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IDENTIFICATION OF KEY GENES IN INTERVERTEBRAL DISK DEGENERATION USING AN IN VIVO RAT MODEL

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ABSTRACT
In the present study, we established a rat model of intervertebral disc degeneration (IDD). RNA sequencing was used to identify differentially expressed genes (DEGs) between the intervertebral discs of IDD model and control rats. Metascape website was used for bioinformatics analyses, and the key genes in IDD were identified. The expression of key genes was examined by qRT–PCR and immunofluorescence staining. A total of 586 DEGs were identified between the IDD and control groups. Bioinformatics analysis revealed that these DEGs were enriched in 13 GO MF terms, 28 GO CC terms, 136 GO BP terms, 33 KEGG pathways, and TNF-α, TLR2, ITGAM, TTN and TCAP were identified as key genes in IDD. QRT–PCR and immunofluorescence staining revealed that the expression levels of TNF, TLR2, ITGAM and TTN were greater in degenerated disc tissues than in normal disc tissues, whereas the expression level of TCAP was lower in degenerated disc tissues than in normal disc tissues. In conclusion, our study revealed that TNF-α and TLR2 are key genes in IDD, and that ITGAM, TTN, TCAP and the calcium signaling pathway may play key roles in IDD. Our research may provide new clues for future studies exploring the pathogenesis of IDD.

KEYWORDS: Intervertebral disk degeneration, rat model, differentially expressed genes, bioinformatics analysis, qRT- PCR, immunofluorescence staining.

INTRODUCTION
A Global Burden Report from 2019 revealed that low back pain has become the main cause of disability worldwide.[1] Degenerative disc disease, which results from intervertebral disc degeneration (IDD), is believed to be the primary cause of low back pain.[2,3] Although the pathophysiology of IDD has been extensively studied[4,5], many unknowns still exist, and the underlying mechanism of IDD has not been fully elucidated. Several researchers have investigated the gene expression profiles of degenerated intervertebral discs via comparisons of the disc tissue of donors after brain death[6], while others have investigated the gene expression profile of the peripheral blood of patients with IDD via comparisons with the peripheral blood of healthy volunteers.[7] However, because early degeneration has been shown to occur in 20% of teenagers[8,9], these studies cannot avoid the bias caused by early degeneration or slight degeneration of human intervertebral disc tissue in the control group due to the age mismatch between the observation group and the control group. Thus, we conducted animal experiments to avoid such bias and obtain and analyze the sequencing data of degenerated intervertebral discs, hoping to further study the pathological changes associated with IDD.

MATERIALS AND METHODS
Animals experiments
The animal experiments in the present study were approved by the Ethics Committee of Sichuan Province Orthopedic Hospital, and national and institutional guidelines for the care and use of laboratory animals were followed. Chengdu Dasuo Experimental Animal Company supplied 8 healthy male 8-week-old Sprague–Dawley (SD) rats (220 ± 30 g; SPF level). SD rats were allowed to adapt to the environment of the

animal center where the animal experiments were conducted for one week and had ad libitum access to standard rat food and water. An SD rat model of IDD was established via anterior approach surgery following a modified procedure introduced by Huang.[10] Four model SD rats and 4 control SD rats were reared for 8 weeks before sampling.

Hematoxylin–eosin (HE) staining

After the discs were fixed with 4% paraformaldehyde, ethylene diamine tetraacetic acid was added, and the mixture was replaced every 3 days. Prior to paraffin embedding and sectioning, decalcification was performed for 1 month. Subsequently, the disc tissue was subjected to HE staining.

RNA extraction and sequencing

Total RNA from 4 control group and 4 IDD model group samples were extracted separately using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. Then, DNase I was added to remove contaminating genomic DNA. The RNA quality and quantity were measured via a NanoDrop spectrophotometer (Thermo Scientific, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, USA). RNA integrity was determined by 1% gel electrophoresis. Equal amounts of total RNA from the intervertebral disk samples were pooled into a model group and a control group.

