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Clinical and biological significance of RNA N6-methyladenosine regulators in Alzheimer disease

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Abstract

RNA N6-methyladenosine (m6A) regulators are essential for a variety of biological functions, such as early development, viral infections, and cancer. However, their roles in Alzheimer disease (AD) are still not very clear. Here, 16 significant m6A regulators were identified using difference analysis between AD patients and non-demented controls based on the GSE132903 dataset from the Gene Expression Omnibus database. Using these 16 m6A regulators, a nomogram model was established to predict the prevalence of AD. We found that patients could obtain a good clinical benefit based on this model. In addition, we revealed 2 distinct m6A patterns and 2 distinct m6A gene patterns in AD and demonstrated their prognostic and risk assessment significance. This present work comprehensively evaluated the functions of m6A regulators in the diagnosis and subtype classification of AD. These results suggested they have potential prognostic and risk assessment significance in AD.

Abbreviations: AD = Alzheimer disease, APP = amyloid-beta precursor protein, DEGs = differentially expressed genes, m6A = N6-methyladenosine, MAPT = microtubule-associated protein tau, NK = natural killer, PC1 = principal component 1, PCA = principal component analysis, PSEN1 = presenilin 1, PSEN2 = presenilin 2, RF = random forest, ssGSEA = single sample gene set enrichment analysis, SVM = support vector machine.

Keywords: AD, diagnostic biomarkers, gene expression analysis, immune microenvironment, RNA N6-methyladenosine regulators

1. Introduction

Alzheimer disease (AD) accounts for a major factor resulting in mortality among elderly people. In 2021, there were about 6.5 million AD patients in America.[1] The number of people who live with AD almost doubles every 5 years after age 65.[2] Since life expectancy around the world has increased steadily over the last century, AD has caused a great burden of morbidity and mortality and has become an escalating burden on society as a whole. Over the last decades, scientists have made tremendous progress in better understanding AD, especially in terms of its neuropathological alterations.[3–6] However, at present, there are still no effective treatment options for AD, and many key questions remain.[7]

In recent decades, increasing importance has been placed on the potential role of epigenetics in AD pathogenesis, as a result, epigenetic mechanisms have different effects on AD occurrence.[8] Epigenetics mostly involves reversible RNA/DNA/histone modifications, and they are inheritable by means of cell division with no change of DNA sequence.[9] To date, >100 RNA posttranscriptional modification types are identified, like N1-methyladenosine, 2-o-dimethyladenosine, and N6-methyladenosine (m6A).[10] m6A is a posttranscriptional RNA modification, as well as a frequently seen RNA chemical modification type, which was associated with diverse biological activities, like early development, viral infections, and cancer.[11] At the molecular level, m6A RNA methylation is under dynamic and reversible modulation via proteins regulating RNA methylation, thereby causing different RNA fates.[12] m6A methylation is catalyzed via a core methyltransferase complex, the "writers" while m6A can also be removed by 2 m6A demethylases, the "erasers." In addition, by binding to the m6A site, another set of proteins, the "readers," can control the fate of the targeted mRNA.[13] Although m6A was recently suggested with critical effects on diverse disorders, ranging from cancer to neurodegenerative diseases to cardiovascular diseases, the clinical and biological significance of m6A regulators in AD is still not clear and needs further investigation.

The present work conducted the comprehensive evaluation of m6A regulators' effects on diagnosing and classifying AD subtypes according to GSE132903 dataset obtained in Gene Expression

ZQ and XB contributed equally to this work.

The authors have no conflicts of interest to disclose.

All data generated or analyzed during this study are included in this published article [and its supplementary information files].

The current analysis does not require ethical approval, because our integrated bioinformatics analysis only collects uploaded data information from the Gene Expression Omnibus (GEO) datasets. The program does not process any patient's personal data and will not cause any patient hurt.

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Omnibus database. In addition, 1 AD risk prediction model was constructed using 16 potential m6A regulators, as a result, this model was utilized for obtaining favorable patient outcomes. Additionally, this work identified 2 different m6A patterns and 2 distinct m6A gene patterns of AD, and both of them have different immune cell infiltration and AD-related gene expression, suggesting their prognostic and risk assessment significance in AD.

2. Methods and Materials

2.1. Data acquisition

GSE132903 dataset containing 98 nondemented controls and 97 AD cases was collected in Gene Expression Omnibus database.[14] Altogether 23 m6A regulators were collected in our enrolled dataset through differential analysis on m6A regulators in AD patients compared with nondemented controls. Such regulators contained 6 writers (METTL3, METTL14, CBLL1, ZC3H13, WTAP, and RBM15B), 2 erasers (FTO and ALKBH5), along with 15 readers (YTHDC1, YTHDC2, FMR1, HNRNPC, YTHDF1, YTHDF2, YTHDF3, LRPPRC, RBMX, HNRNPA2B1, IGF2BP1, IGF2BP2, IGF2BP3, ELAVL1, and IGF2BP1).

