Original Article

Methylation factors as biomarkers of fibromyalgia

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Background: Fibromyalgia (FM) is a common and intractable chronic musculoskeletal pain syndrome, but its exact underlying mechanisms are unknown. This study sought to identify biomarkers of FM and the underlying molecular mechanisms of the disease.

Methods: FM-related gene expression profiles (GSE67311) and methylation profiles (GSE85506) were obtained from the Gene Expression Omnibus database, and a differential expression analysis was performed to identify the methylation factors. Subsequently, an enrichment analysis and gene set enrichment analysis (GSEA) were conducted to examine the methylation factors. In addition, the transcriptional regulators of the methylation factors were predicted, and key methylation factors were identified by a receiver operating characteristic curve analysis and nomogram models. Finally, the relationship between FM and cell death (pyroptosis, necroptosis, and cuproptosis) was assessed by a GSEA and gene set variation analysis.

Results: A total of 455 methylation factors were identified. The enrichment analysis and GSEA results showed that methylation factors were clearly involved in the biological functions and signaling pathways related to neural, immune inflammation, and pain responses. The transcriptional regulator specificity protein 1 (SP1) may have a broad regulatory role. Finally, seven key methylation factors were identified, of which amino beta (A4) precursor protein binding family B member 2 (APBB2), A-kinase anchor protein 12 (AKAP12), and cluster of differentiation 38 (CD38) had strong clinical diagnostic power. In addition, AKAP12 and CD38 were significantly and negatively associated with sepsis, necrotizing sepsis, and cupular sepsis.

Conclusions: Our study suggests that FM is associated with deoxyribonucleic acid methylation. The methylation factors APBB2, AKAP12, and CD38 may be potential biomarkers and should be further examined to provide a new biological framework of the possible disease mechanisms underlying FM.

Keywords: Fibromyalgia (FM); immune; cell death; AKAP12; CD38

Introduction

Fibromyalgia (FM) exists in 2–8% of the population, and is characterized by extensive pain, which is often accompanied by fatigue, memory problems, and sleep disorders (1,2). Although FM has been excluded from the diagnosis of neuropathic pain since the revision of the International Association for the Study of Pain (IASP) definition of

neuropathic pain in 2011, recent studies have found that about 50% of FM patients have neuropathic changes of small and/or large fibers (3,4). One review reported an average prevalence of 2.7% (0.4–9.3%) worldwide, 3.1% in the Americas, 2.5% in Europe, and 1.7% in Asia (5). FM is a complex multisymptomatic long-term disease that places a heavy burden on healthcare systems around the world (6). FM is associated with billions of dollars of annual healthcare spending and a work disability rate of nearly 56% (7). FM lacks relevant specific laboratory tests and other ancillary tests, all tests and analyses will return normal results, and it has no known biomarkers (8). The etiology of FM remains unknown, and its diagnosis is clinical and not based on objective testing (9). Thus, little is known about how it can be effectively diagnosed and how clinicians and patients can use and understand biomarkers.

There are several factors related to the pathophysiology of FM, including central sensitization, the response of the hypothalamus pituitary adrenal axis to pressure, the promotion of inflammation, the reduction of anti-inflammatory cytokines, and interference with neurotransmitters, but the specific mechanism leading to FM is not yet clear (10).

Deoxyribonucleic acid (DNA) methylation mainly occurs in cytosine-C5 in the context of the cytosine-phosphate-guanosine (CpG) dinucleotide, which plays an important role in transcriptional regulation and genome stability (11). DNA methylation has been found in patients with FM (12). Related studies have shown that gene-environment interactions may be the trigger mechanism for FM: in particular, FM appears to be characterised by a pattern of DNA hypomethylation involving genes for stress responses, DNA repair, autonomic nervous system responses and subcortical neuronal abnormalities (13). In previous studies, DNA methylation has been found to be a biomarker for many diseases (14-16), while in FM, DNA methylation changes may reflect the role of immune-inflammatory responses and central sensitization (17). Therefore, the relationship between DNA methylation and FM needs to be further studied, which may help to evaluate and/or diagnose FM.

