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Characterization of immune microenvironment infiltration and m⁶A regulator-mediated RNA methylation modification patterns in osteoarthritis

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Background: Few studies have been reported the potential role of N⁶-methyladenosine (m⁶A) modification in osteoarthritis (OA). We investigated the patterns of m⁶A modification in the immune microenvironment of OA.

Methods: We evaluated the m⁶A modification patterns based on 22 m⁶A regulators in 139 OA samples and systematically associated these modification patterns with immune cell infiltration characteristics. The function of m⁶A phenotype-related differentially expressed genes (DEGs) was investigated using gene enrichment analysis. An m⁶A score model was constructed using principal component analysis (PCA), and an OA prediction model was established based on the key m⁶A regulators. We used real-time PCR analysis to detect the changes of gene expression in the cell model of OA.

Results: Healthy and OA samples showed significant differences in the expression of m⁶A regulators. Nine key m⁶A regulators, two m⁶A modification patterns, m⁶A-related genes and two gene clusters were identified. Some m⁶A regulators had a strong correlation with each other. Gene clusters and m⁶A clusters have high similarity, and cluster A corresponds to a high m⁶A score. Immunocytes infiltration differed significantly between the two clusters, with the m⁶A cluster B and gene cluster B having more types of infiltrating immunocytes than cluster A. The predictive model can also predict the progression of OA through m⁶A regulators expression. The results of real-time PCR analysis showed that the gene expression in the cell model of OA is similar to that of the m⁶A cluster B.

Conclusions: Our study reveals for the first time the potential regulatory mechanism of m⁶A modification in the immune microenvironment of OA. This study also sheds new light on the pathogenesis of OA.

KEYWORDS

m⁶A regulator, immune microenvironment, osteoarthritis, RNA methylation, immunocytes

Introduction

Osteoarthritis (OA) is the most common joint disease worldwide, impacting 240 million individuals (1, 2). OA is a chronic joint disease, that is mainly characterized by pain, stiffness, joint deformity and limited joint activity (3). With the trend of the aging population and the obesity epidemic, this widespread disease and the resulting disability have a great impact on individuals and society (4). The progression of OA is driven by a series of factors, such as gene regulation, biochemical cascades, inflammation and cellular immunity (5, 6). However, the etiology and disease progression mechanisms of OA are still unclear, which limits the development of effective treatment (7).

Many studies have revealed the significance of epigenetics in disease progression (8, 9). Epigenetic modifications such as DNA methylation, RNA modification, histone modification and noncoding RNA modification have also been widely reported (10). Over 100 different types of RNA modifications have been discovered, including N1-methyladenosine (m¹A), N6-methyladenosine (m⁶A), 5-methylcytosine (m⁵C), and N7-methylguanosine (m⁷G) (11). m⁶A RNA methylation occurs on approximately 20%-40% of all transcripts encoded by mammalian cells, and it is the most common type of dynamic and reversible mRNA modification (12, 13). Abnormal m⁶A methylation levels are strongly linked to the progression of cancer, musculoskeletal disorders and other diseases (10, 14). The level of m⁶A methylation is primarily determined by the role of the m⁶A regulator (15). Methyltransferases, demethylases, and binding proteins all play a role in m⁶A modification (16). The m⁶A methyltransferases (writers) include WTAP, METTL3, CBL1, and RBM15B, while demethylases (erasers) consist of ALKBH5 and FTO. YTHDC1, YTHDF1, IGF2BP1, and other binding proteins (readers) can bind to m⁶A and mediate its regulatory function (12, 17). Previous studies have mostly concentrated on the role of m⁶A methylation regulators in tumor development and treatment (18–20). However, the number of studies of m⁶A regulators in nonneoplastic diseases is also increasing (21–23). There are only a few studies on the mechanism of m⁶A regulators and OA at present. Most of these studies clarify the mechanism of FTO and METTL3 in OA, but

they are controversial. The mechanism of m⁶A regulators in OA is still unclear.

Recent research has revealed that m⁶A regulation can mediate some potential immune regulation mechanisms and has a significant impact on adaptive immunity (24, 25). More and more studies have focused on the effects of the immune microenvironment on diseases (26, 27). All types of immune cells are involved in cartilage injury and repair (28). However, the interaction of m⁶A methylation regulators with immune cells in OA is poorly understood (Figure 1).

This study aimed to systematically evaluate the mechanism of m⁶A regulators in OA. We analyzed the gene expression profile of OA by bioinformatics analysis. Subsequently, to further investigate the implication of m⁶A regulators on the immune microenvironment, we investigated the correlations among clustering subgroups, risk mode, and immune cell infiltration. These findings can provide a theoretical basis for the progress and treatment of OA.