2.2. Random forest (RF) and support vector machine (SVM) models establishment

RF and SVM models were built to be the training models for predicting AD prevalence. Receiver operating characteristic curves, boxplots of residual and the reverse cumulative distribution were drawn for model evaluation. RF represents the constituent supervised learning approach, which is suggested to be the decision tree extension. According to our results, the RF model was established using R software RF package (R Foundation, Vienna, Austria) for selecting the potential m6A regulators from those 23 m6A regulators, thus predicting AD risk. This work set mtry and ntrees as 3 and 500, respectively. On the other hand, SVM represents the supervised machine learning approach on the basis of the principle of structural risk minimization following the statistical learning theory. This work drew all data points to be dots within the n-dimensional spaces (with n indicating m6A regulator number).

2.3. Nomogram model establishment

One nomogram model was constructed by using those screened m6A regulators with the use of R package "rms" function for

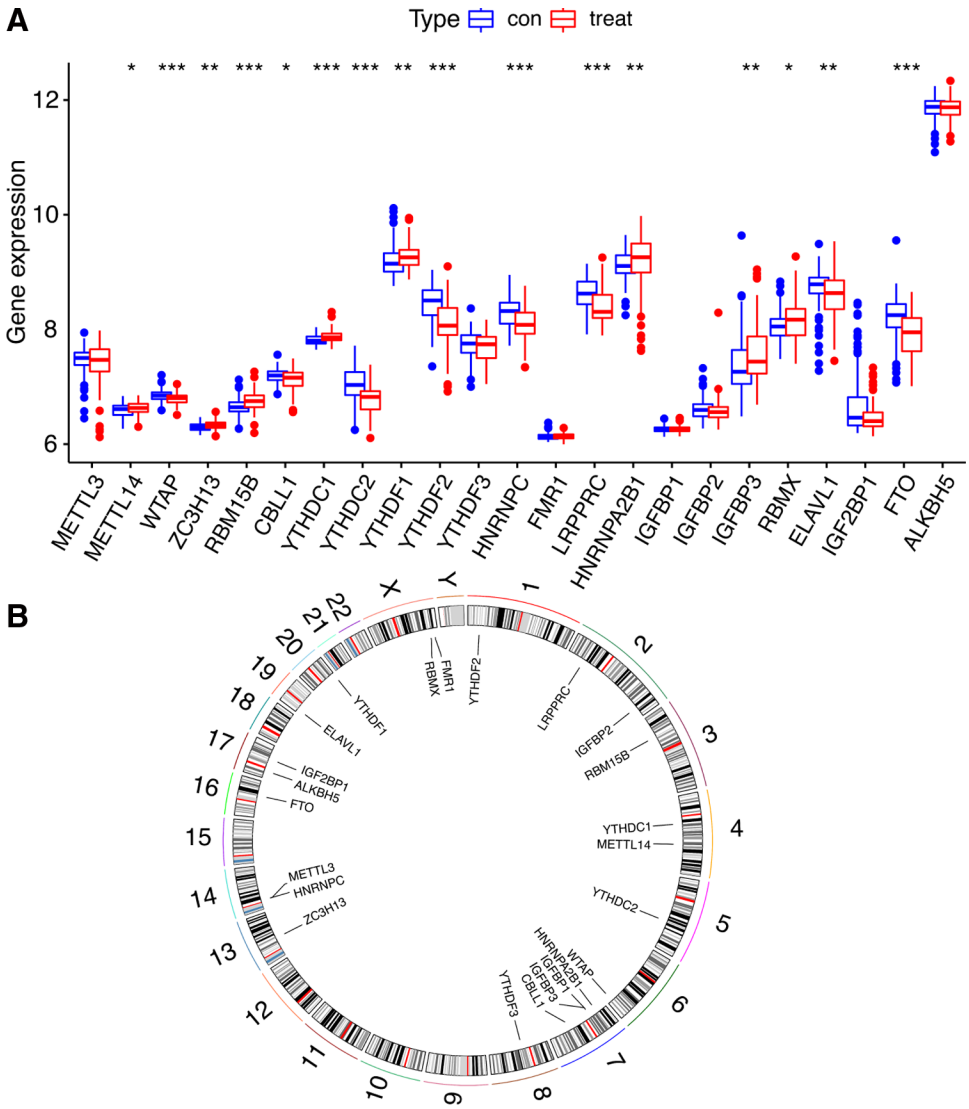

Figure 1. Landscape showing 23 m6A regulators within AD. (A) Histogram showing differential analysis on 21 m6A regulators detected in AD patients compared with nondemented controls. (B) Positions of 23 m6A regulators in chromosomes. * $P < .05$, ** $P < .01$, and *** $P < .001$. AD = Alzheimer disease, m6A = RNA N6-methyladenosine.

predicting AD risk. This work also utilized a calibration curve for evaluating whether the model-predicted results were consistent with real measurements. In addition, the present work drew a clinical impact curve for determining whether our nomogram model was of predicting ability and later conducted decision curve analysis to assess the benefits of our model-based decision-making to patients.[15]

2.4. Molecular subtype identification

Consensus clustering has been developed as the algorithm for identifying different members as well as the subgroup members and for verifying the rationality of clustering on the basis of resampling. Different m6A patterns were identified by consensus clustering by using distinct m6A regulators using the R software ConsensusClusterPlus package.[16]

2.5. Differentially expressed genes (DEGs) identified among different m6A patterns and calculation of the m6A score

This work utilized R software limma package for selecting DEGs among diverse m6A patterns upon $P < .05$ threshold. For quantifying m6A patterns, principal component analysis (PCA) was adopted for calculating m6A scores of different samples. Firstly, m6A patterns were distinguished by PCA. Secondly, this work determined m6A scores as follows, m6A score = PC1i, with PC1 representing principal component 1 (PC1) whereas i representing the DEG level.[17]

