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Research paper

Expression profiles of MiRNAs for intrinsic musculature of the forepaw and biceps in the rat model simulating irreversible muscular atrophy of obstetric brachial plexus palsy

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ABSTRACT

Clinically in obstetric brachial plexus palsy (OBPP), irreversible atrophy of intrinsic musculature of the hand in denervation occurs much earlier than that of denervated arm muscles. With the aim of finding clues to explain this, the miRNA expression profile of denervated intrinsic musculature of the forepaw (IMF) and that of the denervated biceps were examined by microarray screening in the rat model simulating irreversible muscular atrophy caused by pan-plexus lesions of OBPP, and potential targets of specifically dysregulated miRNAs were predicted with use of bioinformatics analysis. It was found that denervated IMF and biceps had their own specifically dysregulated miRNAs, respectively, as compared with control. Analysis of Gene Ontology and of pathway showed that those specifically dysregulated miRNAs and their target genes might participate in self-regulation of neuromuscular junctions (NMJs). These outcomes suggest that self-regulative mechanism of NMJs may be different between denervated IMF and denervated biceps in the rat model simulating irreversible muscular atrophy of OBPP.

1. Introduction

Results of surgical repairs for peripheral nerve injury are, in general, unsatisfactory. One of the major reasons for failures of functional recovery is that the atrophy of denervated target muscles has turned irreversible before regenerative nerve fibers can re-innervate them (Roganovic, 2005). Clinically, the timing of the nerve reconstruction operation for re-innervation of the intrinsic musculature of the hand is different from that of muscles of the arm. As far as obstetric brachial plexus palsy (OBPP) is concerned, it has been a consensus that the nerve reconstruction surgery for the lower trunk should be performed up to three months of age (Chuang et al., 2005); for the upper trunk, however, the nerve repair at 30 months post-natal still has the opportunity of achieving functional recovery (Boome, 2000). Our previous study with the pup rat model for OBPP has indicated that the irreversible atrophy of the denervated intrinsic musculature of the forepaw (IMF) does occur earlier, as compared with that of the denervated biceps (Wu et al., 2013). This implied that after nerve injury, self-regulative mechanism is different between denervated intrinsic musculature of the hand and denervated biceps. Investigation of different regulative mechanisms helps discover biological marks to indicate if atrophy of the denervated muscle is irreversible in OBPP.

MicroRNAs (miRNAs) are endogenous non-coding small RNAs with ~22 nucleotides, and have been identified as post-transcriptional regulators that bind complementarily to specific sequences in target mRNAs for degradation and/or repression of translation of target mRNAs (Ambros, 2004). To date, thousands of genes encoding miRNAs have been discovered. A miRNA maybe targets many different mRNAs and a mRNA can be regulated by different miRNAs in different situations (Leung and Sharp, 2007). It is estimated that miRNAs regulate nearly

Abbreviations: Acsl3, acyl-CoA synthetase long-chain family member 3; Bdnf, brain-derived neurotrophic factor; Ccnd1, cyclin D1; Erbb, erb-b2 receptor tyrosine kinase; FDR, false discovery rate; Fgf9, fibroblast growth factor 9; FGFBP1, fibroblast growth factor binding protein 1; Fgfr2, fibroblast growth factor receptor 2; GO, Gene Ontology; HDAC4, histone deacetylase 4; Igf1, insulin-like growth factor 1; IGF-1/Akt, insulin-like growth factor 1/serine/threonine protein kinase Akt; IMF, intrinsic musculature of the forepaw; Jak-STAT, Janus kinase-signal transducer and activator of transcription; LASS2, ceramide synthase 2; miRNAs, microRNAs; mTOR, mammalian target of rapamycin; Nedd4, neural precursor cell expressed, developmentally down-regulated 4; NMJs, neuromuscular junctions; Nrg, neuroglian; Ntf3, neurotrophin 3; Ntm, neurotrimin; OBPP, obstetric brachial plexus palsy; Rnf10, ring finger protein 10; RT-PCR, real-time polymerase chain reaction; RVM, Random variance modified; SCs, Schwann cells; Sy2a, synaptic vesicle glycoprotein 2a; TGF-β, transforming growth factor, beta; TSCs, terminal Schwann cells; Uba6, ubiquitin-like modifier activating enzyme 6.

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two-thirds of the entire mammalian genome, and this effect of miRNAs has been confirmed to play a key role in cell differentiation, growth, proliferation, and apoptosis (Chen et al., 2009).

The structure and function of the skeletal muscle are maintained by innervation. After nerve injury, denervated skeletal muscle cells undergo a series of changes including inflammation, apoptosis and muscle satellite cell proliferation (Tidball, 2005), which are regulated by many signaling pathways, such as those of IGF-1/Akt, of myostatin, and of WNT (Arthur and Cooley, 2012; Fanzani et al., 2013). Recent studies have revealed that some miRNAs form a feedback loop with regulatory factors relative to muscle atrophy for fine-tuning the output of these signaling pathways mentioned above (Wang, 2013; Hu et al., 2014). In a study of muscle atrophy caused by nerve injury, miR-206 was found to promote, and HDAC4 to impede reinnervation of denervated muscles, respectively, via opposing effects on FGFBP1 that is a muscle-derived regulator of promoting presynaptic differentiation at nerve muscle junctions (NMJs) (Williams et al., 2009).

Considering the faster atrophy of denervated intrinsic musculature of the hand than atrophy of denervated muscles of the arm in OBPP, as well as miRNAs extensively participating in regulation of signaling pathways for denervated muscles, we hypothesized that in OBPP the miRNA expression profile in the denervated intrinsic musculature of the hand was different from that in denervated muscles of the arm. This study tried to test the hypothesis by the rat model simulating irreversible muscular atrophy caused by pan-plexus lesions of OBPP.

