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ORIGINAL RESEARCH

Construction of a Novel Necroptosis-Related Signature in Rat DRG for Neuropathic Pain

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Background: Recent studies have shown necroptosis may play a role in the development of inflammation-associated pain. However, research on the correlation between necroptosis-related genes and neuropathic pain in the dorsal root ganglia (DRG) is limited. This study aims to identify a gene signature related to necroptosis in DRG that can predict neuropathic pain.

Methods: The mRNA expression profiles associated with neuropathic pain (GSE24982 and GSE30691) were acquired from the Gene Expression Omnibus (GEO) database. The Least Absolute Shrinkage and Selection Operator (Lasso) and Support Vector Machine-Recursive Feature Elimination (SVM-RFE) regressions were performed in GSE24982 database to constructed the necroptosis-related diferentially expressed genes (NRDEGs) signature related to neuropathic pain. Nomogram, Receiver Operating Characteristic (ROC), GSE30691 database analysis and basic experiments were used to verify the accuracy of the signature. Go and KEGG analysis, interaction network and immune infiltration were used to analyze the biological function of the signature.

Results: A predictive signature targeting rat DRG for neuropathic pain through a variety of methods to verify the accuracy was developed based on 3 NRDEGs (TLR4, CAPN2, RIPK3). Significantly enriched KEGG and GO pathways, drug target prediction and non-coding RNAs related to the signature holded promise for advancing our understanding of potential avenues for treatment and the mechanisms underlying neuropathic pain. Immune infiltration analysis revealed which types of immune cells related to the NRDEGs signature played an important role in the occurrence and development of neuropathic pain. Basic experiments provided crucial evidence that the 3 NRDEGs in DRG served as important regulators of neuropathic pain.

Conclusion: The prediction signature based on 3 key NRDEGs showed promise in predicting the presence of neuropathic pain, which may open up new avenues for the development of novel therapies for neuropathic pain.

Keywords: DRG, neuropathic pain, necroptosis, signature, prediction

Introduction

Neuropathic pain resulting from injury or disease of the somatosensory nervous system can have a profound impact on an individual's quality of life and has been linked to an increased risk of developing depression, anxiety, and other mental disorders. Epidemiological surveys have indicated that the prevalence of neuropathic pain ranges from 3% to 17%.[1] The current primary approach for treating neuropathic pain in clinical settings is drug therapy. This includes the use of tricyclic antidepressants, serotonin norepinephrine reuptake inhibitors, gabapentin, and similar drugs.[2] However, it's important to note that most of these medications primarily target the central nervous system and often come with a range of side effects. Studies have shown that some drugs targeting the peripheral nervous system, such as dorsal root ganglia (DRG), are sufficient to produce significant analgesic effect without causing noticeable side effects.[3] Hence, it is of

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paramount importance and clinical relevance to explore effective and safe methods of pain management without the undesirable side effects associated with many current treatments.

Cell death plays a crucial role in the occurrence and progression of various human diseases. This includes programmed cell death and uncontrolled death caused by injury. Necroptosis, as an alternative mode of cell death when apoptosis fails, is triggered by a distinct caspase-independent signaling pathway.[4] It is a lytic form of cell death characterized by membrane perforation and mitochondrial dysfunction. The loss of plasma membrane integrity leads to the release of cellular contents into the extracellular environment, thereby exacerbating the inflammatory response. Unlike necrosis, necroptosis follows signal regulation and actively consumes energy.[5] Numerous studies have linked necroptosis to various human diseases, including infection-related diseases, tumors, degenerative diseases, and ischemic diseases.[6,7]

Recent studies have highlighted the role of necroptosis in the controlled release of cellular components, potentially triggering inflammation through distinct signaling pathways.[8] These findings suggest that necroptosis may play a significant role in the development of inflammation-associated pain. Notably, the use of Necrostatin-1, an inhibitor of necroptosis, has been shown to alleviate neuropathic pain induced by peripheral nerve injury by targeting the RIP1/RIP3 pathway in the spinal cord.[9] This implies that necroptosis is indeed a contributing factor to the development of neuropathic pain. However, research on the correlation between necroptosis-related factors in DRG and neuropathic pain is limited, and there is a scarcity of therapeutic targets and diagnostic markers associated with necroptosis in DRG for neuropathic pain. This study aims to identify a gene signature related to necroptosis that can predict the occurrence of neuropathic pain, which seeks to shed light on the underlying pathological mechanisms of neuropathic pain.

Materials and Methods

Reagents and Animals

RNAiso Plus (CAS: 9108/9109), TB Green® Premix Ex Taq™ (CAS: RR420B), and SYBR Premix Ex Taq (CAS: DRR041A) were sourced from Takara Bio Inc. (Japan); PCR primers were purchased from Sangon Biotech (Shanghai) Co., Ltd. (China); siRNAs were supplied by Guangzhou RiboBio Co., Ltd. (China); animals were purchased from the Liaoning Changsheng Biotechnology Co., Ltd. (China).

Screening of Necroptosis-Related Differentially Expressed Genes (NRDEGs) Related to Neuropathic Pain from Adult Rat DRGs

The mRNA expression profiles related to neuropathic pain (GSE24982 and GSE30691) were acquired from the Gene Expression Omnibus (GEO) database, accessible at http://www.ncbi.nlm.nih.gov/geo/. In the case of GSE24982, the expression profiling involved mRNA extracted from adult rat L4 and L5 DRGs in a spinal nerve ligation (SNL) model of neuropathic pain. As for GSE30691, microarrays were conducted on mRNA extracted from adult rat L4 and L5 DRGs following three different sciatic nerve lesions: spared nerve injury (SNI), chronic constriction injury (CCI), and SNL, along with sham control samples.

The GSE24982 and GSE30691 datasets, which are associated with neuropathic pain, were initially normalized using the limma R package (R version 4.1.3). Subsequently, a significance analysis of the neuropathic pain database was conducted on GSE24982 using the limma R package in order to identify differentially expressed genes related to neuropathic pain. These NRDEGs were then visualized in a heat map. Furthermore, correlation analysis among the differentially expressed genes was carried out using the limma and pheatmap R packages.

Go and KEGG Analysis of NRDEGs

The Kyoto Encyclopedia of Genes and Genomes (KEGG) is a comprehensive database that integrates information related to genomics, chemistry, and system functions.[10] On the other hand, the Gene Ontology (GO) system is divided into three categories, molecular function (MF), cellular component (CC), and biological process (BP).[11] To perform GO and KEGG pathway enrichment analysis of the NRDEGs, we utilized several R packages including “clusterProfiler” “org.Hs.eg.db”, “enrichplot”, “ggplot2”, “circlize”, “RcolorBrewer”, “dplyr”, and “ComplexHeatmap”, The criteria for selecting enriched

items were based on a p-value less than 0.05, which was considered statistically significant. Additionally, the p-value correction method applied was the Benjamini-Hochberg (BH) method to account for multiple comparisons.

Identification of Feature Gene Signature

The feature selection process for identifying NRDEGs related to neuropathic pain involved the utilization of two machine learning algorithms, the Least Absolute Shrinkage and Selection Operator (Lasso) regression, which was performed with 10-fold cross-validation, a significance level of 0.05, and 1,000 cycles using the "glmnet" R package. Additionally, the Support Vector Machine-Recursive Feature Elimination (SVM-RFE) technique was implemented with the "e1071" R package. To visualize the intersection of the selected genes from both Lasso and SVM-RFE, a Venn diagram was created using the "VennDiagram" R package. This comprehensive approach helped identify the most relevant and significant NRDEGs associated with neuropathic pain.

Validation of the Feature Gene Signature

Nomograms were constructed using factors identified through multivariate logistic regression analysis with the assistance of the "rms" and "rmda" R packages. Additionally, a calibration curve was plotted to assess the accuracy of the multivariate logistic regression model's predictions. To evaluate the predictive performance of the regression model, a Receiver Operating Characteristic (ROC) curve was generated using the "ROC" R package. Furthermore, the expression levels of feature genes were analyzed in the GSE30691 database using the "limma" and "ggpubr" R packages.

GSEA and GSVA Analysis

Gene Set Enrichment Analysis (GSEA) was employed to evaluate the distribution trends of genes within predefined gene sets and their correlation with phenotypes, helping to determine their contribution to phenotypic differences. The gene sets "c2.cp.kegg.symbols" and "c5.go.symbols" were obtained from the MSigDB database and separately utilized for GSEA in the neuropathic pain and sham control groups. The "clusterProfiler" R package was utilized for this analysis, with a significance threshold set at a p-value < 0.05 to identify statistically significant results. Gene Set Variation Analysis (GSVA), a nonparametric unsupervised analysis method, was used to assess the enrichment of gene sets in the nuclear transcriptome chips. This approach evaluated whether different metabolic pathways were enriched among various samples by transforming the gene set expression matrices. The gene sets "c2.kegg.v7.4.symbols" and "c5.go.v7.4.symbols" were analyzed for GSVA in the context of neuropathic pain and sham controls, and the GSVA enrichment analysis was performed using the "GSVA" R package. The results of this analysis were visualized using the "heatmap" R package.

Construction of Competing Endogenous RNA (ceRNA)-NRDEGs, Drugs-NRDEGs Interaction Networks

In order to analyze the regulatory relationship between NRDEGs and ceRNA, we used 3 network database (miRanda, miRDB, TargetScan) to make miRNA-NRDEGs and miRNA-lncRNA predictions. The DGIdb software (https://dgidb.genome.wustl.edu/) was employed to predict potential drug targets among the NRDEGs. This analysis aimed to explore the direct and indirect interactions between NRDEGs and drugs, providing insights into potential therapeutic strategies. PPI interaction maps were drawn using Cytoscape software.

