Recent advances of m^6^A methylation modification in esophageal squamous cell carcinoma

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Abstract
In recent years, with the development of RNA sequencing technology and bioinformatics methods, the epigenetic modification of RNA based on N^6^-methyladenosine (m^6^A) has gradually become a research hotspot in the field of bioscience. m^6^A is the most abundant internal modification in eukaryotic messenger RNAs (mRNAs). m^6^A methylation modification can dynamically and reversibly regulate RNA transport, localization, translation and degradation through the interaction of methyltransferase, demethylase and reading protein. m^6^A methylation can regulate the expression of proto-oncogenes and tumor suppressor genes at the epigenetic modification level to affect tumor occurrence and metastasis. The morbidity and mortality of esophageal cancer (EC) are still high worldwide. Esophageal squamous cell carcinoma (ESCC) is the most common tissue subtype of EC. This article reviews the related concepts, biological functions and recent advances of m^6^A methylation in ESCC, and looks forward to the prospect of m^6^A methylation as a new diagnostic biomarker and potential therapeutic target for ESCC.

Keywords: N6-methyladenosine, Methylation, Esophageal squamous cell carcinoma

Introduction
Esophageal cancer (EC) is one of the most invasive malignant tumors of digestive tract in the world, and its morbidity and mortality are still high in China [1]. 90% of the histopathological types of esophageal cancer are esophageal squamous cell carcinoma (ESCC) [2]. EC is caused by a variety of causes, among which genetic and epigenetic modifications play a key role in the occurrence and development of ESCC [3, 4]. In recent years, although surgical resection, combined radiotherapy and chemotherapy have improved the prognosis of patients with ESCC, the 5-year overall survival rate is still very low [5], between 20 and 30% [6]. Therefore, there is an urgent need to find new diagnostic biomarkers and potential therapeutic targets for ESCC patients.

In recent years, with the continuous development of tumor epigenetics, N^6^-methyladenine (m^6^A) has not only initiated a new era of post-transcriptional gene regulation in eukaryotes, but also rapidly become a research hotspot in the field of RNA methylation modification. As a reversible RNA methylation modification, m^6^A methylation is dynamically regulated by a variety of regulatory factors [7]. The imbalance of m^6^A methylation regulation changes the biological functions of cell proliferation, migration and invasion, and finally leads to the occurrence and development of tumor.
This article reviews the related concepts and biological functions of m6A methylation and the research progress in ESCC. In addition, it also emphasizes the prospect of m6A methylation as a new diagnostic biomarker and potential therapeutic target for ESCC.

Related concepts and biological functions of m6A methylation
m6A refers to the methylation at the N6 position of adenine, which is mainly concentrated in the 3’ untranslated region near the mRNA Terminator. m6A modification mainly occurs in the RRm6ACH sequence [8]. As one of the most common and abundant internal modifications in mammals and eukaryotes [9], m6A RNA modification involves almost all aspects of RNA metabolism [10]. Its regulation process is mainly dynamically and reversibly regulated by a variety of regulatory factors. The methylated proteins involved in m6A methylation are mainly methyltransferases, demethylases and RNA binding proteins [11–13]. They can add, remove or give priority to recognize m6A sites, which play a key regulatory role in the expression of the whole genome and have a great impact on normal physiological function or pathological status [14].

The written genes are various methyltransferases that promote m6A RNA methylation modification. At present, the components identified by m6A methyltransferase include methyltransferase like 3 (METTL3), METTL14, Wilms’ tumor 1 associated protein (WTAP), and Virilizer like m6A methyltransferase associated protein (VIRMA/KIAA1429), RNA binding motif protein 15 (RBM15), zinc finger CCCH domain-containing protein 13 (ZC3H13), Casitas B-lineage lymphoma-transforming sequence-like protein 1, CBLL1/HAKAI).

m6A modified writing gene (writers)

The basic mechanism of m6A methylation in RNA is shown in Fig. 1.

Fig. 1 Basic mechanism of m6A methylation in RNA. Basic mechanism of m6A. The m6A methylation is catalyzed by the writer complex including METTL3, METTL14, WTAP, VIRMA, RBM15, ZC3H13 and CBLL1. The m6A modification is removed by demethylase FTO or ALKBH5. Reader proteins recognize m6A and determine target RNA nature.
Methyltransferase mainly forms stable complexes through core proteins such as METTL3, METTL14 and WTAP. m^6^A methylation occurs on the bases of mRNA [18], in which METTL3 is a subunit with catalytic activity, METTL14 is responsible for recognizing the substrate, WTAP is mainly responsible for assisting METTL3 and METTL14 to target to nuclear spots, and WTAP has independent methylation sites, which can make some m^6^A sites specific methylation. VIRMA/KIAA1429, another component of methyltransferase complex, is a protein involved in alternative splicing and interacts with WTAP.

m^6^A modified eraser gene (erasers)
The erase gene can remove the m^6^A modification in the RNA molecule by encoding m^6^A demethylase, which is the key to the reversible process of m^6^A modification [19]. m^6^A demethylase has been identified as AlkB homolog 5 (ALKBH5) [20] and fat mass and obesity associated (FTO) [21]. Although they have similar functions, but the action process is different.

