Biological roles of RNA m⁵C modification and its implications in Cancer immunotherapy

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Abstract

Epigenetics including DNA and RNA modifications have always been the hotspot field of life sciences in the post-genome era. Since the first mapping of N6-methyladenosine (m⁶A) and the discovery of its widespread presence in mRNA, there are at least 160-170 RNA modifications have been discovered. These methylations occur in different RNA types, and their distribution is species-specific. 5-methylcytosine (m⁵C) has been found in mRNA, rRNA and tRNA of representative organisms from all kinds of species. As reversible epigenetic modifications, m⁵C modifications of RNA affect the fate of the modified RNA molecules and play important roles in various biological processes including RNA stability control, protein synthesis, and transcriptional regulation. Furthermore, accumulative evidence also implicates the role of RNA m⁵C in tumorigenesis. Here, we review the latest progresses in the biological roles of m⁵C modifications and how it is regulated by corresponding “writers”, “readers” and “erasers” proteins, as well as the potential molecular mechanism in tumorigenesis and cancer immunotherapy.

Keywords: Epigenetics, RNA methylation modification, 5-methylcytosine (m⁵C), Non-coding RNAs, cancer immunotherapy

Overview of RNA 5-methylcytosine (m⁵C) modification

DNA and RNA modifications play crucial roles in various biological processes [1]. Compared with limited modifications detected in DNA, more than 170 modifications have been discovered in RNA including N6-methyladenosine (m⁶A), 5-methylcytosine (m⁵C) and 7-methylguanylate (m⁷G) (Fig. 1). They increase the complexity of the RNA species by acting on the tertiary structure, biogenesis, localization and function of RNA [2–4]. Among these modifications, mRNA N6-methyladenosine (m⁶A) is well acknowledged to be involved in translation, miRNA biosynthesis and gene attenuation [5, 6] (Fig. 1).

The discovery of DNA 5-Methylcytosine (m⁵C) can be traced back to the 1950s, earlier than the discovery of the double helix structure of DNA [7, 8]. In the 1970s, researchers discovered the m⁵C modification. Similar to DNA m⁵C [9], an active methyl-group from the donor, usually S-adenosyl-methionine (SAM), is added to the carbon-5 position of the cytosine base in RNA to form the m⁵C modification, which is likewise a widespread RNA modification detected in messenger RNA (mRNA) and non-coding RNAs including transfer RNA (tRNA), ribosomal RNA (rRNA), long non-coding RNA (lncRNA), small nuclear RNA (snRNA), micro RNA (miRNA) and enhancer RNA (eRNA) [10]. The m⁵C modifications have been reported in many species but its distribution seems to differ. For example, eukaryotic...
tRNA and mRNA have more m5C modification than bacterial mRNA and tRNA [9, 11, 12].

m5C modifications on tRNA and rRNA were extensively studied. In tRNA, m5C has been shown to maintain homeostasis, optimize codon-anticodon pairing, regulate stress response, and control translation efficiency and accuracy [13–18]. rRNA m5C modification is involved in glioma sensitivity to bioactive substrates of the stress-related enzyme NQO1 and structural stability of the tertiary rRNA–tRNA–mRNA complex under stress [19].

Until in the 1970s, researchers discovered the first m5C modification of eukaryotic mRNA, such as hamster cell mRNA and certain viral RNA but not SV40 RNA [20, 21]. Later, with the bisulfite sequencing in the whole transcripts of HeLa, a wide range of m5C modifications in mRNA and ncRNA were discovered. To date, mRNA m5C modifications have been associated with various biological processes, such as mRNA stability, splicing and nuclear–cytoplasmic shuttling [22–24]; DNA damage repair [25]; proliferation, and migration [26]; development, differentiation, and reprogramming of stem cells [27, 28]. Aberrant mRNA m5C modifications have been associated with the etiology of multiple diseases, including arteriosclerosis [29], autoimmune diseases [30] and cancer [31].

5-methylcytosine (m5C) modification in mRNA

With the development of high-throughput sequencing technology and the improvement of liquid chromatography sensitivity, the methods for the identification of the overall level of RNA methylation have been greatly developed. The current technical means used for the identification of the overall level of RNA methylation is mainly liquid chromatography coupled with MS (LC-MS). LC-MS/MS uses tandem mass spectrometry based on liquid-phase mass spectrometry, which is able to obtain both molecular and fragment ion peaks, allowing both qualitative and quantitative analysis of bases [32]. In contrast, bisulfite sequencing is a method that uses methylation to analyze different regions of DNA. In bisulfite sequencing, Bisulfite treatment can convert the unmethylated C bases in the genome into U, which becomes T after PCR amplification to distinguish them from the original C bases with methylation modifications, and then combined with high-throughput sequencing technology, a genome-wide DNA methylation map with single-base resolution can be drawn [33]. Moreover, a novel species-specific computational approach, Staem5, to accurately predict RNA m5C sites in Mus musculus and Arabidopsis thaliana was recently developed [34].

