The roles of RNA N$^\text{6}$-methyladenosine in esophageal cancer

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ABSTRACT

Esophageal cancer is a malignant tumour with a high degree of malignancy and high mortality. Its pathogenesis and treatment strategy remain unclear. N$^\text{6}$-methyladenosine (m$^\text{6}$A) is important for various biological functions in RNA modification and is currently being investigated extensively. It plays an essential role in RNA modification. m$^\text{6}$A modification is a dynamic process that reversibly regulates the target RNA through its regulatory factors and plays an important role in several diseases, especially cancer. However, the role of m$^\text{6}$A in esophageal cancer remains elusive. RNA modification and splicing are regulated by RNA methylation regulators called ‘writers’ (methyltransferases), ‘erasers’ (demethylases) and ‘readers’ (modified RNA-binding proteins). These regulatory factors recognise and bind to RNA methylation sites, regulate biological functions such as RNA splicing and translation and influence the occurrence, development, invasion and metastasis of tumours. Considering the importance of m$^\text{6}$A modification, we reviewed the regulatory mechanisms, biological functions and therapeutic prospects of m$^\text{6}$A RNA methylation regulators in esophageal cancer.

1. Introduction

Esophageal cancer (EC) is one of the most common malignant tumours worldwide, with extremely high mortality and low survival rates. The most common pathological classifications include oesophageal squamous cell carcinoma (ESCC) and oesophageal adenocarcinoma (EAC) [1, 2]. More than half a million people die of esophageal cancer every year, constituting 5.3% of all cancer deaths worldwide. There are significant differences in the incidence of esophageal cancer cases worldwide [3]. In East Asia, the incidence of ESCC is high, which is mainly related to smoking and other factors. EAC is mostly reported in Western countries, and its incidence is positively correlated with obesity-related diseases and gastroesophageal reflux disease (GERD) [4]. Esophageal cancer is an aggressive cancer with a 5-year survival rate of approximately 18.4% owing to its extremely high mortality rate [1]. ESCC remains the most common type of esophageal cancer; however, its incidence has declined in East Asia and increased in Europe and the United States over the past few decades [5]. Differences in lifestyle and genetic background may be the main reasons for these regional differences. At present, surgical resection remains the mainstay of treatment for esophageal cancer [6]. In the United States, only 18% of patients with esophageal cancer were diagnosed without metastases, and 40% of patients were reported to have distant metastases on diagnosis, indicating a poor prognosis of esophageal cancer [7]. It is projected that there will be 15,000 new EAC cases every year in the United States until 2030 [8]. Therefore, in-depth investigation of the pathogenesis of esophageal cancer is significant to improve the survival rate of patients.

Gene transcription abnormalities associated with esophageal cancer, including chromosomal and tumour cell mutations, were a major research focus in the past [9]. Studies have shown that gene mutation is an important mechanism of esophageal cancer, and various genetic mutations are found in patients with esophageal cancer. Of these mutations, TP53 mutation is the most common. High mutation rates of EGFR, CCND1, CDK4/CDK6 and MDM2 genes were reported in ESCC [10]. In addition, CCNE1, cyclin E and MGST1 also have high mutation rates in EAC, and the mutations are closely related to the occurrence and development of
esophageal cancer [11]. Furthermore, the upregulation of vascular endothelial growth factor C (VEGF-C) was positively associated with event-free survival, whereas the allele variation of FLT1 increased the risk of death [10, 12]. It is noteworthy that, in addition to epigenetic changes caused by gene mutations, drug resistance owing to chemical drug therapy for esophageal cancer has become increasingly prominent. Studies on the plasticity and chemotherapeutic resistance of esophageal cancer cells indicate that epigenetic modifications are involved in the regulation of abnormal phenotypic changes [13, 14, 15]. Moreover, epigenetic modifications are reversible, compared with genetic defects; therefore, epigenetic inheritance is considered as a prospective research focus.

Increasing evidence suggests that epigenetic disorders influence the occurrence and development of esophageal cancer [16, 17, 18]. N6-methyladenosine (m6A), or N6-methylated adenosine, has been extensively investigated [19]. m6A methylation regulators consist of three components, namely, methyltransferases (writers), demethylases (erasers) and binding proteins (readers) [20]. Recent studies have demonstrated that m6A either promotes carcinogenesis or inhibits malignant tumours. Zhang et al. demonstrated that m6A-mediated histone modification regulates changes in the tumour microenvironment invasion [21, 22]. In addition, Han et al. reported that m6A modification further enhanced YTHDF1-mediated immune regulation. YTHDF1 can be used as a potential therapeutic target for malignant tumours. Moreover, YTHDF1 also plays an important role in the immune evasion; however, the specific mechanism requires further investigation. m6A modification is important for the prognosis of patients with cancer [23]. The correlation between the expression of m6A methylation regulators and immune invasion in esophageal cancer requires to be investigated comprehensively. Therefore, we conducted a synthetic analysis of m6A modification and analysed the role of m6A modification-mediated epigenetic changes in esophageal cancer. We further elucidated that m6A methylation regulatory factors may serve as diagnostic markers and therapeutic targets for prospective studies on esophageal cancer.

