

## The role of miR-25 in pediatric neuroblastoma.

Chuantao Ren<sup>1,2#</sup>, Chengfei Li<sup>2#</sup>, Hongtao Zhang<sup>2</sup>, Lijian Gao<sup>3</sup>, Aiwu Li<sup>1\*</sup>

<sup>1</sup>Department of Pediatric Surgery, Qilu Hospital of Shandong University, Jinan, PR China

<sup>2</sup>Department of Pediatric Surgery, Dezhou People's Hospital, Dezhou, PR China

<sup>3</sup>Department of Urology, Dezhou People's Hospital, Dezhou City, Dezhou, PR China

#These authors contributed equally to this study.

### Abstract

**Aims:** This study is to investigate the role of miR-25 in pediatric neuroblastoma.

**Methods:** The expression of miR-25 in pediatric neuroblastoma tissues and cell lines was determined by quantitative PCR. SH-SY5Y and IMR32 cells were transfected with miR-25 mimics and inhibitor, respectively. The effect of miR-25 on pediatric neuroblastoma was analysed by CCK-8 assay, flow cytometry and Transwell assay. Western blot was performed to detect the protein expression of *TOB1*, a potential target gene of miR-25. Dual luciferase reporter assay was carried out to determine the direct binding between miR-25 and *TOB1*.

**Results:** The expression of miR-25 was upregulated in pediatric neuroblastoma tissues and positively correlated to distant metastasis and clinical stages. The expression of miR-25 in the metastases derived SH-SY5Y cells was higher than that in the primary tumor derived IMR32 cells. *In vitro* experiments showed that over expression of miR-25 promoted the proliferation, G<sub>1</sub>/S phase transition and metastasis of IMR32 cells, while down regulation of miR-25 inhibited the proliferation, G<sub>1</sub>/S phase transition and metastasis of SH-SY5Y cells. Upregulation of miR-25 decreased the expression of *TOB1* in IMR32 cells, while down-regulation of miR-25 increased the expression of *TOB1* in SH-SY5Y cells. Dual luciferase reporter assay confirmed that miR-25 could bind to the 3'-UTR of *TOB1*, indicating that *TOB1* is the direct target gene of miR-25.

**Conclusions:** The miR-25 expression in neuroblastoma and neuroblastoma cells is upregulated. MiR-25 can promote the proliferation, invasion and metastasis of neuroblastoma cells by regulating *TOB1* expression.

**Keywords:** miR-25, Pediatric neuroblastoma, *TOB1*, Invasion, Metastasis.

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### Introduction

Neuroblastoma (NB) is the most common tumor in children with an incidence rate of 10% [1], which is second only to leukemia and central nervous system tumors [2]. The prognosis of NB is poor [3]. NB belongs to neuroendocrine tumors, which is originated from the neural crest progenitors in the sympathetic nervous system, and usually occurs in the nerve tissues of the adrenal gland, neck, chest, and abdomen [4]. NB has the clinical features of high malignancy and heterogeneity, prone to metastasis and relapse and poor prognosis [5]. The clinical treatment effect for NB is limited and the patients' 5 y survival rate was only 40%, except for a few patients who can spontaneously regress or completely resected at early stage [6,7]. Genetic abnormalities, such as V-Myc Avian Myelocytomatosis Viral Oncogene neuroblastoma derived homolog (MYCN) abnormal expression and missing of tumor suppressor gene sequences lp36.1 and lp36.2, are closely

related to child NB classification, treatment and prognosis [8,9]. In addition, some new biomarkers were reported to be associated with the survival of NB. For instance, upregulation of *ARID1B* gene in NB patients suggests a worse prognosis [10]. The expression level of miR-149 was negatively correlated with the prognosis of NB patients and had potential clinical diagnostic values [11]. However, the molecular mechanisms of the occurrence and development of NB are still unclear.

