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Original Article

Bioinformatic analysis and identification of macrophage polarization-related genes in intervertebral disc degeneration

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Abstract: Background: The relationship between macrophage polarization-related genes (MPRGs) and intervertebral disc degeneration (IDD) is unclear. The purpose of this study was to identify biomarkers associated with IDD. Methods: Three transcriptome sequencing datasets, GSE124272, GSE70362 and GSE56081 were included in this study. Differential expressed genes (DEGs) were obtained by overlapping DEGs1 from the GSE124272 and DEGs2 from the GSE70362. The key module genes associated with the score of MPRGs were identified by weighted gene co-expression network analysis (WGCNA) in GSE12472. Differentially expressed (DE)-MPRGs were acquired by overlapping key module genes and DEGs. Candidate genes were obtained by SVM-RFE algorithm. Biomarkers were obtained by expression level analysis. In addition, immune analysis, enrichment analysis and construction of a ceRNA network were completed. The blood samples from 9 IDD patients (IDD group) and 9 healthy individuals (Control group) were used to verify the expression levels of these biomarkers through RT-qPCR. Results: A sum of 39 DEGs were obtained by overlapping DEGs1 and DEGs2, and 1,633 key module genes were obtained by WGCNA. 9 DE-MPRGs were obtained by overlapping DEGs and key module genes, and ST6GALNAC2, SMIM3, and IFITM2 were identified as biomarkers. These biomarkers were enriched in KEGG_RIBOSOME pathway. Check-point, Cytolytic_activity, T_cell_co-stimulation, Neutrophils, Th2_cells and TIL differed between IDD and control groups. Some relationships such as SMIM3-hsa-miR-107-LINC02381 were identified in the network. Moreover, the functional analysis results of biomarkers showed that FITM2 and SMIM3 could predict IDD and nociceptive pain. The RT-qPCR showed that ST6GALNAC2 and IFITM2 were significantly expressed in IDD group in contrast to the control group. Conclusion: The macrophage polarization related biomarkers (ST6GALNAC2, SMIM3 and IFITM2) were associated with IDD, among which IFITM2 could be considered as a key gene for IDD. This may provide a new direction for the biological treatment and mechanism research into IDD.

Keywords: Intervertebral disc degeneration, macrophage polarisation, immune, enrichment analysis, WGCNA

Introduction

Intervertebral disc degeneration (IDD) is defined as the primary degenerative condition associated with low back pain, which has become an increasing problem worldwide. It has been estimated that more than 70% of the global population suffers from IDD in their lives [1, 2]. IDD is driven by various molecular mechanisms, including abnormal expressions of genes, inflammation, loss of disc matrix, apoptosis of functional cells, and DNA replication errors [3]. Despite relieving the pain to some extent, current therapies for IDD do not target the underlying causes. Moreover, the surgical treatments including disc decompression or fusion are prone to complications. Therefore, there is an urgent need for efficient treatment strategies that can reverse IDD or restore the biological function of the intervertebral disc.

The immune homeostasis plays an important role in the process of IDD and is maintained by a variety of immune cells, such as B cells, T

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cells, and macrophages [4]. Recently, macrophages have received significant attention in the IDD process. Previous studies revealed that macrophage infiltration and polarization occur following the damage to immunologic balance, which can be triggered by end plate micro-fractures or annulus rupture [5]. Additionally, macrophages are notable as the sole inflammatory cells that infiltrate the closed nucleus pulposus and can alter the expression of inflammatory factors such as IL-6 and IL-1β [6, 7]. Animal injury models supported an association between disc degeneration and macrophages, while in humans, the macrophage marker levels were found to positively associate with disc degeneration within the nucleus pulposus and endplate, especially in the unhealthy regions [8, 9]. Another study showed that the intervertebral disc (IVD)-produced IL-1β polarized macrophages to a pro-inflammatory phenotype [10]. Despite these findings, the role of macrophages in disc tissue degeneration has not been thoroughly investigated. Further investigation into macrophage polarizations is crucial for developing effective treatments for IDD.

This study aimed to identify the macrophage polarization-related genes (MPRGs) associated with disc degeneration through a series of bioinformatic approaches such as differential expression analysis. Weighted gene co-expression network analysis, and enrichment analysis were based on transcriptome data from the Gene Expression Omnibus database. Additionally, basic experiments were also performed to validate the bioinformatic-mined markers. The results of this study may promote the understanding of the cytologic mechanism of IDD pathogenesis and facilitate the development of novel treatments for IDD, particularly in its early stages.

Materials and methods

Data source

Three IDD-related datasets (GSE124272, GSE70362 and GSE56081) were acquired from the Gene Expression Omnibus (GEO) database (https://www.ncbi.nlm.nih.gov/gds). Transcriptome data from 8 IDD samples and 8 control blood samples from the GSE124272, 16 IDD samples and 8 control medullary tissue samples from GSE70362, and 5 IDD and 8 control medullary tissue samples from GSE56081 were included in this study. Moreover, 35 MPRGs were acquired from the Molecular Signatures Database (MSigDB).

Differential expression analysis

Differential expression analysis was executed between IDD samples and control samples using limma package [11] in GSE124272 and GSE70362 to screen differentially expressed genes (DEGs)1 and DEGs2 by setting [$\log_2$ (fold change)] > 0.5 and P value < 0.05. Moreover, the intersection of the up- and down-regulated genes of DEGs1 and DEGs2 were taken separately to obtain DEGs.

Weighted gene co-expression network analysis (WGCNA)

Based on GSE124272, the samples were clustered to remove outliers using the WGCNA package [12]. Then, the determination of the soft threshold (β) was performed. According to their proximity, genes were compared for similarity, and a phylogenetic tree of those genes was created. A dynamic tree cutting algorithm was used to separate the modules, with a minimum module size set at 300. The MPRGs score was calculated in a GSE124272 dataset using the single sample gene set enrichment analysis (ssGSEA) algorithm by GSVA package [13]. Pearson correlation analysis was applied to the modules obtained from the WGCNA, using MPRGs scores as scoring traits to obtain key modules. MM (Module Membership) and GS (Gene Significance) values were calculated for each module gene to obtain the key module genes (MM > 0.8 and GS > 0.4).

Identification of DE-MPRGs

Differentially expressed (DE)-MPRGs were obtained by overlapping DEGs and key module genes. GO and KEGG enrichment analysis of DE-MPRGs were completed by ClusterProfiler package [14] (Significance set at P < 0.05).

Identification of biomarkers

The e1071 package was used to rank the importance of DE-MPRGs by Support Vector Machine Recursive Feature Elimination (SVM-RFE) algorithm to obtain candidate genes. Candidate genes in GSE56081 with consistent

Table 1. Primer sequences for PCR in this study

Gene	Sequence of primers
ST6GALNAC2	F: 5'-GCCAGGGACACCACATCATT-3'
	R: 5'-AGAGATTGAACAGGCCACGG-3'
SMIM3	F: 5'-CTGAGAAGCACCGAGCCATC-3'
	R: 5'-TGGCTGACTGCATCCATGTT-3'
IFITM2	F: 5'-AGACTCCCAACACAGGGGA-3'
	R: 5'-ACCCTGTGGGCTTGTTGAAA-3'
GAPDH	F: 5'-CGAAGGTGGAGTCAACGGATTT-3'
	R: 5'-ATGGGTGGAATCATATTGGAAC-3'

F: Forward primers; R: Reverse primers.

and significantly different expression trends from GSE124272 and GSE70362 were used as biomarkers ($P < 0.05$). Moreover, receiver operating characteristic (ROC) curve was drawn to assess prediction accuracy. Spearman correlation analysis was executed between biomarkers and all the genes by psych package and ranked according to the correlation coefficients. Finally, Gene Set Enrichment Analysis (GSEA) of biomarkers was executed by ClusterProfiler package (The value of $P < 0.05$) [14].