Analysis of Immune Infiltration

The CIBERSORT deconvolution algorithm was utilized to estimate the proportions of various immune cell types. In total, 22 different immune cell types were obtained through this analysis. Data with a p-value < 0.05 were retained after CIBERSORT filtering. Subsequently, the percentage of each immune cell type was calculated and visualized in the form of a violin plot image using the "vioplot" R package. Correlation analysis between NRDEGs and immune cells was performed by "tidyverse" "ggplot2" "reshape2" R packages.

Quantitative Real-time PCR (qRT-PCR)

Total RNA was extracted from L3-L5 DRGs of mice using RNAiso Plus (total RNA extraction reagent). Subsequently, cDNA was synthesized with a PrimeScript RT reagent Kit. The reaction conditions for cDNA synthesis were as follows: 37°C for 15 minutes, 85°C for 5 seconds, and termination at 4°C. Real-time PCR was conducted on the Bio-Rad CFX 96 Real-time PCR system (BIO-RAD, USA) using the SYBR Premix Ex Taq (Real-Time PCR Kit), along with specific primers for the target genes: Toll-like receptor 4, TLR4 (Forward: GCATGGATCAGAAACTCAGCAA, Reverse: ACCGATGGACGTGTAAACCA); Receptor-interacting protein kinase 3, RIPK3 (Forward: GAAATGGATTGCCCGAGGGA, Reverse: AGTGCCGTGTCTTCC ATCTC); Calpain 2, CAPN2 (Forward: GGCAAATCTAACCGAACGGC, Reverse: AGCCTTCGGAATCCA TCGTC); GAPDH (Forward: GGAGCGAGATCCCTCCAAAAT, Reverse: GGCTGTTGTCATACTTCTCATGG); The real-time PCR reaction conditions involved an initial denaturation at 95°C for 3 minutes, followed by 35 cycles of 95°C for 30 seconds and 60°C for 30 seconds. Gene expression levels were quantified using the 2^-ΔΔCq method, and statistical differences were assessed using a Two-Sample *t*-Test.

siRNA Injection

All animal procedures are performed in full accordance with the regulations of the Animal Protection and Ethics Committee of Hebei Medical University and the guidelines of the International Association for the Study of Pain (Approval No. IACUC-Hebmu-P2023193). Under deep anesthesia (sodium pentobarbital, 10–20 mg/kg, i.p)., the L4 DRG of about 25 g adult male mouse were accessed by removing transverse processes of the vertebrae. A microinjector (Hamilton Company) was carefully inserted into the ganglion to a depth of approximately 300 μm from the exposed surface. A siRNA solution (20 μM, 2 μL) was injected slowly, and the needle was withdrawn 3 minutes after the injection. The muscles overlying the spinal cord were loosely sutured, and the wound was closed. Mouse RIPK3 siRNA: 5′-GCAGGAAATTTCAGGCCAA-3′; Mouse CAPN2 siRNA: 5′-GCGGTCAGATACCTTCATCAA-3′.

SNL Model and Behavior Test

Before the operation, the laboratory was disinfected with ultraviolet radiation, the operating table was disinfected with 75% alcohol, and the surgical instruments were disinfected at high temperature. Animals were anesthetized and placed on the temperature control panel and given constant temperature control at 37 °C. After the skin was disinfected, the back of the animals was opened to separate the skin and muscle, laminectomy was performed, and the L4 spinal nerve was fully exposed and ligation with No.6 ophthalmic line. The wound was treated with penicillin and then sutured.

Mechanical threshold test: the mice were placed on metal barbed wire, and each mouse was separated by an acrylic transparent box. The experiment began after the mice stopped walking around and looking. Standard Von Frey fibers were used to stimulate the palm of the mouse's right paw. We followed the principle of "up and down" for detection. An animal was tested 60 seconds apart.

Thermal threshold test: the mice were placed on a transparent glass plate and covered with an acrylic transparent box. The intensity of the thermal radiation instrument was adjusted to 15%. The infrared laser was used to irradiate the right paw of the mouse, and paw lifting was regarded as a positive reaction, the latent time shown by the thermal radiation instrument was recorded. The average value of 5 times was the thermal threshold.

Statistics

All expression matrix data were analysed using R software v4.1.3. The data presented in basic experiments were expressed as the mean ± standard error (SE). Two-sample *t*-test was used when comparing two groups of data. A two-way repeated ANOVA followed by Bonferroni's post hoc test was used when comparing groups at different time points. p value < 0.05 was considered significantly.

Results

Screening of NRDEGs Related to Neuropathic Pain

The flowchart of this study was illustrated in Figure 1. The gene expressions in L4 and L5 DRGs were analyzed using the limma R package in the neuropathic pain GSE24982 database to identify differentially expressed genes. In total, 69 genes related to necroptosis were identified from the GSE24982 dataset (Supplementary Table S1), and 28 genes were found to exhibit significant differential expression. Compared with the Control group, 17 of the differentially expressed genes were up-regulated, and 11 were down-regulated in the SNL-neuropathic pain group (Figure 2A and Supplementary Table S2). The correlation analysis, shown in Figure 2B, revealed several strong positive correlations such as RIPK3 and SHARPIN, JAK1 and CAPN2, USP21 and DNM1L, JAK1 and IFNGR1, while strong negative correlations were observed between PGAM5 and RIPK3, IFNGR1 and DNM1L, JAK1 and DNM1L, USP21 and IFNGR1, USP21 and JAK1 (all with $p < 0.001$).

Subsequently, a combined logFC-based GO enrichment analysis was performed using the expression profile data of the 28 NRDEGs from the GSE24982 dataset. The standard scores (Z-scores) for each GO term were calculated based on the molecule's logFC and visualized in a circular diagram (Figure 3A). The top 5 GO pathways were primarily enriched in cytokine-mediated signaling pathways, I-kappaB kinase/NF-kappaB signaling, regulation of DNA-binding transcription factor activity, regulation of I-kappaB kinase/NF-kappaB signaling, and positive regulation of NF-kappaB transcription factor activity (Figure 3B). Additionally, the top 5 KEGG pathways were mainly enriched in cytokine-mediated signaling pathways, I-kappaB kinase/NF-kappaB signaling, regulation of DNA-binding transcription factor activity, regulation of I-kappaB kinase/NF-kappaB signaling, and response to viruses (Figure 3C).

These findings provided valuable insights into the differentially expressed NRDEGs, their correlations, and the enriched pathways associated with neuropathic pain, shedding light on potential molecular mechanisms and pathways involved in this condition.

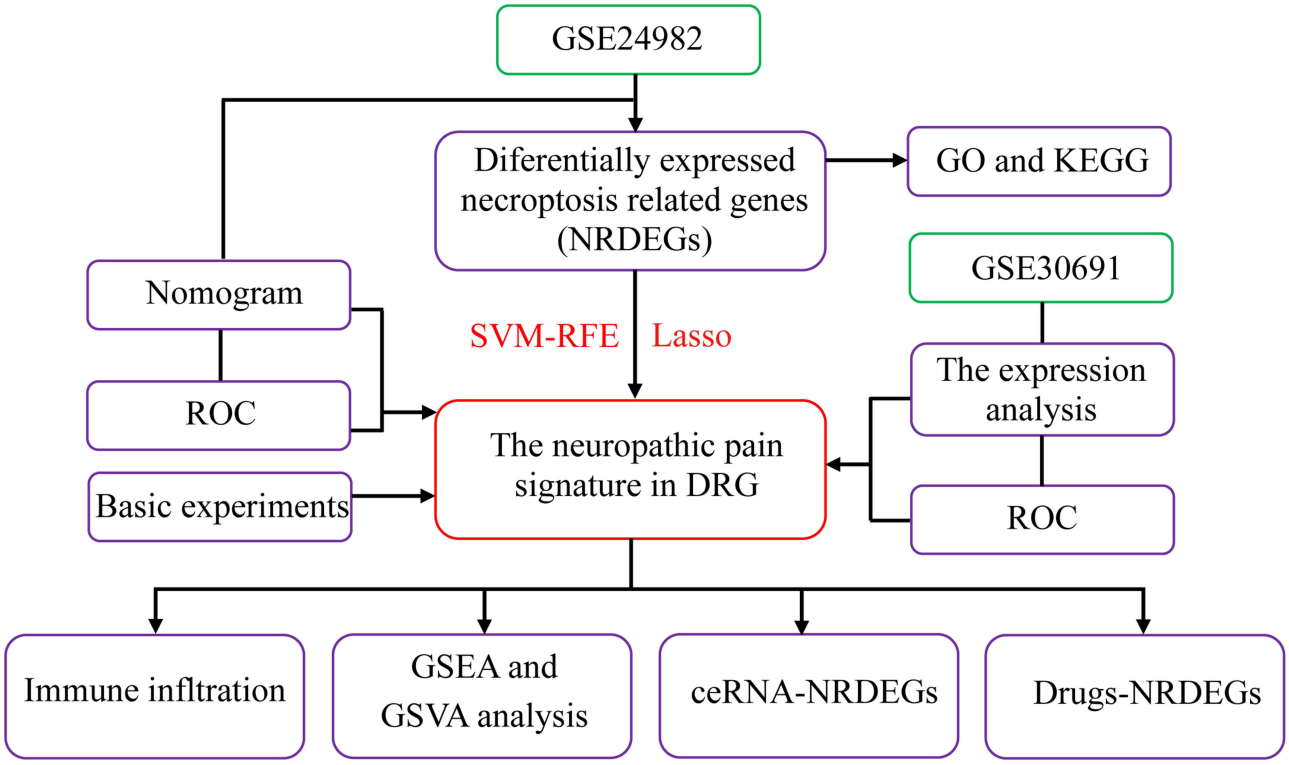

Figure 1 Schematic diagram of the research process.