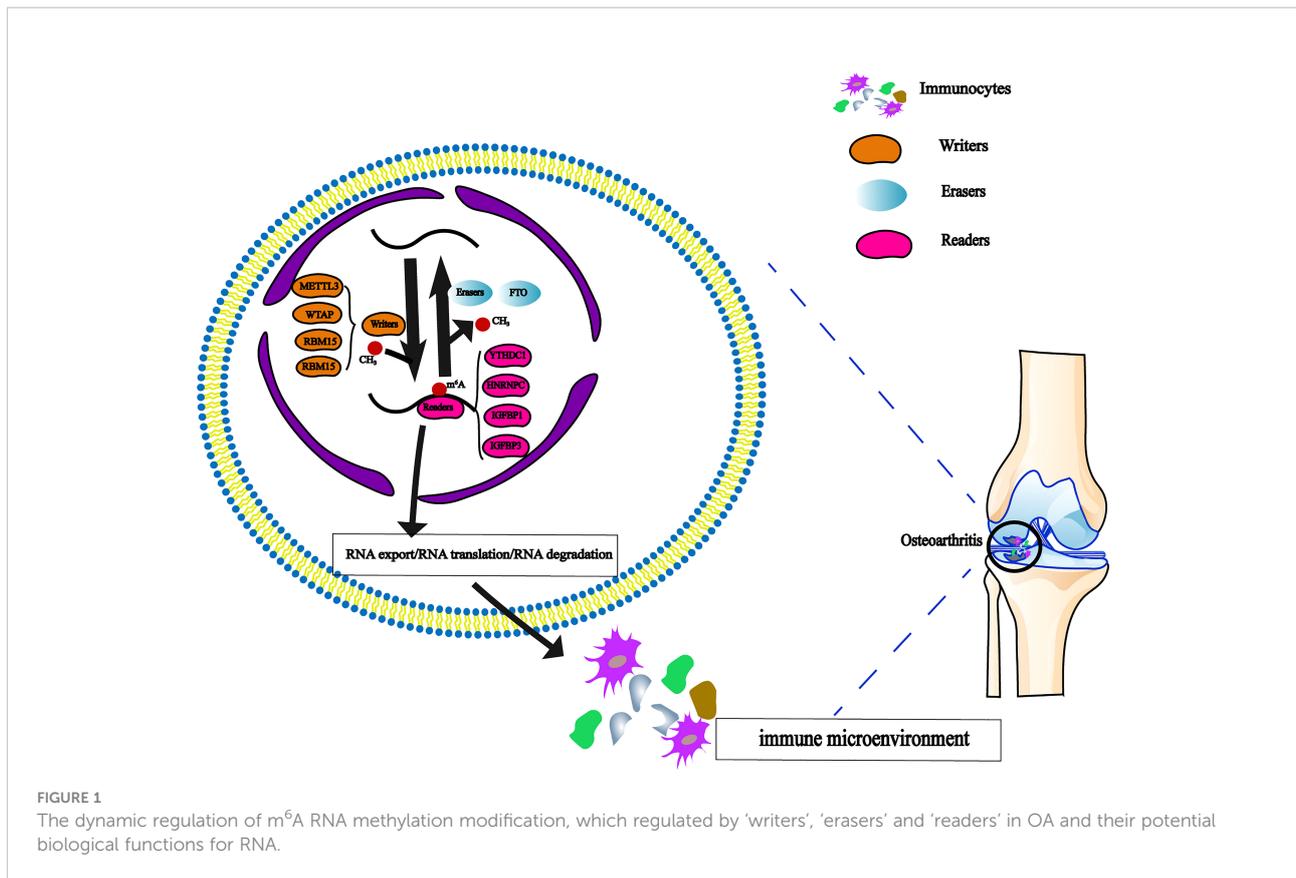
Materials and methods

Datasets preprocess

The GSE48556 datasets were obtained from the Gene Expression Omnibus(GEO) database. Total mRNA was extracted from peripheral blood mononuclear cells and detected using the Illumina HumanHT-12 V3.0 expression beadchip (29). To preprocess the expression value, the “Normalize Between Arrays” function from the “limma” package was utilized.

Analysis of m⁶A regulators between OA patients and healthy controls

We collated 22 recognized m⁶A methylation regulators from published literature (11, 12, 15, 30, 31). The following genes were screened: m⁶A readers (YTHDC1/2, YTHDF1/2/3, HNRNPC, FMR1, LRPPRC, IGF2BP1/2/3, RBMX, ELAVL1, IGF2BP1), m⁶A writers (METTL3, WTAP, RBM15/15B, CBL1, KIAA1429),



and m⁶A erasers (FTO, ALKBH5). The Wilcoxon test was used to compare the expression of 22 m⁶A regulators in OA patients and healthy controls, and the differentially expressed m⁶A regulators were screened with P value < 0.05. The R package “Random Forest” and gene importance plots were used to show the score of differentially expressed m⁶A regulators. A nomogram was used to predict the possibility of OA in patients based on screened m⁶A regulators.

Correlation of m⁶A RNA methylation regulators in OA patients

The correlation between m⁶A regulators in OA patients was investigated by the R package “corrplot” and Spearman’s correlation analysis. The R packages “ggMarginal” and “ggplot” are used to draw the correlation plot of significantly differentially expressed m⁶A regulators.

m⁶A modification pattern identification

Unsupervised clustering analysis was used to detect diverse m⁶A clusters based on the 22 distinct m⁶A regulators’ expression. The number and feasibility of modification

patterns were identified by the consensus clustering algorithm. To categorize OA patients into distinct subtypes, we utilized the “ConsensusClusterPlus” software (1,000 iterations and an 80% resampling rate). The m⁶A-expression pattern was assessed by principal component analysis (PCA). The m⁶A regulators’ differential expression in different m⁶A clusters is shown in the box plot and heatmap. We analyzed the difference in the levels of cytokines among m⁶A clusters.

Immune cell infiltration analysis

We use the ssGSEA algorithm to calculate the enrichment score of immunocytes infiltration in each sample. The gene set of infiltrating immune cells was obtained from previous studies, which included activated B cells (ABCs), type 1/2/17 T helper (Th1/2/17) cells, dendritic cells (DCs), natural killer (NK) cells and other 23 human immune cell subtypes (32). The combined ssGSEA score was used to compare the levels of immunocytes infiltration in distinct m⁶A clusters. The determining criterion of a significant difference was P < 0.05. The correlation between major m⁶A regulators and immunocytes infiltration was determined by Spearman correlation analysis. According to the expression of m⁶A regulators, which are strongly related to immune cell infiltration, OA patients were divided into two

groups. Then, the difference in immunocytes infiltration levels in different subgroups was calculated (P value < 0.05).

Biological enrichment analysis for distinct m⁶A modification patterns

We identified the DEGs among distinct m⁶A subgroups and the overlapping genes were extracted ($|\log FC| > 1$, adjusted P value < 0.05). GO functional analysis and KEGG pathway enrichment analysis were performed to analyze the DEGs *via* R packages (enrichplot, circlize, RColorBrewer, dplyr, ComplexHeatmap, and so on). A P value of 0.05 was used as the cutoff.

Identification and analysis of gene clusters

We used the extracted overlapping genes and the clustering algorithm to determine cluster numbers and stability. We determined the OA gene cluster based on the extracted overlapping genes and unsupervised clustering analysis. We used a box plot diagram to show the m⁶A regulators as differentially expressed in different gene clusters. The degree of immunocytes infiltration and expression of interleukin-associated factors were compared between gene clusters. The method was consistent with the immune infiltration analysis of m⁶A clusters.

Construction of m⁶A gene signature

The m⁶A clusters and gene clusters were identified by previous methods. Then, we constructed the m⁶A gene signature by PCA. As feature scores, principal components 1 and 2 were retrieved. The score was based only on the most significantly correlated genes, while untracked genes' contributions to other set members were weighed. The calculation method for determining the m⁶A gene signature score was based on previous studies ($m^6A_{score} = \sum (PC1_i + PC2_i)$, i = the expression of m⁶A phenotypic related genes) (33, 34). The difference in the m⁶A score in m⁶A clusters and gene clusters was analyzed.

Cell and cell culture

The human chondrosarcoma cell line SW135 (Pricella, China) was maintained in Dulbecco's modified Eagle's medium (DMEM)-high glucose (Gibco, United States) containing 10% Fetal Bovine Serum (FBS, Gibco, United

States) and 1% penicillin/streptomycin. The chondrogenic ATDC5 cell line (Riken Cell Bank, Japan) was cultured in DMEM/F12 (Keygen, China) containing 10% FBS and 1% penicillin/streptomycin. Before the following experiments, all the cells were maintained under standard adherent conditions at 37°C under 5% CO₂ and humidified atmosphere.

Real-time PCR analysis

SW1353 cells and ATDC5 cells were treated with 10 ng/ml recombinant human IL-1 β (Proteintech, Rosemont, IL) for 48 h (35). Total RNA was extracted from cells using RNAiso plus reagent (Takara, Japan) according to the manufacturer's instructions. RNA (1 μ g) was reverse-transcribed to complementary DNA using cDNA Synthesis Super Mix (Trans Gen Biotech, China) according to the protocol of the manufacturer. The primers used for amplification are listed in the table below (Table 1). Green qPCR Super Mix (Trans Gen Biotech, China) was used for real-time PCR using the CFX Connect Real-Time PCR Detection System (Biorad, United States). The thermal cycling conditions were 95°C for 30 s and 42 cycles at 95°C for 5 s and 60°C for 30 s.

Statistical analysis

R 4.1.3 (<https://www.rproject.org/>), Perl 5.32.1 (<https://www.perl.org>) and R Bioconductor packages were used to analyze the data. Statistical analyses of real-time PCR were performed using GraphPad Prism software (version 9.0). Statistical significance was assessed using the student t-test. All experiments were performed independently at least three times. All the statistical P values were bilateral, with P value less than 0.05 considered statistically significant.