2.6. Infiltrating degrees of immune cells

This work conducted single sample gene set enrichment analysis (ssGSEA) for evaluating the abundance of immune cells within the brain tissues from AD cases and nondemented subjects. Firstly, this work adopted ssGSEA for sequencing gene levels within

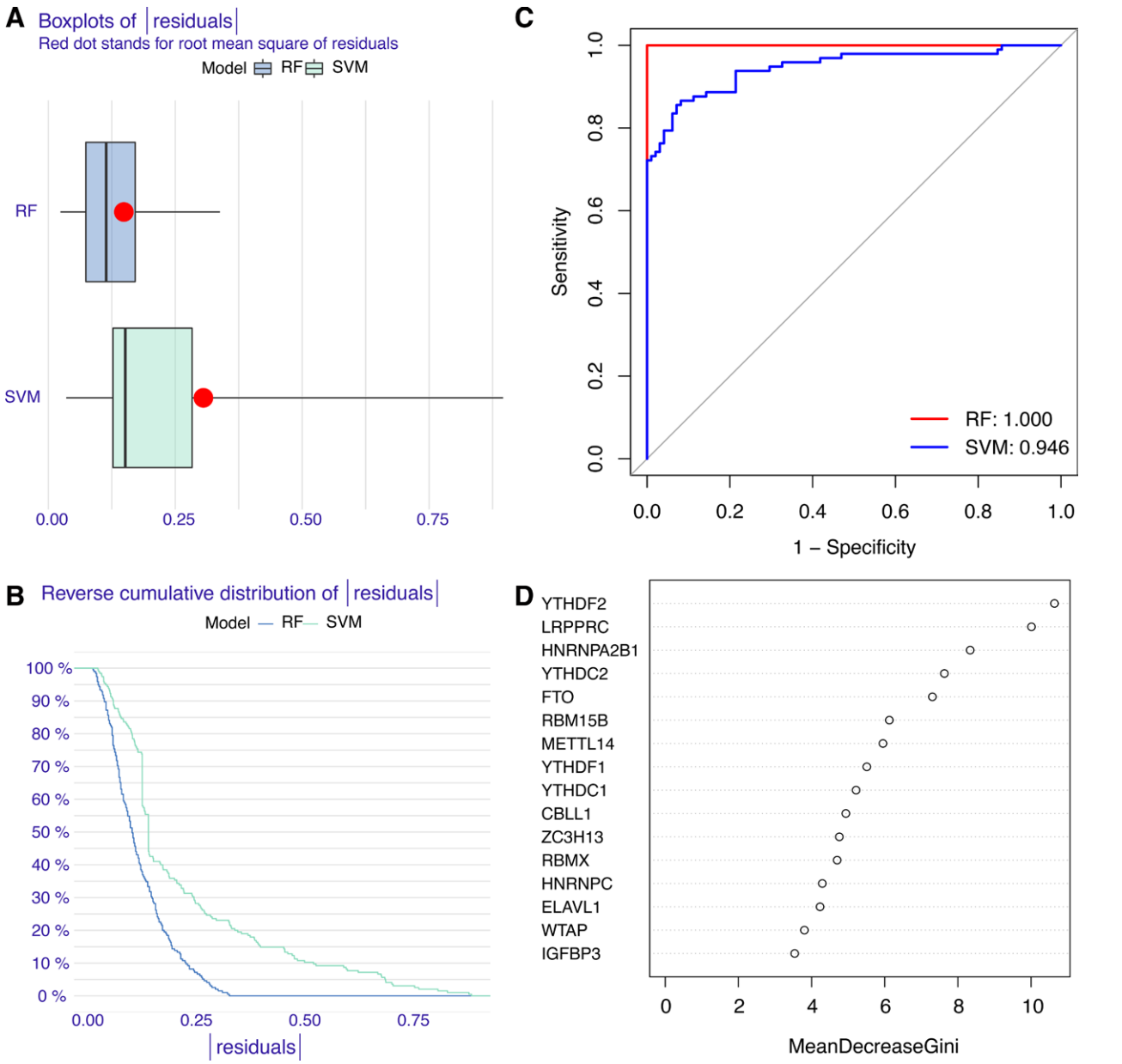

Figure 2. RF and SVM models establishment. (A and B) Residual distribution of the RF and SVM models. (C) ROC curves of RF and SVM models for predicting occurrence of AD. (D) Importance for 16 significant m6A regulators using RF model. AD = Alzheimer disease, m6A = RNA N6-methyladenosine, RF = random forest, ROC = receiver operating characteristic, SVM = support vector machine.

