Neurobiology of Disease 63 (2014) 184–193

Contents lists available at ScienceDirect

Neurobiology of Disease

journal homepage: www.elsevier.com/locate/ynbdi

MiR-139-5p inhibits HGTD-P and regulates neuronal apoptosis induced by hypoxia–ischemia in neonatal rats

Yi Qu [a,b,*,1], Jinlin Wu [a,1], Dapeng Chen [a], Fengyan Zhao [a,b], Junyan Liu [a,b], Chunlei Yang [c], Dapeng Wei [c], Donna M. Ferriero [d], Dezhi Mu [a,b,d,*]

[a] Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu 610041, China
[b] Key Laboratory of Obstetric & Gynecologic and Pediatric Diseases and Birth Defects of Ministry of Education, Sichuan University, 610041 Chengdu, Sichuan, China
[c] West China Medical Center, Sichuan University, 610041 Chengdu, Sichuan, China
[d] Department of Pediatrics and Neurology, University of California, San Francisco, CA 94143, USA

ARTICLE INFO

Article history:
Received 3 August 2013
Revised 21 November 2013
Accepted 26 November 2013
Available online 10 December 2013

Keywords:
MicroRNA
HGTD-P
Brain
Hypoxia–ischemia
Apoptosis

ABSTRACT

Human growth transformation dependent protein (HGTD-P) is a newly identified protein that promotes neuronal apoptosis in hypoxia–ischemia brain damage (HIBD) in neonatal rats. However, the mechanisms regulating HGTD-P expression are not clear. Here we describe microRNAs targeted to HGTD-P and examine their effects on regulating neuronal apoptosis in HIBD. We use samples from cultured neurons after oxygen–glucose deprivation (OGD) and postnatal day 10 rat brains after hypoxia–ischemia (HI). RT-PCR, Western blotting, and immunostaining are used to detect the expression of HGTD-P and cleaved caspase 3, as well as real-time PCR detects microRNA expression. MicroRNA agomir is used to inhibit the expression of HGTD-P, and DAPI, TUNEL, and TTC staining are employed to detect cell apoptosis and brain damage. Moreover, in vitro processing assay is used to examine the mechanism by which HI down-regulates miR-139-5p expression. We found that miR-139-5p is down-regulated in neurons and rat brains after HI treatment. The expression pattern of miR-139-5p correlates inversely with that of HGTD-P. Furthermore, miR-139-5p agomir inhibits neuronal apoptosis and attenuates HIBD, which is concurrent with down-regulation of HGTD-P. Moreover, pre-miR-139 processing activity decreases in extracts from OGD neurons, and OGD neuronal extracts attenuates the processing of pre-miR-139 by Dicer. In conclusion, HI induces inhibitors which block the processing step of pre-miR-139, resulting in the down-regulation of mature miR-139-5p. The down-regulation of miR-139-5p plays a critical role in the up-regulation of HGTD-P expression. MiR-139-5p agomir attenuates brain damage when used 12 h after HI, providing a longer therapeutic window than anti-apoptosis compounds currently available.

Introduction

Hypoxic ischemia (HI) results in severe brain injury in neonates (Ferriero, 2004). In neonatal brains, hypoxic–ischemic brain damage (HIBD) usually causes cell death via either necrosis or apoptosis (Gill and Perez-Polo, 2008). When and by which mechanism the cells undergo cell death depend on the severity of the injury. Up until now, several studies have shown that apoptosis was more frequent in HIBD (Hagberg et al., 2009). Human growth and transformation dependent protein (HGTD-P) is a newly identified proapoptotic protein and an effecter of HI-induced cell death (Lee et al., 2004). Our previous study found that HGTD-P plays a proapoptotic role in developing rat brain subjected to HI by activating caspase 3 and promoting apoptosis inducible factor (AIF) translocation into the neuronal nucleus (Qu et al., 2009). To inhibit HGTD-P induced apoptosis, it is necessary to clarify the mechanism of HGTD-P regulation by HI.

