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SHORT COMMUNICATION

FOXRED1 silencing in mice: a possible animal model for Leigh syndrome

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Abstract

Leigh syndrome (LS) is one of the most puzzling mitochondrial disorders, which is also known as subacute necrotizing encephalopathy. It has an incidence of 1 in 77,000 live births worldwide with poor prognosis. Currently, there is a poor understanding of the underlying pathophysiological mechanisms of the disease without any available effective treatment. Hence, the inevitability for developing suitable animal and cellular models needed for the development of successful new therapeutic modalities. In this short report, we blocked FOXRED1 gene with small interfering RNA (siRNA) using C57bl/6 mice. Results showed neurobehavioral changes in the injected mice along with parallel degeneration in corpus striatum and sparing of the substantia nigra similar to what happen in Leigh syndrome cases. FOXRED1 blockage could serve as a new animal model for Leigh syndrome due to defective CI, which echoes damage to corpus striatum and affection of the central dopaminergic system in this disease. Further preclinical studies are required to validate this model.

Keywords FOXRED1 · Neurodegenerative diseases · Leigh syndrome · Gene silencing

Introduction

In the last few years there has been a growing interest in understanding the complexity of neurodegenerative diseases (Ascherio and Schwarzschild 2016). One hypothesis proposes mitochondrial disruption as a trigger for cell death in many neurodegenerative diseases like Parkinson disease (Haelterman et al. 2014) and aging (Wallace 2005). Mitochondrial oxidative phosphorylation system (OXPHOS) is the main energetic potential that initiates neuronal cells activity (Giachin et al. 2016). The major complex of the mitochondrial respiratory chain is mitochondrial complex I (CI) (Formosa et al. 2015) that forms respirasomes with other respiratory chain complexes (Wittig et al. 2006).

It is interesting to highlight that, the CI protein subunits are encoded by seven mitochondrial (mtDNA) and 38 nuclear DNA (nDNA) genomes (Lazarou et al. 2009; Nouws et al. 2011). During the CI biogenesis, the assembly of subunits into the final mature CI comprises a very complicated stepwise process that involves a group of assembly factors. These assembly factors act as intermediate complexes and not as a part of the final structure of CI signifying their role in CI assembly/stability. However, the exact function of assembly factors is not yet totally understood. They may work as chaperones that stabilize some intermediary complexes in order to react with other subcomplexes to form a mature CI structure (Mimaki et al. 2012).

FOXRED1 is a FAD-dependent oxidoreductase family member; the proteins that contain a Pfam DAO (D-amino acid oxidase; PF01266) domain, and are part of the NADP-Rossmann clan CL0063 (Finn et al. 2013; Lemire 2015a, b). The FOXRED1 gene encodes a 486-amino acid FAD-dependent oxidoreductase domain containing protein. The

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later is localized in the mitochondrion displaying a direct association with complex I assembly intermediates (Andrews et al. 2013; Fassone et al. 2015). Further, Complex I phylogenetic profiling (COPP) and consequent knockdown studies identified FOXRED1 along with other 19 proteins as strong candidate proteins for complex I biogenesis. A reduced level of CI activity was found with knockdown of FOXRED1 using lentiviral-mediated RNAi (Pagliarini et al. 2008). From a clinical perspective, two separate research teams discovered two cases of Leigh syndrome and infantile onset encephalopathy which had mutant p.R352W and p.N430S FOXRED1 genes respectively, resulting in almost non-functional levels of complex I (Calvo et al. 2010; Lienhart et al. 2013). Later in 2016, the third case of FOXRED1 mutation was diagnosed. The patient presented with epilepsy and severe psychomotor retardation along with very similar concentrations of mature complex I (about 9% of normal levels) (Zurita Rendón et al. 2016). Hence, it was hypothesized that FOXRED1 mutations are possible causes of mitochondrial complex-I deficiency (Calvo et al. 2010).

Leigh syndrome (LS), named after the British Pathologist Denis Leigh, is an early onset, progressive neurodegenerative disease. The onset, course and devastating nature of the disease, makes it a crucial problem to patients as well as family (Ruhoy and Saneto 2014). It is well known that LS is a heterogenic disease, hence the difficulty in identification of genes responsible for the disease. As far as we know LS pathology may be related to two genome systems viz.; nuclear and mitochondrial [DiMauro et al. 2013] that should be used in evaluation of commonest pediatric disease of mitochondrial origin (Lake et al. 2016).