This study sought to explore the differential methylation factors between FM patients and control subjects, and identify the biological processes (BPs) involved in the differential methylation factors. By determining the epigenetic factors that form the pathophysiological basis of FM, we identified new indexes for the objective diagnosis of FM. We present the following article in accordance with the TRIPOD reporting checklist (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6631/rc).

Methods

Data sources

The data used in this study were downloaded from the Gene Expression Omnibus (GEO) database. The gene expression data of FM patients were obtained from the GSE67311 data set, which included 67 disease subjects and 75 control subjects. The methylation data were obtained from the GSE85506 data set, which included 24 disease subjects and 23 control subjects. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

Differential expression and methylation analyses

The raw data from the microarray data sets were normalized and log2-transformed. The differentially expressed genes (DEGs) from the GSE67311 data set were calculated with limma package (18). A P value <0.05 was set at the threshold. The differential methylation sites of the GSE85506 data set were calculated using the cAMP package (19). Subsequently, genes differentially expressed in contrast to methylation are used as methylation factors.

Highlight box

Key findings
- Fibromyalgia (FM) may be related to deoxyribonucleic acid methylation, and methylation factors, such as amino beta (A4) precursor protein binding family B member 2 (APBB2), A-kinase anchor protein 12 (AKAP12), and cluster of differentiation 38 (CD38), may be its potential biomarkers.

What is known and what is new?
- AKAP12 can be used as a diagnostic marker of FM, and CD38 and GATA binding protein 2 (GATA2) are also closely related to FM.
- Methylation factors amyloid beta precursor protein binding family b member 2 (APBB2), islet cell autoantigen 1 like (ICA1L), integrin subunit beta 8 (ITGB8) and kinesin light chain 3 (KLC3) may be potential biomarkers of FM.

What is the implication, and what should change now?
- This suggests that further exploration of methylation factors, such as APBB2, ICA1L, ITGB8 and KLC3, is needed to determine whether they can be used as biomarkers of FM.

Functional enrichment analyses

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses of the methylation factors were conducted using clusterProfiler package (20). A gene set enrichment analysis (GSEA) (21) of the methylation factors was carried out with GSEA software. A P value <0.05 was considered statistically significant.

Prediction of TFs

First, all the transcription factors (TFs) that regulate the methylation factors were obtained from TRRUST version 2 (22) human TF target data. The TFs with P values <0.05 according to the hypergeometric tests were used as the transcriptional regulators. All corresponding regulatory relationships in National Center for Biotechnology Information (NCBI) were then screened.

Receiver operating characteristic (ROC) curve analysis

The area under the curve (AUC) values of all the methylation factors were calculated and ROC curves were drawn. Genes with AUC values >0.7 were considered key methylation factors. In addition, the rms package was used to further construct the nomogram and calibration curve to evaluate the ability of key methylation factors to predict FM risk.

Assessment of cell deaths

A GSEA was performed using the clusterProfiler package to assess FM-related cell death (pyroptosis, necroptosis, and cuproptosis). Cell death was quantified by a gene set variation analysis (23). Pearson's correlation coefficients were then used to assess the correlation between genes and cell death, and P value <0.05 was considered statistically significant.

Statistical analysis

For all bioinformatics analysis, P value <0.05 was considered statistically significant.

Results

FM-related methylation factors

To identify the disordered molecules related to FM, we analyzed the DEGs in the GSE67311 data set. A total of 1,787 DEGs were found, which included both upregulated and downregulated genes (*Figure 1A*). To further identify the methylation factors, the different methylation sites of GSE85506 were analyzed. A total of 9,015 methylation genes were identified from the 19,982 methylation sites (*Figure 1B*). After examining the relationship between methylation modification and gene expression, 455 methylation factors were identified (*Figure 1C*). These methylation factors may play an important role in FM.