2. Molecular mechanisms of m6A modification

2.1. m6A writers

m6A modification is dynamic and reversible, and the methyltransferase writers mainly include KIAA1429 (VIRMA), METTL3, RBM15, WTPAT, ZC3H13, METTL16, METTL14 and CBL11 [24]. The main feature of METTL3 and METTL14 is that they both contain a methyltransferase domain (S-adenosylmethionine; SAM), which is used to transfer methyl groups to adenosine at N6 [25]. METTL3 catalyses the formation of the methyltransferase complex, and METTL14 induces serine phosphorylation on METTL3 [26]. In addition, METTL14 expression is upregulated in pancreatic cancer cells, and its downregulation can increase cisplatin-induced apoptosis and autophagy in pancreatic cancer cells [20]. METTL16 is another methyltransferase that binds to non-coding RNAs (ncRNAs) to regulate mRNA transcription and maintain SAM homeostasis [27, 28]. KIAA1429 is a crucial element of the methyltransferase complex, and its main function is to guide regionally selective deposition of m6A [29]. Furthermore, it also regulates the expression of sex-lateral genes by alternative splicing (AS) of pre-mRNA with WTAP [30]. ZC3H13 is a typical CCCH zinc finger protein and a key factor in the nuclear localisation of the ZC3H13–WTAP–Virilizer–Hakai complex [31]. RBM15 is an RNA-binding protein. As a member of the SPEN (split ends) protein family, RBM15 is involved in m6A modification and as regulation and exhibits inhibitory functions in multiple signalling pathways [32]. CBL11 is highly expressed mainly in non-small cell lung cancer cells and promotes tumour proliferation and invasion (Figure 1) [33].

2.2. m6A erasers

To date, only two m6A demethylases have been identified, namely, ALKBH5 and fat mass and obesity-associated gene (FTO) [34, 35], which belong to the ALKB dioxygenase family, and their biological functions are mainly catalysed by Fe2+ and α-ketoglutaric acid [34]. ALKBH5 is a major m6A demethylase that plays an important biological role in human cancers and non-cancer diseases. For example, it plays a dual regulatory role in various cancers and reproductive system diseases [36]. FTO, as an obesity-susceptibility gene, plays a central role in regulating food intake. In addition, FTO performs a peripheral role by influencing lipolysis in adipose tissues [37]. Furthermore, FTO affects mRNA stability and translation efficiency by regulating m6A modification. In addition to FTO and ALKBH5, other m6A demethylases require to be identified (Figure 1).

2.3. m6A readers

m6A-binding proteins mainly include YTS21-B homology (YTH) domain-containing proteins (YTHDC1, YTHDC2, YTHDF1, YTHDF2 and YTHDF3), heterogeneous nuclear ribonucleoprotein (hnRNP) family members (hnRNP A2/B1, hnRNP C and hnRNP G), FXR family members, IGF2BP family member, elf family members and G3BP family members [26, 38]. The main characteristic of m6A-binding proteins is that they all have a conserved m6A-binding domain and bind to the Rrm6ACH sequence [39, 40]. m6A-binding proteins bind to mRNAs that exhibit 10–50 times higher affinity than that exhibited by unmodified mRNA. Moreover, they encode information regarding m6A modification and perform various biological functions by interacting with modified RNA [34]. The hnRNP family is involved in various cellular functions, including the regulation of transcription, mRNA metabolism and translation [41]. hnRNPs represent a large family of RNA-binding proteins (RBPs) that contribute to many aspects of nucleic acid metabolism, including AS, mRNA stabilisation, transcription and translation. Many hnRNPs exhibit common characteristics but differ in domain composition and functional properties [42]. YTH family proteins are the first identified m6A reading proteins, which are involved in the occurrence and development of cancerous tumours by regulating the targeted RNA metabolism, including RNA splicing, RNA output, translation and degradation (Figure 1) [43].

3. Function of m6A on coding and ncRNAs in esophageal cancer

3.1. Function of m6A on mRNA in esophageal cancer

The regulation of post-expression genes is implemented in four processes: transcription, post-transcription, translation and post-translation. m6A regulates RNA transcription and gene expression after RNA transcription by modifying the structure of RNA or by specific binding in the form of binding proteins [44]. The specific mechanism includes the labelling of m6A into the newly formed mRNA after transcription. Furthermore, the post-transcriptional mRNA is recruited to an m6A site or flanking sequence through splicing factors for AS and is then transformed to mature mRNA. Subsequently, mRNAs that contain m6A are recognised by YTHDC1 and transported to the cytoplasm [44]. mRNAs in the tumour cytoplasm are cleaved, matured and positioned on the ribosome for translation. In addition, in the cytoplasm, YTHDF family proteins enhance m6A-mediated mRNA metabolism. YTHDF1 can specifically bind to the m6A site and interact with the initiation factor eIF3 to promote the initiation of translation and protein synthesis [45]. YTHDF3 interacts with YTHDF1 to promote mRNA translation and accelerates the decay of m6A mRNA by interacting with YTHDF2 [46]. Furthermore, METTL3 promotes the translation of m6A-modified mRNAs in the cytoplasm, independent of its methyltransferase activity [47]. Choe et al. reported that METTL3 enhanced translation by interacting with the mRNA near the termination codon and supported the mRNA cyclisation mechanism to achieve ribosome circulation and translation control [47]. In addition, Wang et al. [48] conducted a study with 200 patients with ESCC and reported that METTL3 was upregulated in tumour tissues. METTL3 recruited YTHDF for adenomatous polyposis coli (APC)–mRNA degradation by upregulating m6A of the APC gene. The
expression of APC was decreased, and the expression of β-catenin, cyclin D1, C-MyC and PKM2 was increased, which promoted aerobic glycolysis, proliferation and metastasis of ESCC cells. Moreover, METTL3 promoted ESCC metastasis by enhancing glutaminase-2 (GLS2) expression. This study was the first to demonstrate that GLS2 is regulated by METTL3 through m6A modification. Therefore, METTL3/GLS2 signalling is expected to be a potential therapeutic target for ESCC anti-metastasis strategies [49]. Hou et al. found that METTL3-mediated AKT signalling pathway can promote the occurrence and development of esophageal cancer, and METTL3 upregulation can promote the proliferation, migration and invasion of ESCC cells and inhibit cell apoptosis [50]. Meanwhile, METTL3 can also increase m6A in EGR1 mRNA in a YTHDF3-dependent manner, enhance its stability, and activate EGR1/Snail signal [51]. Thus, METTL3 is upregulated in esophageal cancer, promotes proliferation and metastasis and is expected to be an independent biomarker for prognosis [52, 53, 54].

