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Gene expression signature analysis and Protein-Protein interaction network construction of Spinal Cord Injury

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Abstract. – BACKGROUND: The aim of this study was to investigate the gene expression profile of thoracic propriospinal neurons between post-injury rat and controls.

MATERIALS AND METHODS: Microarray dataset GSE20907 was downloaded from GEO database, including 12 Spinal Cord Injury (SCI) rat and 12 controls. Student's t test was employed to identified differentially expressed genes with a fold-change > 1.2. Then, we used DAVID to perform functional enrichment analysis to uncover dysfunctional biological processes and molecular signatures database (MsigDB) to find any potential relationship between SCI gene expression signature and other published gene expression signature. Protein-Protein interaction (PPI) network was constructed by STRING and visualized in Cytoscape. Functional analysis of the hub protein was performed by BinGO.

RESULTS: The maximum change of gene expression profile was found at 3-days post injury and immune response was found upregulated all tested time points. Interestingly, genes upregulated 2-weeks post injury was found significantly overlapped with genes upregulated in brains from Alzheimer's disease patients. Protein interaction network analysis found that LYN, PTPN6 and SMAD1 could be of great value for further investigation.

CONCLUSIONS: It could be inferred that understanding the underlying molecular mechanism post injury, especially at early moment, may provide novel insight for development of therapeutics strategy.

Keywords:
Protein-Protein interaction network, Gene expression profile, Hub protein.

Introduction

A spinal cord injury (SCI) refers to a sudden, traumatic blow to the spine that caused a damage to the spinal cord[1]. An injury to the spinal cord can damage a few, many and almost all of nerve roots, depending on where the spinal cord and nerve roots are damaged, the symptom can vary widely, from pain to complete paralysis[2]. The damage begins at the moment of injury when displaced bone fragments, disc material, or ligaments bruise or tear into spinal cord tissue[3]. Respiratory complications are often an indication of the severity of spinal cord injury. About one-third of those with injury to the neck area will need help with breathing and require respiratory support.

The use of methylprednisolone after acute spinal cord injury has been under discussion for more than 20 years[4,5]. There is ongoing debate about the efficacy and clinical impact of methylprednisolone in recovery from spinal cord injury, and studies show considerable variability in practice patterns among surgeons. Consensus statements consider methylprednisolone as a treatment option for acute spinal cord injury, but not a standard of care based on available evidence[6].

Advances in the neurosciences have drawn attention to research into spinal-cord injury. Nowadays, advanced interventions provide high hope for regeneration and functional restoration[7]. As scientific advances become more frequent, scepticicism is giving way to the ideas that spinal-cord injury will eventually be repairable and that strategies to restore function are within our

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grasp[8]. Many genes and signaling pathways have demostrated significant changed in the injured spinal cord. A recent study proved that immediate early genes (IEGs), cytokines, neurotrophins, and neurotrophin receptors playing key role in the injured spinal cord[9]. Previous studies have suggested that the Rho signaling pathway regulates the cytoskeleton and motility and plays an important role in neuronal growth inhibition[10]. Recent findings further provide evidence that the Rho signaling pathway is a potential target for therapeutic interventions after spinal cord injury[11].

The classical biomedical researchers, based on molecular biology, cell biology, genetics and other experimental biology, have made significant progress against SCI. However, the researchers on the biomedical area still face the great challenge for against the SCI since the methodology of the classical experimental biology is based on studying individual gene and protein and treat the organisms as a simple and linear system, which is not good enough to solve such problems of the complex diseases. Therefore, it is clear that a new methodology and techniques need to be applied for analyzing the molecular mechanisms of the complex diseases such as SCI, and provide new solutions for preventing and curing the diseases.

In this work, we propose a systems biology approach, which integrates expression profile data, to identify pathways responsible for SCI. This approach consists of three steps: initially, we screened a set of differentially expressed genes (DEGs) using array data sets between normal and SCI samples; further, we identified significant regulated pathways; finally, we constructed the significant regulated pathway network of SCI. Our research highlights tackling the bridge from functional effects of differentially expressed genes into pathways, as well as pathway-pathway interaction networks. It could be inferred that understanding the underlying molecular response post injury, especially at early moment, may provide novel insight for development of therapeutics strategy. Bioinformatics analysis of post-SCI genome-wide expression pattern could perhaps provide such opportunities.

