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N6-Methyladenosine RNA modification in cerebrospinal fluid as a novel potential diagnostic biomarker for progressive multiple sclerosis

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Abstract

Background: Progressive multiple sclerosis (PMS) is an uncommon and severe subtype of MS that worsens gradually and leads to irreversible disabilities in young adults. Currently, there are no applicable or reliable biomarkers to distinguish PMS from relapsing–remitting multiple sclerosis (RRMS). Previous studies have demonstrated that dysfunction of N6-methyladenosine (m6A) RNA modification is relevant to many neurological disorders. Thus, the aim of this study was to explore the diagnostic biomarkers for PMS based on m6A regulatory genes in the cerebrospinal fluid (CSF).

Methods: Gene expression matrices were downloaded from the ArrayExpress database. Then, we identified differentially expressed m6A regulatory genes between MS and non-MS patients. MS clusters were identified by consensus clustering analysis. Next, we analyzed the correlation between clusters and clinical characteristics. The random forest (RF) algorithm was applied to select key m6A-related genes. The support vector machine (SVM) was then used to construct a diagnostic gene signature. Receiver operating characteristic (ROC) curves were plotted to evaluate the accuracy of the diagnostic model. In addition, CSF samples from MS and non-MS patients were collected and used for external validation, as evaluated by an m6A RNA Methylation Quantification Kit and by real-time quantitative polymerase chain reaction.

Results: The 13 central m6A RNA methylation regulators were all upregulated in MS patients when compared with non-MS patients. Consensus clustering analysis identified two clusters, both of which were significantly associated with MS subtypes. Next, we divided 61 MS patients into a training set (n = 41) and a test set (n = 20). The RF algorithm identified eight feature genes, and the SVM method was successfully applied to construct a diagnostic model. ROC curves revealed good performance. Finally, the analysis of 11 CSF samples demonstrated that RRMS samples exhibited significantly higher levels of m6A RNA methylation and higher gene expression levels of m6A-related genes than PMS samples.

Conclusions: The dynamic modification of m6A RNA methylation is involved in the progression of MS and could potentially represent a novel CSF biomarker for diagnosing MS and distinguishing PMS from RRMS in the early stages of the disease.

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Keywords: Progressive multiple sclerosis (PMS), N6-methyladenosine (m6A), Cerebrospinal fluid (CSF), Diagnostic biomarker

Background

Multiple sclerosis (MS) is a complex and disabling disease of the central nervous system (CNS). Disease onset typically occurs between the ages of 20 and 50 years and is driven by complex interactions between underlying genetic and environmental factors [1]. Approximately 80–85% of patients with MS experience a natural course of relapse and remission at disease onset that is referred to as relapsing–remitting MS (RRMS) [1]. Most cases progress steadily into a secondary-progressive disease course after decades without superimposed remissions, a condition known as secondary-progressive MS (SPMS) [2]. Approximately 10–15% of patients initially present with a gradually increasing and irreversible deterioration of neurological functions; this condition is referred to as primary-progressive MS (PPMS) [3]. Although recent studies have shown that low vitamin D concentration, cigarette smoking, and obesity are highly associated with MS, the exact etiology and pathogenesis of MS has yet to be elucidated [1]. The current diagnostic criteria are beneficial to identify MS patients by integrated analysis of clinical manifestations, imaging characteristics, and cerebrospinal fluid (CSF) abnormalities [4]. However, compared to RRMS, the diagnosis of progressive MS (PMS) is usually delayed because of a retrospective history; furthermore, the available disease modifying drugs (DMDs) fail to provide benefit, eventually leading to a poor prognosis [5]. Therefore, there is a need to discover a novel biomarker for the early and accurate diagnosis of PMS to enhance survival with personalized therapeutic management.

N6-Methyladenosine (m6A) is the most common RNA methylation modification and is defined as methylation of the nitrogen-6 position of adenosine in the mRNA via various m6A modification regulators [6]. The specific methylation of mRNA not only influences molecular structure and mRNA-protein interactions, it also causes changes in RNA metabolism and functions. Recent studies have confirmed that neurodegeneration is accelerated in PMS lesions [7], and that dysfunctional RNA modification is related to disease course and reflects prognosis in neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) [8, 9]. However, to our knowledge, the role of dysfunctional RNA modification in MS has not yet been reported. Recent research has identified novel biomarkers for these diseases that are usually detected in the CSF, including beta-amyloid 42, alpha-synuclein, and oligoclonal bands (OB). Therefore,

the aim of the present study was to investigate diagnostic CSF biomarkers for PMS patients based on m6A regulatory genes.

Methods

Data download and preprocessing

First, we screened the Gene Expression Omnibus (GEO) database (<https://www.ncbi.nlm.nih.gov/geo/>) and the ArrayExpress database (<https://www.ebi.ac.uk/array-express/>) for gene array expression files relating to MS using “*Homo sapiens*” (organism), “CSF” (sample), and “RNA assay” (experimental type) as the search criteria. We successfully identified two gene expression microarray datasets: E-MTAB-69 [10] and E-MTAB-2374 [11] from the ArrayExpress database. No datasets were downloaded from the GEO database. The E-MTAB-69 dataset included the molecular profiles of 26 samples from MS cases and 18 samples from non-MS controls (non-inflammatory neurological disorders) while the E-MTAB-2374 dataset contained the expression profiles of 35 MS cases and 13 non-MS controls (non-inflammatory neurological disease controls, including stroke, neurosarcoidosis, and PD). Experiments were conducted on the Affymetrix GeneChip Human Genome U133 Plus 2.0 (*GPL570 Platform, Affymetrix, Inc*). The corresponding annotation file was used to convert identification probes into gene symbols. Mean values were used to determine the gene expression levels when several probes targeted a single gene. The robust multi-array average (RMA) algorithm was then used to obtain log₂ converted and standardized mRNA expression data. Given the limited number of CSF samples from patients with MS, we performed batch-normalization to merge both gene expression profiles; the inter-batch difference was then removed by the *sva* package [12]. Subsequently, a density plot was plotted to evaluate the effectiveness of removing the inter-batch difference.

Selection of m6A RNA methylation regulators and differential expression analysis

A total of 13 currently recognized m6A RNA methylation regulators were extracted for subsequent analysis; these included erasers (ALKBH5 and FTO); readers (HNRNPC, YTHDC1, YTHDC2, YTHDF1, and YTHDF2); and writers (KIAA1429, METTL3, METTL14, RBM15, WTAP, and ZC3H13) [13]. Next, we screened these m6A-related genes to identify differentially expressed genes (DEGs) between MS patients and non-MS controls using the

empirical Bayes (eBayes) methods and the *limma* package. In this study, the cut-offs were set at a $\log_2|\text{fold change (FC)}| > 1$ and a false discovery rate (FDR) < 0.05 to select the DEGs between the MS and non-MS patients. In addition, the Mann–Whitney *U* test (Wilcoxon rank-sum test) was used to confirm significant m6A-related genes between MS and non-MS patients. We also constructed a box-plot of DEGs encoding m6A RNA methylation regulators. Spearman correlation analysis was then carried out to demonstrate interactive associations between each of the m6A-related genes.

Gene functional enrichment analyses

Gene Ontology (GO) annotations were then used to determine the biological processes, cellular components, and molecular functions of the identified DEGs and differentially expressed m6A-related genes [14]. The integrated molecular pathways of these genes were also acquired from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [15]. The significant GO terms and KEGG pathways were considered enrichments when using a specific cut-off (FDR < 0.05) via the *org.Hs.eg.db*, *clusterProfiler*, and *GOPLOT* packages. In addition, protein–protein interaction (PPI) networks were constructed with a high confidence (> 0.7) using the Search Tool for the Retrieval of Interacting Genes (STRING) database [16].