In Leigh syndrome and other mitochondrial disorders, the mesencephalon and brainstem are the most affected structures (Wirtz and Schuelke 2011). The mesodiencephalic dopaminergic (mDA) neurons of the substantia nigra, which characteristically degenerate in Parkinson's disease, and the red, pontine and olivary nuclei are consistently affected in Leigh syndrome (Devi et al. 2008; Lebre et al. 2011). This interaction between dopaminergic pathways and mitochondrial function seems to have a role in suppressing the complex I activity in neuronal cells (Brenner-Lavie et al. 2009). Within this context, the first genetic knock-out model of CI deficiency was introduced by the Palmiter laboratory (Kruse et al. 2008). Recently, animal models of Leigh syndrome (using NDUFS4 silencing) showed striatal affection but, without evident structural damage (Chen et al. 2017; Choi et al. 2017). It is noteworthy that, Leigh syndrome is characterized by diverse clinical presentations (Miyauchi et al. 2018), with more than reported 75 different causative nuclear genes (Lake et al. 2016). Thus, we believe it is imperative to study other possible genes mutations causing this disorder. Within this goal, this report evaluated siRNA-induced blockage of FOXRED1 in mice.

Materials and methods

Twenty C57BL/6 mice (Purchased from the Medical Experimental Research Center [MERC]) were used. Animals treated according to the EU Council Directive 2010/63/EU, and the Guide for the Care and Use of Laboratory Animals (National Research Council 2011). The Mansoura Faculty of Medicine- Institutional Review Board (MFM-IRB) approved all experimental procedures and protocols.

Stereotactic injection

siRNA FOXRED1 (MyBiosource™, Catalog# MBS827329) was prepared in sterile DEPC water (Thermofisher™) per manufacturer recommendations. Mice at 6 months of age were anesthetized by intraperitoneal (i.p.) injection of 0.9% NaCl solution containing 0.2% ketamine and 0.2% xylazine and placed into a stereotactic frame (Stoelting Co. USA).

The intra-cerebral injections were performed with a Hamilton syringe (Stoelting™) according to the following coordinates from bregma: AP: −0.18 cm, ML: +0.15 cm, DV: −0.18 cm (Franklin 1997). Two μL of siRNAs solution containing 250 nM siRNA concentration, as described previously by Xu et al. (2014), were injected at a rate of 0.2 μL/min. For FOXRED1 siRNA-treated mice ($n = 10$), the right cerebral cortices were injected with siRNA targeting FOXRED1. While, control mice (n = 10) were injected with 0.4 nmol of the vehicle (DPEC water).

Behavioral assessment following injections, animals were observed for any abnormal movements. After two weeks, all mice were tested for neurobehavioral performance in "open field test" through automated ANY box and ANY maze enclosures and software (Stoelting Co., USA).

After behavioral testing, half of each mice group were perfused through the aorta with 50 mL of 10 mM phosphate-buffered saline (PBS), followed by 150 mL of a cold fixative [4% paraformaldehyde in 100 mM phosphate buffer (PB)], under deep anesthesia with pentobarbital (100 mg/kg, i.p.). After perfusion, the brains were postfixed for 2 days with paraformaldehyde in 100 mM PB and then transferred to 15% sucrose solution in 100 mM PB containing 0.1% sodium azide stored at 4 °C. The other half of mice were sacrificed and brains were frozen at −20 °C for genetic study.

Semi-quantitative reverse transcription-PCR (RT-PCR)

Total mRNA was extracted by the RNA extraction kit (Promega Corporation, Madison, USA). RT-PCR of FOXRED1 (forward: 5′-CTCCTGATCAGCTTCGGAAC-3′; reverse: 5′-AGGGCACCAAGCTACCTGTG-3′). The following cycles were used: 94 °C/3 min; 1, 94 °C/30 s; 35,

58 °C/30 s; 35, 72 °C/45 s; 35). Mouse Gapdh was taken as an internal control (forward: AGGCCGGTGCTGAGTATGTC; backward: TGCCTGCTTCACCACCTTCT).

Quantitative PCR (qPCR)

The qPCR was performed using Step one Plus real-time PCR system (Life Technologies) by comparative threshold cycle (Ct) method. mRNAs concentrations were assessed by the Nanodrop 2000 (Thermo Fisher Scientific) and 1 μg of total mRNA from each sample was reversely transcripted into total cDNA by the Thermo Scientific™ RevertAid™ First Strand cDNA Synthesis Kit.

Immunohistochemistry

Brain slides were immunostained with the following primary antibodies anti-TH antibody (Novus Biologicals, USA, 1:200 dilution: Catalogue # NB300–109), purified anti-Tau phospho (Thr181) antibody (BioLegend, USA, 1:100 dilution: Catalogue # 846602), anti NeuN for neurons (ABN78, made in rabbit, 1:1000, Millipore) and anti-ionized calcium binding adaptor molecule 1 (Iba-1) (1:200; 019–19,741; Wako Chemicals, Neuss, Germany) followed by biotinylated secondary antibodies for 10 min then the avidin-biotin–peroxidase complex (ABC). The tissues were subsequently examined under the light microscope (CX31RTSF; Olympus, Tokyo, Japan).