Materials and Methods

Microarray dataset

Microarray dataset was downloaded from GEO (GSE20907) including 12 injured rat thoracic propriospinal (TPS) neurons and 12 controls[12]. Samples were divided into three groups based on which comparison of gene expression was made: 1) 3-days post injury versus control; 2) 2-weeks post injury versus control; 3) 1 month post injury versus control. Gene expression data was recalculated and normalized by Robust Multi-array Average (RMA)[13] using an entrezgene-based custom chip description file[14]. Rat Entrez gene IDs were mapped to human gene symbols according to orthologous relationships from MGI (Mouse Genome Informatics)[15].

Identification of gene expression signature of spinal cord injury (SCI)

We identified genes with a fold-change > 1.2 as DEGs. Two-sample unequal variance Student's *t* test was also performed but its results should be interpreted with cautious due to too limited samples involved.

Functional analysis of SCI gene expression signature

Gene expression signature of SCI was submitted to DAVID[16] to perform functional enrichment analysis aiming at uncovering perturbed biological processes. In addition, we submitted the SCI gene expression signature to MSigDB[17] to find any potential relationship between SCI gene expression signature and other published gene expression signature which may inspire novel start points for medical research and therapeutic strategy of SCI.

Protein-protein interaction network construction of SCI gene expression signature

Human protein protein interaction (PPI) data were downloaded from three public databases: MINT[18], BioGrid[19] and HPRD[20]. Only interactions that collected by at least two databases were used in our analysis. PPI network for each time points post injury were constructed separately using interactions that involved with signature genes and visualized in Cytoscape[21]. Hub proteins were identified as those interacted with most partners. Functional analysis of the hub-centered subnetwork was performed by BinGO[22].

Results

Biological interpretation of SCI gene expression signature

The maximum alteration of gene expression occurred 3-days post injury with 1105 gene upregulated and 1232 genes downregulated. Less

Table I. DAVID functional annotation of gene upregulated in TPS neurons 3 days post injury.

Category	*p*-value	Term	FDR
GO:0000279	5.07E-17	M phase	1.33E-13
GO:0000280	1.63E-16	Nuclear division	1.46E-13
GO:0007067	1.63E-16	Mitosis	1.46E-13
GO:0048285	1.80E-16	Organelle fission	1.95E-13
GO:0000087	4.57E-16	M phase of mitotic cell cycle	2.92E-13
GO:0022403	2.40E-15	Cell cycle phase	1.28E-12
GO:0007059	1.20E-13	Chromosome segregation	5.30E-11
GO:0022402	5.48E-12	Cell cycle process	2.06E-09
GO:0000278	2.75E-11	Mitotic cell cycle	9.09E-09
GO:0051301	7.29E-11	Cell division	2.13E-08
GO:0007049	6.89E-10	Cell cycle	1.81E-07
GO:0006954	4.96E-08	Inflammatory response	0.0000119

The *p*-values associated with each terms inside the clusters is *p*-values by the Fisher Exact Test which represents the "degree of enrichment" of the annotation term with the input gene list. Benjamini FDR q-value is the correction for multiple comparison implemented method by Benjamini Y, H.Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. Roy. Stat. Soc, 57.

DEGs could be detected as time went on. For 2-week post injury, 1036 up-regulated gene and 937 down-regulated genes were found while 819 up-regulated gene and 669 down-regulated genes were found 1-month post injury.

For genes altered 3-day post injury, DAVID functional analysis found that the upregulated genes majorly involved in cell cycle and immune response (Table I). Indeed, immune response was captured by gene expression signature through all three time points. Interestingly, at two weeks post injury, downregulated genes majorly involved in olfactory transduction (Table II).

By investigating the relationship between SCI gene signature and the published gene signatures from various studies, we found that genes upregulated 3-days post injury were significantly overlapped with genes upregulated during later stage of differentiation of oligodendroglial precursor cells in response to PD1742[23], an ErbB-family kinase inhibitor (Table III). On the other hand, genes upregulated 2-weeks post injury were significantly overlapped with genes upregulated in brain from patients with Alzheimer's disease[24] (Table IV).

