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Silencing of miR-124 induces neuroblastoma SK-N-SH cell differentiation, cell cycle arrest and apoptosis through promoting AHR

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ABSTRACT

Neuroblastoma is the most common extracranial solid tumor in children. We investigate whether miR-124, the abundant neuronal miRNA, plays a pivotal role in neuroblastoma. Knockdown of miR-124 promotes neuroblastoma SK-N-SH cell differentiation, cell cycle arrest and apoptosis. Further miR-124 is predicted to target aryl hydrocarbon receptor (AHR) which may promote neuroblastoma cell differentiation. We validate that miR-124 may suppress the expression of AHR by targeting its 3′-UTR. These results suggest that miR-124 could serve as a potential therapeutic target of neuroblastoma.

1. Introduction

Neuroblastoma, an embryonic tumor derived from the autonomic nervous system neural-crest tissues, is the second most common extracranial malignant tumor of childhood and the most common solid tumor of infancy [1]. Neuroblastoma arises from fetal precursors of the sympathetic nervous system [2]. Molecular defects in differentiation are one of the causes in neuroblastoma development.

MicroRNAs (miRNAs) are a class of small non-coding RNAs approximately 21 nucleotides in length that regulate diverse cellular processes including decision of cell lineage, stem cell division, development in evolutionarily divergent organisms, tumorigenesis, and tumor cell metastasis [3,4]. These evidences suggest that miRNAs may be valuable therapeutic agents for their roles in modulating the differentiation of human tumor cells. MiR-124 is a conserved miRNA that has been recorded in 60 organisms for 94 sequences in mirBase and been found to be abundant in nerve system, endocrine gland, and embryo [5]. Most studies showed that this neuronal abound miRNA could modulate the neurite outgrowth during neurogenesis and regulate the differentiation in neuron. For instance, miR-124 can inhibit SCP1 expression in embryonal carcinoma cell P19 and shows a complementary expression pattern during spinal cord development [6]. Overexpression of miR-124 in P19 cells contributes to the control of neurite outgrowth by regulation of the cytoskeleton remodeling [7]. In the mouse neuroblastoma cell lines, CAD and Neuro2a, miR-124 promotes the differentiation by targeting PTBP1 mRNA encodes a

Abbreviations: miRNA, microRNA; AHR, aryl hydrocarbon receptor; 3′-UTR, 3′-untranslated mRNA region; Pre, pre-miR-124 precursor; Inh, anti-miR-124 miRNA inhibitor; Mock-Pre, precursor negative control; Mock-Inh, inhibitor negative control

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global repressor of alternative pre-mRNA splicing in nonneuronal cells [8]. During adult neurogenesis in mice, miR-124 is an important regulator of the temporal progression along the subventricular zone stem cell lineage to neurons [9] and during the apical to basal transition of neural precursor cells in the mouse embryonic neocortex [10].

However, the functions of miR-124 in neuronal differentiation remain controversial. Cao et al. showed that experiments to alter expression of miR-124 in neural cells did not appear to affect differentiation in the chick neural tube [11]. In the early developmental stage of optic cup formation in *Xenopus*, miR-124 targeting NeuroD1 plays an anti-neural role in promoting cell proliferation [12]. Furthermore, the role of miR-124 in differentiation shows diversity in a genome wide small RNA sequencing project. Upon differentiation stimulation of retinoic acid (RA), transphorbol ester (TPA), dibutyryl cAMP (dcAMP), or fetal bovine serum (FBS) withdrawn, the expression of miR-124 is upregulated in human SHSY5Y and mouse N1E-115 neuroblastoma cells but downregulated in mouse Neuro2a and NG-108 neuroblastoma cells [13]. Study of dysregulation in miR-124 thus showed decisive in regulation of neuroblastoma.

Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor that residents in the cytoplasm as an inactive form by binding of chaperon proteins such as 90 kDa heat shock protein [14,15] and forms heterodimer with AHR nuclear translocator (ARNT) [16] to activate xenobiotic response element located upstream of the target genes such as CYP1A1 due to activation of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-related ligands binding and translocation into the nucleus [17,18]. Nevertheless, AHR is postulated to play important roles not only in the regulation of xenobiotic metabolism but also in the regulation of differentiation in pro-inflammatory T cells [19], ketarinocytes [20], myeloblastic leukemia cells [21], and Neuro2a cells [22].

Here in this study, we reveal an undescribed regulation of miR-124 in neuroblastoma that loss of miR-124 resulted in upregulation of AHR leading differentiation in the neuroblastoma cell line SK-N-SH.

2. Materials and methods

2.1. Patients and treatment

The clinical evaluation and use of tumor samples in each patient were approved by the Institutional Review Board of National Taiwan University Hospital, Taipei. During a period of 16 years (from December 1990 to December 2006), tissues were immediately frozen and stored at −80 °C prior to real-time RT-PCR analyses.

2.2. Cell culture

Neuroblastoma cell line SK-N-SH and SK-N-BE were maintained in Dulbecco's Modified Eagle's Medium (DMEM)/F12 with 10% (v/v) fetal bovine serum (FBS) (GIBCO, New York, USA). A549 cells were maintained in DMEM. Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

2.3. miRNA transfection

Cells were seeded into six-well plates and transfected using Turbofect™ in vitro Transfection Reagent (Fermentas, Burlington, USA) according to the manual. Pre-miR-124 precursor (Pre), anti-miR-124 miRNA inhibitor (Inh), precursor negative control (Mock-Pre) and inhibitor negative control (Mock-Inh) were purchased from Ambion Inc. (Austin, TX). Each of them was transfected into cells at a concentration of 200 nM as previous described [23].

2.4. RNA extraction and real-time RT-PCR

Total RNA was extracted from cells or tissues with Trizol reagent according to the instruction (Invitrogen, Carlsbad, USA). The miR-124 level was quantified by quantitative real-time RT-PCR using TaqMan MicroRNA Assay kits (Applied Biosystems, Foster City, USA) with U6 small nuclear RNA (snRNA *RNU6B*) as an internal normalized reference. Their relative levels were measured in triplicate on a Prism 7300 real-time PCR machine (Applied Biosystems). For the quantification of AHR, GAP-43, CRT, and NSE, extracted RNA was reverse transcribed to cDNA using an oligo(dT)12 primer and RevertAid™ Reverse Transcriptase (Fermentas). Their relative levels were measured in triplicate on a MyiQ Single-Color Real-Time PCR Detection System (Bio-Rad, Hercules, USA) with GAPDH as internal control.

2.5. Luciferase reporter assay

The reporter constructs containing AHR 3′-untranslated mRNA region (3′-UTR) were cloned into the pMIR-REPORT vector (Ambion) using PCR-generated fragment. The mutated and deleted AHR 3′-UTR were amplified from the Luc-AHR-WT vector. The reporter assays were carried out as described previously with a transfection control [23].

2.6. Western blot analysis

Total cellular protein was separated by electrophoresis in SDS–polyacrylamide gels before transfer to PVDF membrane (Millipore, Bedford, MA, USA). Anti-GAP43, anti-AHR, and anti-Ki67 were obtained from Abcam (Cambridge, UK), Santa Cruz (CA, USA), and Millipore, respectively. Others were purchased from Cell Signaling Technology (MA, USA). Signals were visualized by chemiluminescence using the ECL reagent (Millipore).

2.7. Statistical analysis

Statistical analysis of miRNA expression in tumor cells was performed on $\log_2$ transformed fold change data. Student's *t*-test and *P*-values were tested for multiple comparisons by controlling the false discovery rate.

