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Bioinformatic Analysis of Potential Biomarkers for Spinal Cord–injured Patients with Intractable Neuropathic Pain

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Background: Neuropathic pain is one of the common complications after spinal cord injury (SCI), affecting individuals' quality of life. The molecular mechanism for neuropathic pain after SCI is still unclear. We aimed to discover potential genes and microRNAs (miRNAs) related to neuropathic pain by the bioinformatics method.

Methods: Microarray data of GSE69901 were obtained from Gene Expression Omnibus (GEO) database. Peripheral blood samples from individuals with or without neuropathic pain after SCI were collected. Twelve samples from individuals with neuropathic pain and 13 samples from individuals without pain as controls were included in the downloaded microarray. Differentially expressed genes (DEGs) between the neuropathic pain group and the control group were detected using the GEO2R online tool. Functional enrichment analysis of DEGs was performed using the DAVID database. Protein-protein interaction network was constructed from the STRING database. MiRNAs targeting these DEGs were obtained from the miRNet database. A merged miRNA-DEG network was constructed and analyzed with Cytoscape software.

Results: In total, 1134 DEGs were identified between individuals with or without neuropathic pain (case and control), and 454 biological processes were enriched. We identified 4 targeted miRNAs, including *mir-204-5p, mir-519d-3p, mir-20b-5p, mir-6838-5p*, which may be potential biomarkers for SCI patients.

Conclusion: Protein modification and regulation of the biological process of the central nervous system may be a risk factor in SCI. Certain genes and miRNAs may be potential biomarkers for the prediction of and potential targets for the prevention and treatment of neuropathic pain after SCI.

Key Words: bioinformatics, biomarkers, neuropathic pain, spinal cord injured

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Neuropathic pain is one of the most common symptoms of patients after spinal cord injury (SCI). It is a severe sensory deficit that is experienced and affects about 80% of individuals with SCI.[1,2] The condition may lead to lifelong loss of function, affected quality of life, and increased morbidity and mortality.[3] It is important to clarify molecular mechanism for treatment of neuropathic pain after SCI.

A number of studies are increasingly focusing on pain after SCI. Multiple mechanisms are raised to explain the neuropathic pain, including peripheral and central sensitization as the major issues. For peripheral sensitization, tissue inflammation may change the chemical environment of the peripheral injured site and cause neuropathic pain. Proinflammatory mediators like leukotriene B4 are contributed to the recruitment of inflammatory cells.[4] Expression of the capsaicin-sensitive cation channel transient receptor potential vanilloid type 1 is observed in the dorsal root ganglion neurons after SCI.[5,6] Central sensitization may amplify the synaptic transfer from the nociceptor to the spinal cord. The glutamate-activated N-methyl-D-aspartic acid receptor may be related to the process of central sensitization.[7,8] Neuroimmune interaction with neurotrophic factor (brain-derived neurotrophic factor), substance P, neurokinin 1 (NK1), dynorphin, and cyclooxygenase 2 are also described as chemical signals associated with the central sensitization process.[9–11] However, there have been few studies on microRNAs (miRNAs) involved in neuropathic pain after SCI. Thus, ongoing studies are required to further evaluate potential genes and miRNAs related to neuropathic pain caused by SCI.

In this study, microarray data from the Gene Expression Omnibus (GEO) database, and the differentially expressed genes (DEGs), were identified between individuals with or without neuropathic pain after SCI. miRNAs targeting the DEGs were also included. We aimed to explore new molecular biomarkers or potential therapeutic targets for neuropathic pain after SCI.

METHODS

Microarray Data Search and Selection of Eligible Data Set

We downloaded the microarray data of GSE69901 from the GEO database (www.ncbi.nlm.nih.gov/geo/) with its microarray platform as GPL15207 (Affymetrix Human Gene Expression Array). The gene expression files were uploaded by Yılmaz B and Adıgüzel E. In this microarray, 12 samples of peripheral blood were obtained from individuals with

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neuropathic pain after SCI and 13 samples without pain as controls. Microarray analysis was performed to identify the gene expression pattern.