Immune infiltration analysis and construction of ceRNA network

Enrichment scores for 13 immune-related pathways and 16 immune cells were calculated in GSE124272 by the ssGSEA algorithm. Subsequently, the differences in immune infiltration between IDD and control groups were compared by Wilcoxon test, and Spearman analysis was also executed. The miRNA and lncRNA (clipExpNum > 16) associated with biomarkers were predicted by starBase, and a ceRNA network was established. Additionally, the relationship between biomarkers, pain, and IDD was analyzed using the Comparative Toxicogenomics Database (CTD).

RT-qPCR

The blood samples were collected from the 9 IDD patients in the First Affiliated Hospital of Shandong First Medical University. Additionally, blood samples obtained from 9 healthy individuals were utilized as control samples. The collected blood samples were subjected to reverse transcription-polymerase chain reaction (RT-qRCP). This study was approved by the ethics Committee of the First Affiliated Hospital of Shandong First Medical University (Ethics approval number: No. 2023-255). All patients had signed an informed consent form. The expression of the four biomarkers was further validated by RT-qPCR. Total RNA of 18 samples were extracted using TRIzol (Ambion, Austin, USA) according to the manufacturer's guidance. Reverse transcription of total RNA to cDNA was carried out by using SureScript First-strand cDNA synthesis kit (Servicebio, Wuhan, China) based on the manufacturer's instructions. RT-qPCR was performed utilizing the 2× Universal Blue SYBR Green qPCR Master Mix (Servicebio, Wuhan, China). The primer sequences for PCR are shown in **Table 1**. GAPDH was used as an internal reference gene. The $2^{-\Delta\Delta Ct}$ method was utilized to calculate the expression of biomarkers [15].

Statistical analysis

Statistical analysis was carried out through R software. Differences between nonparametric data were analyzed by the Wilcoxon test. Spearman's correlation analysis was applied to evaluate the correlation between continuous variables. Differences among three or more groups were evaluated using One-way ANOVA analysis followed by LSD test. For the comparison between two groups, the independent samples t-test was performed. $P < 0.05$ was set to indicate statistical significance.

Results

Identification of key module genes related to MPRG score

A total of 2,320 DEGs1 (1,189 up-regulated and 1,131 down-regulated) were screened from the GSE124272 dataset, and 515 DEGs2 (206 up-regulated and 309 down-regulated) were screened from the GSE70362 dataset, as shown in **Figure 1**. 39 DEGs (16 up-regulated and 23 down-regulated) were obtained by overlapping DEGs1 and DEGs2. There were no outlier samples in IDD samples in GSE124272 and the optimal soft threshold (β) was finally chosen as 10 (**Figure 2A**, **2B**). By constructing a co-expression network and setting the minimum number of modules per gene to 300, 12 modules were obtained (**Figure 2C**). Highest correlation between MEturquoise module and MPRGs scores was taken as the key module,

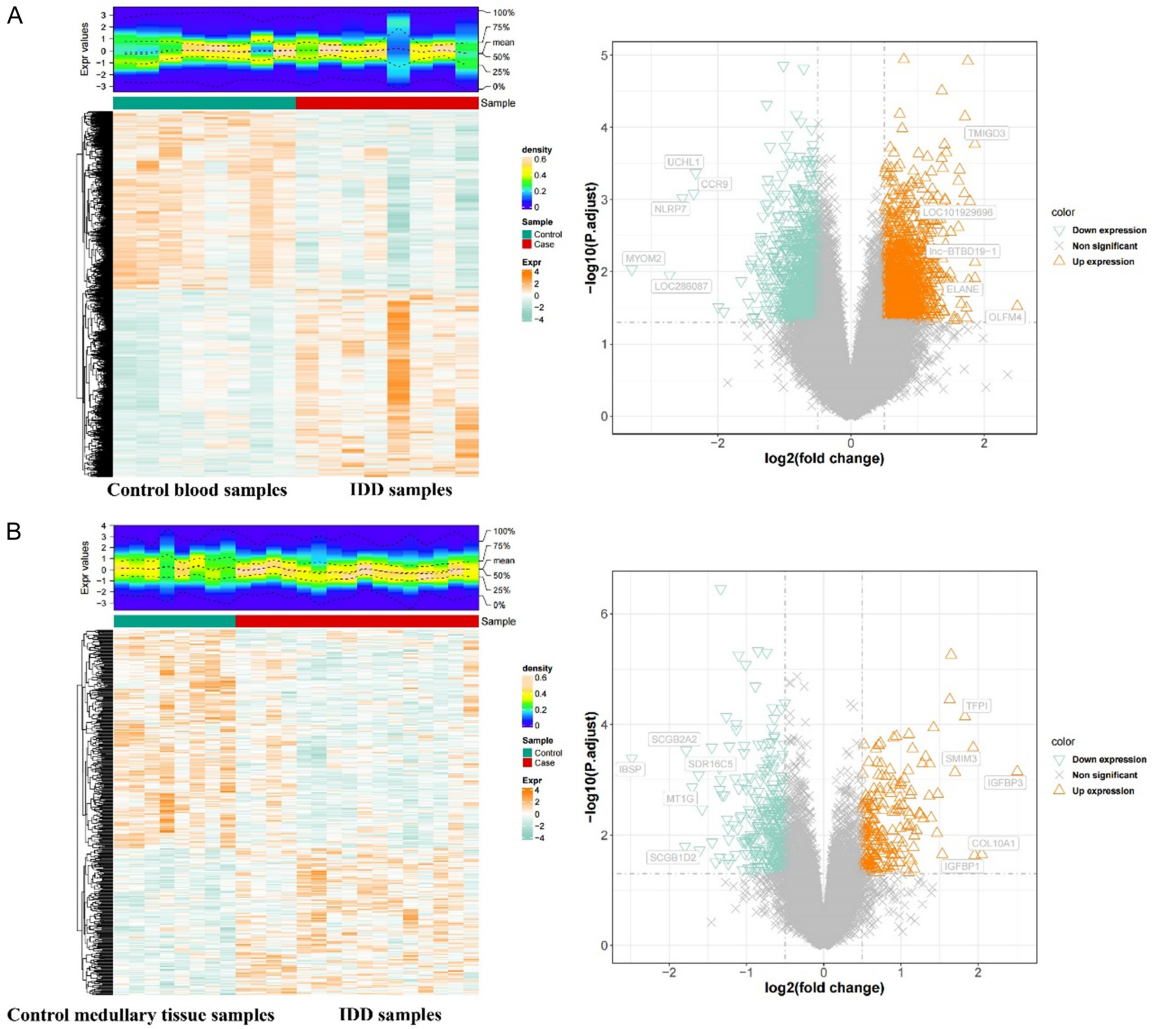

Figure 1. Differentially expressed genes (DEGs) were screened using heatmap and volcano methods between IDD samples and control samples. A: DEGs1; B: DEGs2. IDD: Intervertebral disc degeneration; DEGs: Differentially expressed genes.

which contained a total of 4,512 genes (**Figure 2D**). After screening, a sum of 1,633 key module genes were obtained (MM > 0.8 and GS > 0.4) (**Figure 2E**).