m6A writers and erasers are located in the plaques associated with mRNA splicing factors, suggesting that m6A is functionally associated with mRNA splicing [55]. m6A can exert the splicing function of mRNA on pre-mRNA by recruiting hnRNPB2A1 or altering the local structure and can increase the access of splicing factor hnRNPC/hnRNPG, thus affecting transcription [56]. In view of this, Guo et al. [57] used The Cancer Genome Atlas database and found that m6A is significantly enhanced, m6A regulatory factors are significantly upregulated, and the expression of ALKBH5 and hnRNPA2B1 is upregulated in patients with esophageal cancer, thus suggesting that m6A facilitates the prognosis of patients. hnRNPA2B1 upregulation can promote the proliferation and metastasis of esophageal cancer and the expression of the fatty acid synthase adenosine triphosphate citrate lyase (ACLY) and acetyl–coenzyme A carboxylase-1 (ACCL1). Studies have demonstrated that ACLY and ACC1 are carcinogenic factors that can promote the proliferation and metastasis of esophageal cancer. Therefore, ALKBH5 and hnRNPA2B1 may serve as diagnostic and prognostic markers and therapeutic targets for esophageal cancer. Nagaki et al. reported that ALKBH5 upregulation was associated with a poor prognosis of esophageal cancer, and the inhibition of ALKBH5 delayed the progression of esophageal cancer cell cycle, resulting in cell stagnation at G0/G1 phase. In ALKBH5-deficient cells, CDKN1A (P21) expression was significantly upregulated, and the knockdown of ALKBH5 increased m6A modification and CDKN1A mRNA stability [58]. FTO is an m6A-modified demethylase that has been associated with various tumours. Liu et al. demonstrated that FTO was upregulated in ESCC tissues and promoted the proliferation and migration of ESCC cells by upregulating MMP13 [59]. FTO can also promote the formation of EC cell lipid droplets by enhancing the expression of HSD17B11, thus promoting EC proliferation, invasion and tumorigenicity [60]. By analysing the TCGA database, Xun et al. demonstrated that m6A upregulation resulted in a worse prognosis of patients with ESCC, which may provide important information for diagnosis and treatment strategies [61]. The mechanism of m6A factors that regulate mRNA in esophageal cancer is demonstrated in Figure 2 and Table 1. The study design, key protocols/methods, and materials of m6A regulators in regulating mRNAs as shown in Table 2.

3.2. Function of m6A on ncRNAs in esophageal cancer

ncRNAs are RNAs that do not code for proteins. They include long ncRNAs (lncRNAs), microRNAs (miRNAs) and circular RNAs (circRNAs), with various known and unknown functions. The common feature of these RNAs is that they can be transcribed from the genome but cannot be translated to proteins; therefore, they perform their biological functions at the RNA level. Studies have indicated that m6A methylation regulatory factors are important for regulating ncRNA expression and function [27]. In this review, we have systematically described the regulatory mechanism of m6A modification in ncRNAs. The mechanism of m6A factors that regulate ncRNA in esophageal cancer is demonstrated in Figure 3 and Table 3. The study design, key protocols/methods, and materials of m6A regulators in regulating ncRNAs as shown in Table 4.

3.2.1. miRNAs

miRNAs refer to a class of endogenous small RNAs with a length of about 20–24 nucleotides, which play an important regulatory role in cells and are involved in gene silencing or post-transcriptional gene expression regulation [62]. Li et al. analysed esophageal cancer data from TCGA database and found that the expressions of 25 m6A regulators were increased and positively correlated. hnRNPA2B1 upregulation promotes lymph node metastasis of esophageal cancer and is associated with poor

Figure 1. The regulatory mechanism of m6A RNA methylation regulators in esophageal cancer. m6A modification is regulated by ‘writers’ and ‘erasers’, and m6A can be recognised by various ‘readers’ proteins, thus regulating the biological function of RNA.
Moreover, the knockdown of hnRNPA2B1 significantly downregulated miR-17, miR-18a, miR-20a, miR-93 and miR-106b, resulting in decreased proliferation and metastasis of esophageal cancer cells [63]. In addition, Xue et al. demonstrated that miR-193a-3p was upregulated in ESCC tumour tissues, compared with normal tissues, and promoted invasion and metastasis, and a high expression of miR-193a-3p promoted the recurrence of ESCC. In addition, miR-193a-3p and ALKBH5 can regulate each other by forming a closed loop. Therefore, the mutual regulation of miR-193a-3p and ALKBH5 affects the progression of ESCC [64]. In addition, miR-20a-5p expression was upregulated in esophageal cancer, and METTL3 increased the expression of miR-20a-5p by improving m6A modification. In addition, miR-20a-5p upregulation promotes the invasion and migration of esophageal cancer by targeting NFIC transcription [65]. ALKBH5 has been confirmed to inhibit the development of esophageal cancer mainly through m6A/DGCR8-dependent demethylation of pri-miR-194-2 and inhibition of miR-194-2 biogenesis, followed by inhibition of esophageal cancer progression by ALKBH5/miR-194-2/RAI1 axis [66].

### 3.2.2. LncRNAs

LncRNAs are a group of endogenous RNA molecules defined as ncRNAs with a length of more than 200 nucleotides. They have been reported to play an important role in many diseases, including ESCC [67]. A few studies have investigated the mechanism of m6A modification in lncRNAs. Wu et al. reported downregulation of Y-linked lncRNA LINCO0278 in men with ESCC, which encodes a Yin Yang-1 (YY1)-binding peptide named YY1BM. YY1BM inhibited CDKN1A (p21) expression while FTO upregulated MMP13 expression, promoting proliferation and metastasis of esophageal cancer. As a “Reader”, HnRNPA2B1 can up-regulate the expression of ACLY and ACC1 and promote the increment of esophageal cancer.

