Available online at www.sciencedirect.com

ScienceDirect

www.elsevier.com/locate/jes

JES JOURNAL OF ENVIRONMENTAL SCIENCES

www.jesc.ac.cn

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

Tuan Xu, Heidi Q. Xie, Yunping Li, Yingjie Xia, Rui Sha, Lingyun Wang, Yangsheng Chen, Li Xu, Bin Zhao**

State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: xutuan2012@163.com
University of Chinese Academy of Sciences, Beijing 100085, China

ARTICLE INFO

Article history:
Received 14 February 2017
Revised 16 June 2017
Accepted 30 June 2017
Available online 11 July 2017

Keywords:
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD)
MicroRNA
Acetylcholinesterase (AChE)
Neuron
Transcription regulation
Post-transcriptional regulation

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

* *Corresponding authors.* E-mail: qhxie@rcees.ac.cn (Heidi Q. Xie), binzhao@rcees.ac.cn (Bin Zhao).

http://dx.doi.org/10.1016/j.jes.2017.06.038
1001-0742/

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 per 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

temperature. Cell debris was removed by centrifugation at 10,000 r/min for 1 min. The luciferase activities were quantified using Glomax Multi Detection System (Promega, USA). Relative luciferase activities were determined from three independent transfections and calculated from firefly luciferase activities normalized by renilla luciferase activities.

1.7. Determination of AChE enzymatic activity

AChE enzymatic activity was determined by a modified Ellman method (Ellman et al., 1961). Briefly, transfected cells were washed twice with PBS, then incubated with low-salt lysis buffer (80 mmol/L disodium hydrogen phosphate supplemented with 0.5% Triton X-100 and 2.5 mmol/L benzamidine (Sigma, USA)) for 30 min. Cell debris was removed from the resulting cell lysate by centrifugation at 13,000 r/min for 5 min. About 30 μL of supernatant was incubated with 0.1 mmol/L iso-OMPA (Sigma, USA) and 0.5 mmol/L 5,5′-dithiobis(2-nitrobenzenoic acid) (Sigma, USA) for 30 min to inhibit the butyrylcholinesterase activity. Subsequently, 0.625 mmol/L acetylthiocholine iodide (Sigma, USA) was added to start the AChE-specific reaction. Absorbance at 410 nm was recorded with a multifunctional microplate spectrometer (TECAN Infinite F200 Pro, Switzerland). Optical density (OD) was recorded per 10 min over a period of 1 hr. The velocity of the reaction was calculated from the slope of the fitting line obtained from OD change over the time. Arbitrary units of enzymatic activity were expressed as velocity (mOD per min) per microgram of protein. Protein concentration was determined using the Bradford Protein Assay Kit (Tiangen, China) with bovine serum albumin as the standard.

1.8. Predicted function of hsa-miR-146b-5p

In order to obtain an overview of the biological functions of hsa-miR-146b-5p, target genes were predicted by updated versions of TargetScan v7.0 (updated: Aug 2015, http://www.targetscan.org/vert_71/) and miRDB (updated: Mar 2015, http://www.mirdb.org/miRDB/). Given that the prediction programs often suffer from high false positive rates, only the intersection of predicted target genes from both independent tools were taken into account. The experimentally validated target genes were searched from the literature published in English until October 2016. Articles were primarily identified from database searches in PubMed and Google Scholar, and studies without original data, such as reviews, editorials or conference abstracts, were excluded. The target genes including the predicted and validated genes were submitted to the DAVID functional annotation database (http://david.abcc.ncifcrf.gov/), providing Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) enrichment analyses using the total human genome information as the background.

1.9. Statistical analysis

The data were presented as the means ± SEM from at least three independent experiments. The differences between two groups were analyzed using an unpaired Student's *t*-test using Graphpad Prism version 5 for Windows (GraphPad Software, USA) and $p < 0.05$ (*) or $p < 0.01$ (**) was considered to be statistically significant. The column charts were drawn by Origin 9.0 software (OriginLab, USA).

