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Original Article

miR-483-3p promotes proliferation and migration of neuroblastoma cells by targeting PUMA

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Abstract: Neuroblastoma is the most common extra-cranial solid tumor in infants and children and accounts for about 15% of deaths from childhood cancers. MicroRNAs (miRNAs) have been shown to play an important role in several cellular processes, such as cell proliferation, apoptosis, invasion, metastasis and angiogenesis, and therefore have been implicated in cancer progression. miR-483-3p is associated with neuroblastoma and is found to function as an 'onco-miR' in some malignancies. However, its role in neuroblastoma remains poorly understood. In this study, we confirmed that miR-483-3p is overexpressed in neuroblastoma tissue when compared with normal tissue and miR-483-3p expression is also associated with tumor stage. Overexpression of miR-483-3p substantially enhanced cell proliferation, migration, and invasion of neuroblastoma cells. miR-483-3p also promoted tumor growth of neuroblastoma *in vivo*. Both *in vivo* and *in vitro* experiments showed that the tumor suppressor PUMA was a target of miR-483-3p. Furthermore, down-regulation of PUMA by small interfering RNA (siRNA) exhibited similar effects to those observed as a result of overexpression of miR-483-3p. Our results indicate that miR-483-3p could function as an 'onco-miR' in human neuroblastoma and reveal a new and potentially important target for neuroblastoma anticancer therapy.

Keywords: miR-483-3p, neuroblastoma, proliferation, metastasis, PUMA

Introduction

Neuroblastoma, derived from the neural crestin of infants and children, is one of the most common extracranial solid tumors, and accounts for 7% of childhood malignancies [1]. Invasion and metastasis are the main cause of death in neuroblastoma patients, and these processes are also the major therapeutic challenge for treatment of such cancers. Despite this, treatments have improved significantly in the last 10 years and patients now receive multimodal treatment, such as surgery, chemoradiotherapy, stem cell transplantation, and immunotherapy [2]. However, the 5-year survival rate of high-risk neuroblastoma is still very low and therefore in order to improve treatments, a better understanding of the mechanisms underlying progression and the aggressive nature of neuroblastoma, is required [3].

Insulin-like growth factor 2 (IGF2) is a member of the insulin family and acts as a fetal growth factor in many tumors, such as nephroblastoma, hepatoblastoma, rhabdomyosarcoma, and Wilms' tumor [4]. Aberrant expression of IGF2 also occurs in neuroblastoma cells, in which autocrine/paracrine effects of IGF2 have been demonstrated [5]. However, a transgenic mouse model for IGF2 overexpression exhibited many of the symptoms associated with Beckwith-Wiedemann syndrome, but did not develop tumors [6]. This would suggest that additional cofactors are likely to cooperate with IGF2 to promote cancer in humans.

MicroRNAs (miRNAs) are a class of small endogenous 21-23 nucleotide non-coding RNAs, that can bind to the 3'-untranslated region (3'-UTR) of target mRNAs, leading to translational repression or degradation. Aberrant expression of miRNAs has been detected in several human neoplasms and miRNAs were found to play a central role in all molecular pathways affecting cancer traits, such as proliferation, apoptosis, metastasis, and differentiation [7, 8]. Therefore, miRNAs could be utilized as therapeutics tar-

geting oncogenes and serving as tumor suppressors.

One particular miRNA-miR-338-3p, was found to suppress proliferation, invasion, and migration in neuroblastoma cells, by targeting PREX2a, which in turn affected the PTEN/Akt pathway [9]. Another miRNA (miR-329) suppressed growth and motility of neuroblastoma cells by targeting KDM1A, which drives tumorigenesis via activation of the Wnt/b-catenin signaling pathway [10]. miR-483, harboring in the second intron of the IGF2 locus, was found to play an important role in tumor cell proliferation, apoptosis, and metastasis [11-13]. However, there arefew reports about the precise mechanism of action and regulation of miR-483-3p in neuroblastoma.

In this study, we confirmed that miR-483-3p is upregulated in neuroblastoma tissues compared with adjacent normal tissues. miR-483-3p was found to promote cell proliferation, invasion, and migration in neuroblastoma, and suppress cell apoptosis, at least in part, through repression of PUMA expression.

Materials and methods

Tissue samples

After receiving approval by the Institutional Ethics Committee of Zhujiang Hospital of Southern Medical University, 20 fresh neuroblastoma specimens and 20 matched adjacent normal tissues from patients, which were verified by pathology and immunohistochemistry, were obtained from our department. All the pathological and clinical data, including Edmondson tumor grade, invasion, and metastasis, were recorded. According to the INSS classification, 3 patients were classified as stage 1, 4 patients as stage 2, 3 patients as stage 3, 2 patients as stage 4s, and 8 patients as stage 4. All tissue samples were immediately frozen in liquid nitrogen and stored at -80°C.