Consensus clustering analysis

Next, we investigated the link between m6A RNA methylation regulators and MS classification by clustering the merged dataset into different subgroups using the *ConsensusClusterPlus* package. The unsupervised clustering method was applied to identify different clusters. The clustering algorithm was partitioned around medoids and distances were measured by the Euclidean metric system. Principal component analysis (PCA) was then conducted to verify the classification results. Consequently, the difference in both m6A-related genes and clinical parameters between these clusters were determined using the Chi-square test; these were subsequently presented as a heatmap.

Generation of a random forest (RF) algorithm for feature gene selection and a support vector machine (SVM) classifier

The integrated dataset was randomly divided into a training set for model development (2/3, $n = 41$) and a test set for model validation (1/3, $n = 20$) using the *caret* package. For feature gene selection, the RF algorithm was used to rank the importance of these m6A-related genes; to do this, we used the *randomForest* package. The m6A-related feature genes were identified based on a relative

importance > 0.4 . During model development, we used the selected feature genes to establish a SVM classifier with a C-type classification and a radial basis function kernel. This was measured with a fivefold cross validation via the *e1071* package. Patients were then classified and differentiated by gene expression levels. We also used eigen values to predict the probabilities of patients belonging to the same classification; these values could distinguish and predict the different subtypes of MS. During model validation, we used the test set to verify our previous findings. The area under the receiver operating characteristic (ROC) curve was used to evaluate the effect of classification for both the training and test sets. Moreover, a correlation-based SVM filter was also applied to confirm the feature genes. PCA analysis was applied to evaluate the performance of the correlation-based distances for clustering.

External validation of m6A RNA methylation in MS patients

This study was approved by the Independent Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University, and all patients signed an informed consent form. Patients with a clinical diagnosis of MS were enrolled for external validation between July 2020 and December 2020 at the First Affiliated Hospital, Sun Yat-sen University. The diagnostic criteria were based on the 2017 revisions of the McDonald criteria [4]. Patients who were diagnosed with other autoimmune diseases and had malignant tumors were excluded from this study. Consequently, these patients were automatically divided into RRMS and PMS subgroups. In addition, patients diagnosed with other neurodegenerative disorders between May 2021 and June 2021 were also recruited to act as non-MS controls. A 2 mL sample of CSF was collected via lumbar puncture and placed in a 5-mL RNase/DNase-free centrifuge tube. We excluded CSF samples if they were contaminated by blood. Samples were immediately centrifuged at $500 \times g$ for 10 min at 4°C (ThermoFisher, Sorvall ST40R, USA). Next, total RNA was extracted and purified from CSF cells using the RNeasy Micro Kit (G1AGEN, 74004, Germany) in accordance with the manufacturer's protocol. In brief, CSF cells were disrupted with 350 μl of Buffer RLT, mixed with 350 μl of 70% ethanol, and transferred to a 2 mL RNeasy MinElute spin column, retrospectively. The spin column membrane was then washed with 350 μl of Buffer RW1, and DNA was eliminated by incubation with DNase I mix (10 μl of DNase I stock solution and 70 μl of Buffer RDD) at room temperature (RT) for 15 min. The RNA was then purified with 500 μl of Buffer RPE and 500 μl of 80% ethanol. The flowthrough was removed by centrifugation at $8000 \times g$ for 15 s. Next, 14 μl of RNase-free water was added to the center of a spin column membrane in order to isolate the

RNA. The ratio of absorbance at 260 and 280 nm (A₂₆₀/A₂₈₀) was calculated to evaluate the purity of RNA, with a cut-off value of 2.0 (ThermoFisher, NanoDrop One, USA). The extracted RNA was then stored at -80°C to await further analysis.

First, we measured the global m6A levels in total CSF cell RNA with an m6A RNA Methylation Quantification Kit (Fluorometric; Abcam, ab233491, UK) in accordance with the manufacturer's protocol. Because of the very low and highly variable RNA yield from CSF samples, we used 100 ng of total RNA from each sample. In brief, 2 μl of negative control, 2 μl of diluted positive control, and 100 ng of RNA samples, were added to each well and incubated at 37°C for 90 min after adding 80 μl of binding solution to the 96-well plate. Then, m6A RNA was captured by covering and incubating 50 μl of diluted Capture Antibody at RT for 60 min, 50 μl of diluted Detection Antibody for 30 min, and 50 μl of diluted Enhancer Solution for 30 min, retrospectively. Each well was washed three times with 150 μl of $1\times$ Wash Buffer after each incubation. Signals were then detected by measuring and reading the relative fluorescence units (RFU) on a fluorescence microplate reader (ThermoFisher, Varioskan LUX, USA) at Ex/Em = 530/590 nm after adding 50 μl of Fluoro Developer Mix to each well and incubating at RT for 1–4 min away from the light. We also performed a simple calculation of the proportion (%) of m6A in the total RNA as follows:

$$\text{m6A\%} = \frac{(\text{Sample RFU} - \text{Negative Control RFU}) \div \text{Total Sample RNA}}{(\text{Positive Control RFU} - \text{Negative Control RFU}) \div \text{Total Positive Control RNA}} * 100\%.$$

Then, we synthesized cDNA using the PrimeScript First Strand cDNA Synthesis Kit (Takara, RR047A, Japan). Genomic DNA was first removed from the mixed 10 μl solution with 50 ng of total RNA, 2 μl of $5\times$ gDNA eraser buffer, and 1 μl of gDNA eraser, via polymerase chain reaction (PCR) (Bio-Rad, QX200, USA) at 42°C for 2 min. The 10 μl mixed solution was then reverse transcribed to 20 μl of cDNA by adding 1 μl of PrimeScript RT Enzyme Mix 1, 1 μl of RT Primer Mix, and 4 μl of $5\times$ PrimeScript Buffer 2, via PCR at 37°C for 15 min and 85°C for 5 s. The relative RNA levels of m6A-related genes were then analyzed by quantitative real-time PCR (qRT-PCR) with the SYBR Green detection method (Takara, RR041A, Japan) and a QuantStudio 5 Real-Time PCR System (ThermoFisher, USA). The thermocycling conditions were as follows: a holding stage of 95°C for 30 s; 40 cycles of 95°C for 5 s and 60°C for 30 s, followed by a melting curve stage of 95°C for 15 s, 60°C for 30 s, and 95°C for 15 s. *GAPDH* was used as an internal control. The qRT-PCR reactions were performed in triplicate,

and the results were analyzed using the $\Delta\Delta\text{CT}$ method. The primers are given in Additional file 1: Table S1.

Statistical analysis

Statistical analyses were performed using the R (*version 4.0.2*) and GraphPad Prism (*version 9.0*) software. $P < 0.05$ was considered to indicate statistical significance. Continuous variables were calculated as medians with the standard deviations. Categorical variables were reported as a number with proportions. The *t*-test and Mann–Whitney *U* test were used to analyze the differences in continuous variables. The Chi-squared test was performed to explore differences in categorical variables.

