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Research article

Illumina deep sequencing reveals conserved and novel microRNAs involved in the response to X-ray irradiation after peripheral nerve injury in rats

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ABSTRACT

Peripheral nerve injury is a common disease in clinic practice. Low-dose radiation exposure can increase recovery from peripheral nerve injury, but the mechanism is unclear. The objective of this study was to explore the ways that microRNAs participate in the recovery from peripheral nerve injury in rats. To explore the function of miRNAs in this process, we performed Illumina deep sequencing to investigate miRNA expression in spinal cords after X-ray irradiation. Two miRNA libraries (0 GY and 1 GY) were constructed for Illumina sequencing. The sequencing results showed that a total of 53,342,744 raw reads for the control group −0 Gy and 48,453,367 raw reads for treated group −1 Gy were obtained. After a series of selections, a total of 804 miRNAs were identified. One hundred fifty miRNAs were differentially expressed between the treated group −1 Gy and control group −0 Gy. A total of 23,652 target genes and 48,157 target sites were predicted for the 150 differentially expressed miRNAs. GO and KEGG enrichment analyses revealed that the PI3K-Akt-signaling pathway, neuroactive ligand-receptor interaction, and the MAPK signaling pathway were enriched. We demonstrated that the novelmiRNAs-360,-301,-239, and −400 targeted *Vegfa* and regulated its expression after irradiation. Our study revealed that many novel miRNAs were induced by X-ray irradiation, and were involved in the recovery from peripheral nerve injuries. This study provides a framework for understanding the molecular mechanisms underlying the recovery from peripheral nerve injury following exposure to X-ray irradiation.

1. Introduction

Peripheral nerve injury represents a common disease in clinic practice, and is caused by many factors, including trauma, infection, tumors, and nutrition- and metabolism-related disorders [1,2]. In recent years, microsurgical techniques for repairing peripheral nerve injuries have provided a good foundation for the treatment of this disease [3]. However, the pathology in the peripheral nervous system is complex, and the recovery of peripheral nerve tissues can easily lead to adhesion with the surrounding tissue [4]. The peripheral nerve injury and the delayed regeneration will seriously affect the recovery of sensoryability [5]. Therefore, it is of great importance to promote the regeneration of injured peripheral nerve tissues in the clinic [6]. Peripheral nerve regeneration is based on the interplay between factors that promote and inhibit nerve regeneration [7]. Previous studies have largely focused on ways to improve the activity of factors that promote nerve regeneration, but have ignored the deleterious roles of nerve regeneration played by inhibiting factors [8,9]. Therefore, investigation into the promotion of nerve regeneration and removal of the inhibiting factors is an attractive research direction.

A large number of studies have found that physical therapy following peripheral nerve injury can improve blood circulation and the metabolism of blood vessels, improve tissue growth and the repair function of immune cells, and accelerate the phagocytic function of local tissues [10,11]. Concomitantly, physical therapy can accelerate the regeneration of axons [12] and recovery of the nerve conduction velocity, and improve the curative effect of nerve repair. Several studies have confirmed that low ultra-short wave [13], laser [14], shock wave [15], electromagnetic energy [16], and other physical methods [17] can effectively promote the regeneration of injured peripheral nerves. Because of its safety, reliability, and efficacy, physical therapy is increasingly used in the treatment of peripheral nerve injury.

In previous studies, low-dose irradiation promoted tissue revascularization [18–20]. Furthermore, low-level laser irradiation

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improved functional recovery and nerve regeneration in a sciatic nerve rush rat injury model; the low-dose lasers accelerated peripheral nerve regeneration within a reinforced nerve conduit across a large gap of the transected sciatic nerve in rats. Although low-level laser irradiation did improve nerve regeneration, the mechanism of this process is unknown. The vascular endothelial growth factor(VEGF) could promote the recovery of the ischemic limb and accelerate tissue repair in tissues experiencing trauma [18]. Moreover, it has been found that VEGF is also expressed in neuronal cells. The binding of VEGF to its receptors on the surface of these cells can promote their migration and proliferation, which promote the growth of axons [21,22].

MicroRNAs (miRNAs) are endogenous, small, non-coding RNAs containing 21–24 nucleotides [23]. MiRNAs accurately and effectively regulate gene expression at the post-transcriptional level by either repressing translation or directly degrading target mRNAs [24]. In peripheral nerve injuries, the expression of miRNA is considerably changed. It remains unclear whether these differentially expressed miRNAs are involved in the repair of injured tissues [25]. Low doses of radiation can also cause changes in the differential expression of miRNAs. Whether these differentially expressed miRNAs are involved in the repair of peripheral nerve tissues is insufficiently explored [26,27].