Bisulfite sequencing shows that m5C modification is another abundant mRNA modification and may be another RNA epigenetic marker [35]. Recently, enhanced liquid chromatography-mass spectrometry (LC-MS) has shown that eukaryotes do show methylation and hydroxymethylation of endocytosine. Due to the loss of information about the location of m5C modification in LC-MS, the adaptability of bisulfite sequencing with RNA opens a new possibility of mapping m5C with nucleotide resolution in RNA. Using this method, Lukas Trixl and Alexandra Lusser found ~10,000 sites showing >20% methylation and mapping to ~8500 mRNAs resulting in a rate of 0.43% m5C of all sequenced Cs, and they also published the first cytosine methylome for human cells [36].

Recently, Amort et al. detected about 7500 m5C sites (>20% methylation) corresponding to 1650 mRNAs in mouse embryonic stem cells and 2075 m5C sites corresponding to 486 mRNAs in mouse brain. Their concluded that m5Cs modification mainly exist in the coding region and are enriched around the translation initiation site [37]. Another study also found that m5Cs modification localized to the untranslated regions (UTRs) of mRNA transcripts [38].

Another recently published article on HeLa cells and mouse cytosine, a matrix, identified about 3600 loci in about 2000 genes in HeLa cells and 2500–4400 sites (1000-1655 genes) in different mouse tissues [37, 39]. About 100 m5C sites in Arabidopsis thaliana mRNA were detected by bisulfite sequencing [40], while another study used MeRIP-seq and found that 6045 peaks correspond to 4465 expressed genes [41]. MeRIP-seq was also used to detect the level of m5C in the archaebacterium Sulfolobus solfataricus and budding yeast, and a single site in yeast and 14 m5C modified mRNAs in S. solfataricus [12] were discovered respectively.

However, the exploration of the function of m5C in mRNA is not comprehensive and thorough, while there are some interesting findings, such as Yang et al. demonstrated that during the maternal-to-zygotic transition...
(MZT) of zebrafish, RNA m⁵C modification regulates maternal mRNA stabilization, highlighting the key role of m⁵C mRNA modification in early development [23].

More efforts are needed to uncover the m⁵C pandora-box roles besides in regulating mRNA stability.

**m⁵C modification in ncRNAs**

It was found that m⁵C not only plays a role in regulating the stability of mRNA, but also regulating the stability of rRNA and tRNA. A large number of studies have reported that m⁵C modification is vital for translation regulation of tRNA and rRNA [42, 43].

Most of the researches on m⁵C modification are focused on tRNA. Methylation of tRNA most often occurs on the cytosine at the junction region between the variable ring and the T-stem, as well as on one, two, or three Cs spanning the 47-50 position [44]. They participate in the composition of secondary structure, which is in connection with the codon recognition and stability of tRNA [45]. tRNA methylation mainly affects protein synthesis in mice [46]. Some studies have shown that methylation of C48 is based on augmenting the hydrophobicity of base pairs and promoting base stacking to stabilize this interaction. An unusual “Levitt pair” is formed by C48 and the nucleoside 15 on the D ring, resulting in a characteristic L-shaped three-dimensional structure [47]. Besides C48, C38 is another frequently methylated site in the anticodon loop. Studies have confirmed that methylation of C38 in mouse tRNA^Asp^ can effectively stimulate tRNA charging Asps in vivo and in vitro, hereby promoting the translation of proteins harboring multiple Asps [48]. At the same time, C38 methylation also protects the tRNAs from stress-induced endonuclease-mediated cleavage and corrects the translation and reading of nearly homologous codons [48]. During the decoding of aspartate codons, the loss of m⁵C38 of tRNA^Met^ results in a moderated translational fidelity [49]. At the same time, DNMT2-mediated m⁵C38 tRNA^Asp^ can help to distinguish homologous and near homologous codons, such as different tRNA^Asp^ from tRNA^Glu^, avoiding a false positive rate of amino acids, and improve the accuracy of translation [50]. In the late stage of tRNA^Thr^ and tRNA^Cys^ transcription, the CCA sequence is added at the 3’end and thereafter C72 was confirmed to be methylated [51]. However, there has been no specific function reported about this modification. Other mitochondrial studies have shown that mitochondrial f5C modification stems from m⁵C34 of mt-tRNA^met^, and it play a key role in mitochondrial translation as methionine in decoding unconventional AUA codons [52]. In yeast, wobble of m⁵C34 of tRNA^Leu^ is associated with translation regulation. In oxidative stress responses, its methylation level is significantly increased, which can enhance the translation of uug-codon-rich mRNAs, such as ribosomal protein El22A [47]. In addition, NSUN2-mediated tRNA m⁵C48 and m⁵C49 are located in the junction sequence of the V-loop (VL) and T-stem-loop (TSL), which is essential in accelerating tRNA stability and protein translation (Fig. 2).