Protein-protein interaction network of SCI gene expression signature

Protein interaction network of SCI gene expression signature could reflect the underlying molecular mechanism of cellular response to injury. For the PPI network of 3-days post injury, UBQLN4, VIM and LYN were the three hub genes with most interactions (Figure 1). Gene ontology analysis found that the Lyn-centered subnetwork was enriched with cellular signaling

Table II. DAVID functional annotation of gene downregulated in TPS neurons 2 weeks post injury.

Category	*p*-value	Term	FDR
hsa04740:01	3.55E-09	Factory transduction	5.36E-07
GO:0004984	9.94E-09	Olfactory receptor activity	6.40E-06
GO:0007606	7.23E-09	Sensory perception of chemical stimulus	0.000015
GO:0007608	1.60E-08	Sensory perception of smell	0.000016
GO:0007600	2.54E-07	Sensory perception	0.000178
GO:0050890	3.65E-06	Cognition	0.001930
GO:0007186	0.00006	G-protein coupled receptor	0.028211

The *p*-values associated with each terms inside the clusters is *p*-values by the Fisher Exact Test which represents the "degree of enrichment" of the annotation term with the input gene list. Benjamini FDR q-value is the correction for multiple comparison implemented method by Benjamini Y, H.Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. Roy. Stat. Soc, 57.

Table III. connecting post-injuried TPS neurons gene signature with published gene expression signature. published gene expression signatures were collected by MSigDB in the C2 CGP category. *p*-value was calculated by Fisher's exact test indicating the statistical significance of overlapping between gene signatures upregulated in TPS neurons 3 days post injury and published gene expression signature.

Gene Set Name	Description
PILON_KLF1_TARGETS_DN	Genes down-regulated in erythroid progenitor cells
MARSON_BOUND_BY_E2F4_UNSTIMULATED	Genes with promoters bound by E2F4
BENPORATH_CYCLING_GENES	Genes showing cell-cycle stage-specific expression
SHEDDEN_LUNG_CANCER_POOR_SURVIVAL_A6	Genes in patients with non-small cell lung cancer
RODWELL_AGING_KIDNEY_UP	Genes whose expression increases with age in normal kidney
CHEN_METABOLIC_SYNDROM_NETWORK	Genesforming the macrophage-enriched metabolic network
SCHUETZ_BREAST_CANCER_DUCTAL_INVASIVE_UP	Genes up-regulated in invasive ductal carcinoma
GOLDRATH_ANTIGEN_RESPONSE	Genes up-regulated at the peak of an antigen response of naive CD8+
GOBERT_OLIGODENDROCYTE_DIFFERENTIATION_UP	Genes up-regulated during later stage of differentiation of Oli-Neu cells
HORIUCHI_WTAP_TARGETS_DN	Genes down-regulated in primary endothelial cells

The *p*-values associated with each terms inside the clusters is *p*-values by the Fisher Exact Test which represents the "degree of enrichment" of the annotation term with the input gene list. Benjamini FDR q-value is the correction for multiple comparison implemented method by Benjamini Y, H.Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. Roy. Stat. Soc, 57.

proteins (corrected *p*-value 3.31e-04). For the network of 2-weeks post injury, LYN, PTPN6 and SMAD1 were the three hub genes with most interactions (Figure 2). Gene ontology analysis showed that the LYN-PTPN6 centered sub-network was enriched with proteins involved in signal transduction (corrected *p*-value 3.29e-06) while the SMAD1-subnetwork was enriched with negative regulation of gene expression (corrected *p*-value 1.38e-04). TRAF2, TRAF6 and PTPN6 were the three hub genes with most interactions in the network of 1-month post injury (Figure 3).

Table IV. Connecting post-injuried TPS neurons gene signature with published gene expression signature. published gene expression signatures were collected by MSigDB in the C2 CGP category. *p*-value was calculated by Fisher's exact test indicating the statistical significance of overlapping between gene signatures upregulated in TPS neurons 2 weeks post injury and published gene expression signature.

