

# Characteristics of mRNA dynamic expression related to spinal cord ischemia/reperfusion injury: a transcriptomics study

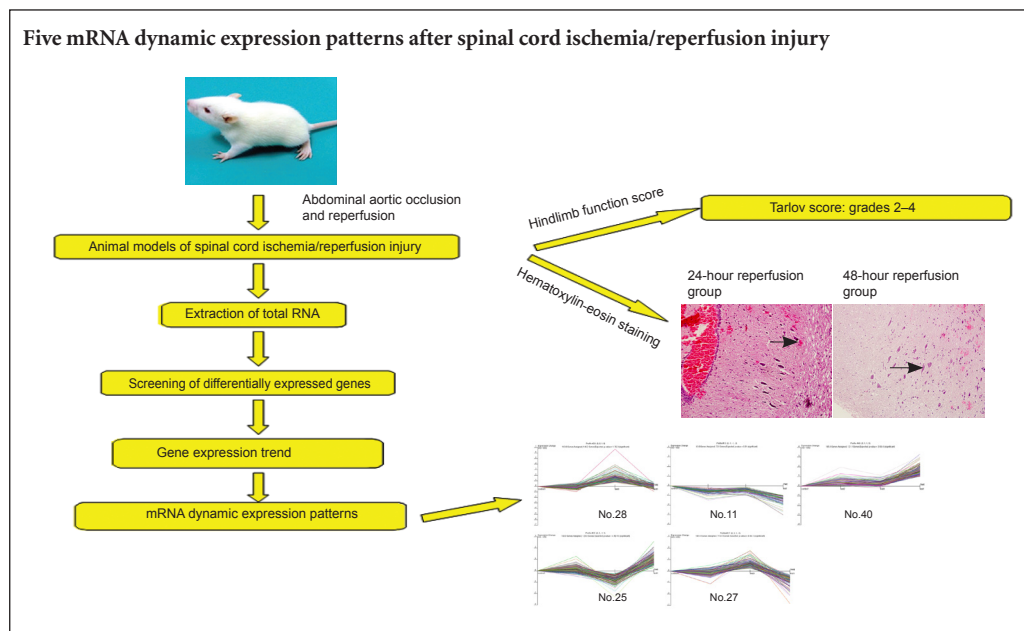
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## Graphical Abstract



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## Abstract

Following spinal cord ischemia/reperfusion injury, an endogenous damage system is immediately activated and participates in a cascade reaction. It is difficult to interpret dynamic changes in these pathways, but the examination of the transcriptome may provide some information. The transcriptome reflects highly dynamic genomic and genetic information and can be seen as a precursor for the proteome. We used DNA microarrays to measure the expression levels of dynamic evolution-related mRNA after spinal cord ischemia/reperfusion injury in rats. The abdominal aorta was blocked with a vascular clamp for 90 minutes and underwent reperfusion for 24 and 48 hours. The simple ischemia group and sham group served as controls. After rats had regained consciousness, hindlimbs showed varying degrees of functional impairment, and gradually improved with prolonged reperfusion in spinal cord ischemia/reperfusion injury groups. Hematoxylin-eosin staining demonstrated that neuronal injury and tissue edema were most severe in the 24-hour reperfusion group, and mitigated in the 48-hour reperfusion group. There were 8,242 differentially expressed mRNAs obtained by Multi-Class Dif in the simple ischemia group, 24-hour and 48-hour reperfusion groups. Sixteen mRNA dynamic expression patterns were obtained by Serial Test Cluster. Of them, five patterns were significant. In the No. 28 pattern, all differential genes were detected in the 24-hour reperfusion group, and their expressions showed a trend in up-regulation. No. 11 pattern showed a decreasing trend in mRNA whereas No. 40 pattern showed an increasing trend in mRNA from ischemia to 48 hours of reperfusion, and peaked at 48 hours. In the No. 25 and No. 27 patterns, differential expression appeared only in the 24-hour and 48-hour reperfusion groups. Among the five mRNA dynamic expression patterns, No. 11 and No. 40 patterns could distinguish normal spinal cord from pathological tissue. No. 25 and No. 27 patterns could distinguish simple ischemia from ischemia/reperfusion. No. 28 pattern could analyze the need for inducing reperfusion injury. The study of specific pathways and functions for different dynamic patterns can provide a theoretical basis for clinical differential diagnosis and treatment of spinal cord ischemia/reperfusion injury.