Cell culture and transfection

The human neuroblastoma cell lines (SH-SY5Y, SK-N-BE, IMR-32) and the human embryonic kidney cell line HEK-293 were obtained from the Cell Bank of Type Culture Collection of Chinese Academy of Sciences (CAS, Shanghai, China) and were maintained in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 mg/ml streptomycin (all purchased from Gibco, New York, USA) and incubated in a humidified atmosphere containing 5% CO_2, at 37°C. Transfections were performed using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions.

Cell transfection

miR-483-3p mimics, miR-483-3p inhibitors, and the relevant negative controls (which were tested to be non-functional), were synthesized by GenePharme (Shanghai, China). Small interfering RNA (siRNA) oligos targeting PUMA and negative control siRNA were also obtained from GenePharma (Shanghai, China).

The siRNA sequences to target PUMA was as follows: siRNA 1 (5'-AAACAGCCAAACTCGACCTTCTTAT-3'); siRNA 2 (5'-CAGCCAAACTCGACCTTCTTATGCA-3'); siRNA 3 (5'-CCAAACTCGACCTTCTTATGCAGAA-3').

RNA preparation and quantitative PCR

Total RNAs were isolated from cells using TRIzol reagent (Invitrogen, USA), and reverse transcriptionwasperformed using TakaraRNA PCR kit (Takara, Dalian, China), following the manufacturer's instructions. The relative level of miR-483-3p wasdetected using SYBR® Premix Ex TaqTM Kits (TaKaRa, Tokyo, Japan) on an ABI 7500 thermocycler (Applied Biosystems, Foster City, CA, USA), with U6 small nuclear RNA (snRNA) as an endogenous control. All primers were purchased from Beijing Genomics institution (Peking, China). The primer sequences for miR-483-3p were as follows: Forward 5'-ATCACTCCTCTCCTCCCGTC-3'; Reverse 5'-TATGGTTGTTCTCGTCTCCTTCTC-3'.

Cell proliferation assays

SH-SY5Y and SK-N-BE cells were seeded in 96-well plates (1×10^4 cells perwell) and treated with miR-483-3p mimics, miR-483-3p inhibitors, and the relevant negative controls. Viability was assessed using a cell counting kit (CCK-8) assay, every 24 h after transfection. CCK-8 was added to each well of the 96-well plates (10 μl) and incubated for 4 h. The optical density (OD) was detected at 450 nm using a