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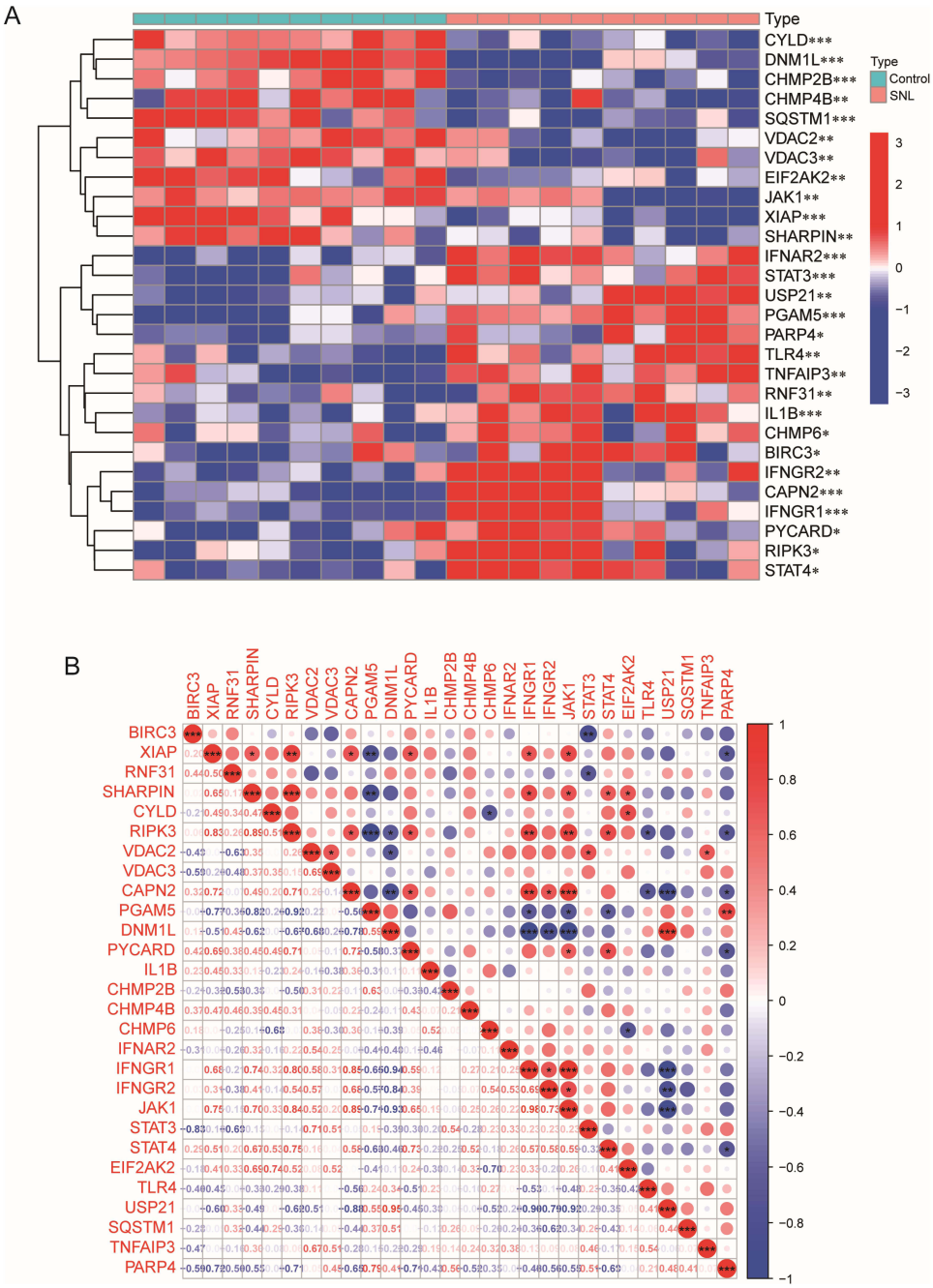

Figure 2 Screening of NRDEGs related to neuropathic pain from the GSE24982 database. **(A)** Differentially expressed NRDEGs between neuropathic pain (SNL model) and Control groups. **(B)** The correlation analysis of the NRDEGs. NRDEGs, necroptosis-related differently expressed genes; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

Construction of the NRDEGs Signature Related to Neuropathic Pain

Based on the expression profiles of the 28 NRDEGs in the GSE24982 database, 2 different feature selection methods were applied to identify potential genes related to neuropathic pain. Lasso regression analysis identified 10 potential feature genes (XIAP, CYLD, RIPK3, VDAC3, PGAM5, DNM1L, CHMP2B, JAK1, TLR4, CAPN2) associated with neuropathic pain (Figure 4A and B). SVM-RFE regression analysis extracted 5 potential feature genes (VDAC2, TLR4, CAPN2, RIPK3, USP21) related to neuropathic pain (Figure 4C and D). The genes that were selected by both the Lasso and SVM-RFE regressions were identified and visualized in the Venn diagram (Figure 4E). Ultimately, a predictive signature targeting DRG for neuropathic pain was constructed based on 3 NRDEGs (TLR4, CAPN2, RIPK3).

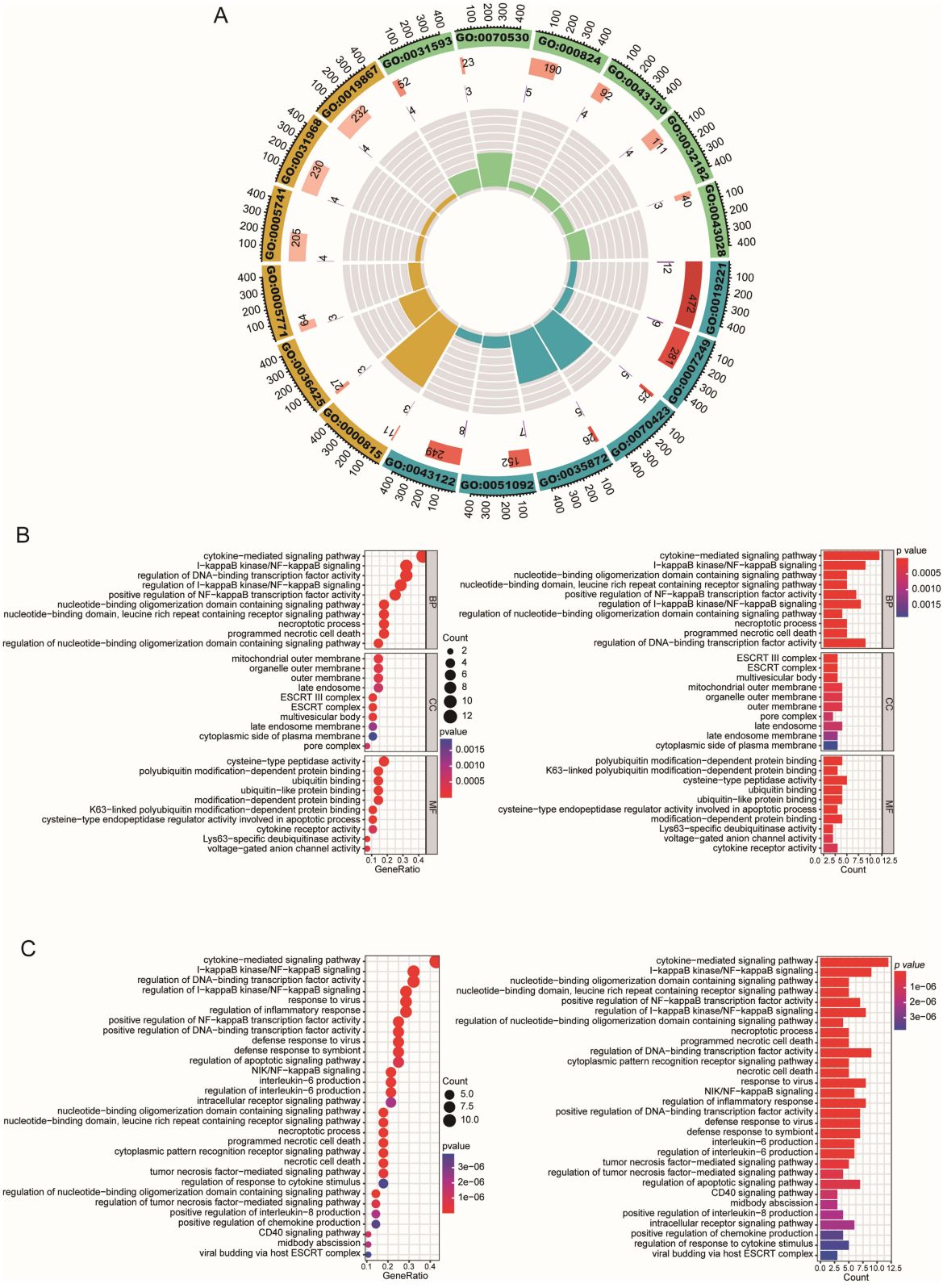

Figure 3 Investigating GO and KEGG pathways in relation to NRDEGs. **(A)** Circle diagram of combined logFC-based GO enrichment analysis. **(B)** GO enrichment analysis. **(C)** KEGG enrichment analysis.