Gene Set Name	Description
PILON_KLF1_TARGETS_DN	Genes down-regulated in erythroid progenitor cells
CHEN_METABOLIC_SYNDROM_NETWORK	Genes forming the macrophage-enriched metabolic network
MCLACHLAN_DENTAL_CARIES_UP	Genes up-regulated in pulpal tissue extracted from carious teeth
FULCHER_INFLAMMATORY_RESPONSE_LECTIN_VS_LPS_DN	Genes down-regulated in monocyte-derived dendritic cells
DACOSTA_UV_RESPONSE_VIA_ERCC3_DN	Genes down-regulated in fibroblasts expressing mutant forms of ERCC3
BLALOCK_ALZHEIMERS_DISEASE_UP	Genes up-regulated in brain from patients with Alzheimer's disease
TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_3D_UP	Genes up-regulated in CD34+
MARKEY_RB1_ACUTE_LOF_UP	Genes up-regulated in adult fibroblasts with inactivated RB1
ZHENG_BOUND_BY_FOXP3	Genes whose promoters are bound by FOXP3
DACOSTA_UV_RESPONSE_VIA_ERCC3_COMMON_DN	Common down-regulated transcripts in fibroblasts

The *p*-values associated with each terms inside the clusters is *p*-values by the Fisher Exact Test which represents the "degree of enrichment" of the annotation term with the input gene list. Benjamini FDR q-value is the correction for multiple comparison implemented method by Benjamini Y, H.Y. (1995) Controlling the false discovery rate: a practical and powerful approach to multiple testing, J. Roy. Stat. Soc, 57.

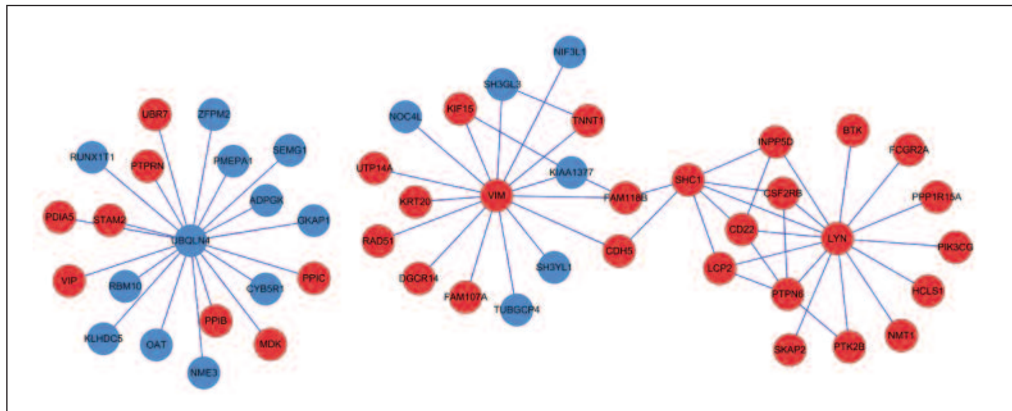

Figure 1. UBQLN4,VIM and LYN were the three hub genes of the PPI network for 3-days post injury. The Lyn-centered subnetwork was enriched with cellular signaling proteins (corrected p-value 3.31e-04). Red nodes represent upregulated genes in injuried TPS neurons while blue nodes represent downregulated genes. Only first neighbors of selected hub genes were showed.

The TRAF2-TRAF6 centered subnetwork was enriched with proteins involved in positive regulation of NF-kappaB transcription factor activity (corrected *p*-value 2.61e-08) while the PTPN6 centered network was enriched with signal transduction (corrected *p*-value 1e-3). Through three time points, a protein-interaction module consist of CD33, LIFR, LYN, PTPN6, CSF2RB and HCLS1 was found (Figure 4) all of which were upregulated in injured neurons. These genes all involved in cellular signal transduction (corrected *p*-value 3.41e-03) and particularly, both LYN and HCLS1 played roles in regulation of JAK-STAT cascade.

