Int. J. Devl Neuroscience 36 (2014) 19–24

Contents lists available at ScienceDirect

International Journal of Developmental Neuroscience

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

MicroRNA-9 regulates neural apoptosis in methylmalonic acidemia via targeting BCL2L11

Yanfei Li[a], Tao Peng[a], Lin Li[b], Xiaohan Wang[a], Ranran Duan[a], Huili Gao[a], Wenjuan Guan[a], Jingjing Lu[a], Junfang Teng[a], Yanjie Jia[a,*]

[a] Department of Neurology, First Affiliated Hospital, Zhengzhou University, Zhengzhou 450052, China
[b] Department of Neurology, Zhengzhou Central Hospital Affiliated to Zhengzhou University, Zhengzhou 450052, China

ARTICLE INFO

Article history:
Received 11 March 2014
Received in revised form 17 April 2014
Accepted 27 April 2014

Keywords:
MicroRNA-9
BCL2L11
Apoptosis
Methylmalonic acidemia

ABSTRACT

Methylmalonic acidemia (MMA) is an autosomal-recessive inborn metabolic disorder that results from a deficiency in methylmalonyl-coenzyme A mutase or its cofactor, adenosylcobalamin. Currently, neurological manifestations in MMA are thought to be associated with neural apoptosis. BCL2L11, which is a proapoptotic Bcl-2 family member, is resident in the outer mitochondrial membrane, where this protein acts as a central regulator of the intrinsic apoptotic cascade and mediates excitotoxic apoptosis. MicroRNAs (miRNAs) are a class of non-coding RNAs that function as endogenous triggers of the RNA interference pathway. Currently, little is known regarding the role of miRNA in MMA. In our previous study, we preliminarily found that the expression of miR-9 was significantly down-regulated in MMA patient plasma and sensitively changed after VitB12 treatment, which may act as a potential "competitor" of gas chromatography-mass spectrometry for the diagnosis of MMA. In the present study, we first confirmed that miR-9 inhibited BCL2L11 expression by directly targeting its 3′-untranslated region, and the up-regulation of miR-9 reduced neural apoptosis induced by methylmalonate via targeting BCL2L11. Taken together, our results suggested that miR-9 might act as a monitor of changes in MMA and might provide new insights into a therapeutic entry point for treating MMA.

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1. Introduction

Methylmalonic acidemia is an inborn error of intracellular cobalamin metabolism with a wide spectrum of clinical manifestations. This metabolic disease is caused by mutations in the methylmalonyl-CoA mutase gene and results in impaired intracellular synthesis of adenosylcobalamin and methylcobalamin, which are cofactors for methylmalonyl-CoA mutase and methionine synthase enzymes. Elevated methylmalonic acid and homocysteine, as well as decreased methionine production, are the biochemical hallmarks of this disorder. Providing a timely diagnosis is necessary to guide the management of affected individuals (Carrillo-Carrasco et al., 2012; Carrillo-Carrasco and Venditti, 2012). The pathophysiology of complications observed in these patients is not fully understood; however, reports have confirmed that MMA might be induced by neuronal apoptosis resulting from mitochondrial dysfunction, impaired methyl group metabolism, and oxidative stress (Mc Guire et al., 2009; Richard et al., 2007, 2009; Wajner and Coelho, 1997).

MiR-9 is important in development and in various diseases via its ability to regulate different targets genes. MiR-9 exerts diverse effects on the proliferation, apoptosis, migration, and differentiation of neural progenitor cells (Delaloy et al., 2010; Zhao et al., 2009). Moreover, the downregulation of miR-9 in postmitotic neurons is also implicated in some neurodegenerative diseases. As previously reported, MMA diagnosis is dependent on the gas chromatography-mass spectrometry method. In our previous study, we found that the expression of miR-9 was significantly down-regulated in MMA patients' plasma and sensitively changed after VitB12 treatment, which suggest that the change in miR-9 expression might act as a potential competitor of gas chromatography-mass spectrometry for the diagnosis of MMA and might reflect the state of the disease (Li et al., 2014).

MiR-9 has many predicted targets, one of which is BCL2-like 11(BCL2L11), as determined by target gene prediction system including Targetscan, miRWalk and miRbase. BCL2L11 is one of the most important apoptosis regulators that mediate excitotoxic apoptosis, mitochondrial depolarization, and apoptosis inducing factor

* Corresponding author. Tel.: +86 371 66862102; fax: +86 371 66862102.
E-mail addresses: jiayanjie1971@aliyun.com, 529307719@qq.com (Y. Jia).

http://dx.doi.org/10.1016/j.ijdevneu.2014.04.005
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translocation (Concannon et al., 2010). In the present study, we conducted a series of experiments to elucidate the role of miR-9 in MMA. Our results suggest that miR-9 could reduce neural apoptosis induced by methylmalonate via targeting BCL11 and thus speculating up-regulation of miR-9 may be a therapeutic entry point for the treatment of MMA.

2. Results

2.1. MiR-9 suppresses neuronal apoptosis induced by methylmalonate

Neurons were transfected with miR-9 mimic or inhibitor and then were exposed to 2.5 mM methylmalonate for 24 h. The MTT method, TUNEL, and Annexin V-FITC staining showed that the cell apoptosis index was higher after the methylmalonate treatment than before the treatment. In addition, the cell apoptosis index was lower in the miR-9 mimic + MMA group, whereas the cell apoptosis index was higher in the miR-9 inhibitor + MMA group when compared with the MMA group and with the control group. And there was no significant difference in apoptosis rate of the siRNA negative control when compared to the control group (Fig. 1). This result indicated that methylmalonate caused neuronal apoptosis and that miR-9 may reduce neuronal apoptosis induced by methylmalonate.