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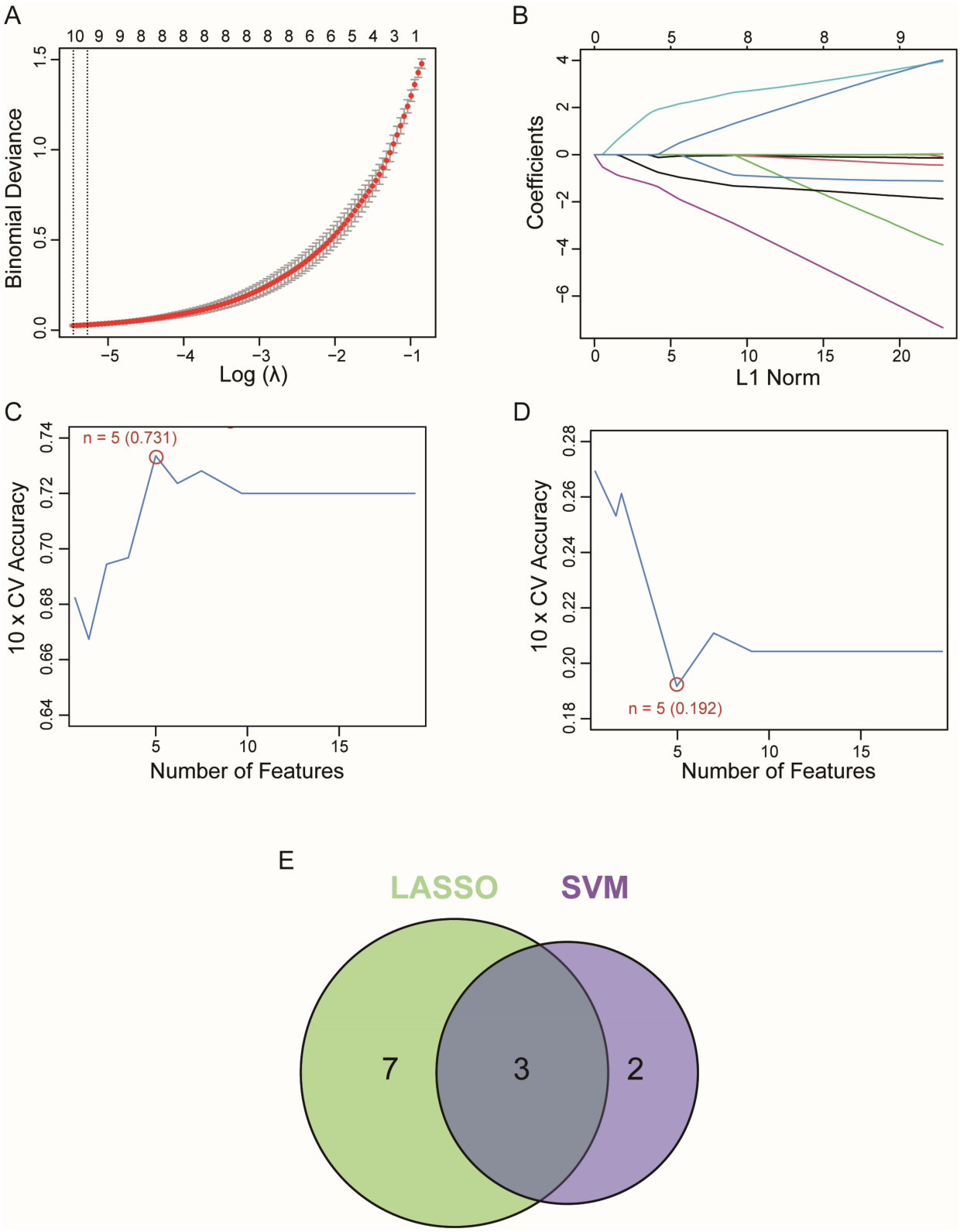

Figure 4 Construction of neuropathic pain signature based on the NRDEGs. **(A** and **B)** The Lasso regression. **(C** and **D)** The SVM-RFE regression. **(E)** The crossed genes (TLR4, CAPN2, RIPK3) shown in the Venn diagram.

Validation of the Neuropathic Pain Prediction Signature

Based on the expression levels of the 3 NRDEGs in the prediction model, a nomogram was established to predict the incidences of neuropathic pain (Figure 5A). To assess the predictive accuracy of the nomogram, a calibration plot was generated, demonstrating that the nomogram had high predictive accuracy (Figure 5B). ROC analysis was performed to evaluate the predictive value of the 3 characteristic genes. The area under the curve (AUC) for TLR4, CAPN2, RIPK3 genes were 0.910, 0.945, 0.800, respectively (Figure 5C). The AUC of the prediction model, incorporating these 3 genes, was 0.956, indicating a high predictive ability (Figure 5D).

To further validate the accuracy of the prediction model, another database (GSE30691) was utilized to analyze the expression of the 3 NRDEGs in neuropathic pain states and their predictive capabilities for neuropathic pain. GSE30691 microarrays were conducted on mRNA extracted from adult rat L4 and L5 DRGs after 3 different neuropathic pain models, including SNI, CCI, and SNL models. In accordance with the results from the GSE24982 database, all 3 NRDEGs were significantly overexpressed in the neuropathic pain group compared to the Control group (Figure 6A). The AUCs for TLR4, CAPN2, and RIPK3 genes were 0.860, 0.758, and 0.871, respectively (Figure 6B), and the AUC of the prediction model was 0.909 (Figure 6C) when analyzing the GSE30691 database. These findings further confirmed that the predictive signature consisting of the 3 NRDEGs in DRG could serve as an independent and robust predictor for neuropathic pain.

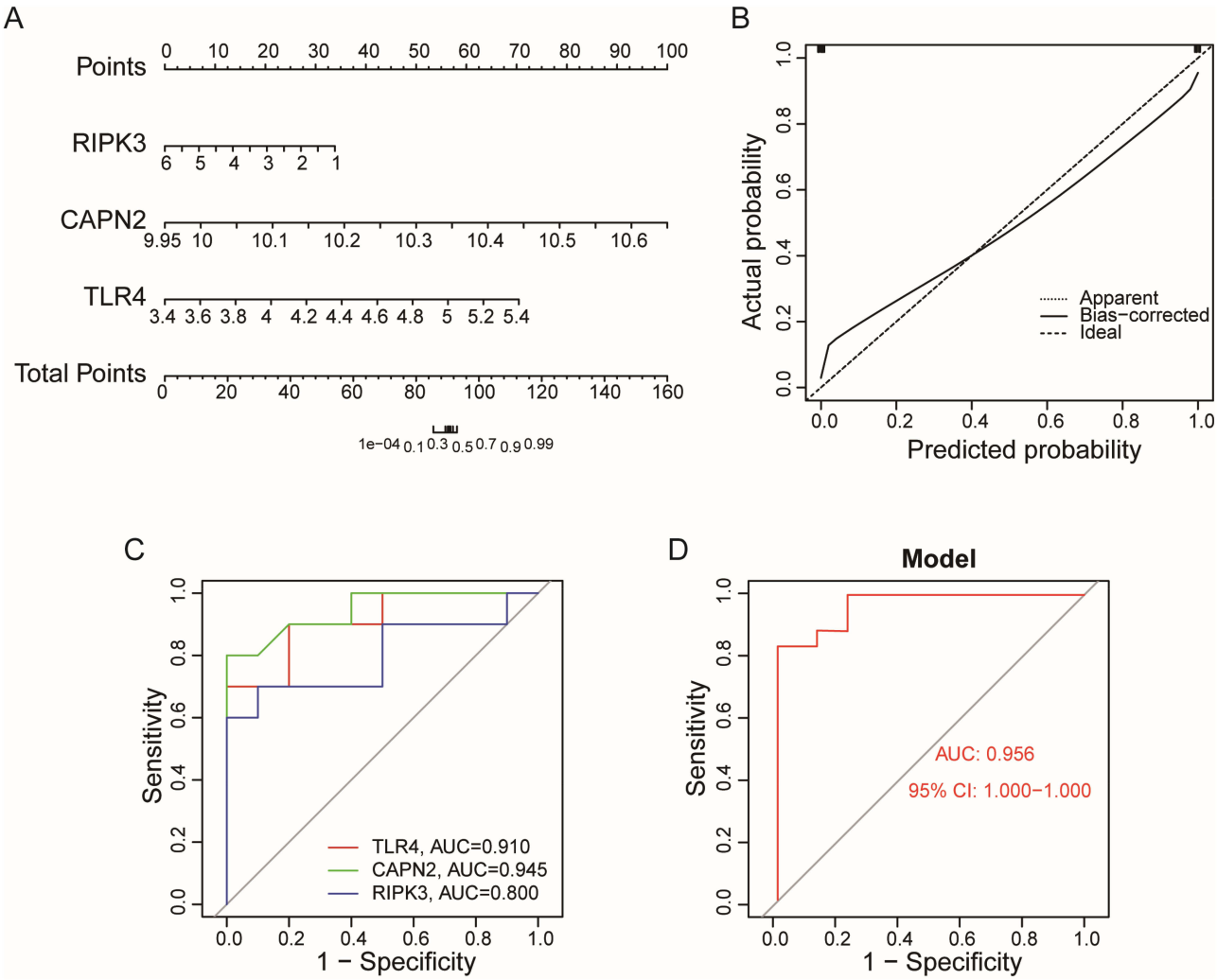

Figure 5 Validation of the neuropathic pain prediction signature. **(A** and **B)** Construction of a nomogram and calibration plot. **(C)** The ROC analysis for the 3 NRDEGs. **(D)** The ROC analysis for the prediction model.