DEGs' Screening

DEGs were screened using the online tool GEO2R/R package limma. In the present study, DEGs between neuropathic pain group and control group were screened and selected by the cut-off point of P-value <0.05 and |logFC| > 0.5.

Functional Enrichment Analysis

The selected DEGs were then deposited to the Database for Annotation, Visualization, and Integrated Discovery (DAVID) version 6.8 Beta (https://david-d.ncifcrf.gov/) for further analysis. The DAVID database offers biological function annotation for researchers.[12,13] In this study, the DAVID database was applied to investigate GO annotation and KEGG pathways of DEGs. The biological processes and pathways might contribute to the neuropathic pain after SCI. P-value <0.05 was chosen as the threshold.

Protein-Protein Interaction (PPI) Analysis

PPI of DEGs was obtained from Search Tool for the Retrieval of Interacting Genes (STRING, http://string-db.org/).[14] The STRING database is an online database resource with comprehensive information of interactions of proteins from prediction or experiments. In our study, confidence score > 0.4 of PPI was the selection threshold to construct the PPI network. The list of PPI pairs was downloaded for further analysis.

miRNAs Targeting the DEGs

We uploaded the DEGs to the database, miRNet, (www.mirnet.ca/faces/home.xhtml)[15] to obtain the miRNAs targeting the screened DEGs. The miRNet database is an online tool with various kinds of information generated from miRs studies. Researchers can build miRNA-target interaction networks in the help of this database. The generated list of miRNA-DEG pairs was preserved for further analysis.

Construction of miRNA-DEG Network

Both miRNA-DEG pairs and PPI pairs were then merged to be the miRNA-DEG network and visualized in Cytoscape software 3.4.0.[16] Module clustering analysis for the network was then performed by the Molecular Complex Detection (MCODE)[17] plugin, to detect the potential functional modules in the network. The degree cut-off value to 2 and the node score cut-off to 0.2 were set in the MCODE process.

RESULTS

DEG Screening Between Patients and Controls

Data normalization and cross-comparability were assessed (Table 1), and then the DEGs analysis was performed. A total of 1134 DEGs were selected by the cut-off point of P-value <0.05 and |logFC| > 0.5, which included 489 upregulated and 645 downregulated DEGs. The most significant top 10 upregulated or downregulated genes are shown in Table 2. The upregulated gene with the smallest P-value ($P = 0.021889$) was GLG1 (Golgi glycoprotein 1), and the downregulated gene with the smallest P-value (0.048081) was HPCAL4 (hippocalcin-like 4).

Functional Enrichment Analysis

Four hundred eighty-nine GO terms ($P < 0.05$) were significantly enriched by upregulated DEGs, whereas 645 genes ($P < 0.05$) were significantly enriched by downregulated DEGs. Many of these enriched terms were associated with neuron cell development or inflammatory processes involved in protein modification and regulation biological processes in the central nervous system. We recognized significant enrichments of the DEGs (top 20, Table 3), which were classified in 454 GO

