# RESEARCH ARTICLE

# miR-200a Inhibits Tumor Proliferation by Targeting AP-2γ in Neuroblastoma Cells

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## Abstract

Background: MicroRNA-200a (miR-200a) has been reported to regulate tumour progression in several tumours but little is known about its role in neuroblastoma. Our aim was to investigate the potential role and mechanism of miR-200a in neuroblastomas. Materials and Methods: Expression levels of miR-200a in tissues were determined using RT-PCR. The effect of miR-200a and shAP-2γ on cell viability was evaluated using MTS assays, and target protein expression was determined using Western blotting and RT-PCR. Luciferase reporter plasmids were constructed to confirm direct targeting. Results were reported as mean±S.E.M and differences were tested for significance using the 2-tailed Students t-test. Results: We determined that miR-200a expression was significantly lower in neuroblastoma tumors than the adjacent non-cancer tissue. Over-expression of miR-200 are reduced cell viability in neuroblastoma cells and inhibited tumor growth in mouse xenografts. We identified AP-2γ as a novel target for miR-200a in neuroblastoma cells. Thus miR-200a targets the 3'UTR of AP-2γ and inhibits its mRNA and protein expression. Furthermore, our result showed that shRNA knockdown of AP-2γ in neuroblastoma cells results in significant inhibit of cell proliferation and tumor growth *in vitro*, supporting an oncogenic role of AP-2γ in neuroblastoma. Conclusions: Our study revealed that miR-200a is a candidate tumor suppressor in neuroblastoma, through direct targeting of AP-2γ. These findings re-enforce the proposal of AP-2γ as a therapeutic target in neuroblastoma.

Keywords: miR-200a - AP-2γ - neuroblastoma - cell proliferation

## Introduction

Recently, microRNAs (miRNAs), have attracted more attention because they have regulatory roles in a broad range of biological processes, including embryogenesis, differentiation, proliferation and apoptosis, as well as in carcinogenesis (Bushati et al., 2007; Davis et al., 2010). MicroRNAs (miRNAs) are a group of endogenously expressed, non-coding small RNAs (20-25 nucleotides in length) known to negatively regulate gene expression by suppressing translation or decreasing the stability of mRNAs by directly binding to the 3'-untranslated region (3'-UTRs) of target mRNAs (Fei et al., 2013). The miR-200 family consists of 5 members (miR-200a, miR-200b, miR-200c, miR-141, and miR-429) that are are key regulators inhibitors of epithelial to mesenchymal transition, and act to maintain the epithelial phenotype by targeting the expression of the E-cadherin transcriptional repressors ZEB1 and ZEB2 (Bracken et al., 2008; Gregory et al., 2008; Korpal et al., 2008). Accordingly, the number of studies reporting down-regulation of miR-200 family expression in cancer is increasing (Dykxhoorn et al., 2009;

Adam et al., 2009; Li et al., 2009; Kong et al., 2009). In addition, members of the miR-200 family have recently been shown to affect other cell behaviors including proliferation, cell cycle and apoptosis (Du et al., 2009; Cochrane et al., 2010; Uhlmann et al., 2010). miR-200a is a miRNA family with tumor suppressive functions in a wide range of cancers, including breast cancer (Yu et al., 2013), colorectal cancer (Paterson et al., 2013), pancreatic cancer (Soubani et al., 2012), and endometrial carcinoma (Bai et al., 2013). , but by now, the role of miR-200a in neuroblastoma remained undefined.