Results

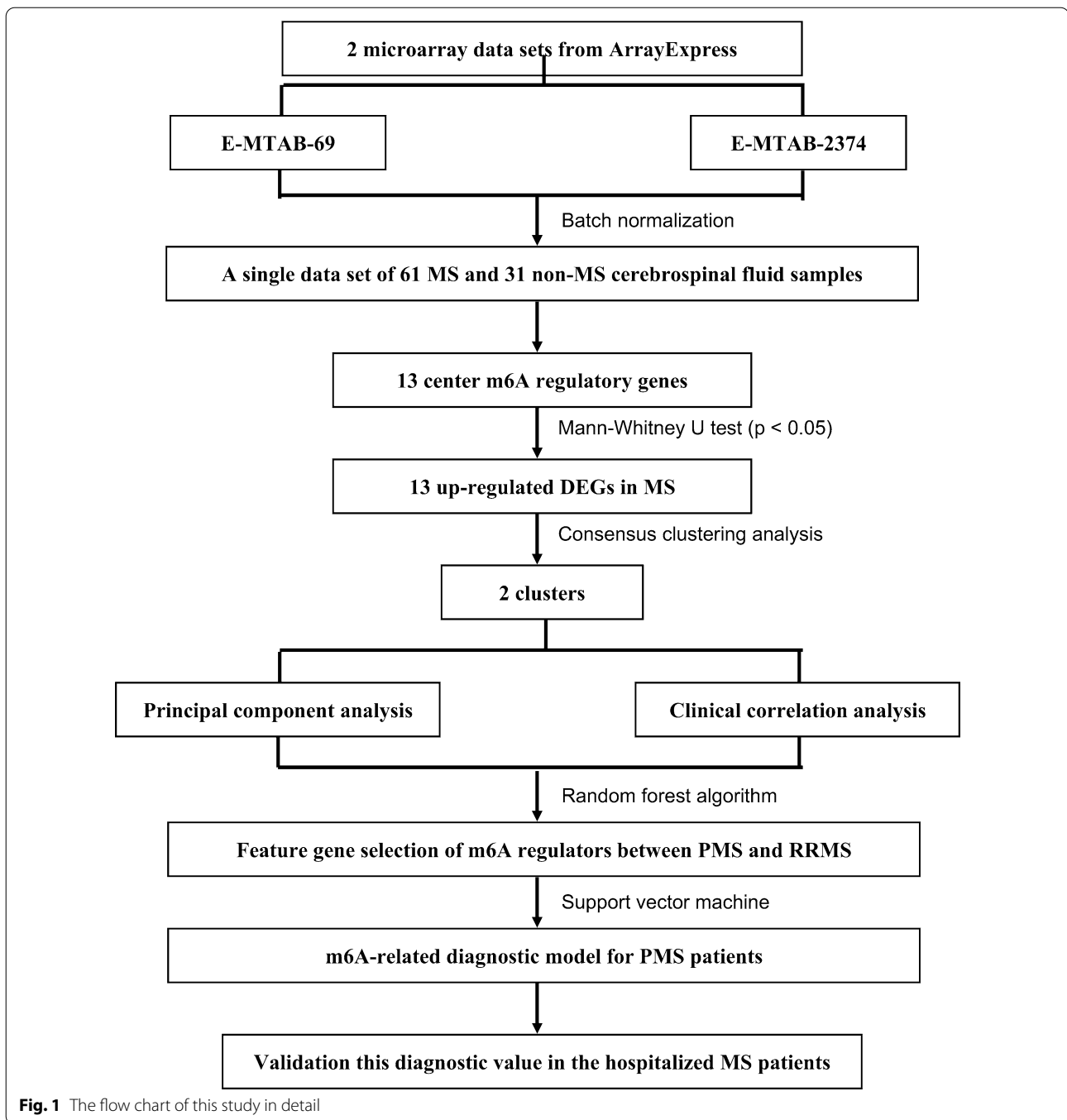
The identification of differentially expressed m6A RNA methylation regulators

A detailed flowchart depicting this study is presented in Fig. 1. Details relating to the E-MTAB-69 and E-MTAB-2374 datasets are available in the ArrayExpress database (Additional file 1: Tables S2, S3). A single dataset was created by batch normalization for background correction and consisted of 61 MS CSF samples and 31 non-MS CSF samples (Additional file 2: Table S4). The demographic and clinical characteristics of both the MS and non-MS patients are presented in Table 1. Interbatch differences were eliminated and the effect was confirmed via density plots that were constructed before

and after batch normalization (Fig. 2a, b). Consequently, the 13 m6A RNA methylation regulators were extracted from the merged dataset (Additional file 2: Table S5). All of the 13 m6A-related genes were identified as significant DEGs between MS and non-MS patients via eBayes methods; a heat map is presented in Fig. 2c (Additional file 2: Table S6). The Mann–Whitney *U* test confirmed that the expression levels of these DEGs were significantly higher in MS patients than in non-MS patients (Fig. 2d). In addition, the Spearman correlation analysis revealed that these DEGs were positively correlated with each other except FTO (Fig. 2e).

Functional analyses of differentially expressed m6A-related genes

The eBayes method identified 5031 DEGs in the merged dataset (Additional file 2: Table S7). Next, we used the ConsensusPathDB interaction database (<http://consensuspathdb.org/>) to construct an integrated plot to analyze the molecular function of the top



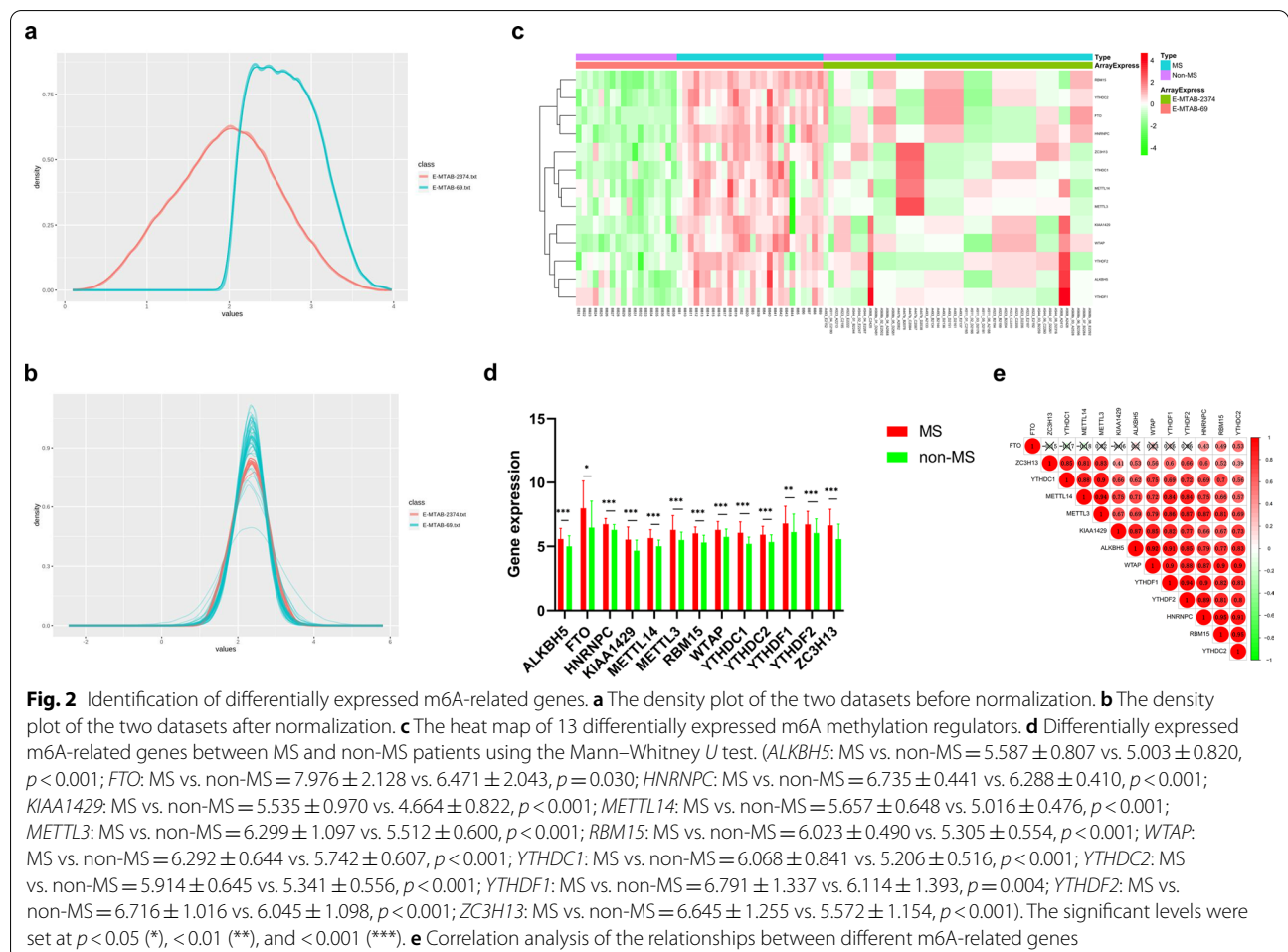
94 DEGs with a $\log_2|FC| > 2$ and the 13 m6A-related genes (Fig. 3a). In addition, we identified 348 significant GO items that were associated with these DEGs (Additional file 2: Table S8), mainly including cellular calcium ion homeostasis, the positive regulation of endocytosis, and the organic acid catabolic process (Fig. 3b). In addition, 157 significant GO items were shown to be associated with m6A-related genes (Additional