For high-throughput sequencing, ribosomal RNA (rRNA) was depleted from total RNA via the Ribo-Zero™ rRNA Removal Kit (Human/Mouse/Rat; Epicenter, USA) according to the manufacturer's protocol. The cDNA libraries were prepared with a ScriptSeq™ v2 RNA-Seq Library Preparation Kit (Epicenter, USA) and sequenced on an Illumina HiSeq X ten paired-end reads at GeneX Health, Beijing, China. RNA-seq read mapping and transcriptome assembly were performed by GeneX Health (Beijing, China).

Differential gene analysis

Fragments per kilobase per million reads were used to normalize the RNA sequence reads of all the samples. R package DESeq. 2 (version v4.3.3) was used to identify the differentially expressed genes (DEGs) between the disc tissues of IDD model SD rats and those of control rats ($p < 0.05$, log 2-fold change (FC) ≥ 1.5 or ≤ -1.5).

Gene Ontology (GO) and Kyoto Encylcopedia of Genes and Genomes (KEGG) pathway analyses

All DEGs were input into the Metascape website for enrichment analysis[11], which included GO enrichment analysis to determine the enriched biological process (BP), cellular component (CC), and molecular function (MF) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis.

Construction of the Protein–protein interaction (PPI) network

A PPI network of DEGs with a combined core > 0.35 was also constructed using the Metascape website. The PPI network was subsequently visualized via Cytoscape software (version 3.10.2; http://www.cytoscape.org/), and the disconnected nodes were excluded. Significant submodules were constructed via the plug‑in MCODE, and the centrality degree of each gene in the PPI network was calculated via the plug‑in CentiScaPe, as we previously described.[12]

Validation by quantitative real-time polymerase chain reaction (qRT–PCR)

QRT–PCR was performed to validate the expression levels of key DEGs from the RNA-seq data analysis via the 2-ΔΔCq method. GAPDH was used as an internal control. Specific primers for the DEGs were designed with Primer 5.0 and synthesized as we previously described.[12]

Immunofluorescence staining

To identify proteins associated with the key DEGs, we performed immunofluorescence staining. Tissues from the intervertebral discs were cut into 5-μm-thick pieces, embedded in paraffin and fixed in 4% paraformaldehyde for 48 hours. An antigen retrieval buffer was added to the sections, which were then blocked for 30 min in 5% BSA. Anti-Tlr 2 (1:100 dilution; Abcam, #AB213676), anti-Itgam (1:100 dilution; Affinity, #DF7585), anti-Titin (1:100 dilution; Affinity, #DF12549), anti-Tcap (1:100 dilution; Affinity, #DF2248), and anti-Tnf-α (1:100 dilution; Boster, #BA0131) antibodies were added and incubated overnight in an enclosed tissue box at 4°C. Before incubation with the secondary FITC-labeled goat anti-rabbit (1:100 dilution; Servicebio, GB22303) antibody for 30 min at room temperature, PBS was used to wash the discal tissue slices. To stain the cells, the tissue sections were incubated with 2 g/mL 2-(4-amidinophenyl)-6 indolecarbamidine dihydrochloride (DAPI; Servicebio, Wuhan, China). Then, the sections were extensively washed with PBS.

RESULTS

Histological staining with HE

HE staining revealed that the intervertebral discs of the control rats were intact, with layered rings and no signs of rupture or disorder, and the central part of the nucleus pulposus was oval-shaped and large (Figure 1 A-a). Additionally, there was no obvious inflammatory cell infiltration and suitable continuity among the cells (Figure 1 A-b). In the model group, the intervertebral disc tissue was stained unevenly, with disordered structures and changes and cracks in the annulus. The nucleus pulposus had shrunk or disappeared, and the collagen content was significantly reduced(Figure 1 B-a), resulting in a large amount of chondrocyte-like necrosis, nuclear fragmentation or lysis and an increased number of eosinophils in the cytosol (Figure 1 B-b).

