug) were subsequently identified to repress FTO expression (Huang et al., 2015; Wang et al., 2015; Wang R. et al., 2018). Similar to MA, MA2, an isomer of MA, interacts with FTO to increase the m^{6}A level but possesses better cell penetration ability. In addition, CHTB, entacapone and N-CDPCB also repress the demethylase activity of FTO (Qiao et al., 2016; Qiao et al., 2017; Peng et al., 2019). Dac51 inhibits the activity of FTO, promotes T cell response and enhances the anti-PD-1 therapy (Liu et al., 2021). Small-molecule FTO inhibitors have been developed as potential drugs not only for tumour therapy but also for the treatment of neurological diseases, cardiovascular disease, metabolic syndrome and autoinflammatory disorders. To date, little is known about specific ALKBH5 inhibitors. However, the imidazobenzoxazin-5-thione MV1035 was found to be a potential candidate ALKBH5 inhibitor (Malacrida et al., 2020). ALK-04 as an inhibitor of ALKBH5 reduces the infiltration of myeloid-derived suppressor cells and Treg cells and suppresses tumour growth by enhancing the therapeutic effect of anti-PD-1 treatment (Li et al., 2020). (Table 2).

6 Conclusions and future prospects

Despite the initial discovery of m^{6}A in 1974, m^{6}A modification has received much less attention than histone and DNA epigenetic modifications. Since the establishment of MeRIP-Seq for mapping m^{6}A deposition at a transcriptome-wide level in 2012, interest in studying m^{6}A methylation has grown rapidly. The level of m^{6}A is dynamically and reversibly regulated by various effectors termed “writers”, “erasers” and “readers”. The METTL3-14-WTAP methyltransferase complex is the core constituent of most m^{6}A writers, whereas FTO and ALKBH5, as m^{6}A demethylases, catalyse the removal of m^{6}A. Currently, emerging evidence has shown that transcription factor and histone modification signatures together shape m^{6}A deposition, suggesting that the contribution of transcription factor histone modification contributes to the modulation of m^{6}A. The interplay between histone H3K36me3 and m^{6}A modification provides a novel layer of gene expression regulation. Further crosstalk between m^{6}A RNA modification and other epigenetic modifications should be carefully elucidated in the future. Nutritional metabolism and metabolites also influence the regulation of m^{6}A. It is possible that tissue-specific m^{6}A levels partially correlate with the metabolic activities of specific organs. Overall, the precise regulatory mechanisms of m^{6}A are still in their infancy and need to be further investigated. Although high-throughput sequencing-based techniques have greatly promoted the research field of m^{6}A methylation, none of the available approaches have simultaneously reached the achievements with single-base resolution, quantification of m^{6}A disposition, and low input of RNA. A future method for the direct sequencing of m^{6}A RNA will be better for illustrating its functions and dynamics *in vivo*.

As mentioned above, abnormal m^{6}A methylation is closely related to various diseases, including cancer, cardiovascular disease, metabolic syndrome, psychiatric disorders and autoinflammatory disorders. Accordingly, m^{6}A methylation is a double-edged sword for tumour: a lack of m^{6}A modification on specific genes accelerates tumour development, while over-m^{6}A modification of other genes also induces tumour progression. In fact, many major challenges remain in elucidating the relationships between m^{6}A and cancer. First, whether the multiple roles proposed in cancer actually rely on m^{6}A modification should be considered. Second, we should not ignore the notion that m^{6}A-related effectors may mediate tumour development and the progression in a m^{6}A-independent manner. Third, it is worth noting that we elucidate the regulatory association of noncoding RNAs and m^{6}A methylation in tumour. Fourth, the effects of m^{6}A regulators on tumor cells and immune cells are complicated and need to be

carefully concerned. Fifth, over 170 RNA modifications have been identified, it is worth evaluating whether other RNA modifications in the same RNA transcripts affects the role of m6A methylation in human diseases. Finally, further studies should be carried out to assess the clinical value of m^{6}A in diseases.

Many studies have demonstrated that m^{6}A regulatory factors are suitable as therapeutic targets, and some inhibitors of m^{6}A-related factors, especially FTO, have been discovered. Nevertheless, no clinical trials using m^{6}A inhibitors for the treatment of cancer and other diseases have been reported yet. We also provide several reasonable strategies for driving m^{6}A-based therapy: 1) Nanoparticles can specifically deliver m^{6}A modification molecules to target immune cells for tumour immunotherapeutic treatment. 2) A programmable m^{6}A gene-editing system by dCas13 or dRCas9 provides a potential tool for the treatment of diseases. 3) Chimeric antigen receptor (CAR) immune cells with lentivirus-mediated gene delivery of m^{6}A effectors are beneficial to cancer immunotherapy. 4) It is feasible to treat cancer and other diseases by using a m^{6}A inhibitor combined with other therapies. Further studies are urgently required for the understanding of RNA m^{6}A modifications and clinical applications.

Author contributions

JL and JS made substantial contributions to conception and design. PL involved in drafting and revising the manuscript, YW revised the manuscript and draw the figures, YS drafted the tables.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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