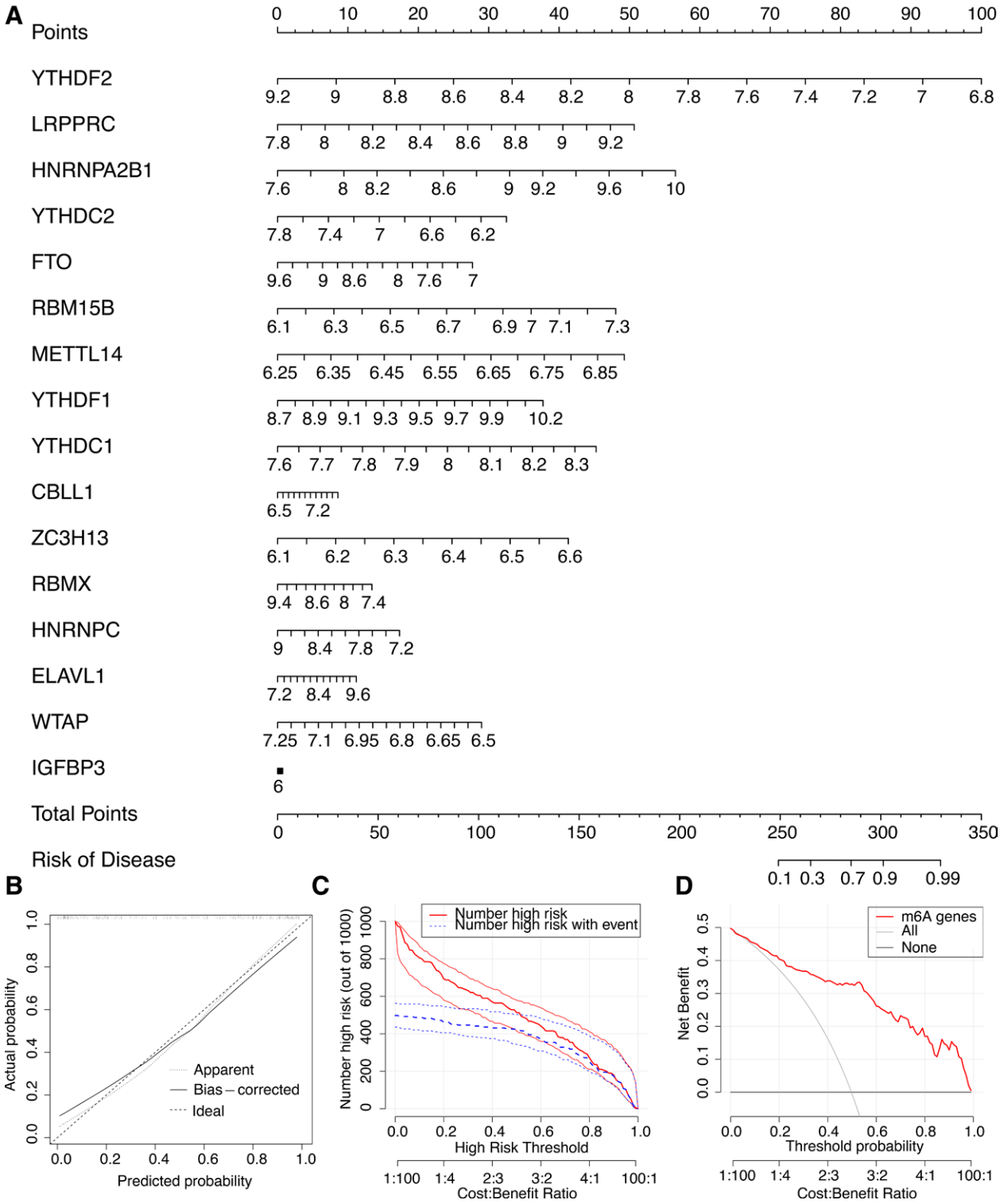

Figure 3. Nomogram model construction. (A) Nomogram model construction by using 16 significant m6A regulators. (B) Predicting ability and (C) clinical impact of our as-constructed nomogram. (D) Our nomogram-based decision-making is beneficial for AD. AD = Alzheimer disease, m6A = RNA N6-methyladenosine.

samples for obtaining the sorting rank. Secondly, the above genes were searched against the input dataset, and then all gene levels were added. According to our assessment, this work quantified the levels of infiltrating immune cells within all samples.

2.7. Statistical analysis

Difference comparison among different groups was completed via Kruskal–Wallis tests. Two-tailed tests were conducted for parametric analysis, and $P < .05$ stood for significance. This work adopted R version 4.0.0 in statistical analysis.

3. Results

3.1. Landscape of the 23 RNA m6A regulators in AD

Using the limma package in R, we first identified a total of 23 m6A regulators from the dataset using difference analysis