Identification of DE-MPRGs

Nine DE-MPRGs were obtained by overlapping DEGs and key module genes (**Figure 3A**). Functional enrichment analysis showed that DE-MPRGs were enriched in biological processes, such as hematopoietic or lymphoid organ development, immune system development, NK and T cell differentiation (GO entries) (**Figure 3B**). Additionally, DE-MPRGs were also enriched in Mucin type O-glycan biosynthesis (KEGG entries) (**Figure 3C**).

Identification of biomarkers: ST6GALNAC2, SMIM3, and IFITM2

A sum of 7 candidate genes were obtained by SVM-RFE algorithm, including ST6GALNAC2, SMIM3, TLR1, IFITM2, ZNF469, SLC40A1 and UBE2D1 (**Figure 4A**). Among these, three biomarkers, ST6GALNAC2, SMIM3 and IFITM2, were highly expressed in IDD (**Figure 4B-D**). The Area Under Curve (AUC) values of these biomarkers in the GSE124272 dataset were all greater than 0.7, indicating high predictive performance. This finding was corroborated by consistent results in GSE70362 (AUC > 0.7) (**Figure 4E**, **4F**). GSEA results showed that ST6GALNAC2 was mainly enriched in GO terms such as GOBP_NCRNA_METABOLIC_PROCESS,

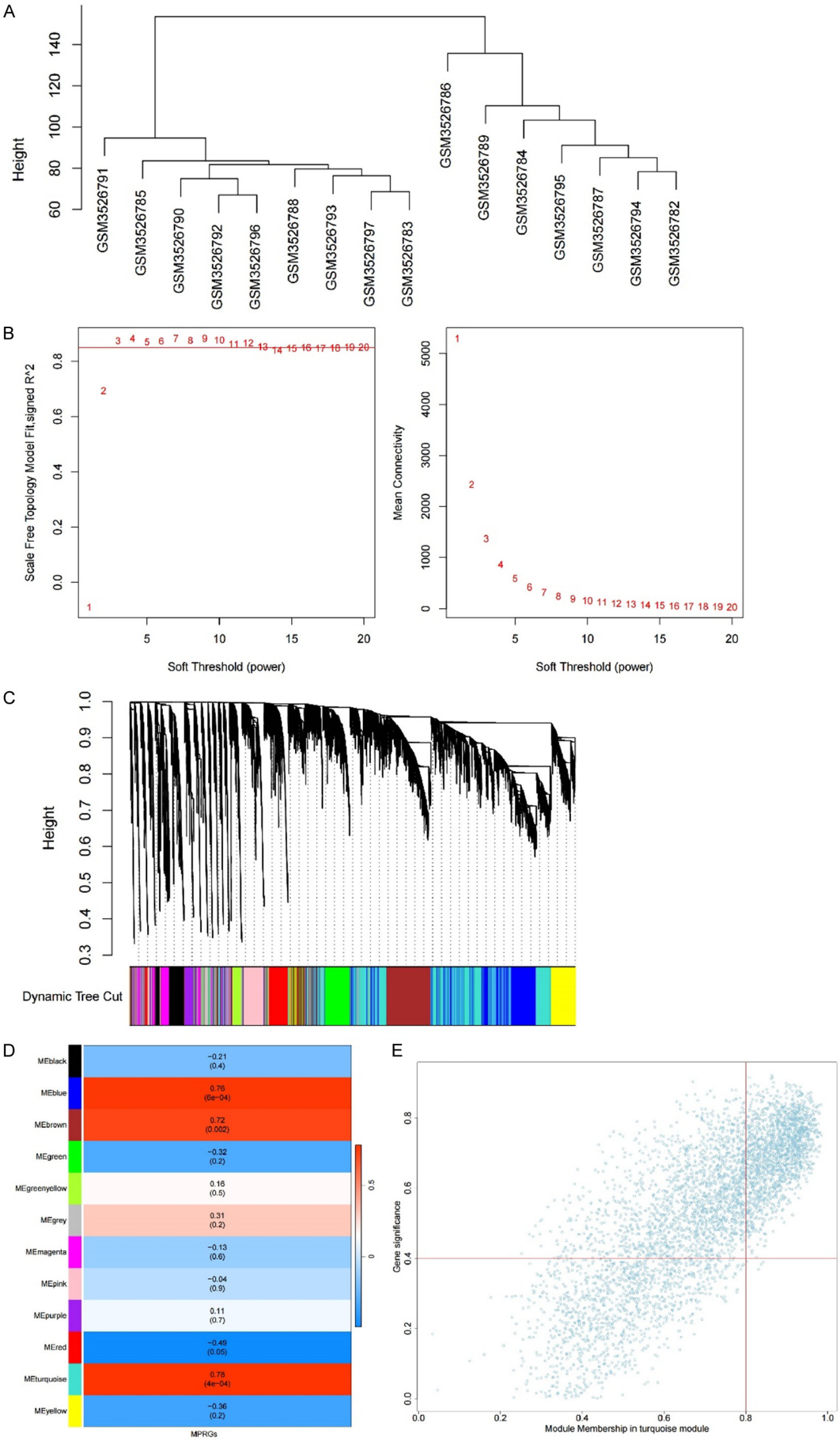
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Figure 2. Key module genes were obtained using weighted gene co-expression network analysis (WGCNA). A: Sample dendrogram and trait heatmap; B: Scale independence and mean connectivity; C: Gene dendrogram and module colors; D: Module-trait relationships; E: Trait MPRGs.

and enriched in KEGG terms such as KEGG_RIBOSOME (**Figure 5**), IFITM2 was mainly enriched in GO terms such as GOBP_NCRNA_METABOLIC_PROCESS, and enriched in KEGG terms such as KEGG_RIBOSOME (**Figure 6**), and SMIM3 was mainly enriched in GO terms such as GOBP_RIBONUCLEOPROTEIN_COMPLEX_BIOGENESIS, and enriched in KEGG terms such as KEGG_RIBOSOME (**Figure 7**).

Functional analysis of biomarkers

There was an evident difference in the infiltration of the three immune-related pathways (Checkpoint, Cytolytic_activity, T_cell_co-stimulation) between the IDD and control groups, and the immune score of three immune-related pathways was higher in the control group (**Figure 8A**). There was a difference in totals of three types of immune cells, with Neutrophils having a high immune score in the IDD group, and Th2_cells and TIL having a high immune score in the control group (**Figure 8B**). Spearman correlation results showed that ST6GALNAC2 had the most negative correlation with B cells. IFITM2 had the highest positive correlation with MHC_class_I and Neutrophils, and the highest negative correlation with Th2_cells. SMIM3 had the highest negative correlation with the checkpoint immune-related pathway (**Figure 8C**). The results of biomarkers in relation to pain and IDD showed that IFITM2 and SMIM3 were associated with IDD and Nociceptive pain, showing the highest Nociceptive Pain score (**Figure 8D**). A total of 18 miRNAs and 50 lncRNAs were acquired, and a network of lncRNA-miRNA-mRNA containing 192 interactive relationship pairs was constructed, including the SMIM3-hsa-miR-107-LINC02381 pair in the network (**Figure 9**).

