Original Article

Expression profiles of long non-coding RNAs in neurogenic bladder of spinal cord injured rats: a transcriptomic analysis

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Background: Growing evidence has indicated that long non-coding RNAs (lncRNAs) are important regulators of pathological and physiological processes through various mechanisms. However, the signature of lncRNA expression and the possible roles of lncRNAs in spinal cord injury (SCI) rat neurogenic bladder (NB) have not been comprehensively explored. In this study, the expression profiles of lncRNAs and mRNAs were explored in the bladder tissue of SCI rats using next-generation sequencing (NGS).

Methods: Twenty female Wistar rats were randomly divided into SCI 1–3 and normal control (NC) groups. The spinal cord was completely transected at the T9–T10 level to establish the SCI model. Bladder tissues were collected on days 7, 14, and 28 after the operation. The expression profiles of lncRNAs were detected by NGS. Differentially expressed lncRNAs (DELs) were chosen for qRT-PCR verification to validate the RNA sequencing results. The functions of the predicted target genes were then evaluated using Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis.

Results: Compared with the NC group, the SCI 1–3 groups had 468, 117, and 408 DELs [fold change (FC) >2], including 247, 38, and 201 up-regulated and 163, 79, and 207 down-regulated lncRNAs, respectively. Likewise, 6,654, 2,133, and 5,706 mRNAs (FC >2) were differentially expressed between SCI 1–3 and NC rats, of which 4,821, 1,195, and 3,695 were up-regulated, and 1,833, 938, and 2,011 were down-regulated, respectively. Specifically, Miat, Mir155hg, and H19 were significant DELs in all SCI groups. Moreover, GO revealed that the DELs were related to several terms, including immune response, and KEGG was mainly enriched in 10 pathways, such as the transforming growth factor β signaling pathway.

Conclusions: The results revealed the expression profiles and possible roles of lncRNAs in SCI rat NB. This study may help identify possible NB mechanisms following SCI from the perspective of lncRNAs and provides new potential lncRNAs for the early diagnosis and treatment of human NB in the future.

Keywords: lncRNAs; spinal cord injury (SCI); neurogenic bladder (NB); next-generation sequencing (NGS)

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Introduction

Spinal cord injury (SCI) has been identified as a global problem, with an estimated 250,000–500,000 cases worldwide (1). It leads to multi-system physiological dysfunction, including neurogenic bladder (NB), disabilities, and potentially fatal complications (2). The overall prevalence and incidence of SCI vary widely, with 12–65 cases/million per year worldwide (3). Following SCI, approximately 95% of patients suffer from different levels of NB, but less than 1% of these patients recover their pre-SCI level of function when discharged from the hospital. SCI usually results in detrusor-sphincter dyssynergia, bladder fibrosis, and detrusor overactivity (DO), leading to urinary dysfunction, such as urinary frequency, urgency, retention, incontinence, and upper urinary tract damage in some cases (4). Bladder fibrosis and DO treatments could decrease detrusor pressure, increase bladder capacity, and reduce incontinence, thus protecting urinary function and improving the long-term prognosis of patients with SCI (5). However, currently available therapeutic options for SCI are unsatisfactory.

NB secondary to SCI is a complex event, and the precise mechanisms have not yet been fully elucidated. Rizwan et al. reported a new spinal reflex circuit mediated by C fibers, which is thought to be responsible for developing NB secondary to SCI (3). Growing evidence has indicated that long non-coding RNAs (lncRNAs) are important regulators of pathological and physiological processes through various mechanisms, including cell proliferation, apoptosis, and differentiation (6). Thus, lncRNAs may also play an important role in the pathophysiology and treatment of NB. The lncRNAs are a class of RNA molecules with a transcription length greater than 200 nucleotides (nt), are structurally similar to messenger RNAs (mRNAs), but do not encode proteins (7). Of all non-coding RNAs (ncRNAs), lncRNAs are the most diverse and complex in the mammalian transcriptome (8). Earlier study reported that lncRNAs are also thought to be associated with the pathophysiology of several diseases, including organ fibrosis and cancer (9). Moreover, in recent study, several lncRNAs such as GAS5, ZEB1-AS1, and NEAT1 are relevant to fibrogenesis in different organs (10). With the rapid development of next-generation sequencing (NGS) technology and transcriptomics, over 50,000 lncRNAs have been identified in humans. Nevertheless, only a small fraction of these genes have been annotated with respect to their functions (11). The biological functions of most lncRNAs have not been completely elucidated. To the best of our knowledge, no one has yet comprehensively explored the signature of lncRNA expression and the possible roles of lncRNAs in SCI rat NB.

To identify the possible mechanisms of NB secondary to SCI, the expression profiles of lncRNAs and mRNAs were explored in the bladder tissue of the SCI model based on NGS. Gene Ontology (GO) terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) were then used to identify target genes and pathways related to NB. Moreover, the co-expression network was analyzed to explore further the potential interactions between differentially expressed lncRNAs (DELs) and target mRNAs. Therefore, this study may help identify possible mechanisms of NB secondary to SCI from the perspective of lncRNAs and provide new potential lncRNAs for the early diagnosis and treatment of human NB in the future. We present the following article in accordance with the ARRIVE reporting checklist (available at https://tau.amegroups.com/article/view/10.21037/tau-21-1161/rc).

Methods

Ethical approval

All experimental procedures were approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital Capital Medical University (No. 20190128), in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals.

Experimental animal model

Adult Wistar rats (female, aged 10–12 weeks, weighing 200–250 g) were provided by Beijing Charles River Laboratories Animal Technology Co., Ltd. A total of 20 rats were randomly divided into four groups: SCI 1–3 and a normal control (NC) (n=5 per group). During the experiments, all the animals were housed in cages at a room temperature of 25 ℃ with 30–70% humidity. All rats had free access to appropriate food and fresh water. Then, we established a transected SCI model as previously reported (12). All SCI rats were anesthetized with isoflurane inhalation (3%) and positioned on the operating tables. A midline skin incision was made over the thoracic vertebrae at T9–T10. The paravertebral muscles were then dissected from the vertebrae to expose the T9–T10 vertebra, and the spinous processes of the T9–T10 vertebrae were surgically exposed.

Subsequently, the spinal cord was completely transected using a sharp scalpel at the T10 segmental level without disrupting the dura. NC group rats had only laminectomy performed. Gelfoam (Ethicon) was packed at the ends of the incision to stop bleeding, and the muscle layer and skin were sutured separately after hemostasis. After the operation, the rats were treated with antibiotics (100 mg/kg ampicillin sodium intramuscularly) for 5 days. To avoid bladder overdistention (13), animals were given manual bladder evacuations every 8 h until the spontaneous reflex of the bladder was re-established.

Bladder tissue collection

According to the previously described group, bladder tissues were collected on days 7, 14, and 28 after the operation. All rats were anesthetized and sacrificed by isoflurane inhalation (3%) and positioned on the operating tables in the supine position. A midline suprapubic laparotomy was then performed to expose the bladder. The intact bladder was surgically removed and divided into two halves sagittally. Three samples are taken from each group. All bladder tissues were stored in liquid nitrogen.