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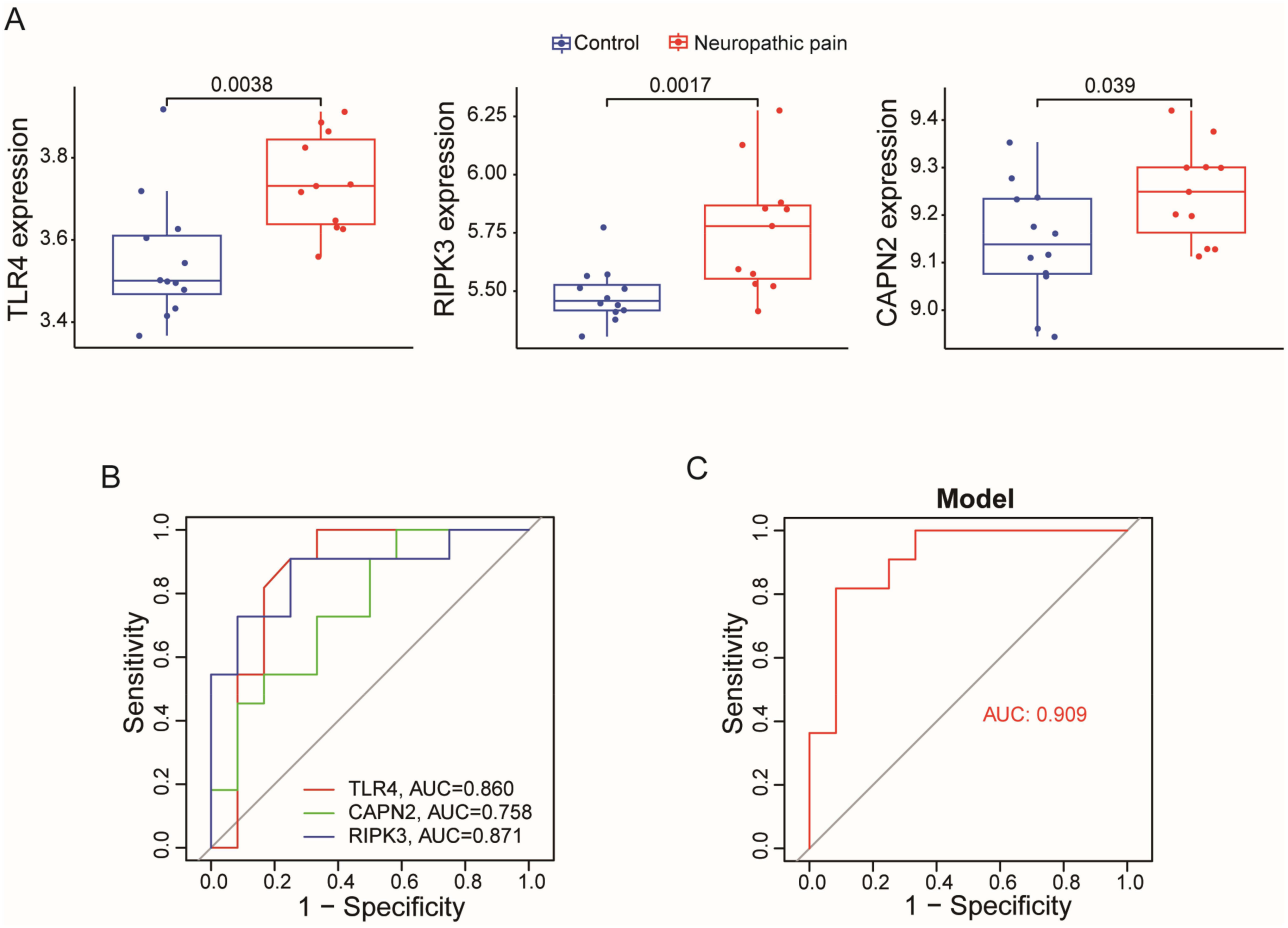

Figure 6 Assessing the signature's validity through the GSE30691 database. **(A)** The expressions of TLR4, CAPN2, RIPK3. **(B)** The ROC analysis for the 3 NRDEGs. **(C)** The ROC analysis for the prediction model.

GSEA and GSVA Analysis

To gain insights into the biological functions associated with the 3 NRDEGs prediction signature, an analysis of significantly enriched KEGG and GO pathways for each NRDEG was conducted using GSEA 4.2.3 software, which involved screening gene sets from c2.cp.kegg.symbols and c5.go.symbols. The top 5 KEGG and GO pathways were presented in Figure 7, with all having $p < 0.05$ and $|NES| > 1.5$.

Subsequently, GSVA enrichment analysis was performed based on the differential expressions of TLR4, CAPN2, RIPK3 genes between the neuropathic pain and sham groups. The results revealed that the top 5 pathways, including neuroactive ligand receptor interaction, maturity onset diabetes of the young, proximal tubule bicarbonate reclamation, glycosphingolipid biosynthesis-lacto and neolacto series, glycosaminoglycan biosynthesis-heparan sulfate, were up-regulated in neuropathic pain group. Conversely, the top 5 pathways, which encompassed cell cycle, pancreatic cancer, ECM-receptor interaction, leishmania infection, complement and coagulation cascades, were down-regulated in the neuropathic pain group (Figure 8).

These analyses provided valuable insights into the potential mechanisms through which NRDEGs could regulate neuropathic pain, shedding light on the underlying biology of this condition.

Construction of Competing Endogenous RNA (ceRNA)-NRDEGs, Drugs-NRDEGs Interaction Networks

The DGIdb software was employed to predict both direct and indirect drug targets of the NRDEGs, aiming to explore potential therapeutic options for neuropathic pain. Specifically, potential inhibitors of the TLR4 gene were predicted, including

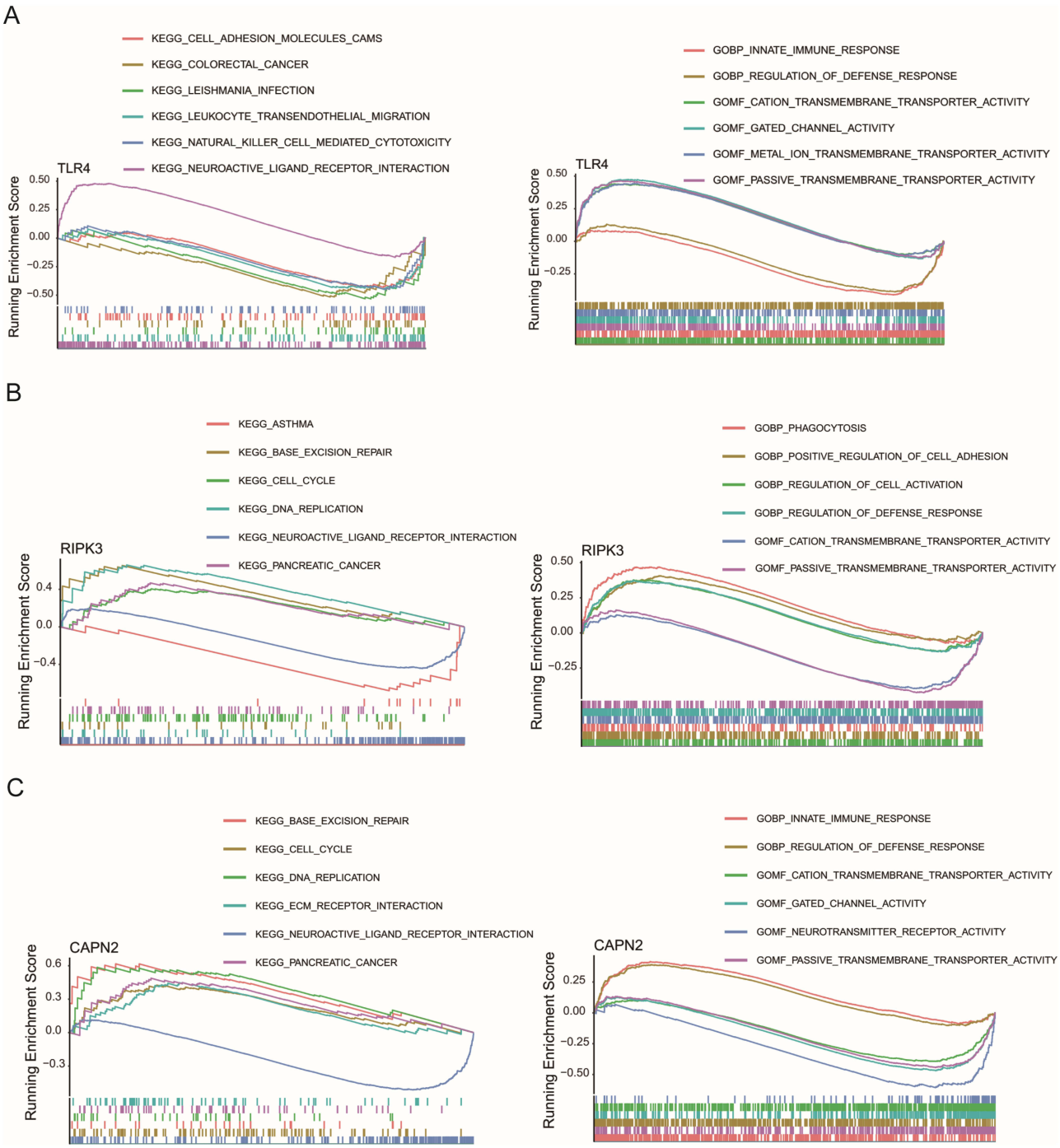

Figure 7 The GSEA analysis based on the NRDEGs signature. (**A-C**) The significantly enriched KEGG and GO pathways analyses for TLR4, RIPK3, CAPN2, respectively.