3. Results and discussion

3.1. MiR-124 regulates neuroblastoma cell differentiation

To investigate the role of miR-124 in neuroblastoma, we performed the loss- and gain-of-function of miR-124 in neuroblastoma cells. It was evident that the miR-124 inhibitor induced an increase in the number of cells bearing neurites compared to control cells (Figs. 1A and S1). The changes in cell morphology and neurite length observed in the presence of Inh suggested induction of neuronal differentiation. To determine whether the Inh-induced morphological changes were accompanied by biochemical patterns resembling the neuronal phenotype, we measured the levels of neuronal-specific markers including growth-associated protein 43 (GAP-43), calreticulin (CRT) and neuron-specific enolase (NSE) by qRT-PCR. These three known differentiation markers were all elevated after inhibition of miR-124 (Fig. 1B). In addition, the protein expression level of GAP43 is also strongly increased after inhibition of miR-124 (Fig. 1C). The results suggest that miR-124 may function as a cell fate regulator which modulates cell differentiation in SK-N-SH cells.

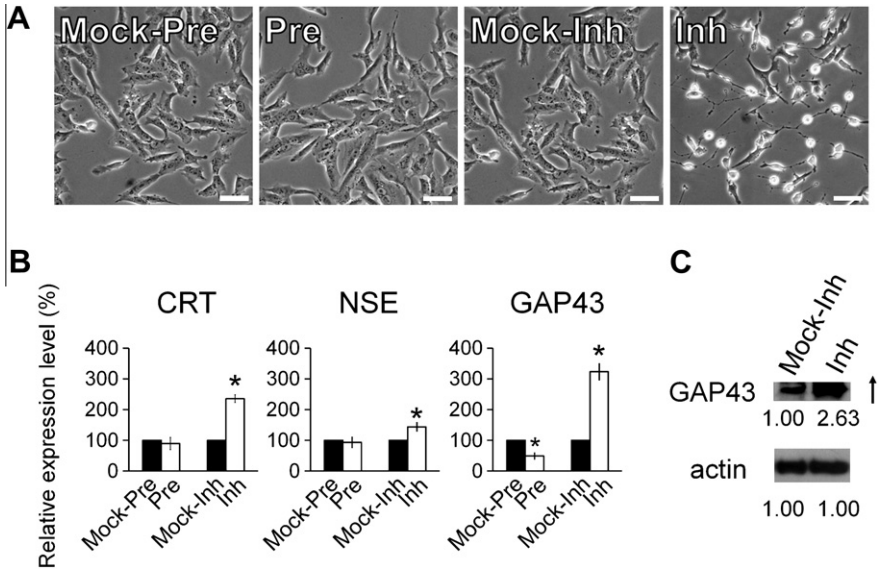

Fig. 1. miR-124 inhibits neuroblastoma cell differentiation. (A) SK-N-SH cells transfected with 200 nM miR-124 precursor (Pre), negative control precursor (Mock-Pre), miR-124 inhibitor (Inh) or negative control inhibitor (Mock-Inh) were observed by phase-contrast microscopy. Bar, 50 μm. (B) qRT-PCR analysis of three neuronal differentiation markers, calreticulin (CRT), neuron-specific enolase (NSE) and growth-associated protein 43 (GAP-43) in SK-N-SH cells after transfected with Inh or Mock-Inh. $^{*}P < 0.05$. (C) The protein expression level of GAP-43 were normalized to actin in SK-N-SH cells after transfected with Inh or Mock-Inh.