The expression levels of the biomarkers

The RT-qPCR results indicated that ST6GALNAC2 and IFITM2 were highly expressed in IDD. However, no difference in SMIM3 expression level was found between the IDD group and control group (**Figure 10**).

Discussion

With the growing prevalence in the aging population, intervertebral disc degeneration (IDD) is one of the most common degenerative diseases. There is a pressing need to identify the most effective treatment for IDD. So far, despite years of attempts and efforts, the exact mechanisms of IDD are still unclear, and effective therapies remain lacking [16]. Although some biomarkers have been obtained in previous research, there has been little focus on a comprehensive exploration of macrophage polarisation-related genes (MPRGs) in the process of IDD [17]. Hence, this study aimed to investigate the biomarkers related to an IDD signature and to further investigate the relationship of MPRG biomarkers with the immune response based on a comprehensive analysis.

We used differential expression analysis, the WGCNA analysis, and SVM-RFE algorithm to screen characteristic MPRGs related to the IDD process. First, we gained 9 MPRGs by overlapping genes from differentially expressed analysis and key modules of the WGCNA. Next, GO and KEGG pathway analyses revealed that these genes were mainly involved in immune system development, and NK and T cell differentiation, suggesting that the inflammatory response might be a crucial pathologic process of IDD. Moreover, ST6GALNAC2, SMIM3, and IFITM2 were identified as biomarkers by expression level analysis. Finally, we discovered three immune-related pathway infiltrates (Checkpoint, Cytolytic_activity, T_cell_co-stimulation) in the IDD process and found that neutrophils Th2_cells, and TIL were closely implicated with immune response. To the best of our knowledge, studies of MPRGs and immune response related to IDD process are scarce [18], and our study may provide new insight into the pathogenesis of IDD by investigating the cross-talk between these biomarkers and immune cells.

The immune response has been considered a vital driver of inflammation, and different immune cells exert various roles in the IDD process. Previous studies found that intervertebral disc could be an immune privileged organ, and the autoimmune reaction was triggered as the rupture of outer annulus [19]. The infiltration of immune cells such as macrophages may release a large amount of proinflammatory mol-

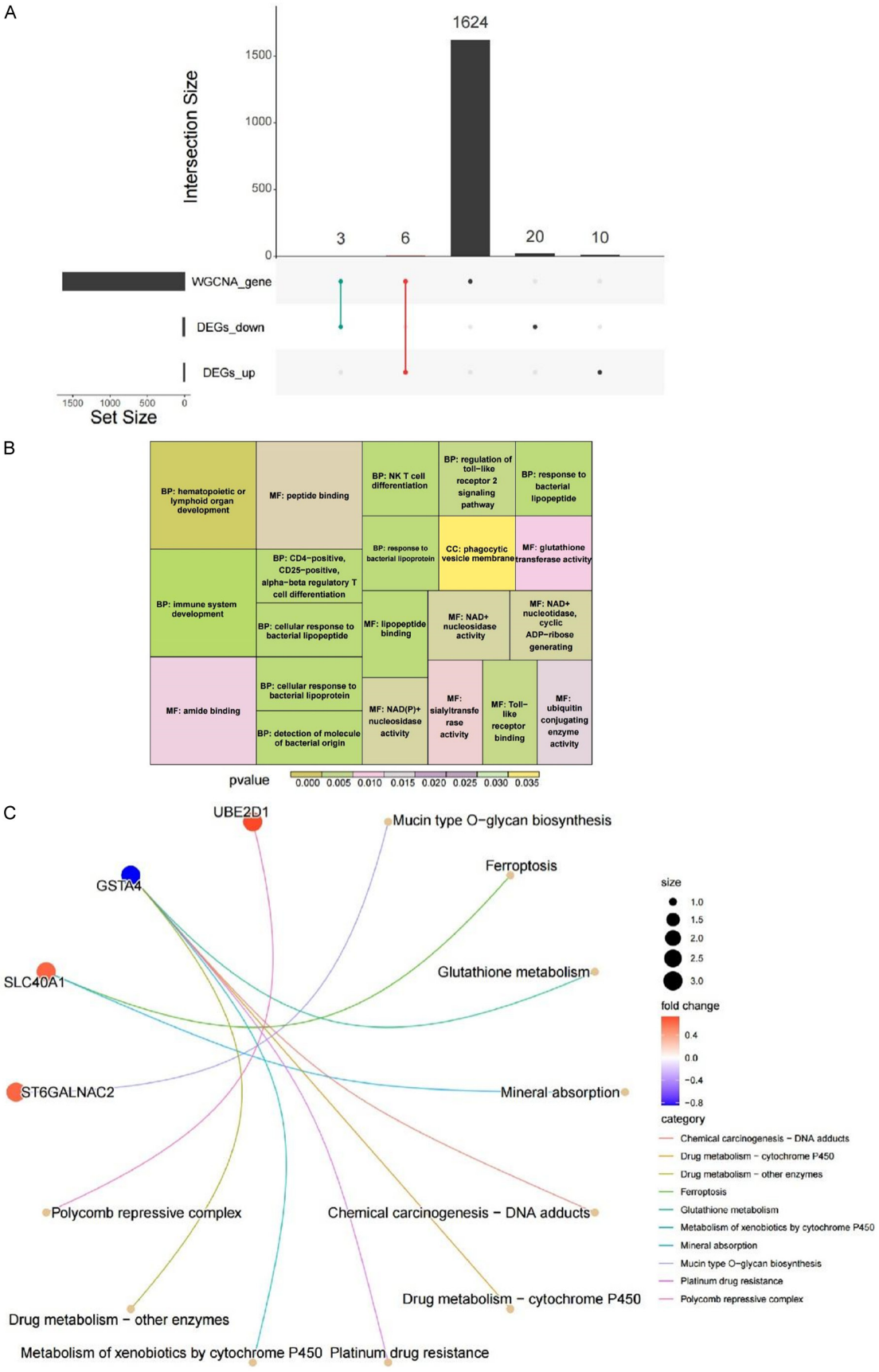


Figure 3. Functional enrichment analysis of DE-MPRG. A: Overlapping genes between DEGs and key module genes; B: GO analysis; C: KEGG analysis.