Infliximab, Methotrexate, Alcohol, Saquinavir, Nelfinavir, Tacrolimus, Eritoran, Pravastatin, Resatorvid, Ritonavir, and Eritoran tetrasodium (Figure 9A). However, it appears that there were no reported drugs associated with the other 2 NRDEGs. Drug target prediction holded promise for advancing our understanding of potential avenues for treatment.

Next, we used 3 network databases (miRanda, miRDB, and TargetScan) to investigate the regulatory relationships between NRDEGs and ceRNAs. PPI networks involving lncRNA-miRNA-TLR4/RIPK3/CAPN2 were visualized in Figure 9B–D. This analysis aimed to further explore the mechanisms targeted by non-coding RNAs related to the 3 NRDEGs underlying neuropathic pain generation.

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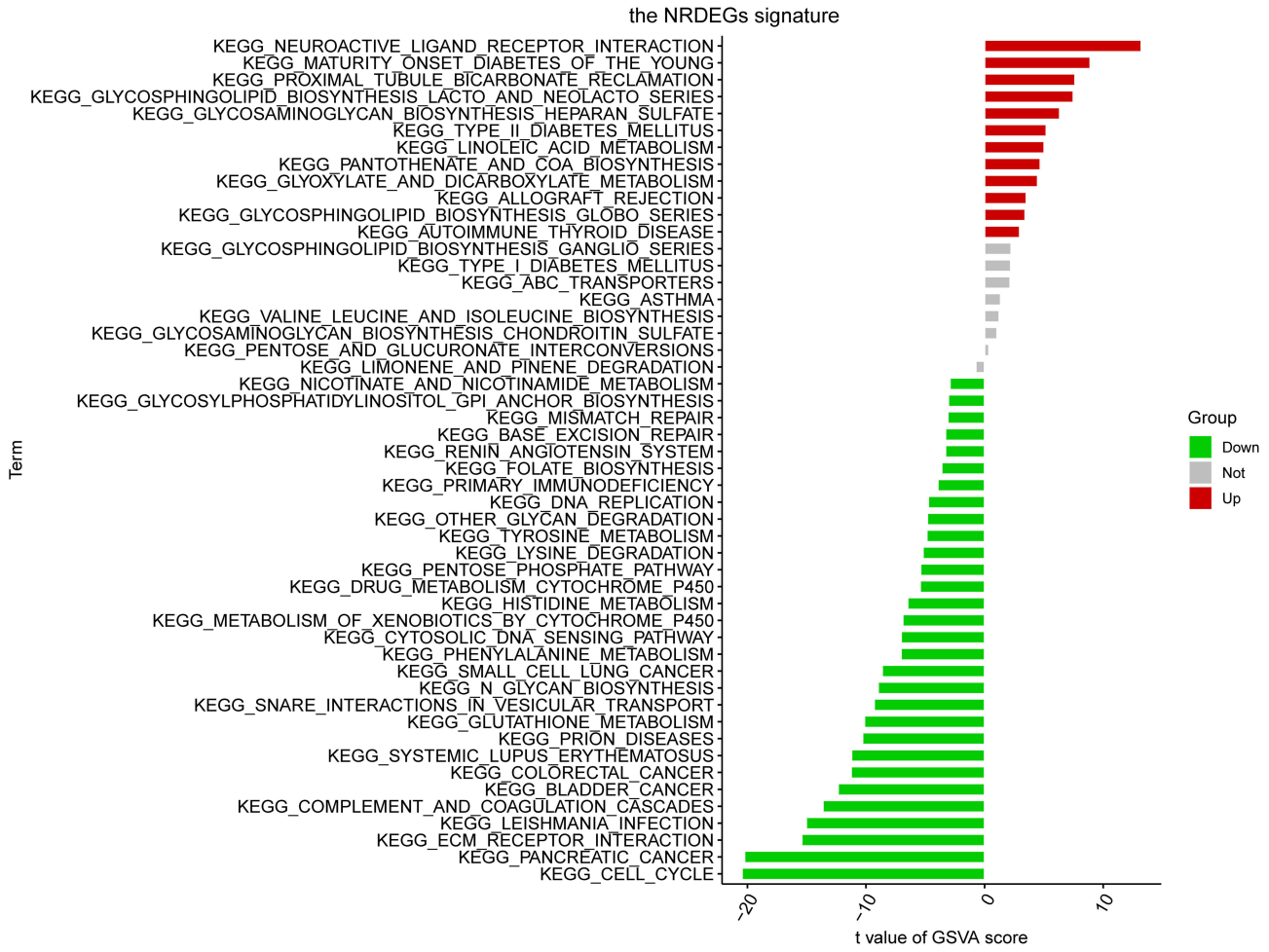

Figure 8 The GSVA analysis based on the NRDEGs signature.

Immune Infiltration Analysis of the NRDEGs Signature

Using the CIBERSORT database for the analysis of immune cell infiltration comprising 22 different immune cell types, the correlation between the NRDEGs signature and the abundance of immune cell infiltration was calculated. Notably, the expressions of dendritic cells resting and monocytes in DRGs of the neuropathic pain group were significantly higher than those in the Control group (Figure 10A).

Further analysis was conducted to investigate the correlations between the degree of immune cell infiltration and each of the 3 NRDEGs separately. The results indicated the following associations: TLR4 was positively regulated with neutrophils and negatively regulated with monocytes. RIPK3 was positively regulated with dendritic cells activated and negatively regulated with T cells CD4 memory activated and plasma cells. CAPN2 was positively regulated with NK cells resting and negatively regulated with T cells CD4 memory activated (Figure 10B). This analysis provided valuable insights into the immune mechanisms underlying neuropathic pain.

The 3 NRDEGs Involved in Peripheral Neuropathic Pain

Ultimately, we further strengthen the validation of the prediction model through basic experiments. The qRT-PCR analysis revealed that the transcription levels of the 3 NRDEGs were significantly increased in the L3-L5 DRGs 5 days after SNL operation in mice, compared to the Control group (Figure 11A).

Given previous reports indicating that local knockdown of TLR4 in DRG could alleviate peripheral neuropathic pain,[12] siRNAs targeting RIPK3 and CAPN2 were used to knock down the expressions of these 2 genes in DRG. Figure 11B was the