MiRNA is a class of small non-coding RNA molecules with lengths of 18-22 nt and can bind to the 3'-UTR region of mRNA to inhibit its translation and regulate the protein expression [12,13]. Evidence shows that miRNA is abnormally expressed in NB, and many miRNA molecules are involved in the proliferation, invasion, metastasis and apoptosis of NB, suggesting that miRNAs play important roles in NB [14,15]. For example, Li et al. found that miR-1303 could promote SH-

SH-SY5Y cell proliferation and metastasis by targeting *GSK3 $\beta$*  and *SFRP1* genes [14]. Li et al. indicated that miR-21 could promote NB cell proliferation and metastasis by regulating *CHL1* gene [13]. MiR-25 belongs to the miR-106b-25 family and locates on human chromosome 7. It is upregulated in many tumors and plays important regulatory roles in the tumor occurrence [16-18]. Petrocca et al. found that miR-106b-25 cluster (miR-106b, miR-25 and miR-93) was highly expressed in gastric cancer cells and regulated by E2F1 regulator. Meanwhile, miR-106b and miR-93 can regulate the expression of E2F1, thereby forming a negative feedback path [19]. In Non-Small Cell Lung Cancer (NSCLC), the expression of miR-25 is upregulated, which promotes the proliferation, invasion and metastasis of tumor cells by targeting F-Box and WD Repeat Domain Containing 7 (*FBXW7*) gene, playing the oncogene functions [20]. These researches indicate that miR-25 plays some important roles in a variety of tumors, and closely related to tumor proliferation, invasion and metastasis. However, the role and mechanism of miR-25 in NB is still unclear.

In this study, the effect and mechanism of miR-25 in NB was investigated. The real-time quantitative PCR, Western blot, Transwell assay and flow cytometry were used.

## Materials and Methods

### Collection of neuroblastoma tissue samples

Fresh NB tissues were collected from 34 patients with NB between May, 2012 and December, 2015. Among these patients, 16 cases had distant metastasis, 22 cases were male and 12 cases were female. The average age of patients was 5.4 y old. All cases were diagnosed as NB by clinical, imaging and histopathological diagnosis. No patient was subjected to radiation therapy before surgery. The patients with stage III NB received chemotherapy and partially relieved before surgery, which complied with the surgical guidelines. NB tissues with 1 cm in diameter were taken during the surgery. The corresponding tumor adjacent tissue was taken 5 cm away from the cancer tissue. The clinical information and pathological data of the patients were collected. Prior written and informed consent was obtained from every patient and the study was approved by the ethics review board of Shandong University.

### Cell culture

NB cell lines SH-SY5Y (metastatic tumor) and IMR-32 (primary tumor) were purchased from Shanghai cell bank of Chinese Academy of Sciences (Shanghai, China). Both cells were cultured in DMEM complete medium at 37°C under an atmosphere containing 5% CO<sub>2</sub>. Cell medium was changed every two days, and the cells were passaged when the confluency reached 80-90%.

### RNA extraction and quantitative RT-PCR

The NB tissues and adjacent tissues were frozen by liquid nitrogen and ground into powder. The tissues were lysed by Trizol<sup>®</sup> isolation reagent, and the total RNAs were extracted by phenol chloroform method. The RNA concentration was determined by electrophoresis and the A260/A280 ratio was measured by UV spectrophotometer to determine RNA quality. RNA (1  $\mu$ g) was then reverse transcribed into cDNA.

To detect miR-25 expression, RT-qPCR was performed. The reaction system (20  $\mu$ L) contained 10  $\mu$ L qRT-PCR-Mix, 0.5  $\mu$ L upstream primer (miR-25, 5'-GCGGAGACTTGGGCAATTG-3'), 0.5  $\mu$ L downstream universal primer (provided by the kit), 3  $\mu$ L cDNA and 6  $\mu$ L ddH<sub>2</sub>O. The reaction protocol was initial denaturation at 95°C for 10 min; 40 cycles of 95°C for 1 min and 60°C for 30 s (iQ5; Bio-Rad, Hercules, CA, USA). The 2<sup>- $\Delta\Delta$ Ct</sup> method was used to calculate the relative expression of miR-25 against internal reference (U6, Forward: CTCGCTTCGGCAGCACA; Reverse: AACGCTTCACGAATTTGCGT). Each sample was tested in triplicate.