In rRNA, m⁵Cs modification in the ribosome function critical region help to keep its conformation stable. In yeast, a methylated nucleotide cluster in the fourth domain of the 25 s rRNA is critical to maintain structural stability of the 60S ribosomal subunits, whereby deletion of m⁵C2278 and ribose methylation of G2288 in 25 s rRNA resulted in severe instability of the 60S ribosomal...
subunits [53]. The modification of m^5C in rRNA is associated with ribosome synthesis and protein translation. Under oxidative stress, m^5C2278 of 25S rRNA in yeast helps to maintain rRNA folding and facilitate selective recruitment and translation of mRNAs for participation in distinct cellular signals [53]. In mitochondrial ribosomes, NSUN4 is also required for m^5C911 12S rRNA. Depletion of NSUN4 caused functional assembly defect and disrupted mitochondrial protein translation [54]. NSUN5-mediated deletion of m^5C3782 28S rRNA hinders the synthesis of whole proteins [55].

It has been reported that if cytosine-5 methylation is absent in the vault RNA, it will be abnormally processed into Argonaute-associated small RNA fragments, which have a similar function as microRNAs [56]. Although m^5C is necessary for stability of vault ncRNAs, m^5C is situated in lncRNA, X-inactive specific transcripts (XIST), and it prevents Polycomb repressing complex 2 (PRC2) complexes from binding in vitro [57]. Hu et al. have investigated the distribution and regulation of lncRNA in colorectal cancer in 5hmC, and they found that the hm^5C is distributed in lncRNA and is positively correlated with lncRNA transcription. Studies have confirmed that hm^5C directly or indirectly regulates dysregulated colorectal cancer lncRNAs by the abnormal activity of superenhancers and promoters modified by the hm^5C. In addition, hm^5C also participates in long-range chromatin interactions at the lncRNA sites. They also found that lncRNAs regulated by different hm^5C markers were associated with different clinical outcomes and tumor status [58].

As a type of RNA modification, m^5C has relatively rich but highly dynamic characteristics, and it constitutes a multifunctional and effective mechanism for coping with constantly changing internal and external environments. It acts by regulating the intracellular RNA metabolism and related functions. m^5C modification is increasingly becoming mainstream in the field of epitranscriptomics.

**Dynamic regulation of m^5C by “writers”, “erasers”, and “readers”**

m^5C modification is mainly mediated by 3 types of proteins, namely the methyltransferases (writers), the demethylases (erasers) and the binding proteins (readers) respectively. The m^5Cs in RNAs are recruited by NOL1/NOP2/SUN (NSUN) protein family, including NSUN1-7 and DNA methyltransferase (DNMT) homologue DNMT2 [59], NSUN1, NSUN2, and NSUN5 are infrequently expressed in eukaryotes, whereas higher eukaryotes have high expressions of NSUN3, NSUN4, NSUN6 and NSUN7 [60] (Fig. 3).

**m^5C writers**

The methyltransferase of m^5C (RCMTs) uses adenosylmethionine as a methyl donor to form m^5C by transferring the methyl group to cytosine [61]. RCMTs are believed to be the writers for catalyzing methylation of cytosine-5, including the NSUN methyltransferases and DNMT2 [62]. More than 10 types of RNA m^5C methyltransferases have been found, including DNMT2, and tRNA-specific methyltransferase (TRDMT) family members, NOL1/NOP2/SUN domain (NSUN) family

![Fig. 3 The summary of discovered writer and eraser proteins of m^5C](image-url)
m\textsuperscript{5}C erasers