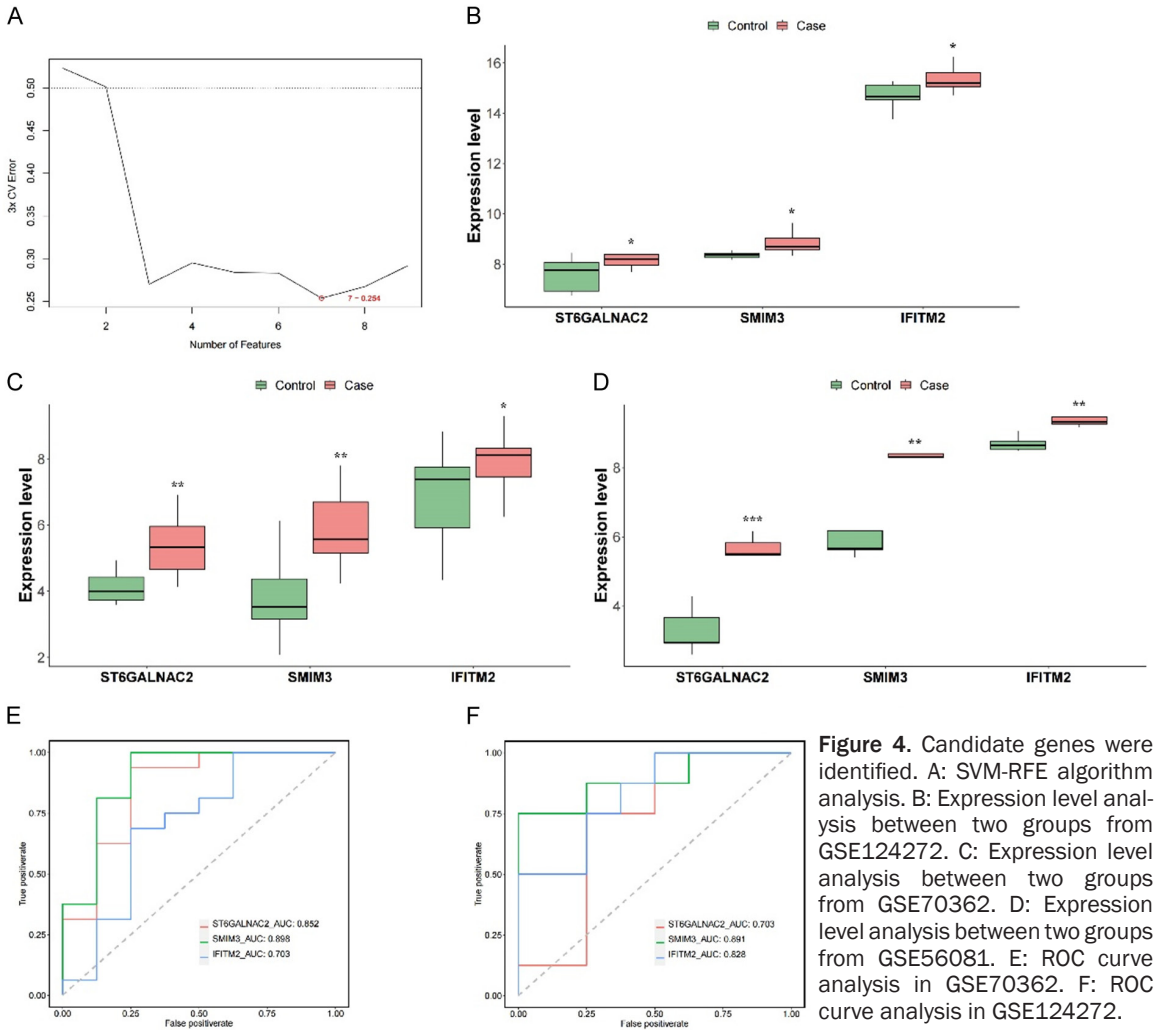


Figure 4. Candidate genes were identified. A: SVM-RFE algorithm analysis. B: Expression level analysis between two groups from GSE124272. C: Expression level analysis between two groups from GSE70362. D: Expression level analysis between two groups from GSE56081. E: ROC curve analysis in GSE70362. F: ROC curve analysis in GSE124272.

ecules and enhance the inflammation cascade within the disc. Some studies demonstrated that the immune infiltration landscape varied obviously between IDD patients and healthy controls, and macrophages were involved in IDD development as a key immune player [20, 21], which is in accordance with this study. Many studies showed that the cross-talk between macrophages and IDD tended to polarize macrophages toward a more pro-inflammatory state, accelerating intervertebral disc degeneration. In response to injury, M1 macrophage polarization presented a proinflammatory effect while M2 macrophage polarization played an anti-inflammatory and remodeling effect [22, 23].

It has been reported that the ST6GALNAC2 influenced metastasis in various tumors, including breast cancer, brain cancer, colorectal cancer, and lung cancer [24, 25]. Another study showed that ST6GALNAC2 was active on glycoproteins [26]. However, its involvement in intervertebral disc degeneration (IDD) has not been previously documented. In this study, ST6GALNAC2 emerged as a macrophage polarization genetic biomarker in the IDD process, showing marked differences in expression between IDD patients and healthy controls. SMIM3 is expressed in various tissues, with highest expression in heart and the lowest expression in skeletal muscle. It has been identified with an important role in cell channel regulation and an association with neuronal differentiation [27]. Some studies showed that SMIM3 could be used as a sensitive and specific biomarker of radiation exposure, with a poor prognosis [28]. Other studies have recognized SMIM3 as a prognostic biomarker in oral squamous cell carcinoma [29]. There is mini-