### Table 1. The role of different m6A regulators in regulating mRNAs in esophageal cancer.

| m6A regulators | Genes     | Location | Role      | Mechanism                        | Function                       | References       |
|---------------|-----------|----------|-----------|----------------------------------|--------------------------------|------------------|
| METTL3        | APC       | mRNA     | Oncogene  | Degrade APC mRNA                 | Promoting EC tumorigenesis and metastasis | [48]             |
| METTL3        | β-catenin | mRNA     | Oncogene  | Enhance expression of β-catenin  | Promoting EC tumorigenesis and metastasis | [48]             |
| METTL3        | cyclin D1 | mRNA     | Oncogene  | Enhance expression of cyclin D1  | Promoting EC tumorigenesis and metastasis | [48]             |
| METTL3        | c-Myc     | mRNA     | Oncogene  | Enhance expression of c-Myc      | Promoting EC tumorigenesis and metastasis | [48]             |
| METTL3        | PKM2      | mRNA     | Oncogene  | Enhance expression of PKM2      | Promoting EC tumorigenesis and metastasis | [48]             |
| METTL3        | GLS2      | mRNA     | Oncogene  | Enhance expression of GLS2       | Promoting EC metastasis         | [49]             |
| METTL3        | AKT       | mRNA     | Oncogene  | Enhance expression of AKT        | Promoting EC tumorigenesis and metastasis | [50]             |
| HNRNPA2B1     | ACLY      | mRNA     | Oncogene  | Enhance expression of ACLY       | Promoting EC tumorigenesis       | [57]             |
| HNRNPA2B1     | ACC1      | mRNA     | Oncogene  | Enhance expression of ACC1       | Promoting EC tumorigenesis       | [57]             |
| ALKBH5        | CDKN1A (p21) | mRNA     | Oncogene  | Degrade CDKN1A (p21) mRNA       | Promoting EC proliferation      | [58]             |
| FTO           | MMP13     | mRNA     | Oncogene  | Enhance expression of MMP13      | Promoting EC proliferation and metastasis | [59]             |
| METTL3        | EGR1      | mRNA     | Oncogene  | Enhance the stability of EGR1-mRNA | Promoting EC proliferation and metastasis | [51]             |
| FTO           | HSD17B11  | mRNA     | Oncogene  | Up-regulated HSD17B11 mRNA      | Promoting EC proliferation and metastasis | [60]             |
| YTHDF1        | HK2       | mRNA     | Oncogene  | Promotes aerobic glycolysis      | Promoting EC proliferation and metastasis | [104]            |
| ALKBH5        | -         | -        | Tumor suppressor | Down-regulated expression in ESCC tissues | Inhibit proliferation and migration | [105, 106]       |
LINC00278 and YY1BM translation [68]. In addition to surgical treatment for ESCC, platino-based drugs in combination with 5-fluorouracil (FP) are the first-line treatment for patients with ESCC, especially for those in advanced stages [69]. A growing body of evidence suggests that m6A modification plays an important role in tumour progression and chemoradiotherapeutic resistance [70]. Zhang et al. reported increased m6A levels and abnormal expression of SNHG3/miR-186-5p in patients with ESCC after platinum therapy. SNHG3/miR-186-5p is involved in the regulation of m6A expression level by targeting METTTL3. Therefore, the regulation of m6A level may offer a new strategy to improve the effects of platinum drugs on patients with ESCC [71]. Another study showed that LncRNA LINC00022 was up-regulated in ESCC, and FTO demethylated LncRNA LINC00022 and inhibited its degradation, promoting tumor growth in ESCC [72]. The expression of IGF2BP2, TK1 and LncRNA CCAT2 were upregulated in ESCC cells and tissues, while the expression of miR-200b was inhibited. CAAT2 bound to miR-200b and reduced its expression, resulting in the upregulated expression of IGF2BP2. IGF2BP2 enhances the stability of TK1 mRNA by recognizing the m6A modification of TK1, thereby enhancing its expression and promoting the migration and invasion of ESCC cells [73].

3.2.3. CircRNAs

circRNA is a new type of ncRNA, which forms a covalently closed continuous loop through reverse splicing, unlike linear RNA. Recently, m6A modification has been reported to be widespread in circRNAs and has the same read–write mechanism as mRNAs [74]. Wang et al. used plasma samples from 10 patients with ESCC, including patients with different tumour, lymph node and metastasis (TNM) stages, and 5 normal controls to screen the expression profiles of circRNAs and analyse the characteristics of circRNAs. They found that plasma circ-SLC7A5 levels in the patients

Table 2. Study design, key protocols/methods, and materials of m6A regulators in regulating mRNAs.

| m6A regulators | Study design | Key protocols/methods | Key materials | References |
|---------------|--------------|------------------------|---------------|------------|
| METTL3        | Bioinformatics database-Clinical sample data-Experimental verification | 1. Tissue microarray construction 2. RNA m6A quantification 3. Dual-luciferase reporter assays 4. MeRIP-qPCR | 1. pcDNA3.1-METTL3 cDNA plasmid 2. Specific shRNA | [48, 49, 50] |
| HNRPA2B1      | Bioinformatics Analysis-Experimental verification | 1. UniCox regression analysis and LASSO (least absolute shrinkage and selection operator) Cox regression model; 2. siRNA Constructs and Transfection | 1. si-HNRPA2B1 #1: GGAGAGTAGTTGAGCCAAA 2. si-HNRPA2B1 #2: GCTACGGAGGTGGTTATGA | [57] |
| ALKBH5        | Analysis of tissue microarray of the tumors-Experimental verification | 1. m6A RNA immunoprecipitation assay (MeRIP) 2. siRNA Constructs and Transfection | 1. si-ALKBH5 (#1): 5'-CAGGGATCTGGAGATGGA-3' 2. si-ALKBH5 (#2): 5'-GCTGCAAGTTCAGGAA'-3' | [58, 105, 106] |
| FTO           | Construct human ESCC tissue microarray (#HneoS160C01)-Experimental verification | 1. Human tissue microarray 2. RNA-seq transcriptome analysis 3. siRNA Constructs and Transfection | 1. siFTo-1: 5'-AAAUGAGGCGUCGUGUGAGA-3' 2. siFTo-2: 5'-GGAUGACUCUCAUCCUGA-3' | [59, 60] |
| YTHDF1        | Bioinformatics Analysis-Experimental verification | 1. Lentiviral vector 2. MeRIP-PCR 3. RIP assays | 1. psi-LVRU6GP lentiviral vector 2. The biotin-conjugated HCP5 probe 3. The Magna MeRIP™ m6A Kit | [104] |