Protein demethylases called erasers, such as the TET family of enzymes, have a reversible effect by mediating the demethylation of written RNA [67]. In recent years, quantitative analysis using LC–MS/MS/MS has shown that TET has the potential as an RNA demethylase, and its overexpression can significantly increase the level of RNA 5hmC in HEK293T cells [68]. The TET (ten-eleven translocation) proteins, TET1, TET2, and TET3, function as DNA dioxygenases catalyzing 5mC to 5hmC on DNA [69]. It has been found that oxidation of 5FC to 5caC in RNA is mediated by TET1 [70]. It is been reported that TET2 mediates the oxidation of m\textsuperscript{5}C, which may inhibit the effect of 5-methylcytosine on the formation of double-stranded RNA in eukaryotes [71]. Besides, TET2 catalyzes RNA 5hmC, which involves degradation of RNA; this indicates that 5hmC plays a key role in post-transcriptional regulation [72]. In recent years, it has been revealed that the identified mitochondria DNA and RNA dioxygenase Alpha-ketoglutarate-dependent dioxygenase ABH1 (ALKBH1) also participates in the demethylation of N1 methyladenosine (m\textsuperscript{1}A) along the cytoplasmic tRNAs [73]. ALKBH1 catalyzes the anticodon modification of m\textsuperscript{5}c34 to hm\textsubscript{5}cm34 (5-hydroxymethyl-20-o-methylcytidine) and f\textsubscript{5}cm34 (5-formyl-20-o-methylcytidine) along mt-tRNA\textsuperscript{met} and cytoplasmic tRNA\textsuperscript{Leu} [74]. Loss of ALKBH1 induce severe deficiency in mitochondrial translation and oxygen consumption, indicating that RNA m\textsuperscript{5}C metabolism by ALKBH1 have great potential in regulating mitochondrial activity. Moreover, ALKBH1 can also specifically act on a histone dioxygenase-histone H2A [75].

m\textsuperscript{5}C readers

Most of the biological functions of RNA modification are related to the protein to which it binds. RNA m\textsuperscript{5}C binding proteins, such as ALYREF and YBX1, are considered to be a type of barcode reader, which exerts biological effects by recognizing and binding to m\textsuperscript{5}C sites [63]. Recently, m\textsuperscript{5}C-modified RNA oligos pull-down coupled with mass spectrometry analysis has been applied to a variety of m\textsuperscript{5}C mRNAs, and two reading proteins of m\textsuperscript{5}C mRNA, ALYREF (RNA and export factor binding protein 2) and YBX1, have been identified [76]. ALYREF is the first mRNA reading protein identified in the nucleus and has the key m\textsuperscript{5}C recognition site of K171. m\textsuperscript{5}C is recognized by the m\textsuperscript{5}C reader ALYREF through the viral RNA transcript installed in the nucleus, which helps them export to the cytoplasm [31]. In order to determine whether m\textsuperscript{5}C installation in viral transcripts occurred in the nucleus or the cytoplasm, Eckwahl and his colleagues performed RNAs isolation from nucleus and cytoplasm separately, followed by bisulfite treatment and Sanger sequencing; they focused their functional investigations on ALYREF reads on the protein. They found that knocking out the m\textsuperscript{5}C reading protein ALYREF has significant impact on viral protein production and viral replication. The depletion of ALYREF decreased the MLV Gag protein and virus released cells by 5-fold. On the contrary, the overexpression of ALYREF increased the Gag protein level. They also examined whether using the plasmid encoding wild- type ALYREF or the known m\textsuperscript{5}C to recognize the impaired ALYREF mutant (K171A) could rescue the 3T3 cells lacking endogenous ALYREF. They found that wild- type ALYREF but not mutant constructs can rescue phenotypic effects [77]. Eckwahl also confirmed that m\textsuperscript{5}C plays an important role in bridging the interaction between ALYREF and MLV RNA through endogenous RNA immunoprecipitation (RIP). They also confirmed that ALYREF knockout decreased the cytoplasmic and nuclear ratio of m\textsuperscript{5}C in viral RNA, due to fewer m\textsuperscript{5}C containing viral transcripts were exported to the cytoplasm.

Unlike ALYREF, YBX1 was identified as a cytoplasmic mRNA m\textsuperscript{5}C reading protein in human cells [78]. Based on the structural analysis of YBX1 protein and isothermal titration thermal analysis, YBX1 recognizes m\textsuperscript{5}C in its cold shock domain through the indole ring of W65 [79]. YBX1 specifically targets several m\textsuperscript{5}C-containing onco-genes, such as HDGF, and promotes the stability of these oncogenes and the subsequent progression of cancer by
recruiting the well-known mRNA stability maintenance factor ELAVL1.

Furthermore, YBX1 could recognize about 60% of m^5^C mRNAs in urothelial bladder cancer (UCB)-derived T24 cells. In zebrafish, the maternal YBX1 loss can increase overall translation, accumulation of unfolded proteins, leading to oogenesis defects and subsequent embryogenesis failure [80]. Recent studies have also found that 87.8% of the mRNAs modified by m^5^C were identified by YBX1. Upon the early development of zebrafish embryos, 397 maternal mRNAs with m^5^C showed significantly decreased expression when YBX1 was knocked out, indicating that these m^5^Cs mediated by YBX1 played a key role in regulating maternal gene clearance during the transition from the mother to the zygote [23]. In Ypsilon Schachtel (YPS) of Drosophila, the YBX1 homologue also acted as the RNA m^5^C reading protein [81]. In Drosophila ovary, YPS promotes the homeostasis, proliferation, and differentiation of germline stem cells (GSCs), which depends on the binding of m^5^C-containing RNAs. Interestingly, like humans and zebrafish YBX1, the highly conserved cold-shock domain (CSD) also contains the YPS m^5^C binding site. Mutation of this site can induce the defective development of GSC, which indicates that RNA m^5^C modification has an effect on the development of adult stem cells [82].