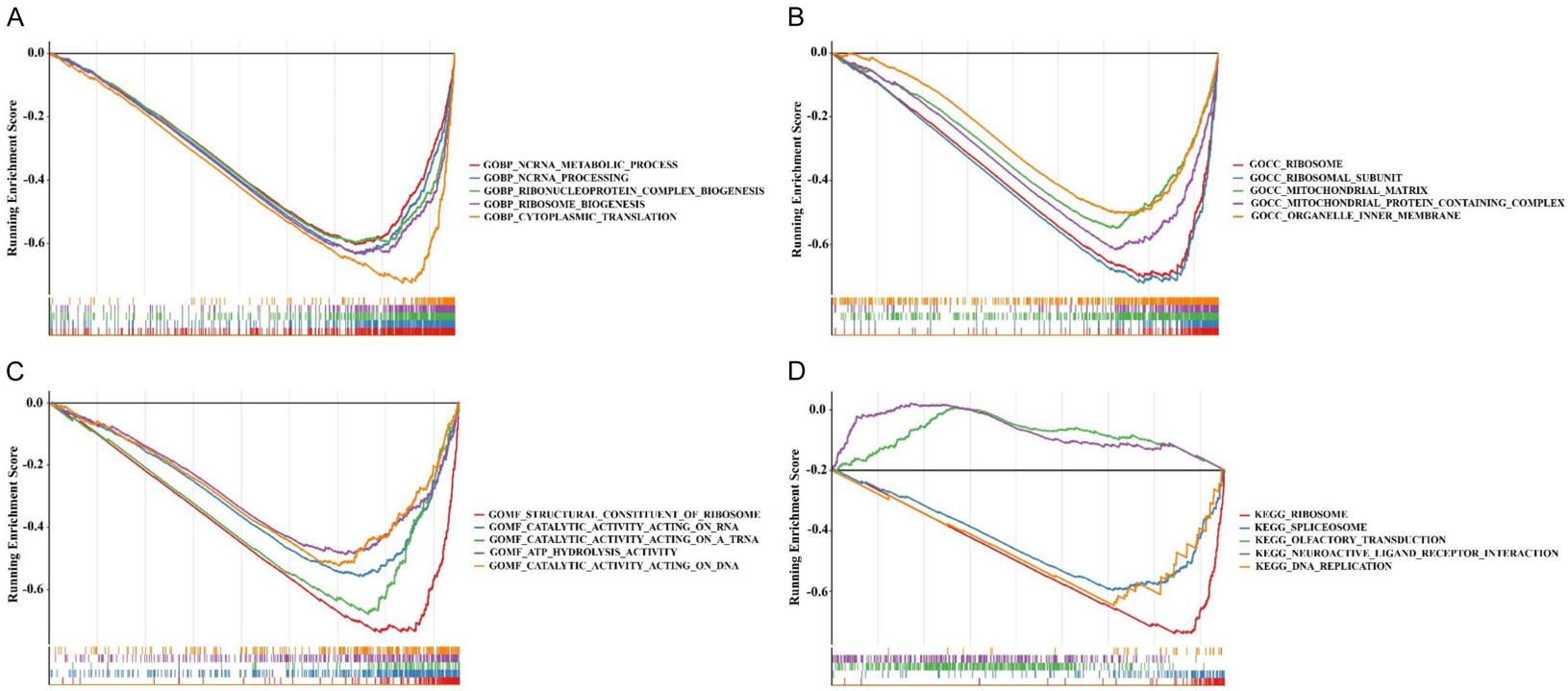


Figure 5. Gene set enrichment analysis (GSEA) of ST6GALNAC2. A: Analysis of Gene Ontology Biological Process. B: Analysis of Gene Ontology Cellular Component. C: Analysis of Gene Ontology Molecular Function. D: Analysis of KEGG. KEGG: Kyoto Encyclopedia of Genes and Genomes.

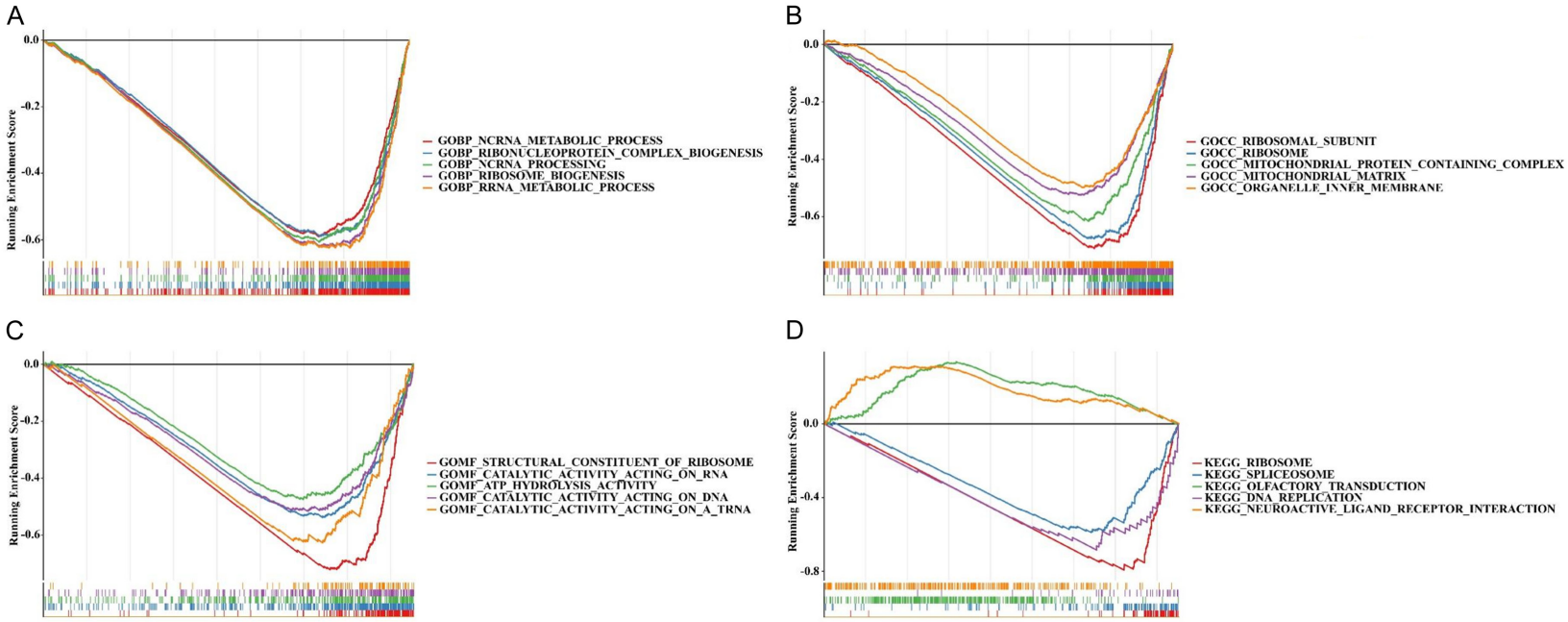


Figure 6. Gene set enrichment analysis (GSEA) of IFITM2. A: Analysis of Gene Ontology Biological Process. B: Analysis of Gene Ontology Cellular Component. C: Analysis of Gene Ontology Molecular Function. D: Analysis of KEGG. KEGG: Kyoto Encyclopedia of Genes and Genomes.