2.2. MiR-9 specifically reduces the expression level of BCL2L11

To explore the possible mechanism by which miR-9 suppresses neuronal apoptosis, we performed an analysis to search for target genes regulated by miR-9 using bioinformatics software. After the preliminary screen, we chose BCL2L11 as a possible target of miR-9 and predicted the target sequence. Then, we constructed a luciferase reporter plasmid and the psiCHECK2 vector containing the 3′-UTR of BCL2L11 with the binding site of miR-9 directly downstream of the luciferase reporter gene. Next, 293T cells were co-transfected with the vector and hsa-miR-9 or the control, and the relative luciferase activity was determined. The result showed that, when compared with the control, the relative luciferase activity was significantly decreased (47% reduction) by miR-9, whereas the luciferase activity was not altered by the vector containing the mutant 3′-UTR (Fig. 2A).

To directly examine how miR-9 regulates BCL2 L11 levels, we transfected the miR-9 mimic and miR-9 inhibitor into neurons. In response, the BCL2L11 level was significantly downregulated in the miR-9 mimic group and was upregulated in the miR-9 inhibitor group when compared with the control group. And there was no significant difference between control group and siRNA negative control group. Therefore, we confirmed that BCL2L11 levels are inhibited by miR-9 (Fig. 2B and C). We confirmed the effective delivery of the mimic and inhibitor into neurons using qRT-PCR; miR-9 levels were enhanced by approximately 7.2-fold in the mimic group and were reduced by 5.9-fold in the inhibitor group (Fig. 2F). Notably, BCL2L11 mRNA levels were also significantly down-regulated following miR-9 mimic transfection, whereas these levels were up-regulated after miR-9 inhibitor transfection (Fig. 2E).

2.3. MiR-9 may reduce neuronal apoptosis in MMA via targeting BCL2L11

After the transfection of the miR-9 mimic and inhibitor, the neurons were treated with methylmalonate. The Western blot results indicated that the BCL2L11 level was lower in the miR-9 mimic + MMA group and higher in the miR-9 inhibitor + MMA group when compared with the non-transfected group. Therefore, we speculated that miR-9 reduced neuronal apoptosis in MMA by suppressing the expression of BCL2 L11 (Fig. 3).

2.4. The expression of miR-9 in neurons with the methylmalonate treatment

As the qRT-PCR results showed, the expression of miR-9 was reduced 22.3-fold in neurons with the methylmalonate treatment when compared with neurons without the methylmalonate treatment (Fig. 4).

3. Discussion

MMA patients present predominantly neurological symptoms, whose pathogenesis is not yet fully established. Currently, neurological manifestations in MMA are thought to be associated with the accumulation of methylmalonate in tissues and in biological fluids with mitochondrial injury occurring through a combination of the inhibition of specific enzymes and transporters, the limitation of the availability of substrates for mitochondrial metabolic pathways, and oxidative stress, which leads to neural apoptosis (Fernandes et al., 2011; Melo et al., 2011). In addition, it has been shown that MMA causes brain injury through glutamatergic mechanisms (de Mello et al., 1996; Malfatti et al., 2003) and through striatal degeneration (Narasimhan et al., 1996). The overexcitation of glutamate receptors, particularly N-methyl-D-aspartic acid (NMDA) receptors, has been implicated in neuronal injury with rapid necrotic death or a more delayed apoptotic injury, which is characterized by cell shrinkage and nuclear condensation (Ward et al., 2007). Additionally, it has been demonstrated that NMDA receptor activation-induced excitotoxicity is due to nitric oxide (NO) generation. Accordingly, the excessive production of NO inhibits the mitochondrial respiratory chain, which leads to oxidative damage in the brain (Stewart and Heales, 2003). A great deal of work has suggested that reactive oxygen species (ROS) generation may underlie the neurotoxic effects of MMA. High levels of ROS induce severe damage in the cell and cause changes in cellular ATP and Ca^{2+} levels, eventually leading to the release of cytochrome c and to the induction of apoptosis.

BCL2L11, which is a proapoptotic Bcl-2 family member, is resident in the outer mitochondrial membrane, where this molecule acts as a central regulator of the intrinsic apoptotic cascade. BCL2L11 is activated in multiple excitotoxicity paradigms, mediating excitotoxic apoptosis, mitochondrial depolarization, and apoptosis inducing factor translocation. Studies have suggested that BCL2L11 activation requires the activation of AMPK and that prolonged AMPK activation is sufficient for increasing BCL2L11 gene expression and for triggering NMDA-mediated excitotoxicity through BCL2L11. FoxO3a has emerged as an important mediator of cell fate, including apoptosis. Recent reports show that BCL2L11 is a direct target of FoxO3a in Aβ-treated neurons, which indicates that FoxO3a is activated, translocated to the nucleus, and mediates neuron death via BCL2L11 in response to Aβ toxicity (Sanphui and Biswas, 2013). Furthermore, FoxO3 activates the overproduction of ROS because of the BCL2L11-dependent impairment of mitochondrial respiration in neuronal cells, which leads to apoptosis (Hagenbuchner et al., 2012). BCL2L11 has an ability to directly activate a voltage-dependent anion channel (VDAC) that regulates the mitochondrial membrane potential and, thus, controls the production of reactive oxygen species and the release of cytochrome C by mitochondria, both of which are potent inducers of cell apoptosis (Sugiyama et al., 2002).

Our study found that miR-9 was down-regulated in neurons treated with methylmalonate, which had a similar trend with

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