In summary, m^5^C modification extensively affects gene expression at multiple levels through interacting with a variety of writer, eraser and reader proteins (Fig. 4).

Therefore, the dynamic modification of m^5^C plays multifaceted roles in various biological processes, including embryonic stem cells self-renewal and differentiation [83], circadian rhythm [84], heat shock [85] or DNA damage response [86], and sex determination [87].

**Aberrant m^5^C in cancers**

It appears clear that the overall modification m^5^C and its regulators, including writers, erasers, and readers, are aberrantly expressed in various types of cancers. Emerging evidence indicates that methylation status is closely associated with the pathogenesis of cancer involving initiation, metastasis, progression, as well as drug resistance and relapse (Fig. 5).

Increased levels of RNA m^5^C can be detected in circulating tumor cells of lung cancer patients [88]. Increased gene expression of NSUN1 and NSUN2 can predict the occurrence and development of cancer [89]. Xue et al. selected m^5^C-regulated genes from the Cancer Genome Atlas (TCGA) database related to HNSCC and performed in-depth sequence analyses. They found that the expression of genes regulated by m^5^C was associated with the copy number variation (CNV) pattern. Gene set enrichment analysis (GSEA) showed that m^5^C reader protein ALYREF can actively promote mRNA export, thereby playing the role of an mRNA export adaptor in vivo and in vitro [90]. Meanwhile, DNMT1 and ALYREF expression levels effectively predicted the risk factors (morbidity and mortality) for patients.

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**Fig. 4** The functional landscape of m^5^C writers, readers, and erasers. m^5^C modification in tRNA or rRNA regulated by NSUN family proteins (NSUN2, NSUN3, NSUN4, NSUN5, NSUN6), NOP2, DNMT2 and ALKBH1 affects the translation. m^5^C modification of IncRNA, miRNA, vRNA and mRNA regulated by NSUN2, YBX1, AlyREF and TET family proteins affects their stability and nuclear export.
with HNSCC. Similarly, based on the TCGA database, He et al. linked the abnormal changes of m\(^5\)C regulatory genes in a variety of tumors with adverse clinical results, and provided a partial explanation for the discussion of cancer pathogenesis and/or survival. He and his colleagues assessed the correlation between the expression of m\(^5\)C regulatory factors and related cancer pathways, and found that mutations in m\(^5\)C regulatory genes and CNVs are significantly related in many cancer types. It is worth noting that this study verified the frequency of m\(^5\)C regulatory patterns in 33 cancers and compared the expression levels of m\(^5\)C regulatory factors in tumors and adjacent tissues, and found that they were overexpressed in tumors [91]. Glioma is the most common primary intracranial tumor, which is difficult to cure and often recurs. Wang et al. obtained the RNA sequence and clinicopathological data of RNA:m\(^5\)C methyltransferase under glioma from the Chinese Glioma Genome Atlas (CGGA) and TCGA data sets. The results showed that RNA:m\(^5\)C methyltransferase was significantly related to the malignant progression of glioma [92].

The NSUN2 gene has the highest mutation rate in gastrointestinal cancers [93]. A high frequency of mutations in m\(^5\)C regulatory genes were found in HCC. Dysregulation of m\(^5\)C-related genes was also associated with a higher HCC stage. Moreover, almost all patients with low survival rate were significantly associated with high expression of m\(^5\)C regulatory factors. High expression of NSUN4 and ALYREF is highly coincident with the survival outcome. GSEA results indicate that methylation and demethylation will lead to high expression of NSUN4 [94]. In addition, the abnormal m\(^5\)C modification of H19 IncRNA mediated by NSUN2 can affect its stability and interaction with the oncoprotein G3BP1, and it has been proved to be related to the poor differentiation of HCC. It indicates that this interaction may serve as a prognostic biomarker for HCC patients [95].
A recent study indicated that m^5C promotes the pathogenesis of human bladder urothelial carcinoma. m^5C stabilizes oncogene-mRNAs through the m^5C-modified sites in their 3' UTRs and promotes the pathogenesis of human bladder urothelial carcinoma by [31]. Zhang et al. found that NSUN2 and YBX1 is responsible for the m^5C methylation in the 3'-untranslated region of HDGF and promote the urothelial carcinoma of the bladder. Moreover, upregulated NUSN2, YBX1, and HDGF expression levels predicted a poorer survival rate in patients with urothelial carcinoma of the bladder [96]. A recent study demonstrated the involvement of m^5C in the development of urothelial carcinoma of the human bladder by stabilizing oncogene-mRNAs through m^5C modified sites in their 3' UTRs [31].