Figure 3. The mechanism of m6A factors that regulate ncRNAs in esophageal cancer. In miRNA module, hnRNPA2B1 up-regulation promotes lymph node metastasis of esophageal cancer, which is associated with poor prognosis. Meanwhile, miR-193a-3p and ALKBH5 can form a closed loop and regulate each other. Therefore, the mutual regulation of miR-193a-3p and ALKBH5 affects the progression of esophageal cancer. LncRNA and CircRNA play a dual regulatory role in esophageal cancer.
were associated with TNM staging. In addition, circ-SLC7A5 contained a large number of m^6^A modification structures and exhibited a high translation potential. Moreover, circ-SLC7A5 exhibited a high affinity for binding to open reading frames and contained more miRNA-recognition elements (MRE), suggesting that circRNAs play a more diverse role in ESCC [75].

### Table 3. The role of different m^6^A regulators in regulating ncRNAs in esophageal cancer.

| m^6^A regulators | ncRNA | Location | Role | Mechanism | Function | References |
|------------------|-------|----------|------|-----------|----------|------------|
| HNRNPA2B1       | miR-17| miRNA    | Oncogene | Enhance expression of miR-17 | Promoting EC proliferation and metastasis [63] |
| HNRNPA2B1       | miR-18a| miRNA    | Oncogene | Enhance expression of miR-18a | Promoting EC proliferation and metastasis [63] |
| HNRNPA2B1       | miR-20a| miRNA    | Oncogene | Enhance expression of miR-20a | Promoting EC proliferation and metastasis [63] |
| HNRNPA2B1       | miR-93 | miRNA    | Oncogene | Enhance expression of miR-93 | Promoting EC proliferation and metastasis [63] |
| HNRNPA2B1       | miR-106b| miRNA   | Oncogene | Enhance expression of miR-106b | Promoting EC proliferation and metastasis [63] |
| ALKBH5          | miR-193| miRNA    | Oncogene | 1. miR-193-3p targeted ALKBH5 and inhibited its expression | Promoting EC proliferation and metastasis [64] |
| ALKBH5          | miR-20a| miRNA    | Oncogene | 2. ALKBH5 in turn inhibited the expression of miR-193-3p | |
| ALKBH5          | miR-194| miRNA    | Oncogene | 3. A positive feedback regulation between miR-193-3p and ALKBH5 | |
| METTL3          | miR-20a| miRNA    | Oncogene | Enhance expression of miR-20a-5p | Inhibit NFI transcription, promoting invasion, and migration. [65] |
| METTL3          | miR-194| miRNA    | Oncogene | Regulating microRNA biogenesis and RAI1 expression | Inhibit the proliferation of EC [66] |
| METTL3          | LINCO0278| lncRNA | Tumor suppressor | Enhance expression of LINCO0278 | Encoding YYIBM, promoting eEF2 activity, leading to EC apoptosis [68] |
| METTL3          | LINCO0278| lncRNA | Tumor suppressor | Enhance expression of LINCO0278 | Encoding YYIBM, promoting eEF2 activity, leading to EC apoptosis [68] |
| METTL14         | LINCO0278| lncRNA | Tumor suppressor | Enhance expression of LINCO0278 | Encoding YYIBM, promoting eEF2 activity, leading to EC apoptosis [68] |
| WTAP            | LINCO0278| lncRNA | Tumor suppressor | Enhance expression of LINCO0278 | Encoding YYIBM, promoting eEF2 activity, leading to EC apoptosis [68] |
| ALKBH5          | LINCO0278| lncRNA | Oncogene | Degrade LINCO0278 | Inhibition of eEF2 activity and EC apoptosis [68] |
| METTL3          | SNHG3  | miR-186-5p| Oncogene | Enhance expression of SNHG3 | Promote the proliferation of EC, inhibit cell apoptosis [71] |
| FTO             | LINCO0022| lncRNA | Oncogene | Promote the demethylation of LINCO0022 | Promote the proliferation of EC [72] |
| IGF2BP2         | CCAT2 | lncRNA    | Oncogene | Inhibit the expression of miR-200b, enhance the stability of TK1-mRNA | Promote the proliferation of EC [73] |
| None            | circ-SLC7A5| circRNA | Oncogene | circ-SLC7A5 had the maximum number of m^6^A modification structure with great translation potential | Promote the proliferation of EC [75] |

