Original Article

Comprehensive analysis of m6A RNA methylation regulators in esophageal carcinoma

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Background: N6-methyladenosine (m6A) is the most pervasive modification of RNA methylation in eukaryotic cells. m6A modification plays a pivotal role in tumorigenesis and progression in many types of cancers. Until now, the role of m6A modification in esophageal carcinoma (ESCA) has remained obscure. The aim of the study was to construct and validate prognostic signatures based on m6A regulators for ESCA.

Methods: Transcriptomic data, somatic mutations and clinical information were obtained from The Cancer Genome Atlas (TCGA). Copy number variations were obtained from the UCSC (University of California, Santa Cruz) Xena database. We curated 21 m6A regulators and performed consensus clustering analysis to quantify the m6A modification pattern.

Results: Of the 184 patients, 23 (12.5%) were genetically altered in m6A regulators, with the highest frequency of mutations in *ZC3H13* and *LRPPRC*. We constructed a m6A score system to investigate the prognosis of ESCA. The m6A score was closely related to immune cell infiltration in the tumor immune microenvironment. Patients with a high m6A score had an unfavorable prognosis. The combination of tumor mutation burden and m6A score would improve the prognostic value.

Conclusions: Our study established and validated a strong prognostic signature based on m6A regulators. This can be used to accurately predict the prognosis of ESCA.

Keywords: N6-methyladenosine (m6A); esophageal carcinoma (ESCA); RNA modification; prognosis; immune cell infiltration

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Introduction

Esophageal carcinoma (ESCA) is a highly aggressive digestive malignancy, with the seventh highest incidence and sixth highest mortality rate worldwide (1,2). According to the National Central Cancer Registry of China (NCCR) statistics, there were approximately 478,000 newly diagnosed ESCA cases in China, which represents a threat to public health (3). The main histological subtypes can be classified into squamous cell carcinoma and adenocarcinoma based on the location of the tumor (4,5). It is challenging to choose an appropriate therapeutic schedule for curing the

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disease. Radical esophagectomy is the standard therapy for ESCA without metastatic disease. Neoadjuvant therapy with chemotherapy or chemoradiotherapy has been utilized as an effective supplemental treatment option to cure ESCA (6). Despite immense improvements in diagnosis and therapy, the prognosis of patients with ESCA remains pessimistic (7). The 5-year survival rate is between 20% and 30% (8,9). Although the TNM staging system (T: extent of the tumor; N: extent of spread to the lymph nodes; M: presence of metastasis) is the most common indicator for prognostic prediction, it is of great importance to develop additional powerful prognostic predictors for precise individualized treatment.

N6-methyladenosine (m6A) modification is one of the most pervasive modifications of RNA methylation in eukaryotic cells and is a dynamic and reversible process involved in RNA splicing, nuclear transport, stability and translation (10,11). It is well known that m6A modification relevant to messenger RNA (mRNA) and noncoding RNA (ncRNA) regulates basic biological processes (BP) and pathological functions, such as cell differentiation, development and tumorigenesis (12,13). Recently, with the continuous development of high-throughput sequencing and tumor epigenetics, m6A modification has gained increasing attention. The m6A modification is regulated by a variety of regulators, such as methyltransferases (writers), demethylases (erasers), and binding proteins (readers). The identification of these m6A regulators brings a new dawn for investigating RNA modification biology (11,14,15). m6A modification is installed enzymatically by a methyltransferase complex comprising writers, such as *METTL3* and *WTAP*. It can be removed by two demethylases, *ALKBH5* and *FTO* (16-18). m6A can be identified by various readers that implement regulatory functions by selectively recognizing methylated RNA (19). Increasing evidence has demonstrated that m6A modification plays an important role in tumorigenesis, cell proliferation and the tumor microenvironment in various malignancies, such as breast cancer, prostate cancer, hepatocellular cancer, thyroid cancer and lung cancer (20-26). A previous study reported that high expression of *WTAP* (one of the "writers") leads to poor prognosis of gastric cancer by influencing T-lymphocyte infiltration, suggested that the m6A modification pattern was significantly associated with the tumor immune microenvironment (TIME) (27). Zhang *et al.* found that m6A modification patterns are strongly related to different TIME cell-infiltrating characteristics, and the evaluation of m6A modification patterns within individual tumors can predict immune infiltration and patient prognosis (28). Han *et al.* reported that *YTHDF1* (one of the "readers") inhibits cross-presentation by dendritic cells by recognizing and binding to m6A-tagged transcripts, thereby inhibiting anti-tumor immunity, blockade of *YTHDF1* can enhance the therapeutic efficacy of immunotherapy (29). Thus, these findings suggest that the m6A modification pattern can affect prognosis and immunotherapeutic efficacy in various cancers. At present, little is known about the biological function of these m6A regulators. There are few studies on the molecular subtypes of m6A regulators in ESCA. The aim of this study was to investigate the landscape of m6A modification and establish a scoring system for accurately predicting the prognosis in ESCA, thereby prolonging the survival time.

In the present study, we downloaded transcriptomic data, clinical information and somatic mutations of ESCA from The Cancer Genome Atlas (TCGA). Copy number variations (CNVs) were obtained from the UCSC (University of California, Santa Cruz) Xena database. The differentially expressed m6A regulators between normal and tumor tissues were identified. Consensus clustering analysis was utilized to establish a scoring system based on these m6A regulators for predicting prognosis. Additionally, we investigated the correlation between m6A modification and immune cell infiltration and tumor mutation burden (TMB). Our observations indicated that m6A modification might exert an essential role in the progression of malignancy, and the prognostic signature may be considered a promising prognostic biomarker and potential therapeutic target in ESCA. We present this article in accordance with the

Highlight box

Key findings

- This study established and validated a strong prognostic signature based on N6-methyladenosine (m6A) regulators.

What is known and what is new?

- m6A modification plays an important role in tumorigenesis, cell proliferation and the tumor microenvironment in various malignancies.
- This study constructed a m6A score system to improve the predictive value of a single m6A regulator, and evaluated the correlation of m6A regulators and immune cell infiltration in the tumor immune microenvironment.