### Transfection of miR-25 mimics and inhibitor into NB cells

The NB cell lines SH-SY5Y and IMR-32 were seeded in 24-well plates. MiR-NC and miR-25 inhibitor were transfected into SH-SY5Y, while miR-25 miR-NC and miR-25 mimics were transfected into IMR-32. Briefly, 1.25  $\mu$ L miR-25 (20  $\mu$ M) mimics/inhibitor, 2  $\mu$ L lipo2000 and 50  $\mu$ L Opti Memi were mixed and added to cells. After 6 h of incubation, cell medium was replaced with fresh medium containing 10% FBS. After another 48 h of incubation, cells were collected for further experiments.

### CCK-8 assay

The cells were seeded in 96-well plates at a concentration of 3000/well. After transfection with miR-25 mimics or inhibitor, the cell proliferation was detected with CCK-8 assay every 24 h. CCK-8 reaction solution was added and incubated for 30 min, and then the absorbance at 450 nm was measured. Cell proliferation was monitored for three consecutive days and a proliferation curve was plotted.

### Transwell assay

A 24-well Transwell chamber (Corning Co., USA) with a diameter of 8  $\mu$ m was used in this experiment. To the upper wells, 200  $\mu$ L of serum free DMEM medium containing 2  $\times$  10<sup>5</sup> cells were added, while 500  $\mu$ L DMEM medium containing 10% FBS was added to each bottom well. After 24 h of incubation, the cells in the upper wells were removed. The cells in the bottom wells were fixed with 4% formaldehyde for 10 min and stained with giemsa. The cells were observed under 200X magnifications. Five views were randomly selected to count the translocated cells in order to evaluate cell metastasis.

To determine cell invasive ability, matrigel (BD biosciences, USA) was used to simulate extracellular matrix environment. The matrigel was taken out from -20°C one day before the experiment and melted at 4°C overnight. The matrigel was diluted with serum free DMEM medium at 1:2 ratio, and 100 µL solution was evenly applied in the upper wells. The chamber was put into 37°C for 60 min to solidify the gel. All operation was performed on ice, and the pipette tips were pre-cooled at 4°C. All other procedures were the same as that of the cell metastasis assay, and the results were obtained after 72 h.

### Flow cytometry

After transfected with miR-25 mimics/inhibitor 24 h,  $1 \times 10^6$  cells were taken from each group and washed with pre-cooled PBS twice. Cell cycle was analysed using a cell cycle assay kit (BD Biosciences, USA) according to the manufacturer's instruction. Briefly, 200 µL solution A was added to the cells and incubated for 10 min, and then 150 µL solution B was added and incubated for another 10 min. Finally, 120 µL solution C was added to incubate for 10 min in the dark. The cell cycle was detected by flow cytometry and the results were analyzed by Modifit software (BD Biosciences, NJ, USA).

### Western blot

Proteins were isolated from cells after lysis with RIPA. Then, the proteins were separated by SDS-PAGE and transferred to PVDF membrane. The membrane was blocked with 50 g/L skim milk for 1 h at room temperature. After that, primary antibodies for Transducer of ERBB (rabbit anti-human *TOB1* polyclonal antibody 1:1000) and mouse anti-human GAPDH monoclonal antibody (1:4000) were added and incubated at 4°C overnight. The membrane was washed with TBST (5 min  $\times$  5 times), and then HRP-labeled secondary antibodies (goat anti-mouse 1:3000, goat anti-rabbit 1:3000) were added and incubated at room temperature for 1 h. After washing with TBST (5 min  $\times$  5 times), the bands were developed with ECL luminous liquid. All antibodies were purchased from Abcam plc. (Cambridge, UK).

### Dual luciferase reporter assay

The target gene of miR-25 was predicted using the Targetscan Online Prediction Software ([www.targetscan.com](http://www.targetscan.com)). According to the bioinformatics prediction results, the wild-type and mutant seed regions of the 3'-UTR of the *TOB1* gene containing miR-25 binding site were chemically synthesized *in vitro*. After added with the cleavage sites of *Spe-I* and *Hind-III* at both ends, they were cloned into the pMIR-REPORT luciferase reporter plasmid. The plasmid containing wild-type and mutant 3'-UTR sequence were transfected together with miR-25 mimics into HEK293T cells using the liposome method. After 24 h of incubation, each group of cells was lysed according to the manufacturer's instruction of Promega dual luciferase reporter assay kit (Promega, Madison, WI, USA). The luminance was recorded by GloMax 20/20 luminometer, using renilla as the internal standard.