2. Results and discussion

2.1. Dioxin increases hsa-miR-146b-5p expression

Alteration of hsa-miR-146b-5p expression upon dioxin exposure was detected using RT-PCR analysis. As shown in Fig. 1a, hsa-miR-146b-5p was found to be significantly increased by approximately 115% after 24 hr exposure of 10^{-10} mol/L TCDD compared with DMSO-treated control. Alteration of hsa-miR-146b-5p expression has been found in different neuronal diseases, such as brain tumors (Table S1). The relationship between dioxin exposure and tumors has been evaluated (Dibenzofurans, 1997). TCDD has been classified as a type I carcinogen (Dibenzofurans, 1997), however, the association of TCDD with certain cancers is still mainly based on animal data due to the lack of data from humans. In rodent nervous system, it was reported that dioxin could activate astrocytes and promote the secretion of tumor necrosis factor-α *via* a PKC/SSeCKS-dependent mechanism in adult female Sprague–Dawley rats (Zhang et al., 2014). Thus, the induction of hsa-miR-146b-5p by dioxin suggests a possible mediating role of this miR in the association of dioxin exposure with brain tumors in human. However, regarding to another hsa-miR-146b-5p-associated pathological condition, AD, there is still no solid evidence showing a clear association of dioxin with the disease. So the AD-related target genes of hsa-miR-146b-5p might be potential dioxin responsive genes valuable for further investigation in order to reveal possible association with AD.

In order to further explore the action mechanism for the dioxin effect on hsa-miR-146b-5p expression, the promoter activity of hsa-miR-146b-5p was examined after dioxin exposure by using the promoter-driven luciferase reporter construct, pmiR-146b-luc (Fig. 1b). The promoter region used here was based on human hsa-miR-146b-5p transcript location (Kutty et al., 2013) and reported promoter region used in a similar assay (Shi et al., 2014). Quiescent SK-N-SH cells were transiently transfected with pmiR-146b-luc 24 hr before the exposures. Promoter activity was determined after 24 hr treatment of 0.1% DMSO or 10^{-10} mol/L TCDD. Consistent with the expression of hsa-miR-146b-5p, its promoter activity was significantly increased by approximately 21% after TCDD exposure compared with DMSO control (Fig. 1c). This data suggests the effect of dioxin on the hsa-miR-146b-5p expression may be attributed to transcriptional regulation. It is well-accepted that dioxin exert their effects by transcriptional regulation of a series of genes *via* the AhR-dependent pathway. The presence of dioxin responsive elements (DREs) in the promoter region is a feature of the dioxin responsive genes (Beischlag et al., 2008). To investigate the presence of putative DREs, core sequence of DRE (5′-GCG TG-3′ or 5′-CAC GC-3′ (Nukaya et al., 2009)) was searched in the promoter region of hsa-miR-146b-5p (−1012 to −67 bp, 947 bp), which has been reported by others (Curtale et al., 2013). As a result, one putative consensus DRE core sequence was found (Fig. 1b),