RNA extraction, library construction, and transcriptome sequencing

Three replicates of each sample were used for RNA sequencing (RNA-Seq). Total RNA was extracted from bladder tissues using TRIzol (Invitrogen) and quantified using a spectrophotometer (ND-2000; NanoDrop, Thermo Fisher Scientific, Inc., USA). Ribosomal RNA was depleted from total RNA using a RiboMinus kit (Life Technologies). RNA-Seq was conducted using a HiSeq 3000 Sequencing System (Illumina Inc., San Diego, CA, USA) with a single-end 50 bp at Ribobio Co. Ltd (Ribobio, China).

The lncRNA and mRNA expression and identification

RNA-seq libraries were constructed using the Illumina TruSeq protocol. Mapping to the genome Rnor_6.0 was performed using TopHat software (version 2.1.0). The Cufflinks algorithm was used to assemble aligned RNA-Seq reads into transcripts. Differentially expressed mRNAs (DEMs) and DELs were determined using DESeq2 (version 1.24.0). All of DELs and DEMs were used to build heatmaps and volcano plot using R software (version 3.22.3). The false discovery rate (FDR) was used to determine the threshold of the P value. Genes with significant expression changes were defined as fold change (FC) >2 and FDR-adjusted P<0.05.

Target genes prediction of DELs

To explore the function of DELs, we predicted target genes of DELs in cis- and trans-regulation. Genes located within 10 kb of DELs were selected.

GO and KEGG enrichment analysis of DELs target genes

The functions of the predicted DEL target genes were evaluated using the GO database and the KEGG database. In addition, the KOBAS program was used for enrichment analysis and to identify the potential functions of the DELs. GO encompasses three domains: molecular function (MF), cellular component (CC), and biological process (BP). The P values denote the significance of GO enrichment or KEGG pathway correlation (P<0.05 was considered statistically significant).

Co-expression analysis between lncRNAs-mRNAs

Co-expression-network reconstruction and analysis were performed to identify the correlated expression of DELs and DEMs. In the process, the Pearson correlation coefficient and P value (P<0.05) of genes were calculated. The R value was used to compare Pearson's correlation coefficients between the genes. DELs and DEMs with Pearson correlation coefficients of 0.8 or greater were selected to construct the network using Cytoscape software (version 3.7.2).

Quantitative real-time polymerase chain reaction (qRT-PCR) validation

The DELs were randomly selected for validation using qRT-PCR. Total RNA was extracted using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc., USA), and reverse transcription reactions were performed using M-MLV Reverse Transcriptase (Promega Corporation, Madison, WI, USA) according to the manufacturer's instructions. *Gapdh* was used as an internal control to normalize the comparative expression levels of lncRNAs or mRNAs with the cycle threshold ($2^{-\Delta\Delta Ct}$) method.

Statistical analyses

Differential expression analysis of lncRNAs was performed using DESeq2 (version 1.24.0), which fits a generalized linear model assuming a negative binomial distribution of RNA-Seq read counts. We determined the statistical significance of the levels of lncRNAs verified by qRT-PCR (normality was confirmed with the Shapiro-Wilk test) using analysis of variance (ANOVA) with SPSS software (version 21.0). Data are expressed as mean ± standard deviation (SD). Statistical analysis was performed using the following thresholds: $P<0.05$; $\log2 |FC| >1$. Statistical significance was set at $P<0.05$.

Results

The lncRNA expression profiles in SCI and NC groups

Total RNA purified from each bladder sample was depleted of rRNA, fragmented, and converted into libraries for high-throughput sequencing. The expression profiles of lncRNAs and mRNAs in the SCI 1–3 and NC groups were examined. We obtained raw data consisting of >91,441,540 clean reads (97.32–97.74%) from the Illumina HiSeq 3000 platform. Following hierarchical clustering, a total of 4,264 lncRNAs were identified by RNA-seq, including 124 annotated lncRNAs and 4,140 novel lncRNAs. An overview of the lncRNA sequencing results is presented in Table S1. Compared with the NC group, the SCI 1–3 groups had 468 (17 annotated), 117 (11 annotated), 408 (13 annotated) DELs (FC >2), including 247 (11 annotated), 38 (5 annotated), 201 (7 annotated) up-regulated and 163 (5 annotated), 79 (6 annotated), 207 (6 annotated) down-regulated lncRNAs, respectively. Compared with the SCI–1 group, the SCI 2–3 groups had 211 and 221 DELs (FC >2), including 53 and 89 up-regulated and 158 and 132 down-regulated lncRNAs, respectively. Compared with the SCI–2 group, the SCI–3 group had 114 DELs (FC >2), including 81 up-regulated and 33 down-regulated lncRNAs. Differentially expressed annotated lncRNAs common to SCI 1–3 groups compared with the NC group are presented in Table S2. In order to identify whether these DELs were able to differentiate SCI from NC, heatmaps and venn diagram (*Figure 1*), and volcano plots (*Figure 2*) were adopted.

mRNA expression profiles in SCI and NC groups

Following hierarchical clustering, 47,483 mRNAs were identified by RNA-seq. The expression profiling data demonstrated that 6,654, 2,133, and 5,706 DEMs (FC >2) were differentially expressed between the SCI 1–3 groups and NC animals, of which 4,821, 1,195, and 3,695 were up-regulated, and 1,833, 938, and 2,011 were down-regulated, respectively. Compared with the SCI–1 group, the SCI 2–3 groups had 3,824 and 4,539 DEMs (FC >2), of which 692 and 1,612 were up-regulated, and 3,132 and 2,927 were down-regulated, respectively. Compared with the SCI–2 group, the SCI–3 group had 2,142 DEMs (FC >2), of which 1,712 were up-regulated, and 430 were down-regulated. To identify whether these DEMs could differentiate SCI from NC, heatmaps and venn diagram (*Figure 3*), and volcano plots (*Figure 4*) were adopted.

Target gene prediction of DELs

To predict the potential target mRNAs of the DELs, we predicted genes of DELs in cis- and trans-targets. A total of 366 DELs (25 annotated) contained 2,845 target genes. Among them, 253 DELs (14 annotated) were predicted for 359 cis-regulated target genes, 25 DELs (7 annotated) were predicted for 558 trans-regulated target genes, and 88 DELs (4 annotated) were predicted for both 116 cis-regulated and 1,812 trans-regulated target genes. The altered expression of target genes may be associated with NB and fibrosis. The target genes prediction of DELs common to SCI 1–3 are presented in Table S3.

GO and KEGG pathway analysis

To identify the potential function of target genes of DELs, GO analysis was performed at the time point of 28 days post SCI, during which MF, CC, and BP were described. The results demonstrated that 810 MFs, 554 CCs, and 4,811 BPs were enriched. In addition, differentially expressed target genes were mainly enriched in the following GO terms: 'enzyme activator activity', 'GTPase activator activity' and 'kinase regulator activity' for the MF terms; 'chromosomal region', 'condensed chromosome', and 'chromosome, centromeric region' for the CC terms; and 'positive regulation of TGF-β1 signaling pathway', 'developmental growth involved in morphogenesis' and 'lymphocyte mediated immunity' for BP terms. The top 10 enriched GO terms for MF, CC, and BP are shown in *Figure 5*.