file 2: Table S9), mainly including RNA modification, methylation, destabilization, and metabolic processes (Fig. 3c). Furthermore, 59 significant KEGG pathways were identified (Additional file 2: Table S10), including lysosomes, cytokine-cytokine receptor interaction, and the JAK-STAT signaling pathway (Fig. 3d). In addition, we used the STRING database to create a PPI network; this showed the relationships between these

Table 1 Comparison of the demographic and clinical characteristics between multiple sclerosis (MS) patients and non-MS controls in the merged dataset and in the validation group

	Datasets			Validation		
	MS (n = 61)	Non-MS (31)	P	MS (n = 11)	Non-MS (n = 3)	P
Age (years)	44.87 ± 15.66	42.71 ± 12.00	0.466	29.73 ± 10.78	42.33 ± 11.85	0.195
Sex (%)						
Female	37 (60.7%)	19 (61.3%)	0.953	8 (72.7%)	2 (66.7%)	0.837
Male	24 (39.3%)	12 (38.7%)		3 (27.3%)	1 (33.3%)	
Subtype (%)						
RRMS	32 (52.5%)	–	–	8 (72.7%)	–	–
PPMS	10 (16.4%)	–	–	1 (9.1%)	–	–
SPMS	19 (31.1%)	–	–	2 (18.2%)	–	–
DMDs (%)	–	–	–	3 (27.3%)	–	–
CSF testing						
OB (%)	–	–	–	1 (16.7%)	–	–
Antibody (%) (anti-AQP4, MOG, MBP)	–	–	–	0 (0.0%)	–	–

In the validation group, six of the 11 MS patients underwent CSF OB testing, and only one patient found positive result. Nine of them measured related antibodies while all reported negative results



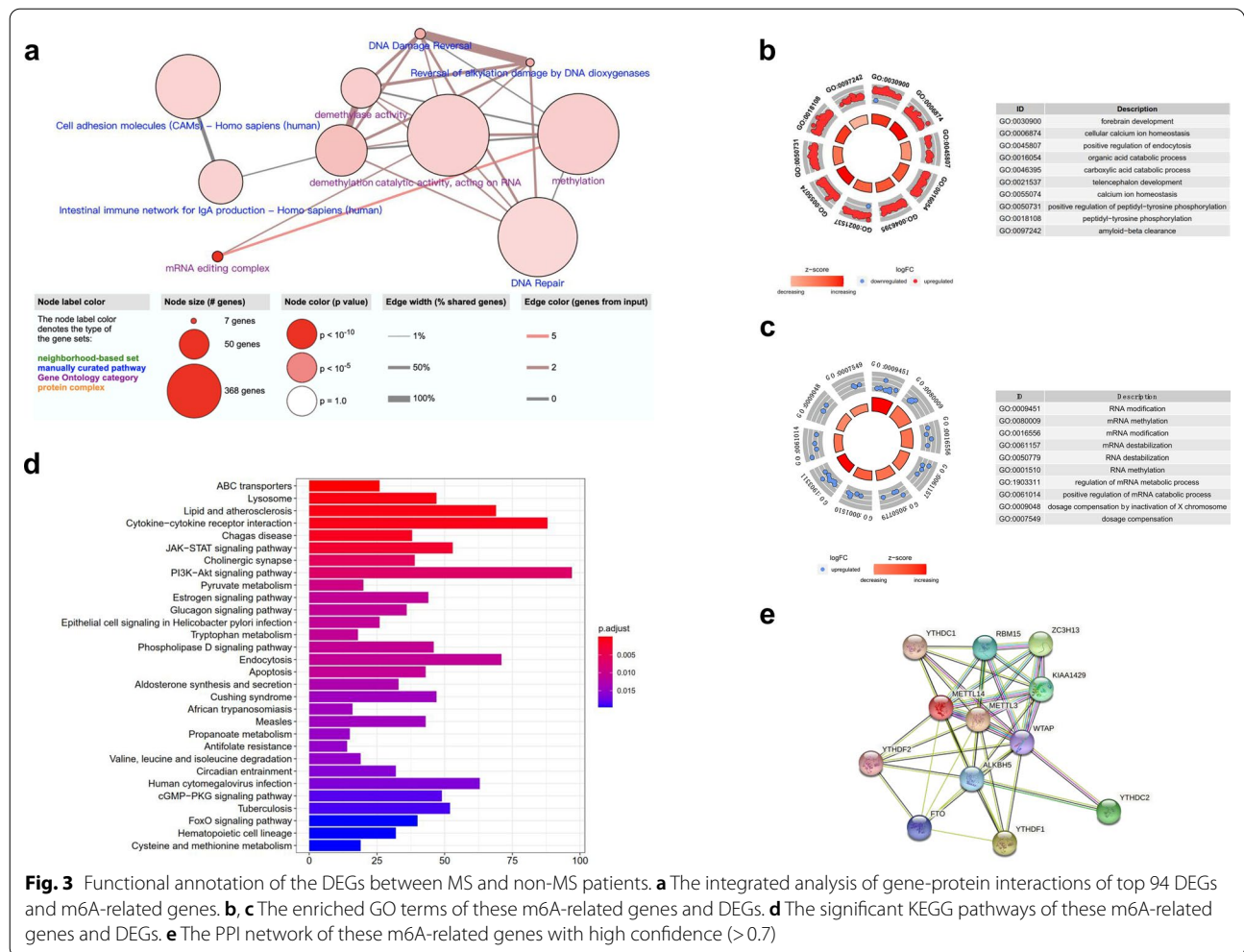


Fig. 3 Functional annotation of the DEGs between MS and non-MS patients. **a** The integrated analysis of gene-protein interactions of top 94 DEGs and m6A-related genes. **b, c** The enriched GO terms of these m6A-related genes and DEGs. **d** The significant KEGG pathways of these m6A-related genes and DEGs. **e** The PPI network of these m6A-related genes with high confidence (>0.7)

m6A-related regulators with high levels of high confidence (Fig. 3e).

