Gene sequence analysis and screening of feature genes in spinal cord injury

ZONGDE YANG[1*], XIN CHEN[2*], REN LIU[3*], CHUANFENG WANG[1], YINCHUAN ZHAO[1], ZHICAI SHI[1] and MING LI[1]

Departments of [1]Spine Surgery and [2]Neurosurgery, Changhai Hospital, Second Military Medical University, Shanghai 200433; [3]Department of Anesthesia, Fuzhou General Hospital of Nanjing Military Command Affiliated to Fujian Medical University, Fuzhou, Fujian 350025, P.R. China

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Abstract. The aim of the present study was to screen for feature genes associated with spinal cord injury (SCI), in order to identify the underlying pathogenic mechanisms. Differentially expressed genes were screened for using pre-processing data. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis was performed to analyze and identify the genes involved in pathways associated with SCI. Subsequently, Gene Ontology enrichment analysis and Uniprot tissue analysis were used to screen out genes specifically expressed in spinal cord tissue. In addition, a protein-protein interaction network was used to demonstrate possible associations among SCI-associated feature genes. Finally, a link was identified between feature genes and SCI by analyzing protein domains in coding areas of the three feature genes. The cytochrome *c* oxidase subunit Va, adenosine triphosphate (ATP) synthase, H+ transporting, mitochondrial F1 complex, α subunit 1 and cardiac muscle and mitochondrial β-F1-ATPase may be downregulated in SCI, resulting in destruction of the mitochondrial electron transport chain and membrane-bound enzyme complexes/ion transporters, thus, affecting the normal function of nerves. The three screened feature genes have the potential to become candidate target molecules to monitor, diagnose and treat SCI and may be beneficial for the early diagnosis and therapeutic control of the condition.

Correspondence to: Dr Zhicai Shi or Dr Ming Li, Department of Spine Surgery, Changhai Hospital, Second Military Medical University, 168 Changhai Road, Shanghai 200433, P.R. China
E-mail: zhicaishizch@163.com
E-mail: minglimml@126.com

*Contributed equally

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Introduction

The spinal cord is a part of the central nervous system in humans and other vertebrates (1). Spinal cord injury (SCI) is damage to the spinal cord, which is categorized according to the extent of loss of function, loss of sensation and the inability of the individual to stand or walk (2). It often results in confinement to a wheelchair and a lifetime of medical comorbidity (3). SCI may result from serious accidents, including road traffic accidents or sports injuries, but may also occur accompanying serious diseases, including developmental disorders, neurodegenerative diseases or demyelinating diseases. Multiple sclerosis, transverse myelitis resulting from stroke or inflammation and vascular malformations can all result in severe consequences and high-disability due to SCI (4).

Several genes and signaling pathways are involved in spinal cord injury (5). Expression of nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), p75 low-affinity nerve growth factor receptor, transforming tyrosine kinase B and interleukin (IL)-6 have been reported to increase in non-neuronal cells and neuronal cells, suggesting that these molecules may be involved in promoting axonal sprouting in the injured spinal cord (6). Furthermore, it has been demonstrated that upregulation of IL-1β, BDNF and NT-3 in the injured spinal cord is attenuated by treatment with high-dose glucocorticoids, with the suggestion that the downregulation of BDNF and NT-3 may be disadvantageous to the survival and axonal sprouting of spinal neurons (7). As for the pathways involved, a previous study revealed that the Rho signaling pathway may be a potential target for therapeutic interventions following SCI (8). In addition, apoptosis signal-regulating kinase 1 and stress-activated mitogen-activated protein kinase pathways, have also been reported to be involved in the transmission of apoptotic signals following SCI (9). However, identification and evaluation of specific and associated genes of SCI, which assist in the clinical diagnosis and treatment of SCI, remain to be elucidated.

In the present study, bioinformatics methods were used to assess the abnormal gene expression in SCI to determine the associated feature genes. Critical genes were screened using expression profiling microarray data. Pathway analysis and protein-protein interaction (PPI) network analysis were performed on the proteins involved in SCI to investigate their

function. The aim of the present study was to explore the molecular mechanisms of SCI and identify potential therapeutic target genes for the treatment of SCI.