What is the implication, and what should change now?

- m6A regulators can serve as potential prognostic biomarkers and promising therapeutic targets against immunotherapy.

TRIPOD reporting checklist (available at https://tcr.amegroups.com/article/view/10.21037/tcr-23-910/rc).

Methods

Data acquisition and processing

Transcriptomic data in the fragments per kilobase million (FPKM) format, somatic mutation and clinical information, including sex, depth of tumor invasion, lymphatic metastasis, distant metastasis and survival information, were downloaded from the TCGA-ESCA database (https://portal.gdc.cancer.gov/). A total of 10 normal samples and 159 tumor samples were enrolled for subsequent transcriptomic analysis. The pathological subtypes included squamous cell carcinoma (81 cases) and adenocarcinoma (78 cases). A total of 184 patients were enrolled for somatic mutation analysis. The CNV dataset was retrieved from the UCSC Xena database (https://xena.ucsc.edu/). Twenty-one well-acknowledged m6A regulators (writers including *METTL3*, *METTL14*, *WTAP*, *VIRMA*, *ZC3H13*, *RBM15* and *RBM15B*; erasers including *FTO* and *ALKBH5*; readers including *YTHDC1/2*, *YTHDF1/2/3*, *HNRNPC*, *FMR1*, *LRPPRC*, *HNRNPA2B1*, *IGF2BP1/2/3*) were selected for subsequent research. This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Survival analysis

We utilized univariate Cox regression analysis and the Kaplan-Meier method to investigate the prognostic effect of candidate genes or prognostic signatures. Survival curves were plotted by the Kaplan-Meier method, and a log-rank P value of less than 0.05 was considered to be statistically significant.

Differential gene and somatic mutation analyses

The limma R package was used to identify m6A regulators that were differentially expressed between normal and tumor samples. The significance criteria were set at the cutoff value of P less than 0.05 and |fold change|=1. We employed the maftools R package to evaluate the differences in somatic mutations.

Consensus clustering analysis

Using the ConsensusClusterPlus R package, we clustered the patients with ESCA into distinct subgroups based on the profiles of 21 m6A regulators and m6A-related differentially expressed genes (DEGs). The K-means clustering method was utilized to decide the number of distinct subgroups. Additionally, we constructed a m6A score system to quantify the m6A modification pattern. All m6A-related DEGs with significant prognoses were dichotomized into two groups according to the hazard ratio with a cutoff value of 1. Then, all patients were divided into low and high m6A score groups on the basis of cumulative hazard ratios <1 or >1. The ggalluvial R package was employed to depict an alluvial diagram to show the correlation between distinct clusters.

Functional annotation

To evaluate the differences in BP between distinct m6A regulator subgroups, we performed gene set variation analysis (GSVA), gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses. Selecting c2.cp.kegg.v7.2.symbols.gmt as the reference gene set, the GSVA R package was utilized to perform GSVA. Based on the coexpressed genes, we utilized the clusterProfiler R package to perform GO and KEGG analyses and utilized the ggplot2 R package to visualize the results.

Correlation analysis between m6A regulators and immune cell infiltration in the TIME

We detected the correlation between m6A regulators and immune cell infiltration (including B cells, $CD4^+$ T cells, $CD8^+$ T cells, macrophages, neutrophils, and dendritic cells) in ESCA by using the TIMER (Tumor Immune Estimation Resource) database (https://cistrome.shinyapps.io/timer/). The TISIDB (Tumor-Immune System Interactions Database; http://cis.hku.hk/TISIDB/) was employed to calculate the correlations between m6A regulators and immune regulators. Meanwhile, we employed the single-sample gene set enrichment analysis (ssGSEA) algorithm to evaluate the infiltration of 28 types of immune cells in the TIME. Additionally, we investigated the correlation between the m6A score and immune cell infiltration in the TIME.

Statistical analysis

The clinicopathological features between distinct subgroups were compared by using the chi-square test. The differences between distinct subgroups were compared by using