ALKBH5 can catalyze the m^6^A modification demethylation of RNA in vitro [20], which can significantly affect the output of mRNA and RNA metabolism as well as the assembly of mRNA processing factors in nuclear spots. FTO has the function of demethylation of m^6^A on single-stranded DNA and RNA, and they are highly expressed in fat, brain and hypothalamus [22], which is vital to metabolism.

m^6^A modified reading protein (readers)
The protein that selectively binds to the post-transcriptional product of m^6^A is called m^6^A reading protein. At present, the research on m^6^A modified reading protein is mainly focused on the YT521-B homology domain family (YTHDF), YT521-B homology domain containing (YTHDC), heterogeneous nuclear ribonucleoprotein binding protein (HNRNP), and insulin like growth factor 2 mRNA binding protein 2 (IGF2BP2), eukaryotic translation initiation factor (eIF3) and ELAV like RNA binding protein 1 (ELAVL1/HuR).

YTHDC1 may affect the splicing of mRNA and its output from the nucleus [15]. YTHDF2, YTHDF3 and YTHDC2, such as YTHDF2, which promote the degradation of mRNA, can selectively bind to methylated mRNA, participate in the storage and degradation of mRNA, affect the decay process of mRNA, and may have a positive effect on human lifespan [23]. It is IGF2BP1/2/3 that maintains the stability of mRNA [24], and it is YTHDF1, YTHDF3, YTHDC2 and IGF2BP1/2/3, that promote the translation of target mRNA. Among them, YTHDF3 can assist YTHDF1 to jointly promote the translation of related mRNA [25].

The role and significance of m^6^A methylation in ESCC
Studies have shown that m^6^A modified mRNA is mal-adjusted in many cancers, and its role in cancer has been gradually confirmed in vivo and in vitro, not only non-coding "writing genes", "erasing genes" and "reading genes", but also other protein factors, including oncoproteins, transcription factors and signal transduction factors. the overexpression or consumption of these m^6^A-related factors may change the m^6^A modification in the tumor and interfere with the progression of cancer. The role of m^6^A regulatory factor in ESCC is shown in Table 1. Therefore, to clarify the molecular mechanism of these changes of m^6^A modified RNA and identifying the abnormal expression of m^6^A regulatory factors in clinical biopsy specimens. It is of great significance for early diagnosis of tumors, prediction of tumor prognosis and provision of new approaches of tumor treatment.

m^6^A modification related protein was downregulated in ESCC.

**ALKBH5**
Previous studies [26] have found that, the expression of ALKBH5 in EC tissue decreased. Functional analysis showed that ALKBH5 could inhibit the proliferation, migration and invasion of EC cells. However, a recent study found that ALKBH5 promotes the proliferation and migration of ESCC [27]. These results contradict previous findings. The reason may be that this study did not compare the differential expression patterns between ESCC and normal esophageal tissue, but only detected the expression of ALKBH5 in ESCC. The results of the cancer genome atlas (TCGA) database through Gene Expression Profiling Interactive Analysis (GEPIA) online tool show that the overall survival time of patients with high expression of ALKBH5 in ESCC. The results of the cancer genome atlas (TCGA) database through Gene Expression Profiling Interactive Analysis (GEPIA) online tool show that the overall survival time of patients with high expression of ALKBH5 is longer than that of patients with low expression, indicating that ALKBH5 plays a tumor inhibitory role in EC [28]. ALKBH5 regulates cell proliferation, migration, invasion, tumor progression, metastasis, tumorigenesis and chemotherapy resistance might by regulating m^6^A methylation.
Yang et al. [29] through the study of the database, it was observed that the expression of YTHDC2 was downregulated in esophageal cancer. In the proliferation experiment, it was found that the low expression of YTHDC2 significantly promoted the growth of cells, suggesting that YTHDC2 may play a role as a tumor suppressor in ESCC. In the further enrichment analysis of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway, it was observed that several pathological pathways related to ESCC, including p53 signal pathway, NF-kappaB signal pathway and JAK-STAT signal pathway, were significantly rich in downregulated genes, thus promoting the proliferation of ESCC cells.