Materials and methods

Data preprocessing and differential expression analysis. The transcription profile of GSE2599 was downloaded from the Gene Expression Omnibus database (http://www.ncbi.nlm.nih.gov/geo/), which was based on the Affymetrix Rat Genome U34 array (Affymetrix, Santa Clara, CA, USA) and deposited by Aimone *et al* (10). A total of six tissue specimens were available for further analysis, including three SCI samples, obtained from female Fischer 344 rats (165-200 g) 35 days after SCI, and three normal tissues, as described in the original experiment (10). The annotation information of all probe sets was provided by Affymetrix, where the raw data (CEL) file was downloaded.

Initially, the probe-level data in the CEL files were converted into expression measures. For each sample, the expression values of all the probes for a particular gene were reduced to a single value by calculating the average expression value. Probes corresponding to more than one gene were discarded. Subsequently, the data with the low signal strength was missing data and the missing data was imputed using the K-nearest neighbor averaging (KNN) method (11) and the complete data were standardized (12). The Samr package in R language (13) was used to identify differentially expressed genes (DEGs) between three samples in the control group (normal specimen) and three samples in the experimental group (samples with SCI). In order to circumvent the multi-test problem, which may induce an excess of false positive results, the Benjamini-Hochberg procedure (14) was used to adjust the raw P-values into false discovery rate (FDR). FDR<0.05 and |logFC|>1.5 were used as the cut-off criteria for DEG identification.

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway and Gene Ontology (GO) enrichment analysis. Based on the deficiency of individual gene analysis, gene set enrichment analysis evaluates differential expression patterns of gene groups to distinguish whether their biological functions and characteristics differ (15). In the present study, the P-value indicated the probability that a gene was randomly endowed a GO function and it was usually used as the criterion for assigning a certain function to a module. A lower P-value increased the probability that the function of a module had not been assigned randomly, but with the purpose of performing a certain biological function, and it has important biological significance (16). The Database for Annotation, Visualization, and Integrated Discovery (DAVID) (17) bioinformatics resource consists of an integrated biological knowledge base and analytical tools aimed at systematically extracting biological meaning from lists of genes or proteins (18). The functional enrichment analysis for the screened DEGs was performed using DAVID, and FDR<0.01 and P<0.05 were selected as the cut-off criteria. Subsequently, KEGG pathway analysis was performed on the upregulated and downregulated genes, obtained using DAVID, to screen for disease-associated pathways.

Uniprot (UP) tissue analysis. GO analysis has become a commonly used approach for functional investigations of large-scale genomic or transcriptomic data (19). DAVID, a high-throughput and integrated data-mining environment, analyzes gene lists derived from high-throughput genomic experiments (20).

In the present study, UP tissue analysis was performed on DEGs in disease-associated metabolic pathways to identify the genes associated with spinal cord tissue. Therefore, the abnormally expressed genes in the injured spinal cord 35 days after injury were selected to distinguish these genes from those, which were expressed not solely in injured spinal cord.

Construction of the PPI network. PPI analysis was performed on the DEGs using Search Tool for the Retrieval of Interacting Genes/Proteins (STRING; http://www.string-db.org/) online database (21). Combined_score was used to measure the strength of the interaction of protein pairs and only the interaction with combined_score > 0.4 was selected as significant. Subsequently, critical genes, which exhibited >45 interactions with other genes, were selected. The feature genes associated with SCI were identified by comparing the critical genes with the DEGs 35 days after SCI. Finally, the PPI network was constructed using Cytoscape software (http://cytoscape.org/) (22,23), based on the STRING database, to determine the association between feature genes and the interacting genes, which may trigger SCI.

Protein domain analysis of specific genes. Coding area prediction of the critical genes associated with SCI was performed using the GENSCAN (http://genes.mit.edu/GENSCAN.html) online software programme (24). Subsequently, the Pfam (25) database was used to examine the protein domain for further protein domain analysis.

Results

Data pre-processing and screening for DEGs. The results of data pre-processing are shown in Fig. 1. Following data pre-processing, the median was almost identical between the samples, indicating good normalization and that the data was suitable for further analysis. A total of 929 DEGs were screened for, including 339 upregulated genes and 590 downregulated genes (Fig. 2).

KEGG pathway enrichment analysis. As shown in Table I, the pathways associated with SCI included Huntington's disease (rno 05016), Parkinson's disease (rno 05012) and Alzheimer's disease (rno 05010). A total of 39 mutual genes were identified between these pathways and all of these genes were downregulated, as shown in Table II.