Results

Expression of m⁶A regulators in OA

The profile expression data consisted of 139 samples, including 106 genetics osteoarthritis and progression (GARP) samples and 33 normal samples. The positions of 22 selected m⁶A regulators on the chromosomes are marked in Figure 2A. The expression levels of 22 m⁶A regulators in healthy patients and OA patients are shown in Figure 2B. There were significant differences in 9 m⁶A regulators (METTL3, WTAP, RBM15/15B, YTHDC1, HNRNPC, IGFBP1/3, and FTO) between normal and OA samples. In OA, four m⁶A regulators (YTHDC1, HNRNPC, METTL3, and WTAP) were downregulated and five m⁶A regulators (RBM15, RBM15B, IGFBP1, IGFBP3, and FTO)

TABLE 1 The sequences of primer pairs used in the study.

Gene	Forward	Reverse
H:GAPDH	5'-GGAAGCTTGTCAATGGAAATC-3'	5'-TGATGACCCTTTTGGCTCCC-3'
H:Mettl3	5'-CAGCACAGCTTCAGCAGTTCC-3'	5'-CGTGGAGATGGCAAGACAGA-3'
H:Wtap	5'-CCAAGAAGGTTTCGATTGAGTGA-3'	5'-CAGACTCCTGCTGTGTGTTGCTTT-3'
H:RBM15B	5'-CACAGCGTATCTGAGGTGGAG-3'	5'-GTTCTGGAACCTTGAGGAAGGCATA-3'
H:Rbm15	5'-GTGAGCGGAGCAAGAAGTTAGG-3'	5'-CTATAACTATGCAAGCGGCTACTG-3'
H:YTHDC1	5'-GAGGGAATTTTCATAACATGGGAC-3'	5'-ATGGTGCTGATAGTAAGGATGGTGT-3'
H:Fto	5'-GCCAGGTGCCAGTCACGAAT-3'	5'-TGTGAGGTCAAACGGCAGAG-3'
H:HNRNPC	5'-CGCTCCATGAACTCCCGTGT-3'	5'-GTTCTGTTACTGACCCGTACATCTC-3'
H:IGFBP1	5'-GCACGGAGATAACTGAGGAGGA-3'	5'-TCTTGTTCAGTTTGGCAGGTA-3'
H:IGFBP3	5'-CTCAGAGCACAGATACCCAGAAC-3'	5'-AGGCTGCCATACTTATCCAC-3'
M:GAPDH	5'-CCTCGTCCCGTAGACAAAATG-3'	5'-TGAGGTCAATGAAGGGTCTGT-3'
M:Mettl3	5'-AGGACTCTGGGCACTTGGATT-3'	5'-ATGGCAAGACGGATGGAAAC-3'
M:Wtap	5'-GCAAGATGACCAACGAAGAACC-3'	5'-TTAACTCATCCCGTCCATAAC-3'
M:RBM15B	5'-CACAGTGTTCGAGGTGGAGC-3'	5'-GCCTTGCCGTAGCCTATCTTT-3'
M:Rbm15	5'-GTTCAAACGCTTCGGTGATGTA-3'	5'-CACAAAGGCTACCCGCTCAT-3'
M:YTHDC1	5'-CCGATACCAGGAAGTGGACAGAC-3'	5'-TGGTGCTGGTAGTAAGGATGGTG-3'
M:Fto	5'-TGAGGTGGAGTTTGTGAGTGGCTG-3'	5'-AGAATCTCACTCCTTTGTTCCACC-3'
M:HNRNPC	5'-ACGGTTCCTCATTTGACTTGG-3'	5'-AACACGCTGACGTTTGAAGG-3'
M:IGFBP1	5'-GCCACGAGCACCTTGTTCAG-3'	5'-GCAGGGCTCCTTCCATTCTT-3'
M:IGFBP3	5'-CTCAAAGCACAGACCCAGAA-3'	5'-GGCGGCACTGCTTCTTCTTAT-3'

(H, Human; Used in the SW1353. M, Mouse; Used in the ATDC5).

were upregulated (Figure 2C). IGFBP3 expression was dramatically higher in the peripheral blood of OA patients ($P < 0.001$). METTL3 and HNRNPC levels in OA patients were considerably lower than those in healthy controls ($P < 0.01$). Thus, these m⁶A regulators are critical in OA.

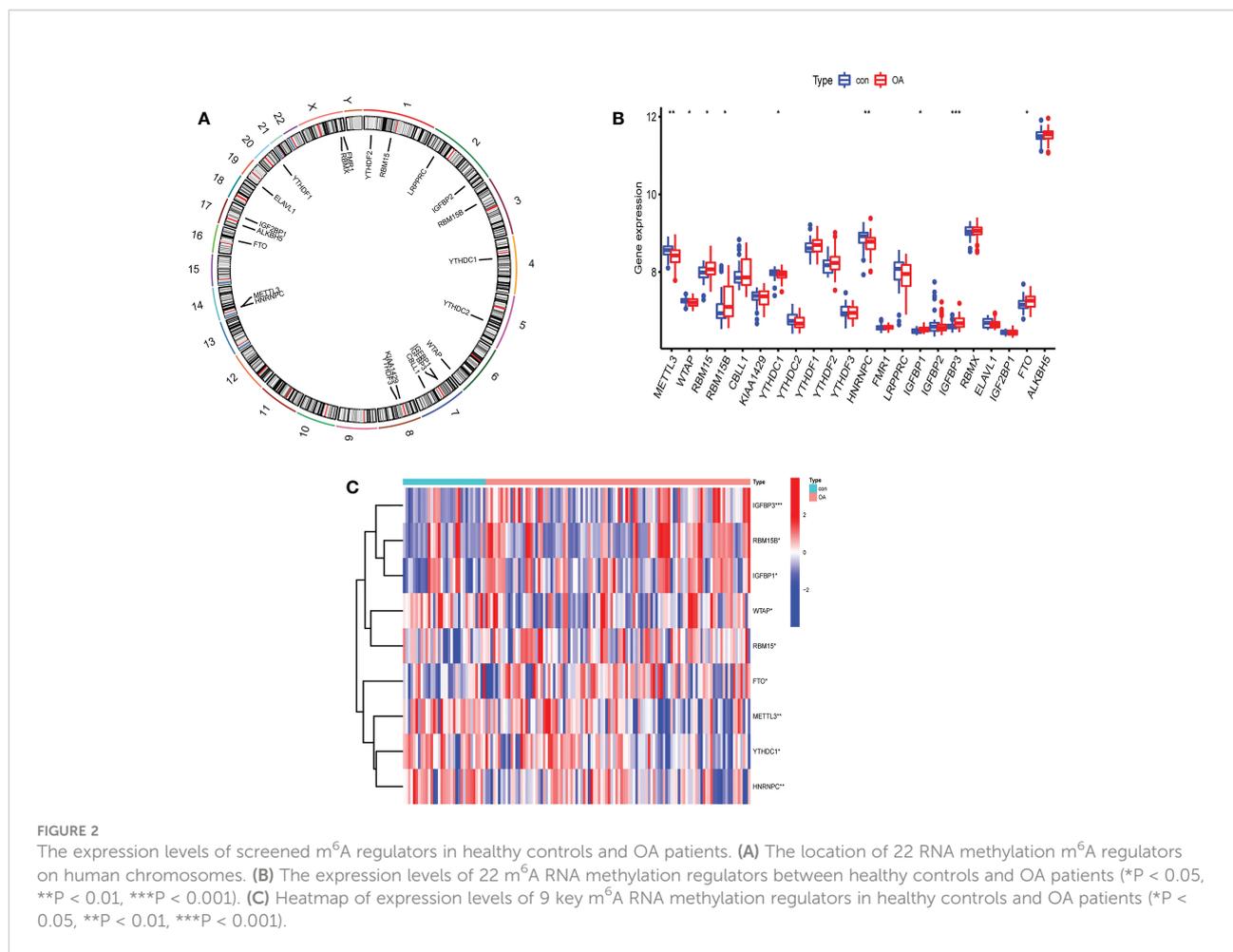
We further analyzed the correlations among the 22 m⁶A regulators in the OA group (Figure 3A). Some m⁶A regulators had a strong correlation with each other (Figures 3B–F: writers and readers, Figure 3G: erasers and writers, Figures 3H, I: erasers and readers).

