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Research paper

Alternative RNA splicing associated with axon regeneration after rat peripheral nerve injury

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ABSTRACT

The intrinsic axon regeneration capacity is crucial for peripheral nerve regeneration after injury. Identifying key molecules involved in this process makes great contribution to the investigation of peripheral nerve injury repair. Alternative splicing (AS) is an important regulation mode of eukaryotic gene expression, which has been widely studied both in physiological and pathological processes. However, less is known about the role of AS in peripheral nerve regeneration. In this work, to identify the AS events associated with axon regeneration capacity, we analyzed the AS events during sciatic nerve injury repair by RNA sequencing (RNA-Seq) and replicate multivariate analysis of transcript splicing (rMATS). The differential AS events were underwent gene ontology enrichment and pathway analyses. Moreover, we identified a significantly increased AS event of neuronal cell adhesion molecule Nrcam (Nrcam-S), and demonstrated down-regulation of Nrcam-S by specific siRNAs inhibited axon regeneration of Dorsal Root Ganglion (DRG) neurons after sciatic nerve injury *in vitro* and *in vivo*. Additionally, we found expression levels of RNA binding proteins (RBPs) CUGBP Elav-like family member 3 (CELF3) and RNA binding protein fox-1 homolog 2 (Rbfox2) were markedly increased after sciatic nerve injury. Our data may serve as a resource useful for further understanding how AS contributes to molecular regulations in DRG during sciatic nerve regeneration.

1. Introduction

Peripheral nerve injury caused by diseases and injury is some of the most prevalent neurological problems (Jones et al., 2016). Different from central nervous system, peripheral nervous system owns stronger ability to regenerate after injury, which refers to axon regeneration in general. Even so, the treatment results are often unsatisfactory and only partially complete, since the axon degeneration leading to atrophy of the target organs and effectors (Faroni et al., 2015; Jiang et al., 2017; Sulaiman and Gordon, 2013). If the axon regeneration is promoted, the consequence will be ameliorated. Axon regeneration is a complicated process controlled by intrinsic and extrinsic factors. Although a large number of studies have identified crucial candidate genes involved in axon regeneration after peripheral nerve injury (Cattin and Lloyd, 2016; Chan et al., 2014; Sun and He, 2010), whose mechanism is not fully understood yet.

Alternative precursor-mRNA (pre-mRNA) splicing (AS) is a key regulation mode of mammalian gene expression, producing diverse mRNAs and proteins, that is thought to contribute to the vital biological functions. In general, AS events could be classified into 7 types, depending on the cutoff location of exonic segments or retained introns: skipped exon (SE), retained intron (RI), alternative 5′ splice site (A5SS), alternative 3′ splice site (A3SS), alternative first exon (AFE), alternative last exon (ALE) and mutually exclusive exons (MXE) (Blencowe, 2006; Matlin et al., 2005). In various species, AS events are conserved, especially in the nervous system, in which high AS frequencies may contribute to the functional complexity (Raj and Blencowe, 2015). Mutations in neural RNA-binding proteins (RBPs) involved in AS regulation and aberrations in neural AS patterns have been linked to neurological disorders and disease (Licatalosi and Darnell, 2006). As for axon regeneration, much work has focused on several AS isoforms of a few individual genes (Amiri et al., 2009; Struebing et al., 2017; Zhou et al., 2013). However, genome-wide alternative splicing profiling of the DRGs during the peripheral nerve injury repair is unclear.

In this study, we investigated the dynamics of AS of DRGs during rat sciatic nerve injury repair by RNA-Seq and bioinformatics analysis.

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Then, we determined whether the genes of differentially expressed AS during sciatic nerve injury repair were enriched for axon regeneration related specific functions, in which we focused on several AS events and determined their expression levels. We identified a key alternative splicing event of Nrcam (Nrcam-S) as an important candidate in promoting axon regeneration and determined the outcome of repairing rat sciatic nerve injury with siRNAs targeting Nrcam-S *in vitro* and *in vivo*. In addition, we detected expression levels of 24 RBPs following sciatic nerve injury and found marked increases in CELF3 and Rbfox2 expression levels, which indicates the two RBPs may play vital roles in sciatic nerve regeneration.

2. Materials and methods

2.1. Animal surgery and tissue preparation

All animal procedures were performed in accordance with Institutional Animal Care guideline of Nantong University, and were ethically approved by the Administration Committee of Experimental Animals, Jiangsu Province, China. Male Sprague-Dawley (SD) rats (8 weeks) underwent surgery of sciatic nerve crush injury as previously described (Yi et al., 2015). The animals were anaesthetized by an intraperitoneal injection of complex narcotics (85 mg/kg trichloroacetaldehyde monohydrate, 42 mg/kg magnesium sulfate, and 17 mg/kg sodium pentobarbital), and the sciatic nerve was exposed by a small incision on the skin. The left sciatic nerve at 10 mm above the bifurcation into the tibial and common fibular nerves was crushed with a forceps at a force of 54 N for 3 times (a period of 10 s for each time). 36 animals were then randomly divided into 6 groups and were killed by cervical dislocation at 0 h (h), 3 h, 9 h, 1 day (d), 4 d and 7 d after surgery. The L4–6 DRGs were harvested from animals in different groups and stored at −80 °C.