In recent years, a class of small non-coding molecules has been found to regulate gene expression, microRNA (miRNAs). MicroRNAs represent endogenously expressed, small non-coding RNAs that regulate gene expression (Battel, 2004). After sequential processing steps, mature microRNAs are incorporated into the RNA-induced silencing complex (RISC) and target specific messenger RNAs (mRNAs). Consequently, the translation of these mRNAs is inhibited or destabilized resulting in down-regulation of the encoded protein (Chua et al., 2009). Until now, several microRNAs have proven to be regulated by hypoxia, and responsible for the hypoxia induced bio-behavioral change of cells and tissues (Gorospe et al., 2011). Therefore, we hypothesize that the alteration of specific microRNAs is the potential mechanism

Abbreviations: HGTD-P, human growth transformation dependent protein; HIBD, hypoxia–ischemia brain damage; OGD, oxygen–glucose deprivation; HI, hypoxia–ischemia; AIF, apoptosis inducible factor; RISC, RNA-induced silencing complex; 3′UTR, 3′-untranslated regions; CCA, common carotid artery; CC3, cleaved caspase 3; AI, apoptotic index; TTC, triphenyltetrazolium chloride; tPA, tissue plasminogen activator.

* Corresponding authors at: Department of Pediatrics, West China Second University Hospital, Sichuan University, Chengdu, Sichuan 610041, China. Fax: +86 28 85559065.
E-mail addresses: quyi712002@163.com (Y. Qu), mudz@scu.edu.cn (D. Mu).

Available online on ScienceDirect (www.sciencedirect.com).

[1] Theses authors contributed equally to this report.

0969-9961/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.nbd.2013.11.023

to regulate HGTD-P expression in HIBD. In this study, we identify microRNAs targeted to HGTD-P, show that these microRNAs are new molecules that regulate HGDT-P in the HI brain, and explore the feasibility of using microRNAs to inhibit cell apoptosis induced by HI in neonatal rats.

Materials and methods

Primary culture of rat cortical neurons

All animal protocols were approved by the Sichuan University Committee on Animal Research. Cortical neurons were prepared from brains of 18-day-old Sprague–Dawley rat embryos. Cells were plated in neurobasal medium supplemented with 2% B27 (Invitrogen) on plates coated with poly-D-lysine. Neurons were cultured at 37 °C in a humidified 5% CO_2 atmosphere and used after 10 days in vitro.

To initiate oxygen–glucose deprivation (OGD), cortical neurons were exposed to DMEM without serum or glucose in a humidified atmosphere containing 95% nitrogen and 5% CO_2. After 3 h of OGD, neurons were fed with serum and glucose-supplemented original medium, and returned to the incubator under normoxic conditions.

MicroRNA microarray assay and analysis

The microRNA microarray was performed by Kangchen Com (China) to detect microRNA profiling in neurons with OGD treatment. Total RNA was harvested using TRIzol (Invitrogen, USA) and miRNeasy mini kit (Qiagen, Germany) according to the manufacturer's instructions. After having passed RNA quantity measurement using a NanoDrop 1000, the samples were labeled using the miRCURY™ Hy3™/Hy5™ Power labeling kit and hybridized on the miRCURY™ LNA Array (v.18.0). Following the washing steps the slides were scanned using the Axon GenePix 4000B microarray scanner. Scanned images were then imported into GenePix Pro 6.0 software (Axon, USA) for grid alignment and data extraction. Replicated microRNAs were averaged and microRNAs that intensities ≥30 in all samples were chosen for calculating normalization factor. Expressed data were normalized using the Median normalization. After normalization, significant differentially expressed microRNAs were identified through Volcano Plot filtering. The fold change values presented are the mean of 3 independent microarray (3 chips for each sample) experiments.

Luciferase reporter assay

The miR-139-5p binding site of HGTD-P was amplified by PCR from genomic DNA of rat blood cell that was extracted with a QIAamp DNA Mini Kit (Qiagen, Germany). The PCR products were gel purified, digested and inserted into the pMIR-RB-REPORT™ vector (Ribobio, China), the reconstituted plasmid was titled as pWT. The miR-139-5p binding site mutations were introduced using the Multisite-Quickchange (Stratagene, USA) according to the manufacturer's protocol and cloned into the pMIR-RB-REPORTTM vector (Ribobio, China), the reconstituted plasmid was titled as pMUT. All inserted or mutated sequences were confirmed by sequencing. Then HeLa cells were transfected with miR-mimics and pWT by the induction of lipofectamine (Invitrogen, USA) according to the manufacturer's instructions. Meanwhile, miR-mimcs and pMUT, or miR-control and pWT, or miR-control and pMUT were also transfected into Hela cells as the controls. The cells were lysed 48 h after the transfection for measurement of luciferase activity. Dual luciferase assay was used to quantify the effects of miR-139-5p interaction with the 3′-untranslated regions (3′UTR) of HGTD-P. In all experiments, transfection efficiencies were normalized to hLuc luciferase that was constitutively expressed by pMIR-RB-REPORT™ vector. Experiments were performed in triplicate, and 3 independent experiments were performed.

