



RAPID COMMUNICATION

Profiling the m⁶A-regulated RNA expression patterns and alternative splicing features in esophageal carcinoma



The m⁶A modification involves almost all aspects of RNA biology, including the alternative splicing of mRNA precursors, mRNA transport and stability, and miRNA processing and regulation of target genes.^{1,2} Alternative splicing controlling the information storage and RNA translation involves the regulation of various biological processes.^{3–5} Here, we integrated the genomic information of 161 esophageal cancer (EC) samples to comprehensively evaluate the m⁶A modification patterns and correlated the m⁶A modification pattern with the prognosis of EC patients, where two distinct m⁶A modification patterns were proposed. The combined effects of high m⁶Ascore and low TMB correlated with a better prognosis of EC patients. In addition, we found an inherent correlation between m⁶A modification and the occurrence of alternative splicing events in EC patients. Altogether, we established a scoring system to quantify the m⁶A modification pattern with RNA alternative splicing events in individual EC patients (Fig. S1).

The RNA m⁶A methylation dynamically regulates different biological functions of RNA (Fig. 1A). Here we identified a total of 22 m⁶A regulators, including 7 writers, 2 erasers, and 13 readers, and predicted a comprehensive landscape of their interactions, and connections, as well as their prognostic significance for EC patients. We also found that the expression of the 22 m⁶A regulators was positively correlated with each other (Fig. 1B; Fig. S2, 3).

Using unsupervised clustering based on the expression of m⁶A regulators, two distinct modification patterns were eventually identified, in which 49 cases were classified in pattern A and 112 cases in pattern B (Fig. 1C; Fig. S4). Notably, 22 m⁶A regulators were highly expressed in pattern A patients who had a worse prognosis. In contrast, the 94 EC

patients clustered in gene cluster B had a worse prognosis (Fig. S4).

The following GSVA analyses revealed that the cluster A modification pattern was significantly associated with RNA alternative splicing. The cross-talk among the regulators of writers, readers, and erasers may play critical roles in forming different m⁶A modification patterns and the prognosis of EC patients.

We also confirmed that DEGs (different expression genes) were characterized by the status of cell cycle and RNA localization (Fig. 1E). Consistent with the above findings, patients with DNA_REPAIR, CELL_CYCLE, G2M_CHECKPOINT, and DNA_REPLICATION pathways were classified into gene cluster A, which was relevant to the poor survival outcome (Fig. S5, 6 and Table S1–4).

Considering the individual heterogeneity and complexity of m⁶A modification, we constructed a scoring system based on these phenotype-related genes to quantify the m⁶A modification pattern of individual EC patients, which was termed as m⁶Ascore. We also tested the correlation between the known features and the score of m⁶A, such as the prognosis of patients and tumor staging. The Kruskal–Wallis test revealed that m⁶A cluster A showed a higher median score than gene cluster B. Moreover, the clustering of EC patients with high m⁶Ascore has a better prognosis. It suggested that the m⁶Ascore could better indicate the m⁶A modification patterns of an individual tumor, and further evaluate the tumor prognosis (Fig. 1F; Fig. S7–10).

Moreover, we found that patients with both low m⁶Ascore and high TMB score had the poorest prognosis, while patients with both high m⁶Ascore and low TMB score had the best prognosis. These results suggested that TMB and m⁶Ascore which are relatively independent (Fig. 1G; Fig. S10), cooperatively affect the prognosis of EC patients. To further construct the risk model of RNA alternative splicing, we used multi-Cox to screen the key RNA alternative editing target molecules. The patients with a higher