2. Materials and methods

2.1. Animal surgery and tissue preparation

15 Sprague–Dawley rats weighing 11 g to 13 g at the age of seven days were enrolled in this study. The anesthesia was performed by an intraperitoneal injection of 10% chloral hydrate with the dose of 300 mg/kg, and the rat was placed supinely. Under aseptic techniques and an operating microscope, a supraclavicular incision was made to expose the right brachial plexus. One of the commonest pathological types in OBPP was created, that is, the spinal nerves C5C6 being divided distal to the intervertebral foramen and C7C8T1 avulsed. The distal ends of divided or avulsed spinal nerves were embedded in neighboring soft tissue, by some stitches of 11/0 nylon, to prevent re-innervation of these distal stumps by regenerating axons. The brachial plexus on the left was undisturbed so that the corresponding innervated muscles were taken as control. After recovering from anesthesia, pup rats were put back to their mothers. In our previous model study with rats for irreversible muscular atrophy of OBPP, it was shown that the irreversible atrophy of denervated IMF would occur unless the ulnar nerve was reconstructed up to five weeks after injury, while the atrophy of the denervated biceps was reversible even if the musculocutaneous nerve was reconstructed ten weeks after injury. Hence, the examination of these two kinds of denervated muscles in this study was performed at five weeks after injury. The rats were killed by cervical dislocation to collect IMF and biceps from both sides. The muscle specimens of 15 rats were stored at −80 °C until analysis: those of nine rats were taken for miRNA microarray screening and those of the other six rats were used for real-time quantitative RT-PCR analysis to validate results of microarray screening. For miRNA microarray screening, harvested IMF and biceps from every three rats were pooled together, respectively, to generate sufficient RNAs for labeling. For RT-PCR analysis, muscle specimens were tested individually. Total RNAs for miRNAs' microarray screening and RT-PCR analysis were extracted and purified using the mirVana™ miRNAs' Isolation Kit (Ambion, Austin, TX, USA) according to the manufacturer's instructions. The integrity of the purified RNAs was evaluated on a Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA). All surgical procedures and protocols in this study were in accordance with the Guidelines for Ethical Care of Experimental Animals approved by the International Animal Care and Use Committee.

2.2. MiRNA microarray screening

Microarray assay was performed using a service provider (Genminix, Shanghai, China). Total RNAs (1 μg) from each sample were labeled using the Flash-Tag RNA Labeling Kit (Genisphere, Hatfield, PA) and hybridized to microarray as described by the manufacturer (Affymetrix, Santa Clara, CA). Based on 100% Sanger miRBASE version 17 (www.mirbase.org) by a one-color approach, the microarray contains 19, 724 probe sets covering 153 species, and in humans it can detect 1, 733 mature miRNAs. After hybridization, the fluorescence on the array was scanned using an Affymetrix Gene Array Scanner. The scanned images were processed using the AGCC (Affymetrix GeneChip Command Console) software, and CEL files were imported into Partek H Genomics Suite TM software (Partek, Inc., MO, USA). The Robust Multichip Analysis (RMA) algorithm was applied for generation of signal values and normalization.

2.3. Real-time quantitative RT-PCR

Real-time quantitative RT-PCR was used to validate the authenticity of miRNA expression screened by microarray assay. We randomly chose specifically dysregulated miRNAs, which were exclusively expressed in denervated IMF or in the denervated biceps, for the performance of this validation. Sample total RNAs (10 ng) were reversely transcribed into cDNA using specific stem-loop primers (Table 2) and TaqMan® MicroRNA Reverse Transcription Kit. With cDNA taken as the template, Taq-Man MicroRNA Assay (Foster City, CA) and TaqMan® Universal PCR Master Mix were used for performance of real-time quantitative RT-PCR according to the manufacturer's directions. Each sample was run in triplicate. Signals normalized to U6 housekeeping miRNAs ran simultaneously. A comparative threshold cycle method (Livak and Schmittgen, 2001) was applied to calculate relative miRNA expression of denervated muscles and of control.

2.4. Bioinformatics analysis

Potential targets of specifically dysregulated miRNAs in denervated IMF and denervated biceps were predicted using the combined miRNA target prediction databases including miRanda and miRDB (http://www.mirdb.org) (Hsieh et al., 2010). For the purpose of minimization of the false positivity, only the overlapped genes that were identified in both databases were considered as potential target genes regulated by specifically dysregulated miRNAs. Functional annotation on target genes of specifically dysregulated miRNAs was performed by Gene Ontology (GO) analysis to determine biological effects of these targets. Meanwhile, pathway analysis was carried out to identify the enriched pathways of target genes of specifically dysregulated miRNAs by the KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database. The Fisher's exact and chi-square tests were used for the identification of the significance of GO terms and of pathways, and false discovery rate (FDR) was calculated to correct the p-value (Benjamini et al., 2001). We chose only GO terms and pathways that had a p-value of <0.05 and a FDR of <0.05. For each GO term and pathway of specifically dysregulated miRNA targets, the enrichment was acquired as previously reported in literature (Guo et al., 2009; Garbacki et al., 2011).

MiRNA-target gene network was built to identify relationships among the specifically dysregulated miRNAs and their target genes in GO and pathway categories. Based on different expression values of specifically dysregulated miRNAs and interactions between miRNAs and their target genes in miRanda and miRDB miRNAs databases, relationships of these miRNAs and their target genes were evaluated to establish miRNA-target gene network (Joung et al., 2007; Cai et al., 2013). In this network, the square represented miRNAs, the circle stood for genes, and one edge indicated their relationship. The size of the center of the network reflected the degree. The higher the degree of miRNAs, the more links with which a miRNA contributed to surrounding genes.