Biological effects of methylation factors

Changes in the methylation spectrum between FM patients and normal control subjects may reveal important biological functions. The enrichment analysis showed that methylation factors were significantly involved in 599 BPs, 76 cell components (CCs), 151 molecular functions (MFs), and 9 KEGG signaling pathways. The statistical analysis showed that methylation factors are mainly involved in the regulation of neuron project development, the regulation of small molecular metallic process, and other biological functions (*Figure 2A-2C*). Moreover, methylation factors are mainly involved in the immune inflammation and metabolism related KEGG pathway (*Figure 2D*). In addition, the GSEA identified four KEGG signalling pathways involving the methylation factors, including the neural system and pain response–related pathways (*Figure 2E*).

TFs regulate methylation factors

To identify the methylated integrated regulatory network of FM, we predicted the regulatory TFs of the methylated factors. After filtering the regulatory relationships reported in NCBI, a total of 18 TFs were found to regulate the methylation factors (*Figure 3A*). Among these, specificity protein 1 (SP1) and signal transducer and activator of transcription 3 (STAT3) regulate most of the methylation factors. In addition, we found a strong coupling relationship between TFs, particularly between SP1 and metastasis associated 1 (MTA1), suggesting that these two genes may play a synergistic regulatory role in the induction of FM (*Figure 3B*).

Identify key methylation factors

To identify the methylation factors with a clinical significance, the AUC values were calculated. The AUC
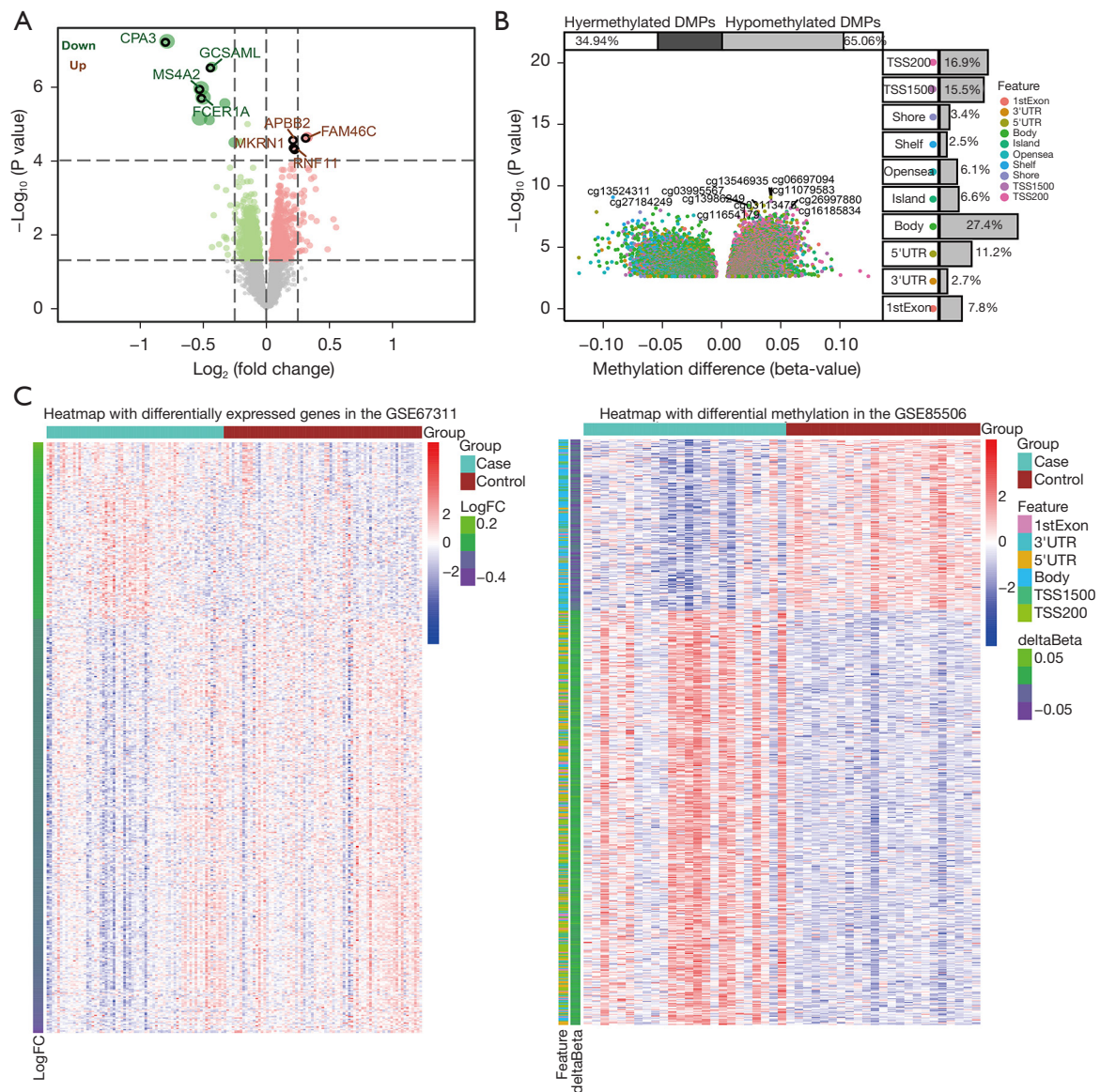
Figure 1 Methylation factors associated with FM. (A) The DEGs of FM in GSE67311. (B) The differential methylation sites of FM in GSE85506. (C) Expression and methylation of methylation factors in FM. DMP, differential methylation sites; UTR, untranslated regions; FM, fibromyalgia; DEG, differentially expressed gene; FC, fold change.