The AP-2 family of transcription factors is involved in the regulation of embryonic development, cell proliferation and tumorigenesis. To date, five members of the AP-2 family have been identified: AP-2α, AP-2β, AP-2γ, AP-2δ and AP-2ε (Eckert et al., 2005). To date, little is known on the interaction between AP-2 transcription factors and miR-200 family; AP-2γ is a member of the developmentally regulated AP-2 transcription factor family that was originally identified from a MCF-7 cDNA library (McPherson et al., 1997). It is an important regulator of embryogenesis and tumor

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regenesis (Pellikainen et al., 2007). Recently, AP-2γ has been implicated in mammary carcinogenesis, where it is required for oestrogen mediated proliferation of breast cancer cells and tumour growth (Woodfield et al., 2007). This is in concordance with high expression level of the gene in breast tumours which is correlated with resistance to anti-oestrogen, oestrogen deprivation, and poor patient survival outcome (Gee et al., 2009). AP-2γ has been characterized as a transcriptional activator in breast cancer cells, directly upregulating genes such as HER2 (Perissi et al., 2000). But for the role of AP-2γ in neuroblastoma in neuroblastoma remained undefined.

Therefore, we proposed that miRNA might also regulate AP-2γ. Using several computational programs, we identified a potential binding site (miRNA response element, MRE) of miR-200a in the 3'UTR of the AP-2γ gene. The miRNAs miR-200a share a common seed sequence of AAUACUG and are highly expressed in endometrial cancers than in normal endometrial tissues (Park et al., 2008; Lee et al., 2011). In this report, We determined that miR-200a expression was significantly lower in neuroblastoma tumors than the adjacent non-cancer tissue. Over-expression of miR-200 are reduced cell viability in neuroblastoma cells and inhibit tumor growth in mouse xenografts. We identified AP-2γ as a novel target for miR-200a in neuroblastoma cell. and miR-200a targets the 3'UTR of AP-2γ and inhibits its mRNA and protein expression. Furthermore, our result showed that shRNA knockdown of AP-2γ in neuroblastoma cell results in significant inhibit of cell proliferation and tumor growth *in vitro*, supporting an oncogenic role of AP-2γ in neuroblastoma.

## Materials and Methods

### Cell lines and culture

The neuroblastoma cell line SK-N-AS (American Type Culture Collection) was maintained in DMEM media (Life) containing 10% fetal bovine serum (Gibco), and 100U penicillin/streptomycin (life) at 37°C with 5% $CO_2$.

### Luciferase assays

The double firefly luciferase reporter plasmids contained the entire wild-type 3'UTR of AP-2γ or a mutated derivative deleted for the 7 bp seed sequence deleted constructed and purchase from Gene Copoeia company. The plasmids (1μg) were cot-ransfected into SK-N-AS cells either with 20 nM of as-miR-200a ( Ambion), miR-200a (Dharmacon) or non-targeting control (NC) (Ambion) using Lipofectamine™ 2000 (Life) to the cells. The AP-2γ luciferase activity of the luciferase vector construct only (EV) was normalized to one and the other transfection combinations were compared with EV. Cells were harvested 48 h after transfection and assayed using the Dual Luciferase Reporter Assay System (Promega).

### Cell viability assays

Viability of cells was measured by MTS-formazan reduction using 6-well plates or 24-well plates Aqueous One Solution Cell Proliferation Assay (Promega, USA) at 48 hr and 72 hr post transfection with 20nM miRNAs,

antisense (as)-miRNAs or siRNAs using the siPORT NeoFX transfection agent (Ambion). Absorbance was measured at 490 nm using a Synergy Multi-Mode PlateReader (Boitek, USA).

### RNA analysis

RNA was purified by the Trizol method (Life), treated with RNase-free DNase (Ambion), and reverse transcribed with using SuperScript III RT (Life) to generate cDNA. RNA levels were determined by SYBR Green-based real-time PCR of the cDNA, with the level of GAPDH used as a loading control. Each sample was run in triplicate, and the data represent the mean±SD of three independent experiments. The primers used were the following: AP-2γ5'-TGACCAAGAACCCTCTGAACCT-3', 5'-CCAG GGACTGAGCAGAAGAC-3' and β-actin used as an internal standard 5'-ACCCAGAAGACTGTGGATGG-3' and 5'-ACGCCTGCTTCACCACCTTC-3'. The resulting products were electrophoresed on 2% agarose gels. The bands and Band density were visualized using the KODAK Image Station 4000MM Digital Imaging System (Stratagene, USA).