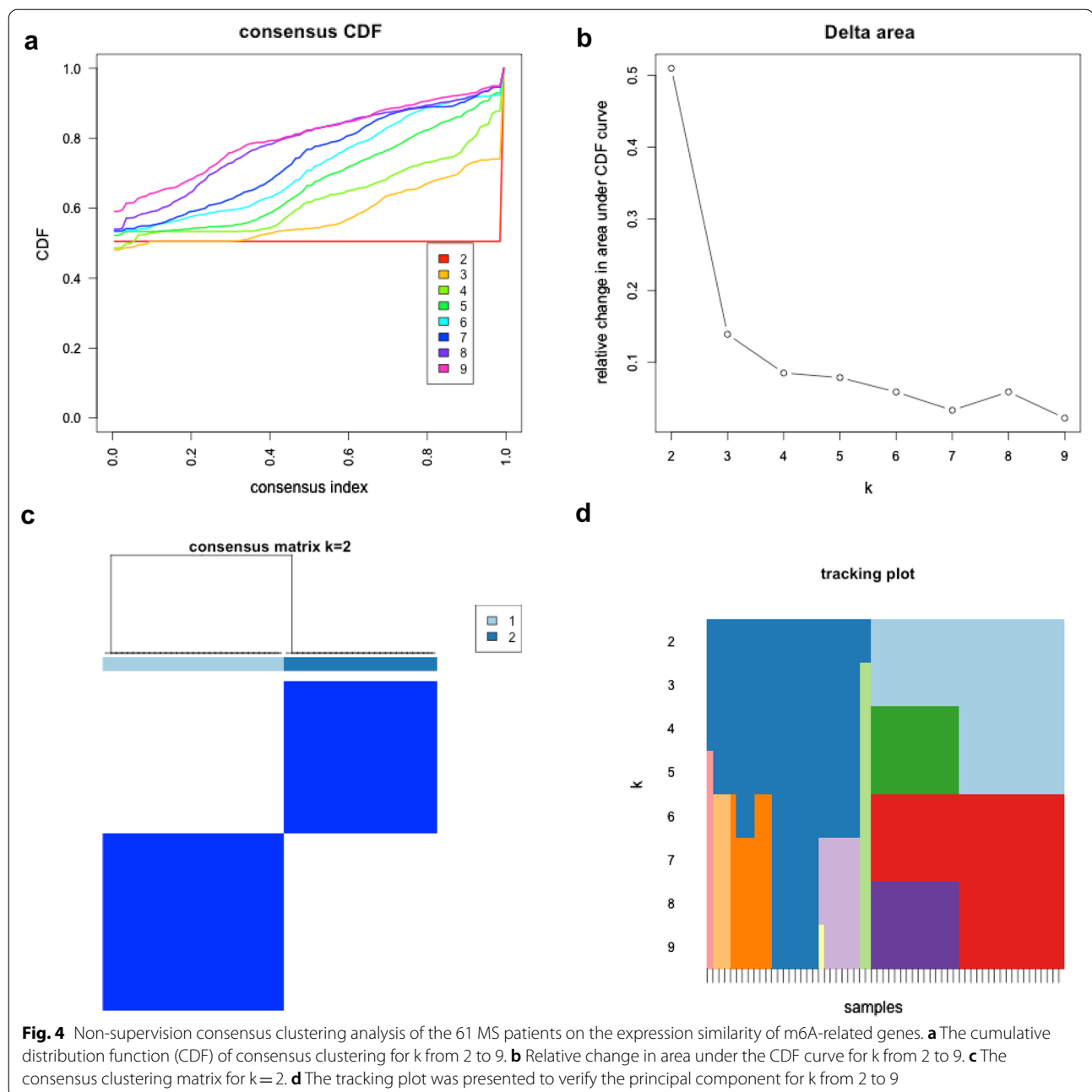
Non-supervision consensus clustering analysis identified two clusters of patients with MS

The total gene expression levels of these 13 m6A RNA methylation regulators were used to classify the 61 MS patients into different clusters on the basis of non-supervision consensus clustering analysis. When the clustering index “k” increased from 2 to 9, k=2 was demonstrated to be the optimal point with which to identify the largest differences and the smallest interferences between clusters (Fig. 4). Consequently, the 61 MS patients were automatically classified into two clusters: cluster 1 and cluster 2. Next, we used a PCA plot was used to verify the effect of classification, as shown in Fig. 5a. A count plot was used to confirm the different quantification of m6A RNA methylation between clusters (Fig. 5b). We also plotted a heatmap to express the differences in gene expression of these 13 m6A-related genes and together

with demographic and clinical characteristics between the clusters (Fig. 5c). Interestingly, all of the patients with PMS were classified into cluster 1 while most of the patients with RRMS were divided into cluster 2; this suggested that the dynamic m6A RNA modification in CSF might be a diagnostic biomarker with which to distinguish PMS from RRMS. Differences in the expression of these m6A-related genes between the PMS and RRMS subgroups were statistically significant, as determined by the Mann-Whitney *U* test (Fig. 5d); there were no statistical differences between the SPMS and PPMS groups (Fig. 5e).

The identification and evaluation of an m6A-related diagnostic gene signature

In this study, we used the Spearman’s correlation analyses to investigate the effect of influence of age and gender on the expression of key genes. There was no significant correlation between gender and any of the m6A-related genes. However, age exhibited a negative



relationship with *ALKBH5*, *HNRNPC*, *KIAA1429*, *METTL14*, *METTL3*, *YTHDC2*, *YTHDF1*, *YTHDF2*, and *WTAP* (Additional file 3: Fig. S1). Indeed, in order to eliminate the effects of this factor, we randomly divided these MS patients into a training set for model development (2/3, $n=41$) and a test set for model validation (1/3, $n=20$). There were no significant differences in terms of demographic and clinical characteristics when compared between the training set (Additional file 2: Table S11) and the test set (Additional

file 2: Table S12), as shown in Table 2. The RF algorithm was applied to the training set to identify key parameters with the nTree set to 1000; this analysis revealed that the error was small and stable after the 400 nTree (Fig. 6a). Then, we calculated and ranked the importance of the 13 m6A-related genes (Fig. 6b). A total of eight feature genes were selected using a cut-off value of 0.4: *KIAA1429*, *WTAP*, *YTHDF1*, *ALKBH5*, *YTHDF2*, *HNRNPC*, *METTL3*, and *YTHDC2*. Subsequently, an m6A-related diagnostic gene signature was constructed