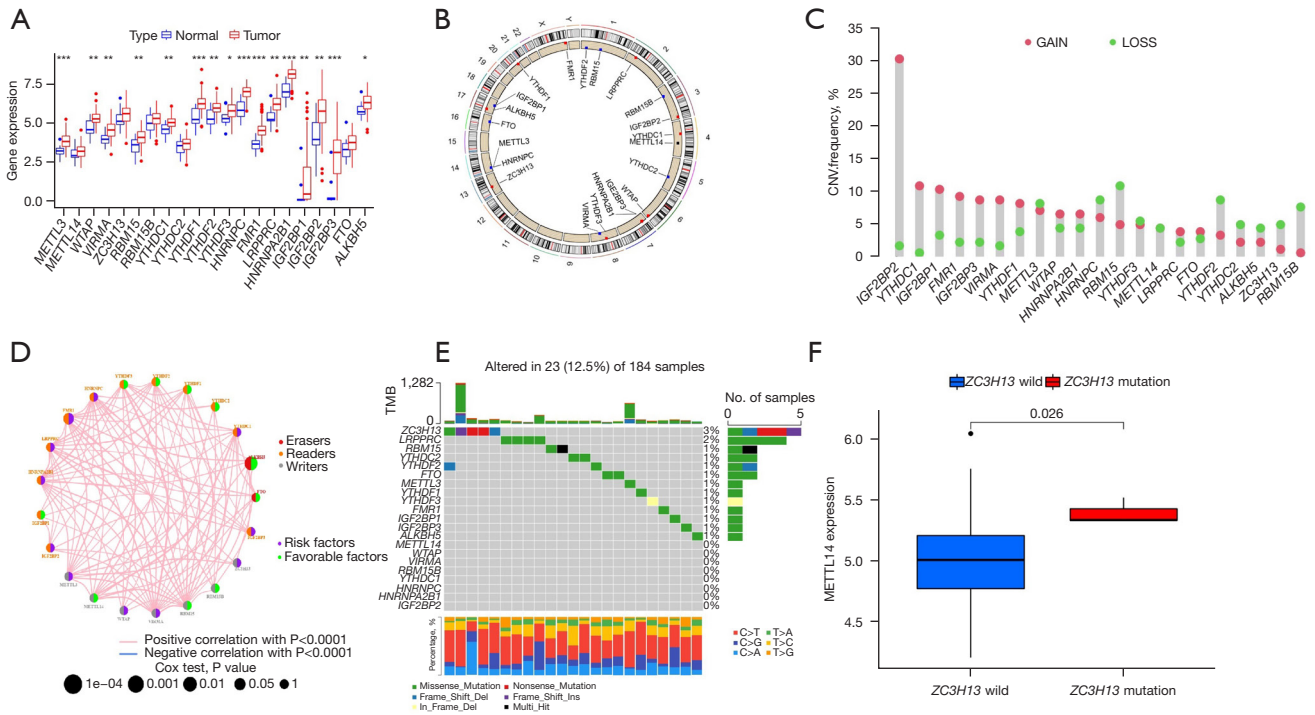

Figure 1 Genetic alterations of 21 m6A regulators in ESCA. (A) Expression pattern of m6A regulators in normal and tumor tissues. (B) The location of CNV alterations of m6A regulators on chromosomes. (C) CNV mutation frequency of 21 m6A regulators in ESCA. (D) The landscape of the interactions and their prognostic significance between 21 m6A regulators in ESCA. (E) Somatic mutation frequency of 21 m6A regulators. (F) Correlation analysis between *ZC3H13* mutation and *METTL14* expression. *, P<0.05; **, P<0.01; ***, P<0.001; ns, no significance. m6A, N6-methyladenosine; ESCA, esophageal carcinoma; CNV, copy number variations.

Student's *t*-test. We utilized univariate Cox regression analyses to evaluate the prognostic factors in patients with ESCA. Survival differences between distinct subgroups were compared by using the Kaplan-Meier method and log-rank test. SPSS version 21.0 (IBM Corp, Armonk, NY, USA) was employed to perform all statistical tests.

Results

Overview of m6A regulator expression in ESCA

First, we investigated the differences in 21 m6A regulators between normal and tumor tissues. The expression analysis revealed that *METTL3*, *WTAP*, *VIRMA*, *RBM15*, *YTHDC1*, *YTHDF1/2/3*, *HNRNPC*, *FMR1*, *LRPPRC*, *HNRNPA2B1*, *IGF2BP1/2/3*, and *ALKBH5* were elevated in tumor tissues, and the remaining five m6A regulators showed no significant differences between normal and tumor tissues (*Figure 1A*). Using the GEPIA database by matching TCGA-ESCA normal and GTEx data, eight m6A regulators, including *ZC3H13*, *RBM15*, *FTO*, *YTHDF1*, *YTHDF3*, *LRPPRC*, *IGF2BP2* and *IGF2BP3*, were differentially expressed in tumor tissues (Figure S1A-S1U). Compared with paired normal tissues, differentially expressed m6A regulators, including *METTL3*, *YTHDF1*, *HNRNPC*, *FMR1*, *HNRNPA2B1*, *IGF2BP2* and *IGF2BP3*, were observed in tumor tissues (Figure S2A-S2U). The circle map shows the locations of CNV alterations of 21 m6A regulators on chromosomes (*Figure 1B*). Further analysis revealed that 21 m6A regulators in ESCA had prevalent CNV alterations. *IGF2BP2*, *YTHDC1* and *IGF2BP1* showed extensive CNV amplification. In contrast, *RBM15*, *METTL3*, *HNRNPC*, *YTHDF2* and *RBM15B* had widespread CNV deletions (*Figure 1C*). A network map was constructed to illustrate the comprehensive landscape of the interactions between the m6A regulators and their prognostic significance in ESCA (*Figure 1D*). Additionally, we evaluated the prevalence of somatic mutations in 21 m6A regulators in ESCA. Among 184 samples, 23 samples (12.5%) experienced genetic mutations, mainly missense mutations and nonsense