3.2. Inhibition of miR-124 induces cell-cycle arrest and apoptosis in SK-N-SH

Cell cycle arrest and cell death are tightly coupled to terminal differentiation of neuroblastoma cells. Since neuroblastomas, as well as their derived cell lines, maintain the potentiality of terminal differentiation, we subsequently testify whether Inh can also cause anti-proliferative effects including cell cycle arrest and apoptosis along with cell differentiation. To confirm this possibility, SK-N-SH cells were treated with Inh or Mock-Inh and subsequently processed for FACS and immunoblotting analysis. Cytofluorimetric analysis revealed that there was an accumulation of a G_0/G_1-phase population in miR-124 downregulated cells (Fig. 2A). Ki-67 is routinely used as a marker of cell cycle progression and proliferation. We found that the expression level of Ki-67 was decreased in Inh-transfected cells (Fig. 2B). We next investigated whether cell cycle proteins were modulated in Inh-treated SK-N-SH cells by immunoblotting experiments. Fig. 2B shows that Inh induced dramatically decrease in cyclin D_1 and CKD4. Moreover, the expression profile of cyclin and CDK was in agreement with the hypo-phosphorylated/active form of Rb (Fig. 2C). We also observed an increasing number of cells progressing programmed cell death by annexin V/PI, DAPI staining, and immuneblotting in SK-N-SH cells transfected with Inh. The Inh treated cells distributed increase in annexin V positive population (Fig. 3A), showed shrinkage morphology in nucleus (Fig. 3B) and decreased in the protein expression of procaspases and pro-PARP (Fig. 3C). These data revealed that apoptosis was undergone in SK-N-SH cells after cell differentiation through a caspase dependent pathway.

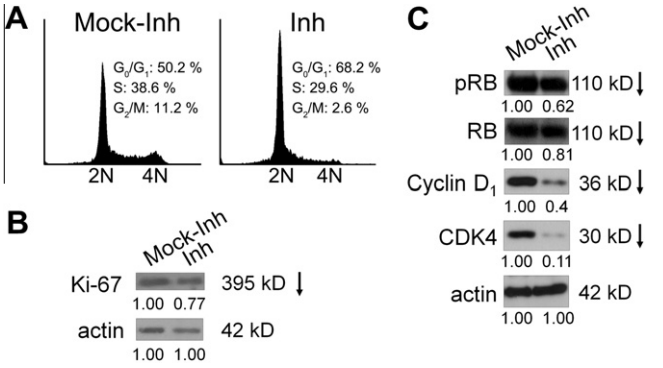

Fig. 2. Anti-miR-124 induces cell cycle arrest in SK-N-SH cells. SK-N-SH cells were transfected with 200 nM miR-124 inhibitor (Inh) or negative control inhibitor (Mock-Inh). (A) DNA distributions of SK-N-SH cells after transfection were analyzed by flow cytometry and quantified by ModFit program. (B) The level of proliferation marker Ki-67, (C) cell cycle regulators pRB, RB, cyclin D_1, and CDK4 were examined by Western blot and normalized to actin.

3.3. AHR is a direct target of miR-124

Using TargetScan 5.1 [24], miR-124 was predicted to target the AHR 3′-UTR (Fig. 4A). Since AHR has been shown to promote neuroblastoma cell differentiation [18,25,26], it is plausible that miR-124 may affect neuroblastoma cell differentiation by targeting on AHR. To demonstrate whether there was a direct targeting of AHR by miR-124, Pre was cotransfected with a luciferase reporter plasmid carrying 3′-UTR of AHR in the form of wild type (WT), mutation in the first four miR-124 targeting site (mut), and deletion of the seed region binding site (del) (Fig. 4A). The luciferase assay was performed in a neuroblastoma cell line SK-N-SH and a lung adenocarcinoma cell line A549 lacking of endogenous miR-124 expression. In both cell lines, miR-124 only inhibited the luciferase activity by approximately 30–40% in WT AHR, compared with the activity of a negative control miRNA, which does not recognize any potential target (Fig. 4B). Next, we determined whether changes in miR-124 expression would modulate AHR expression. To this end, we overexpressed miR-124 with Pre and knocked down its levels with Inh in both SK-N-BE and SK-N-SH cell lines. The expression level of miR-124 was confirmed by qRT-PCR after transfection (Fig. 4C). The mRNA (Fig. 4C) and protein (Fig. 4D) expression level of AHR was inhibited by Pre and elevated by Inh. The results showed that miR-124 indeed regulated expression of AHR by directly targeting its 3′-UTR.