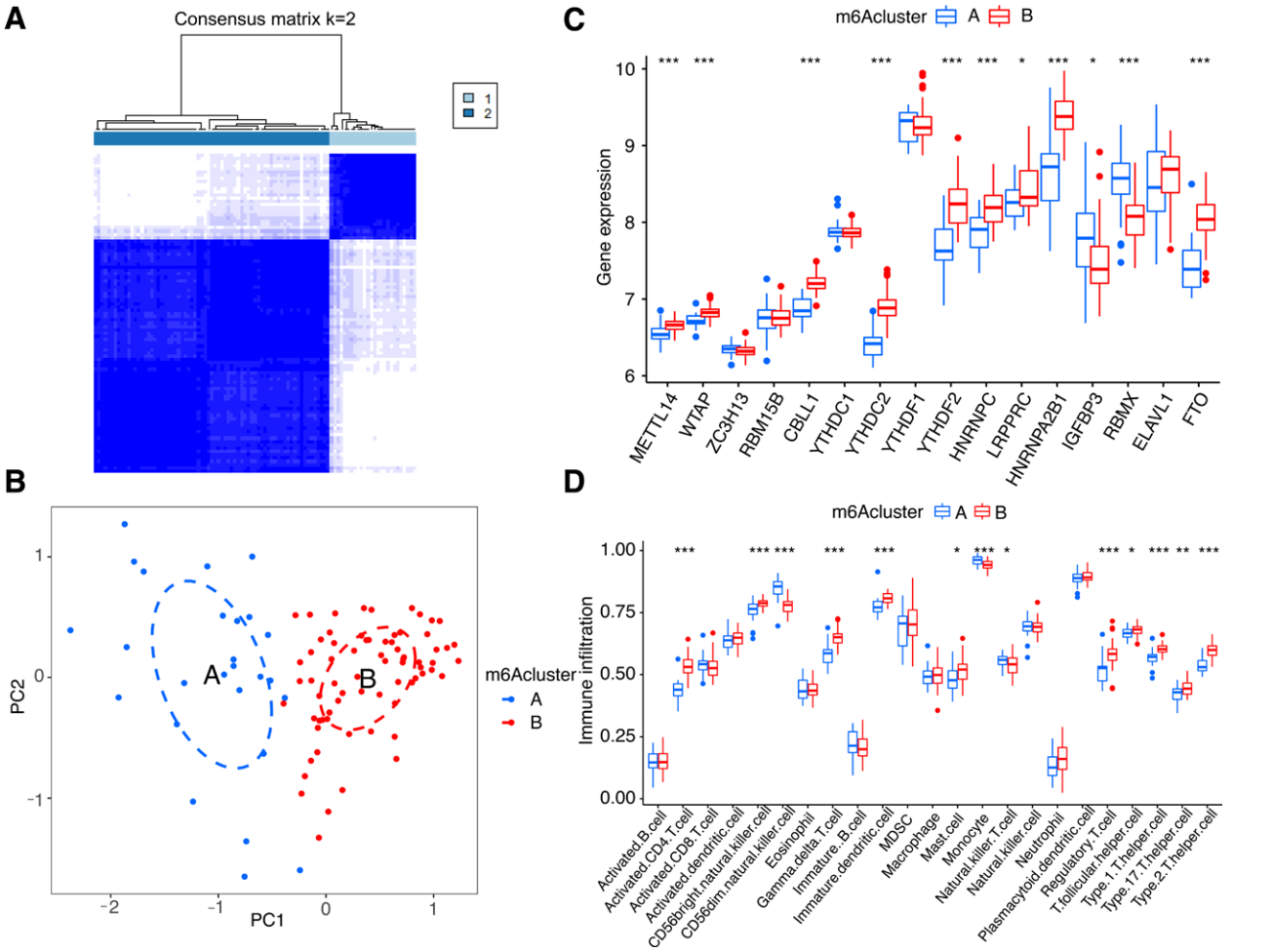

Figure 4. Identification of 2 distinct m6A patterns. (A) Consensus matrices for 16 distinct m6A regulators at $k = 2$. (B) Principal component analysis for 16 distinct m6A regulators expression profiles in the 2 m6A patterns. (C) The expression levels of 16 distinct m6A regulators within Clusters A and B. (D) Different immune cell infiltrating levels in Clusters A and B. * $P < .05$, ** $P < .01$, and *** $P < .001$. m6A = RNA N6-methyladenosine.

between AD patients and nondemented controls. Then, 16 significant m6A regulators were further screened, and 8 of them (METTL14, ZC3H13, RBM15B, YTHDC1, YTHDF1, HNRNPA2B1, IGFBP3, and RBMX) were overexpressed in AD patients, while 8 of them (WTAP, CBLL1, YTHDC2, YTHDF2, HNRNPC, LRPPRC, ELAVL1, and FTO) displayed decreased expression in AD patients compared to nondemented controls (Fig. 1A). Furthermore, by virtue of the RCircos package, we visualized the chromosomal positions of these 23 m6A regulators (Fig. 1B).

3.2. RF and SVM models construction

To determine possible m6A regulators that can be used for predicting the occurrence of AD from these 23 m6A regulators, we established RF and SVM models in AD. According to both boxplots of residual (Fig. 2A) and residual reverse cumulative distribution (Fig. 2B), RF model showed the minimum residuals, besides, many samples from RF model showed low residuals. Moreover, this work drew receiver operating characteristic curve for model evaluation. As revealed by area under the curve, RF model outperformed SVM model in terms of accuracy (Fig. 2C). Therefore, it was suggested that RF model performed well in predicting AD risk. Also, those top 16 m6A regulators were selected in line with importance-based gene ranking, which were regarded as candidate genes for further investigation. These 16 genes were YTHDF2, LRPPRC, HNRNPA2B1, YTHDC2, FTO, RBM15B, METTL14, YTHDF1, YTHDC1, CBLL1, ZC3H13, RBMX, HNRNPC, ELAVL1, WTAP, and IGFBP3 (Fig. 2D).