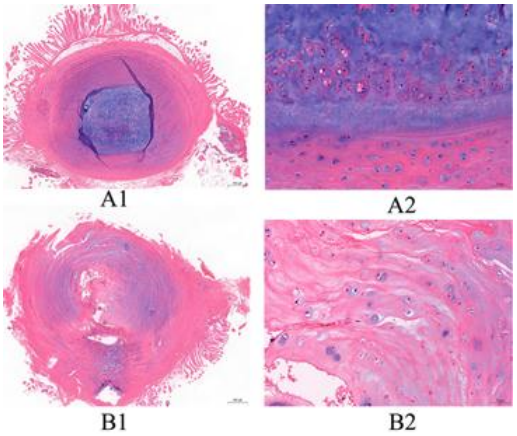

Figure 1: HE staining was performed to evaluate the pathological changes of intervertebral disc degeneration. A: normal intervertebral disc; B: degenerated intervertebral disc.

DEGs

A total of 586 DEGs were identified between degenerated and control discs, including 269 upregulated genes and 317 downregulated genes. Figure 2 shows a heatmap of DEG expression in both groups.

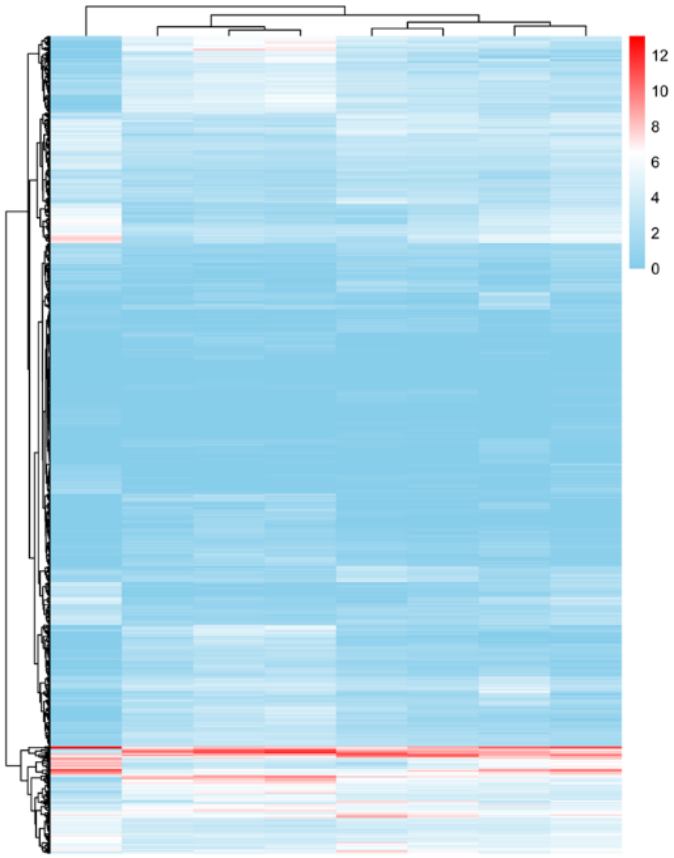

Figure 2: Heatmap of differentially expressed genes.

Enrichment analysis of DEGs

Enrichment analysis of DEGs via Metascape revealed 13 GO MF terms, 28 GO CC terms, 136 GO BP terms, and 33 KEGG pathways. The results of the Metascape analysis suggested that the significantly enriched GO terms were muscle structure development and myofibrils and that the significantly enriched KEGG pathways were the calcium signaling pathway and pathways related to hypertrophic cardiomyopathy (Figure 3).