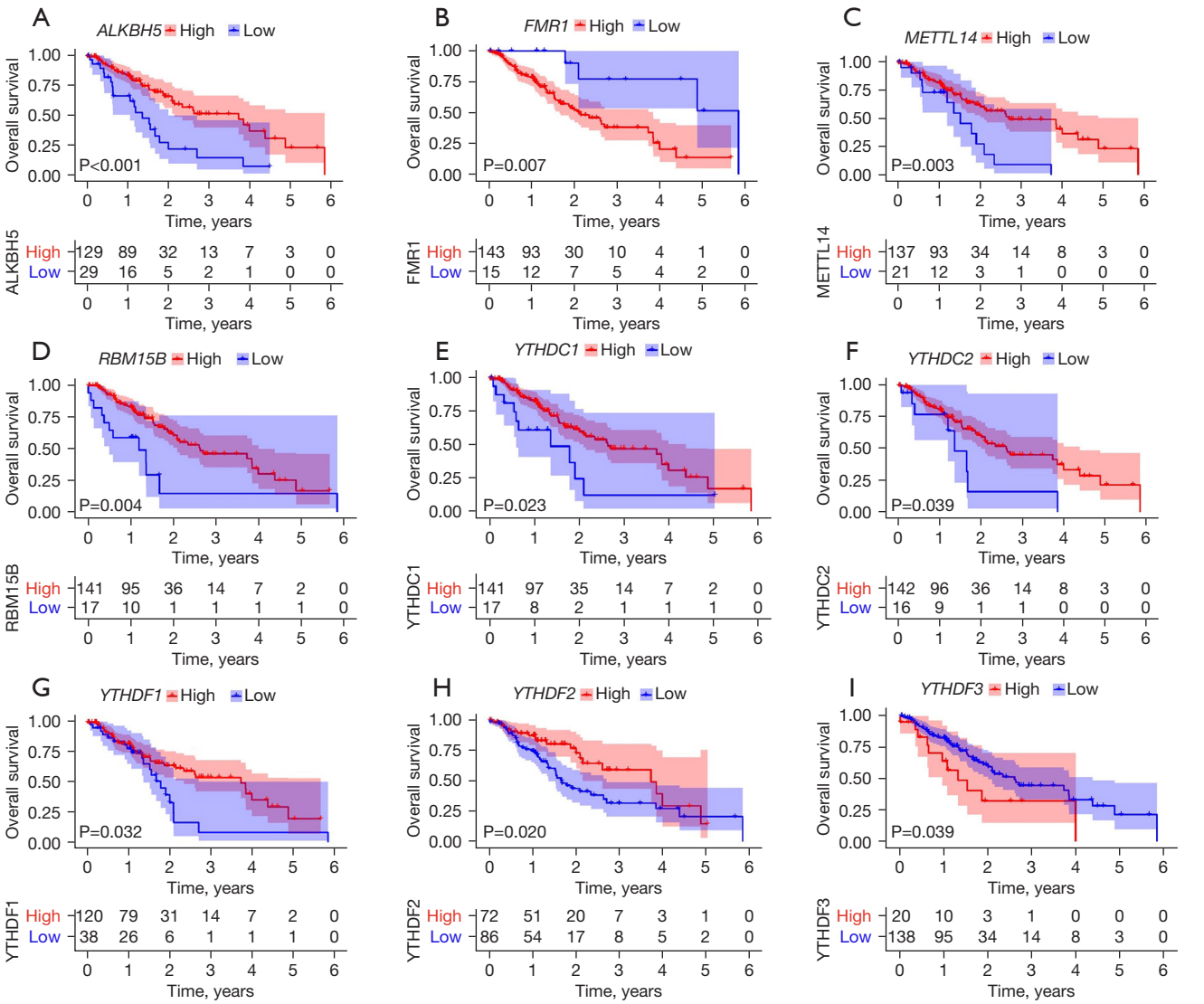

Figure 2 Survival analysis of m6A regulators that are strongly associated with prognosis. (A) *ALKBH5*. (B) *FMR1*. (C) *METTL14*. (D) *RBM15B*. (E) *YTHDC1*. (F) *YTHDC2*. (G) *YTHDF1*. (H) *YTHDF2*. (I) *YTHDF3*. m6A, N6-methyladenosine.

mutations. *ZC3H13* had the highest mutation frequency, followed by *LRPPRC* (*Figure 1E*). We also employed correlation analysis to investigate the mutual regulation among these m6A regulators. *METTL14* was significantly upregulated in *ZC3H13* mutation samples (*Figure 1F*). However, other correlation analyses showed no apparent differences (data not shown). Survival analysis demonstrated that 9 m6A regulators were significantly associated with prognosis. Upregulation of *FMR1* and *YTHDF3* was remarkably related to shorter survival time, while *ALKBH5*, *METTL14*, *RBM15B*, *YTHDC1*, *YTHDC2*, *YTHDF1* and *YTHDF2* were significantly associated with prolonged survival time (*Figure 2A-2I*). These results revealed that m6A regulators, including writers, erasers and readers, were implicated in the m6A modification pattern, thereby simultaneously affecting the prognosis of ESCA.

Identification of m6A modification patterns in ESCA

To evaluate the m6A modification pattern in ESCA, consensus clustering analysis was performed to stratify all samples. By using the K-means clustering method, two clusters with distinct m6A regulators were finally identified, including 47 cases in the m6A cluster A subgroup and 111 cases in