**Key Words:** nerve regeneration; spinal cord injury; ischemia/reperfusion injury; messenger RNA; transcription; oligonucleotide sequence; microarray; transcriptome; cDNA sequence; NADPH oxidase; neural regeneration

## Introduction

Spinal cord ischemia/reperfusion injury is defined as the inability to improve neuronal function in spinal cord neurons after the removal of factors that cause spinal cord ischemia and the recovery of spinal cord blood supply (Simon et al., 2008; Smith et al., 2012; Kuhn, 2014; Zhang et al., 2015). Understanding the time and mechanism of reperfusion injury has been a challenge. Neuroethology, pathology and proteomics have been conventionally used to investigate spinal cord ischemia/reperfusion injury. Previous studies have proposed the hypothesis of disturbance of microcirculation, apoptosis and inflammatory response (Temiz et al., 2013; Gong et al., 2014; Guven et al., 2015; Sezer et al., 2015). Conventional methods can analyze spinal cord reperfusion at a specific time at the level of the protein, but cannot ascertain the dynamic evolution of gene and mRNA during reperfusion. It is predicted that bioinformatic analysis of the dynamic transcriptome and construction of expression regulation may become an important strategy for future research to reveal the mechanism of reperfusion injury.

Transcriptomics is an important technology in the post-genomic era and has unique advantages in the study of spinal cord ischemia/reperfusion injury (Costa et al., 2010; Yang et al., 2010). Firstly, transcriptomics is highly dynamic. Under normal conditions, most cells share the same set of genes, but their transcription is highly specific in both space and time. Thus, transcriptomics can reflect the dynamic evolution of mRNA in continuous time. Secondly, changes in transcript levels embody genomic information. The transcriptome can be seen as a precursor for the proteome. Thus, transcriptomics can be used to further study the mechanism of reperfusion injury. There are no reports concerning the study of spinal cord ischemia/reperfusion injury using transcriptomics. Reperfusion injury is the whole process of pathological cascade involved in the endogenous damage system, and thus high-throughput transcriptomic analysis can directly reflect dynamic changes of gene in reperfusion injury.

The aim of this study is to screen differentially expressed mRNA during reperfusion injury using transcriptomics, further establish the highly dynamic expression patterns of mRNA, analyze dynamic expression patterns with obvious clinical significance, and clarify mRNA changes during spinal cord ischemia/reperfusion injury.

## Materials and Methods

### Animals

Twenty-four clean healthy adult male Sprague-Dawley rats weighing 280–300 g were provided by the Beijing HFK Bioscience Co., Ltd., Beijing, China (animal license No. SCXK (Jing) 2009-0004). Rats were housed in individual cages under a 12-hour light/dark cycle and in a dry and ventilated room at 23–25°C, with free access to food and water. All surgery was performed under anesthesia, and all efforts were made to minimize pain and distress in the experimental animals. All procedures were carried out in accordance with the United States National Institutes of Health Guide for the Care and Use of Laboratory Animal (NIH Publication No.

85-23, revised 1986). Protocols were approved by the Animal Ethics Committee of Jilin University of China.

### Establishment of spinal cord ischemia/reperfusion injury rat models and group assignment

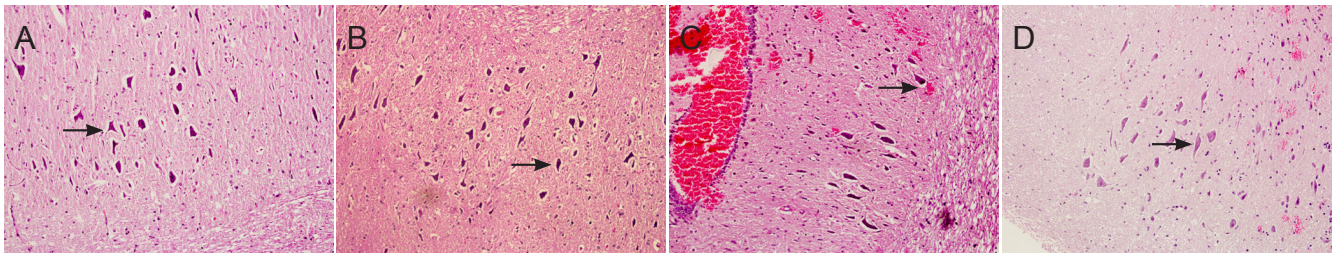
In accordance with simple random sampling, 24 rats were equally and randomly assigned to sham group, 90-minute ischemia (I90R0) group, 90-minute ischemia 24-hour reperfusion (I90R24) group and 90-minute ischemia 48-hour reperfusion (I90R48) group. Models were established in accordance with a previous study (Bowes et al., 1994). Briefly, rats were anesthetized intraperitoneally with chloral hydrate (0.3 mL/100 g). A median longitudinal incision was made on the belly and the abdominal aorta was exposed. In the sham group, the abdominal aorta was exposed for 90 minutes without obstruction before the incision was sutured. In the I90R0 group, the abdominal aorta was blocked with a vascular clamp for 90 minutes; after occlusion, the left kidney had changed from bright red to dark red, and the abdominal aortic pulse had disappeared. In the I90R24 and I90R48 groups, the abdominal aorta was blocked with a vascular clamp for 90 minutes, and then the vascular clamp was removed. The incision was sutured after revascularization under direct vision.