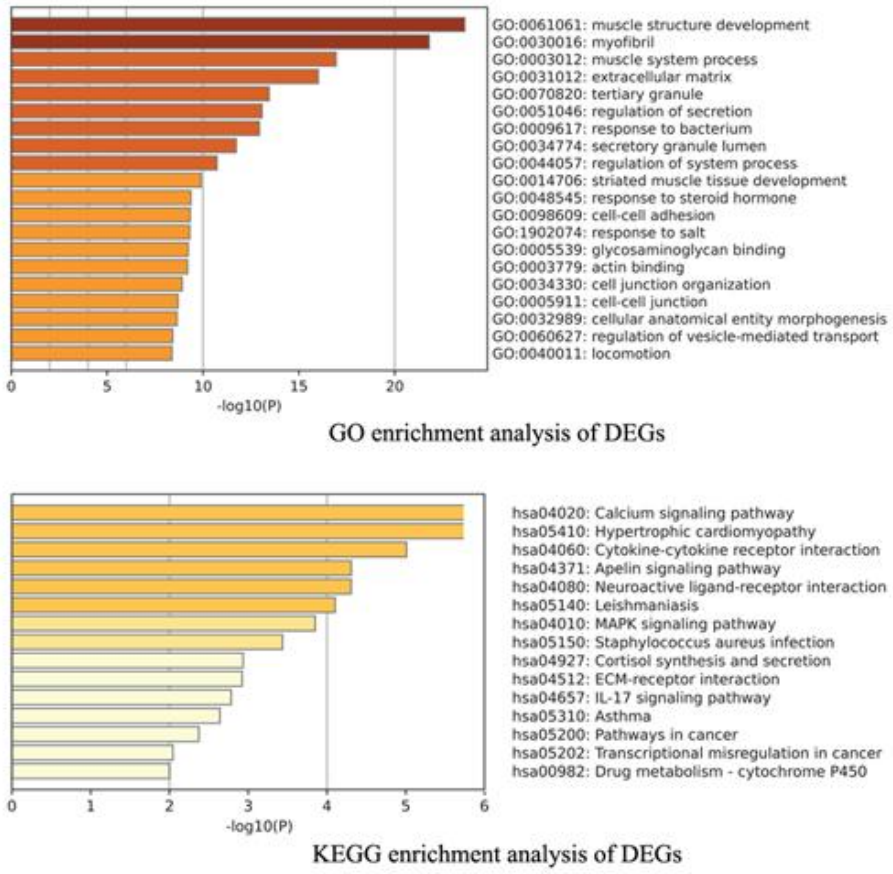

Figure 3: Top 20 significant enriched Gene Ontology terms and top 15 significant enriched KEGG pathways according to the P-values.

By representing each enriched term as a node and connecting pairs of nodes with kappa scores above 0.3, a network of the top 20 significant clusters of enriched GO terms was constructed in Metascape (Figure 4).

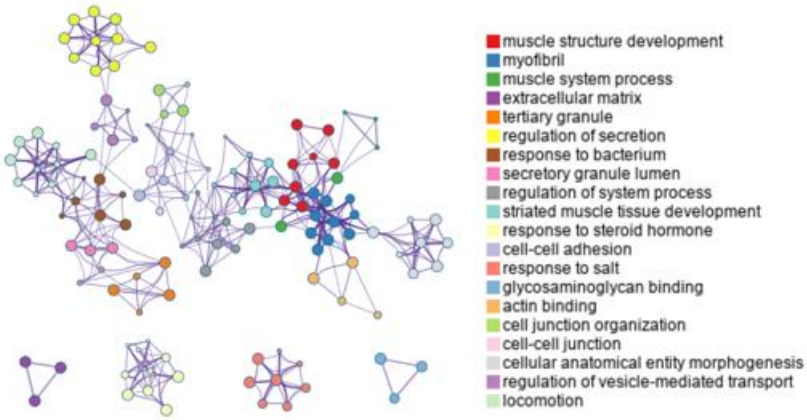

Figure 4: Network of the top 20 significant clusters of the enriched terms according to P-value. Enrichment networks were created by representing each enriched term as a node and connecting pairs of nodes with kappa scores above 0.3. Each node represents an enriched term and is col-oured by cluster.

PPI network of DEGs

A total of 1066 edges and 414 connected nodes composed a PPI network of the DEGs (Figure 5). Twelve submodules separated by MCODE analysis are displayed in different colors in Figure 5.

