Genes & Diseases (2023) 10, 1812–1815

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RAPID COMMUNICATION

# Profiling the m$^6$A-regulated RNA expression patterns and alternative splicing features in esophageal carcinoma

The m$^6$A 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 m$^6$A modification patterns and correlated the m$^6$A modification pattern with the prognosis of EC patients, where two distinct m$^6$A modification patterns were proposed. The combined effects of high m$^6$Ascore and low TMB correlated with a better prognosis of EC patients. In addition, we found an inherent correlation between m$^6$A modification and the occurrence of alternative splicing events in EC patients. Altogether, we established a scoring system to quantify the m$^6$A modification pattern with RNA alternative splicing events in individual EC patients (Fig. S1).

The RNA m$^6$A methylation dynamically regulates different biological functions of RNA (Fig. 1A). Here we identified a total of 22 m$^6$A 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 m$^6$A regulators was positively correlated with each other (Fig. 1B; Fig. S2, 3).

Using unsupervised clustering based on the expression of m$^6$A 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 m$^6$A 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 m$^6$A 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 m$^6$A modification, we constructed a scoring system based on these phenotype-related genes to quantify the m$^6$A modification pattern of individual EC patients, which was termed as m$^6$Ascore. We also tested the correlation between the known features and the score of m$^6$A, such as the prognosis of patients and tumor staging. The Kruskal–Wallis test revealed that m$^6$A cluster A showed a higher median score than gene cluster B. Moreover, the clustering of EC patients with high m$^6$Ascore has a better prognosis. It suggested that the m$^6$Ascore could better indicate the m$^6$A 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 m$^6$Ascore and high TMB score had the poorest prognosis, while patients with both high m$^6$Ascore and low TMB score had the best prognosis. These results suggested that TMB and m$^6$Ascore 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

Peer review under responsibility of Chongqing Medical University.