### Western blotting

Total protein (60μg) from neuroblastoma cells was isolated by standard methods in RIPA buffer (25 mM Tris.HCl pH7.6, 150 mM NaCl, 1% NP-40, 1% sodium, deoxycholate, 0.1% SDS), electrophoretically separated, and transferred to nitrocellulose filters. The filters were incubated overnight at 4°C with anti-AP-2γ (1:500; Abcam.) and anti-β-actin (1:2000; Sigma). The density of the bands was quantified and normalized by the loading control β-actin .

### Xenograft experiments

SK-N-AS cells ($5 \times 10^6$) were injected into the right flank of nu/nu mice (Guangdong Medical Laboratory Animal Center), all of which developed tumors in 12 days with size of 100 mm3. The mice were randomly distributed into groups (5 mice per group) and treated with miR-200a (100 nM), as-miR-200a (100 nM), miRNA negative control (miR-NC; 100 nM), AP-2γshRNA (100 nM). All treatments were given by intraperitoneal (i.p) injection every four days. Tumor volumes were monitored every four days. Tumors were harvested on day 36 for weights and mRNA analysis of AP-2γ. All mouse experiments were performed according to the Institutional Animal Care and Use Committee procedures and guidelines .

### Statistical analysis

All statistical analysis was performed using SPSS16.0 software (GraphPad Software, USA) A P-value of <0.05 was regarded as statistically significant (*$p < 0.05$).

## Results

### miR-200a inhibits AP-2γ expression in neuroblastoma cell

We examined whether AP-2γ is a direct target of miR-200a in a cancer Cells. Luciferase reporter plasmids containing the wild-type 3'UTR sequence of AP-2γ (WT) or a deletion mutant (lacking the 7-bp seed sequence) were
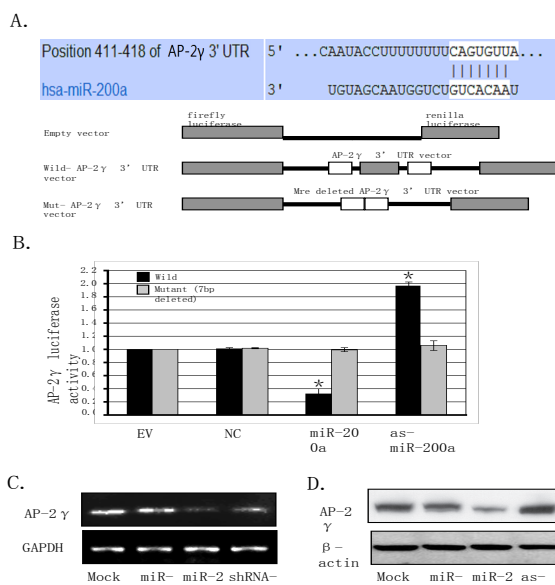
**Figure 1. miR-200a Inhibits AP-2γ Expression in Neuroblastoma Cell. A)** Complementarity sequence (vertical lines showing the seed sequence between positions 411-418) between miR-200a and AP-2γ. **B)** Luciferase activity of reporters containing the wild-type or mutant type (7-bp deleted 3'UTR of AP-2γ ) when 48h after transfection with miR-200a, antisense against miR-200a (as-miR-200a) or miR negative control (NC) or non-transfected cells (EV). **C)** AP-2γ mRNA levels in SK-N-AS cells transfected with miR-200a or AP-2γ shRNA .;The GAPDH serve as a loading control. **D)** Western blot showing AP-2γ protein levels in cells transfected with the indicated RNAs; levels of β-Actin serve as a loading control