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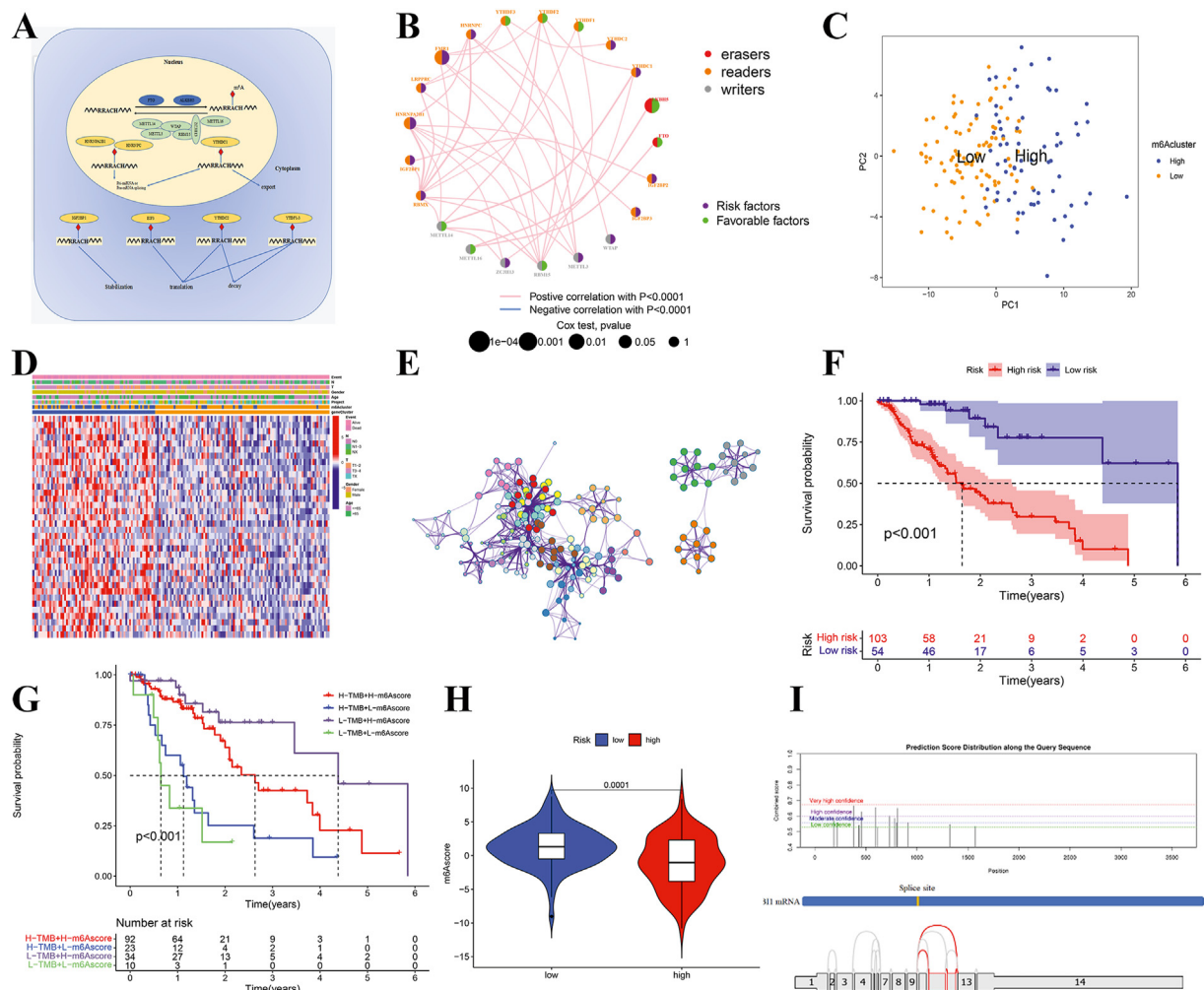


Figure 1 Profiling the m⁶A-regulated RNA expression patterns and alternative splicing features in esophageal carcinoma. (A) Summary of the dynamic reversible process of m⁶A RNA methylation mediated by regulators “writers”, “erasers”, and “readers” and their potential biological functions for RNA. (B) The interaction between m⁶A regulators in esophageal cancer. The circle size represented the effect of each regulator on the prognosis, and the range of values calculated by Log-rank test was $P < 0.001$, $P < 0.01$, $P < 0.05$, and $P < 0.1$, respectively. Green dots in the circle represent risk factors of prognosis. Black dots in the circle represent protective factors of prognosis. The lines linking regulators showed their interactions, and thickness showed the correlation strength between regulators. Negative correlation was marked with blue and positive correlation with red. (C) Principal component analysis for the m⁶A phenotype-related genes of two m⁶A modification patterns, showing a remarkable difference on transcriptome between different modification patterns. (D) Unsupervised clustering of 22 m⁶A regulators in the TCGA-ESCA cohort. The m⁶Acluster, ESCA molecular subtypes, tumor stage, survival status, and age were used as patient annotations. Red represented high expression of regulators and blue represented low expression. (E) Functional annotation for m⁶A-related genes using GO enrichment analysis and KEGG enrichment analysis by Metascape. (F) Prognostic signatures based on TMB and m⁶Ascore in ES for overall survival. (G) DFS-related prognostic model. High-risk and low-risk groups were divided based on the median value of risk score. The upper plot illustrated assignment of patients’ survival status and survival times, the middle plot showed the risk score curve, and the bottom heatmap depicted splicing distribution of the AS in compound prognostic models. Color transition from blue to red indicates the increasing PSI value of corresponding AS event from low to high. (H) Differences in m⁶Ascore among high- and low-risk clusters in TCGA cohort. The Kruskal–Wallis test was used to compare the statistical difference between three gene clusters ($P < 0.001$). (I) m⁶A modification site of ABI1 gene is close to the region where ABI1-ES variable editing occurs and the expression of ALKBH5 was correlated with ABI1.