### Modified Tarlov scoring

At 24 and 48 hours after suturing the incision in the I90R24 and I90R48 groups, and when the rats had regained consciousness in the sham and I90R0 groups, three investigators who had not participated in the model establishment performed hindlimb motor function testing using the modified Tarlov score as follows (Cheng et al., 1996): grade 0, no activity, totally paralyzed, no response to acupuncture; grade 1, no activity, totally paralyzed, response to acupuncture; grade 2, active, cannot load; grade 3, hindlimb can load, cannot walk; grade 4, can walk, but unsteady, ataxia; grade 5, can walk, but not flexible, no ataxia; grade 6, normal walking. The mean value from the three investigators was calculated and recorded.

### Hematoxylin-eosin staining

Spinal cord tissue was obtained 90 minutes after exposing and occluding abdominal aorta in the sham and I90R0 groups, and 24 and 48 hours after reperfusion in the I90R24 and I90R48 groups. L<sub>2-5</sub> spinal segment was cut and 0.8 cm L<sub>3-4</sub> spinal segment was obtained. A part of spinal segment was fixed in 4% paraformaldehyde for 24 hours, embedded in paraffin, stained with hematoxylin and eosin. Another part was washed with PBS, immersed in liquid nitrogen for 2–3 hours, and placed at –70°C for further use. Paraffin-embedded tissues were sliced into 4 μm thick serial sections. The sections were treated with xylene for 5–10 minutes, immersed in the mixture of xylene and pure ethanol (1:1) for 5 minutes, in 100%, 95%, 85%, and 70% ethanol, each for 2–5 minutes. After washing with distilled water, the samples were immersed in staining solution. After removal of the staining solution, samples were placed in hematoxylin without stirring

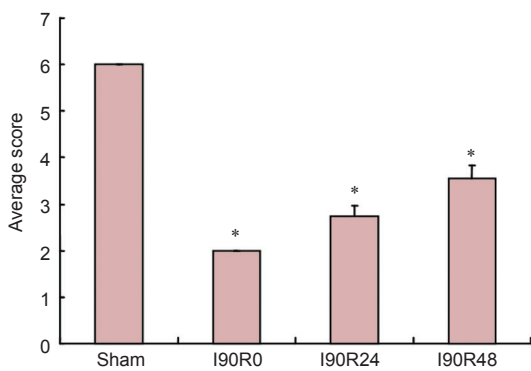


**Figure 2 Pathological changes in the injured spinal cord of rats under a light microscope (hematoxylin-eosin staining, × 200).** (A) Sham group: distinct processes, darkly stained plump nuclei. (B) I90R0 group: slightly dense cytoplasm, lightly stained swollen nuclei. (C) I90R24 group: neurons were spindle-shaped; nuclei shifted, became small, and fragmented. (D) I90R48 group, neuronal cells became spindle-shaped; nuclei fragmented and disappeared. Sham, I90R0, I90R24 and I90R48: sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively. Arrows point to neurons.