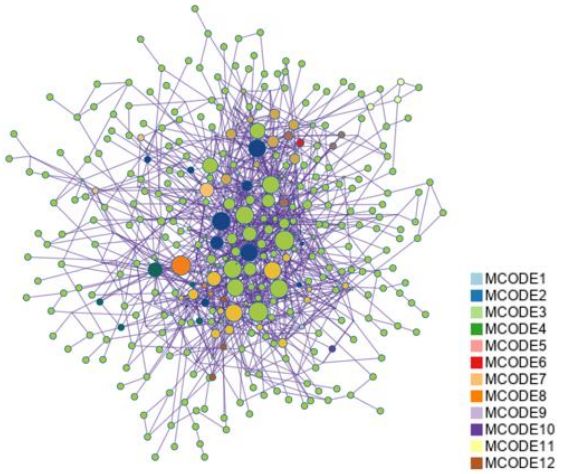

Figure 5: PPI network of differentially expressed genes. MCODE analysis identified 12 clusters.

The genes in the PPI network were sorted by the centrality degree of each node calculated by the plug-in CentiScaPe, and the top 5 genes with the highest centrality degree were identified as key genes in IDD (Table 1).

Table 1: The top five genes with the highest centrality degree.

Gene	Centrality Degree	Cluster
Tnf-α	84	Cluster 3
Tlr2	47	Cluster 2
Itgam	41	Cluster 2
Ttn	39	Cluster 1
Tcap	30	Cluster 1

Validation of the mRNA expression levels of the hub genes

The gene chip hybridization results of tumor necrosis factor-alpha (TNF-α), toll-like receptor 2 (TLR2), integrin subunit alpha M (ITGAM), titin (TTN) and titin-cap (TCAP) were verified by qRT–PCR(Figure 6).

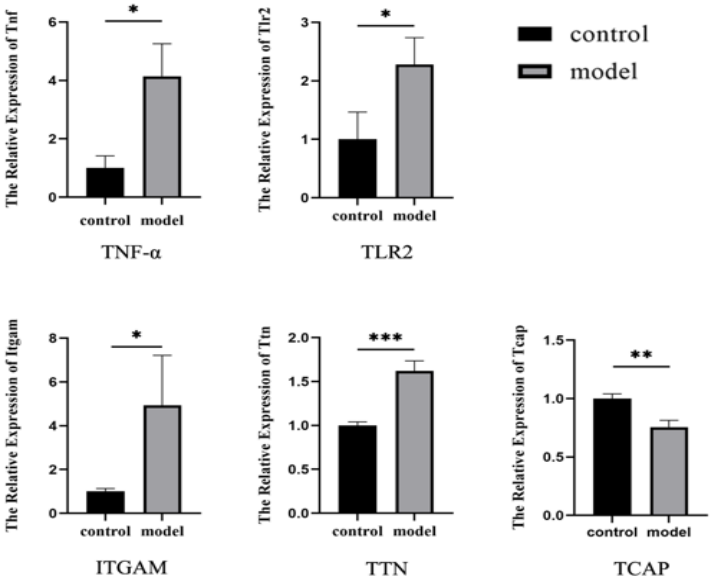

Figure 6: Expression of key genes.

The expression levels of TNF, TLR2, ITGAM and TTN were greater in degenerated disc tissues than in normal disc tissues. The expression level of TCAP was lower in degenerated disc tissues than in normal disc tissues. The primers designed for qRT–PCR are listed in Table 2.

Table 2: Sequences of primers used for quantitative real-time polymerase chain reaction.