In addition, it has been shown that m^5C and its writer proteins are associated with lineage-specific chromatin structure and treatment response/resistance, and they may be an epigenetic driver of leukemia cytochemical resistance. Studies have shown that NSUN3 and DNMT2 forms 5-azacytidine (5-AZA) sensitive chromatin structure through binding to the RNA binding protein hnRNPK, complexed with GATA1 and SPI1/PU.1, which interacts with CDK9/P-TEFB to recruit RNA polymerase-II in the nascent RNA. However, NSUN1 is recruited to the active chromatin structure formed by BRD4 and RNA polymerase-II. Though this chromatin structure is insensitive to 5-AZA, it is highly sensitive to the BRD4 inhibitors JQ1 and NSUN1 knockdown. Furthermore, RNA m^5C and NSUN1−/BRD4 related active chromatin were enriched in 5-AZA-resistant leukemia cells and 5-AZA-resistant myelodysplastic syndrome and acute myeloid leukemia specimens [97].

In summary, aberrant expressions of m^5C writer, eraser and reader proteins are found in various human cancers (Fig. 6, Table 1).

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**Fig. 6** Role of aberrant expressions of m^5C writer, reader and eraser proteins in various cancer types. NSCLC: Non-Small Cell Lung Cancer; BRC: Breast Cancer; AML: Acute myeloid leukemia; BLC: Bladder Cancer; GBM: Glioblastoma; GC: Gastric Cancer; GNC: Gynecologic Cancer; CRC: Colorectal Cancer; HCC: Hepatocellular carcinoma; PRC: Prostate Cancer; HDFs: Human diploid fibroblasts; U87: Human glioma cell line U87; ATX: Autotaxin
The concept of protein replacement therapy using a gene was first proposed 30 years ago [106]. However, due to the fact that RNA molecule is unstable and can induce immunogenicity, this approach did not gain propagation. Later, Kariko group incorporated m5C into mRNA therapy either separately or in combination, and it reduced the toll-like receptor (TLR)-mediated immune effects of mRNA [9]. This marks the breakthrough in the RNA and immunotherapy field that base modification in mRNA alters the immune activity. Andries et al. [107] believed that m5C-mRNA can be used as a new approach in the field of mRNA-based therapeutics, partially due to that m5C-modified mRNA may evade the innate immune system through inhibition of endosomal Toll-like receptor 3(TLR3) (Fig. 7).

### m5C implications in tumor immune microenvironment

Increasing evidence based on bioinformatic analysis implicate the significance of m5C in tumor immune microenvironment. Diverse methylation regulators can be used as prognostic and diagnostic markers of cancer [108, 109]. Oral Squamous Cell Carcinoma (OSCC) patients with higher expression levels of m5C regulators had lower immune activity in TILs such as DCs, NK cells, and CD8+ T-cells [110]. Papillary thyroid cancer (PTC) patients with low m5C-score contained higher resting CD4+ memory T cells and CD8+ T cells and had better prognosis, while activated NK cells and monocytes were mostly enriched in the high m5C-score patients with worse prognosis [111]. In triple-negative breast cancer, the changes in the expression of m5C RNA methylation regulators, the up-regulation of NSUN2 expression and the down-regulation of NSUN6 expression, which can objectively predict the clinical prognostic risk of TNBC patients to a large extent. Therefore, it may become new prognostic markers of TNBC and provide clues for understanding the RNA epigenetic modification of TNBC [102]. Related studies have also confirmed that NSUN3 and NSUN4 can predict the prognosis of lung squamous cell carcinoma and regulate the immune microenvironment [112]. In lung adenocarcinoma patients, different m5C pattern had different TME immune cell infiltration and high m5C score group had a better prognosis [113]. Besides, m5C-regulated lncRNAs also predict the overall survival of lung adenocarcinoma patients and affect the tumor immune microenvironment [114]. In pancreatic cancer patients, m5C-related lncRNAs showed prognostic value. With TIDE (Tumor Immune Dysfunction and Exclusion) algorithm, high-m5C-lncRNA scores had a better response to immunotherapy [115]. In another study of pancreatic ductal adenocarcinoma (PDAC), relationships between m5C-related lncRNAs and PDAC-infiltrating immune