GO enrichment analysis. DAVID was used to identify over-represented GO categories among the genes (Table II) and P<0.05 and FDR<0.01 were selected as thresholds. The most markedly enriched five terms among these genes in the PPI network were all associated with the chondriosome (Table III). GO terms associated with the mitochondria, which were enriched in the network, included the 'mitochondrial inner membrane', 'organelle inner membrane' and 'mitochondrial envelope'.

Table I. Kyoto Encyclopedia of Genes and Genomes pathway enrichment analysis.

Term	P-value	False discovery rate	Up/downregulated
rno03010: Ribosome	3.22 E-12	3.79 E-09	Upregulated
rno04612: Antigen processing and presentation	4.13 E-08	4.85 E-05	Upregulated
rno00190: Oxidative phosphorylation	2.50 E-23	2.98 E-20	Downregulated
rno05016: Huntington's disease	1.94 E-21	2.31 E-18	Downregulated
rno05012: Parkinson's disease	9.73 E-21	1.16 E-17	Downregulated
rno05010: Alzheimer's disease	7.00 E-20	8.33 E-17	Downregulated

Table II. Downregulated genes in Huntington's disease, Parkinson's disease and Alzheimer's disease.

Uqcrc2	Atp5o	Ndufb5	Cox7a2
Atp5d	Atp5j	Ndufb6	Ndufa3
Atp5b	Ndufb10	Ndufb8	Ndufa8
Cyc1	Cycs	Ndufb9	Ndufa9
Ndufab1	Ndufc2	Cox7b	Ndufa6
Cox5a	Cox4i1	Atp5g1	Sdha
Uqcrfs1	Uqcr	Ndufb2	Ndufv2
Cox5b	Atp5c1	Ndufa4	Cox6a1
Ndufs7	Ndufb3	Loc688869	Atp5a1
Ndufs5	Ndufb4	Ndufa5	

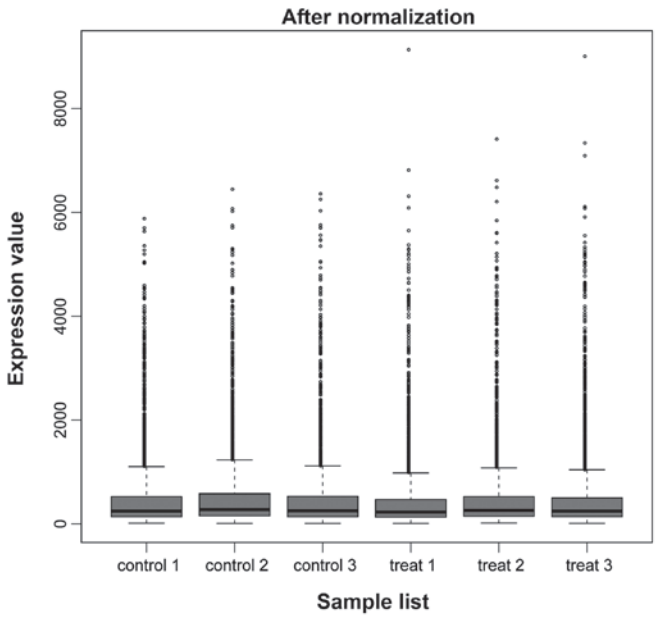

Figure 1. Box pattern of expression data following normalization. The horizontal axis indicates different samples and the vertical axis indicates the expression value. The black line within each box indicates the median of each group of data, which revealed the extent of normalization of the data. The black lines were almost on the same straight line, indicating a high level of normalization.

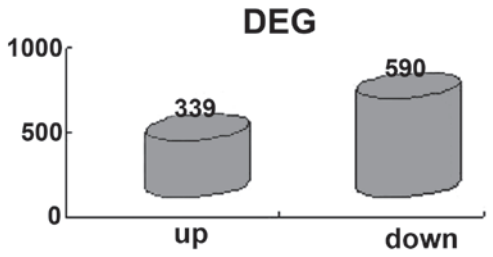

Figure 2. Number of differentially expressed genes (DEGs) that were upregulated (up; 36.5%) and downregulated (down; 62.5%) in spinal cord injury tissue compared with that of normal tissue.

Identification of feature genes in SCI. Downregulated genes, which were abnormally expressed following SCI, were also involved in several known nerve disease pathways, including Huntington's disease (rno 05016), Parkinson's disease (rno 5012) and Alzheimer's disease (rno 05010) KEGG pathways. Combined with the annotation information of spinal-cord-specific expressed genes from the Uniprot database, a candidate set of SCI-associated feature genes was obtained, including Sdha, Uqcrc2, Ndufa5, Atp5b, Atp5a1 and Cox5a.