values of 7 methylated genes [i.e., A-kinase anchor protein 12 (AKAP12), amino beta (A4) precursor protein binding family B member 2 (APBB2), cluster of differentiation 38 (CD38), GATA binding protein 2 (GATA2), islet cell autoantigen 1 like (ICA1L), integrin subunit beta 8 (ITGB8), and kinesin light chain 3 (KLC3)] were >0.7, and thus have high clinical diagnostic ability (*Figure 4A*). We also observed the expression of these genes in the GSE67311 data set (*Figure 4B*). The disease risk prediction

ability of the 7 methylation factors was further evaluated by a nomogram (*Figure 4C*). Among them, APBB2, AKAP12 and CD38 showed better predictive power compared to other methylation factors and could be further investigated as potential biomarkers for FM. The calibration chart showed that the nomogram performed well compared to the ideal model (*Figure 4D*). The GO functions of APBB2 and AKAP12 are very similar, suggesting that they may be involved in similar BPs in affecting FM (*Figure 4E*).
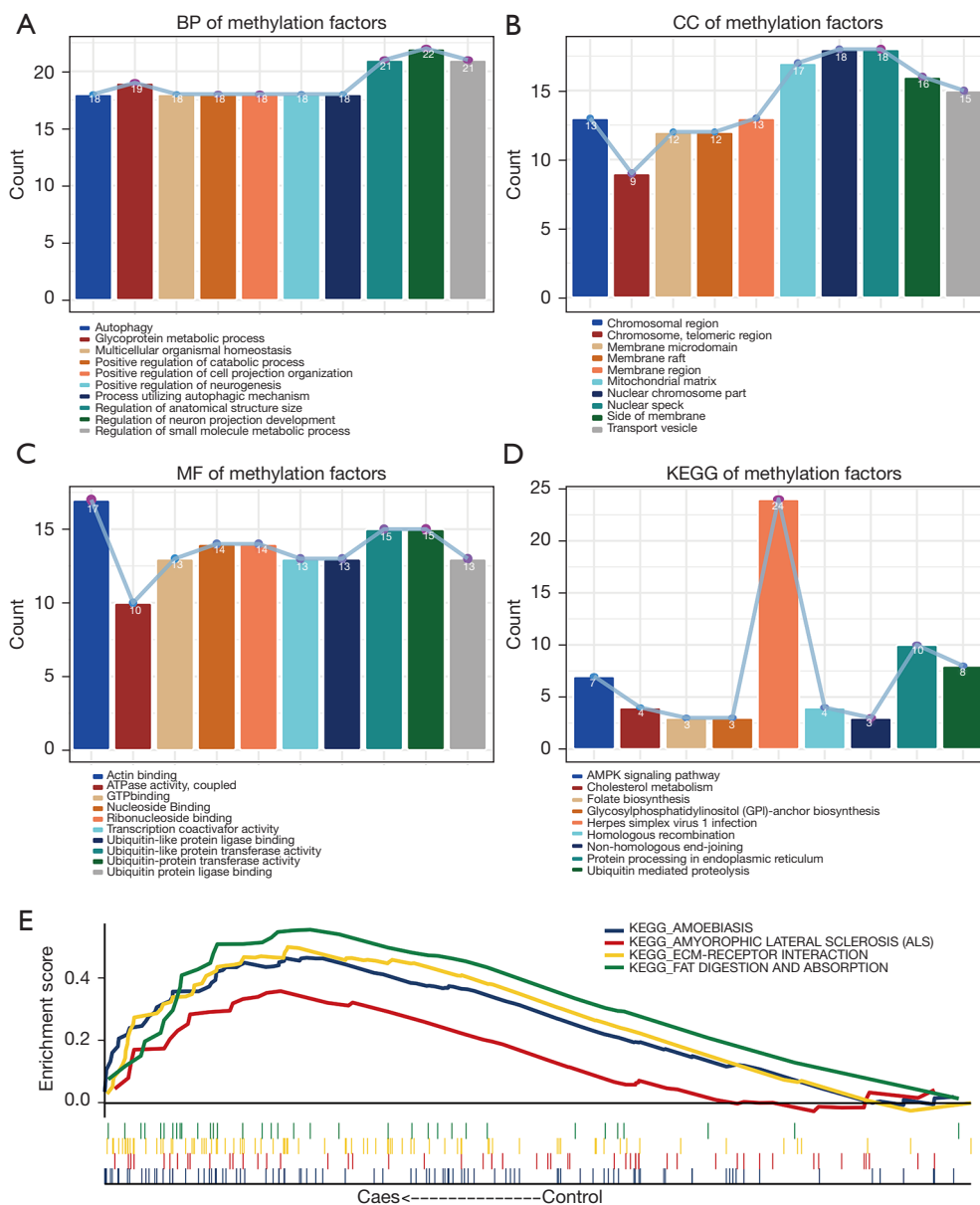
Figure 2 The methylation factors involved in the GO functions and KEGG pathways. (A-C) The BPs (A), CCs (B), and MFs (C) significantly enriched by the methylation factors. (D) The KEGG pathway significantly enriched by the methylation factors. (E) The KEGG pathway enriched by the methylation factors according to the results of the GSEA. BP, biological process; CC, cell component; MF, molecular function; KEGG, Kyoto Encyclopedia of Genes and Genome; GO, Gene Ontology.

Cell deaths influenced by key methylation factors

To evaluate whether cell death is present in FM patients, we performed a GSEA. The results showed that pyroptosis, necroptosis, and cuproptosis were significantly inhibited in FM (*Figure 5A*). The correlation results revealed a significant negative correlation between AKAP12 and all

3 types of cell death, and a significant negative correlation between CD38 and cell death (*Figure 5B*).

Discussion

In this study, we used bioinformatics methods and identified a set of potential molecular diagnostic markers

Figure 3 Transcription regulatory network of methylation factors. (A) The network of TFs that regulate the methylation factors. The round nodes represent the target genes, the nuclei represent the methylation level, and the edges represents the expression levels. Blue represents down, red represents up. (B) There was a coupling relationship between the TFs. ns, not significant; FDR, false discovery rate; TF, transcription factor.

for FM. Currently, the diagnosis of FM is based solely on a comprehensive clinical assessment and no validated biomarkers associated with FM have been identified. Therefore, the FM biomarkers reported here are of great clinical significance. In particular, our study showed that the 7 differential methylation factors in the peripheral blood of the FM patients had good clinical diagnostic ability. Notably, transcriptomic studies have shown that between 35% and 80% of known transcripts are present in brain and blood tissue samples (24), and therefore blood samples can be a reliable and easy source of FM biomarkers. Some studies had measured the excitability parameters of the cerebral cortex in FM patients and control subjects and found that the results were parallel to the changes in the peripheral blood methylation levels in FM patients (12). These findings highlight the importance of peripheral blood DNA methylation in future FM biomarker studies.