Transfection of microRNA agomir or antagomir into neurons

The cells were seeded at a density of 1.0×10^5 (24-well plates). The dosage of microRNA agomir or antagomir (Ribobio, China) was determined according to the manufacturer's protocol. MicroRNA agomir (6 nM, final concentration) or antagomir (20 nmol, final concentration) in 50 μl of Opti-MEM was complexed with 1 μl of lipofectamine in 50 μl of Opti-MEM (Ambion, USA). The cells were transfected with these complexes and maintained for 8 h, then neurons were fed with serum and glucose-supplemented original medium and cultured in normoxic conditions for another 24 h prior to OGD insult. In all experiments, an equal concentration of scrambled non-targeting controls (agomir or antagomir-negative control, Ribobio, China) was used as a control for non-sequence-specific effects in microRNA experiments.

In vitro processing assay

To synthesize microRNA precursors, DNA templates encoding the sequences of various microRNAs were transcribed using T7/SP6 in vitro transcription kit (Roche, USA). The DNA templates were annealed to a T7 runoff DNA oligonucleotide (5′AATTTAATACGACTCACTATAGG3′) that spans the T7 promoter site. Digoxigenin (DIG)-UTP was used for in vitro transcription to label the pre-miR with DIG. The sequences used were as follows:

Rno-pre-miR139
5′gttactccaacagggccgcgtctccagcctccgagccacactggagacacgtg
cactgtagaatacacCCTATAGTGAGTCGTATTAA-3′;
Rno-pre-miR-124a-1:
5′cagccccattcttggcattcaccgcgtgccttaattgtatggacatttaaatcaaggt
ccgctgtgaacacggagagagaggcctCCTATAGTGAGTCGTATTAA-3′.

These synthetic precursors were folded into their hairpin-shaped structure by heating for 1 min at 95 °C and cooling slowly to room temperature. Cytoplasmic extracts for in vitro processing reaction were prepared in lysis buffer (0.5% Nonidet P-40, 150 mM NaCl, 20 mM Tris–HCl, pH 7.5, 2 mM MgCl2, 10 mM NaF, 1 mM DTT, 20% glycerol, and protease inhibitors). For processing reactions, 10 μg protein was incubated for 90 min at 37 °C with 20 nM RNA in 75 mM NaCl, 20 mM Tris–HCl (pH 7.5), 3.0 mM $MgCl_2$, and 0.1 U/μl RNAsin. Digestion with recombinant Dicer (0.1 U/reaction) was performed according to the manufacturer's recommendations (Stratagene, USA). For mixed processing reactions, precursors were used at a concentration of 20 nM and were pretreated for 10 min at 30 °C with increasing amounts of OGD neuronal extract (2, 4, 6, 8, 16, 24, and 32 μg), then recombinant Dicer was added and incubated at 37 °C for another 2 min. The reaction products were separated on a 15% denaturing PAGE and transferred to the nylon membrane, the fragments of RNA signals in the reaction products were tested using the DIG luminescence detection kit (Roche, USA) according to the manufacturers' instructions. NIH image was used to measure the densities of the band signals after scanning.

Animal protocols

Post-natal day 10 Sprague–Dawley rats (male) were anesthetized and then subjected to hypoxia and ischemia using previously described methods (Qu et al., 2009). Briefly, the right common carotid artery (CCA) was exposed and ligated with a 7–0 silk suture through a midline cervical incision. Surgery lasted less than 5 min. Rats with excessive bleeding were excluded. After ligation of the CCA, the rats were returned to the cage for 1 h to recover from anesthesia. Rats were then exposed to hypoxia (8% O_2/92% N_2) for 2.5 h, and returned to their cage. Body temperature of the rats was maintained at about 37 °C using an infrared heating lamp and monitored with a thermal probe. Rats were killed at the indicated time points after HI and their