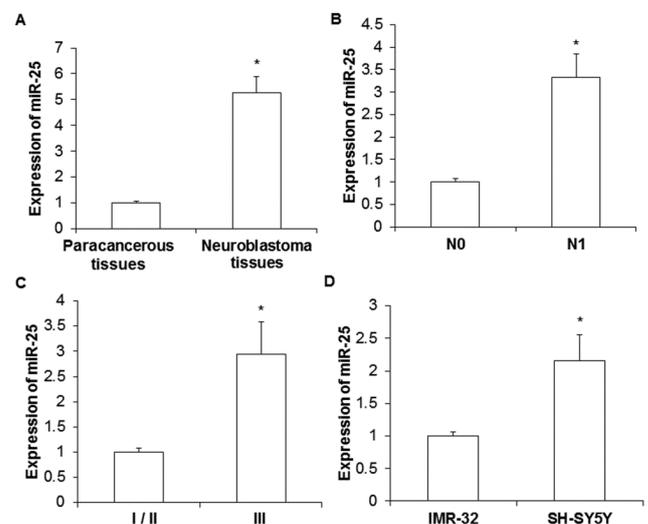
### Statistical analysis

The statistical analysis was performed with the statistical software SPSS 17.0. All data was expressed as mean  $\pm$  Standard Deviation (SD). The t-test was used for comparisons between groups, and a p value less than 0.05 was considered as statistically significant.

## Results

### The expression of miR-25 in NB tissues and cells and the clinical significance

To determine the expression level of miR-25, real-time quantitative PCR was performed. As shown in Figure 1A, the expression of miR-25 in NB tissues was significantly increased compared to that in paracancerous tissues ( $P < 0.01$ ). The miR-25 expression in the tissues of NB patients with distant metastasis ( $N_1$ ) was significantly higher than that of NB patients without metastasis ( $N_0$ ,  $P < 0.05$ ) (Figure 1B). For the comparison between different clinical stages, it was found that miR-25 expression in the tissues of stage III NB was significantly higher than those in the tissues of stage I/II NB ( $P < 0.05$ ) (Figure 1C). In addition, the expression of miR-25 in the metastases-derived NB cell line SH-SY5Y was obviously higher than that in the primary tumor derived IMR-32 cells ( $P < 0.05$ ) (Figure 1D).

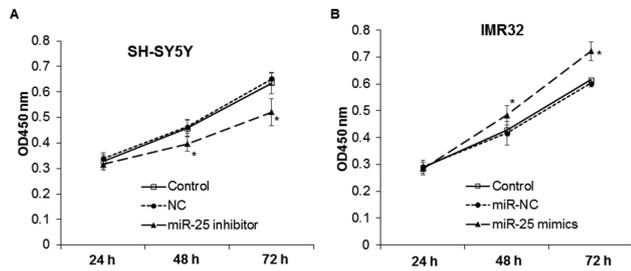


**Figure 1.** The expression of miR-25 in neuroblastoma tissues and cell lines. The expression of miR-25 was determined by q-PCR in (A) NB and adjacent tissues; (B) NB tissues with different metastasis degrees; (C) NB tissues at different clinical stages; and (D) different NB cell lines. Compared to the corresponding control group, \* $P < 0.05$ .