**Table 1 Identification of RNA extracted from the injured spinal cord of rats**

Sample ID	$A_{260\text{ nm}/280\text{ nm}}$	$A_{260\text{ nm}/230\text{ nm}}$	Conc. (ng/ $\mu\text{L}$ )	Volume ( $\mu\text{L}$ )	Quantity (ng)	Result
Sham-1	2.04	2.31	649.81	20	12 996.2	Pass
Sham-2	2.04	2.35	619.59	20	12 391.8	Pass
Sham -3	1.97	2.32	520.72	20	10 414.4	Pass
I90R0-1	1.98	2.26	583.79	10	5 837.9	Pass
I90R0-2	2.00	2.32	690.03	10	6 900.3	Pass
I90R0-3	1.97	2.32	597.39	10	5 973.9	Pass
I90R24-1	1.99	2.28	575.32	10	5 753.2	Pass
I90R24-2	1.90	2.28	311.50	10	3 1150	Pass
I90R24-3	1.98	2.29	421.42	10	4 214.2	Pass
I90R48-1	2.01	2.31	561.32	10	5 613.2	Pass
I90R48-2	2.01	2.31	572.32	10	5 724.3	Pass
I90R48-3	1.99	2.32	601.27	10	6 012.7	Pass

Sham, I90R0, I90R24 and I90R48: sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively  $A_{260\text{ nm}/280\text{ nm}}$  and  $A_{260\text{ nm}/230\text{ nm}}$  were more than 1.8 in each group. RNA extraction could satisfy the requirements of the follow-up experiment. A: Absorbance.



**Figure 1 Neurological function in rats with ischemia/reperfusion injury (Tarlov score).**

\* $P < 0.05$ , vs. sham group (mean  $\pm$  SD,  $n = 6$ , one-way analysis of variance and Tukey-Kramer *post hoc* test). Sham, I90R0, I90R24 and I90R48: Sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively.

for 15 minutes, treated with 0.5–1.0% ethanol hydrochloride for a moment, washed with running water for 15–30 minutes, washed with distilled water, and stained with 0.1–0.5% eosin for 1–5 minutes. All samples were dehydrated with 70%, 85%, 95%, and 100% ethanol, each for 2 or 3 minutes, permeabilized with xylene, mounted, and observed with a light microscope (BH-2; Olympus, Tokyo, Japan).