TABLE 1. Data Normalization and Cross-comparability

Groups	Lower Whisker	Lower Hinge	Median	Upper Hinge	Upper Whisker	Mean	SD	N
Control								
GSM1712184	4.72	3,666,068.5	5,017,354	6,919,015.5	9,999,881	5,028,338.01	2,545,534.91	49,395
GSM1712185	9.5	3,642,136	5,001,089	6,903,982.5	9,999,678	5,008,402.98	2,555,861.14	49,395
GSM1712186	5.69	3,636,448	4,964,987	6,888,151.5	9,999,997	4,990,430.25	2,570,535.07	49,395
GSM1712187	4.59	3,670,260.5	5,029,147	6,886,073	9,999,775	5,017,970.9	2,545,510.92	49,395
GSM1712188	3.85	3,675,823.5	5,013,726	6,897,734	9,999,963	5,011,778.49	2,554,123.45	49,395
GSM1712189	4.56	3,700,229	5,066,739	6,909,265.5	9,999,789	5,039,620.5	2,539,008.86	49,395
GSM1712190	3.36	3,642,463	4,968,945	6,851,610.5	9,999,907	4,979,644.99	2,547,492.5	49,395
GSM1712191	3.42	3,637,891	4,979,304	6,879,673.5	9,999,531	4,994,329.63	2,556,272.61	49,395
GSM1712192	2.99	3,680,093.5	5,035,185	6,923,889.5	9,999,833	5,036,412.77	2,557,605.85	49,395
GSM1712193	5.48	3,655,661	5,029,109	6,923,814	9,999,848	5,014,179.82	2,568,544.99	49,395
GSM1712194	4.24	3,699,317	5,052,407	6,881,152	9,999,776	5,028,067.6	2,541,273.1	49,395
GSM1712195	4.06	3,709,656	5,049,326	6,873,823	9,999,408	5,029,736.16	2,532,686.58	49,395
GSM1712196	3.81	3,681,355	5,047,544	6,864,883.5	9,998,966	5,016,205.59	2,538,709.09	49,395
Case								
GSM1712172	3.86	3,677,131.5	5,052,405	6,862,898	9,999,974	5,011,561.86	2,541,648.62	49,395
GSM1712173	3.07	3,650,665.5	5,012,139	6,920,339.5	9,998,799	5,015,405.3	2,562,831.7	49,395
GSM1712174	5.32	3,676,019	5,059,126	6,866,129.5	9,999,894	5,017,484.29	2,538,816.09	49,395
GSM1712175	3.97	3,775,161	5,144,407	6,856,996	9,999,437	5,064,520.44	2,497,354.14	49,395
GSM1712176	4.14	3,621,528	4,964,224	6,910,990.5	9,998,967	5,001,747.03	2,559,535.45	49,395
GSM1712177	4.42	3,632,421.5	4,966,668	6,862,025.5	9,999,502	4,972,279.14	2,565,699.14	49,395
GSM1712178	3.55	3,676,787	5,010,466	6,884,470.5	9,999,877	5,023,454.48	2,539,103.2	49,395
GSM1712179	4.72	3,657,787.5	5,033,792	6,894,234	9,999,776	5,016,640.07	2,547,930.76	49,395
GSM1712180	4.09	3,636,030	4,980,927	6,873,579	9,999,155	4,996,770.11	2,553,905.39	49,395
GSM1712181	3.58	3,621,665	4,954,772	6,853,315	9,999,494	4,967,557.49	2,557,504.22	49,395
GSM1712182	5.49	3,695,330.5	5,082,481	6,913,891	9,999,473	5,037,741.75	2,545,464.19	49,395
GSM1712183	4.1	3,677,958.5	5,026,092	6,870,587	9,999,387	5,013,058.25	2,543,174.23	49,395

TABLE 2. The 10 Most Strongly Upregulated Genes or Downregulated Genes in SCI

ID	P	logFC	Gene Symbol	Stage
11717835_a_at	0.02089	3.46	*GLG1*	Up
11744156_a_at	0.002739	2.96	*RPS6KA1*	Up
11754395_a_at	0.001272	2.74	*BRD4*	Up
11740230_a_at	0.045202	2.57	*PHEX*	Up
11741375_a_at	0.047445	2.56	*CADM1*	Up
11716567_a_at	0.001363	2.5	*BAT2L2*	Up
11725071_at	0.026417	2.42	*PHF17*	Up
11738143_x_at	0.015512	2.32	*SSX2IP*	Up
11720407_x_at	0.016032	2.3	*PPP6R2*	Up
11754361_s_at	0.01944	2.25	*GSK3B*	Up
11729106_a_at	0.048081	−1.30E-01	*HPCAL4*	Down
11754279_a_at	0.040999	−1.44E-01	*MYEF2*	Down
11759586_s_at	0.030052	−1.52E-01	*RAB18*	Down
11746506_a_at	0.048036	−7.32E-01	*SPP1*	Down
11715494_s_at	0.046183	−8.09E-01	*HBG1*	Down
11742281_at	0.049698	−8.11E-01	*NAF1*	Down
11722874_s_at	0.049822	−8.20E-01	*MSL3*	Down
11726038_s_at	0.049825	−8.20E-01	*C3orf38*	Down
11718089_at	0.04697	−8.21E-01	*TNKS*	Down
11720996_at	0.048861	−8.22E-01	*ADCY2*	Down