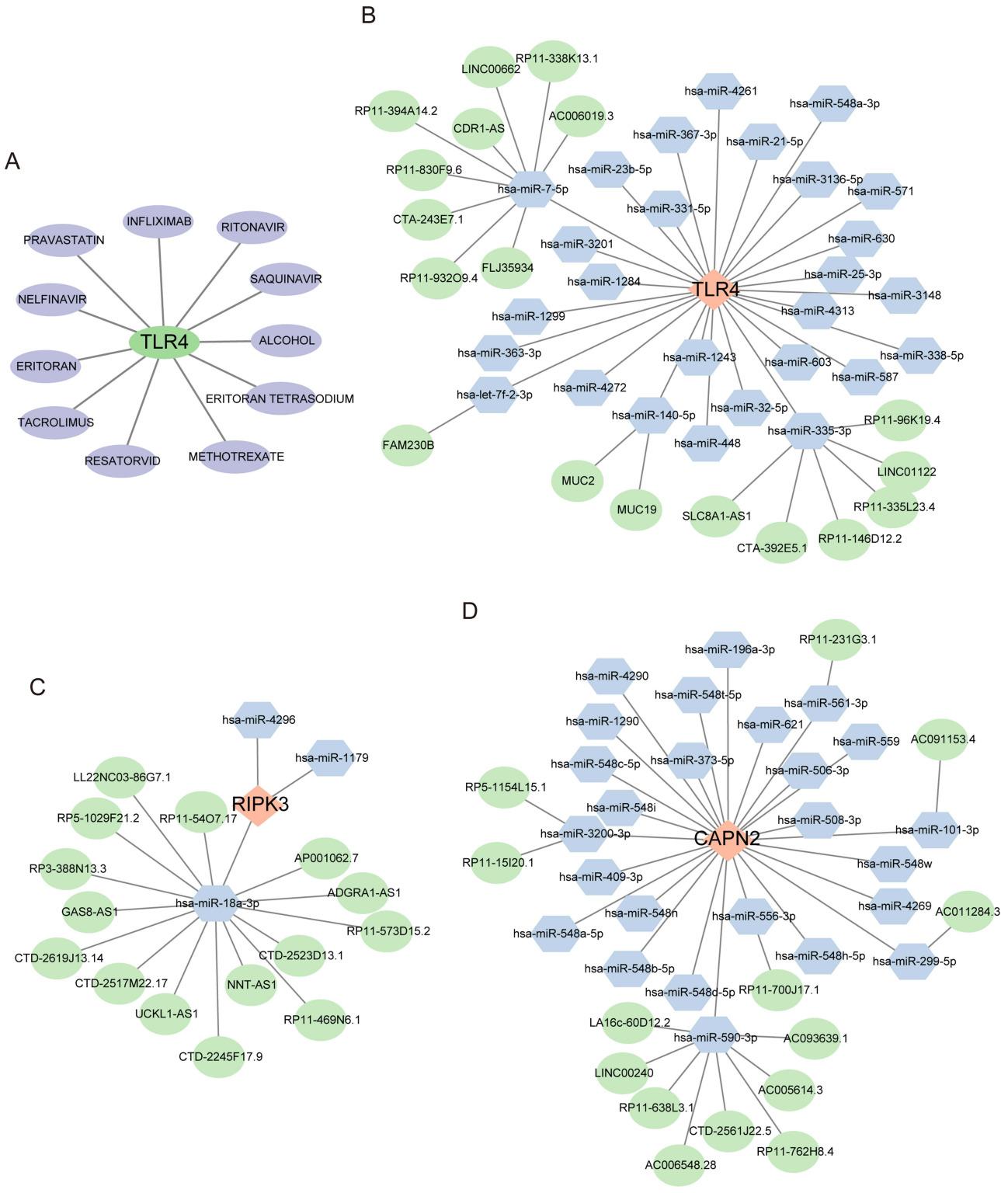

Figure 9 Construction of ceRNA-NRDEGs, drugs-NRDEGs interaction networks. **(A)** Drugs-TLR4 interaction network. **(B-D)** PPI networks of lncRNA-miRNA-TLR4 /RIPK3/CAPN2.

cartoon diagram of siRNA injection into L4 DRG in mice. qRT-PCR experiment confirmed that the mRNA expression levels of RIPK3 or CAPN2 in L4 DRG on the 5th day after siRNA injection was reduced by about 50%, compared with the Negative Control group (Figure 11C). The schematic diagram of behavioral and surgical experiments was shown in

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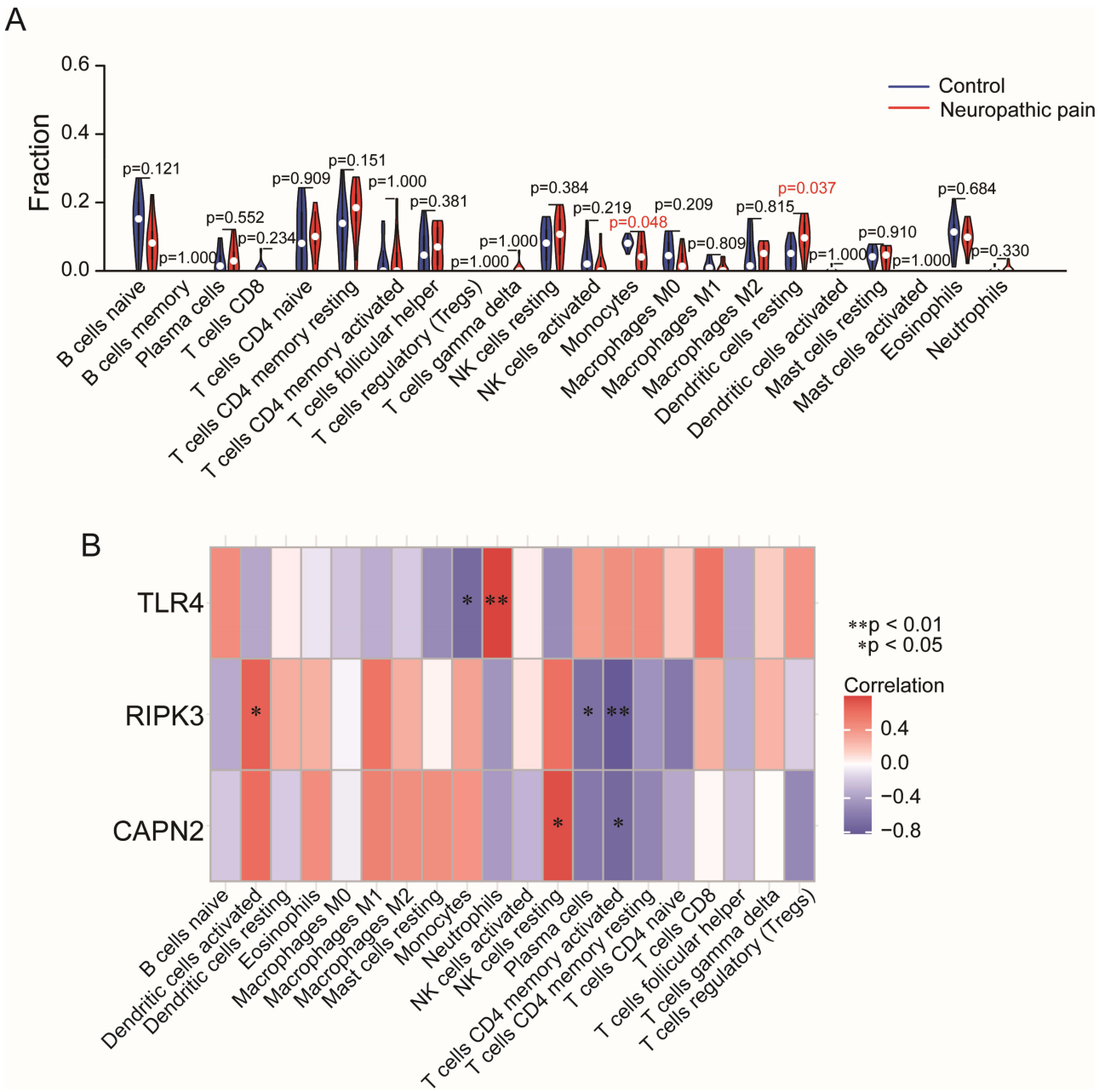

Figure 10 Immune infiltration analysis. **(A)** The correlation between the NRDEGs signature and the abundance of immune cell infiltration. **(B)** The correlation between the degree of immune cell infiltration and the 3 NRDEGs. *$p < 0.05$; **$p < 0.01$.

Figure 11D. By injecting siRNA into the L4 DRG of the mice, it was observed that knockdown of RIPK3 or CAPN2 significantly relieved the mechanical and thermal sensitization behavior induced by SNL (Figure 11C and E).

These basic experiments provided crucial evidence that the 3 NRDEGs in DRG served as important regulators of neuropathic pain. Furthermore, they strongly supported the high accuracy and reliability of the neuropathic pain signature.

Discussion

DRG, where primary sensory neurons converge, serves a crucial role in transmitting and regulating somatosensory information.[13] Recent studies have shed light on the fact that both acute and chronic nerve injuries can lead to alterations in the excitability of DRG neurons. This, in turn, activates various molecules and pathways that are closely associated