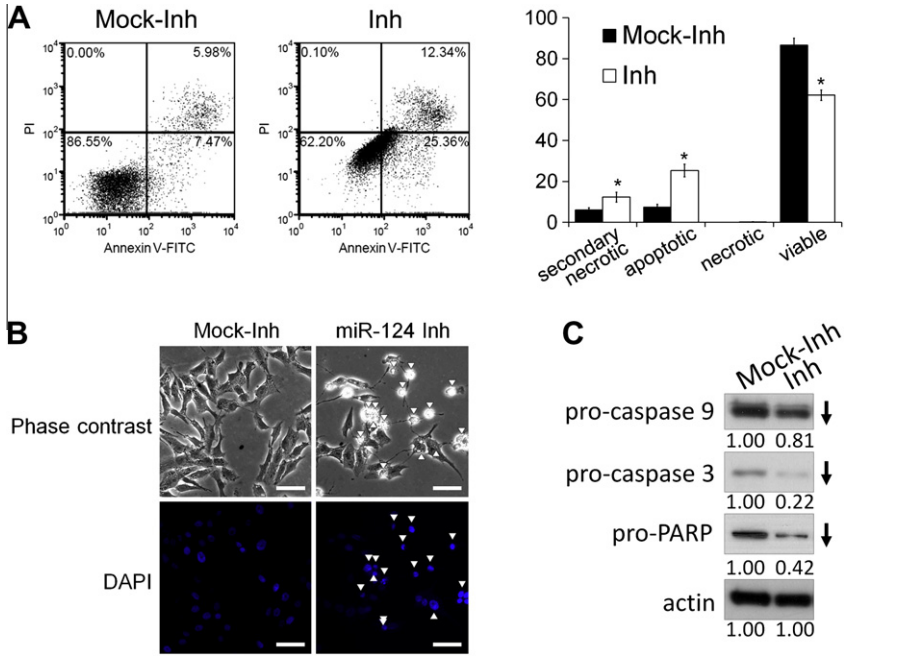

Fig. 3. Anti-miR-124 induces apoptosis in SK-N-SH cells. After transfected with miR-124 inhibitor (Inh) or the negative control inhibitor (Mock-Inh) for 72 h, cells were double-stained with annexin-V-FITC (AV) and propidium iodide (PI) for flow cytometry analysis and stained with DAPI for nucleus observation. (A) Lower left quadrant shows viable cells; lower right, AV^+/PI^- cells (early apoptosis); upper left, AV^-/PI^+ cells (necrosis); upper right, AV^+/PI^+ cells (late apoptosis). The percentage of cells in the four quadrants were quantified and plotted. $^*P < 0.05$. (B) Cells transfected with Inh showed shrinking morphology and condensed chromosome in nuclei were indicated by white arrows. (C) The protein level of procaspase-9 and 3 and pro-PARP were examined by Western blot and normalized to actin.