3.3. Nomogram establishment

According to these 16 possible m6A regulators, this work built a nomogram model with R software rms package for predicting AD occurrence (Fig. 3A). As revealed by calibration curves, our as-constructed nomogram showed high accuracy in predictability (Fig. 3B). Based on clinical impact curve, our constructed nomogram had remarkable predictive power (Fig. 3C). In addition, in decision curve analysis curve, the red line was always above black and gray lines in 0 to 1, which suggested that the nomogram-based decision-making was beneficial for AD cases (Fig. 3D).

3.4. Two different m6A pattern identification

To identify different m6A patterns according to 16 distinct m6A regulators, this work used consensus clustering by R software ConsensusClusterPlus package, which discovered 2 m6A patterns (Clusters A and B) (Fig. 4A). Cluster A contains 26 cases, and Cluster B contains 71 cases. According to PCA, 16 distinct m6A regulators were able to differentiate 2 m6A patterns (Fig. 4B). Then, we investigated differential expression of 16 distinct m6A regulators between these 2 clusters. METTL14,

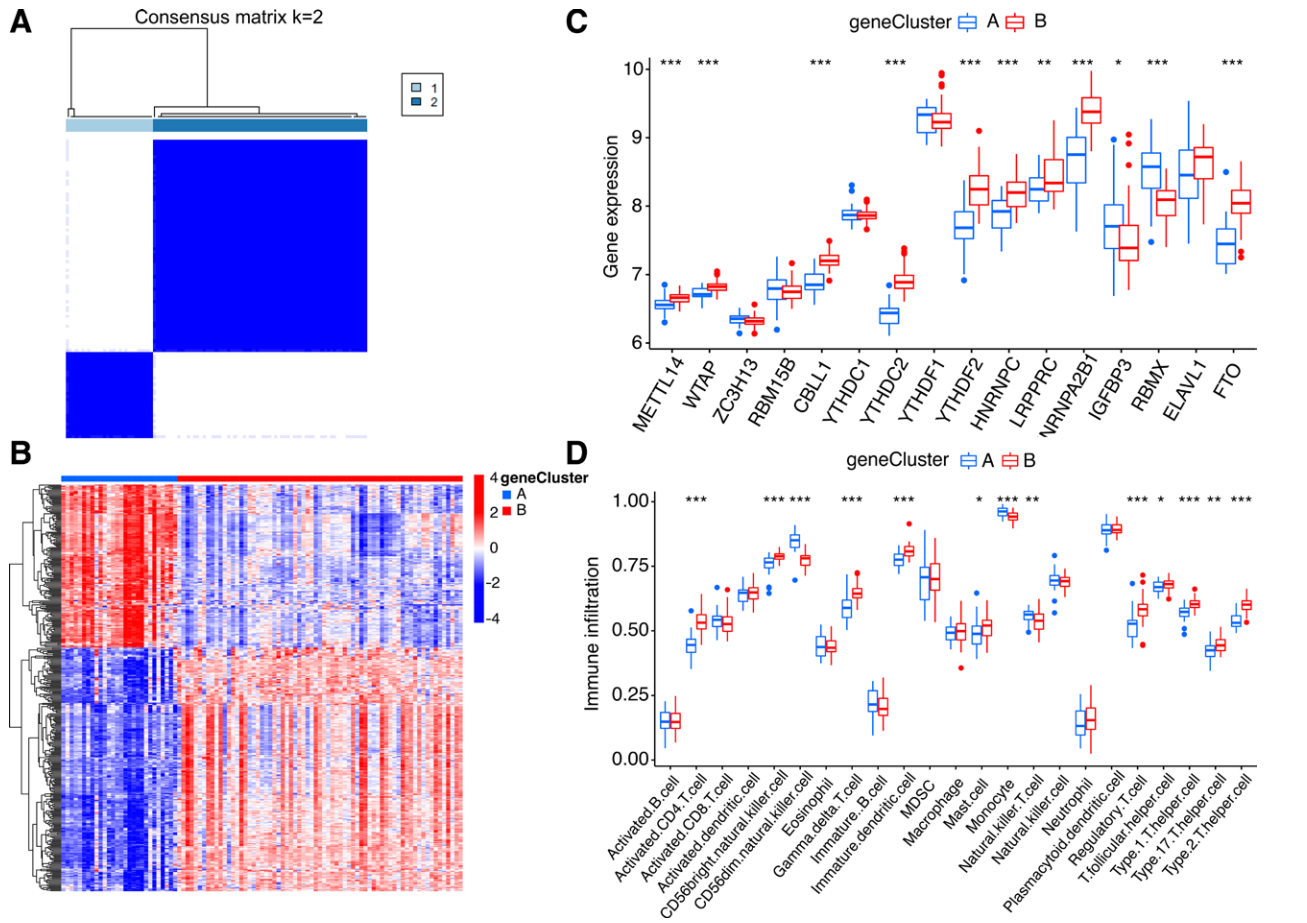

Figure 5. Identification of 2 distinct m6A gene patterns. (A) Consensus matrices for 397 m6A-associated DEGs at $k = 2$. (B) Heatmap of 397 m6A-associated DEGs levels. (C) The expression levels of 16 distinct m6A regulators within gene Clusters A and B. (D) Different immune cell infiltrating degrees within gene Clusters A and B. * $P < .05$, ** $P < .01$, and *** $P < .001$. DEGs = differentially expressed genes, m6A = RNA N6-methyladenosine.