Pathway analysis was carried out based on the KEGG database at the time point of 28 days post SCI. Thirty

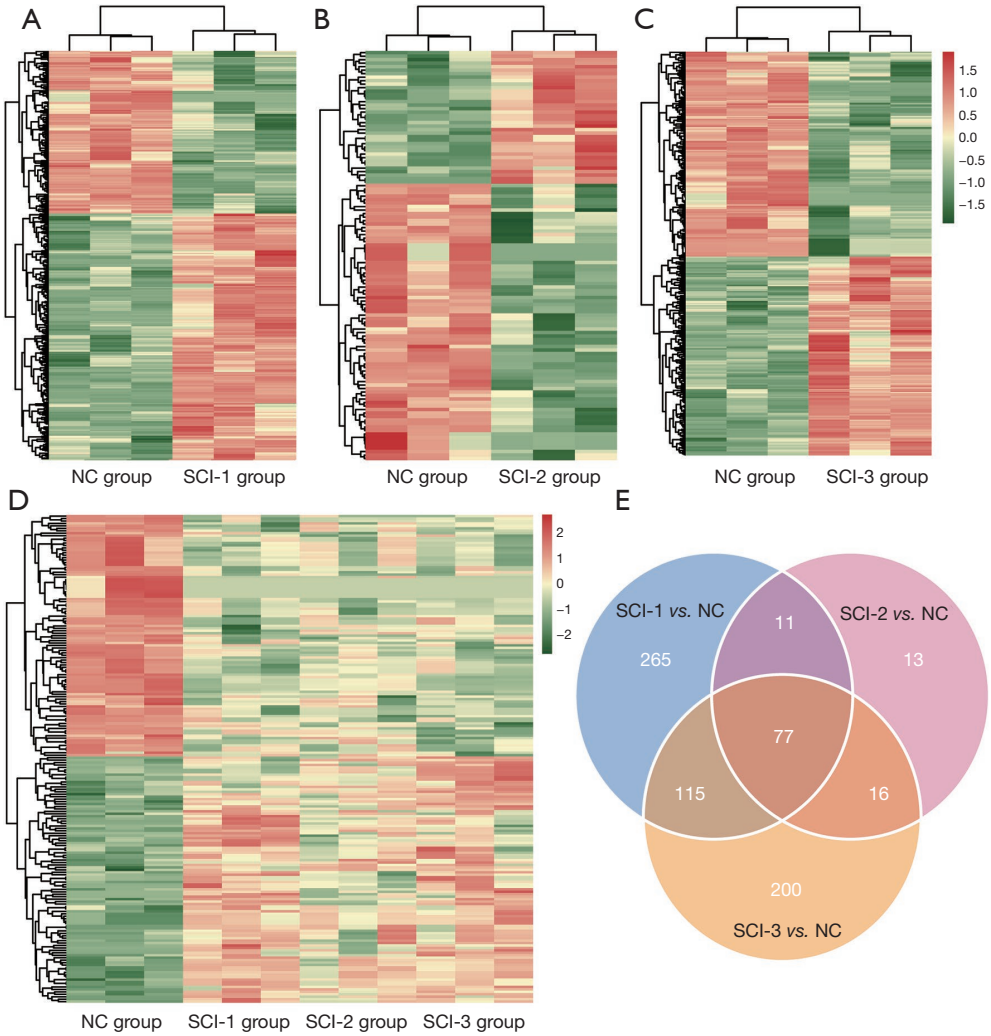

Figure 1 Heatmap and venn diagram of DELs in SCI 1–3 groups bladder samples compared to NC group bladder samples. (A) SCI–1 group *vs.* NC group. (B) SCI–2 group *vs.* NC group. (C) SCI–3 group *vs.* NC group. (D) SCI 1–3 groups *vs.* NC group. (E) Venn diagram of SCI 1–3 groups *vs.* NC group. Relative lncRNA expression is depicted according to the color scale. Red indicates relatively higher expression, and green indicates lower expression. DELs, differentially expressed lncRNAs; SCI, spinal cord injury; NC, normal control.

pathways showed statistical differences (P<0.05) in target genes between the SCI rat NB tissues and normal rat bladder tissues. As revealed in *Figure 6*, the target genes of DELs were predominantly enriched in the following pathways: 'Osteoclast differentiation' and 'Relaxin signaling pathway' for the Organismal Systems; 'Purine metabolism' and 'Nicotinate and nicotinamide metabolism' for the Metabolism; 'Herpes simplex virus 1 infection' and 'Human papillomavirus infection' for the Human Diseases; 'Proteasome' and 'Homologous recombination' for the Genetic Information Processing; 'Cytokine-cytokine receptor interaction', 'TNF signaling pathway', 'TGF-beta signaling pathway', and 'JAK-STAT signaling pathway' for the Environmental Information Processing. However, these results require further validation.

Co-expression analysis between DELs-DEMs

The gene co-expression network was analyzed to explore further the potential interactions between eight annotated DELs and 54 target mRNAs. Each lncRNA was co-expressed with one or multiple target mRNAs (*Figure 7*). This result showed that LOC102554317, Mir155hg, and LOC100910945 may play a more significant role in the

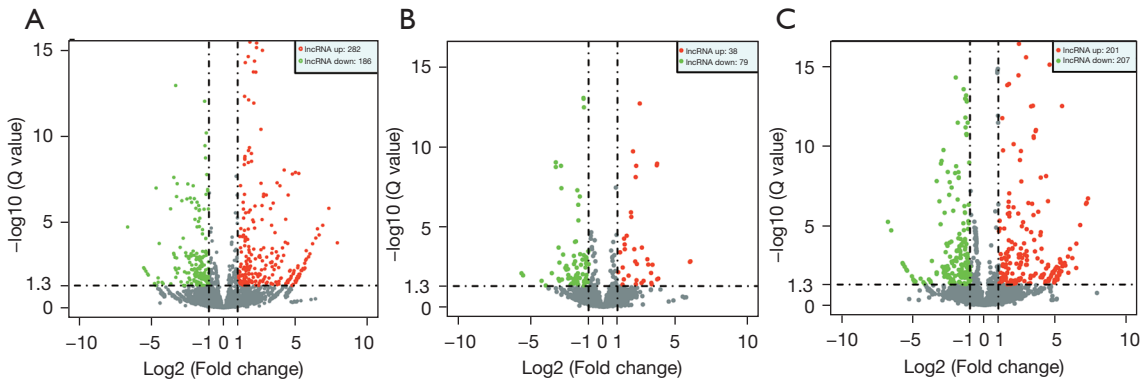

Figure 2 Volcano plot of DELs in SCI 1–3 groups bladder samples compared to NC group bladder samples. (A) SCI–1 group *vs.* NC group. (B) SCI–2 group *vs.* NC group. (C) SCI–3 group *vs.* NC group. Red and green points represent higher and lower expression of DELs with statistical significance (FC ≥ or ≤2, respectively; P≤0.05). DELs, differentially expressed lncRNAs; SCI, spinal cord injury; NC, normal control; FC, fold change.

network, as 2, 11, and 36 mRNAs were targeted by these three lncRNAs, respectively.