**m^6A** regulators in ESCC

| m^6A writer | METTL3 | Upregulated | Promote | In vivo: human | The malignant phenotype of ESCC cells was significantly inhibited by down-regulating PI3K/AKT signal pathway | [30, 31] |
| m^6A eraser | ALKBH5 | Downregulated | Inhibition | – | There is a positive feedback regulation node between miR-193a-3p and ALKBH5 in esophageal cancer cells | [26] |
| m^6A reader | YTHDC2 | Downregulated | Promote | In vitro: KYSE150, Eca-109, EC9706 | Up-regulation of MMP13 promotes cell proliferation and migration in esophageal squamous cell carcinoma | [32] |
| m^6A reader | HNRNPA2B1 | Upregulated | Promote | In vitro: HEEpiC, ECA109, TE10 | rs2416282 participates in the risk of esophageal cancer by regulating the expression of YTHDC2 | [31] |
| m^6A reader | HNRNPC | Upregulated | Promote | – | HNRNPC may be the promoter of ESCA carcinogenesis | [34] |

**YTHDC2**

A modification related protein expression upregulated in ESCC

**METTL3**

METTL3 is the core catalytic component of methyltransferase complex. Xia et al. [30] detected the expression of METTL3 in 207 patients with ESCC. The results of open data set and immunohistochemistry showed that the expression of METTL3 in tumor tissues was up-regulated compared with normal tissues adjacent to cancer, and the higher the expression level of METTL3 was, the worse the survival time was. In addition, it was also found that the expression level of METTL3 was an independent predictor of disease-free survival and overall survival in patients with ESCC. Another study [31] showed that small interfer RNA (siRNA) gene knockout of METTL3 inhibited the survival, colony formation, migration and invasion of EC cells, induced apoptosis, and significantly inhibited the malignant phenotype of ESCC cells by down-regulating PI3K/AKT signal pathway. In conclusion, METTL3 is a good predictor of ESCC and can be used as a potential biomarker for the prognosis of ESCC.

**FTO**

A study [32] through immunohistochemistry and data mining of 80 pairs of ESCC tissues, it was found that the expression of FTO in ESCC tissues was higher than that in adjacent normal tissues. The survival curve shows that the high expression of FTO has the trend of poor prognosis. A large number of evidence in the study confirmed that the downregulated expression of FTO can significantly inhibit the proliferation and migration of ESCC.
In addition, FTO promotes cell proliferation and migration in ESCC by upregulating matrix metalloproteinase 13 (MMP13). Therefore, FTO acts as a tumor promoter in the progression of ESCC.

**HNRNPA2B1**

Guo et al. [33] found that the expression of m^6^A and its regulatory factor HNRNPA2B1 was significantly increased in ESCC tissues, and the high expression of HNRNPA2B1 was positively correlated with tumor diameter and lymph node metastasis of ESCC. In addition, functional studies have shown that HNRNPA2B1 gene knockout inhibits the proliferation, migration and invasion of ESCC. In terms of mechanism, HNRNPA2B1 promotes the progress of ESCC by up-regulating the expression of fatty acid synthase ATP citrate lyase (ACYL) and aminocyclopropane-1-carboxylate (ACC1), indicating that HNRNPA2B1, as a carcinogenic factor, promotes the progression of ESCC by accelerating fatty acid synthesis, and may become a prognostic biomarker and therapeutic target of ESCC.

In addition, ALKBH5 and HNRNPA2B1 are effective indicators for predicting Overall Survival (OS) in patients with ESCC. High expression of HNRNPA2B1 and low expression of ALKBH5 are risk factors for ESCC survival. The combination of these two factors shows better predictive ability than using these two factors alone.

**HNRNPC**

Studies have shown that HNRNPC is overexpressed in ESCC tissues, and its expression is negatively correlated with the overall survival of patients with ESCC [34]. The double gene prognostic markers composed of ALKBH5 and HNRNPC have been proved to be a good predictor of survival outcome in ESCC.

**m^6^A methylation as a therapeutic strategy for ESCC**

More and more studies have found that microRNAs [35, 36] and circular RNAs [37] can be used as potential biomarkers for prognosis, diagnosis and treatment of EC. However, due to the high morbidity and mortality of EC, it is necessary to find new anticancer drugs, such as curcumin [38], which can be potentially used in chemotherapy and chemoprevention of EC by regulating miRNAs. Allicin [39] can achieve its anticancer effect by inhibiting cell growth and inducing apoptosis.