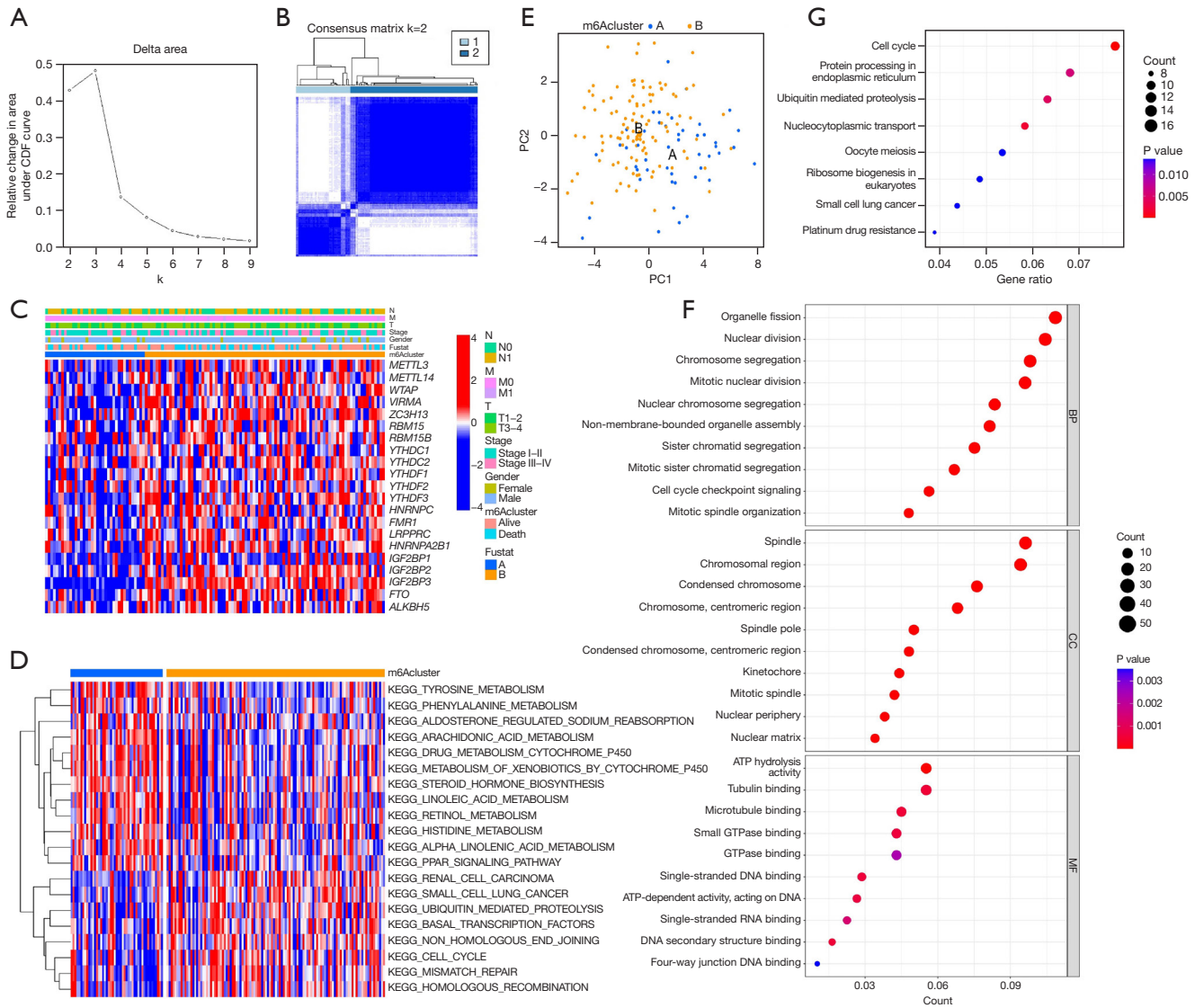

Figure 3 Consensus clustering analysis based on 21 m6A regulators and analysis of the overlapping DEGs. (A) The area under the curve in consensus clustering analysis. (B) The consensus fraction matrix of all samples when k=2. (C) Heatmap of the expression of 21 m6A regulators in each sample. (D) Heatmap demonstrating the GSVA score of representative pathways in distinct m6A cluster subgroups. (E) Principal component analysis of distinct m6A cluster subgroups. (F) Bubble diagram of GO enrichment analysis. (G) Bubble diagram of KEGG pathway analysis. CDF, cumulative distribution function; m6A, N6-methyladenosine; DEGs, differentially expressed genes; GSVA, gene set variation analysis; GO, gene ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; BP, biological process; CC, cellular component; MF, molecular function.

the m6A cluster B subgroup (*Figure 3A*,*3B*). A heatmap of 21 m6A regulators revealed that there were apparent differences in distinct m6A cluster subgroups (*Figure 3C*). In addition, we performed GSVA enrichment analysis to evaluate the differences in biological function among distinct m6A cluster subgroups. The results showed that there were dramatic differences in tumor progression, such as in the PPAR signaling pathway and cell cycle (*Figure 3D*).

Functional annotation

Moreover, we utilized principal component analysis (PCA) to evaluate the differences among distinct m6A cluster subgroups and discovered that it was obvious to distinguish

between the two m6A cluster subgroups (*Figure 3E*). By using univariate Cox analysis, a total of 519 DEGs between the two m6A cluster groups were identified. We performed GO enrichment and KEGG pathway analyses to evaluate the potential function. The top five BP, including organelle fission, nuclear division, chromosome segregation, mitotic nuclear division and nuclear chromosome segregation, were enriched. The category of cell components (CC) was enriched in spindle, chromosome region, condensed chromosome, centromeric region, and spindle pole. The top five enriched molecular functions (MFs) included ATP hydrolysis activity, tubulin binding, microtubule binding, small GTPase binding, and GTPase binding (*Figure 3F*). KEGG pathway analysis revealed that the top five processes, including the cell cycle, protein processing in the endoplasmic reticulum, ubiquitin-mediated proteolysis, nucleocytoplasmic transport and oocyte meiosis, were significantly enriched (*Figure 3G*).

Construction of the m6A score system

On the basis of the DEGs, we also performed consensus clustering analysis to further investigate m6A modification. By using the K-means method, two gene cluster subgroups were finally identified (*Figure 4A,4B*). The gene cluster A subgroup was remarkably related to m6A cluster A (*Figure 4C*). There were no significant differences in *RBM15B* and *YTHDF2* among distinct gene cluster subgroups. The remaining 19 m6A regulators in the gene cluster B subgroup were significantly expressed in tumor tissues compared with normal tissues (*Figure 4D*). We constructed a m6A score system to subsequently evaluate the prognosis of ESCA. All patients were dichotomized into low and high m6A score subgroups. The alluvial diagram was utilized to show the correlation of distinct clusters (*Figure 4E*). Additionally, correlation analysis indicated that the m6A cluster A and gene cluster A subgroups were significantly associated with high m6A score (*Figure 4F,4G*). Between the high and low m6A score subgroups, we compared somatic tumor mutation profiles to investigate the relationship between m6A score and TMB. Somatic mutation analysis revealed that *MUC16*, *CSMD3*, *ZNF804B* and *DYNC2H1* had more mutations in the high m6A score subgroups, while *FLG* and *PCLO* had more mutations in the low m6A score subgroups (*Figure 4H,4I*). Further analysis indicated that patients who died had higher m6A score (*Figure 4J*). On the other hand, we found that surviving patients accounted for 79% of the low m6A score subgroup (*Figure 5A*). Survival analysis indicated that patients in the high m6A score or high TMB subgroup experienced unfavorable prognoses (*Figure 5B,5C*). Survival analysis combined with TMB and m6A score demonstrated that patients in the high TMB and high m6A score subgroup had the worst prognosis (median survival time 12 months), while patients in the low TMB and low m6A score subgroup had the best prognosis (median survival time 48 months) (*Figure 5D*).