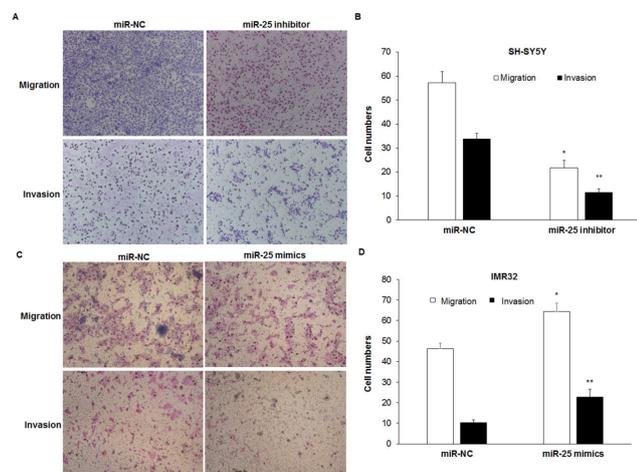
### Effect of miR-25 on the proliferation of NB cells

To determine the effect of miR-25 on cell proliferation, CCK-8 assay was performed. Compared with control and NC, the proliferation of NB cell line SH-SY5Y was significantly reduced after transfected with miR-25 inhibitor ( $P < 0.05$ ) (Figure 2A). This indicates that down-regulation of miR-25

may inhibit the proliferation of NB cell SH-SY5Y. On the other hand, expression of miR-25 mimics in IMR-32 cells significantly promoted cell proliferation ( $P < 0.05$ ) (Figure 2B).



**Figure 2.** The effect of miR-25 on the proliferation of NB cells. The cell proliferation was measured by CCK-8 assay after the cells were transfected with (A) miR-25 inhibitor or (B) mimics. Compared to the NC group, \* $P < 0.05$ .



**Figure 3.** The effect of miR-25 on the invasion and metastasis of NB cells was measured by Transwell assay. (A) Invasion and metastasis of SH-SY5Y cells after transfected with miR-25 inhibitor. (B) The number of invasion and metastasis SH-SY5Y cells after transfected with miR-25 inhibitor. (C) Invasion and metastasis of IMR-32 cells after transfected with miR-25 mimics. (D) The number of invasion and metastasis IMR-32 cells after transfected with miR-25 mimics. Compared to the NC group, \* $P < 0.05$ .

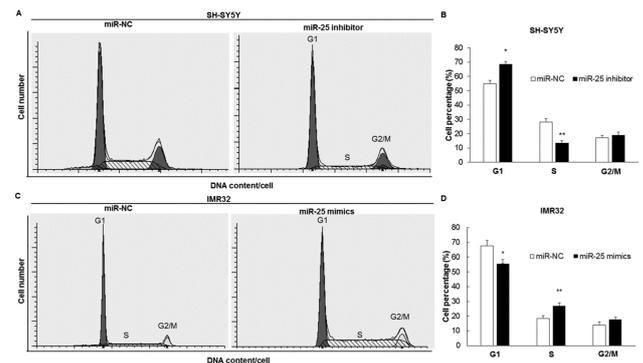
### Effect of miR-25 on the invasion and metastasis of NB cells

Transwell assay was performed to determine cell invasion and metastasis after transfection with miR-25 inhibitor or mimics. The metastasis ability of SH-SY5Y cells was significantly inhibited after transfected with miR-25 inhibitor, as the number of cells migrated through well membranes was less than that in the NC group ( $57.3 \pm 4.5$  vs.  $21.7 \pm 3.2$ ) ( $P < 0.05$ ) (Figures 3A and 3B). The invasion experiment results showed that the number of SH-SY5Y cells invaded the Transwell membranes decreased ( $33.8 \pm 2.34$  vs.  $11.5 \pm 1.6$ ) ( $P < 0.05$ ). However, when miR-25 expression in IMR32 cells was increased, the metastasis of cells enhanced, as the number of cells migrated the well membranes was higher than that of the NC group ( $64.3 \pm 4.2$  vs.  $46.3 \pm 2.6$ ) ( $P < 0.05$ ) (Figures 3C and 3D). The invasion experiment results showed that the number of cells

invaded the well membranes in the miR-25 mimics transfection group was higher than that in the NC group ( $22.7 \pm 3.9$  vs.  $10.3 \pm 1.25$ ) ( $P < 0.05$ ).