Validation of lncRNA expression by qRT-PCR

To validate the RNA-seq results, eight annotated DELs were chosen for qRT-PCR verification, including four highly up-regulated and four highly down-regulated lncRNAs. Total RNA from the bladder tissue samples of the NC and SCI 1–3 groups were used. As shown in *Figure 8*, the qRT-PCR analysis demonstrated that lncRNA LOC100910973, Mir155hg, LOC102554317, and H19 were up-regulated significantly, and LOC100910558, LOC100910945, Miat, LOC100911717 expression was down-regulated significantly in the bladder tissues of the SCI group compared with their levels in the NC group, which agreed with the RNA-Seq results (P<0.05). Specifically, the expression level of lncRNA Miat and LOC100911717 were gradually decreased, and that of Mir155hg was gradually increased. The expression level of LOC100910558 was the lowest in SCI–1 group, and then increased gradually. H19 was highest in SCI–1 group and then decreased gradually. The expression level of LOC100910945 and Mir155hg decreased overall but fluctuated between the SCI 1–3 groups. Although the FC values differed in DELs expression between the groups, the RNA-seq and qRT-PCR results showed the same trends. These results were considered to verify the reliability of RNA-Seq successfully.

Discussion

NB secondary to SCI is an intractable disease with few treatment options. This disease causes significant personal and financial burdens. To enhance the therapeutic effect and improve the prognosis of patients with NB, it is vital to discover the expression of sensitive and specific biomarkers of NB secondary to SCI. Personalized treatment of NB could be made possible in the near future if researchers can fully modulate RNA expression. Currently, lncRNAs have been studied as potential tools to determine the biological behavior of illness and remedial goals due to their relatively high stability (14,15). NB is a complex and dynamic continuous process. Detrusor insufficiency and the formation of bladder diverticulum could have resulted from an overactive bladder, ultimately leading to smooth muscle cell apoptosis and further to bladder fibrosis (16). Information regarding NBs and lncRNAs remains limited, and the biological functions of most lncRNAs have not been completely elucidated. As such, identifying possible mechanisms of NB secondary to SCI from the perspective of lncRNAs would be of importance for the exploration of early diagnosis and treatment.

The suitability of the animal model plays a decisive role in the research process. The lncRNA detection time points are also important in experimental research. Compared with other SCI models, contusion SCI has long been recognized as the classic SCI model, closely resembling histopathology and clinical situation. Specifically, the contusion SCI

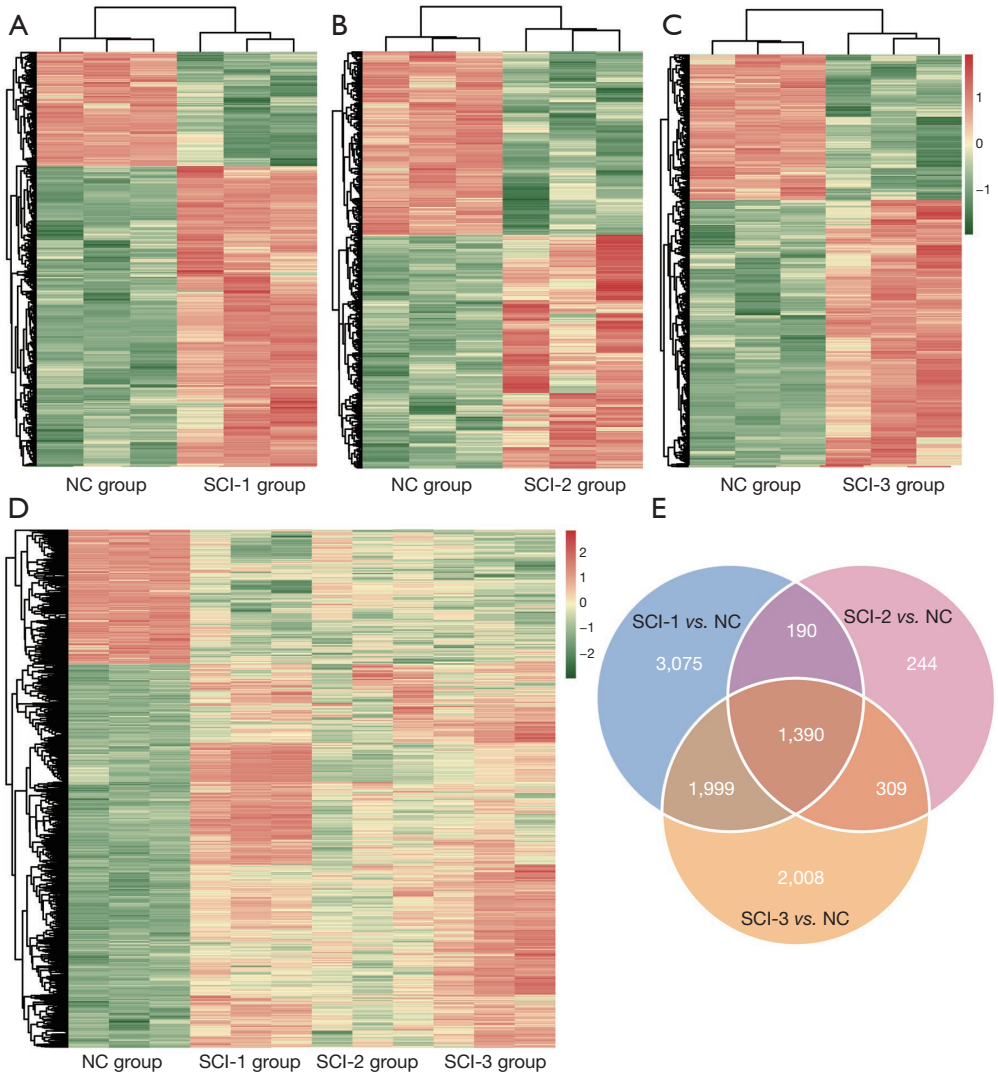

Figure 3 Heatmap and venn diagram of DEMs in SCI 1–3 groups bladder samples compared to NC group bladder samples. (A) SCI–1 group *vs.* NC group. (B) SCI–2 group *vs.* NC group. (C) SCI–3 group *vs.* NC group. (D) SCI 1–3 groups *vs.* NC group. (E) Venn diagram of SCI 1–3 groups *vs.* NC group. Relative mRNA expression is depicted according to the color scale. Red indicates relatively higher expression, and green indicates lower expression. DEMs, differentially expressed mRNAs; SCI, spinal cord injury; NC, normal control.

model comprises spinal tissue destruction, extruded disk tissue, and bony fragments. Regarding the assessment of nerve regeneration and the recovery of spinal function, the contusion SCI model exhibits some disadvantages due to the variation in residual tissues. However, this problem is well solved in a complete transection SCI model. Secondary damage caused by SCI is central to the pathophysiology of NB and may help to determine an appropriate temporal window for both diagnosis and treatment (17). Tissue necrosis and cell apoptosis were the most prominent changes and peaked seven days post-SCI. Therefore, we collected bladder tissues and analyzed lncRNA expression profiles on days 7, 14, and 28 after complete spinal cord transection to better analyze lncRNA expression in NB (17).