With the rapid development of next generation high-throughput sequencing technologies, RNA-Seq has become an important technique in transcriptome research [28]. In this study, we detected the expression of miRNAs in irradiated rats following sciatic nerve injury by RNA-Seq, and explored the mechanism underlying miRNA function in irradiated rats after sciatic nerve injury.

2. Materials and methods

2.1. X-ray-treated animals and samples

Eight male Sprague Dawley (SD) rats (Suzhou University Animal Experimental Center), weighing around 200–250 g were used. All experiments were performed in accordance with the animal care guidelines of Soochow University. Rats were anesthetized using 3.6% chloral hydrate (1 mL/100 g body weight). After sciatic nerve exposure, the sciatic nerves were transected at the midpoint between the sciatic nerve pelvic outlet and the tibiofibular nerve bifurcation, and then nerve epicardium was sutured in situ under a 9.0 magnification microscope (Leica). The process was performed under sterile conditions.

The rats were randomly divided into two groups: the treated group −1 Gy and the control group −0 Gy (n = 4). The treated group −1 Gy was irradiated with1 Gy, while the control group −0 Gy was not irradiated. X-ray irradiation was performed using 1 Gy (6 MV X-ray, dose rate: 2000 cGy/min), with the X-ray irradiation being generated by a medical linear accelerator (Siemens Primus, Siemens Medical Systems, Concord, CA, USA). The skin suture of the transection joint on the left side of the sciatic nerve of the irradiated rats was exposed to X-ray irradiation for 3 s. After 72 h of irradiation, 4–6 segments of the spinal cord together with dorsal root ganglia and connected sciatic nerves (left side) were collected for the next experiment. In the electrophysiological nerve testing, the amplitude and conduction velocity of motor neurons in the irradiation group were higher than those in the control group, which suggested that irradiation-treated animals had better recovery than the non-treated animals.

2.2. Construction of the transcriptome library and sequencing

Total RNA for each sample was extracted using Trizol Reagent (Life Technology, USA), according to the manufacturer's instructions. The miRNA transcriptome library was constructed as previously described [29].

2.3. Bioinformatics analysis and identification of miRNA targets

Two rat miRNA transcriptome libraries were sequenced using the Illumina HiSeq 2000 platform. High quality 20 ± 21 nucleotide-long reads were subjected to the CleaveLand pipeline for small RNA target identification, as previously described [29]. Sequences for rRNAs, tRNAs, snoRNAs, and snRNAs were retrieved from the Rfam database (http://www.sanger.ac.uk/Software/Rfam/). The control group −0 Gy and treated group −1 Gy data were analyzed separately.

2.4. Construction of miRNA-target networks

The obtained profiles were used for building and visualizing miRNA-mRNA global interaction networks using the Cytoscape 3.2.1 software, as previously described [30]. In this network, nodes represent miRNAs or target genes. The network structure is formed of basic elements (target genes and miRNAs; named nodes) and the connections represent miRNA-target interactions (named edges). If two genes were annotated as related, an edge was added between them in the network.

2.5. Functional annotations of miRNA targets

Gene Ontology (GO) annotations, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis (http://www.kegg.jp/kegg/pathway.html), and the Non-Redundant (NR) Protein Database were employed to annotate and classify target genes using the DAVID gene annotation tool. For enrichment analysis, a hypergeometric distribution-based statistical test (with level of significance set at 0.05) was performed to reject the chances of randomness in the associations of miRNAs to target genes with their corresponding ontology term.

2.6. Luciferase activity assay

A segment of the 3′UTR of *Vegfa* was cloned into the pMir-reporter (Ambion). The mutated 3′UTR of *Vegfa* was introduced into the potential novel miRNAs-360,-301,-239, and −400 binding sites, using a two-step PCR approach. The reporter vector containing the wide-type or mutant 3′ UTR of *Vegfa* and the novel miRNAs-360,-301,-239, and -400 mimics were co-transfected into 293T cells. The control group was transfected with a reporter vector containing the wild-type copy of the *Vegfa* 3′UTR and a control mimic. After 24 h, luciferase activity was measured using a dual-luciferase reporter assay system (Promega).

2.7. miRNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

MiRNAs from spinal cord tissues were extracted using the miRNeasy Serum/Plasma Kit (Qiagen), according to the manufacturer's instructions. Subsequently, miRNA was reverse transcribed into cDNA, using the One Step PrimeScript & reg miRNA cDNA Synthesis Kit (TAKARA).The real-time PCR reaction was performed and analyzed using an ABI 7000 real-time PCR system (ABI, USA) according to the Prim-Script RT reagent kit (Takara, Japan) protocol. The primer list is provided in the Supplemental Word 1.

2.8. Statistics

Data are presented as means ± SD. Statistical differences between two groups were determined by the Student's *t* test. All experiments were repeated at least three times, and representative experiments are shown. Differences were considered to be significant at $p < 0.05$.