### MiR-25 promotes NB cell G1/S phase transition

Flow cytometry was used to detect the effect of miR-25 on the cell cycle transition of SH-SY5Y and IMR-32 cells. The results showed that G<sub>1</sub>/S phase arrest occurred in the SH-SY5Y cells of the miR-25 inhibitor group (Figures 4A and 4B) ( $P < 0.05$ ), while G<sub>1</sub>/S phase transition was promoted in the IMR-32 cells of the miR-25 mimics group ( $P < 0.05$ ) (Figures 4C and 4D).



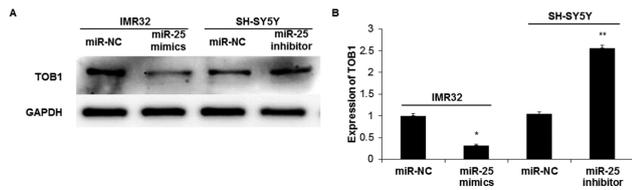
**Figure 4.** The effect of miR-25 on the cell cycle of NB cells was detected by flow cytometry. (A) The effect of cell cycle of SH-SY5Y cells after transfected with miR-25 inhibitor. (B) Percentage of cells at each cell cycle phase after transfected with miR-25 inhibitor. (C) The effect of cell cycle of IMR-32 cells after transfected with miR-25 mimics. (D) Percentage of cells at each cell cycle phase after transfected with miR-25 mimics. Compared to the NC group, \* $P < 0.05$ ; \*\* $P < 0.01$ .

### The expression of TOB1 protein

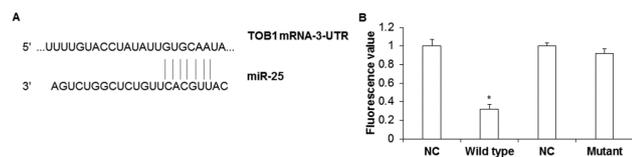
Bioinformatics predicted that the proliferation-related gene *TOB1* was one of the target genes of miR-25. To further verify this, Western blot was used to detect *TOB1* protein expression. The results showed that the expression of *TOB1* in IMR-32 cells was significantly decreased when miR-25 was overexpressed ( $P < 0.05$ ) (Figures 5A and 5B). On the contrary, when miR-25 was down-regulated, the expression of *TOB1* in SH-SY5Y cells significantly increased ( $P < 0.05$ ).

### Dual luciferase reporter assay

To determine whether *TOB1* gene was the target gene of miR-25, dual luciferase reporter assay was performed. The binding sequence of miR-25 on *TOB1* gene was shown in Figure 6A. The results showed that the fluorescence value of the wild type group was significantly lower than that in the NC group ( $P < 0.05$ ), while there was no obvious difference between the luminance in the mutant group and NC group ( $P > 0.05$ ) (Figure 6B). This indicates that miR-25 can directly bind to the 3'-UTR of wild type *TOB1*, while mutation of the 3'-UTR of *TOB1* can inhibit miR-25 binding to *TOB1*. Thus, miR-25 can directly regulate the expression of *TOB1* gene.



**Figure 5.** The effect of miR-25 on the expression of TOB1 protein. (A) The expression of TOB1 in SH-SY5Y and IMR-32 cells was detected by Western blot after transfected with miR-25 mimics and inhibitor, respectively. GAPDH was used as the internal standard. (B) Quantification of the expressions of TOB1 protein in the corresponding groups. Compared to the NC group, \* $P < 0.05$ ; \*\* $P < 0.01$ .



**Figure 6.** Dual luciferase reporter assay identified that TOB1 was the target gene of miR-25. (A) A prediction for the binding site of miR-25 to TOB1. (B) Dual luciferase reporter assay to determine the binding site of miR-25 to TOB1. Compared to the NC group, \* $P < 0.05$ .

**Discussion**

In this study, the expression of miR-25 in NB tissues was detected by quantitative PCR and found to be upregulated. The expression of miR-25 was positively correlated to the distant metastasis and clinical stage of NB. In the *in vitro* experiment, miR-25 could obviously promote NB cell proliferation, invasion and metastasis. Bioinformatics predicted that TOB1 might be the target gene of miR-25. Upregulation and down-regulation of miR-25 could cause the decrease and increase of TOB1 expression, respectively. Dual luciferase reporter assay confirmed that miR-25 could directly bind to the 3'-UTR region of TOB1 to regulate its expression. Therefore, we suppose that miR-25 may play a role as an oncogene in NB and may promote the occurrence and development of NB through inhibiting TOB1 expression.