Gene	Sequence (5' to 3')
Tnf-α	F: AGAAGTTCCCAAATGGCCTC
	R: TACAACCCATCGGCTGGCAC
Tlr2	F: AGAAAGCTCCTTGCAGGGAC
	R: TGATCCATTTGCCCGGAACA
Itgam	F: GCTCCTCAAGGTCGTTGTGA
	R: AGATGGCGTACTTCACAGGC
Ttn	F: AGACAATTGCTGCATCCGTG
	R: TGCAGAGCCGTGATGATGTG
Tcap	F: TTCAGAGCTGAGCTGCCAAG
	R: GGTGGTAGGTCTCATGCCTC

Immunofluorescence staining

To explore the protein expression of key DEGs in the intervertebral disc tissue of both groups, TLR-2, TNF-α, TITIN, TCAP and ITGAM in rat disc tissue were detected via immunofluorescence staining. As shown in Figure 7, TNF-a-, TLR-2-, ITGAM- and TTN-positive cells (green fluorescence) were detected in the discs of model group rats but rarely in those of control group rats. Moreover, TCAP-positive cells (green fluorescence) were detected in the discs of control group rats but rarely in those of model group rats. These results demonstrated that disc degeneration increased TNF-α, TLR-2, ITGAM and TTN protein expression and decreased the protein expression of TCAP in intervertebral discs.

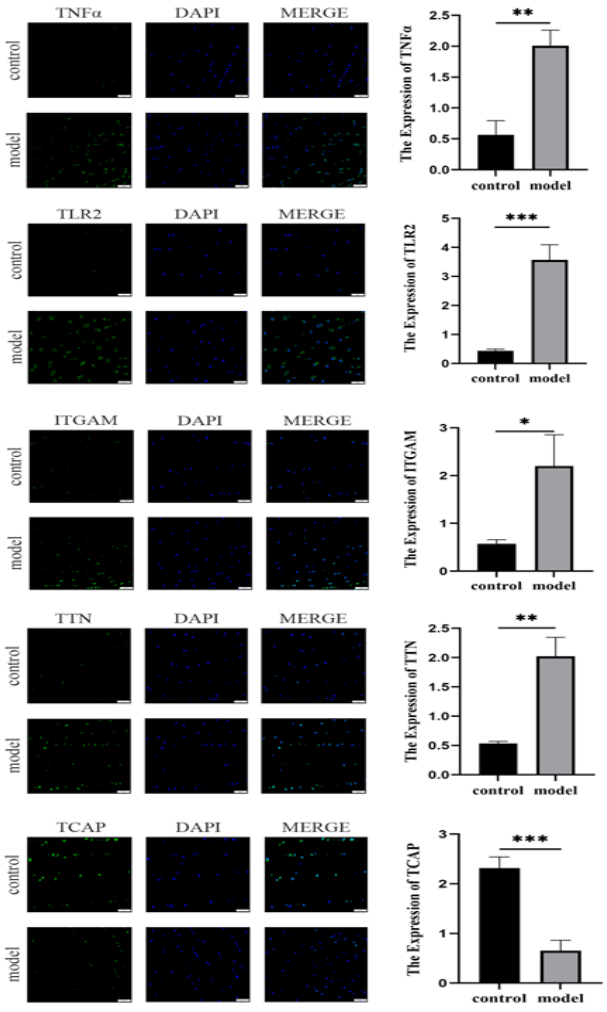

Figure 7: Key genes were detected by immunofluorescence combined with DAPI staining.

DISCUSSION

The molecular mechanisms of IDD have been widely studied, however, IDD is a condition that involves multiple factors[13,14], resulting in complex mechanisms. Animal experiments that reduce the influence of external factors on IDD are important for exploring the

pathogenesis of IDD. Successful animal experiments are highly dependent on effective and suitable animal models. In the present study, we followed a surgical video and protocol of an improved anterior approach to establish a mouse puncture model of IDD for investigating the underlying mechanisms of IDD(10). We identified 586 DEGs between the disc tissues of IDD model SD rats and control rats. These DEGs were significantly enriched in the GO terms muscle structure development and myofibrils, suggesting that these terms may play a role in the pathology of IDD. KEGG pathway enrichment analysis revealed 15 potential pathways that may play a crucial role in IDD. Through protein–protein interaction (PPI) network analysis, we revealed that TNF-α, TLR2, ITGAM, TTN and TCAP are key genes involved in IDD. The most important key genes identified in this study are important inflammatory factors, and the inflammatory cascade is one major process of IDD. Inflammation combined with senescence and apoptosis causes cell loss and extracellular matrix degradation, resulting in IDD.[15]