WTAP, CBLL1, YTHDC2, YTHDF2, HNRNPC, LRPPRC, HNRNPA2B1, and FTO displayed higher levels within Cluster B, while IGFBP3 and RBMX displayed increased expression within Cluster A. Regarding the expression levels of ZC3H13, RBM15B, YTHDC1, YTHDF1, and ELAVL1, there were no significant differences between Clusters A and B (Fig. 4C). By applying ssGSEA, we then calculated immune cell abundances within brain samples of AD patients and analyzed the difference in immune cell infiltrating degrees of both m6A patterns. As a result, there were more T follicular helper cells, activated CD4 T cells, gamma delta T cells, CD56 bright natural killer (NK) cells, mast cells, regulatory T cells, immature dendritic cells, Type 1 T helper cells, Type 2 T helper cells, and Type 17 T helper cells in Cluster B, while there were more CD56 dim NK cells, monocytes and NK T cells within Cluster A (Fig. 4D).

3.5. Detection of 2 m6A gene patterns

To validate 2 m6A patterns described above, this work adopted consensus clustering for classifying AD cases into distinct genomic subtypes by using 397 DEGs between Clusters A and B (DEGs associated with m6A). Two different m6A gene patterns were identified (gene Clusters A and B), conforming to 2 m6A patterns grouped (Fig. 5A). Using a heatmap, we also illustrated these 397 m6A-related DEGs expression between 2 gene clusters (Fig. 5B). Additionally, those 16 distinct m6A regulators showed diverse gene levels between 2 gene clusters, along with different immune cells levels (Figure 5C, D). Moreover, the difference between these 2 distinct m6A gene patterns was similar to the difference between the 2 m6A patterns, which suggested that consensus clustering was accurate in grouping.

3.6. Prognostic significance of different m6A or m6A gene patterns

To further investigate the prognostic significance of different m6A patterns, we first calculated the m6A scores in different samples with PCA algorithm. By comparing m6A scores between 2 clusters and 2 gene clusters, we found that m6A scores of Cluster B and gene Cluster B increased relative to Cluster A and gene Cluster A (Figure 6A, B). Relationship between the m6A scores and different m6A patterns and different m6A gene patterns was visualized in a Sankey diagram (Fig. 6C). As the expression levels of presenilin (PSEN)-1, PSEN2, amyloid-beta precursor protein (APP), and microtubule-associated protein tau (MAPT) were reported to be closely related to the risk of AD onset,[18–20] we investigated the relation of different m6A patterns with MAPT, APP, PSEN1, and PSEN2 expression. The results demonstrated that the MAPT, APP, and PSEN1 expression increased within Cluster B and gene Cluster B samples compared with Cluster A and gene Cluster A, as a result, Cluster B and gene Cluster B have higher AD susceptibility than the other 2 clusters (Figures 6D, E).

4. Discussion

Recently, substantial efforts are undertaken to reveal the potential role of m6A in a broad range of physiological and pathological processes.[21–23] As a most common type of various kinds of