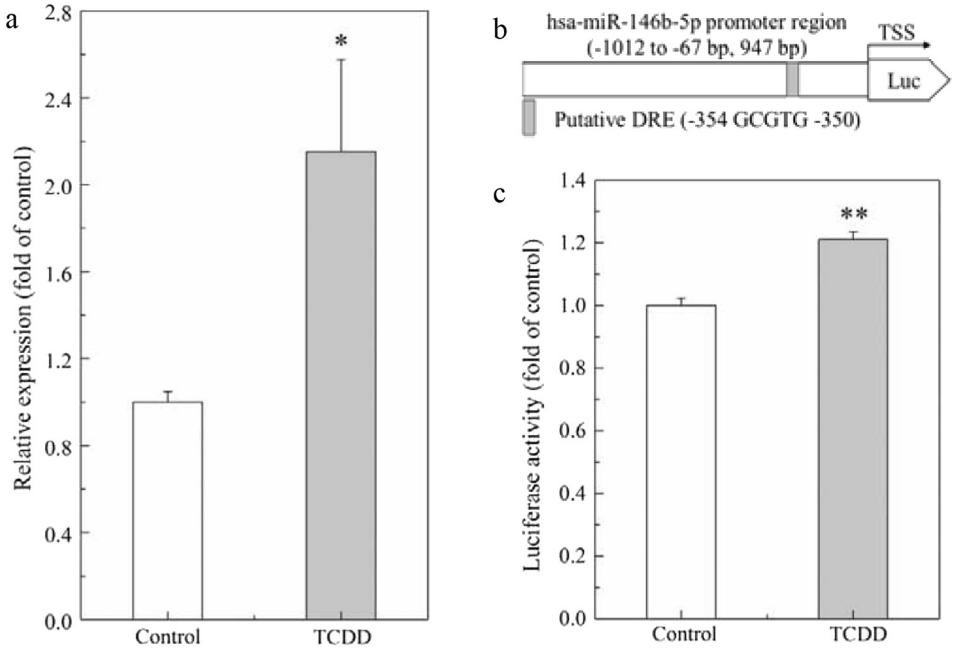

Fig. 1 – TCDD induces hsa-miR-146b-5p expression and its promoter activity in SK-N-SH cells. (a) Cells were exposed to 10^{-10} mol/L or 0.1% DMSO for 24 hr, and Hsa-miR-146b-5p expression was analyzed by RT-PCR, and U6 rRNA was used as an internal control; (b) A schematic diagram showing the putative promoter region and a putative DRE in pmiR-146b-luc construct used in (c); (c) pmiR-146b-luc was transiently transfected into cells 24 hr before the treatments of 10^{-10} mol/L TCDD or DMSO, and promoter activity was determined by the Dual-Glo luciferase assay after 24 hr-treatment. Values were expressed as mean ± SEM (n = 3) and each independent sample was detected in triplicate. Statistical analysis was done by t-test, and $^{*}p < 0.05$ compared with control (DMSO), $^{**}p < 0.01$ compared with control (DMSO). TCDD: 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin; DMSO: dissolved in dimethyl sulfoxide; miR: microRNA; RT-PCR: Real-Time quantitative PCR; DRE: dioxin responsive element.

which suggests that the AhR-dependent signaling pathway may be involved in the transcriptional alteration of hsa-miR-146b-5p by dioxin.

2.2. *Hsa-miR-146b-5p suppresses AChE expression*

MiRs, as negative regulators, can silence or suppress gene expression predominantly by binding to the 3′-UTR of target mRNAs (Krol et al., 2010). After over-expression of hsa-miR-146b-5p by transient transfection of hsa-miR-146b-5p mimics, AChE enzymatic activity was significantly decreased by approximately 17% compared to control in the SK-N-SH cells, indicating that hsa-miR-146b-5p is a suppressor of AChE expression (Fig. 2a). Since $AChE_T$ is the major splicing variant of *ACHE* in neurons, we next confirmed whether hsa-miR-146b-5p could target this AChE subunit. For this purpose, a reporter assay involving the 3′-UTR of $AChE_T$ was employed (Fig. 2b). After co-transfection of pACHE-3′-UTR with the hsa-miR-146b-5p mimics, luciferase activity was significantly decreased by approximately 14% compared with control, showing the ability of hsa-miR-146b-5p to bind to the 3′-UTR of $AChE_T$ (Fig. 2c). However, its role in expression of other minor neuronal AChE subunits, such as AChE R subunit, which has different sequences of 3′-UTR from that of $AChE_T$, needs further investigation. Thus, these results suggested that hsa-miR-146b-5p could cause down-regulation of AChE at least partially through targeting the $AChE_T$ subunit.

The expression of AChE can be regulated through transcriptional and post-transcriptional mechanisms (Xie et al., 2016). Multiple miRs can target one single gene to produce identical expression signatures (Ambros, 2004; Filipowicz et al., 2008). It has been reported that the expression of AChE was negatively regulated by miR-608 in human histiocytic lymphoma cell lines, miR-132 in human inflammatory bowel disease or Chinese Hamster Ovary cells, or miR-212 in human non-small cell lung cancer cell lines through targeting the AChE 3′-UTR (Hanin et al., 2014; Lu et al., 2013; Maharshak et al., 2013; Shaked et al., 2009). To our knowledge, the present study provides the first data demonstrating the role of hsa-miR-146b-5p in $AChE_T$ regulation. On the other hand, given the induction effect of TCDD on hsa-miR-146b-5p expression, this relationship between hsa-miR-146b-5p and $AChE_T$ provide a possibility that dioxin could down-regulate $AChE_T$ expression indirectly through a post-transcriptional down-regulation mechanism involving the enhancement of hsa-miR-146b-5p expression. Based on the well-accepted action mechanism for miRs to cause down-regulation of the target genes (Luo et al., 2015), we speculated that mRNA expression of $AChE_T$ might be decreased as a result of the interaction of hsa-miR-146b-5p with $AChE_T$ subunit. As the major splicing variant of AChE in neurons, this down-regulation of $AChE_T$ might in turn lead to the decrease of the protein expression and enzymatic activity. Nevertheless, hsa-miR-146b-5p may not be the only one involved in such indirect post-transcriptional

