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Research paper

The mechanism of action of FXR1P-related miR-19b-3p in SH-SY5Y

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ABSTRACT

The biological effects of microRNAs (miRNAs) in the Fragile X Syndrome (FXS) have been widely studied. Dysregulation of miRNAs plays a critical role in the progression of nervous system diseases and in cell proliferation and differentiation. Our previous study validated that miR-19b-3p was associated with *FXR1* (Fragile X related gene 1), one of homologous genes of *FMR1* (Fragile X mental retardation 1). The purpose of this study was to investigate the relationship of *FXR1* and miR-19b-3p, and the crucial role of miR-19b-3p in FXS and to validate whether miR-19b-3p could regulate the growth of SH-SY5Y cells. We determined that miR-19b-3p could regulate the expression of not only *USP32*, *RAB18* and *Dusp6* but also *FXR1*, and *FXR1* could in turn regulate the expression of miR-19b-3p. What's more, the overexpression of miR-19b-3p significantly inhibited the proliferation, contributed the apoptosis and slowed down the cycle of SH-SY5Y cells. Taken together, our results indicate that miR-19b-3p plays a significant role in the molecular pathology of FXS by interacting with *FXR1* and influencing the growth of SH-SY5Y cells.

1. Introduction

Fragile X Syndrome (FXS), the most common inherited mental retardation disorder and a childhood-onset neurodevelopmental disorders associated with autism spectrum disorders (ASD) (Baumgardner et al., 1995; Devitt et al., 2015), is caused by a loss of function of the X-linked fragile X mental retardation protein (FMRP) (Hagerman and Polussa, 2015 Mar; Wang et al., 2012). What's more, FXS is also related to other neurological and psychiatric disorders such as Fragile X-associated tremor/ataxia syndrome (FXTAS), Fragile X-associated Primary Ovarian Insufficiency (FXPOI), Attention deficit hyperactivity disorder (ADHD), Autism spectrum disorders (ASD) and so on (Lai et al., 2016; González-Pérez et al., 2016). Individuals with FXS display widely levels of cognitive and learning impairment. FMRP, as a RNA binding protein, can repress the translation and protein synthesis of mRNA relating to the development of dendritic spine and synaptic plasticity (Kelley et al., 2012). *FMR1*, who encodes FMRP, has two highly similar genes in sequences, fragile X related gene 1 (*FXR1*) and fragile X related gene 2 (*FXR2*), and their encoding products respectively are FXR1P and FXR2P, which are both RNA-binding proteins with similar functional domains, and constitute the fragile X protein family together, however, they diverge in the nucleolar localization signal sequence and in the C-terminal, suggesting that they possess both common and distinct functions (Darnell et al., 2009; Li and Zhao, 2014 Jul).

miRNAs, a class of small non-coding RNAs (approximately 22 nt in length) relating to mostly all biological processes, can negatively regulate gene expression by binding to sites in the 3′UTR of target mRNAs. It has been calculated that above 45,000 miRNAs were discovered in human. Although the regions of human genome encoding miRNAs are only about 2%–3%, but according to the prediction, above 60% of human protein-coding genes are probably regulated by multiple miRNAs; miRNAs can regulate temporal transitions in gene expression associated with cell fate progression and differentiation throughout animal development by binding to mRNA specific sites of their target genes, thus inhibiting translation or inducing the degradation of mRNA (Ambros, 2011). What's more, miRNAs can also regulate the transcription and expression of genes, participate in cell differentiation and tissue differentiation, regulate the normal development of

Abbreviations: mRNA, messenger ribonucleic acid; miRNA, micro ribonucleic acid; TNF-α, tumor necrosis factor-alpha; SH-SY5Y, cells SH-SY5Y neuroblastoma cells; Ago2, Argonaute 2; USP32, ubiquitin specific peptidase 32; RAB18, member RAS oncogene family; Dusp6, dual specificity phosphatase 6; Cdkn1a, cyclin-dependent kinase inhibitor 1A; Bcl-2, B-cell lymphoma-2; FMRP, fragile X mental retardation protein; FXR1P, fragile X-related 1 protein; FXR2P, fragile X-related 2 protein; 3′UTR, 3′untranslated regions; CDS, coding sequence; FACS, fluorescence activated cell sorter; PMSF, phenylmethanesulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; ANOVA, analysis of variance; SD, standard deviation; PCR, polymerase chain reaction.

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organism. At the same time, miRNAs are involved in multiple biological processes, including transcription, chromosome structure, the formation and modification of RNA, the stability and translation mRNA as well as the transportation and stability of protein (Iwakawa and Tomari, 2015; Afonso-Grunz and Müller, 2015). Taken together, those functions of miRNAs presented here have important implications for the prevention and treatment of human diseases and exploring the process of biological evolution.

FXR1P, encoded by *FXR1*, whose distribution is more widely than FMRP, can express in cerebrum, cerebellum, liver, kidney, testis, skeletal muscle, myocardium and other tissues, especially in skeletal muscle and myocardium where FMRP is not produced, FXR1P is particularly abundant. FXR1P plays an important role in normal muscle development and has been involved in Facioscapulohumeral muscular dystrophy (FSHD). The loss of FMRP or FXR1P could led to the down-regulation of the brain specific miRNAs, which were crucial to several aspects of neuronal development and function, in addition, *FXR1* could be regulated by miRNA (Xu et al., 2011; Siew and Tan, 2013) and interact with the target gene protein of miRNA (Cheever et al., 2010). For example, miRNA can bind to *TNF-α* mRNA and then combine with Ago2 to suppress the expression of FXR1P (Vasudevan and Steitz, 2007; Marianthi Kiriakidou et al., 2007). Cheerver et al. found that the overexpression of miR-367 could significantly decrease the expression of endogenous FXR1P in human HEK-293T and HeLa cell lines, and demonstrated that FXR1P was regulated through miRNA binding with one site being the miR-25/32/92/363/367 seed sequence (Cheever et al., 2010). The loss of *FMR1* or *FXR1* changed the expression level of miR-130a, miR-200b, miR-96, miR-196a and other miRNAs, resulting in abnormal eye development as well as defects in cranial cartilage in *Xenopus laevis* (Gessert et al., 2010). These findings reveal differential roles of fragile X protein family in controlling the expression levels of brain-specific miRNAs. miR-19b-3p was found to be possibly regulated by *FXR1* in our previous study, however, the mechanism of action between the two needs to be further studied.