In the whole life process, the expression of genes changes are partly affected by epigenetic mechanisms, such as DNA methylation (25). Among the seven key methylation factors we identified, the *APBB2* gene is known to be related to Alzheimer's disease, which affects the cognitive ability of patients (26,27). In this study, APBB 2 had low methylation and high expression, which may be related to the memory and sleep problems of FM patients. AKAP12 is a new and effective scaffold protein, which plays an important role in protein kinase C, protein kinase A, cyclin, F-actin, and other

key signal factors (28). *AKAP12* gene knockout has been shown to promote the infiltration of inflammatory cells, the production of reactive oxygen species, and autophagy (29). The low expression of AKAP12 in FM patients may lead to the increase of pro-inflammatory cytokines, which was related to the pathophysiology of FM (30). Moreover, CD 38 is also considered a candidate gene for FM (31). The absence of CD 38 will lead to the development of astrocytes and oligodendrocytes in mice and affect the function of the central nervous system (32).

In addition, by predicting transcription regulators, we obtained the key regulator SP1 and STAT3. SP1 can regulate gene expression positively and negatively by binding to CpG rich sites, and is affected by the methylation status of these regions (33). STAT3 plays a key role in inflammation and immune control and mutations in it have been associated with diseases such as immunodeficiency, autoimmunity and cancer (34). It is suggested that SP1 and STAT3 may participate in the KEGG pathway related to immune inflammation and metabolism by regulating methylation factors, thus affecting the development of FM.