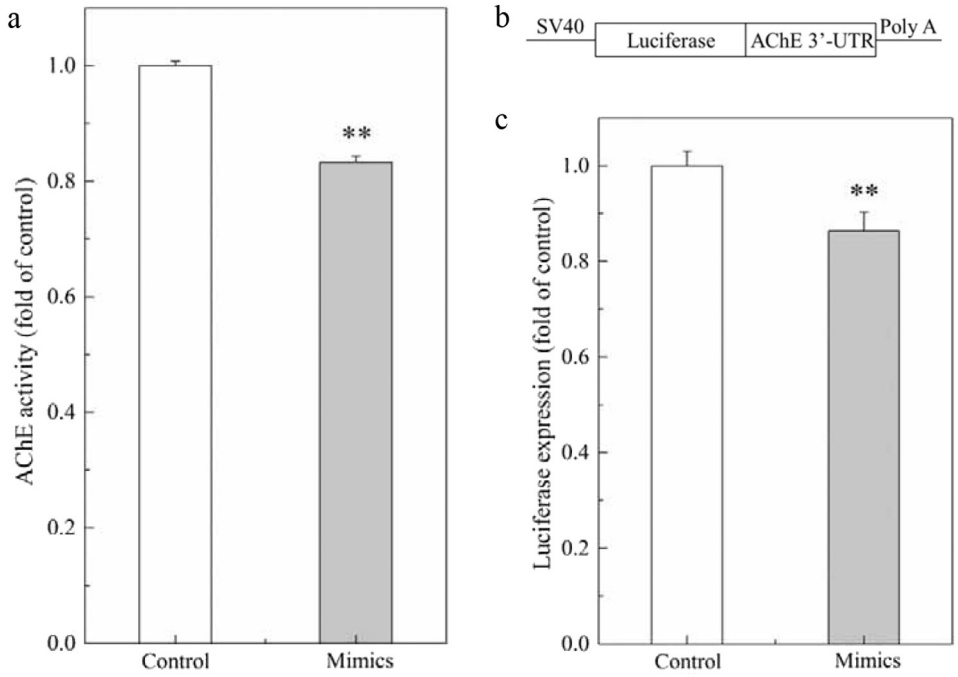

Fig. 2 – Hsa-miR-146b-5p mimic suppresses AChE expression *via* binding to AChE-3′-UTR. Hsa-miR-146b-5p mimic or control (mimic negative control) were transiently transfected into cultured SK-N-SH cells for 24 hr, respectively. (a) AChE enzymatic activity was determined according to the Ellman method; (b) A schematic diagram showing the constructed pAChE-3′-UTR plasmid including 3′-UTR of $ACHE_T$ (226 bp) located downstream of the luciferase reporter gene; (c) pAChE-3′-UTR was co-transfected with the hsa-miR-146b-5p mimic into the cells. Luciferase assays were performed using the Dual-Glo luciferase assay. Values were expressed as mean ± SEM (n = 3) and each independent sample was detected in triplicate. Statistical analysis was done by t-test. $^{**}p < 0.01$, compared with control (DMSO). AChE: acetylcholinesterase; UTR: untranslated region.

regulation. However, these assumptions need further investigation. Thus, the present data support the notion that dioxin could induce dysregulation of AChE *via* complex multi-level mechanisms involving, at least, AhR-dependent transcriptional regulation and miR-mediated post-transcriptional regulation (Xie et al., 2013, 2016). On the other hand, hsa-miR-146b-5p expression was found to be decreased in AD patients (Cogswell et al., 2008; Wang et al., 2011). Thus, the role of hsa-miR-146b-5p in the dysregulation of AChE gene expression in AD patient brain is merit for further investigation.

2.3. *Functional analysis of hsa-miR-146b-5p*

In order to reveal the toxicological significance of the hsa-miR-146b-5p induction caused by dioxin exposure, functional analysis of hsa-miR-146b-5p was performed based on target gene prediction supplemented with bioinformatics analysis for functional clustering. There were 13 predicted target genes and 34 validated target genes for hsa-miR-146b-5p (Table S2, Fig. S1 and Table S3). After removing duplicate genes, 42 unique target genes were subjected to bioinformatics analysis. Totally 25 KEGG pathways and 414 GO terms (including 377 Biological Processes (BP), 19 Molecular Functions (MF) and 18 Cellular Component (CC)) were found to be related to these genes using DAVID online software analysis (Tables S4 and S5).

Eight functional clusters related to brain function or cyto-toxicities are summarized in Fig. 3 and Table S6, in which three categories of the target genes were involved, including predicted target genes, validated target genes reported in nervous system and validated target genes reported in non-nervous system. Some of the predicted functional clusters are consistent with reported functions of hsa-miR-146b-5p in the nervous system, such as in glioma (Li et al., 2013a; Liu et al., 2015; Srinivasan et al., 2011), locomotion (Li et al., 2013a) and neurotransmission. Moreover, most of the functional clusters are closely related to the reported effects or toxicities of dioxin in the nervous system, particularly effects on behavior (Vreugdenhil et al., 2002), synapse transmission (Xie et al., 2013), neuron differentiation (Collins et al., 2008), apoptosis (Li et al., 2013b) and cell cycle (Jin et al., 2004). The consistency in hsa-miR-146b-5p functions and dioxin effects suggested that dysregulation of this miR may participate in some interfering effects of dioxin on the nervous system. From the left panel of Fig. 3, we found that most of the target genes related to the functions of interest were not validated in the neuronal system. It is notable that there were no behavior related target genes being validated in the nervous system which might be a valuable aspect for further study (Fig. 3 and Table S6). The relationship of those target genes with AhR was also summarized based on literature reports, in which more than half of the genes did