SCI indicates spinal cord injury.

categories. Most of the categories were about the nervous system, and the extremely significant enrichment GO category was nervous system development with a *P*-value of 6.31E-07. Other significant categories covered epithelial cell migration and cellular response chemical stimulus processes. To further research the functions of the DEGs, we represented significant enrichments of the DEGs to the KEGG database (top 20 Table 4). We identified

TABLE 3. The Top 20 Enriched GO Terms Among the DEGs in SCI

GO Terms	Count	P	FDR
Nervous system development	174	6.31E-07	0.001243484
Brain development	69	6.53E-06	0.012860518
Head development	71	1.03E-05	0.020229937
Neuron development	88	1.04E-05	0.020445904
Intracellular signal transduction	195	1.36E-05	0.026786425
Generation of neurons	113	2.29E-05	0.04503678
Regulation of epithelial cell migration	25	2.52E-05	0.049598
Neuron projection development	76	2.89E-05	0.056921098
Regulation of cell development	78	3.12E-05	0.061397173
Neuron differentiation	104	3.14E-05	0.061763371
Neurogenesis	118	3.65E-05	0.071885493
Central nervous system development	81	5.40E-05	0.106397122
Response to abiotic stimulus	92	5.44E-05	0.107213467
Regulation of neurogenesis	65	6.00E-05	0.118238555
Cell surface receptor signaling pathway	189	6.45E-05	0.127091114
Neuron projection morphogenesis	53	9.07E-05	0.178565503
Response to organic substance	205	9.58E-05	0.18852846
Cellular response to chemical stimulus	190	1.38E-04	0.272230269
Cell morphogenesis involved in neuron differentiation	49	1.41E-04	0.27791141
Cell morphogenesis involved in differentiation	67	1.48E-04	0.290962114

DEG indicates differentially expressed gene; FDR, false discovery rate; SCI, spinal cord injury.

TABLE 4. The Top 20 Most Significant Pathways Identified in the KEGG Database

KEGG Term	Count	P	FDR
Dopaminergic synapse	22	1.87697E-05	0.024604275
Cholinergic synapse	18	0.000276271	0.361585263
Estrogen signaling pathway	16	0.000702049	0.916482827
Aldosterone synthesis and secretion	14	0.000907645	1.183404423
Circadian entrainment	14	0.003953894	5.061250907
Adrenergic signaling in cardiomyocytes	18	0.005897437	7.461388865
Notch signaling pathway	9	0.006854023	8.622007309
Protein processing in endoplasmic reticulum	19	0.011580811	14.16216121
Hepatitis B	17	0.012235287	14.90431337
Thyroid hormone signaling pathway	14	0.017679066	20.85159691
Platelet activation	15	0.022310411	25.60648089
Osteoclast differentiation	15	0.023675291	26.95657246
Oxytocin signaling pathway	17	0.025722009	28.93874929
Sphingolipid signaling pathway	14	0.025932372	29.13963675
Glucagon signaling pathway	12	0.032909097	35.51236463
Prostate cancer	11	0.035757792	37.95876114
Retrograde endocannabinoid signaling	12	0.037356015	39.29345131
cGMP-PKG signaling pathway	17	0.038299087	40.06852402
Amphetamine addiction	9	0.04130641	42.47949262
Pathways in cancer	33	0.043002293	43.79925259

FDR indicates false discovery rate.

21 significant pathways based on KEGG database analysis. The most significant pathway in our KEGG analysis was dopaminergic synapse with a *P*-value of 1.88E-05.