PPI network analysis. Feature genes were obtained by further analysis of abnormally expressed genes in the injured spinal cord. Subsequently, a PPI network was constructed, as shown in Fig. 3, which revealed that Atp5b, Atp5a1 and Cox5a, all downregulated genes, were closely associated with the SCI when examined 35 days after the SCI. Additionally, the majority of genes interacting with these three genes were also downregulated.

Protein-domain analysis. The protein domain in the coding area of the Atp5b, Atp5a1 and Cox5a feature genes, among

Table III. The five most enriched genes in Gene Ontology enrichment analysis.

Category	GO term	P-value	FDR
CC	0005743: Mitochondrial inner membrane	4.40 E-53	4.14 E-50
CC	0019866: Organelle inner membrane	4.34 E-52	4.09 E-49
CC	0031966: Mitochondrial membrane	3.90 E-49	3.67 E-46
CC	0005740: Mitochondrial envelope	4.49 E-48	4.22 E-45
CC	0044429: Mitochondrial part	1.43 E-43	1.34 E-40

GO, Gene Ontology; CC, cellular component; FDR, false discovery rate.

Table IV. Protein domain in coding areas of feature genes associated with diseases 35 days after spinal cord injury.

Gene	Family	Description	P-value
Cox5a	COX5A	Cytochrome *c* oxidase subunit Va	2.50 E-58
Atp5a1	ATP-synt_ab_N	ATP synthase α/β family, β-barrel domain	3.60 E-17
Atp5b	ATP-synt_ab	ATP synthase α/β family, nucleotide-binding domain	2.50 E-72
	ATP-synt_ab_C	ATP synthase α/β chain, C terminal domain	1.50 E-26

ATP, adenosine triphosphate.

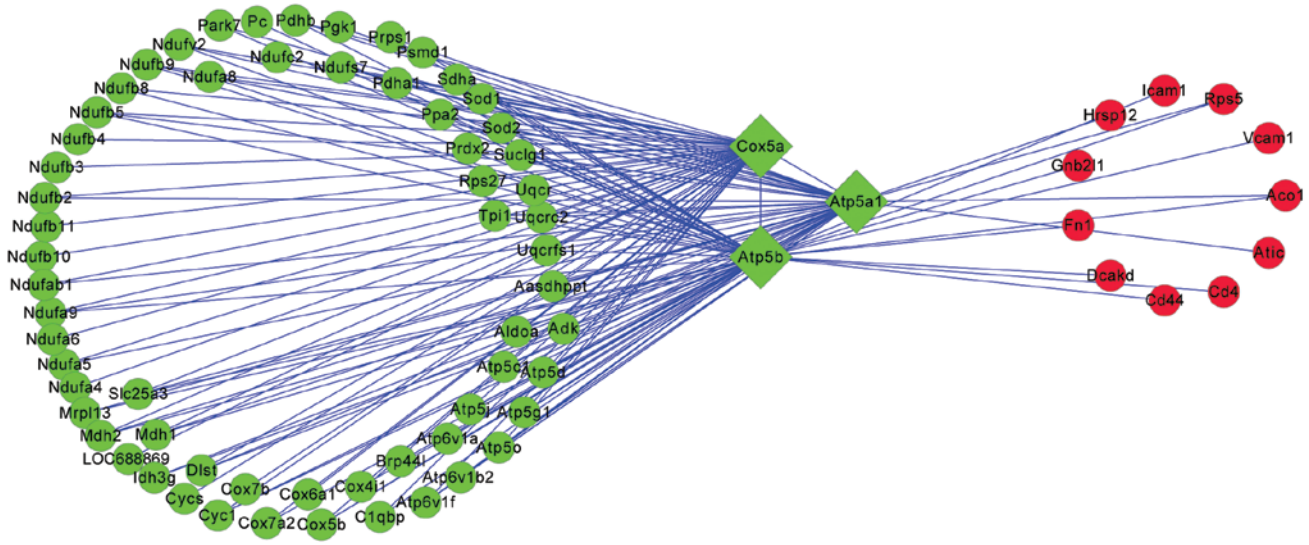

Figure 3. Interaction network of feature genes associated with disease 35 days after spinal cord injury. The rhombi indicate feature genes and circles indicate interacting genes. The green color indicates downregulated genes, while the red color indicates upregulated genes.

genes that may be associated with the disease at 35 days after the spinal cord injury are shown in Table IV.