2.2. Lumbar (L) 4/L5 intra-DRG injection

Male Sprague-Dawley (SD) rats (8 weeks) were randomly divided into 2 groups ($n = 5$). Following an incision along the dorsal midline, the left L4 and L5 DRGs were exposed by removal of the lateral processes of the vertebrae. The epineurium lying over the DRG was opened, and the glass needle inserted into the ganglion. For direct DRG injections, 2OMe + 5Chol + 5Cy5 modified siRNA (2 nmol in 4 μl) was injected over a period of 5 min (min) into the L4/L5 DRGs through the indwelling catheter attached to a 10-μl Hamilton syringe. After a delay of 2 min, the needle was removed. After 2 d, the left sciatic nerve was crushed, and obtained for analysis another 3 d later.

2.3. RNA extraction and RNA-Seq

Total RNA was isolated from DRG tissues using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and then purified with RNeasy spin columns (Qiagen, Valencia, CA) to remove contaminating DNA. After the total RNA extraction and DNase I treatment, magnetic beads with Oligo (dT) are used to isolate mRNA. Mixed with the fragmentation buffer, the mRNA is fragmented into short fragments. Then cDNA is synthesized using the mRNA fragments as templates. Short fragments are purified and resolved with EB buffer for end reparation and single nucleotide A (adenine) addition. After that, the short fragments are connected with adapters. After agarose gel electrophoresis, the suitable fragments are selected for the PCR amplification as templates. At last, the library could be sequenced using Illumina HiSeqTM 2000. Raw reads are subjected to quality control that determines if a resequencing step is needed. After quality control, raw reads are filtered into clean reads which will be aligned to the reference sequences with TopHat v2.0.13 (Trapnell et al., 2009). The alignment data were utilized to calculate distribution of reads on reference genes and perform coverage analysis. If alignment result passes QC, we would proceed with downstream analysis.

2.4. Real-time PCR (qRT-PCR) analysis

The purified total RNA was reverse transcribed using the PrimeScript RT Reagent Kit according to manufacturer instructions. Candidate splicing events were characterized by PCR using the primers given in Supplementary File S1 followed by agarose gel electrophoresis. For quantitative RT-PCR, 5 ng cDNA were amplified in a 10 μl reaction containing SYBR Premix Ex Taq using a 2-step procedure. Melt curve analysis was enabled at the end of amplification. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control for normalization. The primers of RBPs are given in Supplementary File S2. The primers for all Nrcam transcripts except for Nrcam-S (Nrcam-E) are as follows: Forward primer, CACCCAAATGGTATCCTGAC; reverse primer, GAAGAATACCAGCTTCGTCCAC. The primers for all Nrcam transcripts (Nrcam) are as follows: Forward primer, TCCTCCGAAGTG GTCAGTCA; reverse primer, TTCAGTCCTTGGGAAACCCG. All experiments were done in triplicate.

2.5. Alternative splicing analysis

The replicate multivariate analysis of transcript splicing (rMATS) v4.0.2 (Shen et al., 2014) was used to screen differential alternative splicing events across different samples. First, we used TopHat v2.0.13 (Trapnell et al., 2009) to align the reads to the *Rattus norvegicus* reference sequence using default parameters except up to two mismatches allowed. Second, the aligned data were run on rMATS for AS analysis, and the 0 h group was treated as a control group. The obtained AS events were classified into five groups: skipped exon (SE), retained intron (RI), alternative 5′ splice sites (A5SS), alternative 3′ splice site (A3SS) and mutually exclusive exons (MXE). Then we calculated the differential AS events with the threshold of $|\Delta$ Percent spliced in (PSI)$| > 0.05$ and FDR < 0.1. The PSI scores named Inclevel Differences were estimated as: Inclevel Differences = IncLevel 1 − IncLevel 2; IncLevel = (IJC_SAMPLE/IncFormLen)/[(IJC_SAMPLE/IncFormLen) + (SJC_SAMPLE/SkipFormLen)]. IJC_SAMPLE_x: inclusion junction counts for SAMPLE_x; SJC_SAMPLE_x: skipping junction counts for SAMPLE_x; IncFormLen: length of inclusion form. SkipFormLen: length of skipping form. *P* value: Significance of splicing difference between two sample groups. FDR: False Discovery Rate calculated from *p*-value. IncLevel1: inclusion level for SAMPLE_1. IncLevel2: inclusion level for SAMPLE_2.

2.6. Gene ontology enrichment and pathway analysis

The genes lists were submitted to the database for annotation, visualization and integrated discovery (DAVID 6.8) for GO enrichment and KEGG pathway analysis (Huang et al., 2009). $P < .05$ was used as a criterion for the determination of whether the enrichment analysis was significant.

2.7. Primary DRG neuron culture and transfection

Adult (8 weeks) rat DRGs were dissected in cold HBSS and then digested with 0.5 mg/ml collagenase (Roche) for 2 h at 37 °C followed by 0.125% trypsin digestion for 30 min at 37 °C. Tissues were triturated in culture medium (Neurobasal medium with 2% B27, 1% glutamine, Thermo fisher scientific) with 1 ml tips and passed through a 0.45 μm cell strainer. Cells passing through the strainer were re-suspended in culture medium and plated to 24-well plates pre-coated with Poly-L-Lysine. At the time of plating, neurons were transfected with siRNAs targeting Nrcam-S (Si-1: CAACTGTGGACGAAGGTAA; Si-2: CTGTGGA CGAAGGTAAGAA; Si-3: GAAGGCTGGTATTCTTCCA; Ribobio, Guangzhou, China) using Lipofectamine RNAiMAX reverse transfection reagent (Invitrogen), according to the manufacturer's instructions. After being cultured in transfection reagent overnight (12 h), neurons were