Construction of miRNA-DEG Network Analysis

Taking the selected 1134 DEGs into account, we identified 2236 PPI pairs by STRING, as well as 1269 miRNA-DEG pairs by miRNet through the software of Cytoscape, and 2 big pairing pictures were generated. The pictures mentioned above were merged, and an intact tremendous miRNAs-DEG network was generated in Cytoscape. To assess the key functional modules of this network, module clustering based on the miRNA-DEG network mentioned above was then performed by the MCODE plugin of Cytoscape. Three modules were identified and showed DEGs (Fig. 1), such as *IL22RA1, IFNA21, IFNA2, NUP50, SSU72, USP42, FZD1*, and *CSNK1A1*, and targeted miRNAs, such as *mir-204-5p, mir-519d-3p, mir-20b-5p*, and *mir-6838-5p*. Two outstanding hub DEGs, *FZD1* and *IL22RA1* (black rhombus, Fig. 1) in these modules also occurred in the GO terms enriched above, and their interacted DEGs, *CSNK1A1* and miRNA like *mir-204-5p* (white rectangle linked with black rhombus, Fig. 2), *IFNA21, IFNA2* (white rectangle linked with black rhombus, Fig. 3), associated with inflammatory or neurons development of protein modification and regulation processes. We also observed a key hub miRNA, *mir-6838-5p* (black rhombus, Fig. 1), that interacted with DEGs, including *NUP50, SSU72*, and *USP42* (white linked with black rectangle, Fig. 4).

DISCUSSION

Neuropathic pain following SCI is caused by dysfunction of the nervous system or damage of nervous cells.[1]