m⁶A methylation modification patterns mediated by regulators

According to m⁶A regulator expression, the clustering stability was analyzed with cluster numbers from 2 to 9 (Figure 4A and Figure 1S). The optimal k value was determined, and k = 2 was eventually selected as the optimal cutoff (Figure 4B). PCA revealed that OA patients could be classified into two m⁶A clusters that did not intersect. Thus, two is an appropriate cluster number (Figure 4C). There were 45 OA patients in cluster A and 61 in cluster B. IGFBP1, IGFBP3, RBM15B, RBM15 and WTAP were highly expressed in cluster A. FTO, HNRNPC, METTL3 and YTHDC1 were expressed at lower levels in cluster A than in cluster B (Figures 4D, E).

Immune microenvironment features related to two m⁶A modification patterns

The expression of immune cells was examined to demonstrate the changes in immune microenvironment features in distinct m⁶A modification patterns. Between the two m⁶A clusters, there were substantial variations in virtually all immune cells observed (Figure 5A). Compared with m⁶A cluster A, the cluster B had a higher level of ABCs, activated CD8⁺ T cells, activated DCs, eosinophils, immature B cells, immature DCs, macrophages, plasmacytoid DCs, regulatory T cells, Th1 cells and Th2 cells. Activated CD4⁺ T cells, MDSCs, mast cells, monocytes, NK T cells, NK cells, neutrophils, T follicular helper cells and Th17 cells were enriched in cluster A. More immune cells are enriched into the cluster B. The correlation between the screened m⁶A regulators and immunocytes was investigated. The infiltration of immunocytes was substantially linked with the levels of IGFBP1 and RBM15B (Figure 5B). OA patients were separated into two groups based on the difference in the expression of IGFBP1 or RBM15B. Although 9 types of immunocytes were enriched in the 2 IGFBP subgroup, the high IGFBP expression group may have a higher level of immunocytes infiltration (Figure 5C). In addition, the high RBM15B expression group had fewer kinds of immune cells (Figure 5D).



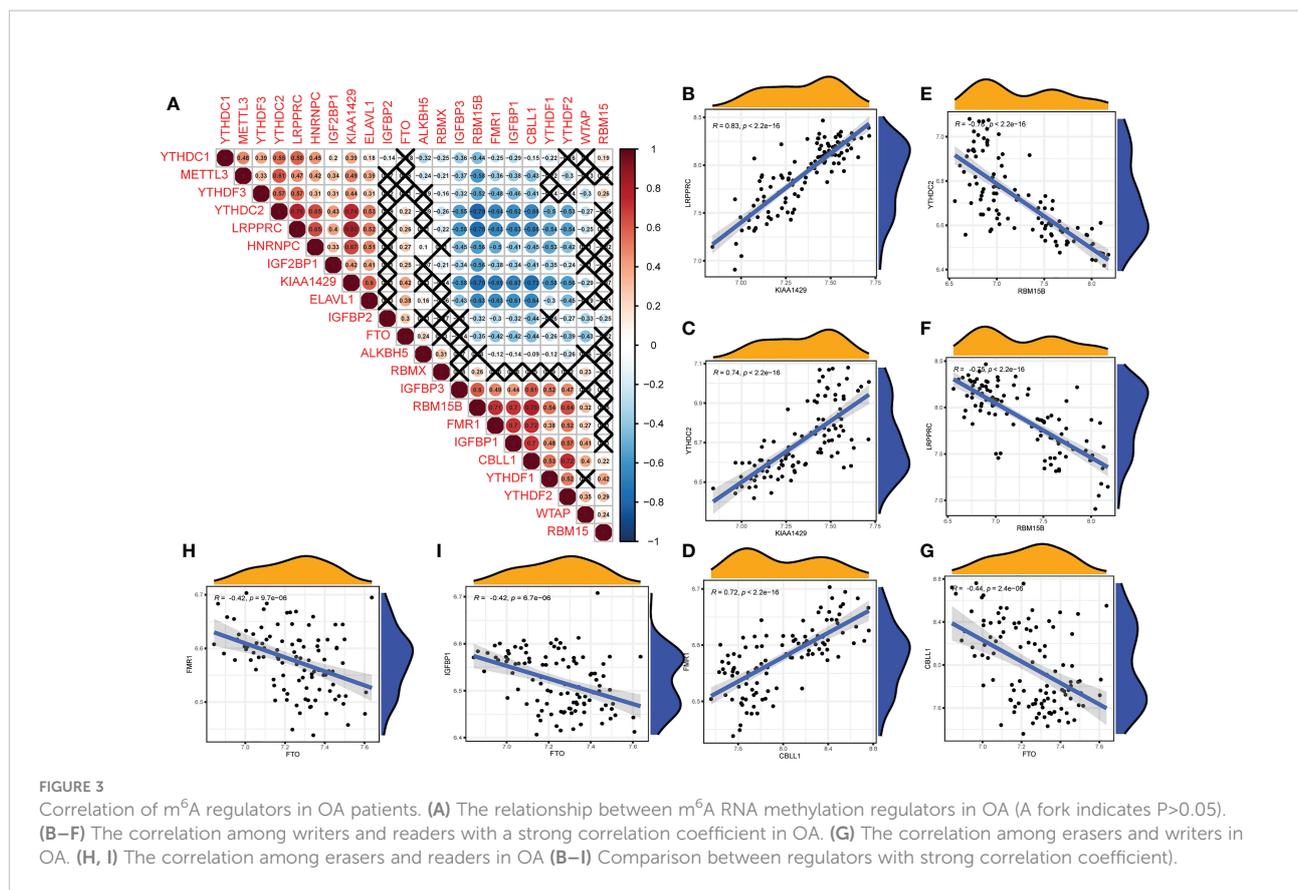
Biological characteristics of m⁶A modification patterns

Between m⁶A clusters A and B, 303 genes (m⁶A phenotype-related genes) were differentially expressed. GO enrichment analysis was used for analysis of enriched biological processes, molecular functions, cellular components. These genes are mostly associated with amoeboid-type cell migration (biological process), basal plasma membrane (cell component), and lyase activity (molecular function) (Figures 6A, B). To investigate the relevant activities and pathways of m⁶A phenotype-related DEGs, we employed KEGG pathway enrichment analysis (Figure 6C).