The destruction of the epigenetic mechanism is related to various immune, neurological, and endocrine diseases (35). In this study, the enrichment analysis of the methylated factors revealed more biological functions and signaling pathways related to the nerves, immune inflammation, and metabolism. Previous studies had shown
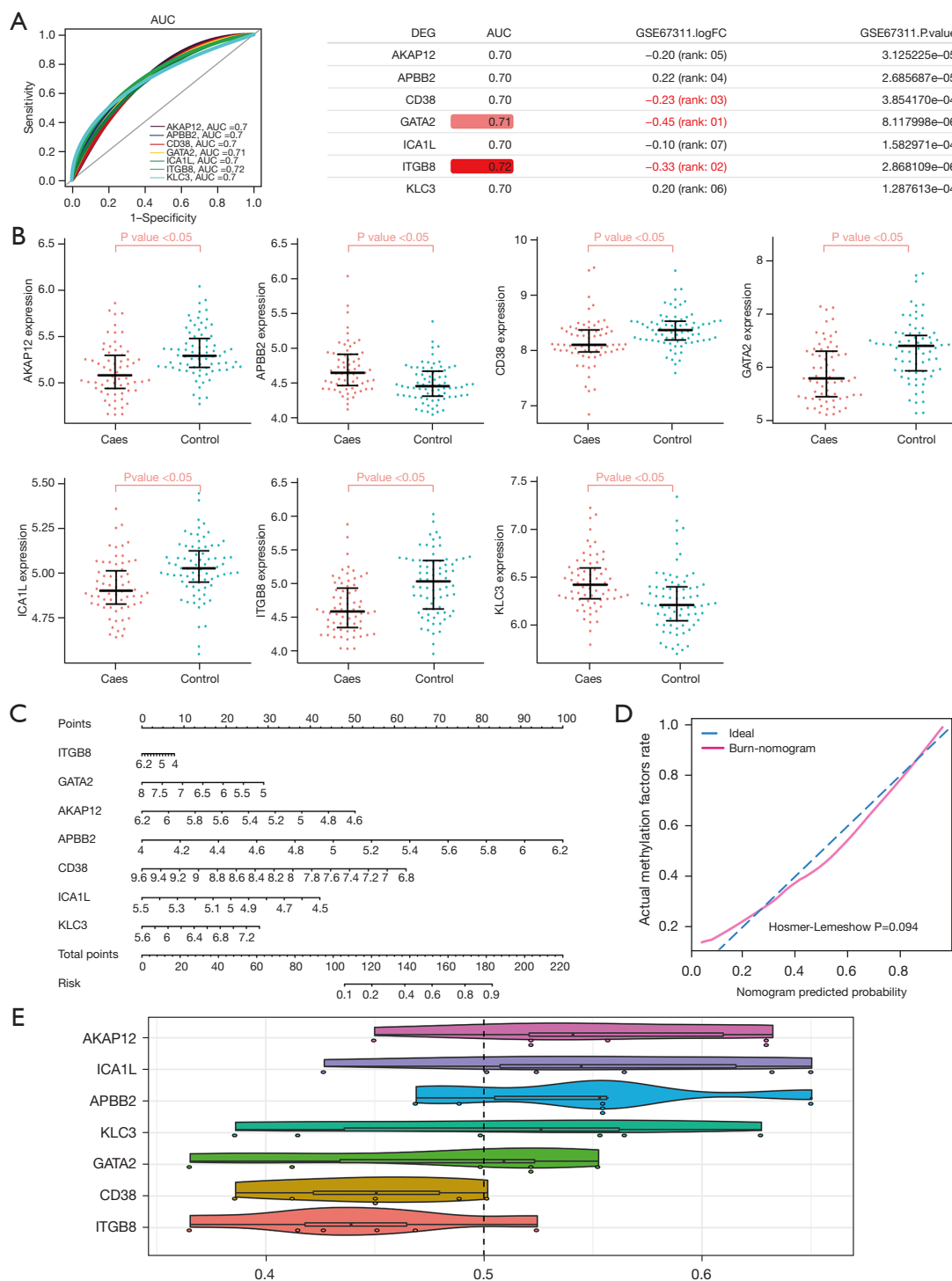
Figure 4 Identifying the marker methylation factors for FM. (A) ROC curves and AUC values of methylation factors with AUC values >0.7. (B) Expression values of key methylation factors in GSE67311. (C) Nomogram of key methylation factors to predict the risk of FM. (D) Plots depicting the calibration of each model in