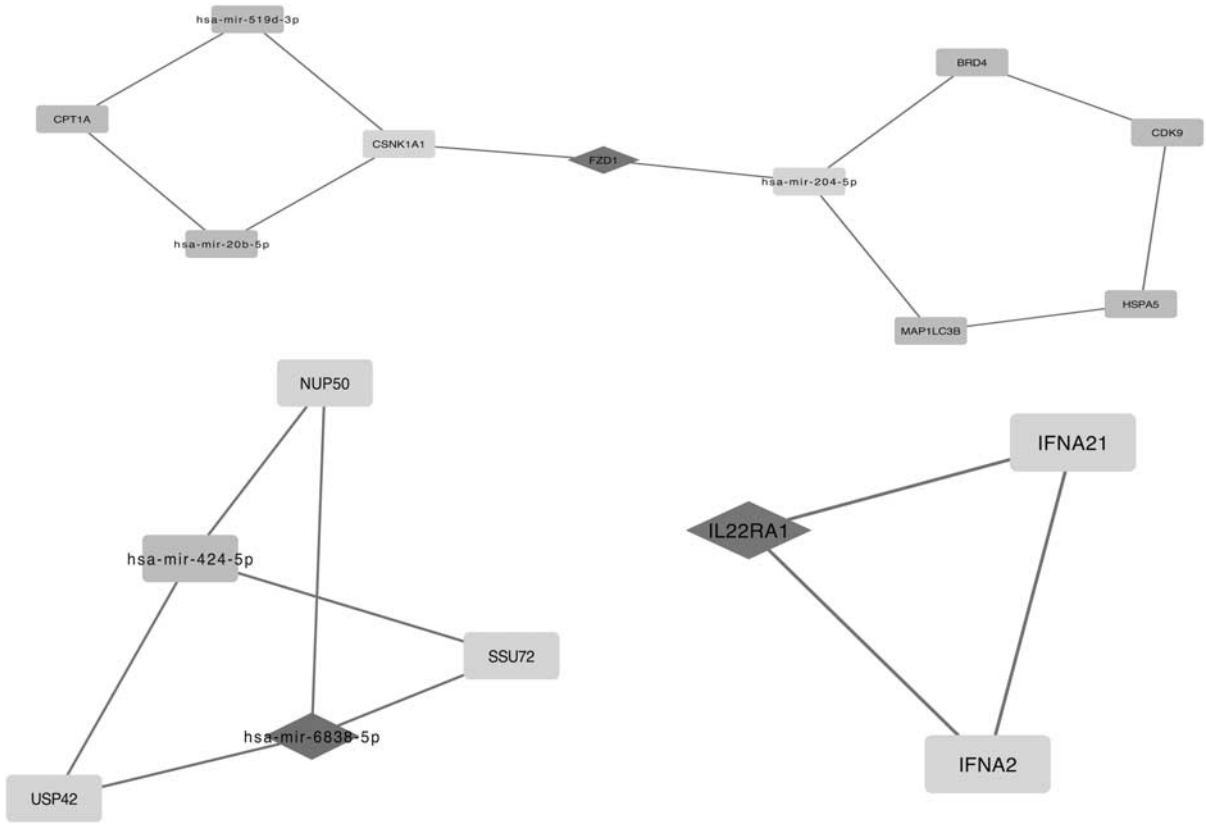

FIGURE 1. 2236 protein-protein interaction pairs by STRING, as well as 1269 microRNAs-differentially expressed gene (miRNA-DEG) pairs by miRNet through the software of Cytoscape, and 2 big pairing pictures were generated. Pictures above were merged, and an intact tremendous miRNAs-DEG network was generated in Cytoscape. Three modules above were identified and showed DEGs, such as *IL22RA1, IFNA21, IFNA2, NUP50, SSU72, USP42, FZD1*, and *CSNK1A1*, and targeted miRNAs, such as *mir-204-5p, mir-519d-3p, mir-20b-5p*, and *mir-6838-5p*.

Almost one third of people with SCI will experience continuous neuropathic pain. It is difficult to treat and reduces the quality of life for these individuals.[18] It is a pity that nonpharmacological approaches may be more useful to neuropathic pain, and the same current management does not achieve satisfactory pain reduction.[3] It is necessary to share many more quality data about SCI between different research centers or countries, to better recognize how to treat SCI, as well as more clinical studies or novel management methods to evaluate the effect of treatments.[19]

As the miRNA-DEG network analysis shows, neuropathic pain after SCI was closely related to the *FZD1* gene. Previous studies showed that *FZD1* is the transmembrane receptor of Wnt signaling pathways,[20,21] Wnt-protein family played an important regulation role in the neural development of the hippocampus in the human central nervous system.[22] Wnt proteins were involved in almost all aspects of development in the nervous system.[23] Researches had shown that the Wnt signaling was associated with the maintenance of neural stem cells by Muriel et al,[24] and the study also found that Wnt

FIGURE 2. An outstanding hub differentially expressed gene (DEG), *FZD1* (black rhombus), its interacted DEG such as *CSNK1A1*, and miR such as *mir-204-5p* (white linked with black rectangle).

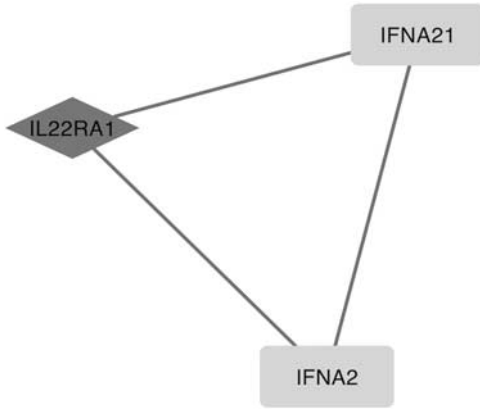

FIGURE 3. Another outstanding hub differentially expressed gene (DEG), *IL22Ra1* (black rhombus), and its interacted DEG such as *IFNA21* and *IFNA2* (white linked with black rectangle).

signaling pathways regulated new neurons generated from neural stem cells in adult hippocampus. *FZD1* was an important receptor for Wnt signaling, highly expressed in the dentate gyrus neural stem cells.[24] *FZD1* knockdown led to a marked decrease in the differentiation of neural stem cells into neurons, and increased the generation of astrocytes, significantly. Studies had demonstrated that stem cells could be used to treat some common forms of neuropathic pain, including SCI and sciatic nerve injury; this research was confirmed in animal models by Sudhakar et al.[25] Moreover, a large number of studies had shown that astrocytes played an important role in the central sensitization of pain, especially for chronic pain.[26–28] Thus, we speculated that *FDZ1* could regulate the development of neural stem cells, and inhibit the production of astrocytes, relieving the inflammation response triggered by SCI, and alleviate neuropathic pain caused by SCI through Wnt signaling pathways.