Correlation analysis between m6A regulators and immune cell infiltration

First, we investigated the correlation between m6A regulators and six types of immune cells by using the TIMER database. Our results revealed that B cells were positively associated with *METTL3*, *METTL14*, *ZC3H13*, *RBM15*, *RBM15B*, *YTHDC1*, *YTHDF1* and *LRPPRC*. $CD8^+$ T cells were positively related to WTAP and negatively related to *RBM15B*. Macrophages were positively correlated with *METTL3*, *ZC3H13*, *FTO*, *YTHDC1*, *YTHDF1* and *FMR1*. Neutrophils were positively associated with WTAP and negatively related to *LRPPRC*. Dendritic cells were negatively related to *VIRMA*, *ZC3H13*, *RBM15*, *RBM15B*, *YTHDF1*, *YTHDF3*, *HNRNPA2B1* and *LRPPRC* (Figures S3-S9). Then, we utilized the TIMER database to analyze the influence of somatic CNVs on immune cell infiltration and demonstrated that CNVs significantly affected the abundance of six types of infiltrating immune cells (Figure S10). Based on distinct m6A cluster subgroups, ssGSEA revealed that activated B cells, activated CD8 T cells and neutrophils were upregulated in the m6A cluster A subgroup. In contrast, activated CD4 T cells, type 2 T helper cells and memory B cells were reduced in the m6A cluster A group (*Figure 5E*). In terms of the m6A score, neutrophils and monocytes were positively correlated with the m6A score, while CD4 T cells, type 2 T helper cells and memory B cells were negatively associated with the m6A score (*Figure 5F*). Meanwhile, we evaluated the relationships between the abundance of 28 types of tumor-infiltrating immune cells and m6A regulators by using the TISIDB database. This observation showed that m6A regulators were significantly associated with distinct tumor-infiltrating immune cells (Figure S11A). Correlation analysis indicated that most m6A regulators were significantly related to immunoinhibitors and immunostimulators (Figure S11B,S11C). To further evaluate the effects of m6A regulators on the tumor immune response, we also analyzed the correlation between m6A regulators and major histocompatibility complex (MHC) molecules and found that WTAP and *ZC3H13* were positively related to

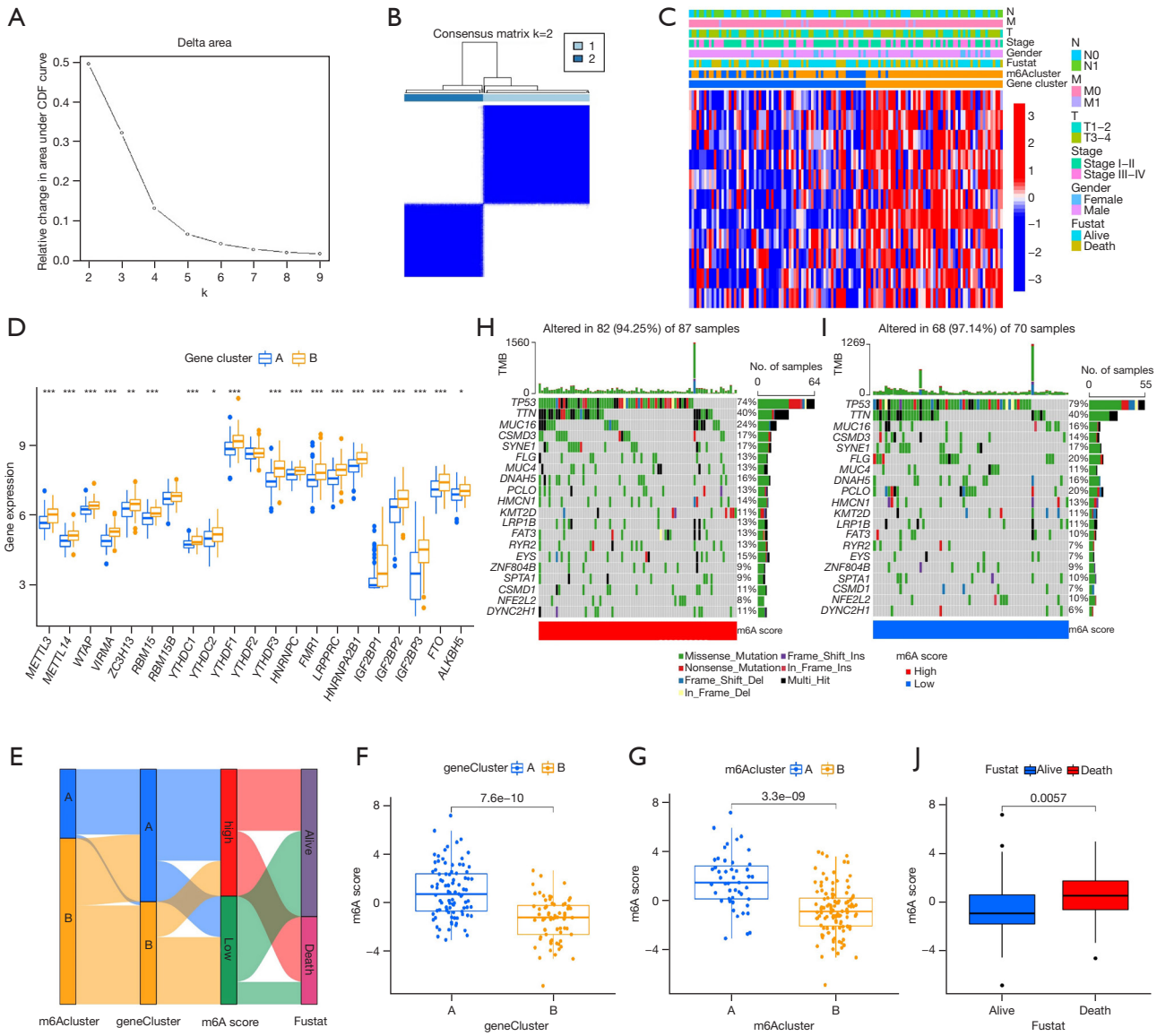

Figure 4 Consensus clustering analysis based on the overlapping DEGs and construction of the m6A score system. (A) The area under the curve in consensus clustering analysis. (B) The consensus fraction matrix of all samples when k=2. (C) Correlation analysis between clinical features and distinct clusters. (D) Expression pattern of m6A regulators in distinct gene cluster subgroups. (E) Sankey plots of distinct molecular subgroups. (F) Correlation analysis between the m6A score and gene cluster. (G) Correlation analysis between m6A score and m6A cluster. (H) Mutation landscape of the high m6A score subgroup. (I) Mutation landscape of the low m6A score subgroup. (J) Correlation analysis between the m6A score and survival status. *, P<0.05; **, P<0.01; ***, P<0.001; ns, no significance. CDF, cumulative distribution function; m6A, N6-methyladenosine; DEGs, differentially expressed genes.

