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Dioxin induces expression of hsa-miR-146b-5p in human neuroblastoma cells

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ABSTRACT

Dioxin can cause a series of neural toxicological effects. MicroRNAs (miRs) play important roles in regulating nervous system function and mediating cellular responses to environmental pollutants, such as dioxin. Hsa-miR-146b-5p appears to be involved in neurodegenerative diseases and brain tumors. However, little is known about effects of dioxin on the expression of hsa-miR-146b-5p. We found that the hsa-miR-146b-5p expression and its promoter activity were significantly increased in dioxin treated SK-N-SH cells, a human-derived neuroblastoma cell line. Potential roles of hsa-miR-146b-5p in mediating neural toxicological effects of dioxin may be due to the regulation of certain target genes. We further confirmed that hsa-miR-146b-5p significantly suppressed acetylcholinesterase (AChE) activity and targeted the 3′-untranslated region of the AChE T subunit, which has been down-regulated in dioxin treated SK-N-SH cells. Functional bioinformatic analysis showed that the known and predicted target genes of hsa-miR-146b-5p were involved in some brain functions or cyto-toxicities related to known dioxin effects, including synapse transmission, in which AChE may serve as a responsive gene for mediating the effect.

Introduction

Dioxin, a persistent organic pollutant, is mainly formed by combustion and then released into the environment, and bioaccumulates in the adipose tissue of exposed human and other animals through the food chain (Van den Berg et al., 2006). Prenatal exposure to dioxin are thought to exert their toxic effects on neural cells of fetal brain directly by crossing the immature blood brain barrier resulting in abnormal brain development and maturation (Nishijo et al., 2013; Pontillo et al., 2013; Powers et al., 2005). Air Force veterans serving in the Vietnam War who were exposed to high level dioxin had decreases in memory functions (Barrett et al., 2001). Alteration of gene expression caused by dioxin is considered as an important mechanism for the biological and toxicological effects of dioxin (Sans et al., 2016). It is generally accepted that dioxin can transcriptionally regulate gene expression *via* the aryl hydrocarbon receptor (AhR) pathway (Beischlag et al., 2008).

MicroRNAs (miRs) are small endogenous noncoding RNAs of ~22 nucleotides in length that post-transcriptionally regulate gene expression (Selbach et al., 2008). Accumulating evidence suggests that miRs play roles in neuronal development, function and plasticity (Fiore et al., 2008; Im and Kenny, 2012; Loohuis et al., 2012). Hsa-miR-146b-5p is evolutionarily conserved in mammals and its expression appears to be altered in Alzheimer's disease (AD), Parkinson disease and glioma

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patients (Cogswell et al., 2008; Dong et al., 2016; Li et al., 2013a; Liu et al., 2015; Srinivasan et al., 2011; Wang et al., 2011). In the nervous system, hsa-miR-146b-5p inhibits glioma migration and invasion by targeting epidermal growth factor receptor (EGFR), which was also induced by dioxin (Campion et al., 2016; Joiakim et al., 2016; Katakowski et al., 2010). However, little is known about the effect of dioxin on the expression of hsa-miR-146b-5p in the human nervous system.

Acetylcholinesterase (AChE) is a well conserved glycoprotein, which functions to hydrolyse acetylcholine in the nervous systems (Xu et al., 2015). The dysregulation of AChE is associated with neurodegenerative diseases, such as AD (Zemek et al., 2014). Recently, we found dioxin could transcriptionally down-regulate AChE expression in a human neuroblastoma cell line, SK-N-SH (Xie et al., 2013; Xu et al., 2015). Meanwhile, another group demonstrated that dioxin exposure triggered the up-regulation of mmu-miR-132, which caused AChE down-regulation in murine cell lines (Shaltiel et al., 2013). Therefore, it is interesting to investigate whether hsa-miR-146b-5p could target AChE, if dioxin could alter the expression of hsa-miR-146b-5p.

Therefore, in the present study, we examined dioxin effects on the expression of hsa-miR-146b-5p, and revealed potential roles of hsa-miR-146b-5p on AChE expression in human neuroblastoma cells. Finally, based on the experimental data, the possible involvement of hsa-miR-146b-5p in neuronal interferences by dioxin was proposed using bioinformatics tools.