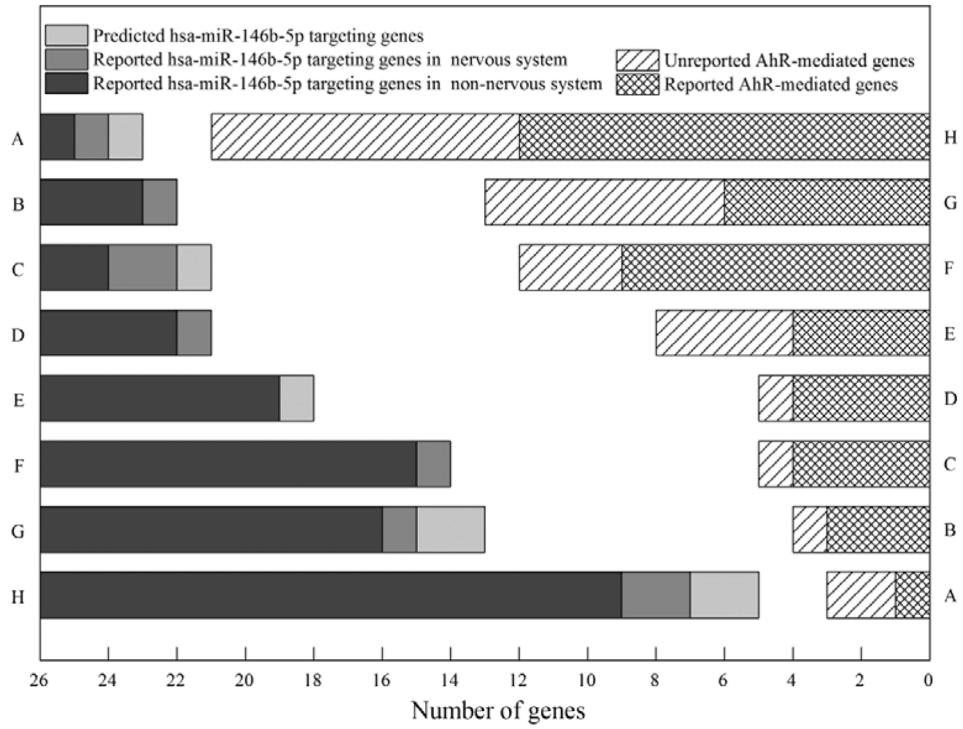

Fig. 3 – Functional enrichment of the target genes of hsa-miR-146b-5p. The clustering genes consisted of predicted and experimental validated target genes of hsa-miR-146b-5p, and they were subjected to GO and KEGG enrichment analysis using DAVID online software with the total human genome information as the background. Gene classification was based on literature reports. A: gliogenesis, B: glioma, C: regulation of synaptic transmission, D: regulation of locomotion, E: behavior, F: cell cycle related, G: neuron differentiation, H: apoptosis; KEGG: Kyoto Encyclopedia of Genes and Genomes; GO: Gene Ontology.

not have any report showing such relationship (Fig. 3, right panel). Given the induction of hsa-miR-146b-5p by dioxin, its target genes with unknown correlation with AhR could be candidates to find new dioxin responsive genes associated with the neuronal interfering effects.

Here, we reported hsa-miR-146b-5p as a novel regulator of $AChE_T$ subunit. Whether the functions of hsa-miR-146b-5p could be mediated by the dysregulation of $AChE_T$ is still an open question. Among the predicted functional annotations of hsa-miR-146b-5p target genes, AChE is closely associated with the annotation of synapse transmission, in particular cholinergic neurotransmission (Xie et al., 2013). Apart from this possible mediating role of AChE in neurotransmission, emerging non-classical functions of AChE may provide new insight into the involvements of AChE, such as in cell cycle, neuron differentiation and apoptosis, which were also present in the functional clusters of hsa-miR-146b-5p target genes (Fossati et al., 2015; Jiang and Zhang, 2008; Layer et al., 2013; Pérez-Aguilar et al., 2015; Shaked et al., 2009; Silman and Sussman, 2005). However, the potential roles of AChE in hsa-miR-146b-5p related neuronal functions still need further validations. As shown in Table 1, the validated target genes of hsa-miR-146b-5p suggest involvements of this miR in some brain functions or dioxin induced cyto-toxicities, which merit further investigation.