m⁶A phenotype-related DEGs in OA

The 303 m⁶A phenotype-associated DEGs were analyzed by unsupervised clustering analysis. It was most appropriate to divide the sample into two gene clusters (Figure 2S). There were

considerable discrepancies between the two gene clusters, and only one m⁶A methylation-related gene was highly expressed in cluster B (Figure 7A). The evident difference in m⁶A phenotype-related gene expression between the two gene clusters was significant in controlling the establishment of immune cell infiltration (Figure 7B). The m⁶A regulators also had significantly different expression in different gene clusters (Figure 7C). However, these assays were unable to predict the m⁶A methylation pattern in particular individuals. We developed an m⁶A scoring system to quantify the pattern of m⁶A modifications in each OA patient. m⁶A cluster A had a considerably higher m⁶A score than m⁶A cluster B. Furthermore, the m⁶A score was considerably higher in gene cluster A. The m⁶A score in gene cluster A was significantly higher than that in gene cluster B (Figure 7D). An alluvial plot was used to display the characteristic variations in each patient with OA. The results of m⁶A regulators typing were similar to those of genotyping (Figure 7E). We also analyzed the differential expression of immune regulatory genes and immune checkpoints in m⁶A clusters and gene clusters. There are



significant differences in the expression of immune regulatory genes and immune checkpoints between A and B (Figures 7F, G).

Predictive model in OA

We compared the advantages of the random forest model (RFM) and support vector machine model (SVM). The RFM was better than the SVM (Figures 8A–C). The optimal cutoff point was selected for analysis according to the random forest tree model. The importance score of m⁶A regulators was obtained (Figures 8D, E). An OA prediction model was established and evaluated. The calibration curve, clinical influence curve and decision curve all showed that the model was accurate (Figures 8F–H). The predictive model can predict the incidence of OA disease by score (Figure 8I).

Expression of m⁶A regulator in two chondrocyte lines after the treatment of IL-1β

After we found that m⁶A regulators play an important role in OA, we established an *in vitro* cell model of OA by treating

SW1353 cells and ATDC5 cells with IL-1β. The differences in m⁶A regulator gene expression before and after cell treatment were assessed. After treatment of IL-1β, three m⁶A regulators (IGFBP3, RBM15, and WTAP) were down-regulated and two m⁶A regulators (METTL3 and YTHDC1) were up-regulated in ATDC5. In SW1353 cells after treatment with IL-1β, the expression of FTO, HNRNPC, and IGFBP1 was down-regulated, and the expression of WTAP was up-regulated (Figures 9A, B). It appeared that the gene expression in the OA model established with cell lines is similar to that of the m⁶A cluster B. These may be related to the accumulation of macrophages in the m⁶A cluster B, and IL-1 is mainly secreted by macrophages.

Discussion

OA is a prevalent degenerative joint disease that can be painful and uncomfortable (36). Increasing evidence shows that m⁶A modification is critical for inflammation and innate immunity by interacting with a variety of m⁶A regulators. Abnormal modification of the m⁶A gene may lead to disorders of important genes and dynamic balance, resulting in disease (37). In recent years, there have been many discussions about the

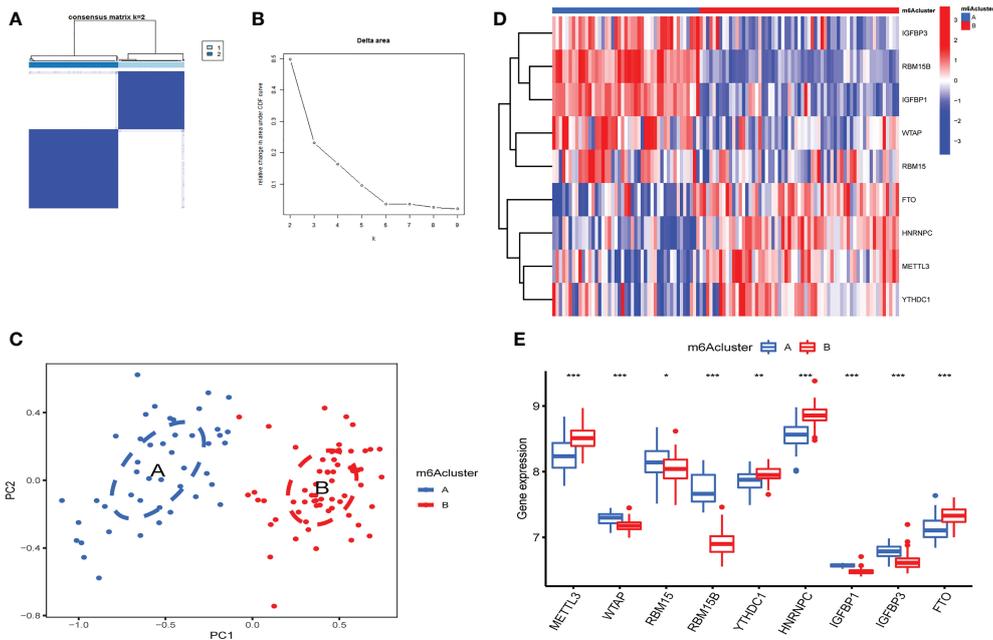


FIGURE 4 Identification of 2 distinct m⁶A modification patterns in OA. **(A)** Consensus clustering matrix (CCM) with K=2. **(B)** Change in the area under the cumulative distribution function (CDF) curve for k from 2 to 9. **(C)** PCA showed that two m⁶A clusters did not intersect (2 modification patterns in OA are an appropriate choice). **(D)** Heatmap of the expression of 9 key m⁶A RNA methylation regulators in 2 m⁶A clusters. **(E)** Box plot of the expression of 9 key m⁶A regulators in the 2 m⁶A modification patterns (*P < 0.05, **P < 0.01, ***P < 0.001).