1. Materials and methods

1.1. Cell culture

SK-N-SH, a cell line derived from human neuroblastoma cells, was purchased from the cell resource center of the Chinese Academy of Medical Sciences (Beijing, China). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco, UK), supplemented with 10% fetal bovine serum (FBS, Corning, USA), and 1% penicillin–streptomycin (Gibco, Scotland). Cells were cultured at 37°C in a 5% CO_2 humidified incubator.

1.2. Exposure experiments

2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), the most potent congener of dioxin, was purchased from Wellington Laboratories Inc. (Ontario, Canada) and dissolved in dimethyl sulfoxide (DMSO, Sigma, USA). TCDD was employed at the low concentration of 10^{-10} mol/L, close to environmental relevant level. DMSO was present at 0.1% in all treatments. After 24 hr-exposure, sample cells were washed with phosphate-buffered saline (PBS, pH 7.4) and prepared for Real-Time quantitative PCR (RT-PCR).

1.3. MiR isolation and RT-PCR

Total RNA was extracted from SK-N-SH cells treated with 0.1% DMSO or 10^{-10} mol/L TCDD for 24 hr using the Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturer's protocol. Total RNA was reverse transcribed to cDNA using the TaqMan MiR Reverse Transcription Kit (Applied Biosystems, USA). Afterwards, 1.33 μL of the cDNA solution was amplified using 1× TaqMan Universal PCR Master Mix (Applied Biosystems, USA). Quantitative PCR was run on a LightCycler 480 Instrument (LC-480II, Roche, USA) using a two-step PCR protocol with an initial denaturation step at 95°C for 10 min, followed by 40 cycles with a denaturation step at 95°C for 15 sec, and an annealing/elongation step at 60°C for 60 sec. The cycle threshold (Ct) values were calculated with the Roche 480 instrument with software (LC-480II, Roche, USA). U6 small nuclear RNA was used as an endogenous control. The data were analyzed using the ΔΔCt method (Livak and Schmittgen, 2001).

1.4. Reporter gene construction

A fragment consisting of the predicted promoter region (−1012 to −67 bp, 947 bp in length) of hsa-miR-146b was obtained by PCR using the following primers modified from Shi et al. (2014): forward 5′-GGA GCT CGA GTT TTC AGG CAG AGT AGA GAG A-3′ and reverse 5′-CAT G AA GCT TCC AGG ATG AGT AAG TTG AGG C-3′. The PCR product were cloned upstream of a firefly luciferase gene in the pGL3-Basic vector (Promega, Madison, USA) using Xho I/Hind III restriction sites. The resulting construct is designated as pmiR-146b-luc. The 3′-untranslated region (UTR) of the AChE T subunit ($AChE_T$), a major splicing variant of *ACHE* gene in vertebrate nervous system, was amplified using following primers modified from Lu et al. (2013): forward 5′-CTA ATC TAG ACC CCG GCG GGA CCC CCA T-3′ and reverse 5′-GGC TCT AGA TGG CTG TAA CAG TTT ATT GGC AGC CC-3′. The PCR product was cloned downstream of the firefly luciferase gene in the pGL3-Promoter vector (Promega, USA) using XbaI single restriction site. The resulting construct was designated as pACHE-3′-UTR. All constructs were confirmed by sequencing.

1.5. Cell transfection

Cultured cells with good growth status were seeded in 24-well plates at 1×10^5 cells/well 24 hr before transfection. In order to detect the effects of dioxin on hsa-miR-146b-5p promoter activity, purified plasmid pmiR-146b-luc (0.5 μg/well) was co-transfected with pRL-SV40 (10 ng/well) into cells using Lipofectamine LTX and PLUS reagent (Invitrogen, USA) according to the manufacturer's protocol. After 24 hr, the transfected cells were treated with 0.1% DMSO or 10^{-10} mol/L TCDD for 24 hr. To assess the effects of hsa-miR-146b-5p on AChE expression, hsa-miR-146b-5p mimics (60 pmol/L/well) (Ambion, USA) were transfected into cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen, USA) according to the manufacturer's instruction. For luciferase reporter assay, pACHE-3′-UTR (0.5 μg/well) and an internal control construct, pRL-SV40 (10 ng/well), were co-transfected into cells using Lipofectamine LTX and PLUS reagent (Invitrogen, USA).

1.6. Luciferase assay

The firefly and renilla luciferase activities were determined by using the Dual-Glo Luciferase Assay System (Promega, USA) according to the manufacturer's protocol. Briefly, cells were washed twice with PBS and lysed for 10 min at room

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