### Table 4. Study design, key protocols/methods, and materials of m^6^A regulators in regulating ncRNAs.

| m^6^A regulators | Study design | Key protocols/methods | Key materials | References |
|------------------|--------------|-----------------------|---------------|------------|
| HNRNPA2B1       | Bioinformatics Analysis-Experimental verification | 1. Bioinformatics Analysis 2. Cell Transfection | 1. siRNA-HNRNPA2B1: CCUAUCCAUCACAGGAU 2. siRNA-Control: GGAAUACCAUUAGGAGAU | [63] |
| ALKBH5          | Clinical sample data-Experimental verification | 1. MeRIP 2. Luciferase reporter assay 3. Transfection | 1. siALKBH5: CCAAGTACTTCCTGCAGGGA 2. pmrGLO-ALKBH5-WT 3. pmrGLO-ALKBH5-MUT | [64, 66] |
| METTL3          | Clinical sample data-Experimental verification | 1. Dual luciferase reporter gene assay 2. RNA pull-down assay 3. Mass spectrometry | METTL3 and miR-186-5p up and down regulated lentivirus | [65, 71] |
| YTHDF1          | Clinical sample data-Experimental verification | 1. Microarray data analysis 2. Preparation of cigarette smoke condensate 3. Mass spectrometry analysis 4. Production of YY1BM knockout and FLAG knockin cells | 1. Cas9/gRNA (puro-GFP) vector 2. Thermo Fisher LTQ Orbitrap ETD mass spectrometry 3. Cigarette smoke condensate | [68] |
| FTO             | Bioinformatics Analysis-Clinical sample data-Experimental verification | 1. Bioinformatics Analysis 2. Plasmids and siRNAs transfection 3. M6-methyladenosine modification prediction | 1. GEO and TCGA databases 2. LINCO0022 (si-022#1, si-022#2, si-022#3) 3. Online tools RMBase v2.0 and SRAMP | [72] |
| IGF2BP2         | Bioinformatics Analysis-Clinical sample data-Experimental verification | 1. Bioinformatics Analysis 2. Dual Luciferase Reporter Assay 3. MeRIP | 1. RNA22, mirWalk, GEPIA and R language database 2. Ualcan, LinkedOmics, GEPIA and MEM database 3. CCAT2 luciferase reporter plasmid | [73] |

### 4. Mutants of m^6^A sites and m^6^A regulators in esophageal cancer

Maladjustment of m^6^A regulators leads to abnormal m^6^A modification in key transcripts and abnormal regulation of the expression of these cancer-related genes. Therefore, m^6^A site mutations may play a role in cancer by interfering with m^6^A deposition [44]. Yang et al. assessed the
relationship between m^A modification gene variation and ESCC risk and reported the single nucleotide polymorphism rs2416282 in the promoter region of YTHDC2, which was significantly associated with the occurrence and development of ESCC. Furthermore, rs2416282 regulated YTHDC2 expression, and the knockout of YTHDC2 significantly promoted the proliferation rate of ESCC cells by affecting various cancer-related signalling pathways [76]. As a risk variant of YTHDC2, rs2416282 alters YTHDC2 expression and reduces ESCC risk. Therefore, gene variation plays an important role in the regulation of m^A modification. However, further investigation is required to comprehensively understand the pathogenesis of ESCC.

Although m^A modification does not change base pairing and coding, it broadly affects gene expression in multiple levels through interacting with diverse reader proteins and associated complexes. Therefore, dynamic m^A modification is critical for many normal bioprocesses, including self-renewal and differentiation of embryonic stem cells and hematopoietic stem cells, tissue development, circadian rhythm, heat shock or DNA damage response, and sex determination [77]. Recently, abnormal reductions or increases in m^A abundance have been found in some types of cancer, and this disorder may be associated with cancer progression and clinical outcomes. For example, it has been reported that m^A abundance (detected by m^A spot blotting or ELISA-like colorimetry) was significantly increased in mRNA or total RNA in human gastric cancer tissues compared with normal control tissues [78]. It is now understood that abnormal regulation of m^A regulators, particularly writer and erasers, leads to abnormal modification of m^A in key transcripts and to abnormal post-transcriptional regulation of the expression of these cancer-related genes. Therefore, it is reasonable to speculate that m^A mutations in these transcripts may interfere with m^A deposition and thus play a role in cancer. It has been reported that codon NO.273 of TP53 pre-mRNA G>A mutation (resulting in R273H mutation) makes colon cancer cells resistant in an m^A-dependent manner [79]. More recently, a large-scale population study identified a missense variant, the rs8100241 variant located in the exon of ANKLE1 with a G > A change (resulting in Ala > Thr change) in CRC, and showed that it was associated with decreased risk of CRC [80]. Mechanically speaking, compared with rs8100241[G] allele, rs8100241[A] allele can be methylated by m^A MTC and recognized by YTHDC1, thereby increasing the protein expression of ANKLE1. ANKLE1 is a potential tumour suppressor that inhibits cell proliferation by maintaining genomic stability [80]. In addition to the acquisition of new m^A sites due to cancer mutations, mutations that lead to loss of m^A modifications may also exist and contribute to cancer development and drug response. Several online tools combine m^A site information with SNP information to facilitate the investigation of functional m^A site mutations in cancer [81].

5. Immune infiltration of m^A in esophageal cancer

The immune system is the host's defence against infection and disease. Immunotherapy is a new cancer treatment strategy that has been widely used in the treatment of various solid tumours, including gastrointestinal tumours [82, 83]. In recent years, m^A regulatory factors have been extensively investigated to determine their function in tumour immunotherapy and immune avoidance. Currently, tumour immunotherapy and m^A modification are the most promising therapeutic strategies that are under investigation in studies concerning the pathogenesis and prognosis of esophageal cancer. Liu et al. used the Tumor Immune Estimation Resource (TIMER) database to analyse the relationship between glucose transporter-1 (GLUT1) expression, m^A modification and immune infiltration in esophageal cancer. They found that GLUT1 expression was upregulated in esophageal cancer and was associated with infiltration of various immune cells. For example, when GLUT1 expression was upregulated, the number of memory B cells was reduced. Therefore, upregulated GLUT1 expression in patients with esophageal cancer may trigger an anti-tumour immune response and play an important role in the regulation and recruitment of infiltrating immune cells in esophageal cancer [84]. There has been encouraging progress in immunotherapy for esophageal cancer, and some related immunosuppressors have entered clinical trials and exhibited persistent anti-tumour activity and controllable adverse reactions [85]. m^A modification has been reported to play an important role in tumour immunotherapy and immune avoidance, and therapeutic strategies that target m^A regulators have become the focus of current and future research [23]. Guo et al. analysed the transcriptional sequencing data and clinical information regarding 20 m^A methylation regulators from 453 patients with ESCC and found that METTL12, WTAP, IGF2BP3, YTHDF1, hnRNPA2B1, hnRNPC and PD-L1 were significantly upregulated in the patients. Immune scores showed a significant increase in the number of CD8+ T cells, stationary mast cells and regulatory T cells (Treg) in the patients. These results indicated that m^A regulators are the key mediators of PD-L1 expression and immune cell infiltration, which is expected to be an important target of ESCC immunotherapy [86]. In addition, Zhao et al. also demonstrated that the abnormal expression of m^A regulators was significantly correlated with the expression of ESCC immunomodulators (immunosuppressants, immunostimulants and major histocompatibility complex [MHC] molecules) and the level of immune infiltration [87]. YTHDF1 recognizes m^A-labeled transcripts encoding lysosomal proteases and increases their translation in dendritic cells. Loss of YTHDF1 promotes cross-expression of tumor antigens and cross-initiation of CD8+ T cells in vivo. In addition, the absence of YTHDF1 enhances the therapeutic effect of PD-L1 checkpoint blockade [23]. In melanoma, increased FTO levels promote tumor growth by decreasing m^A methylation in PD-1 (PDCD1), CXCR4, and SOX10 and preventing YTHDF2-mediated RNA decay. FTO knockout in melanoma cells sensitized tumor cells to interferon γ (IFNγ) in vitro and promoted mouse melanoma response against PD-1 antibodies [88]. METTL3 promotes circRNA2BP3 cycling and protects PD-L1 from proteasome-mediated degradation in a YTHDC1-dependent manner, thereby negatively regulating CD8+ T cell infiltration and promoting immune evasion of non-small cell lung cancer (NSCLC) cells [89]. In addition, m^A modification was positively correlated with the number of CD8+ T cells in pancreatic cancer immune microenvironment, suggesting that m^A modification may regulate CD8+ T cell aggregation [90]. These data suggest that regulation of m^A regulators can be combined with anti-PD-1/PD-L1 blockade to improve anticancer immunotherapy. Therefore, future studies should be focused on the function of m^A methylation regulators in ESCC tumour immunotherapy.