terms of agreement between the predicted and observed risk of FM. Model performance is shown by the plot, relative to the 45-degree line, which represents perfect prediction. (E) Cloud rain map showing the GO functional similarity of key methylation factors. AUC, area under the curve; DEG, differentially expressed gene; FM, fibromyalgia; ROC, receiver operating characteristic; GO, Gene Ontology.
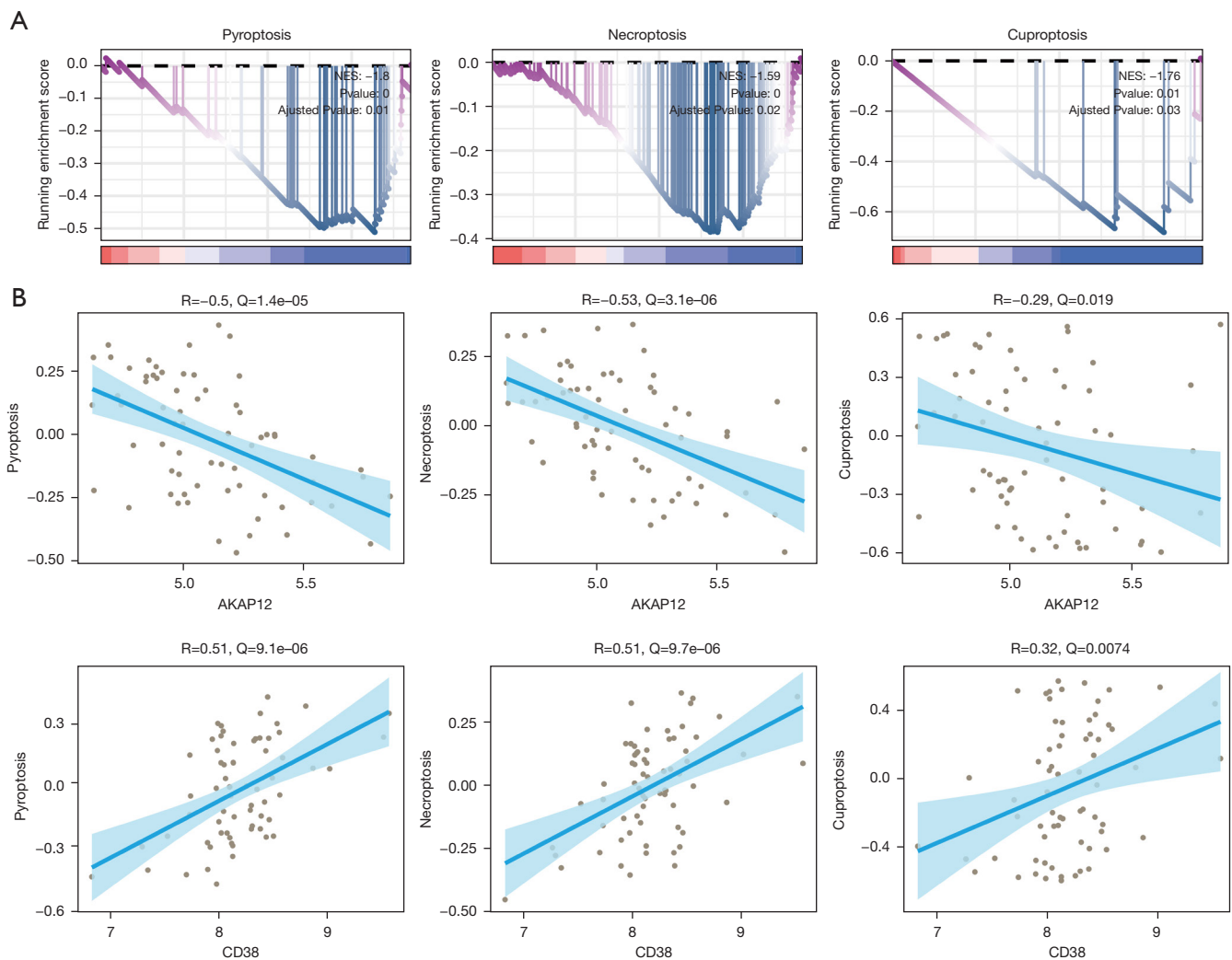
Figure 5 Cell deaths in FM. (A) GSEA evaluates cell deaths that were significantly altered in the disease. (B) Correlations between significantly altered cell deaths and key methylation factors. FM, fibromyalgia; GSEA, gene set enrichment analysis.