As expected, inflammatory factors and mediators, such as interleukins, cyclooxygenase 2, calcitonin gene-related peptide, tumor necrosis factor-α, were involved in the occurrence and development of pain.[29–31] In our study, through functional enrichment analysis, we found that *IL22RA1* connected with neuropathic pain after SCI. *IL22RA1* was a unique receptor of IL-22.[32] IL-22 was an IL-10 family cytokine generated by Th17, Th22, and Th1 cells, and IL-22 played an significant role in T cell-mediated inflammatory diseases.[33] *IL22RA1* and its associated genes through cytoscape analyzing, including *IFNA21* and *IFNA2*, mainly related to inflammation reaction in the GO-BP database. They may be the key genes of initiating the inflammatory response. Through MCODE analysis, we found that *mir-6838-5p* which may be the core of the module. *Mir-6838-5p* was associated with genetic RPS6KB1. The studies had shown that RPS6KB1 was closely related to the inflammatory and immune response.[34,35] *Mir-6838-5p* together with its targeted genes, such as *NUP50, SSU72*, and *USP42*, were closely associated with protein modification and biosynthesis through GO-BP database analysis. Therefore, we argued that *IL22RA1* and *mir-6838-5p* played a vital role as inflammation reaction mediators in neuropathic pain induced by SCI.

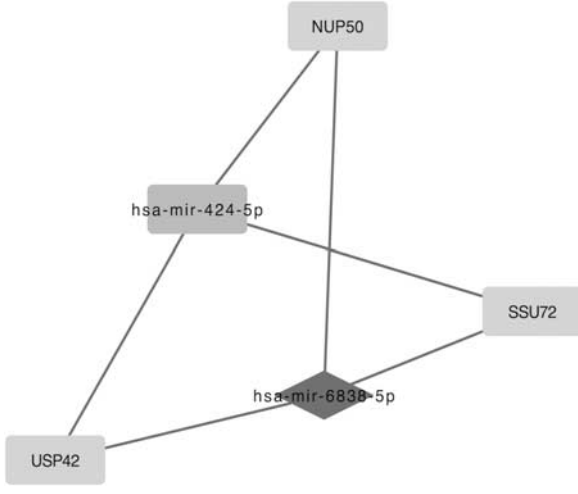

FIGURE 4. A key hub microRNA, *mir-6838-5p* (black rhombus) and its interacted differentially expressed genes (DEGs) such as *NUP50, SSU72*, and *USP42* (white linked with black rectangle).

In conclusion, FDZ1 could regulate the differentiation of neural stem cells into neurons and induce astrocyte proliferation. An important supporter of neuron cells, astrocytes can generate inflammatory mediators and mediate central sensitization of pain.[36,37] Numerous researches suggest that astrocytes have also played a key role in immunoreaction.[38–40] As a member of the IL-10 family, IL-22 has an effect in the inflammatory response. *Mir-6838-5p* could induce an immunoreaction through immunoregulation. Hence, we argue that astrocytes can be activated, releasing inflammatory mediators or immunomodulatory factor when the spinal cord is injured, inducing inflammation or immune response, resulting in central sensitization of acute pain and then producing chronic pain. Down-regulation of FDZ1 would inhibit astrocytes activation, inflammatory medium (IL22) release and some genes expression (*mir-6838-5p*, *IL22RA1*). Thus, it may stop acute pain to translate into chronic pain. This would be beneficial for the treatment of intractable neuropathic pain induced by SCI.

Nevertheless, the currently popular molecules such as mitogen-activated protein kinase and nuclear factor kappa beta, are all involved in the formation and development of inflammation response pain or neuropathic pain.[41–44] But in our study, these genes were not being filtered, it did not mean these signaling molecules or pathways not participation in neuropathic pain after SCI, it maybe not in the core position in the development of developing the pain, or further studies are needed in the future.

CONCLUSION

In conclusion, *FZD1, IL22RA1*, and *mir-6838-5p* might become potential biomarkers for the prediction of and new targets for prevention and treatment of neuropathic pain after SCI. However, further studies are necessary for verifying the clinical applications of these findings.

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