The current study is the first to explore the lncRNA expression profiles in NB following SCI thoroughly. The findings indicated that 4,264 lncRNAs were identified by RNA-seq, including 124 annotated lncRNAs and 4,140 new lncRNAs. To validate the RNA-seq results, eight annotated DELs were chosen for qRT-PCR verification, including

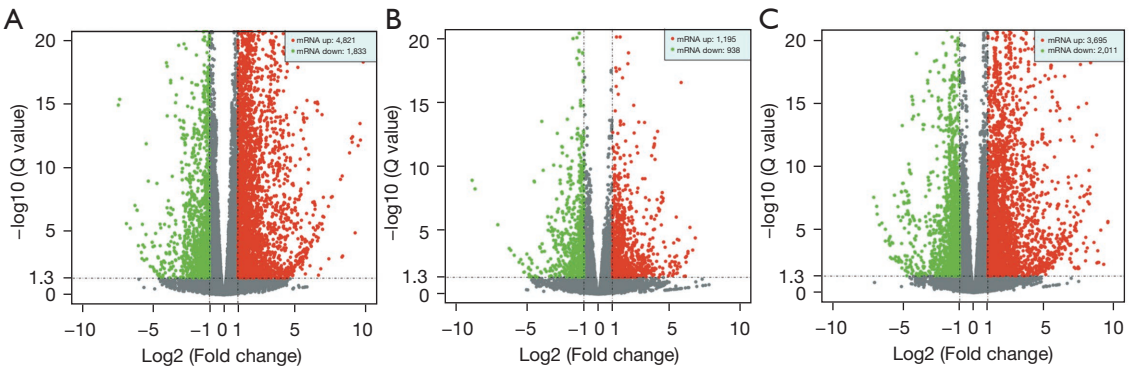

Figure 4 Volcano plot of DEMs in SCI 1–3 groups bladder samples compared to NC group bladder samples. (A) SCI–1 group *vs.* NC group. (B) SCI–2 group *vs.* NC group. (C) SCI–3 group *vs.* NC group. Red and green points represent higher and lower expression of DEMs with statistical significance (FC ≥ or ≤2, respectively; P≤0.05). DEMs, differentially expressed mRNAs; SCI, spinal cord injury; NC, normal control; FC, fold change.

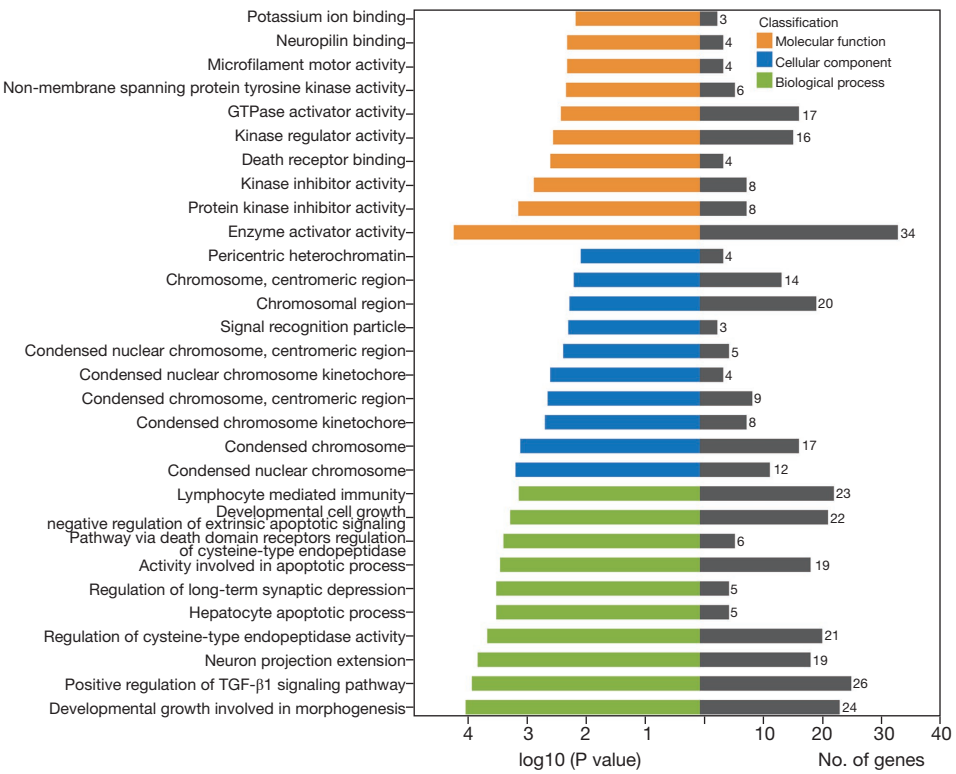

Figure 5 GO analysis of DELs in SCI group NB compared to NC group. Top 10 enriched GO terms of MF, CC, and BP. GO, Gene Ontology; DELs, differentially expressed lncRNAs; SCI, spinal cord injury; NB, neurogenic bladder; NC, normal control.

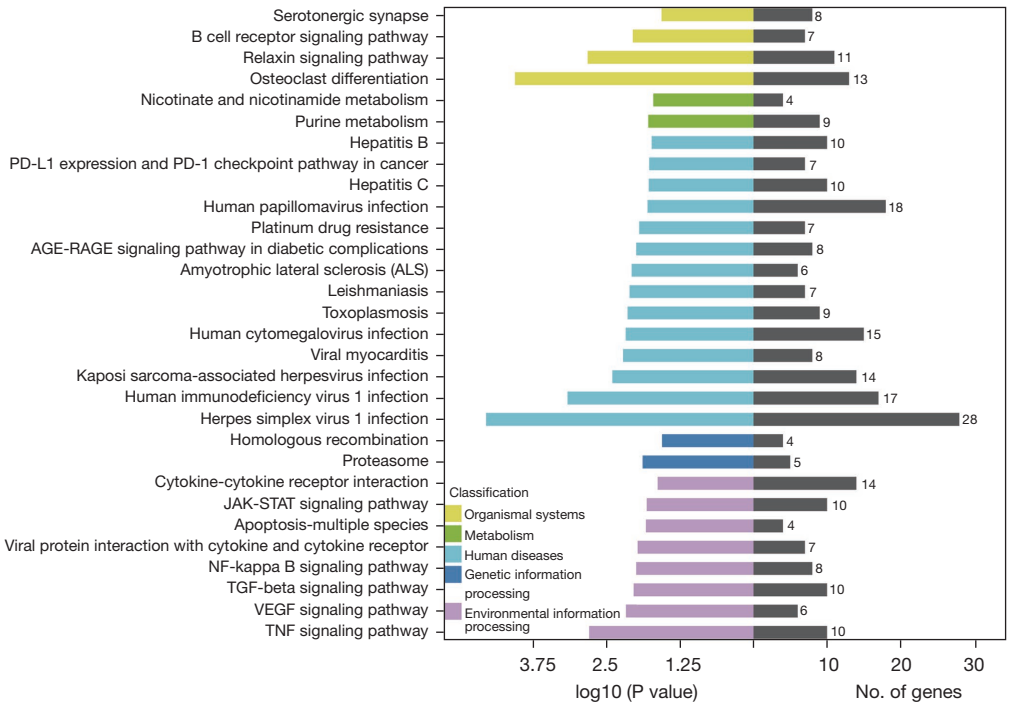

Figure 6 KEGG pathway analysis of DELs in SCI group NB compared to NC group. KEGG, Kyoto Encyclopedia of Genes and Genomes; DELs, differentially expressed lncRNAs; SCI, spinal cord injury; NB, neurogenic bladder; NC, normal control.