It has long been known that intervertebral disc cells secrete TNF-α[16], which play an important role in intervertebral disc degeneration through multiple pathways.[17] TNF-α levels in intervertebral discs increase with the degree of disc degeneration, as does age.[18] TNF-α levels are also increased in symptomatic patients with lumbar disc herniation.[19] TNF-α is one of the most studied cytokines involved in the process of disc degeneration because of its strong proinflammatory nature. Stimulating degenerated intervertebral disc cells with TNF-α increases the levels of IL-6, IL-8, IL-17, substance P, nitric oxide and prostaglandin E2[20,21], as well as the levels of the chemokines CCL2, CCL3, CCL20, CXCL2 and CXCL5.[22] By activating the NF-κB/MAPK signaling pathway, TNF-α prompts degradation of the extracellular matrix. With increasing TNF-α levels in the degenerated intervertebral discs, the levels of various thrombospondin motifs and metalloproteinases in the degenerated intervertebral discs increase, which subsequently mediate the degradation of collagen and aggrecan.[23,24] TNF-α exerts a proapoptotic effect on nucleus pulposus cells, leading to disc degeneration.[25] Both anti- and proapoptotic proteins are regulated by TNF-α through the NF-κB and JNK/ERK-MAPK signaling pathways, and a trend toward promoting apoptosis has been observed in degenerated intervertebral discs.[26] TNF-α promotes or inhibits cell proliferation in a concentration-dependent manner[27], and these effects are achieved mainly through the JNK, NF-κB and p38 MAPK signaling pathways.[28] In addition, TNF-α increases the expression levels of autophagy-related genes in intervertebral discs[29], thus cause cell death exacerbating IDD.[30] Moreover, TNF-α may cause cell senescence in intervertebral discs.[31,32] These reports demonstrated that TNF-α plays an important role in multiple areas of IDD. However, the specific role of TNF-α in the process of IDD described above remains largely unknown, and further research is still needed.

TLR2 is an inflammatory regulator of the toll-like receptor family, which is also deeply involved in the pathogenesis of IDD.[33] The upregulation of TLR2 has been confirmed in degenerated intervertebral discs.[34] Moreover, TLR2 activates the MAPK pathway to produce cytokines involved in IDD under the stimulation of various factors.[35] JNK is activated following the activation of TLR2 and subsequently affects the apoptosis of intervertebral disc cells.[36] In disc cells with increased TLR2 expression, the number of senescence-associated secretory factors increases, and in the context of IDD, TLR2 plays a crucial role in the activation of inflammation-related signaling pathways to participate in defense; moreover, the increase in senescent cells in degenerative discs suggests that there is a positive correlation between cell senescence and IDD.[37]

In addition to TNF-α and TLR2, two factors that have been extensively studied, and the role of these two factors in IDD has been partially clarified, we found that ITGAM, TTN and TCAP are key genes in IDD. However, the specific mechanism by which these genes are involved in IDD has not yet been reported. Exploration of these newly identified key genes associated with IDD may be needed in the future. Our study also revealed that the calcium signaling pathway may play an important role in IDD, but how this signaling pathway regulates IDD is still unknown. Further research is needed to determine the role of the calcium signaling pathway in the process of IDD.

In conclusion, consistent with existing reports, the present study revealed that TNF-α and TLR2 are key genes involved in IDD. As a supplement to existing research findings, our study revealed that the ITGAM, TTN, TCAP and calcium signaling pathways may play key roles in IDD. Our research may provide new clues and directions for future studies exploring the mechanisms of IDD.

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Data Availability Statement: The datasets used and analyzed during the current study are avail-able from the corresponding author on reasonable request.

Conflicts of Interest: The authors declare no conflicts of interest.

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