2. Materials and methods

2.1. Cell culture

SH-SY5Y cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, SiJiQing), 0.29 mg/mL glutamine (Sigma) and 1% penicillin/streptomycin (Beyotime) at 37 °C in an atmosphere of humidified air with 5% CO_2 and saturated in a cell culture incubator.

2.2. RNA isolation and quantitative real-time reverse transcriptase PCR

Total RNA was extracted from SH-SY5Y cells using Trizol Reagent (Invitrogen) and reversely transcribed into complementary DNA (cDNA) using miRNA Stem-loop RT-primer. To quantify the expression of miR-19b-3p as well as its targeted genes, qRT-PCR were performed according to the manufacturer's instruction of Two Step Stemaim-it miRNA qRT-PCR Quantitation Kit (Shanghai Novland Co., Ltd). Briefly, following first-strand cDNAs synthesis, Quantitative Real-time amplification was carried out using MX3000P Quantitative Real-time PCR (Agilent, Germany) by miRNA Stem-loop RT-primer. Gene-specific RT-PCR primers came from Shanghai Novland Co. The RT-PCR procedure was as follow: 16 °6 for 30 min; 42 °i for 30 min; 85 °i for 10 min. Then detecting the expression levels of miR-19b-3p and its targeted genes using qRT-PCR Quantitation Kit including miRNA and genes qRT-PCR Primer, and U6 rRNA and GAPDH mRNA were used as internal control respectively. All samples were normalized based on internal control, and fold changes were calculated through relative quantification ($2^{-\Delta\Delta Ct}$).

2.3. Cell transfection

Cells were seeded into 6-well plate at $1 * 10^5$ cells/mL and incubated for 24 h. Cells were transfected with plasmid, miR-19b-3p mimic, mimic negative control, miR-19b-3p inhibitor mimic as well as inhibitor negative control (Ribo Bio) by lipofectamine 2000 (Invitrogen, USA) following the manufacturer's instructions when cells confluence was about 70%–80%. 5 h later, replace the incomplete medium with complete. After 72 h, transfected cells were analyzed by qRT-PCR、MTT, FACS and Western blotting.

2.4. Luciferase assay

Dual-Luciferase Reporter Assay System (Promega, USA) was used to measure Firefly and *Renilla* luciferase activities consecutively after transfected for 36h. Experiments were carried out. The activities of *Renilla* luciferase and Firefly luciferase were detected by a Modulus™ single tube multimode reader (Turner Biosystems, USA) according to the manufacturer's instructions. The relative activities ($hRluc/hluc^+$) are analyzed as mean ± SD.

2.5. Cells proliferate, apoptosis and cell cycle were detected by MTT and FACS

The proliferate of SH-SY5Y cells was detected by a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) after transfected with miRNA mimic for 0, 24, 48, 72, and 96 h respectively according to the manufacturer's instructions (Beyotime, China), then harvested with Trypsin–EDTA Solution, and recorded the absorbance values at 550 nm by an ELISA reader. The apoptosis of SH-SY5Y cells was measured using an Annexin V-FITC/propidium iodide (PI) staining assay, while cell cycle was measured using PI (4A biotech Co, China), and the FITC and PI fluorescent signals were analyzed by flow cytometry.

2.6. Protein isolation and western blot analysis

Total proteins were extracted from SH-SY5Y cells with cell lysis buffer (Beyotime) containing 1M PMSF (Beyotime, China), and the protein concentrations in the fractions were determined by the bicinchoninic acid assay (BCA, Beyotime, China). Then 50 μg extracted proteins were separated by SDS-PAGE and transferred to membrane before incubated with FXR1, RAB18, USP32, Dusp6 and GADPH. All of the procedures were carried out following the manufacturer's instructions.

2.7. Statistical analysis

All experiments were performed at least in triplicate and results were expressed as means ± SD. Statistical analysis was performed using ANOVA or Student's *t* test with SPSS 18.0 statistical program. $P < 0.05$ (*) or $P < 0.01$ (**) was statistically considered to be statistically significant.

3. 3. Results

3.1. FXR1, USP32, RAB18 and Dusp6 are target genes of miR-19b-3p

According to several publicly available bioinformatics web sites including Target Scan, miRanda, miRBase and so on, *FXR1*, *USP32*, *RAB18*, *CD47*, *Cdkn1a* and *Dusp6* may be direct targeted genes of miR-19b-3p in SH-SY5Y cells. Fluorescence microscopy demonstrated a relatively high transfection efficiency (approximately 80%) of both the mimic and the inhibitor at 50 nM concentration into SH-SY5Y cells (Fig. 1). Using qRT-PCR analysis, we detected the mRNA transcripts of *FXR1*, *USP32*, *RAB18*, *CD47*, *Cdkn1a* and *Dusp6* (Table S1). Compared with miRNA NC mimic, the mRNA expressions of *RAB18* and *USP32*