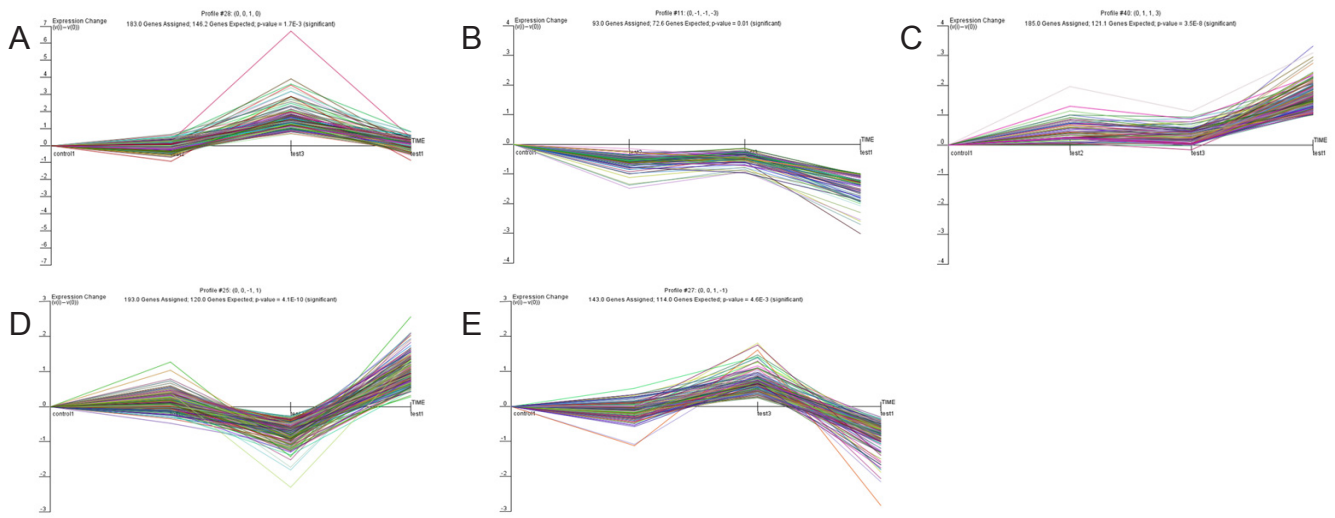
**RNA extraction**

Spinal cord tissue was triturated with a pestle, and homogenized with a Mini-Bead-Beater-16 homogenizer (Biospec, Bartlesville, Okla, USA) for 1–2 minutes. The samples were maintained in place for 5 minutes to completely dissociate nucleic acid-protein complex, and then incubated with TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and chloroform. 700  $\mu\text{L}$  of the samples were transferred to RNeasy Mini spin columns, placed in 2 mL of collecting duct, centrifuged at 8,000 $\times g$  for 15 seconds for washing the column. After adding 500  $\mu\text{L}$  RNasey Mini Kit (Qiagen p/n 74104, Germany), the samples were centrifuged at 8,000 $\times g$  for 2 minutes. Absorbance (A) values were measured at 230 nm, 260 nm and 280 nm with a spectrophotometer (NanoDropND-1000; Nanodrop, Wilmington, DE, USA) in three samples of each group. The ratio of  $A_{260\text{ nm}/280\text{ nm}}$  and  $A_{260\text{ nm}/230\text{ nm}}$  was calculated, and total RNA content and purity were measured.

**A DNA microarray and data analysis**

Quick Amp Labeling Kit (Agilent p/n 5190-0442, Santa Clara, CA, USA) and RNeasy Mini Kit (Qiagen p/n 74104) were used for labeling of mRNA and cRNA, respectively. Agilent Gene Expression Hybridization Kit (Agilent p/n 5188-5242) was used for mRNA hybridization. Agilent Microarray Scanner (Agilent p/n G2565BA) was used to read microarrays.

Data were extracted with Agilent Feature Extraction



**Figure 3** Five mRNA dynamic expression patterns related to ischemia/reperfusion injury.

(A) No. 28 pattern: mRNA expression presents a trend in up-regulation at 24 hours after reperfusion (0, 0, 1, 0). (B) No. 11 pattern: the number of mRNAs became small with prolonged time of reperfusion in a trend of (0, -1, -1, -3). (C) No. 40 pattern: the number of mRNA increased with prolonged time of reperfusion in a trend of (0, 1, 1, 3). (D) No. 25 pattern: mRNA expression down-regulated at 24 hours, but up-regulated at 48 hours in a trend of (0, 0, -1, 1). (E) No. 27 pattern: mRNA expression up-regulated at 24 hours, but down-regulated at 48 hours in a trend of (0, 0, 1, -1). On the X-axis, control1: sham group; test2: I90R0 group; test3: I90R24 group; test1: I90R48 group. On the Y-axis, numerical values present the altered range of differential expression of mRNAs in other groups compared with the sham group. Sham, I90R0, I90R24 and I90R48: sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively.

**Table 2** 16 mRNA dynamic expression patterns related to ischemia/reperfusion injury

No.	Sham	I90R0	I90R24	I90R48	Genes_assigned	Genes_expected	P
36	0	1	0	1	2096	536.3	0.00
6	0	-2	0	-1	726	188.1	0.00
19	0	-1	1	-1	660	192.9	0.00
33	0	1	-1	1	643	188.0	0.00
37	0	1	0	2	585	202.4	0.00
16	0	-1	0	-1	1064	536.3	0.00
20	0	-1	1	0	306	114.0	0.00
3	0	-2	-1	-2	382	188.1	0.00
43	0	2	0	1	370	193.0	0.00
15	0	-1	0	-2	324	183.2	0.00
46	0	2	1	2	319	191.8	0.00
25	0	0	-1	1	193	120.0	0.00
40	0	1	1	3	185	121.1	0.00
28	0	0	1	0	183	146.2	0.00
27	0	0	1	-1	143	114.0	0.01
11	0	-1	-1	-3	93	72.6	0.01

Numerical value is a sign of change and grading. Genes assigned represents the number of mRNAs in the pattern. Genes expected represents the number of mRNAs in the pattern in random distribution. P value represents the significance level between genes assigned and genes expected. When expression pattern was significantly affected by timing change of reperfusion, P value was small. Sham, I90R0, I90R24 and I90R48: sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively.

(Agilent). With Multi-Class Dif analysis,  $P < 0.05$  and false discovery rate  $< 0.05$ , significant differentially expressed mRNAs were obtained. Data were calculated with Serial Test Cluster (Ernst et al., 2005). Among 80 default patterns,  $P < 0.05$  was used to obtain dynamic expression patterns of differential genes. Figures were used to quantify the dynamic changes.

**Statistical analysis**

Measurement data are expressed as the mean  $\pm$  SD, and analyzed with SPSS 17.0 software (SPSS, Chicago, IL, USA).