MiR-25 belongs to the miR-106b family, which is one of the recently discovered tumor-related miRNA molecules [21]. MiR-106b-25 family locates on Human chromosome 7 and encodes three mature miRNAs, including miR-106b, miR-25 and miR-93. MiR-106b and miR-93 have similar seed sequences and cancer-promoting gene function. However, miR-25 has a different sequence, suggesting it may have different functions [22]. MiR-25 is abnormally expressed in a variety of tumors and involved in the proliferation, invasion and metastasis of tumor. For example, Xiang et al. found that miR-25 was upregulated in Non-Small Cell Lung Cancer (NSCLC), and promoted cell proliferation and metastasis by regulating the expression of FBXW7 [20]. Chen et al. indicated that miR-25 could inhibit the apoptosis of NSCLC cells by regulating the expression of regulator of G-protein signaling 3 (RGS3) [23]. Peng et al. found that miR-25 could promote the proliferation and metastasis of gliomas by target regulating

neurofilament (NEFL) gene [24]. These results demonstrate that miR-25 plays a role as oncogene. The present study indicated that miR-25 expression in NB tissues was obviously increased and positively correlated to the distant metastasis and clinical stages of the tumor. The expression of miR-25 in the metastases-derived NB cell line SH-SY5Y was obviously higher than that in the primary tumor cell line IMR32, suggesting miR-25 expression is correlated to the metastasis of NB. *In vitro* experiment showed that down-regulation of miR-25 expression could inhibit the proliferation, invasion and metastasis of SH-SY5Y, while upregulation of miR-25 could promote the proliferation, invasion and metastasis of IMR32. Although the results from cell lines could not reflect the actual status of *in vivo* tumor tissues, it still to some extent suggests that miR-25 may play an oncogene function in NB.

MiRNAs exert their biological functions through binding to the 3'-UTR region of target genes. We predicted the potential target gene of miR-25 by Targetscan (<http://www.targetscan.org>), and found that TOB1 could be the potential target gene. TOB1 belongs to the Anti-Proliferative gene (APRO) family, containing TOB/BTG domain at the N-terminus, which is the main domain to exert biological functions [25]. It can regulate cell cycle through the Ras/MAPK signal pathway, and the combination with CCR4-Caf1 to form a complex and other ways. TOB1 expression in tumor is down-regulated, which will accelerate the cell cycle and promote tumor proliferation. Current researches [26] show that TOB1 could inhibit tumor cell proliferation. TOB1 is the transducer of ErbB-2 and exert functions through their interaction [26]. Wu et al. found that TOB1 could enhance the resistance to radiation therapy of breast cancer by regulating JNK and p38 signaling pathway [27]. Li et al. indicated that miR-25 could promote the proliferation, invasion and metastasis of gastric cancer [28]. Cheet al. also reported that overexpression of TOB1 could promote the resistance to radiation therapy of bronchial epithelial cells through MAPK/ERK pathway [29]. The above results indicate that TOB1 can inhibit tumor. However, whether TOB1 is mutated and its effects on NB are still not reported. In addition, there have been no reports of TOB1 mutation in any tumors, and whether TOB1 was regulated by miR-25 has not been reported either. In this study, the results demonstrated that miR-25 could regulate TOB1 expression in NB cells. Dual luciferase reporter assay further showed that miR-25 could directly bind to the 3'-UTR region of TOB1, indicating TOB1 is the direct target gene of miR-25.

**Conclusions**

In conclusion, miR-25 is upregulated in NB and NB cells. And, miR-25 can directly target TOB1. Additionally, miR-25 could promote the proliferation, invasion and metastasis of NB cells by regulating TOB1 expression.

**Acknowledgement**

None

## Disclosures

All authors declare no financial competing interests.

All authors declare no non-financial competing interests.

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**\*Correspondence to**

Aiwu Li  
Department of Pediatric Surgery  
Qilu Hospital of Shandong University  
PR China