transfected into the SK-N-AS neuroblastoma cancer cell line with miR-200a or an anti-sense RNA against miR-200a (as-miR-200a). The AP-2γ luciferase activity of the wild-type reporter is reduced 3-fold upon miR-200a overexpression, whereas it is increased by 60% upon miR-200a inhibition (Figure 1B). In contrast, no changes in AP-2γ luciferase activity are observed in the mutant reporter plasmid upon overexpression of miR-200a or as-miR-200a. As expected, Overexpression of miR-200a results in decreased levels of AP-2γ mRNA (Figure 1C). In addition, AP-2γ protein levels are decreased upon overexpression of miR-200a and increased upon addition of antisense against miR-200a (Figure 1D). Thus, miR-200a inhibits AP-2γ expression in neuroblastomas cells.

*miR-200a inhibits neuroblastoma cell proliferation in vitro and tumor growth in mouse xenografts*

We investigated the role of miR-200a in neuroblastoma cell proliferation by overexpressing either miR-200a or its antisense RNA. Overexpression of miR-200a inhibits cell growth, whereas overexpression of as-miR-200a increases cell proliferation (Figure 2A). More importantly, in mouse xenografts involving the neuroblastoma cell line, administration of miR-200a, but not a control miRNA, strongly reduces tumor growth, whereas tumor growth is enhanced by treatment with as-miR-200a (Figure 2C, 2D and 2E). These observations are indicative of a tumor suppressive role for miR-200a in neuroblastomas. Lastly, in tumors generated by injection of SK-N-AS cells in nude mice, AP-2γ mRNA expression is reduced 3-fold in tumors
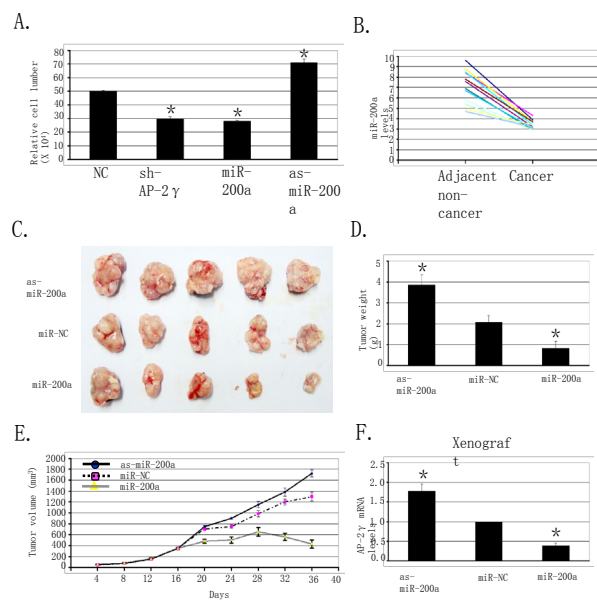


**Figure 2. miR-200a Inhibits Neuroblastoma Cell Proliferation *in vitro* and Tumor Growth in Xenografts. A)** Relative number of viable SK-N-AS cells that were transfected with the indicated RNAs for 48 hours and then allowed to grow for an additional 24 hours. NC indictes untreated (no siRNA). **B)** miR-200a RNA levels in neuroblastoma and adjacent non-cancer tissues from 12 patients, with each line representing an individual patient. **C)** Tumor growth of mouse xenografts containing neuroblastoma (SK-N-AS) cells after intraperitoneal treatment with miR-200a, as-miR-200a or control miRNA on the indicated number of days after the initial injection of cancer cells. **D)** Tumor weights (mean±SD) in mouse xenografts containing SK-N-AS cells that were or were not treated with miR-200a, as-miR-200a or control miRNA (n=5). **E)** Tumor volume (mean±SD) in mouse xenografts containing neuroblastoma SK-N-AS cells that were or were not transfected with the indicated RNAs. **F)** AP-2γ mRNA levels in mouse xenografts that are or are not injected with miR-200a, as-miR-200a or control miRNA