One-way analysis of variance and Tukey-Kramer *post hoc* test were used to compare the difference of intergroup data. A value of  $P < 0.05$  was considered statistically significant.

**Results**

**Neurological function of rats with ischemia/reperfusion injury**

Hindlimb function was normal in the sham group at various time points, but markedly impaired in the other groups. Tarlov scores were graded 2–4. With prolonged time



**Table 3 Corresponding genes of differentially expressed mRNAs in the No. 28 pattern**

Gene		Sham	I90R0	I90R24	I90R48
PARK2	Parkin 2	0	0.05	1.02	-0.21
PAK2	The p21 activated kinases 2	0	-0.15	1.27	-0.22
FSTL1	Follistatin-related protein 1	0	0.14	1.28	-0.02
STEAP4	STEAP family member 4	0	-0.64	1.39	-0.31
SEL1L	Protein sel-1 homolog 1	0	-0.23	0.94	0.13
SPINT3	Kunitz-type protease inhibitor 3	0	-0.45	1.23	-0.13
SLC2A4	Glucose transporter type 4	0	-0.29	0.89	-0.08
EIF5A2	Eukaryotic translation initiation factor 5A-2	0	0.12	1.29	0.10
ZFP187	zinc finger 187	0	-0.38	1.34	-0.07
ADAMTS1	A disintegrin and metalloproteinase with thrombospondin motifs 1	0	-0.40	1.02	-0.07
LCLAT1	lysocardiolipin acyltransferase-1	0	-0.21	1.09	-0.17
MUP5	Major urinary proteins 5	0	-0.13	3.54	-0.27
MXD1	MAD protein	0	-0.14	0.84	-0.17
ITGB8	Integrin beta-8	0	0.65	2.57	0.58
LOC687022	-	0	-0.52	1.68	-0.07
RUP2	-	0	0.25	1.25	0.00
SULT1B1	family cytosolic 1B member 1	0	0.34	2.49	0.42
PTPRC	phosphatase, receptor type, C	0	-0.26	1.16	0.12
LIMA1	actin-binding protein 1	0	-0.19	2.09	0.24
SLC4A4	cotransporter 1	0	-0.04	1.08	-0.31
AP4M1	AP-4 complex subunit mu-1	0	-0.3	1.58	0.19
FGB	The Fibrinogen beta chain	0	-0.32	2.85	-0.01
SCN8A	-	0	-0.13	0.96	0.16
NREP	-	0	-0.06	0.96	-0.19
SYNGR2	-	0	-0.34	0.67	-0.12
NOTCH2	Neurogenic locus notch homolog protein 2	0	0.22	1.37	0.01
MYO5C	-	0	-0.22	1.03	-0.28
AFB2	AF4/FMR2 family member 2	0	-0.24	1.1	0.08
SH3BGRL	SH3 domain-binding glutamic acid-rich-like protein	0	0.03	1.53	0.35
GRAMD4	-	0	-0.43	1.96	0.24
USP22	Ubiquitin specific peptidase 22	0	0.22	1.34	-0.21
UHMK1	U2AF homology motif (UHM) kinase 1	0	-0.33	1.1	-0.10
NAV3	-	0	-0.37	0.94	-0.05
DHCR24	24-dehydrocholesterol reductase	0	-0.18	1.07	-0.28
OLR1748	-	0	0.29	1.14	0.09
LOC298109	-	0	0.21	2.86	0.41
ITGB1BP2	-	0	-0.29	0.87	-0.23
RMND5A	Required for meiotic nuclear division 5 homolog A	0	-0.42	1.06	-0.23
SLC10A1	Sodium/bile acid cotransporter	0	0.46	1.51	0.38
BPIFA1	Palate, lung, and nasal epithelium clone protein	0	-0.29	0.84	0.04
GNA12	Guanine nucleotide-binding protein subunit alpha-12	0	-0.18	1.15	0.08

Sham, I90R0, I90R24 and I90R48: sham, 90-minute ischemia 0-hour, 24-hour and 48-hour reperfusion groups, respectively. Numerical value is a sign of change and grading. “-”: unknown full name.

of reperfusion, the degree of nerve injury was reduced gradually (**Figure 1**).