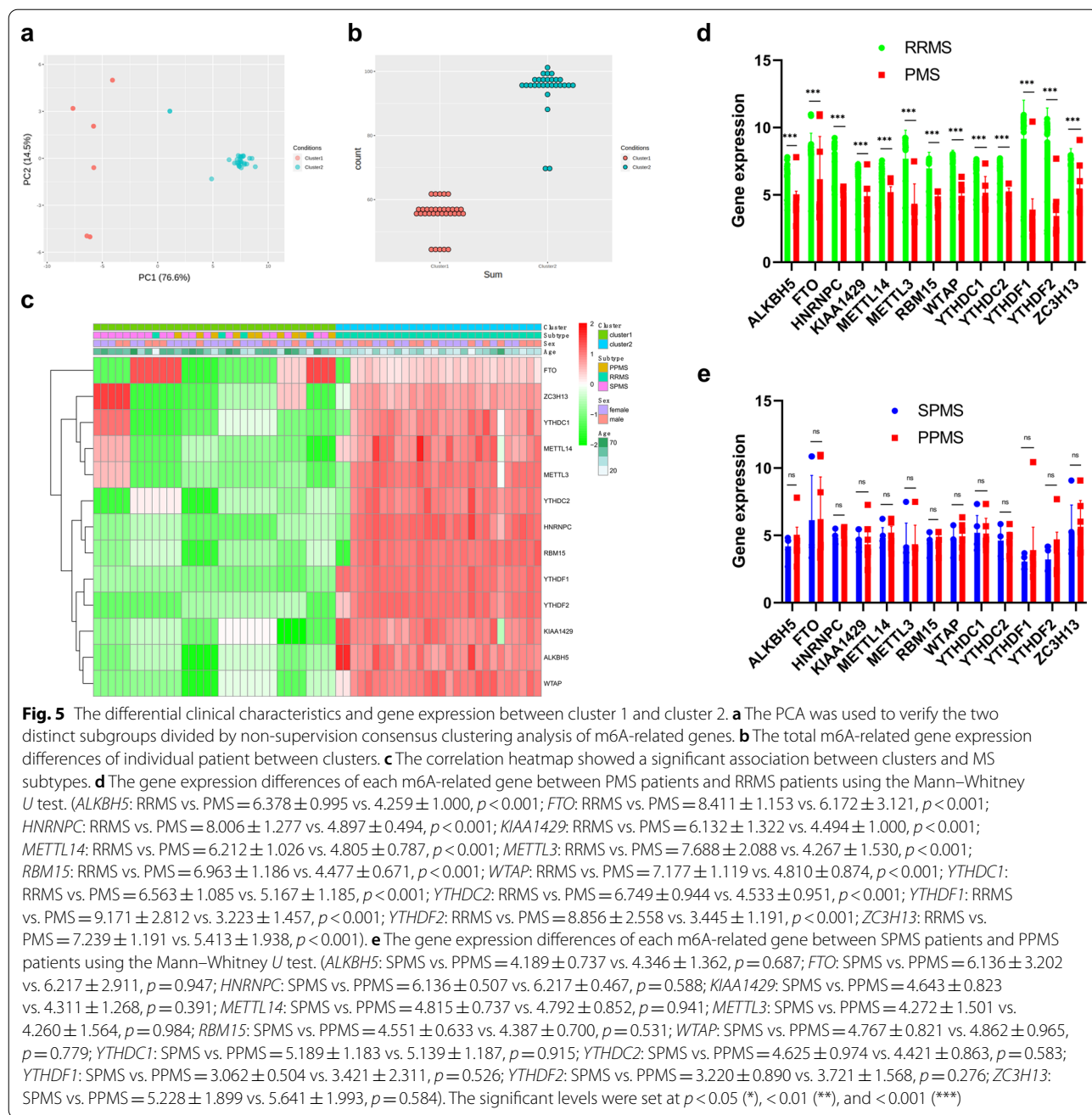
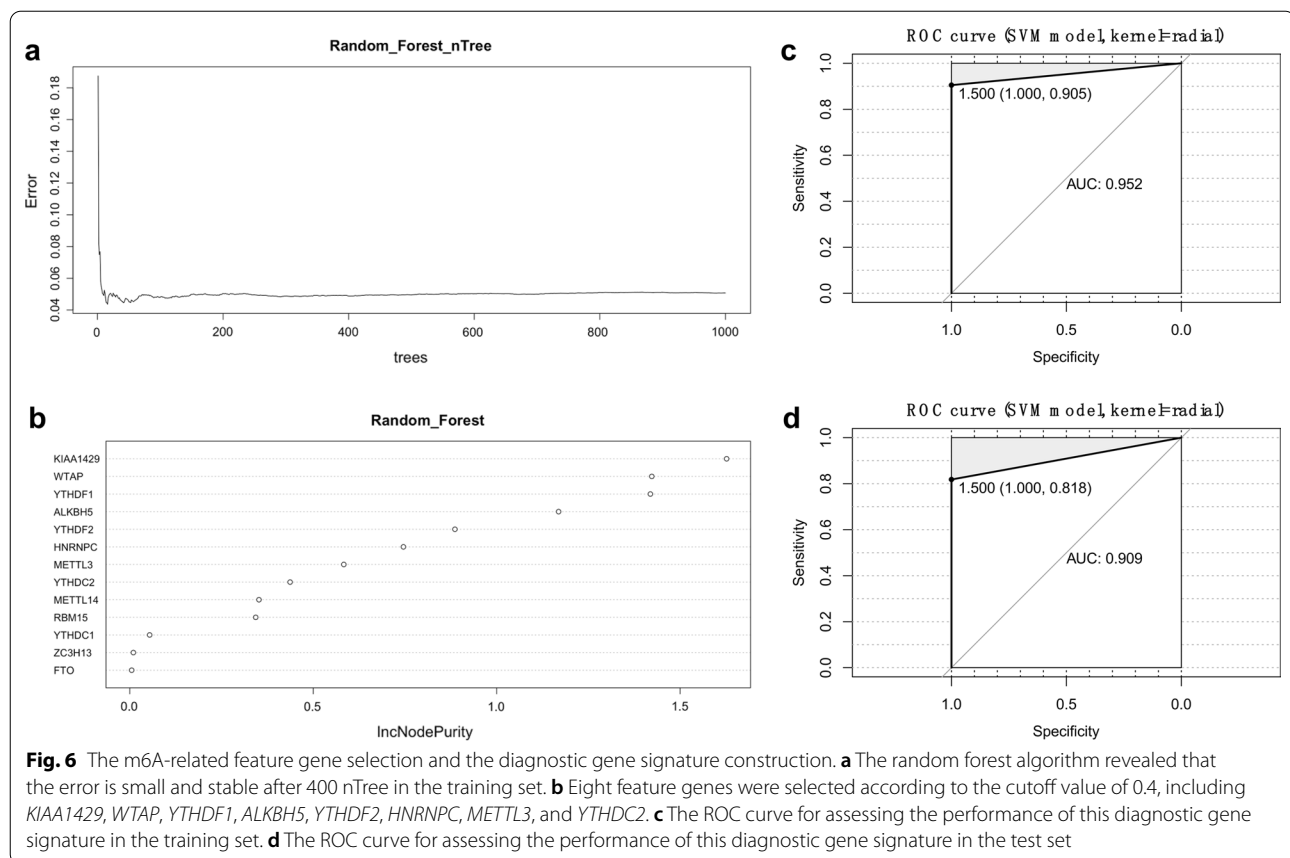


Table 2 Comparison of the demographic and clinical characteristics between training set and test set

	Training (n = 41)	Test (n = 20)	<i>P</i>
Age (years)	45.0 ± 14.5	44.7 ± 18.2	0.954
Female (%)	26 (63.4%)	11 (55%)	0.528
Diagnosis (%)			
RRMS	21 (51.2%)	11 (55.0%)	0.959
SPMS	13 (31.7%)	6 (30.0%)	
PPMS	7 (17.1%)	3 (15.0%)	

in the training set of data; for this, we used the SVM method with a type of *C*-classification and a kernel of radial. Next, we validated the diagnostic model in the test set. ROC curve analysis yielded an AUC of 0.952 in the training set and an AUC of 0.909 in the test set, thus demonstrating that the diagnostic gene signature performed well (Fig. 6c, d). Next, we applied PCA analysis to evaluate the performance of the SVM classifiers based on the correlation-based distances. This diagnostic gene signature was successfully able to distinguish



PMS cases from RRMS in both the training and test sets (Additional file 4: Fig. S2).