four highly up-regulated and four highly down-regulated lncRNAs. The qRT-PCR analysis demonstrated that lncRNA LOC100910973, Mir155hg, LOC102554317, and H19 were up-regulated significantly, and LOC100910558, LOC100910945, Miat, LOC100911717 expression was down-regulated significantly in the SCI group bladder tissues as compared with their levels in the NC group, which agreed with the RNA-Seq results. As expected, compared with NC rats, several lncRNAs exhibited elevated expression or decreased expression following SCI. In addition, some of these DELs were associated with particular biological events and signaling pathways, indicating that they may serve as candidate biomarkers for NB following SCI.

Moreover, GO, and KEGG pathways were performed to identify the possible function of target genes of DELs in SCI rat NB tissues. To date, no one has yet comprehensively explored the signature of lncRNA expression and relevant enrichment pathways in complete transection SCI rat NB. A study indicated that target DEMs were predominantly enriched in cell adhesion, inflammatory response, and metabolic processes in contusion SCI. In addition, the target genes of DELs were predominantly enriched in the following KEGG pathways: the PI3K-Akt signaling pathway, neuroactive ligand-receptor interactions, and metabolic pathways (18). Conversely, the present study in GO enrichment analysis found that differentially expressed target genes were predominantly associated with enzyme activator activity, GTPase activator activity, kinase regulator activity, condensed chromosome, chromosome segregation, developmental growth involved in morphogenesis, and lymphocyte-mediated immunity. Similarly, KEGG enrichment analysis indicated that the target genes of DELs were predominantly enriched in the relaxin signaling pathway, purine metabolism, nicotinate and nicotinamide metabolism, proteasome, homologous recombination, cytokine-cytokine receptor interaction, TNF signaling pathway, signaling pathways regulating pluripotency of stem cells, and JAK-STAT signaling pathway. As the link between relaxin and TGFβ signaling pathway with fibrotic changes in bladder, a previous study revealed the expression of relaxin receptor in the urothelium, lamina propria, and muscularis propria of bladder. Stimulation of normal human bladder smooth muscle cells with relaxin affects tissue remodeling and expression of fibrosis-related proteins via TGF-β/Smad signaling pathway (19). Therefore, we

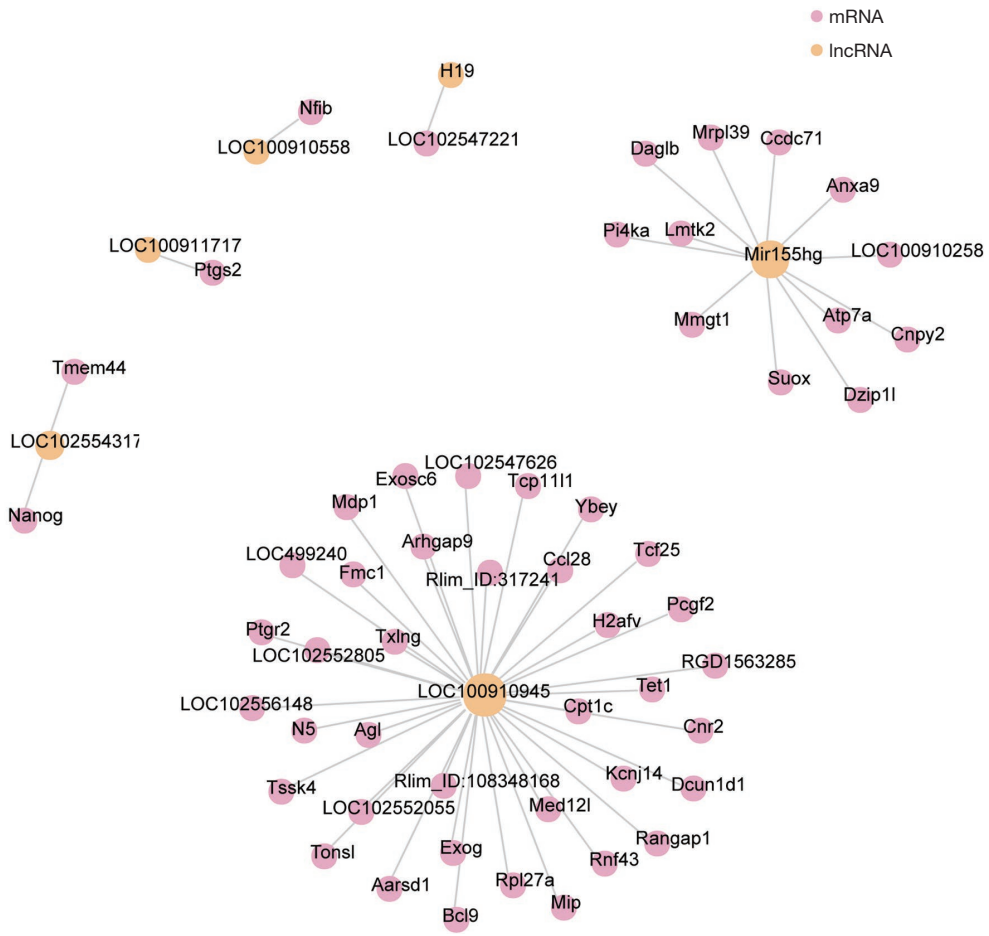

Figure 7 Construction of a lncRNA-mRNA co-expression network. The yellow marked nodes represent lncRNAs; the red marked nodes represent target mRNAs.

suspected these DELs might play a significant role in the process of bladder fibrosis. These pathways are mostly related to metabolic, inflammatory, and immune reactions. The results further underline the significant role of metabolism and immuno-inflammatory responses in NB secondary to SCI.

SCI usually results in detrusor-sphincter dyssynergia, bladder fibrosis and DO, leading to urinary dysfunction (4). The lncRNAs regulate gene expression through different mechanisms involving transcription factors sequestration, transcriptional activation, and sequestration of complementary microRNAs by base pairing (20). To date, the biological functions and possible mechanisms of most lncRNAs have not been completely elucidated. Importantly, lncRNA Miat (NR_111959.1) is highly expressed in many metabolic pathways and organs, including the heart, kidney, and endometrium, and has been widely explored in some important biological processes such as organ fibrosis (9). Notably, recent study on diabetic cardiomyopathy have also demonstrated that lncRNA Miat is involved in cardiac fibrosis via silencing of miR-214-3p (21). In addition, the Miat/miR-133a-3p axis may be related to atrial fibrillation-induced myocardial fibrosis (22). The lncRNA Miat also improved endometrial fibrosis by targeting miR-150 and ameliorating fibrosis in diabetic nephropathy by regulating the expression of miR-147a and E2F3 (21,23). In this study, we found that lncRNA Miat was expressed at low levels in SCI rat bladder tissue. The results showed that lncRNA Miat might play a significant role in the process of bladder fibrosis.