Image analysis

The following areas were subjected to image analysis:

1) **Striatal TH-fiber density measurement.** In order to evaluate the TH-positive fiber innervation in the striatum, mean optical density measurements were performed using the Image J software version 1.33–1.34 (NIH, USA, http://reb.info.nih.gov/ij/) as described previously (Carlsson et al. 2006).
2) **Dopaminergic neurons in Substantia Nigra pars compacta (SNpc).** TH-positive cells in the SNpc of both hemispheres were counted, in every fourth section, throughout the entire nucleus as described previously by Blandini et al. (2010). Results were expressed as the percentage of TH-positive cells in the siRNA injected side SNpc compared to control side.
3) **Cerebral cortex and hippocampus:** they were scanned for abnormal tau aggregates (anti-Tau), neuronal loss (NeuN) and inflammatory response to stereotaxic injection (anti-Iba1).

Results

Suppression of FOXRED1 expression

Intra-cerebral injection of 250 nM siRNA FOXRED1 led to about 70% reduction in FOXRED1 mRNA compared to vehicle treated controls ($p < 0.001$) as verified by PCR.

Behavioral changes

Following siRNA injection, mice developed severe tremors that continued during the study period. Moreover, their performance in open field test, revealed behavioral changes (in the form of immobility episodes, total immobility time, freezing episodes and, freezing time, latency to immobility and freezing and efficient paths) compared to controls (Fig. 1).

Immunohistochemical study

Staining brain sections with anti-TH revealed severe degeneration in the striatum of injected side compared to other side and controls as evidenced by the significant decrease in dopaminergic fiber density revealed by image analysis (Table 1, Fig. 1). On the other hand, substantia nigra preserved dopaminergic neurons compared to the control side (Table 1, Fig. 1). This was confirmed by quantification of neurons as described previously.

No abnormal tau aggregates (anti-Tau), neuronal loss (anti-NeuN) or inflammation (anti-Iba1) were observed in the injected side compared to the contra-lateral side. The absence of inflammation or neuronal loss on the injected side confirms successful siRNA injection without injury of brain regions.

Discussion

The present work reports the effects of in vivo blocking of FOXRED1 in C57bl6 mice brains. FOXRED1 blockage led to locomotor disturbances and structural changes in the corpus striatum. However, dopaminergic neurons in SNpc were preserved.

FOXRED1 is known for its role as CI assembly factor. The commonest manifestation of FOXRED1 defects is Leigh syndrome, which is a fatal disorder, caused by the affection of mitochondrial respiration due to mutations in CI controlling genes e.g. FOXRED1 and NDUFS4. Patients with this syndrome are usually characterized by motor and behavioral affection with brain stem and basal ganglia lesions (Arii and Tanabe 2000). More interestingly, recent clinical reports show parkinsonian manifestations in some cases of Leigh syndrome (Martikainen et al. 2013). This juncture with the dopaminergic system in LS, however, was not totally recapitulated in available animal models. Previous models for Leigh syndrome, using NDUFS4 defective mice, showed brainstem lesions with some striatal changes (Quintana et al. 2010, 2012). The