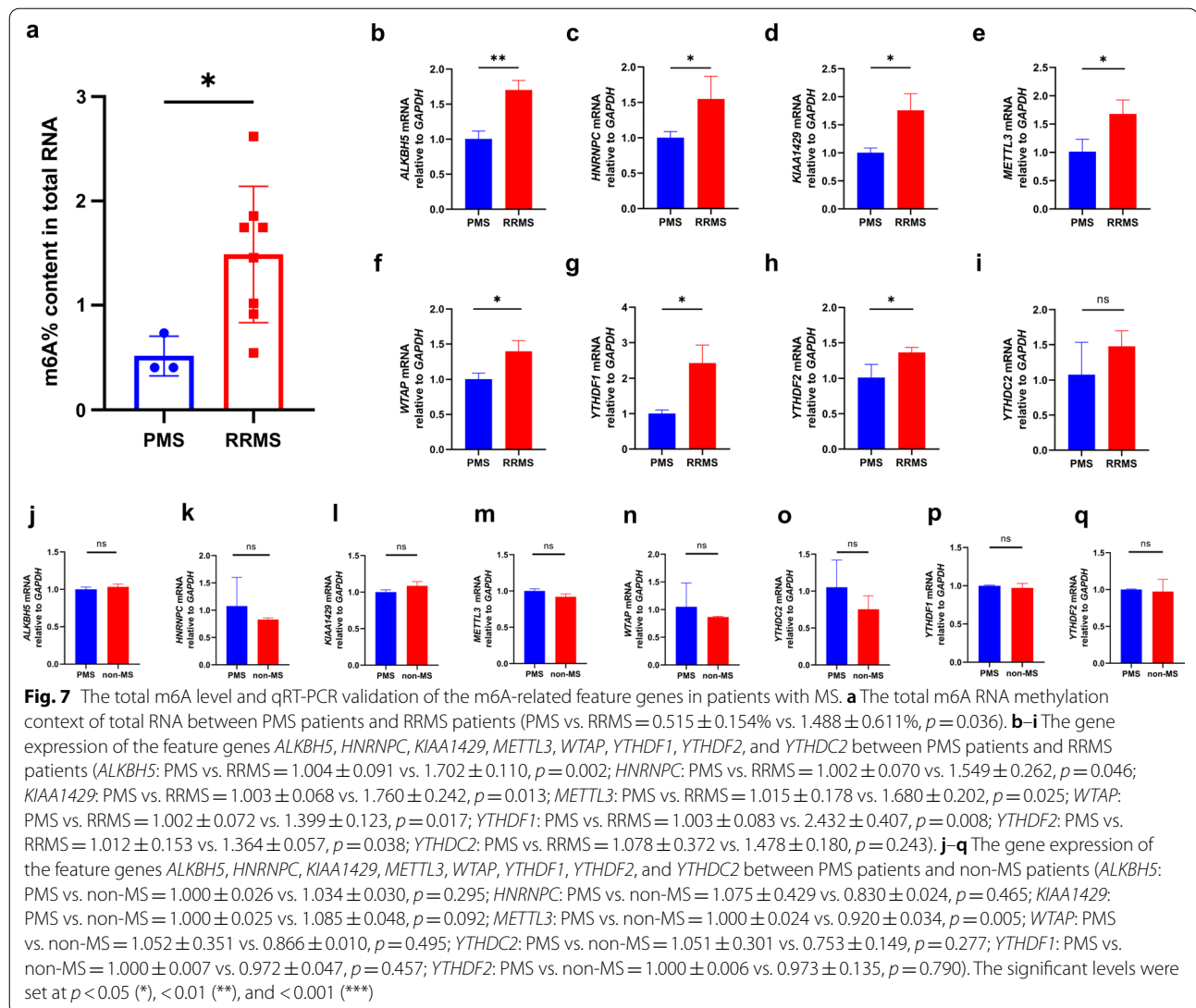
External validation confirmed the performance of the diagnostic model in MS and non-MS patients

A total of 20 patients diagnosed with MS underwent lumbar puncture in our hospital between July 2020 and December 2020. We excluded CSF samples from nine patients because of very low total RNA concentration and/or poor RNA quality. Of the 11 remaining patients, one had PPMS, two had SPMS, and eight had RRMS. The clinical and demographic characteristics of the external validation cohort are presented in Table 1. The quantification of total m6A RNA methylation was evaluated by reading individual RFU data with a fluorescence microplate reader. The proportion (%) of m6A in total RNA was calculated and is presented in Fig. 7a; this data confirmed that the total levels of m6A RNA methylation were relatively lower in patients with PMS than those with RRMS. In addition, seven of the eight feature m6A-related genes were verified by qRT-PCR (Fig. 7b–h). RRMS patients exhibited higher expression levels of *YTHDC2* than those with PMS, although this was not statistically significant (Fig. 7i). Nevertheless, we also recruited three

patients who had been diagnosed with neurodegenerative disorders [including one case of PD and two cases of amyotrophic lateral sclerosis (ALS)] as non-MS controls between May 2021 and June 2021. These feature genes were also tested; however, the expression levels of these genes did not differ significantly when compared between PMS patients and non-MS patients (Fig. 7j–q). In short, this diagnostic model seems to be a potential tool to help to distinguish MS subtypes.

Discussion

In this study, we found that 13 central m6A RNA methylation regulators were all upregulated in the CSF of MS patients when compared with non-MS patients. Non-supervision consensus clustering analysis further identified two clusters of MS samples according to different m6A RNA modification levels; these two clusters were significantly associated with MS subtypes. The RF algorithm and SVM methodology successfully identified an m6A-related diagnostic gene signature. Further evaluation, using both training and test sets, showed that this diagnostic model exhibited well performance. In addition, we also quantified total m6A RNA methylation levels and carried out qRT-PCR to verify these findings in a



small external validation cohort that included 11 patients with MS and 3 non-MS patients.

To our knowledge, this is the first report to describe m6A RNA methylation changes in MS. Previous studies have only demonstrated elevated levels of DNA methylation and the dynamic changes of differentially methylated regions in MS patients; these changes were significantly and positively associated with the expanded disability status scale (EDSS) score and progression index (PI) [17–19]. Previous research demonstrated an association between pathological MS lesions, DNA methyltransferase, and hypermethylated oligodendrocyte survival genes, thus suggesting that changes in methylation could represent a potential target that can accelerate the course of MS disease [20–22]. In contrast, Singhal et al. [23] found that betaine, a methyl donor, played a

neuroprotective role in the cuprizone mouse model of MS by increasing the rate of methylation and by preventing mitochondrial impairment. Consistent with this result, we observed reduced levels of methylation in PMS patients when compared with RRMS patients, thus indicating that elevated methylation levels might provide neuroprotection for patients with MS. A previous study showed that fumaric acid esters exhibit a direct and dose-dependent effect on hypermethylation to protect MS patients from relapse [24]. Another study demonstrated that global methylation levels were negatively correlated with treatment duration in MS patients who were administered with IFN- β , thus suggesting that total methylation levels are a potential and reliable biomarker of the clinical response to DMDs [25]. In addition, cigarette smoking is understood to promote the disease process in

MS patients via DNA methylation [26]. Increased body weight may also alleviate the course of disease by regulating ceramide-induced anti-proliferative gene methylation to modulate the infiltration of monocytes [27]. Consequently, these previous studies have highlighted the possible importance of methylation modification in the pathogenesis of MS.