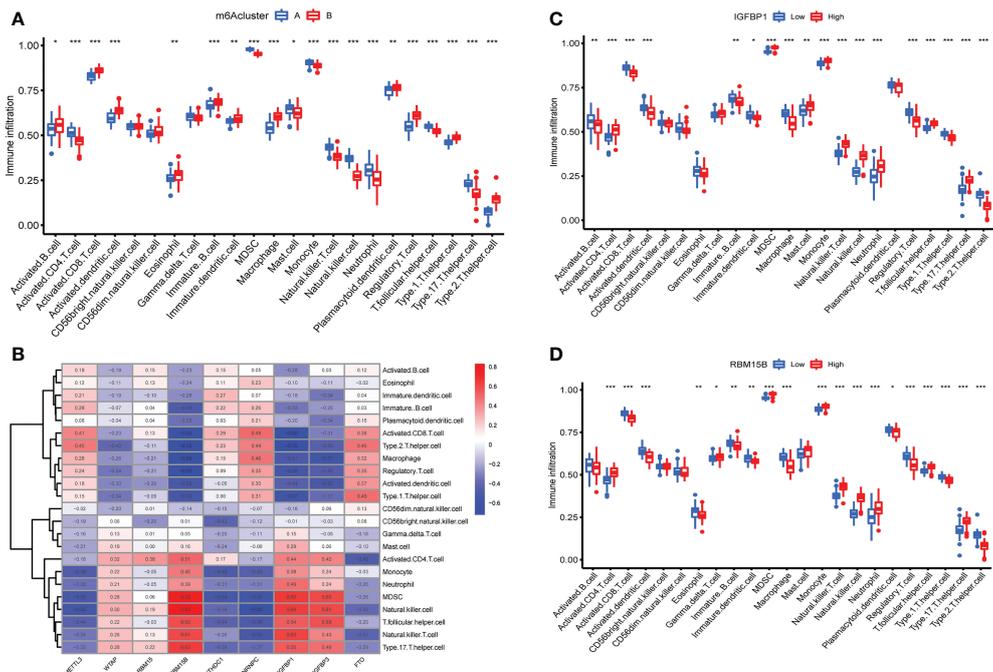
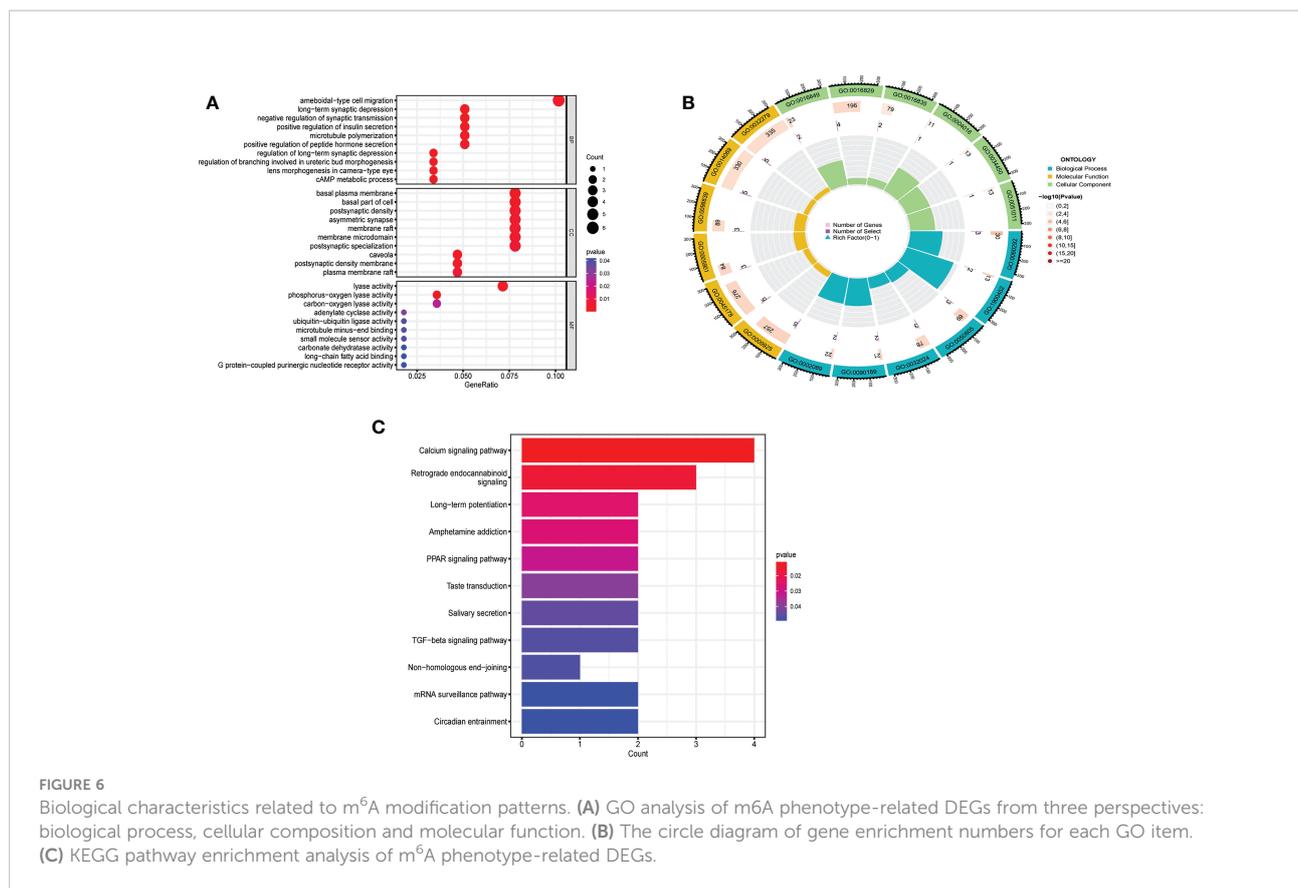


FIGURE 5 Immune microenvironment features in two m⁶A modification patterns. **(A)** Levels of infiltrating immunocytes in two m⁶A clusters in OA (*P < 0.05, **P < 0.01, ***P < 0.001). **(B)** Correlation between 9 key m⁶A regulators and immune cells. **(C)** The effect of high and low expression of IGFBP1 on immune infiltration (*P < 0.05, **P < 0.01, ***P < 0.001). **(D)** The effect of high and low RBM15B expression on immune infiltration ns, P>0.05, No statistical difference; (*P < 0.05, **P < 0.01, ***P < 0.001).



relationship between inflammatory factors and the pathogenesis and progression of OA. OA is now regarded as an inflammatory illness defined by inflammatory factors rather than a degenerative disease (38). The inflammatory environment is critical to the progression of OA (39). At present, researchers are interested in the association between m⁶A modifications and OA. Thus, future studies will focus on the link between epigenetic regulation and inflammatory factors in OA.

We systematically investigated the mechanism of the modification mode of m⁶A in the immune microenvironment of OA. First, we discovered that the expression of the majority of m⁶A regulators changed between healthy controls and OA patients, indicating that m⁶A regulators were implicated in the occurrence and progression of OA. Previous studies have indicated that METTL3 plays a significant role in the pathogenesis of osteoarthritis. Liu's study demonstrated that METTL3 regulates OA pathogenesis by promoting inflammation and extracellular matrix (ECM) synthesis (7). Jiangdong Ren showed that METTL3-mediated LINC00680 accelerates OA (40). However, Sang. W showed that overexpression of METTL3 leads to reduced inflammatory cytokine levels and regulates the TIMP/MMP balance (14). There are a few studies on the relationship between FTO and

OA. However, there is also controversy between these studies (41, 42). In our study, both FTO and METTL3 were found to be key m⁶A regulators in OA. Our study shows that METTL3 is downregulated and FTO is upregulated in OA.

We further classified OA patients according to the screening results for 9 key m⁶A regulators. Furthermore, several m⁶A regulators exhibited expression correlations with each other, which revealed the m⁶A modification regulatory network. Third, the impact of m⁶A modification patterns on immunocytes infiltration was determined to strengthen the knowledge of the interaction between m⁶A RNA and the immunological response. We found that IGFBP1 and RBM15B were strongly correlated with infiltrating immunocytes in OA. Lange-Brokaar's study showed that the immunocytes participating in cartilage injury and repair mostly comprise T cells, B cells, NK cells, DCs and macrophages (43). Our study also showed that these immunocytes play a significant role in distinct m⁶A modification patterns. The m⁶A cluster B had more kinds of infiltrating immunocytes than cluster A, and the degree of immune cell infiltration could not be judged. However, these validated the accuracy of our immunophenotypic categorization through key m⁶A regulators. It also showed that there is a certain correlation between the m⁶A regulator and immune infiltration.