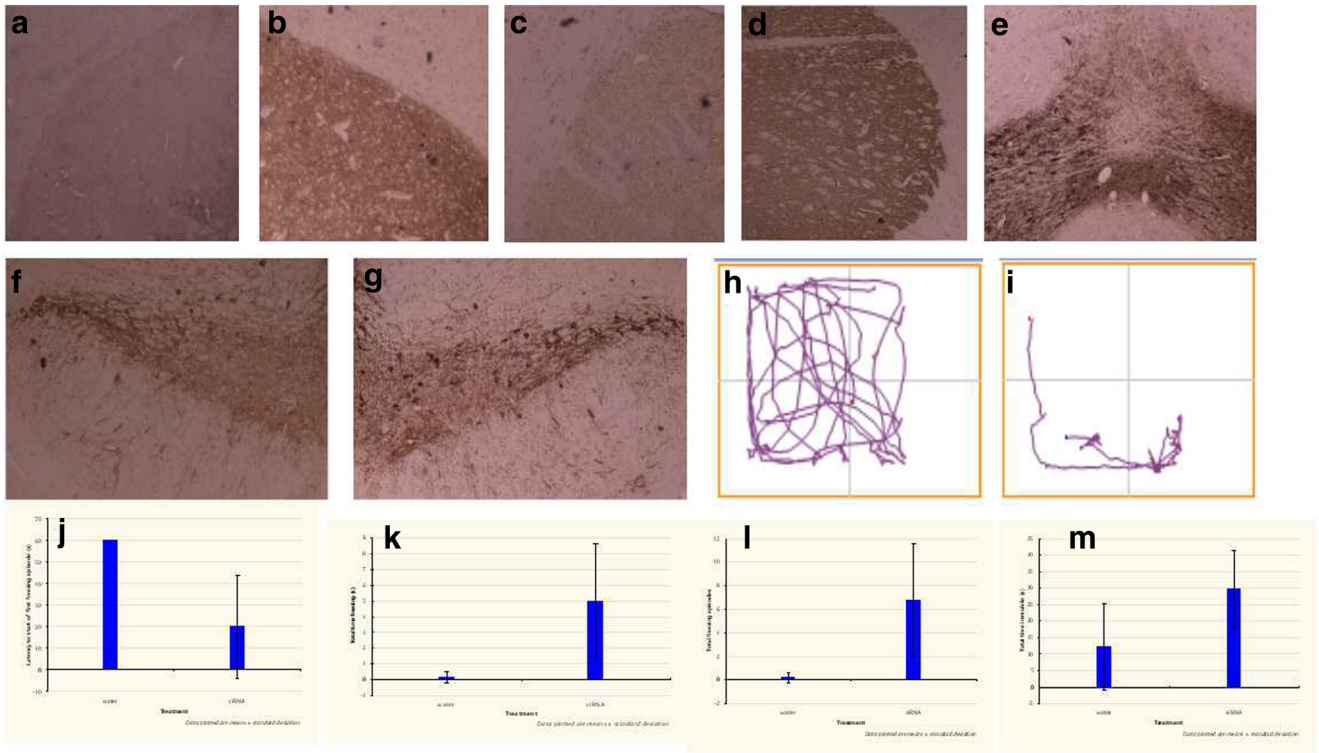

Fig. 1 Photomicrographs for anti-TH staining of corpus striata of siRNA FOXRED1 injected right side (**a**, **c**) and non injected sides (**b**, **d**), showing a decrease in dopaminergic fibers density in the injected side. On the other hand, substantia nigra photos (**e**, **f**, **g**) showed similar dopaminergic neuron distribution between two sides. Behavioral tests showed a difference regarding efficient path for control (**h**) compared to injected mice (**i**). Moreover, injected mice showed significant difference regarding latency to start immobility episodes (**j**), total freezing time (**k**), total episodes of freezing (**l**) and total time of immobility (**m**)

changes in the striatum in the previous models, however, showed decreased brain dopamine level without structural damage (Quintana et al. 2010). Since striatal affection seems to play a central role in clinical features characterizing Leigh syndrome, the delay in the appearance of striatal lesions in NDUFS4 models has been attributed to milder impairment in mitochondrial capacity (Chen et al. 2017). We believe that neurodegenerative diseases models should reiterate all disease features (Salama and Arias-Carrio'n 2011), hence, in disorders with heterogenic nature, different models should be available to study all features of the disease and evaluate proposed therapies on different levels.

Table 1 Comparison between the percentage of dopaminergic neurons (SNpc) and fibers (Striatum) in the injected (right side) to the non-injected (left side) in studied groups

	Control	FOXRED1 siRNA
Striatal optical density percentage of right side compared to the left side	98.9 + 2.03	32.5+ 2.32*
Right SNpc neuronal quantification percentage compared to left side.	97.4 + 1.83	96.6+ 3.31*

*$P < 0.001$ compared to control side

In this report, we show for the first time that blockage of FOXRED1 displayed behavioral changes and striatal affection. Compared to previous NDUFS4 models, FOXRED1 blockage showed severe degeneration in the striatum (Chen et al. 2017). This would highlight the diversity in causes and presentations in Leigh syndrome as well as its relation to CI induced degenerative diseases with emphasizing on the different roles both factors; NDUFS4 and FOXRED1, play. Although both are considered assembly factors for Complex I, FOXRED1 proved to have more functions recently (Lienhart et al. 2013). These findings also, underline the fact that Leigh syndrome (as in most neurodegenerative diseases) has different genetic causes as well as different phenotypic features. We believe that FOXRED1 blockage could serve as a new animal model for Leigh syndrome due to defective CI, which restates damage to corpus striatum and affection of the central dopaminergic system in this puzzling disease. There are many future steps needed to fully validate this model to understand the molecular mechanisms underlying the LS unique pathology especially with the heterogeneous and not yet fully determined genetic basis of LS. Needless to say, our proposed model may help in future therapeutic trials and translational approaches.

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