**Pathological morphology of injured spinal cord in rats**

Spinal cord tissue was collected 90 minutes after exposing and occluding the abdominal aorta in the sham and I90R0 groups. Staining was observed at 24 and 48 hours after reperfusion in the I90R24 and I90R48 groups. In the sham group, processes were distinct; nuclei were darkly stained, intact and rounded and no edema was found (**Figure 2A**). In the I90R0 group, cell bodies were irregular; the cytoplasm was slightly dense; nuclei began to swell and were weakly

stained, and slight edema was observed (**Figure 2B**). In the I90R24 group, neurons were spindle-shaped; nuclei were displaced, became small, and fragmented, and the edema worsened (**Figure 2C**). In the I90R48 group, neuronal cells became spindle-shaped; nuclei fragmented and disappeared; edema was still visible in interstitial tissue (**Figure 2D**).

**Identification of RNA in the spinal cord of rats with ischemia/reperfusion injury**

A values of total RNA were more than 1.8 in each group (**Table 1**), confirming that RNA extraction could satisfy the requirements of the follow-up experiment.

### Screening results of differentially expressed mRNAs

At 0 minute of ischemia, 0 hour, 24 hours and 48 hours of reperfusion, 8,242 differentially expressed mRNAs were obtained using Multi-Class Dif analysis, taking  $P < 0.05$  and false discovery rate  $< 0.05$  as a screening condition.

### mRNA dynamic expression patterns related to ischemia/reperfusion injury

At 0 minute of ischemia, 0 hour, 24 hours and 48 hours of reperfusion, expression patterns of injury-related mRNA were classified and the significance was analyzed. Among 80 default patterns, 50 different expression patterns were found. At  $P < 0.05$ , 16 significant expression patterns were obtained. The number and changes of differentially expressed mRNAs in each pattern are listed in **Table 2**. Of them, five mRNA dynamic expression patterns were significant for differential diagnosis (**Figure 3**). In the No. 28 pattern, all differential genes were detected in the 24-hour reperfusion group, and their expression presented a trend in up-regulation (0, 0, 1, 0) (**Figure 3A**). In the No. 11 and No. 40 patterns, mRNA showed a decreasing or increasing trend with prolonged time (0, -1, -1, -3) and (0, 1, 1, 3) respectively (**Figure 3B, C**). In the No. 25 and No. 27 patterns, differential expression appeared only in the 24-hour and 48-hour reperfusion groups, showing a trend of (0, 0, -1, 1) and (0, 0, 1, -1) (**Figure 3D, E**). In the No. 28 pattern, 183 differentially expressed mRNAs were detectable. Due to the limitation of the length of writing, 30 mRNAs were displayed in **Table 3**. Protein kinase p21-activated kinase 2 (PAK2) gene was also noticeably up-regulated.

### Discussion

This study investigated spinal cord ischemia/reperfusion injury from the aspects of etiology, pathology and transcriptomics. Hindlimb function was evaluated following spinal cord ischemia/reperfusion injury. Results showed that hindlimb function score was evidently lower in the reperfusion groups than in the sham group. Hindlimb function score was obviously lower in the I90R24 group than in the I90R48 group. Neurological function was gradually restored with prolonged time of reperfusion. Hematoxylin-eosin staining demonstrated neuronal cell injury in gray matter and white matter edema which were observed immediately after ischemia, and aggravated apparently 24 hours after reperfusion. Neuronal cell injury was still visible but edema was lessened 48 hours after reperfusion. Behavioral changes after reperfusion were associated with pathological changes in the spinal cord. Behavioral and pathological changes can reflect whether model establishment was successful and can evaluate the degree of injury. Differentially expressed mRNAs during reperfusion were successfully screened out, and 16 mRNA dynamic expression patterns were obtained by Serial Test Cluster. Five expression patterns with important significance for differential diagnosis of spinal cord ischemia/reperfusion injury were determined, including No. 28, No. 11, No. 40, No. 25 and No. 27. Of these, No. 28 pattern was of particular interest.