In this study, we developed an m6A-related diagnostic gene signature that would allow us to distinguish PMS from RRMS; most of these feature genes were m6A readers and writers (with relatively lower gene expression levels in PMS). These m6A readers and writers have previously been reported to act as key epigenetic factors in neurodevelopment, synaptogenesis, axon guidance, and neural repair [28]. Knockout of the m6A reader *METTL3* is known to inhibit neural proliferation and maturation while loss of the m6A reader *METTL14* is known to reduce the regeneration of functional axons [29, 30]. Mutation of the m6A reader *YTHDF1* is known to delay pre-crossing axonal guidance by influencing the expression levels of *Robo3.1* mRNA [31]. In addition, recent studies have demonstrated that the dysregulation of RNA methylation is associated with multiple biological processes in neurodegenerative diseases. For example, *METTL3* was shown to be upregulated in brain tissues and positively correlated with the concentration of Tau protein [8, 28]. Levels of the m6A eraser *FTO* were shown to be significantly reduced in AD patients while risky genetic variations were correlated with approximately 8% and 12% of brain volume deficits in the frontal and occipital lobes of patients with AD, respectively [29, 30]. In addition, Hess et al. [31] found that the inactivation of *FTO* had a negative impact on the dopamine receptors in a mouse model of PD, thus leading to a reduction in quinpirole-mediated motion function and increased levels of adenosine methylation in the *FTO*-deficient mice, thus indicating that m6A-related genes regulated the RNA methylation of hub genes to control the dopamine transmission in PD [31]. Collectively, these studies provided reliable evidence to prove that alterations in m6A RNA methylation are highly associated with neurodegenerative disorders. However, in contrast to the scenario observed for AD and PD, PMS patients exhibited methylation levels that were lower than those with RRMS. Inflammatory and demyelination are known to cause several pathological hallmarks, including axonal loss, gray matter pathology, and immune cell infiltrations in RRMS patients; however, PMS patients do not exhibit an obviously active immunization status. Current evidences proves that the methylation of m6A RNA is highly associated with immune recognition, the activation of innate and adaptive immune responses, and cell fate decisions [32]. Thus, the extent of total m6A RNA methylation

was relatively higher in patients with RRMS. In addition, because of the ethical considerations related to the use of lumbar puncture on healthy controls, we were only able to obtain control CSF samples from patients with neurodegenerative disorders. Consequently, there were no obvious differences between controls and PMS patients in the external validation set (which only featured a small number of samples), thus suggesting that the expression levels of m6A-related genes were similar.

PMS is an uncommon and severe subtype of MS that leads to a gradual decline and irreversible disabilities without appropriate treatment. It is important to diagnose PMS at disease onset; however, this represents a significant challenge. Current diagnostic criteria can usually extend the course of disease, as determined by retrospective history. Neurofilament light chain (NfL) was previously reported to be a reliable biomarker with which to diagnose RRMS [33]. However, levels of NfL are also known to be increased in a variety of neurological diseases associated with axonal injury or degeneration, including inflammatory, neurodegenerative, traumatic, and cerebrovascular diseases [34]. Therefore, NfL cannot be used for the specific diagnosis of RRMS. In contrast, several studies have reported significant differences in NfL levels when compared between PMS and RRMS; thus, NfL may have the potential to predict conversion from RRMS to SPMS [35, 36]. Although NfL may exhibit diagnostic and prognostic value for PMS, this hypothesis should now be tested with a large sample size [37–39]. In addition, the integrated analysis of 11 radiomics, metabolomics, and proteomic characteristics was shown to lead to an earlier diagnosis of SPMS in a previous limited cohort of patients, although the high cost and stringent conditions required rendered this analysis difficult to apply for PPMS [40]. Presently, we know very little about the true diagnostic value of biological biomarkers for PMS; as such, we do not yet have an efficient tool with which to specifically diagnose PMS. Therefore, personalized therapeutic advice for preventing neurological deterioration in patients with PMS is not yet evidence-based. In the present study, we identified possible diagnostic biomarkers for PMS from CSF samples based on m6A regulatory genes. Validation tests demonstrated that this gene signature showed good performance for distinguishing between PMS and RRMS.

Our study has some limitations that should be considered. First, the complete clinical characteristics of MS patients were not available in the original datasets; PMS patients usually have a higher EDSS, and a shorter disease duration, than RRMS patients. Furthermore, DMDs have been shown to be beneficial for prolonged transitional disease duration in SPMS [41]. Thus, future research should consider the precise relationships between these

observations and the expression of m6A-related genes. Second, this m6A-related gene signature was only verified in a small cohort. A randomized control study, with a larger sample size, should now be conducted to validate our findings. Third, lumbar puncture is an invasive assessment for MS patients. Consequently, future research should investigate the expression of these biomarkers in whole and/or peripheral blood mono-nucleic cells (PBMCs). Finally, the therapeutic effect of DMDs on the methylation of m6A RNA methylation should be assessed in order to identify effective targets.

Conclusions

In conclusion, this preliminary study suggested that the dynamic modification of m6A RNA methylation is involved in the progression of MS and is likely to represent a novel CSF diagnostic biomarker for distinguishing PMS from RRMS at early disease onset.

Abbreviations

MS: Multiple sclerosis; CNS: Central nervous system; RRMS: Relapsing–remitting multiple sclerosis; SPMS: Secondary progressive multiple sclerosis; PPMS: Primary progressive multiple sclerosis; PMS: Progressive multiple sclerosis; CSF: Cerebrospinal fluid; DMDs: Disease modifying drugs; m6A: N⁶-Methyladenosine; AD: Alzheimer’s disease; PD: Parkinson’s disease; OB: Oligoclonal bands; GEO: Gene Expression Omnibus; RMA: Robust multi-array average; DEGs: Differentially expressed genes; eBayes: Empirical Bayes; FC: Fold change; FDR: False discovery rate; GO: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; PPI: Protein–protein interaction; STRING: Search Tool for the Retrieval of Interacting Genes; PCA: Principal component analysis; RF: Random forest; SVM: Support vector machine; ROC: Receiver operating characteristic; RT: Room temperature; RFU: Relative fluorescence units; PCR: Polymerase chain reaction; qRT-PCR: Quantitative real-time PCR; ALS: Amyotrophic lateral sclerosis; EDSS: Expanded disability status scale; PI: Progression index; Nfl: Neurofilament light chain; CDF: Cumulative distribution function; PBMCs: Peripheral blood mono-nucleic cells.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12967-021-02981-5>.

Additional file 1: Table S1. The primers of the 13 m6A-related genes. **Table S2.** The original gene expression matrix of E-MTAB-69 from ArrayExpress database. **Table S3.** The original gene expression matrix of E-MTAB-2374 from ArrayExpress database.

Additional file 2: Table S4. The merged gene expression matrix from E-MTAB-69 and E-MTAB-2374 via batch-normalization. **Table S5.** The 13 m6A RNA methylation regulators in the merged data set. **Table S6.** The differentially expressed analysis of the 13 m6A RNA methylation regulators between MS patients and non-MS controls. **Table S7.** Identification of differentially expressed genes (DEGs) between MS patients and non-MS controls. **Table S8.** The gene ontology (GO) analysis of the DEGs. **Table S9.** The gene ontology (GO) analysis of the 13 m6A RNA methylation regulators. **Table S10.** The Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways of the DEGs. **Table S11.** The clinical characteristic in the training set (n = 41).

Additional file 3: Figure S1. Correlation analysis of the relationships between different m6A-related genes and demographic characteristics (age or sex).

Additional file 4: Figure S2. The PCA analysis was applied to evaluation of the performance of SVM classifiers based on the correlation-based distances in the training set and in the test set.

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Authors’ contributions

FY designed and performed the study, contributed to the discussion, and wrote the manuscript. TW analyzed the data, contributed to the discussion, and wrote the manuscript. XW collected study samples, performed the study, and analyzed the data. JLi and JLi contributed to the discussion. WS designed the study and revised the manuscript. All authors read and approved the final manuscript.

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Availability of data and materials

The datasets in this study can be found in the ArrayExpress database.

Declarations

Ethics approval and consent to participate

This study was approved by the Independent Ethics Committee of the First Affiliated Hospital of Sun Yat-sen University, and all patients signed an informed consent form.

Consent for publication

All authors gave their consent for publication.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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