As shown in Table IV, the protein domain in the coding areas of Cox5a belonged to the COX5A family, 13 sub-unit complex, EC: 1.9.3.1, which is the terminal oxidase in the mitochondrial electron transport chain (26). By contrast, the protein domain in the coding areas of Atp5a and Atp5b belong to the ATP synthase α and β family, including ATP-synt_ab_N, ATP-synt_ab and ATP-synt_ab_C. The ATP synthase α/β family includes the ATP synthase α and β subunits and ATP synthase, associated with flagella (27).

Discussion

In the present study, it was demonstrated that the three feature genes, Cox5a, Atp5al and Atp5b, in the injured spinal cord, were rapidly downregulated 35 days after the onset of injury, resulting in the destruction of the mitochondrial electron transport chain and membrane-bound enzyme complexes/ion transporters. These genes have been reported to be involved in the pathways of several types of neurological disease, including Huntington's disease, Parkinson's disease and Alzheimer's disease (28,29). Since these processes are associated with the

transportation of energy in biological bodies, changes to these processes 35 days after SCI may result in disruption in the transport of energy.

COX5A is a protein-coding gene. It is a multi-subunit enzyme complex, which couples the transfer of electrons from cytochrome *c* to molecular oxygen and contributes to a proton electrochemical gradient across the inner mitochondrial membrane (26). Diseases associated with COX5A include acquired idiopathic sideroblastic anemia and cardioencephalomyopathy (30). Its associated super-pathways include the electron transport chain and metabolic pathways. GO annotations associated with this gene include electron carrier activity and cytochrome *c* oxidase activity (31). This indicates that COX5A may be important in the regulation and assembly of the complex in the human mitochondrial respiratory chain enzyme, thus, affecting energy supply in SCI. A previous study revealed that COX5A is associated with the migration, invasion and prediction of distant metastasis (32). In the present study, COX5A was markedly downregulated in SCI, therefore, it was hypothesized that the downregulation of COX5A in SCI caused the interdiction of energy transportation, interrupting the metabolic process.

ATPases, or ATP synthases, are membrane-bound enzyme complexes/ion transporters, which combine ATP synthesis and/or hydrolysis with the transport of protons across a membrane. ATPases harness the energy from a proton gradient, using the flux of ions across the membrane via the ATPase proton channel, to drive the synthesis of ATP (33). Atp5a1 (34) and Atp5b (35) are also protein-coding genes. Super-pathways associated with the genes include the electron transport chain and adenosine ribonucleotides *de novo* biosynthesis. GO annotations associated with ATP5A1 include eukaryotic cell surface binding and ATPase activity (36), while those for ATP5B include transmembrane transporter activity and transporter activity (37,38). Deregulated energy metabolism is a marker of malignant disease, which offers possible future targets for treatment (39). Polymorphism and association analysis has revealed that mutations in Atp5a1 and Atp5b genes may be potential markers of diseases associated with the destruction of energy transport (40). Atp5a1 and Atp5b, which are involved in energy transportation in mitochondria, may be critical genes and certain variations of these genes may lead to increased risk in SCI (40).

In addition, the results obtained from GO enrichment analysis of the PPI network in the present study demonstrated that most enriched GO terms of the DEGs in SCI were associated with mitochondria, including 'mitochondrial electron transport chain', 'mitochondrial membrane' and 'mitochondrial envelope'. This suggested that the majority of DEGs in SCI were associated with energy transportation and that the progression of SCI may be affected by the genes expressed differently in the tissue. Therefore, the 39 mutual genes in Huntington's disease, Parkinson's disease and Alzheimer's disease, which coordinate with genes in SCI, may assist in defining the origins of malignancies and offer promise for earlier diagnosis and improved treatment of SCI.

In conclusion, the results of the present study presented a comprehensive bioinformatics analysis of genes and pathways, which may be involved in the progression of SCI. A total of 929 DEGs were identified from GSE2599, and PPI networks were constructed using these DEGs. Furthermore, the Cox5a, Atp5al and Atp5b genes, which were downregulated in SCI, were found to result in the destruction of the mitochondrial electron transport chain and membrane-bound enzyme complexes/ion transporters, thus affecting the normal function of nerves. These genes can be identified as feature genes of SCI and assist in the early diagnosis and improved treatment of SCI.

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