Table 1 – Hsa-miR-146b-5p validated target genes and their functional enrichment.

Gene ID	Symbol	Description	Functional enrichment
4857	NOVA1	NOVA alternative splicing regulator 1	Behavior
6478	SIAH2	Siah E3 ubiquitin protein ligase 2	Apoptosis related, cell cycle
7189	TRAF6	TNF receptor associated factor 6	Apoptosis related

3. Conclusions

In human neuroblastoma cells, dioxin significantly increased hsa-miR-146b-5p expression through a transcriptional mechanism. Hsa-miR-146b-5p significantly suppressed the enzymatic activity of AChE probably by targeting the 3′-UTR of $AChE_T$, the major AChE subunit in neurons. Functional analysis suggests that hsa-miR-146b-5p may be involved in dioxin effects on the nervous system, such as synapse transmission, in which AChE may serve as a responsive gene for mediating the effect.

Acknowledgments

This work was supported by the Strategic Priority Research Program of the Chinese Academy of Sciences (Nos. XDB14030401, XDB14030402), the National Natural Science Foundation of China (Nos. 21177150, 21377160, 21525730), and we thank Dr. Marjorie A. Phillips from UC Davis for comments during the manuscript preparation.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jes.2017.06.038.

REFERENCES

Ambros, V., 2004. The functions of animal microRNAs. Nature 431 (7006), 350–355.

Barrett, D.H., Morris, R.D., Akhtar, F.Z., Michalek, J.E., 2001. Serum dioxin and cognitive functioning among veterans of operation ranch hand. Neurotoxicology 22 (4), 491–502.

Beischlag, T.V., Morales, J.L., Hollingshead, B.D., Perdew, G.H., 2008. The aryl hydrocarbon receptor complex and the control of gene expression. Crit. Rev. Eukaryot. Gene Expr. 18 (3), 207–250.

Campion, C.M., Leon, Carrion S., Mamidanna, G., Sutter, C.H., Sutter, T.R., Cole, J.A., 2016. Role of EGF receptor ligands in TCDD-induced EGFR down-regulation and cellular proliferation. Chem. Biol. Interact. 253, 38–47.

Cogswell, J.P., Ward, J., Taylor, I.A., Waters, M., Shi, Y., Cannon, B., et al., 2008. Identification of miRNA changes in Alzheimer's disease brain and CSF yields putative biomarkers and insights into disease pathways. J. Alzheimers Dis. 14 (1), 27–41.

Collins, L.L., Williamson, M.A., Thompson, B.D., Dever, D.P., Gasiewicz, T.A., Opanashuk, L.A., 2008. 2,3,7,8-Tetracholorodibenzo-*p*-dioxin exposure disrupts granule neuron precursor maturation in the developing mouse cerebellum. Toxicol. Sci. 103 (1), 125–136.

Curtale, G., Mirolo, M., Renzi, T.A., Rossato, M., Bazzoni, F., Locati, M., 2013. Negative regulation of toll-like receptor 4 signaling by IL-10-dependent microRNA-146b. Proc. Natl. Acad. Sci. U. S. A. 110 (28), 11499–11504.

Dibenzofurans, P., 1997. Polychlorinated dibenzo-para-dioxins and polychlorinated dibenzofurans. IARC Monogr. Eval. Carcinog. Risks Hum. 69.

Dong, H., Wang, C., Lu, S., Yu, C., Huang, L., Feng, W., et al., 2016. A panel of four decreased serum microRNAs as a novel biomarker for early Parkinson's disease. Biomarkers 21, 129–137.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem. Pharmacol. 7, 88–95.

Filipowicz, W., Bhattacharyya, S.N., Sonenberg, N., 2008. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat. Rev. Genet. 9, 102–114.

Fiore, R., Siegel, G., Schratt, G., 2008. MicroRNA function in neuronal development, plasticity and disease. Biochim. Biophys. Acta 1779 (8), 471–478.

Fossati, S.M., Candiani, S., Nödl, M.T., Maragliano, L., Pennuto, M., Domingues, P., et al., 2015. Identification and expression of acetylcholinesterase in *Octopus vulgaris* arm development and regeneration: a conserved role for AChE? Mol. Neurobiol. 52 (1), 45–56.

Hanin, G., Shenhar-Tsarfaty, S., Yayon, N., Yau, Y.H., Bennett, E.R., Sklan, E.H., et al., 2014. Competing targets of microRNA-608 affect anxiety and hypertension. Hum. Mol. Genet. 23 (17), 4569–4580.

Im, H.I., Kenny, P.J., 2012. MicroRNAs in neuronal function and dysfunction. Trends Neurosci. 35 (5), 325–334.