By regulating RNA transcription, splicing, processing, translation and decay, m^A modification is involved in the occurrence and metastasis of various malignant tumours. As the modification of m^A and its mechanism are involved in the occurrence, development, maintenance and drug resistance of various types of diseases, the study of m^A regulatory factors in immune response is still in its infancy. m^A gene modification supports rapid phenotypic variation in the disease. In malignant tumors, m^A regulators contribute to changing transcriptional patterns of gene expression in malignant tumors, which may contribute to tumor immune evasion and persistence in other diseases. However, these meaningful conclusions are due to the absence of one of the regulatory factors of m^A modification, while other mRNA or molecular component methylation levels associated with key phenotypes remain to be explored. Currently, most studies that rely on m^A immunophenotypes use cells in which an important component of the m^A regulatory complex has been deleted. Determining which m^A regulatory factors drive the observed phenotypes and whether other regulatory factors co-regulate the results remains to be studied. In the context of immune regulation, how immune signals can be gene-specific for specific m^A regulatory factors. Some m^A-mediated transcripts are immune-related genes that may drive phenotypic changes in the immune system. In addition, m^A modification inhibits the expression of MHC I on the surface of cancer cells, meaning that tumors can avoid inherent and adaptive immune responses. The m^A regulatory factor FTO also upregulates immune checkpoints and promotes immune escape. m^A seems to increase the activity of immunosuppressive cells, suppressing the activity of immune-promoting cells.
The same result was found for chemokines. At present, numerous studies have reported the relationship between m6A methylation and TME immune cell infiltration. For example, METTL3-mediated mRNA m6A methylation promotes dendritic cell activation and function, and knockdown of METTL3 in dendritic cells results in impaired phenotype and functional maturation of dendritic cells, decreased expression of costimulatory molecules CD40 and CD80 and cytokine IL-12, and reduced ability to stimulate T cell responses [91]. Yin et al. found that ablation of METTL3 in myeloid cells promoted the infiltration of tumor-associated macrophages and regulatory T cells into tumors [92]. Dong et al. demonstrated that down-regulation of METTL14 expression leads to differentiation of CD8+ T cells along a dysfunctional trajectory, promoting immune escape [93]. In addition, knockdown of ALKBH5 in GBM cells significantly inhibited the recruitment and immunosuppression of tumor-associated macrophages, and the expression and secretion of CXCL8/IL8 were also significantly inhibited [94]. These results suggest that m6A modification may play an active role in macrophage activation and the pathogenesis of various inflammatory diseases [95]. In addition, although much progress has been made in m6A modification, there is still a gap in understanding how m6A modification affects immune response, especially immune avoidance.

At present, immunotherapy has become the most popular cancer treatment method, among which targeting immune checkpoints is also closely related to m6A [96]. In the above, we discussed various mechanisms of immune evasion and immune regulation by mRNA modification in cancer. Among the many immunotherapy strategies, immune checkpoint blockade has shown remarkable efficacy. Studies have shown that m6A modification is involved in the regulation of PD-1 and PD-L1, and affects the tumor response to anti-PD-1 therapy. For example, in melanoma, METTL13/14, ALKBH5, and FTO can all modulate tumor response to anti-PD-1 therapy [97]. Inhibition of FTO can reduce tumor resistance to PD-1 treatment [88]. In addition to immunotherapy targeting PD-1, m6A modification is also involved in the regulation of cytotoxic T-lymphocyte antigen 4 (CTLA-4) targeted therapy [96]. In addition, m6A modification has also been implicated in the regulation of TIM3 (T-cell immunoglobulin-3), TIGIT (T Cell Immunoreceptor With Ig And ITIM Domains) and LAG3 (Lymphocyte Activation Gene3) [96]. Although the relationship between these immune checkpoints and m6A has not been thoroughly studied, their potential impact on tumor immunity should not be ignored. These findings suggest that the development of specific inhibitors targeting m6A regulators may be helpful for antitumor therapy. Regulation of m6A is closely related to the development and function of immune cells. Targeted regulation of m6A can enhance the function and infiltration of immune cells in the tumor microenvironment and enhance the tumor response to anti-PD-1 therapy through a variety of signaling pathways. It can be seen that the use of specific inhibitors targeting m6A regulators can combine antitumor activity with antitumor immunity and exert a comprehensive therapeutic effect. This will also be the hot direction of future studies on m6A modification involved in immunotherapy.