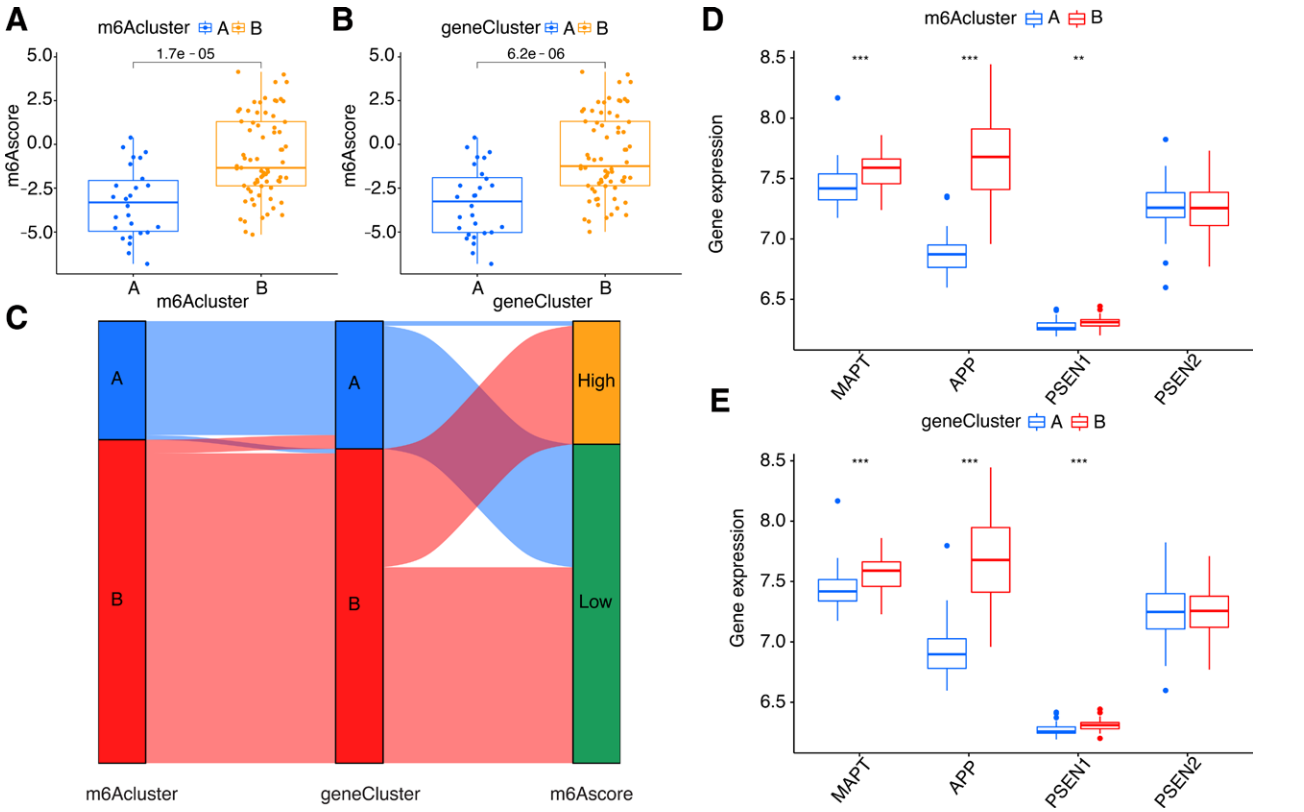

Figure 6. Prognostic significance of different m6A or m6A gene patterns. (A) Different m6A scores in Clusters A compared with B. (B) Different m6A scores in gene Clusters A compared with B. (C) Relation of m6A scores, m6A patterns, and m6A gene patterns as illustrated by the Sankey diagram. (D) The expression levels of APP, PSEN1 and PSEN2 within Clusters A and B. (E) APP, PSEN1 and PSEN2 expression within gene Clusters A and B. *P < .05, **P < .01, and ***P < .001. APP = amyloid-beta precursor protein, m6A = RNA N6-methyladenosine, PSEN = presenilin 1.

modifications present on mRNA, m6A can regulate the fate of mRNA.[24–26] Although prior works suggest that m6A could also play a crucial role in neurodegenerative diseases, its potential role in AD is still not very clear.[27–29] Thus, this work investigated m6A regulators' effects on AD.

First, 16 distinct m6A regulators were discovered from 23 m6A regulators by means of differential analysis in AD patients compared with nondemented controls. By comparing the established RF and SVM models, this work found that the RF model was a better model for predicting AD risk. According to the 16 screened significant m6A regulators, we further constructed a nomogram model. It was confirmed that this model has accurate predictability, which was beneficial for decision-making in AD cases.

As previous studies have shown that the consensus clustering method could be used for subgrouping analysis, we tried to identify different m6A patterns through this method.[16,30] According to those 16 distinct m6A regulators, this work performed consensus clustering, and identified 2 m6A patterns (Clusters A and B). It is obvious that these 2 m6A patterns not only have distinct expression of 16 m6A regulators but also have distinct immune cell levels within the brain microenvironment of AD patients. Basically, Cluster B has increased T-cell infiltration compared with Cluster A, such as gamma delta T cells, T follicular helper cells, activated CD4 T cells, regulatory T cells, Type 1 T helper cells, Type 2 T helper cells, and Type 17 T helper cells. This result suggested that Cluster B is more closely involved in T-cell-mediated immunity than Cluster A. Considering that increasing evidence suggests that AD pathogenic mechanism may be restricted to the neuronal compartment, and is strongly related to brain immunological mechanisms,[31–34] according to our results, different AD subgroups could have different immune cell infiltrations. In addition, we further validated these 2 distinct m6A patterns by dividing the AD cases as diverse genomic clusters (gene Clusters A and B) by using 397 DEGs between Clusters A and B through consensus clustering. Regarding 16 significant m6A regulators and immune cell infiltration, there were similarities in m6A compared with m6A gene patterns, which suggested that our grouping by consensus clustering was accurate. By calculating the m6A scores of AD patients, we found that m6A scores in Cluster B and gene Cluster B increased compared with the other 2 clusters. Finally, we investigated the relations of different m6A or m6A gene patterns with the expression levels of several risk genes of AD. We demonstrated that MAPT, APP, and PSEN1 expression increased relative to Cluster B and gene Cluster B, which suggested that Cluster B and gene Cluster B have increased AD susceptibility compared with Cluster A and gene Cluster A AD patients.

Overall, this present work comprehensively evaluated the functions of m6A regulators in the diagnosis and subtype classification of AD. We not only established a gene model for predicting AD susceptibility based on sixteen candidate m6A regulators but also revealed 2 distinct m6A patterns and 2 distinct m6A gene patterns of AD and both of them have important prognostic and risk assessment value in AD.

As the database we used in this study only contains 98 nondemented controls and 97 AD patients, more patients need to be included in our evaluation process for more reliable conclusions. In addition, even though we have demonstrated the importance of the 16 significant m6A regulators, we have not investigated the biological function of these m6A regulators in AD. Thus, more biochemical and molecular biology experiments need to be done for revealing their exact roles in the process of AD. Considering that this study is just a primary investigation of the role of m6A regulators in AD, the results from this study

definitely provide us a good reason to research deeper in this direction in the future.

Author contributions

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Methodology: Zhiqiang Qiu, Xuanyang Bai, Xinye Han.
Writing – original draft: Xiang Wang, Yuxia Lv.
Writing – review & editing: Zhiqiang Qiu, Xuanyang Bai, Yihua An.

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