MHC molecules, and the remaining m6A regulators were negatively associated with MHC molecules (Figure S11D). A previous study conducted by Thorsson *et al.* revealed that tumor tissues can be divided into six immune subtypes: wound healing, IFN-gamma dominant, inflammatory, lymphocyte depleted, immunologically quiet, and TGF-b dominant (30). Our results demonstrated that *HNRNPC*, *FMR1*, *LRPPRC*, *HNRNPA2B1*, *IGF2BP1* and *IGF2BP2* were implicated in immune subtypes (Figure S12). *METTL3*, *WTAP*, *RBM15*, *FTO*, *ALKBH5*, *YTHDF1*,

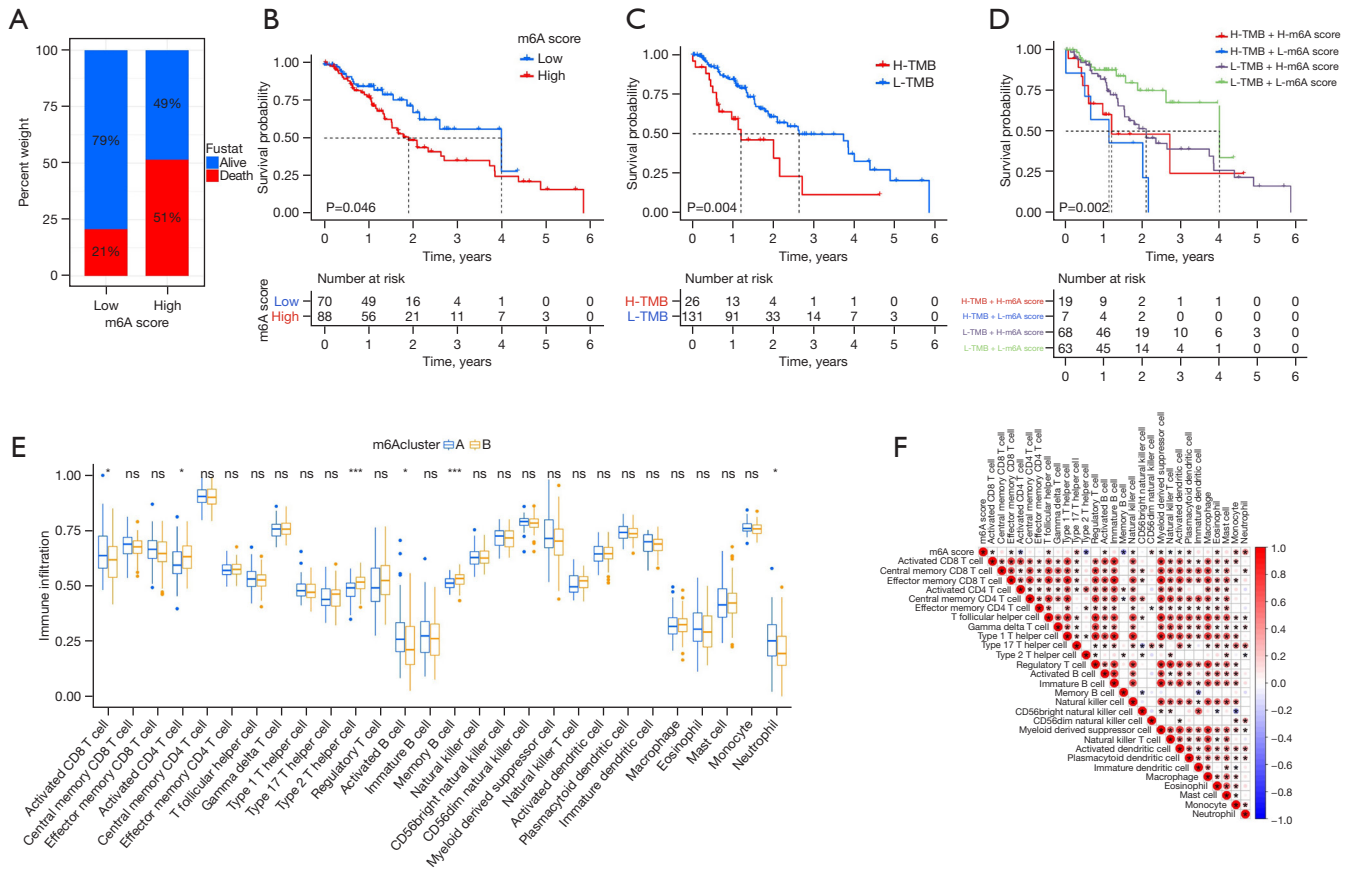

Figure 5 Exploration of the clinical significance of the m6A score and correlation analysis between m6A regulators and immune cell infiltration. (A) Correlation analysis between the m6A score and the prognosis of ESCA. (B) Kaplan-Meier curves for distinct m6A score subgroups. (C) Kaplan-Meier curves for distinct TMB subgroups. (D) Kaplan-Meier curves for distinct m6A scores combined with TMB subgroups. (E) Correlation analysis between m6A clusters and immune cell infiltration. (F) Correlation analysis between the m6A score and immune cell infiltration. *, P<0.05; ***, P<0.001; ns, no significance. m6A, N6-methyladenosine; TMB, tumor mutation burden; ESCA, esophageal carcinoma.

YTHDF2 and *HNRNPC* were significantly associated with molecular subtypes of ESCA (Figure S13). In conclusion, our studies revealed that the m6A regulators were closely related to immune cell infiltration in the tumor immune microenvironment.