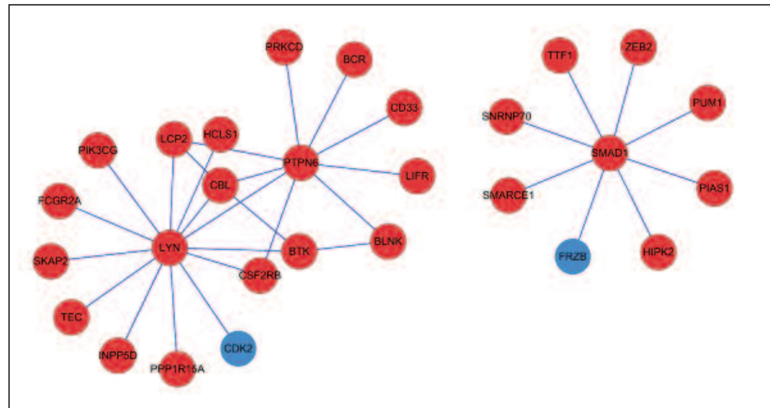

Figure 2. LYN, PTPN6 and SMAD1 were the three hub genes of the network for 2-weeks post injury. Gene ontology analysis showed that the LYN-PTPN6 centered subnetwork was enriched with proteins invovled in signal transduction (corrected p-value 3.29e-06) while the SMAD1-subnetwork was enriched with negative regulation of gene expression (corrected p-value 1.38e-04). Red nodes represent upregulated genes in injuried TPS neurons while blue nodes represent downregulated genes. Only first neighbors of selected hub genes were showed.

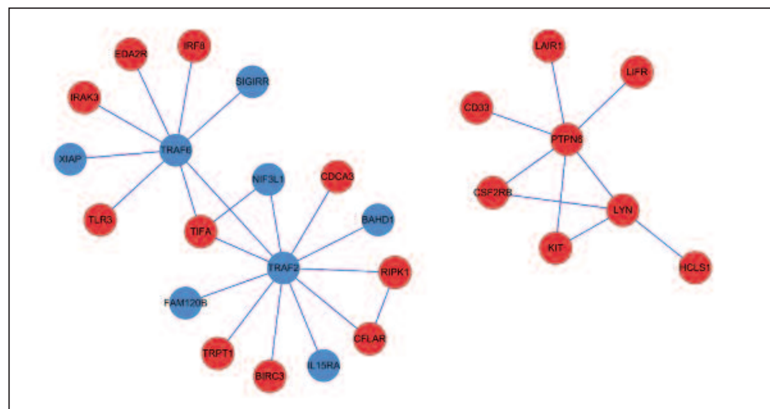

Figure 3. TRAF2, TRAF6 and PTPN6 were the three hub genes of the PPI network for 1-month post injury. The TRAF2-TRAF6 centered subnetwork was enriched with proteins involved in positive regulation of NF-kappaB transcription factor activity (corrected p-value 2.61e-08) while the PTPN6 centered network was enriched with signal transduction (corrected p-value 1e-3). Red nodes represent upregulated genes in injuried TPS neurons while blue nodes represent downregulated genes. Only first neighbors of selected hub genes were showed.

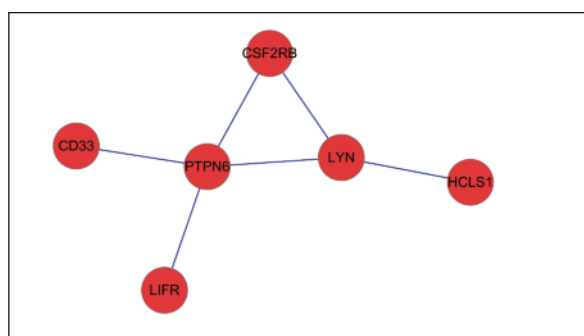

Figure 4. A core PPI-subnetwork consisting of six genes all of which were upregulated post injury through three timepoints (3-days, two-weeks and one month). These genes all involved in cellular signal transduction and particularly, both LYN and HCLS1 played roles in regulation of JAK-STAT cascade.

Discussion

Lyn, a non-receptor Src-like tyrosine kinase (SLK), was an important signaling intermediary, relaying and modulating different inputs to regulate various outputs, such as proliferation, differentiation, apoptosis, migration and metabolism[25]. It plays an important role in oligodendrocyte differentiation and myelination in the brain[26]. Inhibitory molecules in myelin in central nervous system might present one of the major hurdles for successful axon regeneration[27]. Hossain et al[26] suggested a role for SLKs in the initiation of peripheral myelination via the activation of p38, Akt and ERK, which regulate Krox-20 expression and peripheral myelination. Chudakova et al[28] found that ligation of the integrin with fibronectin results in higher expression of activated Lyn kinase. Both in oligodendrocytes and in the mouse brain, Lyn selectively associates with alpha(v)beta(3) integrin, not with alpha(v)beta(5) integrin, leading to suppression of acid sphingomyelinase activity and preventing ceramide-mediated apoptosis. They showed that knockdown of Lyn resulted in oligodendrocytes apoptosis with concomitant accumulation of C(16)-ceramide due to activation of acid sphingomyelinase (ASMase) and sphingomyelin hydrolysis.