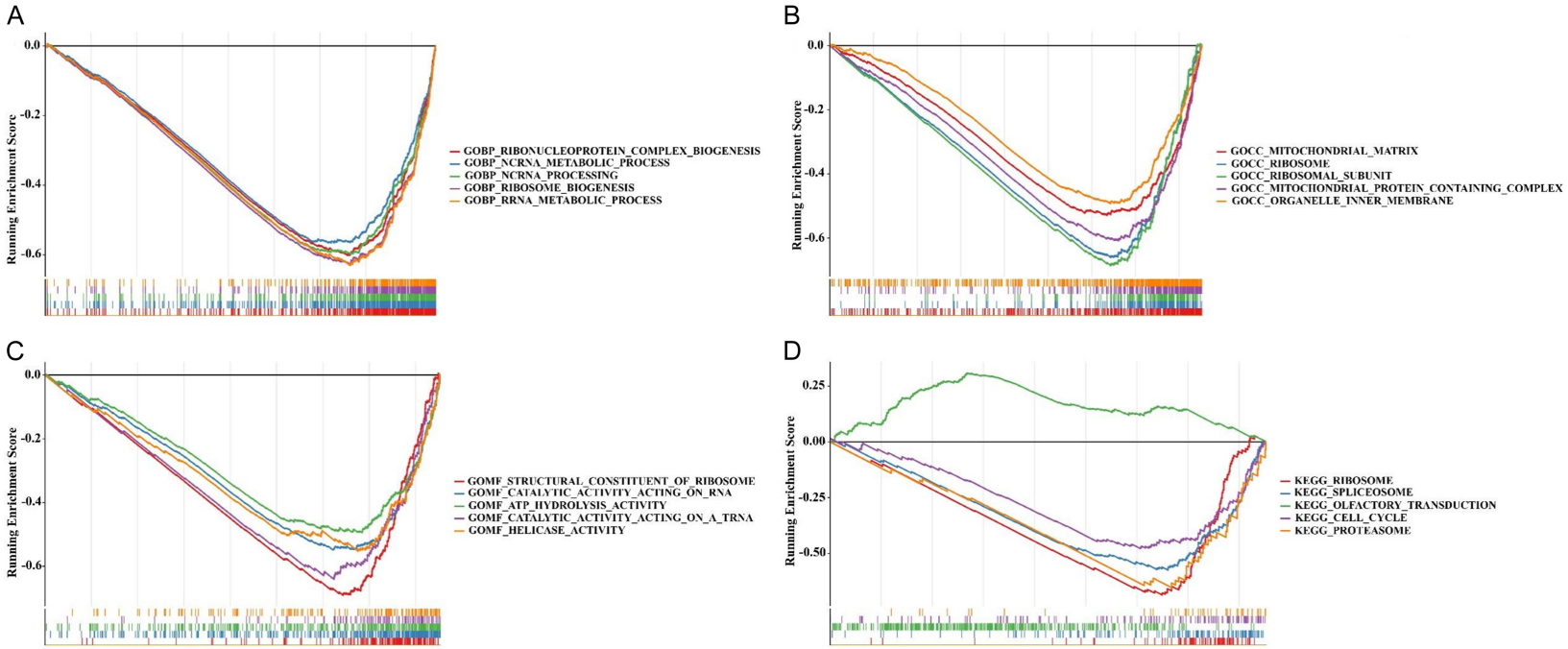
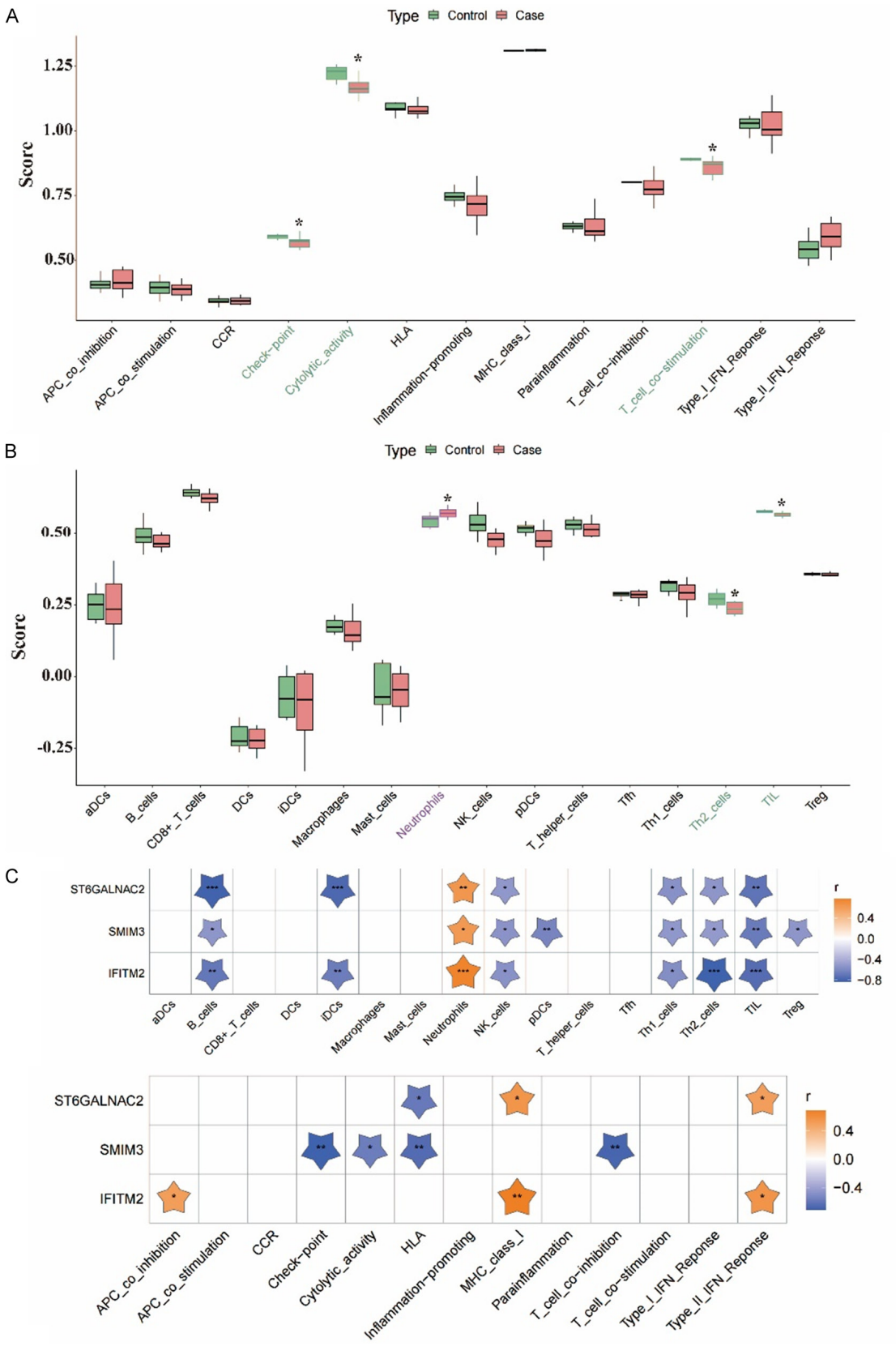


Figure 7. Gene set enrichment analysis (GSEA) of SMIM3. A: Analysis of Gene Ontology Biological Process. B: Analysis of Gene Ontology Cellular Component. C: Analysis of Gene Ontology Molecular Function. D: Analysis of KEGG. KEGG: Kyoto Encyclopedia of Genes and Genomes.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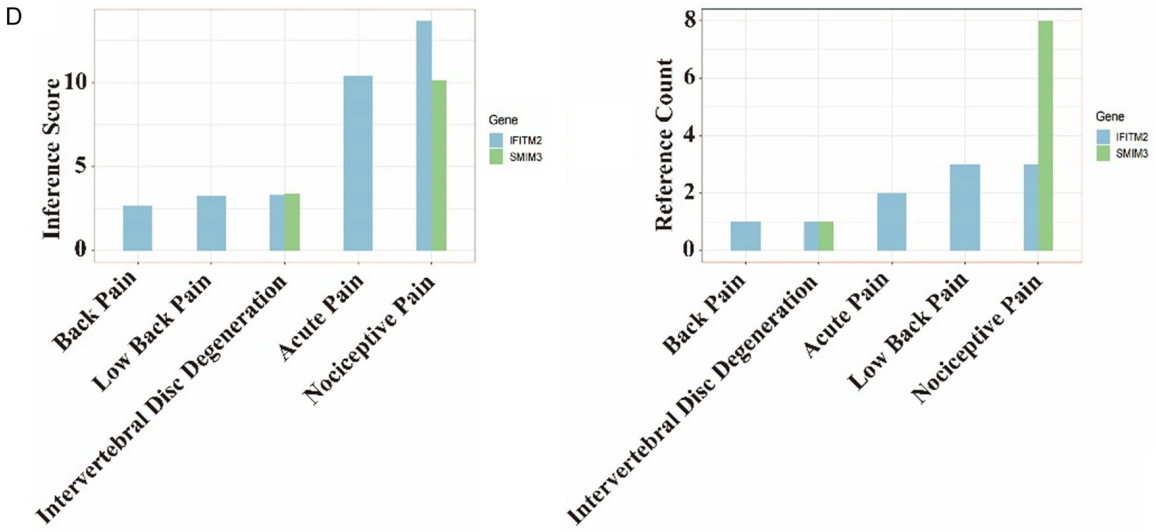

Figure 8. Functional analysis of biomarkers. A: Significant differences for three immune-related pathways were observed between IDD group and control group, *P < 0.05 vs control group. B: Significant differences for three types of immune cells were found between the IDD group and control group, *P < 0.05 vs. control group. C: Spearman correlation results of ST6GALNAC2, IFITM2, and SMIM3. D: IFITM2 and SMIM3 could predict IDD and nociceptive pain.