LncRNAs have been identified to exert both promotive and inhibitory effects on the multifaceted processes

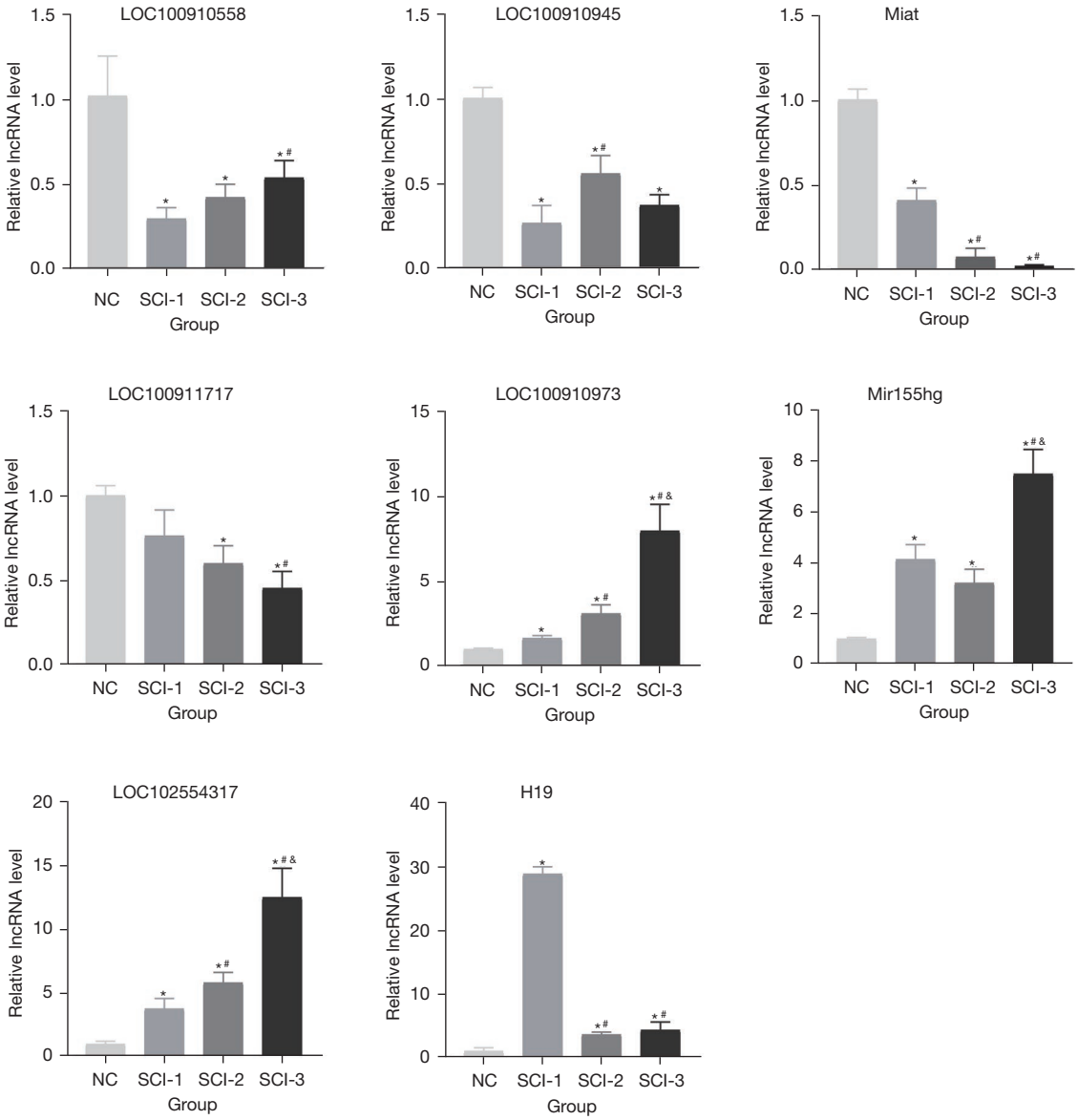

Figure 8 Quantitative real-time polymerase chain reaction validation of 8 DELs between different groups. Data are presented as the mean ± SD. *, P<0.05 *vs.* NC group; #, P<0.05 *vs.* SCI–1 group; &, P<0.05 *vs.* SCI–2 group. DELs, differentially expressed lncRNAs; SD, standard deviation; NC, normal control; SCI, spinal cord injured.

of fibrosis. Among these lncRNAs, lncRNA H19 (NR_027324.1) was the most appreciably up-regulated lncRNA in SCI rat bladder tissue. Although its level tended to follow a decreasing tendency within 14 days after SCI and remained relatively stable after that, it was also appreciably higher than that in the normal rats. Notably, the expression of lncRNA H19 was up-regulated by 4.83-fold in the SCI–1 group compared with that in the NC group. Several studies have reported the important effects of H19 during the complex process of epithelial-mesenchymal transition (EMT) (24,25). EMT is a complicated process during which epithelial cells gradually lose their characteristics and functionality and eventually transform into mesenchymal-like cells. This is a crucial mechanism underlying multiple organ fibrosis, including that of the bladder (26). Our previous miRNA study showed that miR-139-5p might retard the process of EMT and fibrosis through certain signaling pathways (27). These lncRNA-mRNA interactions

require further exploration to identify their exact roles and dynamic expression. It has been demonstrated that lncRNA H19 promotes EMT as a significant regulator both *in vitro* and *in vivo* (28). Moreover, lncRNA H19 might exert its effect as a competitive endogenous RNA (ceRNA) to regulate EMT by sequestering miR-29b-3p (29). Likewise, the upregulation of lncRNA H19 has a potential role in bladder fibrosis following SCI. LncRNA H19 has been verified to be involved in EMT and multiple organ fibrosis with different mechanisms, revealing that lncRNA H19 possibly serves as an important therapeutic target for organ fibrosis (30). Nevertheless, further studies are required to investigate the possible mechanisms between lncRNA H19 and EMT in bladder fibrosis.