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**Table 1** The role and mechanism of m5C in cancer

| m5C regulators | Position | Function | Potential mechanism | Refs |
|---------------|----------|----------|---------------------|------|
| ALYREF, NSUN5, DNMT1 | HNSCC | Participate in progress and predict prognosis | Involved in regulating tumor energy metabolism and protein synthesis | [90] |
| NSUN2 | Gastric Cancer | Cell Proliferation | Regulate downstream gene p57Kip2 | [98] |
| NSUN2 | HDFs | Cell Proliferation and Senescence | Regulate the expression of p27 and CDK1 | [99] |
| NSUN2 | U87 | Cell Migration | Participate in regulation by methylating ATX mRNA | [100] |
| NSUN2 | HCC | Cell Differentiation | Recruit oncoprotein G3BP1 | [95] |
| NSUN2, ALYREF | Bladder Cancer | Cell Proliferation and Migration | Regulation of mRNA stability through a new binding protein YBX1 in the cytoplasm | [31] |
| NSUN6 | Pancreatic Cancer | Cell Proliferation | Regulates cell cycle and G2M checkpoints | [91] |
| NSUN4, ALYREF | HCC | Tumor progression | High expression of NSUN4 and ALYREF is significantly correlated with survival outcome | [94] |
| NSUN2, NSUN6 | TNBC | Tumor proliferation Occurrence and Metastasis | Affect tumor development and tumor immune microenvironment | [102] |
| NSUN2 | Gastrointestinal Cancer | Tumor progression and pathological staging | Regulates GSK3ß and participates in the Erbb/P38-Akt signaling pathway | [103] |
| DNMT3A | Glioblastoma Multiforme | Tumor progression | Inhibit the formation of mRNA/mRNA duplexes, loss of tumor suppression kinetic energy | [104] |
| ALYREF | Bladder Cancer | Glycolysis and Tumorigenesis | Overexpression of ALYREF promotes the proliferation of bladder cancer cells through PKM2-mediated glycolysis. | [105] |
cells were evaluated. Naïve B cells, CD8+ T cells, Treg cells, and resting NK cells had a higher expression level in the low-risk group while the M0 and M2 phenotype macrophages had a higher expression level in the high-risk group, suggesting that m5C-related lncRNAs may regulate pancreatic cancer progression via promoting M2 phenotype macrophage polarization or infiltration in PDAC [116]. Related expression also exists in the research of Head and Neck Squamous Cell Carcinoma [117], hepatocellular carcinoma [118], breast cancer [119], human high-grade serous ovarian cancer [120], cutaneous melanoma [121] and colon cancer [122].

**m5C writers in regulation of cancer immunotherapy**

Immune escape is a hallmark of human cancer. T-cell exhaustion mainly accounts for escape of tumors from immune surveillance. NSUN2, a tRNA methyltransferase, modifies tRNA and mRNA by increasing the methylation of m5C. NSUN2 is an important factor in maintaining stem cell self-renewal and differentiation [123]. In head and neck squamous cell carcinoma (HNSCC), the combination of NSUN2 expression and T-cell activation correlates with patient survival regardless of the HPV status, indicating that NSUN2 may serve as a potential biomarker for immune-checkpoint blockade [124]. It has been reported that NSUN2 methylates ICAM-1 (Intercellular Adhesion Molecule 1) mRNA and promotes its translation [29], thus inhibiting M2-macrophage polarization and suppressing tumor metastasis (Fig. 7). Moreover, targeting NSUN2 expression may also improve the outcome of immunotherapy in HNSCC [125]. Nonetheless, a larger sample size is necessary to further validate how the NSUN2 affects immune checkpoint blockade outcome [126].

**m5C readers in regulation of cancer immunotherapy**

YBX1 protein is the most common tumor-associated antigen and can also induce T cell response [127]. Therefore it can be used as a target of effector immunity and a candidate vaccine for evaluation [128]. Chemotherapy induces an immunosuppressive microenvironment in tumors and induces immune evasion through YBX1-mediated upregulation of PD-L1 (programmed death-1 ligand 1) (Fig. 7). YBX1 is upregulated in chemoresistant HCC cells. YBX1 knockout reverses chemoresistance by blocking PD-L1 expression and activating T cells in a tumor microenvironment [129]. Upregulation of functional cytotoxic CD8+ T cells and downregulation of myeloid-derived suppressor cells and regulatory T cells are associated with the overcome of the tumor immunosuppressive environment and immune escape status.
YBX1 drives signal transduction in a tumor immunosuppressive microenvironment and immune escape pathway. In addition, YBX1 knockout can reverse HCC drug resistance by blocking PD-L1 expression and activating T cells in a tumor microenvironment. Data presented by Tao et al. showed that both tumor immune evasion and multidrug resistance can be reversed by targeting the YBX1 signaling cascade, suggesting an effective treatment regimen against tumor chemoresistance [130].

In conclusion, these data implicate that modulation of m5C regulators could be a useful strategy.

**Conclusions and perspectives**

m5C modifications of RNA have been demonstrated as a new molecular mechanism that controls eukaryotes gene expression. How the modification of m5C affects the ncRNA biogenesis, localization, and function, and how these effects are associated with etiology of cancer are to be elucidated. In recent years, extensive efforts have been devoted to the study of m5C modifications in cancer. In this review, particular attention was focused to Writer-NSUN2, the Eraser-TET family, and Reader-YBX1..