The No. 28 expression pattern altered in a trend like fashion (0, 0, 1, 0), indicating that all mRNAs were expressed at 24 hours. 24 hours of reperfusion is an important stage of

cell injury and edema, and a key time point of spinal cord ischemia/reperfusion injury (Zhu et al., 2013). mRNA expression in the No. 28 expression pattern is probably associated with secondary damage and is of great significance for the screening of reperfusion injury. In the No. 28 pattern, an up-regulation of PAK2 gene has attracted more and more attention (Itakura et al., 2013; Elsherif et al., 2014). PAK2 belongs to type I P21-activated kinase, and has been shown to be strongly associated with an inflammatory response. An up-regulation in PAK2 expression can noticeably improve the direction and speed of inflammatory cells migrating to the site of injury, and promote inflammatory response at the site of injury (Itakura et al., 2013). PAK2 and PAK1 are important upstream regulatory members of NADPH oxidase (NOX) (Chang et al., 2009). The major function of NOX as a multienzyme complex is to produce reactive oxygen species. With the continued accumulation of NOX, reactive oxygen species are increased gradually and generate a large amount of oxygen free radicals. NOX overexpression has been shown to cause myocardial ischemia/reperfusion injury and inflammation (Sareila et al., 2011; Webster, 2012). An obvious up-regulation in PAK2 expression mediates a large number of downstream NOX activation and expression, and leads to reactive oxygen species accumulation which can induce oxygen free radical overload, mediate inflammatory responses and finally result in secondary spinal cord reperfusion injury. Monasky et al. (2012) demonstrated PAK1 mediated NOX pathway in myocardial ischemia-reperfusion injury. Because of the similar effects of PAK2 and PAK1 in the inflammatory response (Elsherif et al., 2014), we still consider the effect of PAK2 on spinal cord ischemia/reperfusion injury worthy of concern. PAK2 is activated during apoptosis and alters cell morphology through proteolysis, induces the occurrence of apoptosis, and is strongly associated with apoptotic bodies (Vilas et al., 2006). Previous studies have shown that chemokine (C-X-C motif) ligand 1 (CXCL1) up-regulates the expression of inflammatory mediators mediated by PAKs (Chen et al., 2004; Monaco et al., 2004). These results indicate that the 24 hours is the key time point of reperfusion injury. Secondary damage is strongly associated with inflammatory response, oxygen free radical overload, and apoptosis, which is consistent with previous studies (Simon et al., 2008; Kuhn, 2014).

Genes screened out in No. 11 and No. 40 patterns can be compared with the physiological state of genes in the spinal cord, and certain genes have the potential to identify spinal cord injury. In the comparison of genes in No. 28 pattern and the physiological state, genes only specifically expressed in secondary damage can distinguish secondary damage, such as PAK2. Moreover, PAK2 has been shown to be strongly associated with inflammatory reaction and oxidative stress. Thus, significant up-regulated PAK2 expression can reflect the characteristics of histological changes during reperfusion injury. Genes screened out by transcriptomics provide some new targets for the study of differential diagnosis and mechanisms of reperfusion injury.

No. 11 and No. 40 patterns represent a series of mRNAs expressed from ischemia to reperfusion. mRNAs in No. 11 and No. 40 expression patterns can distinguish spinal cord

injury from normal tissue. With the progression of injury, mRNA expression in the No. 11 and No. 40 patterns decreased or increased respectively, and peaked at 48 hours after reperfusion, suggesting that relevant mRNA presented significant cumulative effect in the whole process of evolution. Up-regulation expression of mRNA occupied obvious advantages, which suggested that up-regulated mRNA expression played a leading role in the progression of spinal cord injury. In the No. 11 and No. 40 patterns, mRNA expression was consistent between simple ischemia and 24-hour reperfusion, but altered markedly at 48 hours. The above results indicated that mRNAs mainly affected late reperfusion, and were possibly associated with nerve repair. No. 25 and No. 27 patterns represent a series of mRNAs that specially expressed in ischemia and reperfusion, but did not express in normal tissue and ischemic tissue. These mRNAs could be used to distinguish simple ischemia from ischemia and reperfusion, which may have an important reference value for injury and repair mechanisms during reperfusion.

In summary, this study explained the level of gene transcription during spinal cord ischemia/reperfusion injury using transcriptomics with the high-throughput continuous method, and established five mRNA dynamic evolution patterns with important significance of differential diagnosis. Results of this study provided important reference values for comparing the differences between spinal cord with ischemia/reperfusion injury and normal spinal cord, and among different stages of reperfusion. However, this study has some limitations. The first is how to establish gene regulatory networks with obtained mRNA data so as to better understand pathogenesis of spinal cord ischemia/reperfusion injury. The second is how to diminish the cost of transcriptomics. Our team hopes to provide references for the progression and differential diagnosis of spinal cord ischemia/reperfusion injury through transcriptomics analysis.

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