risk score showed a poor prognosis. Furthermore, we calculated the ROC curves of clinical features, AS prognostic model, and the nomogram in the training group. Further analysis showed a significant negative correlation between risk score and m⁶Ascore. The multi-Cox analysis established a risk model that had a significant negative

correlation with m⁶Ascore and m⁶A demethylase ALKBH5 (Fig. 1H; Fig. S11, 12 and Table S6).

Interestingly, we found that the m⁶A modification site of ABI1 gene is close to its variable editing region, in which the expression of ABI1 was correlated with ALKBH5 (Fig. 1I; Fig. S13–16). According to the above results, the occurrence of

ABI1|11037|ES alternative splicing is closely related to the demethylation of ALKBH5, which is conducive to a better prognosis of EC patients.

Two different patterns of m⁶A methylation modification were identified, which represent distinct types of RNA alternative splicing. Enrichment of m⁶A regulators leads to a relatively complicated interaction pattern between RNA alternative splicing activation and cardiac activation. We screened the genes closely related to the prognosis from the differential genes in the two clusters, and constructed the m⁶Ascore model according to the expression of these genes. Interestingly, the high or low m⁶Ascore model corresponds to the patients in the two clusters. This phenomenon indicates that the m⁶Ascore model could better evaluate the impact of m⁶A regulators on the prognosis of patients. The cluster with high TMB and low m⁶Ascore has a worse prognosis, whereas the cluster with low TMB and high m⁶Ascore has a better prognosis. This further suggests that the prognosis of tumor patients is determined by many external independent but internal related factors.

The expression of ALKBH5 is positively correlated with a better prognosis in esophageal cancer patients. We further analyzed the RNA alternative splicing in all esophageal cancer samples, screened alternative splicing genes closely related to the prognosis of patients, and constructed a stable risk assessment model through multiple regression analysis. As we know, the probability of ES events is much higher than that of other alternative splicing types. However, the high-risk factor determining the poor prognosis of patients with esophageal cancer is other alternative splicing types such as AT or AA. We speculate that this phenomenon may be caused by the effects of different alternative splicing types on gene function. What's more, we found that there was a close correlation between alternative splicing risk score and m⁶Ascore ($P = 0.0001$), which means that m⁶A modification may directly affect the occurrence of these key alternative splicing events. The results revealed a close correlation between ALKBH5 and ABI1|11037|ES, and the occurrence of ABI1|11037|ES events was closely related to the good prognosis of EC patients, which is further confirmed by the *in vitro* proliferation and apoptosis assays (Fig. S17, 18).

We constructed a risk model of alternative splicing by multi-Cox analysis, which can predict the prognosis of EC patients. Further studies showed that the alternative splicing risk model is negatively correlated with the m⁶Ascore model. In addition, the risk model was found to negatively correlate with ALKBH5 expression, and ABI1-ES and SDCBP-ES events. However, only ABI1-ES events are closely related to the poor prognosis of EC patients. These results suggested that the alternative splicing risk model can effectively evaluate the prognosis of EC patients.

Author contributions

YGP and LSK designed this work. CBZ, QYY, FG, FFZ, RX, CFC, WXW, DBH and ZYL integrated and analyzed the data. LSK, YGP and CBZ wrote this manuscript. YGP and LSK edited and revised the manuscript. All authors approved this manuscript.

Conflict of interests

All authors declare that there is no conflict of interests.

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Abbreviations

ABI1	Ablinteractor 1
ALKBH5	Alkylation repair homolog protein 5
EC	Esophageal cancer
FTO	Fat-mass and obesity-associated protein
GDC	Genomic Data Commons
GSVA	Gene set variation analysis
IGF2BP1	Insulin-like growth factor 2 mRNA binding protein 1
IGF2BP2	Insulin-like growth factor 2 mRNA binding protein 2
m ⁶ A	N ⁶ -methyladenosine
METTL14	Methyltransferase-like 14
PCA	Principal component analysis
PSI	Percent spliced-in
TCGA	Cancer Genome Atlas
TMB	Tumor mutational burden
WTAP	Wilms tumor 1 associated protein
YTHDF1	YTH N ⁶ -methyladenosine RNA binding protein 1
YTHDF2	YTH N ⁶ -methyladenosine RNA binding protein 2
YY1	Yin Yang-1

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.gendis.2022.12.009>.

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