that the central nervous system may play an important role in the occurrence and maintenance of FM (36). In FM patients, pain is not only related to noxious stimulation, but is also related to central pain management abnormality (37). The pathophysiology of FM is not clear, but it may involve the immunoinflammatory pathway (38). Metabonomic results have shown that the levels of arginine and ornithine in patients with FM are increased (39). Interlukin-1β triggers NLR family pyrin domain containing 3 (NLRP3) inflammasome activation, inducing inflammatory cell death, which in turn participates in chronic pain responses (40). These results suggested that the differential methylation of genes play an important role in the development and

maintenance of the nervous system, immune inflammation and pain response, and may be an important factor in FM.

This study provided a new model for understanding the dynamic interactions among the stochastic, environmental, genetic, and epigenetic factors that influence pain perception and expression in FM patients by examining the patterns of DNA methylation modifications that regulate gene expression profiles. Our results may serve as a starting point for further large-scale and independent population-based research on DNA methylation as a possible disease mechanism for FM. Moreover, an understanding of the key methylation factors of FM may lead to new diagnosis and treatment options. However, the sample included in our

study analysis was relatively small, so the findings need to be analyzed in a larger sample and further validated using experiments.

Conclusions

Our study suggests that FM is associated with DNA methylation. The methylation factors APBB2, AKAP12, and CD38 should be further investigated as potential biomarkers. Our findings may provide a new biological framework for the possible disease mechanisms underlying FM.

Acknowledgments

Footnote

Reporting Checklist: The authors have completed the TRIPOD reporting checklist. Available at https://atm.amegroups.com/article/view/10.21037/atm-22-6631/rc

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://atm.amegroups.com/article/view/10.21037/atm-22-6631/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work, including ensuring that any questions related to the accuracy or integrity of any part of the work have been appropriately investigated and resolved. The expression profiles used in this study was downloaded from the GEO database (access Nos. GSE67311, GSE85506). The corresponding ethical approval and informed consent of patients have been provided in the database. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013).

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