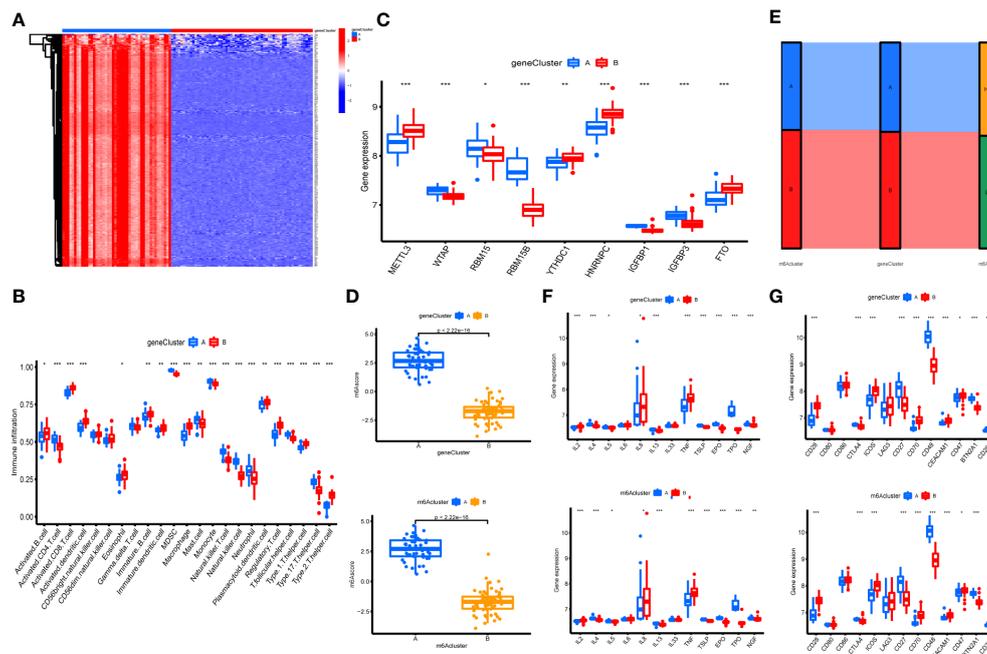


FIGURE 7

Construction of m^6A signatures. (A) Heatmap of m^6A phenotype-related in different gene clusters. (B) The immunocytes infiltrating levels in distinct gene clusters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (C) The expression levels of 9 key m^6A regulators in different gene clusters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (D) The m^6A score in different gene clusters and m^6A clusters. (E) Alluvial diagram showing the changes in m^6A clusters, gene clusters, and m^6A score. (F) Differences in the expression of immune regulatory genes in different gene clusters and m^6A clusters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$). (G) Differences in the expression of immune checkpoints in different gene clusters and m^6A clusters (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

Different immune microenvironments has different effects on osteoarthritis, which also provides a basis for immune research in osteoarthritis.

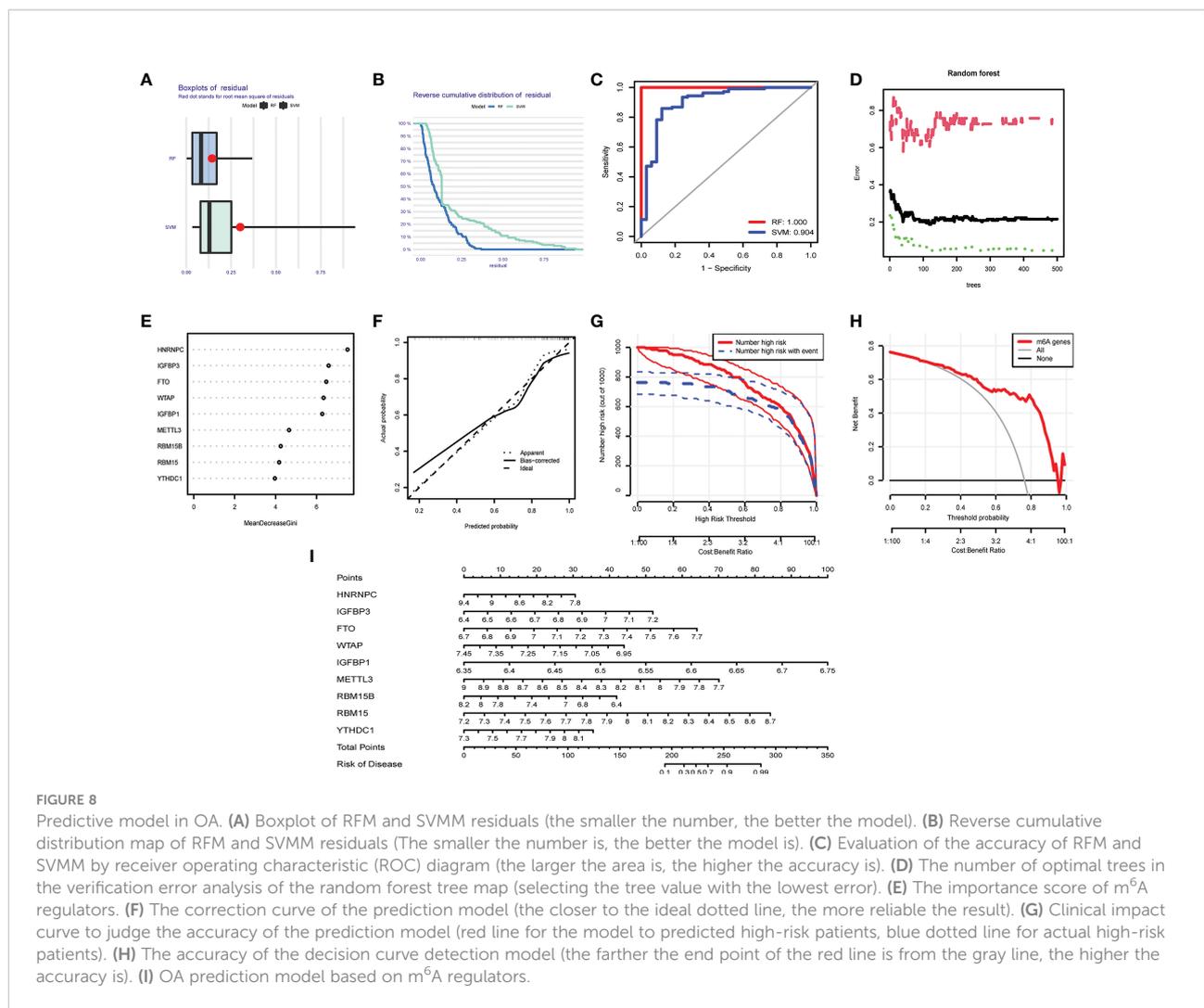
Furthermore, the m^6A phenotype-related DEGs and their biological functions were identified. Our study confirmed that m^6A phenotype-related DEGs were significantly related to immune cell infiltration. We further found that the different expression levels of immune regulatory and immune checkpoints in different m^6A clusters and gene clusters. This finding may provide new ideas for the study on treatment of OA. We set up an m^6A score model to assess m^6A modifications in individual OA patients. We also found that the m^6A clusters and gene clusters contained almost the same sets of patients. Then, we established a prediction model that can predict the occurrence and progression of OA. We can score patients according to the expression of the m^6A regulator, and then get the probability of OA by score. Finally, we tried to use the OA cell model to verify whether the changes in the m^6A gene were consistent with our bioinformatics analysis. The results of real-time PCR analysis were not consistent with the differences in m^6A regulator expression between OA patients and healthy patients. This may be due to the fact that the cell model was

different from the human body and that the samples came from the peripheral blood of the patient. But the change of m^6A regulators' expression proved that these genes play a role in the progression of OA. More importantly, we found the change in gene expression in the OA cell model was almost consistent with that of the m^6A cluster B. We believed that this phenomenon is due to the fact that we used IL-1 β for cell modeling, and the macrophages that mainly secrete IL-1 were mainly enriched in the B cluster.