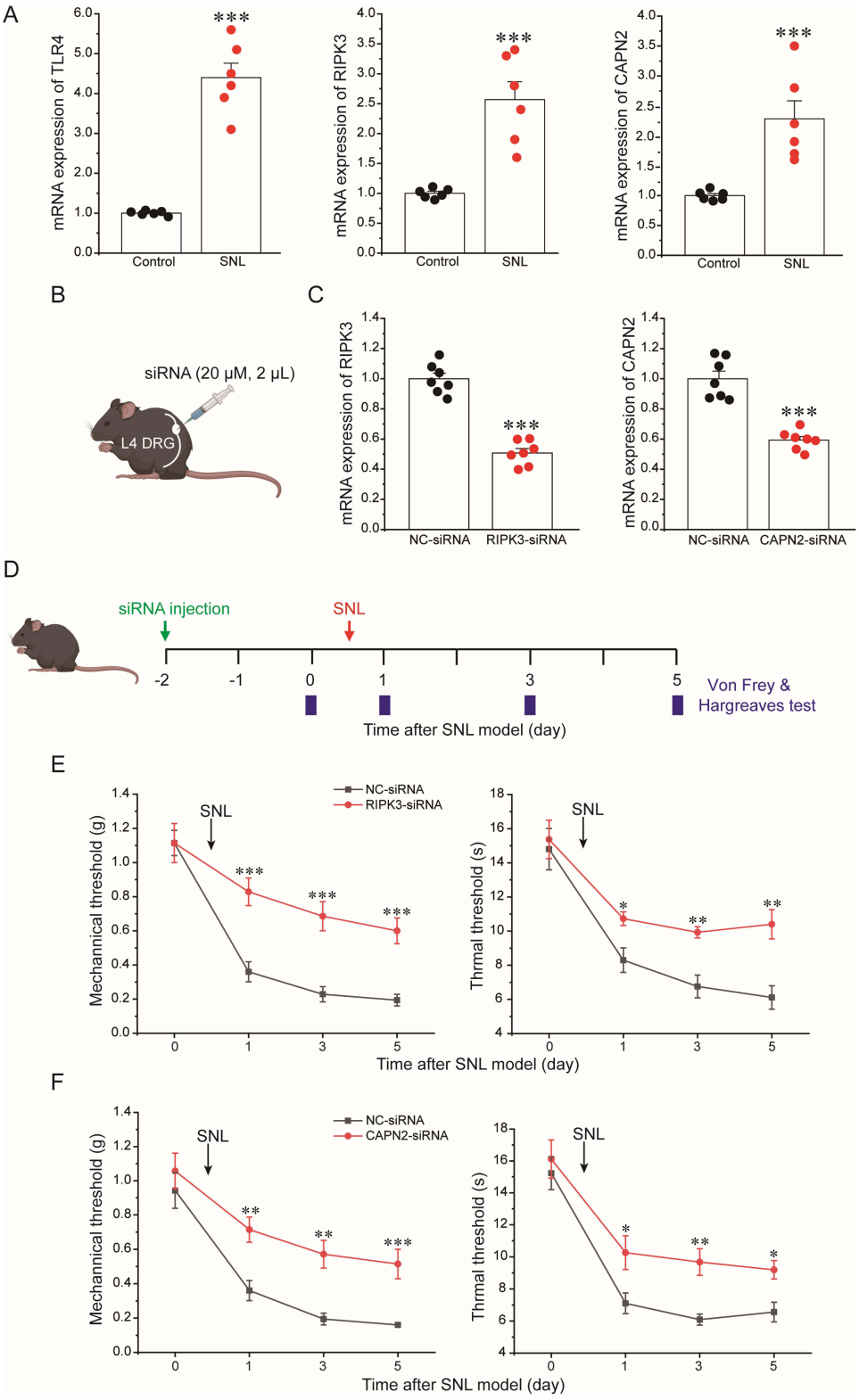

Figure 11 Confirmation of the signature's validity via foundational experiments. **(A)** mRNA expressions of the 3 NRDEGs were significantly increased in the L3-L5 DRGs 5 days after SNL operation in mice via qRT-PCR analysis. **(B)** The cartoon diagram of siRNA injection into L4 DRG of mouse. **(C)** mRNA expression level of RIPK3 or CAPN2 in L4 DRG on the 5th day after siRNA injection was significantly reduced than the Negative Control group. **(D)** Schematic diagram of behavioral experiments. **(E** and **F)** RIPK3 or CAPN2 knockdown in DRG could significantly relieve the mechanical and thermal sensitization behavior induced by SNL; asterisks indicate the difference between groups within the corresponding time point. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

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with pain mechanisms.[14,15] Consequently, DRG-targeted analgesia has emerged as a prominent area of research and interest in the treatment of neuropathic pain.

Necroptosis is a form of regulated cell necrosis that occurs independently of caspases. Studies have reported that necroptosis can lead to the release of cellular components, potentially triggering inflammation in a programmed manner through specific signaling mechanisms.[8] However, whether necroptosis-related factors expressed in peripheral nervous system DRG? Whether these factors involved in the occurrence and development of neuropathic pain? Whether a necroptosis-related feature gene signature in DRG could be developed to predict neuropathic pain? This study aims to provide comprehensive answers to these questions by investigating the expression and potential involvement of necroptosis-related factors in the DRG and their association with neuropathic pain.

We identified a total of 69 necroptosis-related genes that were expressed in the L4 and L5 DRGs from the GSE24982 dataset. Using Lasso and SVM-RFE regression analyses, we constructed a neuropathic pain prediction model based on 3 NRDEGs (TLR4, CAPN2, RIPK3). The nomogram established by the model and the ROC analysis of the GSE24982 and GSE30691 dataset confirmed that the 3 NRDEGs prediction signature could be the independent and predominant predictor for neuropathic pain.

More evidences proved that TLR4 could be one of the key receptors that promote the neuroinflammatory response associated with the pathogenesis of chronic pain.[16] TLR4 is previously found to be widely expressed in microglia, astrocytes, and peripheral primary sensory neurons. In terms of TLR4 expressed by neurons, it is mainly expressed in primary sensory neurons of DRG and trigeminal ganglion (TG), and can participate in the activation or sensitization of primary sensory neurons, and directly contribute to the occurrence of nociceptive transmission.[17,18] TLR4 is expressed on capsaicin-sensitive small diameter neurons and co-expressed with transient receptor potential vanillate 1 (TRPV1) and ATP-gated purinergic receptor P2X3.[19] TLR4 agonist lipopolysaccharide (LPS) can enhance the firing frequency of $TRPV1^+$ sensory neurons, and therefore directly affect the hyperexcitability of primary sensory neurons through a TLR4-dependent mechanism.[20]

RIP3, also known as RIPK3, is a crucial regulatory molecule in the programmed necrosis pathway, and it plays a significant role in various cellular processes, including inflammation. In particular, RIPK3 has been shown to activate NLRP3 (NACHT, LRR, and PYD domains-containing protein 3), which in turn promotes the activation of caspase-1, leading to the formation of the inflammasome and the secretion of inflammatory factors such as TNF-α and IL-1β.[21] Studies have indicated that RIPK3 in DRG is involved in the development of peripheral pain. Research by Ma et al in 2022 demonstrated that paclitaxel treatment could lead to macrophage infiltration in DRG, resulting in the release of TNF-α, IL-1β and the initiation of neuronal necroptosis via the RIPK3/MLKL pathway.[22] In our experiment, we used siRNA to locally down-regulate the expression of RIPK3 in the L4 DRG of mice and observed a significant analgesic effect. This suggests that reducing RIPK3 expression in DRG can alleviate pain symptoms, supporting the theory that RIPK3 is involved in the regulation of neuropathic pain. However, it's important to note that the molecular mechanisms underlying the involvement of RIPK3 in neuropathic pain require further investigation.

CAPN2, a member of the Calpains family, has been associated with the development and progression of various diseases, including tumors, synaptic plasticity, and neurodegenerative diseases.[23–25] However, its role in pain regulation has been less explored. Chen et al in 2018 reported an association between CAPN2 in DRG and the regulation of TNF-α expression related to neuropathic pain following motor nerve injury. They found that MDL28170, a CAPN2 inhibitor, could attenuate ventral root transection-induced hypersensitivity and reduce TNF-α overexpression.[26] However, there have been questions regarding the specificity of MDL28170 as a CAPN2 inhibitor. In our study, we demonstrated that knockdown of CAPN2 expression in the DRG using siRNA injection could alleviate neuropathic pain behavior. This further suggests a role for CAPN2 in the regulation of pain, but the specific molecular mechanisms involved in CAPN2's contribution to neuropathic pain require further investigation. Understanding how CAPN2 interacts with other signaling pathways and molecules in the context of pain regulation is essential for a comprehensive understanding of its role in this process.

In this study, we not only developed a signature based on NRDEGs for accurate prediction of neuropathic pain but also identified potential drugs targeting these NRDEGs for the treatment of neuropathic pain. Among these drugs, Saquinavir, Nelfinavir, and Eritoran are of particular interest, as they have not been extensively studied in the context of pain management. Saquinavir is primarily known as an HIV protease inhibitor, used to block the biological activity of

HIV proteases and halt HIV virus replication. Beyond its antiviral properties, Saquinavir has demonstrated beneficial effects in cancer, inflammation, and immune-mediated diseases.[27,28] These additional properties make it an intriguing candidate for neuropathic pain treatment. Further research is needed to understand how Saquinavir may influence pain-related mechanisms and its potential role in pain management. Nelfinavir is another HIV protease inhibitor commonly used in AIDS treatment. Current studies have revealed that Nelfinavir can induce endoplasmic reticulum stress, autophagy, and apoptosis, which can slow the growth of cancer cells and inhibit the activity of multiple myeloma cells.[29] These findings suggest that Nelfinavir may have broader effects beyond its antiviral activity, and its potential in modulating neuropathic pain mechanisms should be explored further. Eritoran is a second-generation synthetic analogue of lipid A, and it is closely linked to its anti-inflammatory activity. It selectively binds to the TLR4 receptor on the cell surface, blocking TLR4-mediated inflammatory responses.[30] Given its anti-inflammatory properties and potential involvement in modulating TLR4-associated pathways, Eritoran presents a compelling candidate for investigating its impact on neuropathic pain.

However, our study is not without its limitations. Firstly, we establish a predictive model for neuropathic pain using the GSE24982 dataset, and utilize GSE30691 dataset to validate this model. To further ascertain the accuracy of our predictive model, a broader range of datasets should be utilized to comprehensively evaluate the model's performance. Secondly, we recognize the necessity for more in-depth research into the specific mechanisms by which TLR4, CAPN2, RIPK3 are involved in the initiation and progression of neuropathic pain. To summarize, our study provides significant contributions to the diagnosis and management of neuropathic pain by focusing on necroptosis related factors, and acknowledges the opportunity for future studies to broaden and enhance our comprehension in this area.

Conclusions

In summary, this study identified specific genes related to necroptosis in DRG that were dysregulated in neuropathic pain, and a prediction model based on 3 key genes (TLR4, CAPN2, RIPK3) showed promise in predicting the presence of neuropathic pain, which could open up new avenues for the development of novel therapies for neuropathic pain.

Abbreviations

DRG, dorsal root ganglia; GEO, Gene Expression Omnibus; Lasso, Least Absolute Shrinkage and Selection Operator; SVM-RFE, Support Vector Machine-Recursive Feature Elimination; NRDEGs, necroptosis-related diferentially expressed genes; ROC, Receiver Operating Characteristic; SNI, spared nerve injury; CCI, chronic constriction injury; SNL, spinal nerve ligation; GSEA, Gene Set Enrichment Analysis; GSVA, Gene Set Variation Analysis; ceRNA, competing endogenous RNA.

Ethics Statement

All the animal experiments were carried out with the approval of Laboratory Animal Ethical and Welfare Committee of HeBei Medical University. The ethics approval number is IACUC-Hebmu-P2023193.

Data Sharing Statement

The datasets used and analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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Disclosure

The authors declare no conflict of interest.

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