In addition, we also found that lncRNA LOC100910973 was significantly up-regulated, suggesting that it might contribute to the process of NB secondary to SCI and offers us inspiration for further exploration. Recently, Ding *et al.* revealed the expression profiles of lncRNAs and mRNAs in a rat model of SCI. The study reported that LOC100910973 was significantly up-regulated in SCI rats and might exert an essential effect on the immuno-inflammatory response following SCI (31). The other lncRNA identified in this study, Mir155hg, was originally thought to be involved in several immune and inflammatory processes (32). Mir155hg is a vital regulatory factor implicated in multiple physiological and pathological processes, including immune responses, inflammation, and organ fibrosis (33). Several previous studies have shown that Mir155hg is involved in regulating macrophage polarization and innate immunity and in regulating acute rejection and graft loss (34-36). The lncRNA Mir155hg has been identified as the primary miRNA of miR-155 and is known to regulate hematopoiesis, organ fibrosis, and tumorigenesis (37). We know that Mir155 originates from Mir155hg and that Mir155hg performs a critical function by interacting with Mir155 (38). Thus, we inferred that Mir155hg might affect different biological processes by interacting with Mir155. Mir155 acts as a crucial regulator to directly modulate the expression of numerous immune-specific transcripts, including T-cell differentiation, DC maturation, B-cell proliferation, and antibody production (39,40). Moreover, it has been shown that the expression of Mir155hg is activated by many transcriptional regulatory factors, including activator protein 1, nuclear factor-kappa B, and Smad4 (38,41,42). Recent studies have shown that a regulatory loop is formed by the MIR155HG/miR-627/HMGB1/NF-κB axis, which regulates transforming growth factor β1 (TGF-β1)-induced NHLF activation. Specifically, MIR155HG could inhibit the expression of miR-627 via directing binding (43). The role of MIR155HG in NB has not been well studied. GO enrichment and KEGG pathway analysis in the current study indicated that the co-expression between MIR155HG and mRNAs was predominantly enriched in immune-related terms and immune-related pathways, which shows that MIR155HG might be closely associated with immunity. Furthermore, some of these signaling pathways have been previously reported to be involved in bladder dysfunction.

The TGF-β1 signaling pathway is critically involved in the development and progression of bladder fibrosis. Recent studies have highlighted that overexpression of TGF-β1 could activate the Smad signaling pathway, a critical molecular mechanism contributing to smooth muscle cell apoptosis and bladder fibrosis (44). In addition, several previous studies have indicated that the TGF-β/Smad signaling pathway might be closely associated with chronic bladder outlet obstruction (BOO), which further causes bladder fibrosis. This is mainly because the high intravesical pressure could cause additional detrimental effects on bladder smooth muscle cells, and protein kinases could be further activated to modulate cell function and promote bladder fibrosis progression (45,46). Several studies on the heart and liver showed similar findings that high pressure could induce myocardial fibrosis, stress-activated protein kinases, and initiate hepatic fibrosis (47,48). The idea that lncRNAs participate in the regulation of NB is relatively recent, and therefore their importance in the NB microenvironment and their potential clinical utility are not yet well known.

In this study, bioinformatics analysis revealed that the expression profiles of numerous lncRNAs and mRNAs were significantly altered in the bladder tissue secondary to SCI. Although we initially predicted the possible biological function of lncRNA by NGS, it is still too early to use these DELs as primary targets for therapeutic intervention or as possible NB biomarkers. There are some limitations to this study. First, the interactions between lncRNAs and mRNAs in NB were not investigated in detail. Whether these DELs may also serve as possible NB biomarkers or as primary targets for therapeutic intervention requires further study. Therefore, further in-depth studies should explore these interactions and their possible mechanisms. Second, NB tissue may need to be collected 60 days or more after complete transection SCI to study lncRNAs that may be functional at that time. We will further explore the

lncRNA profiles in the separated layers of bladder mucosa and detrusor muscles. Third, the sample size was small, and more prospective studies that include more rats or human samples are needed to verify this result. Because of the limitations of this study, the biological functions and possible mechanisms of NB secondary to SCI from the perspective of lncRNAs require further investigation.

Conclusions

We have revealed the signature of lncRNA expression and the possible roles of lncRNAs and mRNAs in SCI rat NB through NGS analysis and qRT-PCR validation. The results may help identify possible mechanisms of NB secondary to SCI from the perspective of lncRNAs and provide some new potential lncRNAs for the early diagnosis and treatment of human NB in the future.

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Footnote

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Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://tau.amegroups.com/article/view/10.21037/tau-21-1161/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All experimental procedures were implemented in compliance with the National Institute of Health Guidelines for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee of Xuanwu Hospital Capital Medical University (No. 20190128).

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Supplementary

Table S1 Overview of lncRNAs sequencing results

Groups	LncRNAs classification	Total	Differential expression	Up-regulated	Down-regulated
SCI–1 *vs.* NC	Annotated	74	16	11	5
	Non-annotated	3,958	452	271	181
SCI–2 *vs.* NC	Annotated	66	11	5	6
	Non-annotated	2,943	106	33	73
SCI–3 *vs.* NC	Annotated	74	13	7	6
	Non-annotated	3,958	395	194	201
SCI–2 *vs.* SCI–1	Annotated	74	12	4	8
	Non-annotated	3,960	199	49	150
SCI–3 *vs.* SCI–1	Annotated	77	12	6	6
	Non-annotated	4,046	209	83	126
SCI–3 *vs.* SCI–2	Annotated	69	9	5	4
	Non-annotated	3,405	105	76	29

SCI, spinal cord injury; NC, normal control.

Table S2 Differentially expressed annotated lncRNAs common to SCI 1–3 compared with NC

LncRNAs	Ref. ID	Log2 (FC)			Regulation	P value
		SCI–1 *vs.* NC	SCI–2 *vs.* NC	SCI–3 *vs.* NC		
LOC100910558	NR_110680.1	–1.546415992	–1.366787363	–1.287343456	Down	<0.05
LOC100910945	NR_131086.1	–1.055060514	–0.547077347	–0.720552987	Down	<0.05
Miat	NR_111959.1	–0.36042155	–0.842292570	–3.707241104	Down	<0.05
LOC100911717	NR_132640.1	–0.317800792	–0.704729328	–0.113700253	Down	<0.05
LOC100910973	NR_102351.1	1.082645959	1.484233505	3.464149930	Up	<0.05
Mir155hg	NR_132107.1	2.463915289	2.030949228	2.822043830	Up	<0.05
LOC102554317	NR_133666.1	2.801305823	3.400502277	5.458524688	Up	<0.05
H19	NR_027324.1	4.830080779	1.327515764	2.425336332	Up	<0.05

SCI, spinal cord injury; NC, normal control; FC, fold change.

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Table S3 Target gene prediction of differentially expressed lncRNAs

LncRNAs	Ref. ID	Cis-regulation	Trans-regulation	Cis-target gene	Trans-target gene
LOC100910558	NR_110680.1	+	–	*Nfib*	
LOC100910945	NR_131086.1	+	+	*Tet1*	*Kcnj14, Pcgf2, Exosc6, Exog, Cpt1c, Aarsd1, LOC499240, H2afv, Agl, Txlng, Ptgr2, Rpl27a, Tcp11l1, Cnr2, LOC102547626, RGD1563285, Ccl28, Rlim_ID:108348168, Tssk4, LOC102552055, Ybey, Bcl9, Arhgap9, Fmc1, Med12l, Tonsl, Dcun1d1, LOC102556148, Mdp1, Tcf25, Mip, LOC102552805, Rangap1, Rlim_ID:317241, N5, Rnf43*
Miat	NR_111959.1	–	–		
LOC100911717	NR_132640.1	+	–	*Ptgs2*	
LOC100910973	NR_102351.1	–	–		
Mir155hg	NR_132107.1	+	+	*Mrpl39*	*Pi4ka, Ccdc71, Dzip1l, Daglb, Cnpy2, Anxa9, Lmtk2, Atp7a, LOC100910258, Suox, Mmgt1*
LOC102554317	NR_133666.1	–	+		Nanog, Tmem44
H19	NR_027324.1	+	–	LOC102547221	

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