The study of m^6A regulators is widely used in the field of oncology, and better treatment can be achieved through molecular subtyping (44, 45). In the field of OA, however, few studies have focused on the mechanism of m^6A regulators. We are the first to systematically analyze the mechanism of m^6A in OA. We proved that m^6A modification has a role in regulating the OA immune microenvironment.

Limitations of the study

These results will provide new inspiration for the study of the pathogenesis and treatment of OA. However, there are still some

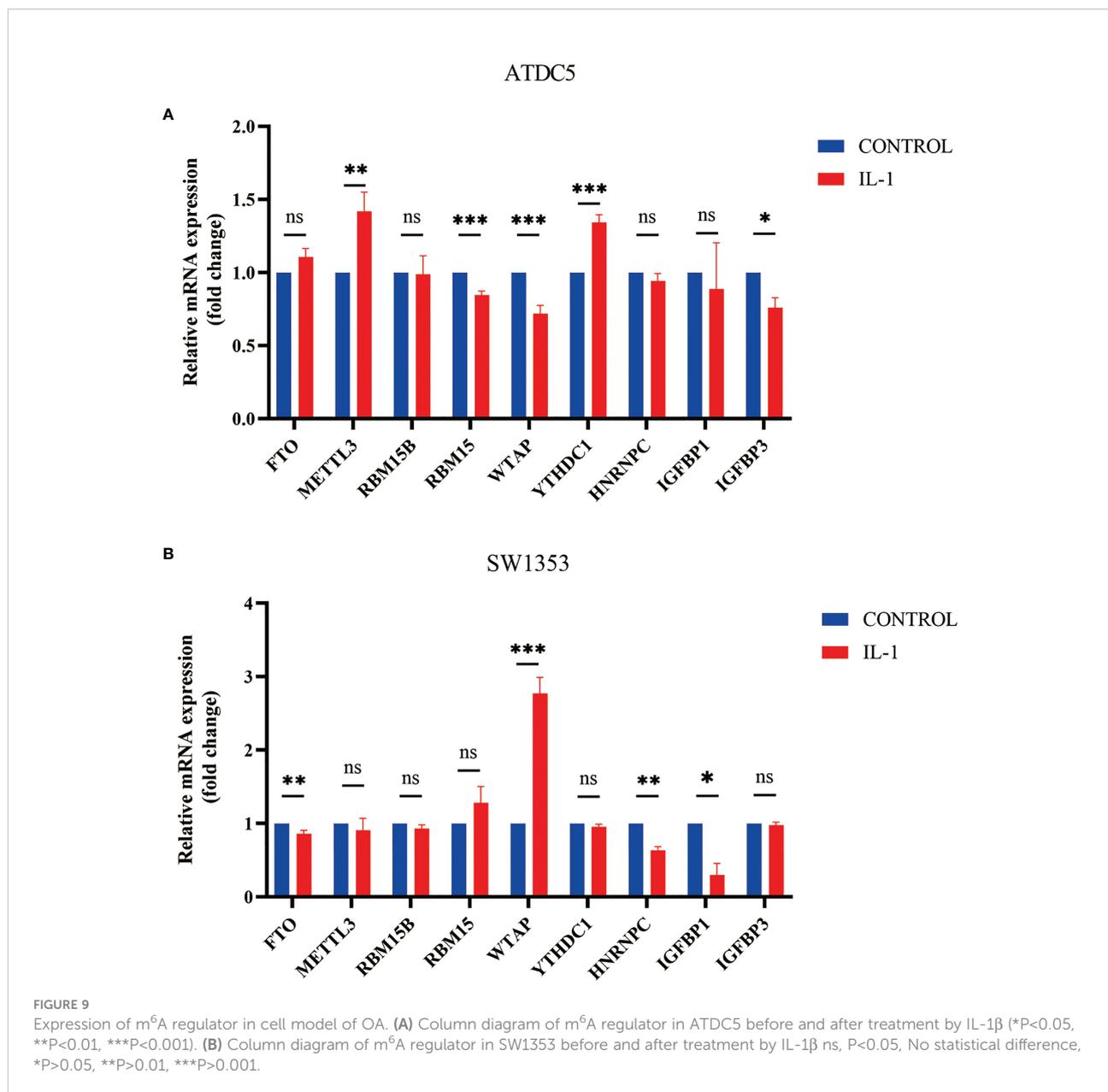


deficiencies in our research. First, our study is based on bioinformatics analysis, and although our results are accurate and reliable in theory, they need to be verified by more experiments. In the future, we hope that single-cell RNA seq can be performed to better understand how m⁶A modification affects immune cell infiltration in OA patients. In addition, the expression profile dataset (GSE48556) contains only 139 samples, and a larger sample size would be more beneficial for bioinformatics analysis. Therefore, we hope that more OA expression profile data and studies with larger sample sizes will become available. Third, because OA is a nonneoplastic disease, its survival curve is rarely studied, so it is impossible to establish a model considering the m⁶A score and survival. However, the severity of knee joint damage can be studied, and more clinical data will establish the relationship between the m⁶A score and disease progression. In this way, m⁶A score will be critical in predicting the progression of the disease. But the accuracy of m⁶A

scoring model need validated by a large number of patient samples. Finally, there were few genes in the GO and KEGG analyses of m⁶A phenotype-related DEGs, which could be due to the limited sample size. However, some of the enriched biological functions mentioned in our study have been proven to participate in OA by other studies, which indicates that our results are worth using for reference. We hope that the expression profile datasets with a larger sample size become available.

Conclusion

In summary, our study reveals for the first time the potential regulatory mechanism of m⁶A methylation modification in the immune microenvironment of OA. This study also sheds new light on the pathogenesis of OA. The difference in m⁶A modification mode is a significant contributor to the



complexity of the OA microenvironment. Individual OA patients can utilize the m⁶A score to assess their m⁶A modification pattern and immune cell infiltration features. The prediction model we have established is helpful in predicting disease development in OA patients. Additional studies are needed to fully clarify the molecular mechanisms of m⁶A regulation and biological function during OA.

Data availability statement

Publicly available datasets were analyzed in this study. This data can be found here: The GSE48556 datasets were obtained

from the GEOdatabase(<https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE48556>).

Author contributions

GS and YO designed this study. TW, HF and SC conducted literature searches and management. YO, YT and HM were responsible for data management and statistical analysis. GZ, ZW, WY interpreted the findings. YO performed the manuscript writing. The manuscript was revised by all authors, and the final version was approved by all. All authors contributed to the article and approved the submitted version.

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Conflict of interest

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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Supplementary material

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fimmu.2022.1018701/full#supplementary-material>

FIGURE 1S

The best m6A cluster number was evaluated by the CCM. (A–G) K=3–9 CCM.

FIGURE 2S

The best gene cluster number was evaluated by the CCM. (A–I) K=2–9 CCM. (J) Relative change in the area under the CDF curve for k from 2 to 9.

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