Some proof-of-concept studies have revealed that mal-adjusted m5C modulators targeted by small-molecule inhibitors have therapeutic potential for cancer treatment. As of now, specific m5C inhibitors have not been developed. In DNA methylation, the chemicals initially developed to target these proteins, such as 5-aza-2-deoxycytidine (decitabine) or 5-azacytidine (Vidaza), have been approved to treat myelodysplastic syndrome by the FDA, but their usage in solid tumor treatments is limited.

Bioinformatic research in RNA m5C modification will also be critical in both identifying the m5C sites from Single-molecule sequencing and in prediction of m5C sites in different RNA species [131]. Several machine-learning algorithms have recently been developed to predict the m5C sites in Homo sapiens, Mus musculus and Arabidopsis thaliana [34, 132]. With the fast development of computational approaches, it can be expected that the m5C epitranscriptomics will be fully understood and its relation with cancer will be uncovered.

In the future, the detailed roles of RNA m5C in immune system regulation and tumor immune microenvironment will be important directions. As for RNA m5A, depletion of m5A writers, readers or erasers had significant phenotypic consequences in the cellular response to infection and in a few immune cell types. Whether RNA m5C exerts similar functions in the immune system deserves deep efforts. More important, how different RNA modifications combat or combine with each other in immune regulation needs a long way to explore.

Different from m5A, the RNA m5C modification partially shares the same enzymatic machinery including DNMT1 with DNA m5C. This mechanism may also play critical roles during the tumor development and progression. m5C can operate with the tumor cells and immune microenvironment to brake or accelerate the tumorigenesis. It will be interesting to identify the immune checkpoint molecular target of m5C in the tumor microenvironment, so that targeted therapy can be used in combination with checkpoint immunotherapy to better treat cancer. In general, the investigation of novel m5C epigenetic modification in cancer not only will provide new insights into the molecular mechanisms of cancer biology and immune response, but also will pave the way to the develop new promising therapies.

**Abbreviations**
m5A: N6-methyladenosine; m5C: 5-methylcytosine; UTRs: Untranslated regions; tRNA: Transfer RNA; rRNA: Ribosomal RNA; mRNA: Messenger RNA; snRNA: Small nuclear RNA; miRNA: Micro RNA; IncRNA: Long non-coding RNA; eRNA: Enhancer RNA; ShmC: 5-hydroxymethylcytosine; SFC: S-formylcytosine; ScaC: S-carboxycytosine; DNMT: DNA methyltransferase; LC-MS: Liquid chromatography-mass spectrometry; CD5: Coding sequence; RCMTs: RNA:m5C methyltransferases; TRDMT: TRNA-specific methyltransferase; TET: Ten-eleven translocation; ALKBH1: Alpha-ketoglutarate-dependent dioxygenase ABH1; hmsCrm34: 5-hydroxymethyl-20-o-methylcytidine; fscm34: 5-formyl-20-o-methylcytidine; RIP: RNA immunoprecipitation; GSCs: Germline stem cells; CSD: Cold-shock domain; VL: V-loop; TSL: T-stem and loop; XIST: X-inactive specific transcripts; PRC2: Polycomb repressing complex 2; CNV: Copy number variation; GSEA: Enrichment analysis; Tfs: Transcription factors; 5-AZA: 5-aza-2-deoxycytidine; 5-azacytidine; FDA: Food and Drug Administration; MD5: Myelodysplastic syndrome; AML: Acute myelogenous leukemia; SGC: Structural Genome Consortium; SAM: S-adenosylmethionine; SFU: 5-fluourouracil; GSC: Germline stem cell; S1P: Phosphoglycerate-1-phosphate; SPHK: Phosphoglycerase kinase; BC: Breast cancer; PCa: Prostate cancer; TLR: Toll-like receptor; SLE: Systemic lupus erythematosus; CR: Coding region; HNSCC: Head and neck squamous cell carcinoma; CTLA4: Cytotoxic T lymphocyte antigen 4; PD-1: Programmed cell death 1; ICAM-1: Intercellular adhesion molecule 1; PD-L1: Programmed death-1 ligand 1.

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H.S., B.L., and T.M. conceived this review and critically revised the manuscript. H.S. and B.L. drafted the manuscript. J.X., B.C., H.Y. and J.S. drew the figures. H.S., B.L., and T.M. conceived this review and critically revised the manuscript. J. Z. and X.Y. supervised and revised the manuscript. All authors read and approved the final manuscript.

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**Availability of data and materials**

The authors confirm that the data support the findings of this study are available within the article.
Declarations

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