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

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**A R T I C L E I N F O**

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

**A B S T R A C T**

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.

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**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; Joakim 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% CO2 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. 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 co-transfected into cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen, 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 10 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 GGA TTT TCC AGG CAG AGT AGA GAG A-3′ and reverse 5′-CAT GAA GAT TCC AGG AAT TTG AGG C-3′. The PCR product was 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 (AChET), a major splicing variant of AChE gene in vertebrate nervous system, was amplified using primers modified from Lu et al. (2013): forward 5′-CTA ATC TAG ACC CCC GCG GGA CCC CCA T-3′ and reverse 5′-GCC TCT AGA TGG CTG TAA CAG TTT ATT GCC 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 × 105 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 co-transfected into cells using Lipofectamine RNAiMAX transfection reagent (Invitrogen, USA) according to the manufacturer’s protocol. 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-nitrobenzoic 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 multi-functional 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 10⁻¹² 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 1 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′-GGG 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),
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 AChET 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 AChET 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 AChET (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 AChET, needs further investigation. Thus, these results suggested that hsa-miR-146b-5p could cause down-regulation of AChE at least partially through targeting the AChET 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 AChET 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 AChET provide a possibility that dioxin could down-regulate AChET 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 AChET might be decreased as a result of the interaction of hsa-miR-146b-5p with AChET subunit. As the major splicing variant of AChE in neurons, this down-regulation of AChET 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
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 cytotoxicities 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
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 AChET subunit. Whether the functions of hsa-miR-146b-5p could be mediated by the dysregulation of AChET 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.

### 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 AChET, 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.

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

<table>
<thead>
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<th>Gene ID</th>
<th>Symbol</th>
<th>Description</th>
<th>Functional enrichment</th>
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</thead>
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<tr>
<td>4857</td>
<td>NOVA1</td>
<td>NOVA alternative splicing regulator 1</td>
<td>Behavior</td>
</tr>
<tr>
<td>6478</td>
<td>SIAH2</td>
<td>Siah E3 ubiquitin ligase 2</td>
<td>Apoptosis related, cell cycle</td>
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<tr>
<td>7189</td>
<td>TRAF6</td>
<td>TNF receptor associated factor 6</td>
<td>Apoptosis related</td>
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</table>
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.

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