### 6. Other antitumor therapies mediated by m6A modification

Although many scholars have reported studies on m6A modification in tumor therapy, precise targeted therapy of m6A needs to be explored due to the complex mechanism of tumor formation. It affects the expression of a series of downstream oncogenes or transcription factors by changing the m6A level of some specific genes corresponding to mRNA in cells. Regulating the level of m6A in tumor cells may be the entry point for radiotherapy, chemotherapy and drug therapy. So far, more studies have focused on the identification of m6A-modified mRNA targets in cancer, while fewer studies have focused on m6A modification on ncRNAs, which is one of the future research directions. Thus, the treatment of cancer still has a long way to go in the future. Studying how m6A modification affects ncRNA production, cell location, and function, as well as how these processes are related to cancer, will greatly improve our understanding of m6A cell function and the currently unrecognized function of ncRNA. Recently, modifications of m6A have been found on chromosomal associated regulatory RNAs (carRNAs), including promoter associated RNAs, enhancer RNAs, and repeat RNAs, and have been shown to regulate chromatin state and transcription [98]. Whether these carRNA species play a role in cancer remains an interesting topic for further study. In addition to immune checkpoints and cell therapy, some regulatory cells related to tumor immunity also have the potential to be targets [99]. For example, regulatory T cells (Tregs) in the tumor microenvironment inhibit the body’s immune response to the tumor, which is a factor hindering immunotherapy. It can change the tumor response to immunotherapy through the regulation of m6A [100]. In addition, knockdown of ALKBH5 reduces Treg infiltration in melanoma and enhances the response to anti-PD-1 therapy [101].

In addition, since m6A modification also plays an important role in mediating tumor responses to chemotherapy, radiotherapy and immunotherapy, targeted therapies targeting m6A regulatory factors can also be combined with chemotheraphy, radiotherapy or immunotherapy in clinical practice to improve tumor treatment in the near future. In addition, similar to genome editing, external transcriptome editing can restore or remove functional essential m6A sites that are mutated or maladjusted in cancer, and such editing may also have clinical applications in future cancer treatments. In conclusion, studying the modification of m6A in cancer is a new frontier in cancer research. It not only reveals new aspects of epigenetic regulation in cancer, thereby contributing to the understanding of tumogenesis, immune response, and drug resistance, but will also lead to the development of effective new therapies. Targeting malregulated m6A regulators with effective inhibitors (or targeting mutated or dysfunctional m6A sites by targeting external transcriptomic editing), alone or in combination with other therapies, may have potential therapeutic potential for treating various types of cancer, particularly those that are resistant to existing therapies.

### 7. Conclusions and perspectives

RNA m6A modification has attracted increasing attention as a prime focus for epigenetic research, and its involvement in various biological processes and disease progression is also being increasingly investigated. From an epigenetic perspective, m6A modification provides new insights into the pathogenesis of many diseases, especially tumours. m6A plays a dual role by either promoting or inhibiting the occurrence and development of tumours by regulating mRNA and ncRNA levels of oncogenes or tumour suppressor genes [102]. This article reviews the role, mechanism and clinical applications of m6A in esophageal cancer. Recent studies have demonstrated that the role of m6A modification in the occurrence, development and biological function of esophageal cancer is complex, which is a novel research direction in epigenetics. The complexity of m6A modification may be related to different tumour stages, tissue types and complex RNA metabolism in the inflammatory and immune microenvironments of esophageal cancer. Furthermore, m6A modification is also a key factor in the diagnosis, treatment and prognosis of patients with esophageal cancer.

Several studies have been conducted to investigate the effect of m6A modification on esophageal cancer, and some m6A factors have been reported to regulate immune filtration and serve as diagnostic and prognostic markers and therapeutic targets for esophageal cancer; however, certain concerns regarding their function warrant further investigation. m6A modification may serve as an early diagnostic marker and a therapeutic target for esophageal cancer. METTL3, hsnRNA2B1, ALKBH5 and FTO have been confirmed to be upregulated in esophageal cancer and are expected to act as biomarkers for the diagnosis and prognosis of esophageal cancer. However, current literature is still preliminary and limited, and sufficient samples have not been obtained for testing. Therefore, further studies and large-scale clinical trials of m6A are required. Furthermore, mutations in some m6A regulatory factors increase the complexity of m6A-mediated epigenetic modification
regulation. For instance, as a YTHDC2 risk variant, rs2416282 alters YTHDC2 expression and reduces ESCC risk [76]. Currently, no other studies have further investigated the mechanism of m^6^A modification variation, and its complex mechanism in esophageal cancer remains unknown. In addition, current research on m^6^A modification is limited to the interaction mechanism of esophageal cancer, and studies on medical transformation remain elusive. Moreover, targeted therapy and immunotherapy offer great prospects for the treatment of esophageal cancer [103]. Therefore, more translational studies are required to promote the clinical applications of m^6^A modification, such as the combination of m^6^A with chemotherapy and immunotherapy. In conclusion, research on m^6^A modification in esophageal cancer is progressing rapidly and is a prospective approach for investigating clinical medicine transformation in future studies.

Finally, the immune system is the host’s defense against infection and disease. Meanwhile, immunotherapy is a new cancer treatment strategy, which has been widely used to treat various solid tumors, including various gastrointestinal tumors. In recent years, m^6^A regulatory factors have been widely studied in tumor immunotherapy and immune evasion. In addition, tumor immunotherapy is the most promising therapeutic strategy. Currently, immune invasion mediated by m^6^A modification is becoming a hot field of studying the pathogenesis and prognosis of esophageal cancer. However, the study of immune invasion mediated by m^6^A modification in esophageal cancer is still in its infancy, and m^6^A modification is expected to make new breakthroughs in this field in future studies.

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Author contribution statement

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