Discussion

Tumorigenesis is a multistep and multifactor process that involves genetic alteration, epigenetics, posttranscriptional regulation and immune evasion (31,32). m6A is the most pervasive modification of human RNA and is dramatically correlated with various physiological and pathological processes, such as obesity and cancer (33,34). Growing evidence has revealed that abnormal expression of m6A regulators is remarkably implicated in cell proliferation, migration and invasion, thereby leading to tumorigenesis and progression (9). Until now, very few studies have focused on ESCA. Therefore, it is imperative to further investigate the underlying functional role of m6A regulators in ESCA and the relationship between m6A regulators and immune cell infiltration in the TIME. With the development of high-throughput sequencing and other biological techniques, the biological function of m6A modification in ESCA has been gradually disclosed.

In this study, we evaluated the expression pattern and prognostic significance of 21 m6A regulators and found that most m6A regulators were upregulated in ESCA tumor

tissues compared with normal tissues. Survival analysis of a single m6A regulator revealed that nine genes were closely related to survival time. By using consensus clustering analysis, all included patients with ESCA were divided into two subgroups. Patients in the low TMB and low m6A score subgroup had the best prognosis. Moreover, we constructed a human m6A score system to further evaluate prognosis and immune cell infiltration. Based on the m6A score system, distinct subgroups can be completely distinguished. Additionally, we also investigated the correlations of m6A regulators and immune cell infiltration in the TIME. Dendritic cells were negatively associated with most m6A regulators. Neutrophils and monocytes were positively related to the m6A score. $CD4^+$ T cells and type 2 T helper cells were negatively associated with the m6A score.

An increasing number of studies have demonstrated that m6A regulators have an important regulatory effect on immunity (35). Previously, the therapeutic schedule was mainly based on the TNM staging system and pathological classification. To date, with the development of research, it has been found that the status of the host immune system is closely related to the prognosis of various cancers, such as colorectal cancer, lung cancer and ESCA. The inherent aptitude of the immune system is to distinguish it from healthy self-tissues or foreign substances and to recognize and eradicate tumor cells. Nevertheless, tumor cells can escape immune surveillance by cancer immunoediting, where the immune system can hold back or accelerate tumor growth (36,37). Accumulating evidence has revealed that the TIME plays a crucial role in mediating tumor proliferation and affecting the response to immunotherapy (38). Recently, immunotherapies, including PD-1 antibodies and dendritic cell vaccines, have been used in clinical trials for patients with advanced malignancy for whom first-line chemotherapy failed (39,40). Our study demonstrated that most m6A regulators were negatively related to dendritic cells, indicating that m6A regulators contribute to dysfunction of the immune system. Dendritic cells, as robust antigen-presenting cells, take part in mediating the initiation of adaptive immune responses and innate immune responses (41).

At present, the relationship between m6A regulators and immune cell infiltration in the tumor microenvironment remains obscure. In this study, we also found that TP53 and TTN displayed the highest incidences of somatic mutations, and *IGF2BP2* showed the most extensive CNV amplification. *IGF2BP2*, as a reader, plays a pivotal role in mRNA localization, stability and translation. A previous study demonstrated that higher expression of *IGF2BP2* was observed in hepatocellular carcinoma and significantly related to unfavorable prognosis, indicating that *IGF2BP2* serves as an oncogene and could promote tumor progression via a m6A-FEN1-dependent mechanism (42). Another study revealed that upregulation of *IGF2BP2* facilitates lung cancer proliferation and angiogenesis (43). Meanwhile, our study found that *IGF2BP2* was overexpressed in ESCA tumor tissues and closely related to immune subtypes, thereby hinting that *IGF2BP2* is involved in the prognosis of ESCA. A previous study revealed that the mRNA and protein levels of *METTL3* were significantly upregulated in esophageal squamous cell carcinoma (ESCC) tissues, and inhibition of *METTL3* could impede tumor proliferation (44). In our study, we confirmed that *METTL3* was upregulated in tumor tissue, indicating that it acts as an oncogene. Growing evidence has reported that *YTHDF1* has an important role in the antitumor immune response. A study conducted by Han *et al.* reported that knockdown of *YTHDF1* can elevate the antitumor response of $CD8^+$ T cells and PD-L1, implicating *YTHDF1* as a candidate therapeutic target in antitumor immunotherapy (29). Our research revealed that CNV of m6A regulators, including arm level again, high amplification and arm level deletion, could affect the abundance of immune cell infiltration in ESCA. This observation confirmed that m6A regulators are likely to display a vital regulatory effect on immune cell infiltration in ESCA. The role of the m6A demethylase *ALKBH5* in ESCA is controversial (9). A previous study investigated the functional role of *ALKBH5* in ESCC and found that upregulation of *ALKBH5* was dramatically related to unfavorable prognosis. Knockdown of *ALKBH5* impeded tumor cell proliferation and migration *in vitro* and *in vivo* and accumulated tumor cells in the G0/G1 phase (45). In contrast, another study reported that overexpression of *ALKBH5* reduced tumor cell migration and invasion in ESCC (46). Similar to this research, other studies reported that *ALKBH5* can suppress the malignancy of esophageal cancer *in vivo* and *in vitro*, indicating that *ALKBH5* takes part in an inhibitory effect in ESCA (47,48).

It is noteworthy that there are several limitations in this study. First, we did not separately investigate the distinct histological types due to the small sample size. The difference between squamous cell carcinoma and adenocarcinoma could affect the consistency of our results. In addition, these results were obtained by integrated bioinformatics analyses, lacking validation *in vivo* and *in vitro*. To further verify these conclusions, subsequent studies in cells, animal models and clinical experiments need

to be implemented.

Conclusions

In summary, the present study systematically investigated the expression pattern of m6A regulators, constructed a m6A score system to improve the predictive value of a single m6A regulator, and evaluated the correlation of m6A regulators and immune cell infiltration in the TIME. Our results provide important insights into the underlying role of m6A regulators and suggest that m6A regulators can serve as potential prognostic biomarkers and promising therapeutic targets against immunotherapy.

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Footnote

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Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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