As described in Table 1, m^6^A plays different regulatory roles in ESCC through different biological functions. In addition, a study [40] constructed and verified prognostic markers of ESCC based on m^6^A RNA methylation regulators, which may be a promising tool for predicting patient survival and provide important information for ESCC to make diagnosis and treatment strategies.

In recent years, many studies have revealed that m^6^A plays an important role in the formation of many kinds of tumors, such as breast cancer [41, 42], ovarian cancer [43, 44], cervical cancer [45], acute myeloid leukemia [46–48], glioblastomas [49–51], non-small cell lung cancer [52–55], hepatocellular cancer [56–59], gastric cancer [60], colorectal cancer [61], pancreatic cancer [62], etc. Furthermore, it is found that m^6^A modification plays a key role in tumor radiotherapy, chemotherapy and drug therapy. Table 2 summarizes the mechanism and drug resistance of m^6^A regulatory factors in related tumors, which may provide clinical reference value and significance for the treatment of esophageal cancer in the future.

Although many scholars have reported the study of m^6^A modification in tumor therapy, because the mechanism of tumor formation is very complex, accurate m^6^A targeting therapy needs to be explored. By changing the m^6^A level of some specific genes corresponding to mRNA in cells, it affects the expression of a series of downstream oncogenes or transcription factors. It is possible that regulating the level of m^6^A in tumor cells will become the entry point of tumor radiotherapy, chemotherapy and drug therapy [63, 64]. There is still a long way to go in the treatment of tumor in the future.

**Conclusion**

In summary, with the development of biological techniques such as high-throughput sequencing, the role of m^6^A methylation in ESCC has been gradually revealed, at present, it has been found that there are abnormal expressions of METTL3, ALKBH5, FTO, YTHDC2, HNRNPA2B1 and HNRNPC in ESCC, mainly by affecting the stability of mRNA, regulating cancer cell proliferation and affecting tumor cell metastasis and invasion.

The discovery of m^6^A opens a new way for the study of epigenetics and tumor-related diseases, but the study of m^6^A modification is still in its infancy and there are still many challenges. The aim is to further study the role of epigenetic network in the occurrence and development of ESCC and to strengthen the evaluation of the safety and effectiveness of m^6^A-related regulatory factors and pathways as new targets for tumor therapy. To further explore the correlation between m^6^A and drug sensitivity and long-term prognosis of patients with ESCC, and to realize the application of m^6^A from basic research to clinical drug development as soon as possible.
| Related tumors                | m6A regulator | Roles          | Study model       | Mechanism                                                                 | Resistance                  | Ref.     |
|------------------------------|---------------|----------------|-------------------|---------------------------------------------------------------------------|-----------------------------|----------|
| Breast cancer                | METTL3        | Oncogene       | In vitro: MCF-7   | METTL3, hepatitis B virus X protein binding protein (HBXIP) and miRNA let-7 g form a positive feedback loop | Tamoxifen                   | [41]     |
|                              | ALKBH5        | Oncogene       | In vivo: mice     | Deme ethylation of NANOG and increase of mRNA level                       |                             |          |
| Ovarian cancer               | YTHDF1        | Oncogene       | In vitro: SKOV3, A2780 | TRIM29 may be used as an oncogene                                         | Cisplatin                   | [43]     |
|                              | FTO/ALKBH5    | Oncogene       | In vitro: PEO1    | Up-regulation of Wnt/β-catenin pathway by stabilizing FZD1                | Olaparib                     | [44]     |
| Cervical cancer              | FTO           | Oncogene       | In vitro: SiHa    | Regulation of β-catenin/ERCC1 axis                                        |                             | [45]     |
|                              | METTL3        | Oncogene       | In vitro: MOLM13, THP-1, MV4-11, NOMO-1, HL-60, EOL-1, KG-1, RN2c, HEL, JURKA T, LOUCY, K562 | Regulating the expression of c-Myc, Bcl-2 and PTEN                         |                             | [46]     |
|                              | METTL14       | Oncogene       | In vivo: human    | Enhanced self-renewal of hematopoietic stem cells and inhibition of bone marrow cell differentiation through SPI1-METTL14-MYB/MYC axis |                             | [47]     |
|                              | WTAP          | Oncogene       | In vitro: K562, HL-60, OCI-AML3, Ba/F3 | Regulating WT1 pathway to promote cell proliferation                      |                             | [48]     |
| Glioblastomas (GBMs)         | METTL3        | Oncogene       | In vivo: human    | Inhibition of tumorigenesis and self-renewal / proliferation of MSCs       |                             | [49]     |
|                              | METTL14       | Suppressor     | In vivo: human    | It is possible to target ADAM19 to inhibit tumorigenesis and self-renewal / proliferation of glioma stem-like cells (GSCs) |                             | [50]     |
|                              | FTO           | Oncogene       | In vivo: human    | The inhibitory effect of drugs on FTO can inhibit the formation of m6A demethylation gene in glioblastoma |                             | [50]     |
|                              | ALKBH5        | Oncogene       | In vivo: mice     | Deme thylated FOXM1 promotes tumorogenicity of GSC                       |                             | [51]     |
| Non-small cell lung cancer   | METTL3        | Oncogene       | In vitro: A549, H1299, Calu6, H520,95-D, PC9,HCC827 | SUMO promotes tumor growth of lysine residues K177, K211, K212 and K215 in NSCLC | Cisplatin/ Gefitinib         | [52, 53] |
|                              | Wtap          | Oncogene       | In vitro: H1299, A549, EBC-1, HCC827, CALU-3, H661, H596, H358, H660, H1650, H1975, HI 395, H292 | Down-regulation of c-MET expression                                      | Crizotinib                  | [54]     |
|                              | YTHDF1        | Suppressor     | In vitro: HEK293T, H1975, A549, NCI-H838, H1299, NCI-H1650, GLC-82, SPC-A1 | regulating the translational efficiency of CDK2, CDK4, and cyclin D1       | Cisplatin                   | [55]     |
| Hepatocellular cancer        | METTL3        | Oncogene       | In vitro: HepG2, Huh-7, MHCC977L, HepG-2, Hepa1-6, HEK-293T, WRL68, HuVEC, SMMC-7721, Bel7402, HepG-2, WRL68, HEK-293T | Reduce the stability of SOCS2 mRNA                                       | Sorafenib                   | [56, 57] |
|                              | METTL14       | Oncogene       | In vivo: mice     | Progress in regulating miR-126 through DGCR8                              | Sorafenib                   | [58]     |
|                              | YTHDF2        | Oncogene       | In vitro: HepG2,293T | MIR-145 regulates m6A level by targeting YTHDF2 mRNA 3-UTR in hepatocellular carcinoma cells |                             | [59]     |
Table 2 (continued)