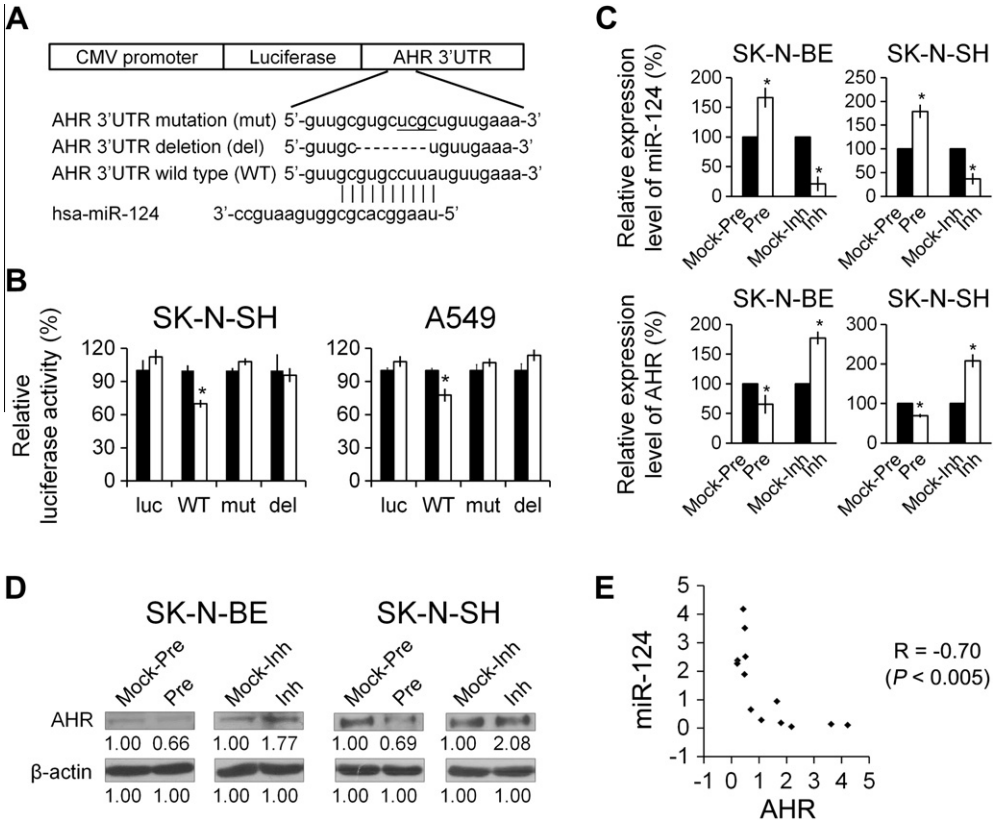

Fig. 4. Down-regulation of AHR by miR-124 at mRNA and protein levels via direct targeting of 3′-UTR. (A) The full length wild type AHR 3′-UTR (WT), seed region mutated (mut) and deleted (del) AHR were cloned into pMIR reporter. (B) Target of AHR 3′-UTR by miR-124 were examined by luciferase reporter assay in SK-N-SH and A549 cells. Both SK-N-SH and A549 cells were cotransfected with miR-124 precursor (Pre, open bar) or the scramble negative control (Mock-Pre, solid bar). Levels of (C) miR-124 expression and *AHR* mRNA and (D) AHR protein were examined after transfected with Pre, Mock-Pre, miR-124 inhibitor (Inh) or negative control inhibitor (Mock-Inh). (E) The mRNA expression levels of *AHR* and miR-124 was assessed by qRT-PCR from thirteen neuroblastoma patients. Pearson's correlation analysis was carried out to evaluate the relationship between *AHR* and miR-124. The miR-124 and *AHR* mRNA expression levels were normalized to snRNA *RNU6B* and *GAPDH*. AHR protein levels were normalized to actin. $^*P < 0.05$.

3.4. Clinical evidences for association between miR-124 and AHR

Since AHR is one of the targets of miR-124, we next clarify the association between miR-124 and AHR in vivo from 13 patients with neuroblastoma. We found a significantly reverse correlation between miR-124 and AHR (Fig. 4E, Pearson's correlation coefficient = −0.7 with a *P*-value less than 0.005). The results show that the expressions miR-124 and AHR are complementary in clinic and suggest that miR-124 might negatively regulate the expression of AHR in neuroblastoma cells not only in vitro but also in vivo.

3.5. Concluding remarks

To date, miR-124 is a highly conserved miRNA that specifically expressed in the nervous system of all animals studied. The role of miR-124 is not only critical in the neuronal development, but also in tumorigenesis. Recently, many reports show that AHR possesses anti-cancer effects [25,26]. These studies support our findings that inhibition of miR-124 caused increasing in AHR and resulted in cell differentiation.

In summary, we found that withdrawal of miR-124 in SK-N-SH neuroblastoma cells increases the protein expression level of AHR with followed by inhibition of cell proliferation, blockage of cell cycle at G_0/G_1 phase, differentiation in neuroblastoma cells, and occurrence in programmed cell death. Herein, we suggest that the involvement of miRNAs in neuroblastoma development can be taken into consideration when applying differentiation therapy in cancer treatments.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2011.10.025.

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