Jiang, H., Zhang, X.J., 2008. Acetylcholinesterase and apoptosis. FEBS J. 275 (4), 612–617.

Jin, D.Q., Jung, J.W., Lee, Y.S., Kim, J.A., 2004. 2, 3, 7, 8-Tetrachlorodibenzo-p-dioxin inhibits cell proliferation through arylhydrocarbon receptor-mediated G1 arrest in SK-N-SH human neuronal cells. Neurosci. Lett. 363 (1), 69–72.

Joiakim, A., Mathieu, P.A., Shelp, C., Boerner, J., Reiners Jr., J.J., 2016. Epidermal growth factor receptor kinase inhibitors synergize with TCDD to induce CYP1A1/1A2 in human breast epithelial MCF10A cells. Drug Metab. Dispos. 44 (5), 665–671.

Katakowski, M., Zheng, X., Jiang, F., Rogers, T., Szalad, A., Chopp, M., 2010. MiR-146b-5p suppresses EGFR expression and reduces *in vitro* migration and invasion of glioma. Cancer Investig. 28 (10), 1024–1030.

Krol, J., Loedige, I., Filipowicz, W., 2010. The widespread regulation of microRNA biogenesis, function and decay. Nat. Rev. Genet. 11 (9), 597–610.

Kutty, R.K., Nagineni, C.N., Samuel, W., Vijayasarathy, C., Jaworski, C., Duncan, T., et al., 2013. Differential regulation of microRNA-146a and microRNA-146b-5p in human retinal pigment epithelial cells by interleukin-1beta, tumor necrosis factor-alpha, and interferon-gamma. Mol. Vis. 19, 737–750.

Layer, P.G., Klaczinski, J., Salfelder, A., Sperling, L.E., Thangaraj, G., Tuschl, C., et al., 2013. Cholinesterases in development: AChE as a firewall to inhibit cell proliferation and support differentiation. Chem. Biol. Interact. 203 (1), 269–276.

Li, Y., Wang, Y., Yu, L., Sun, C., Cheng, D., Yu, S., et al., 2013a. MiR-146b-5p inhibits glioma migration and invasion by targeting MMP16. Cancer Lett. 339 (2), 260–269.

Li, Y., Chen, G., Zhao, J., Nie, X., Wan, C., Liu, J., et al., 2013b. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces microglial nitric oxide production and subsequent rat primary cortical neuron apoptosis through p38/JNK MAPK pathway. Toxicology 312, 132–141.

Liu, J., Xu, J., Li, H., Sun, C., Yu, L., Li, Y., et al., 2015. MiR-146b-5p functions as a tumor suppressor by targeting TRAF6 and predicts the prognosis of human gliomas. Oncotarget 6 (30), 29129–29142.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(−Delta Delta C(T)) method. Methods 25, 402–408.

Loohuis, N.O., Kos, A., Martens, G., Van Bokhoven, H., Kasri, N.N., Aschrafi, A., 2012. MicroRNA networks direct neuronal development and plasticity. Cell. Mol. Life Sci. 69 (1), 89–102.

Lu, L., Zhang, X., Zhang, B., Wu, J., Zhang, X., 2013. Synaptic acetylcholinesterase targeted by microRNA-212 functions as a tumor suppressor in non-small cell lung cancer. Int. J. Biochem. Cell Biol. 45 (11), 2530–2540.

Luo, X., Yang, B., Nattel, S., 2015. MicroRNAs and atrial fibrillation: mechanisms and translational potential. Nat. Rev. Cardiol. 12 (2), 80–90.

Maharshak, N., Shenhar-Tsarfaty, S., Aroyo, N., Orpaz, N., Guberman, I., Canaani, J., et al., 2013. MicroRNA-132 modulates cholinergic signaling and inflammation in human inflammatory bowel disease. Inflamm. Bowel Dis. 19 (7), 1346–1353.

Nishijo, M., Anh, N.T.N., Maruzeni, S., Nakagawa, H., Van Luong, H., Anh, T.H., et al., 2013. Dioxin exposure in breast milk and infant neurodevelopment in Vietnam. Occup. Environ. Med. 70 (9), 656–662.

Nukaya, M., Moran, S., Bradfield, C.A., 2009. The role of the dioxin-responsive element cluster between the CYP1A1 and CYP1A2 loci in aryl hydrocarbon receptor biology. Proc. Natl. Acad. Sci. U. S. A. 106 (12), 4923–4928.

Pérez-Aguilar, B., Vidal, C.J., Palomec, G., García-Dolores, F., Gutiérrez-Ruiz, M.C., Bucio, L., et al., 2015. Acetylcholinesterase is associated with a decrease in cell proliferation of hepatocellular carcinoma cells. Biochim. Biophys. Acta 1852 (7), 1380–1387.