https://doi.org/10.1016/j.gendis.2022.12.009

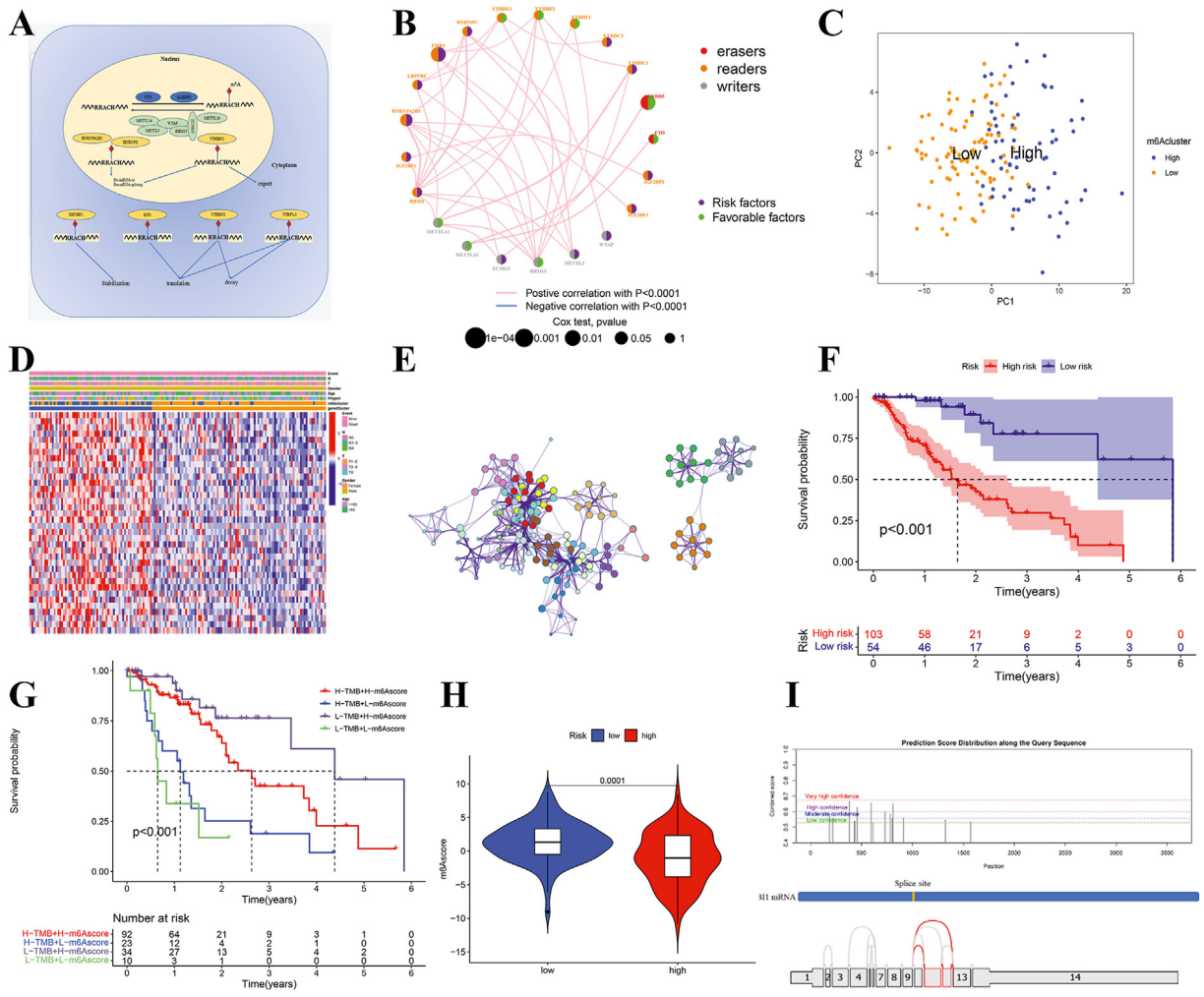


**Figure 1** Profiling the m$^6$A-regulated RNA expression patterns and alternative splicing features in esophageal carcinoma. **(A)** Summary of the dynamic reversible process of m$^6$A RNA methylation mediated by regulators "writers", "erasers", and "readers" and their potential biological functions for RNA. **(B)** The interaction between m$^6$A 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 m$^6$A phenotype-related genes of two m$^6$A modification patterns, showing a remarkable difference on transcriptome between different modification patterns. **(D)** Unsupervised clustering of 22 m$^6$A regulators in the TCGA-ESCA cohort. The m$^6$Acluster, 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 m$^6$A-related genes using GO enrichment analysis and KEGG enrichment analysis by Metascape. **(F)** Prognostic signatures based on TMB and m$^6$Ascore 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 m$^6$Ascore 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)** m$^6$A 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 m$^6$Ascore. The multi-Cox analysis established a risk model that had a significant negative correlation with m$^6$Ascore and m$^6$A demethylase ALKBH5 (Fig. 1H; Fig. S11, 12 and Table S6).

Interestingly, we found that the m$^6$A 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 m$^6$A methylation modification were identified, which represent distinct types of RNA alternative splicing. Enrichment of m$^6$A 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 m$^6$Ascore model according to the expression of these genes. Interestingly, the high or low m$^6$Ascore model corresponds to the patients in the two clusters. This phenomenon indicates that the m$^6$Ascore model could better evaluate the impact of m$^6$A regulators on the prognosis of patients. The cluster with high TMB and low m$^6$Ascore has a worse prognosis, whereas the cluster with low TMB and high m$^6$Ascore 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 m$^6$Ascore ($P = 0.0001$), which means that m$^6$A 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 m$^6$Ascore 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.

## Funding

This work was supported by the 2022 Anhui Health Research Project Key Project (China) (No. AHWJ2022a017), the Anhui Provincial Natural Science Foundation of China (No. 2008085MH299). The fundamental Research Funds for the Central Universities (China) (No. WK9110000008, WK9110000090, WK9110000132 and WK9110000086). The Postdoctoral Research Funding of Anhui Province in 2019 (China) (No. 2019B371). The Youth Fund of Anhui Cancer Hospital (China) (No. 2018YJQN017, 2018YJQN004, 2020YJQN003 and 2018YJQN004) and the Youth Technical Backbone Fund of West Branch of the First Affiliated Hospital of USTC granted to CBZ and LSK, respectively.

## 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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25 April 2022
Available online 13 January 2023

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