injected intra-tumorally with miR-200a, but not with the control miRNA (Figure 2F). Thus, miR-200a inhibits AP-2γ expression in neuroblastomas mouse xenografts.
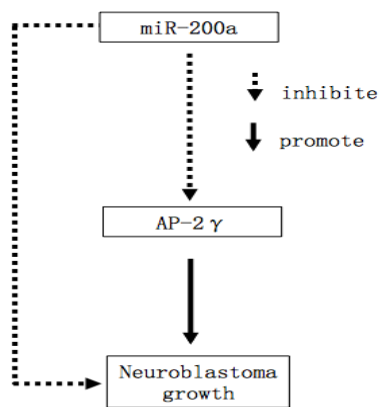
*miR-200a levels are reduced in neuroblastoma tissues*

To examine whether the tumor-suppressor effects of miR-200a in neuroblastoma cell lines are relevant to the human disease, we measured miR-200a RNA levels in tissue samples from human patients. In all 12 cases tested, miR-200a levels in neuroblastoma tissue were 2 fold lower than in the adjacent non-cancer tissue (Figure 2B). Thus, reduced levels of miR-200a are associated with neuroblastoma progress.

AP-2γ plays a tumor-promoting role in neuroblastoma The functional role of AP-2γ activation during cancer development remains controversial, in part because the experiments have been performed with AP-2γ agonists or antagonists that may mediate their effects through non-AP-2γ mechanisms. To avoid this problem, we inhibited expression of the AP-2γ gene by an shRNA and found that this resulted in reduced AP-2γ protein expression (Figure 3A)in neuroblastoma cell. In accord with these experiments, transfect to these neuroblastoma cells with the AP-2γ shRNA inhibits cell growth *in vitro*

A.

AP-2γ

β-acti

Mock    shRNA-NC  shRNA-AP-2γ

B.



C.

shRNA-NS

shRNA-AP-2γ

D.



E.
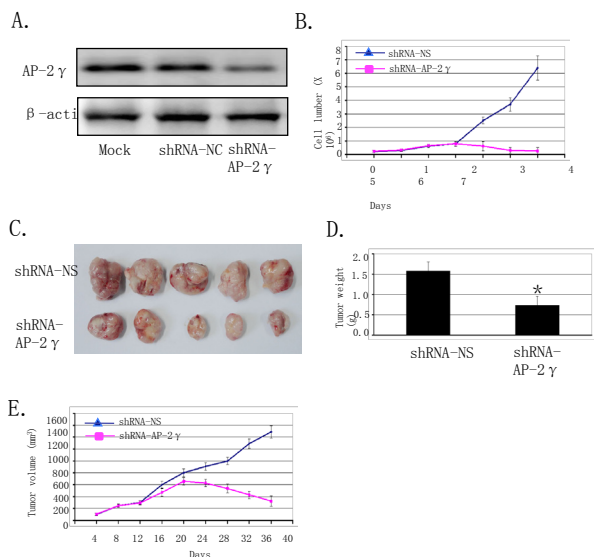


**Figure 3. AP-2γ Plays a Tumor Promoting Role in Neuroblastoma. A)** AP-2γ protein levels in SK-N-AS cells that were or were not treated with AP-2γ shRNA or control miRNA. **B)** Relative number of viable SK-N-AS cells that were transfected with AP-2γ shRNA or control miRNA for 48 hours and then allowed to grow for an additional 24 hours. **C)** Tumor growth of mouse xenografts containing neuroblastoma (SK-N-AS) cells after intraperitoneal treatment with AP-2γ shRNA or control miRNA on the indicated number of days after the initial injection of cancer cells (n=5). **D)** Tumor weights (mean±SD) in mouse xenografts containing neuroblastoma SK-N-AS cells that were or were not treated with AP-2γ shRNA. **E)** Tumor volume (mean±SD) in mouse xenografts containing neuroblastoma SK-N-AS cells that were or were not treated with AP-2γ shRNA