Human IgMs could promote remyelination and stimulate an increase of remyelinated axons in animal models of multiple sclerosis[29-32]. rHIgM22 is a recombinant form of human IgM identified from a patient with Waldenström macroglobulinemia[33,34]. Watzlawik et al[35] found that Lyn expression was 9-fold up-regulated and Lyn activation was 3-fold higher in rHIgM22-treated oligodendrocytes and they identified Lyn as a key player of actions mediated by rHIgM22. They concluded that rHIgM22 prevents apoptotic signaling and inhibits oligodendrocytes differentiation by Lyn and suggested that IgM-mediated remyelination is due to protection of oligodendrocyte precursor cells (OPC) and oligodendrocytes rather than promotion of OPC differentiation[35] .

SMAD1, a BMP signaling downstream transcription factor, can be phosphorylated and activated by the BMP receptor kinase. Parikh et al[36] showed that Smad1-dependent bone morphogenetic protein (BMP) signaling is developmentally regulated and governs axonal growth in dorsal root ganglion (DRG) neurons. Reactivating Smad1 selectively in adult DRG neurons results in sensory axon regeneration in a mouse model of spinal cord injury. Smad1 signaling can be effectively manipulated by an adeno-associated virus (AAV) vector encoding BMP4. Sun et al[37] showed that both SMAD1 and SMAD4 could bind to CRMP2 promoter in the neocortex and overexpression of SMAD1 and SMAD4 *in vivo* suppresses CRMP2 expression. RNA interference of CRMP2 and overexpression of dominant negative forms of CRMP2 in utero cause accumulation of multipolar cells in the ventricular, subventricular, and intermediate zones and suppresses neurite outgrowth. They suggested that CRMP2 is required for multipolar to bipolar transition for directional neuronal migration and neurite outgrowth. Ji et al[38] examined retrograde signaling mechanisms that specify neuronal identity in the trigeminal ganglia and found that neuron specification requires the sequential action of two target-derived factors, BDNF and BMP4. BDNF induces the translation of axonally localized SMAD1/5/8 transcripts. Axon-derived SMAD1/5/8 is translocated to the cell body, where it is phosphorylated to a transcriptionally active form by BMP4-induced signaling endosomes and mediates the transcriptional effects of target-derived BDNF and BMP4[38].

PTPN6, also known as SHP-1, is a member of the protein tyrosine phosphatase (PTP) family. Previous studies[39-41] have demonstrated that SHP-1 is upregulated in a subset of non-dividing astrocytes after CNS injury and may play a role in limiting injury-induced astrocyte proliferation. Results by Beamer et al[42] demonstrated that loss of SHP-1 results in greater healing of the infarct due to less apoptosis and more neuronal survival in the ischemic core and suggests that pharmacologic inactivation of SHP-1 may have potential therapeutic value in limiting CNS degeneration after ischemic stroke.

Marsh et al[43] identify SHP-1 promoted neuronal survival by negatively regulating TrkA. They found that SHP-1 formed complexes with TrkA and dephosphorylated it. Inhibition of endogenous SHP-1 with a dominant-inhibitory mutant stimulated basal tyrosine phosphorylation of TrkA, thereby, promoting neuronal survival which was dependent on nerve growth factor (NGF) and causing sustained and elevated TrkA activation in the presence of NGF.

Conclusions

In this study, we propose a systems biology approach, which integrates expression profile data, to identify genes and pathways responsible for SCI. In our research, based on online software DAVID, we identified many widely studied SCI related pathways, such as cell cycle, immune response, olfactory transduction. Furthermore, We also screened hub proteins with protein-protein interaction network of SCI gene expression signature, such as UBQLN4,VIM and LYN; LYN, PTPN6 and SMAD1; TRAF2, TRAF6 and PTPN6. Through three time points, a protein-interaction module consist of CD33, LIFR, LYN, PTPN6, CSF2RB and HCLS1 was found all of which were upregulated in injured neurons. These genes all involved in cellular signal transduction, which provided a new solutions for preventing and curing the diseases.

Acknowledgements

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Conflict of interest

The Authors declare that they have no conflict of interests.

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