Pontillo, C.A., Rojas, P., Chiappini, F., Sequeira, G., Cocca, C., Crocci, M., et al., 2013. Action of hexachlorobenzene on tumor growth and metastasis in different experimental models. Toxicol. Appl. Pharmacol. 268 (3), 331–342.

Powers, B.E., Lin, T.M., Vanka, A., Peterson, R.E., Juraska, J.M., Schantz, S.L., 2005. Tetrachlorodibenzo-*p*-dioxin exposure alters radial arm maze performance and hippocampal morphology in female ahr mice. Genes Brain Behav. 4 (1), 51–59.

Sans, J.D.P., Clements, K.J., Suvorov, A., Krishnan, S., Adams, H.L., Petersen, S.L., 2016. Developmental exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin may alter LH release patterns by abolishing sex differences in GABA/glutamate cell number and modifying the transcriptome of the male anteroventral periventricular nucleus. Neuroscience 329, 239–253.

Selbach, M., Schwanhäusser, B., Thierfelder, N., Fang, Z., Khanin, R., Rajewsky, N., 2008. Widespread changes in protein synthesis induced by microRNAs. Nature 455 (7209), 58–63.

Shaked, I., Meerson, A., Wolf, Y., Avni, R., Greenberg, D., Gilboa-Geffen, A., et al., 2009. MicroRNA-132 potentiates cholinergic anti-inflammatory signaling by targeting acetylcholinesterase. Immunity 31 (6), 965–973.

Shaltiel, G., Hanan, M., Wolf, Y., Barbash, S., Kovalev, E., Shoham, S., et al., 2013. Hippocampal microRNA-132 mediates stress-inducible cognitive deficits through its acetylcholinesterase target. Brain Struct. Funct. 218 (1), 59–72.

Shi, C., Zhu, L., Chen, X., Gu, N., Chen, L., Zhu, L., et al., 2014. IL-6 and TNF-alpha induced obesity-related inflammatory response through transcriptional regulation of miR-146b. J. Interf. Cytokine Res. 34 (5), 342–348.

Silman, I., Sussman, J.L., 2005. Acetylcholinesterase: 'classical' and 'non-classical' functions and pharmacology. Curr. Opin. Pharmacol. 5 (3), 293–302.

Srinivasan, S., Patric, I.R.P., Somasundaram, K., 2011. A ten-microRNA expression signature predicts survival in glioblastoma. PLoS One 6 (3), e17438.

Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., et al., 2006. The 2005 world health organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol. Sci. 93 (2), 223–241.

Vreugdenhil, H.J., Slijper, F.M., Mulder, P.G., Weisglas-Kuperus, N., 2002. Effects of perinatal exposure to PCBs and dioxins on play behavior in Dutch children at school age. Environ. Health Perspect. 110 (10), A593–A598.

Wang, W.X., Huang, Q., Hu, Y., Stromberg, A.J., Nelson, P.T., 2011. Patterns of microRNA expression in normal and early Alzheimer's disease human temporal cortex: white matter versus gray matter. Acta Neuropathol. 121 (2), 193–205.

Xie, H.Q., Xu, H.M., Fu, H.L., Hu, Q., Tian, W.J., Pei, X.H., et al., 2013. AhR-mediated effects of dioxin on neuronal acetylcholinesterase expression *in vitro*. Environ. Health Perspect. 121 (5), 613–618.

Xie, H.Q., Xu, T., Chen, Y., Li, Y., Xia, Y., Xu, S.L., et al., 2016. New perspectives for multi-level regulations of neuronal acetylcholinesterase by dioxins. Chem. Biol. Interact. 259 (Pt B), 286–290.

Xu, M.L., Luk, W.K., Lau, K.M., Bi, C.W., Cheng, A.W., Gong, A.G., et al., 2015. Three N-glycosylation sites of human acetylcholinesterase shares similar glycan composition. J. Mol. Neurosci. 57 (4), 486–491.

Zemek, F., Drtinova, L., Nepovimova, E., Sepsova, V., Korabecny, J., Klimes, J., et al., 2014. Outcomes of Alzheimer's disease therapy with acetylcholinesterase inhibitors and memantine. Expert Opin. Drug Saf. 13 (6), 759–774.

Zhang, Y., Nie, X., Tao, T., Qian, W., Jiang, S., Jiang, J., et al., 2014. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin promotes astrocyte activation and the secretion of tumor necrosis factor-alpha via PKC/SSeCKS-dependent mechanisms. J. Neurochem. 129 (5), 839–849.