| Related tumors       | m^6A regulator | Roles      | Study model                           | Mechanism                                                                 | Resistance          | Ref.   |
|----------------------|----------------|------------|---------------------------------------|---------------------------------------------------------------------------|---------------------|--------|
| Gastric cancer       | METTL3         | Suppressor | In vitro: AGS, HGC-27, MKN-45         | mediated this process occurred on the A879 locus of pri-miR-17-92         | Everolimus          | [60]   |
| Colorectal cancer    | YTHDF1         | Oncogene   | In vitro: SW480, CaCO2, HT29, RKO, DLD-1, KM12SM, HCT-116, LoVo | C-Myc promotes the expression of YTHDF1 and affects the proliferation and chemosensitivity of colorectal cancer | Oxaliplatin/ 5-Fu   | [61]   |
| Pancreatic cancer    | METTL3         | Oncogene   | In vitro: MIA PaCa-2                  | METTL3 is associated with mitogen-activated protein kinase cascades, ubiquitin-dependent process and RNA splicing and regulation of cellular process | Cisplatin/ Fu / Y-Irradiation | [62]   |
Abbreviations
m6A: N6-methyladenosine; miRNAs: Messenger RNAs; EC: Esophageal cancer; ESCC: Esophageal squamous cell carcinoma; METTL3: Methyltransferase like 3; WTAP: Wilms' tumor 1 associated protein; VIRMA: Virilizer like m6A methyltransferase; RBM15: RNA binding motif protein 15; ZC3H13: Zinc finger CCCH domain-containing protein 13; CBBL1: Casitas B-lineage lymphoma-transforming sequence-like protein 1; ALKBH: ALkB homolog 5; FTO: Fat mass and obesity associated; YTHDF: YTS21-B homology domain family; YTHDC: YTS21-B homology domain containing, HNRNP: Heterogeneous nuclear ribonucleoprotein binding protein; KEGG: Kyoto Encyclopedia of Genes and Genomes; siRNA: Small interfering RNA; MMP13: Upregulating matrix metalloproteinase 13; ACLY: ATP citrate lyase; ACC1: Aminocyclopropane-1-carboxylate; QSO: Overall survival.

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Authors’ contributions
XZ and NL conceived, designed and writing of the manuscript. LW and YW performed the literature retrieval, data analysis and interpretation. ML and YZ participated in revising the manuscript. MC, MZ and LZ critically reviewed and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Not applicable.

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All authors have reviewed the manuscript and agree to publish it in its current form.

Competing interests
The authors declare no conflict of interest.

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