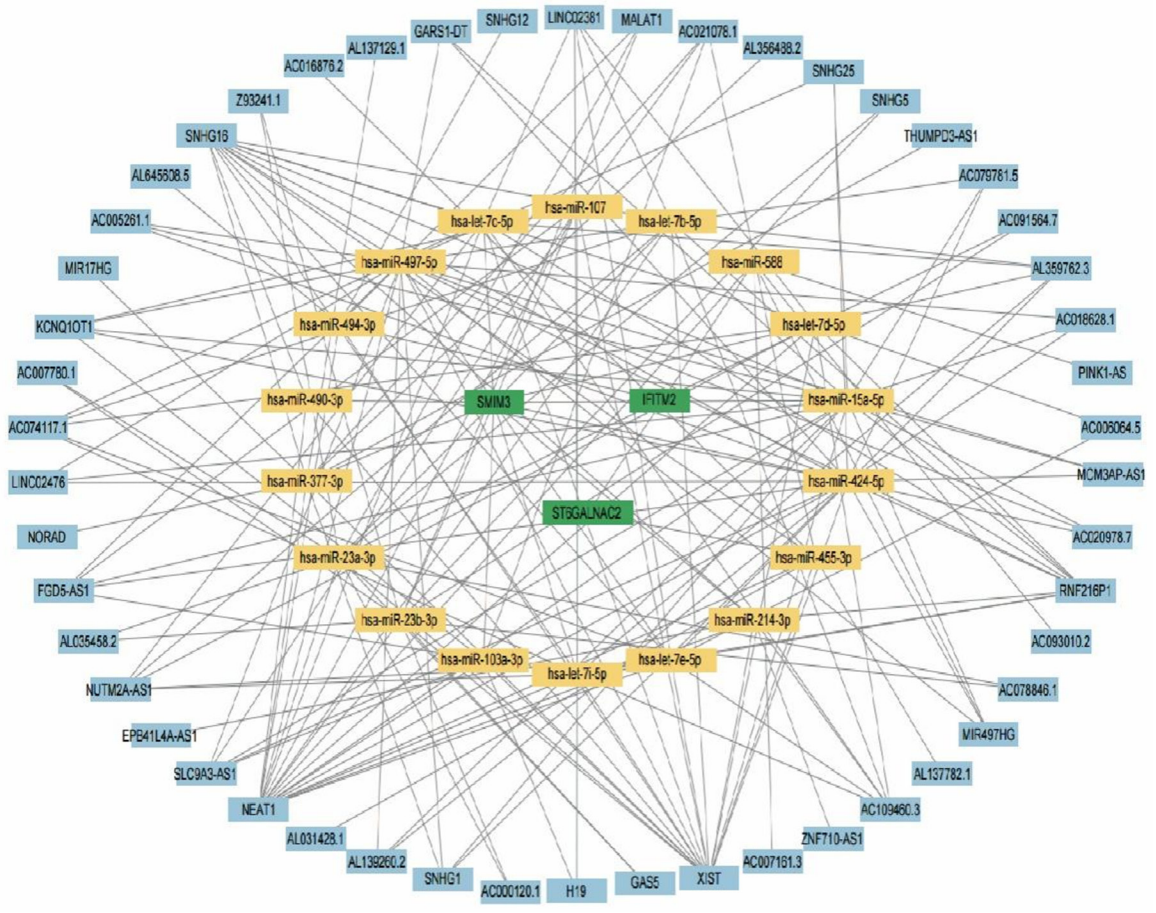

Figure 9. Network of lncRNA-miRNA-mRNA.

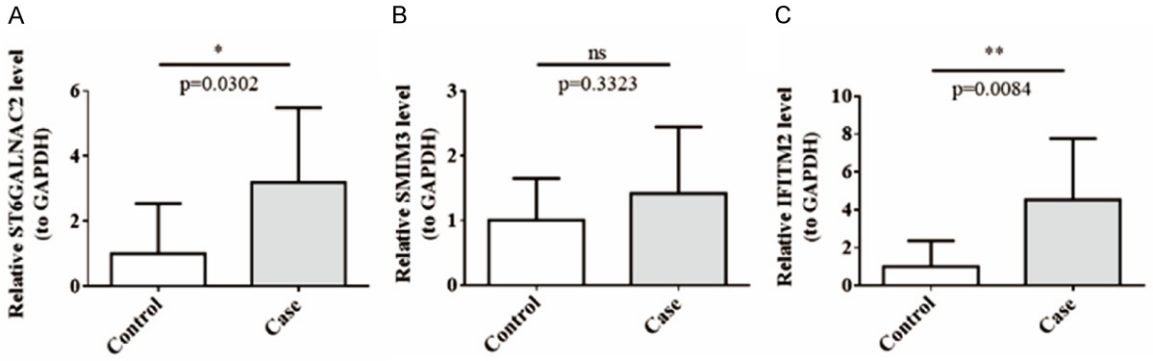

Figure 10. Expression levels of the biomarkers. A: The expression level of ST6GALNAC2, *P < 0.05 vs. control. B: The expression level of SMIM3. C: The expression level of IFITM2, **P < 0.01 vs. control.

mal research on SMIM3, and it had not been investigated in IDD process. In this study, SMIM3 was found to be a biomarker for the development of IDD. IFITM2 is one of human IFITM family members, which consisted of five proteins. IFITM are thought not only to act by antagonizing virus-cell membrane fusion, but also to have a role in regulation of immune responses, such as innate antiviral and inflammatory responses, and adaptive T-cell and B-cell responses [30]. Some studies showed elevated inflammatory signaling in IFITM-deficient models [31]. Other studies provided evidence for a proinflammatory role of IFITMs in an allergic airway inflammation model [32]. It was found that IFITM2 was expressed in peripheral T cells, with its expression regulated by TCR/CD28 ligation, which rapidly regulates IFITM2 [33]. Another study indicated that IFITM2 was critical for Th2 differentiation and inhibition of Th1 differentiation [34]. In this study, IFITM2 was highly expressed in IDD patients compared to the control group, and it was closely associated with the IDD process through regulating the immune response.

Notably, there are still few studies on the interaction of MPRGs and immune response in the pathogenesis of IDD. The rigorous bioinformatic analysis provided reliable MPRG-related biomarkers in the process of IDD and paved the way for future treatment strategies studies for IDD by targeting the MPRGs and immune modulation. Nevertheless, there are some limitations in this study. First, this research was completely based on the public datasets with a small sample size, which may lead to a biased explanation. Second, RT-qPCR analysis was used to confirm the biomarker expression, and *in vitro* and *in vivo* studies further for validation were lacking. Therefore, in-depth biological experiments on macrophage polarization and inflammatory response are needed.

In conclusion, the present study found that the expression level of MPRGs significantly differed between healthy control and IDD patients. Additionally, based on this comprehensive bioinformatic analysis, ST6GALNAC2, SMIM3 and IFITM2 were identified and the correlations between these biomarkers and immune-related pathways or immune cells were analyzed. These findings may extend our knowledge regarding the inflammatory response in IDD patients and provide new targets for IDD.

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Disclosure of conflict of interest

None.

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