**Figure 4. In Neuroblastoma, miR-200a levels are Reduced, and miR-200a Acts as a tumor Suppressor, Providing an Independent Line of Evidence that Reduction of AP-2γ Levels is Associated with Reduced Neuroblastoma Cell Proliferation and tumor Growth.** Collectively these results strongly suggest that AP-2γ has a growth-stimulating and tumor-promoting role in neuroblastoma

(Figure 3B) and in mouse xenografts (Figure 3C, 3D and 3E). Lastly, as mentioned above, miR-200a levels are reduced in neuroblastoma tissues, and miR-200a acts as a tumor suppressor, providing an independent line of evidence that reduction of AP-2γ levels is associated with reduced cancer cell proliferation and tumor growth. Collectively these observations strongly suggest that AP-2γ has a growth-stimulating and tumor-promoting role in neuroblastoma .

## Discussion

The dys-regulation of miRNAs is a key mechanism involved in the pathogenesis of neuroblastoma, with several tumor suppressor miRNAs having been identified (Jian et al., 2012; Lynch et al., 2012; Chen et al., 2013; Qiao et al., 2013; ). Here we determined that expression of miR-200a, another potential tumor suppressor in neuroblastoma, was significantly lower in tumors than that adjacent non-tumor tissue in our patient cohort. Similarly, miR-200a expression has been associated with improved patient survival in other cancers, including breast and colorectal cancer, suggesting a wider tumor suppressor role for this miRNA. Ectopic expression of miR-200a decreased breast cancer cell proliferation and increased apoptosis in colorectal cancer cell lines (Uhlmann et al., 2010; Stratmann et al., 2011). Similarly, we observed a significant increase in cell proliferation in neuroblastoma cell lines following over-expression of as-miR-200a *in vitro* and inhibits tumor growth in mouse xenografts.

To date, the characterisation of miR-200a function has not been fully extensive, although several targets have been identified with a key role in differentiation , cell apoptosis, migration, Invasion, EMT and survival pathways, including Grb2, BCL2, E-cadherin, ZEB1, SIP1, β-catenin and TGF-β (Bracken et al., 2008; Korpal et al., 2008; Saydam et al., 2009; Lynch et al., 2012; Su et al., 2012; Liu et al., 2013). We have identified a novel target of miR-200a, AP-2γ, a member of the developmentally regulated AP-2 transcription factor family that was originally identified from a MCF-7 cDNA library (McPherson et al., 1997). It is an important regulator of embryogenesis and tumor regenesis (Pellikainen et al., 2007). Recently, AP-2γ has been implicated in mammary carcinogenesis, where it is required for oestrogen-mediated proliferation of breast cancer cells and tumour growth (Perissi et al., 2000; Woodfield et al., 2007). However, a recent report showed that AP-2γ can also function as a transcriptional repressor, directly inhibiting the expression of the growth inhibitory CDKN1A gene in breast cancer cells (Williams et al., 2009). AP-2γ has been associated with survival in several other cancer types including glioblastoma, malignant melanoma and breast cancer (Hasleton et al., 2003;Gabriely et al., 2011; Osella-Abate et al., 2012). This is consistent with our findings. Inhibition of AP-2γ results in reduced cell growth *in vitro* and tumor growth in mouse xenografts.

In conclusion, Our study identifies a molecular pathway important for growth and tumor progression of neuroblastomas cells (Figure 4). Specifically, miR-200a functions as a tumor suppressor by directly inhibiting the expression of AP-2γ. Inhibition of AP-2γ by miR-200a or sh-RNA reduces the cell proliferation and tumor growth. Furthermore, inhibition of AP-2γ results in reduced cell growth *in vitro* and tumor growth in mouse xenografts, indicating that AP-2γ functions as a tumor-promoting factor in neuroblastomas, but the mechanism of AP-2γ tumor-promoting is not known .Our results do not exclude additional cancer-inhibit functions for miR-200a or for AP-2γ tumor-promoting in neuroblastoma.

## Acknowledgements

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