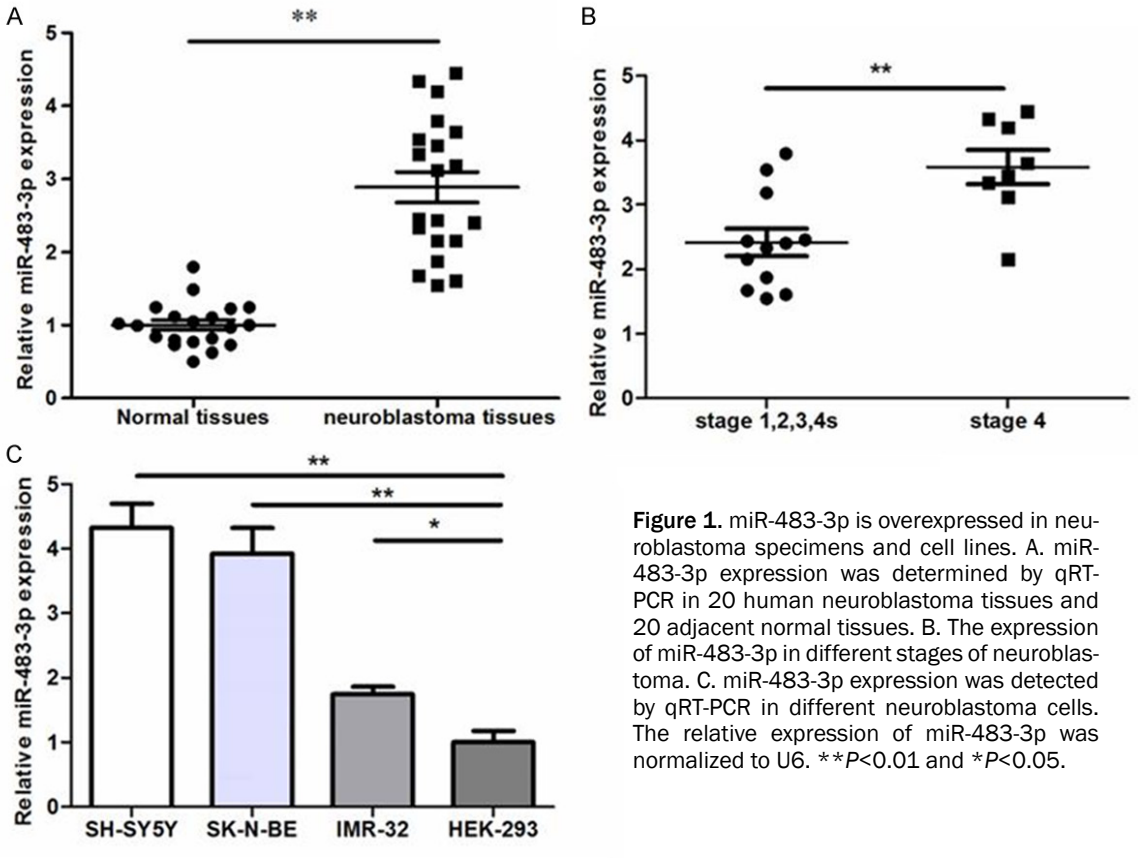

Figure 1. miR-483-3p is overexpressed in neuroblastoma specimens and cell lines. A. miR-483-3p expression was determined by qRT-PCR in 20 human neuroblastoma tissues and 20 adjacent normal tissues. B. The expression of miR-483-3p in different stages of neuroblastoma. C. miR-483-3p expression was detected by qRT-PCR in different neuroblastoma cells. The relative expression of miR-483-3p was normalized to U6. $^{**}P<0.01$ and $^{*}P<0.05$.

Microplate Reader (Bio-Tek Instruments, Winooski, VT, USA). All experiments were carried out using 8 wells for each different condition, and repeated at least three times.

Colony formation assay

Transfected SH-SY5Y and SK-N-BE cells were seeded in 6-well plates (1×10^3 cells/well), in DMEM supplemented with 10% FBS, and cultured at 37°C with 5% CO_2. When most of the clones contained more than 50 cells, the cells were washed twice with ice-cold PBS, and fixed in methanol, then stained with 0.5% crystal violet for 5 min. Colony numbers were then counted under a microscope.

Cell-cycle analysis

SH-SY5Y and SK-N-BE cells were treated with miR-483-3p mimics, miR-483-3p inhibitors, and the relevant negative controls, for 24 h. The cells were then harvested, washed twice with PBS and adjusted to a concentration of 5×10^5 cells/ml with PBS. Cell suspensions (200 µl) were then added to Tris-HCl buffer (pH 7.4) supplemented with 100 µg/mL RNase A, and labeled with propidium iodide for 15 min at room temperature, in the dark. Cells were then assayed using flow cytometry (Becton Dickinson, San Jose, CA). All data were collected, stored, and analyzed by ModFit software (Verity Software House, USA).

Cell migration and invasion assays

Cell migration and invasion assays were carried out using BD BioCoat™ Growth Factor Reduced MATRIGEL™ Invasion Chamber (BD Biosciences, San Jose, CA, USA), according to the manufacturer's instructions. SH-SY5Y and SK-N-BE cells were harvested and resuspended in serum-free medium after pre-treatment for 24 h with miR-483-3p mimics, miR-483-3p inhibitors, and relevant negative controls. Cells were then plated into the top well of a Transwell migration chamber (for a migration assay) or an extracellular matrix-coated invasion chamber (for an invasion assay). After incubation for 24 h, cells that had migrated or invaded into the lower cham-

ber were fixed with methanol, stained with 0.5% crystal violet, and photographed under the microscope.

Fluorescent reporter assays

Human PUMA (BBC-3) 3'-UTR harboring miR-483-3p potential binding sequences and mutated PUMA 3'-UTR, which was not complementary with miR-483-3p, were both synthesized by GenePharma (Shanghai, China). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega, Beijing, China). Promoter activities were expressed as the ratio between Firefly luciferase activity and Renillaluciferase activity.

Western blotting

Treated cells were washed twice with ice-cold PBS. The cells were then lysed using RIPA buffer (1 mM $MgCl_2$, 10 mMTris-HCl (pH 7.4), 1% Triton X-100, 0.1% sodiumdodecyl sulfate (SDS), 1% NP-40). Protein extracts were separated on a 10% SDS-PAGE gel and transferred to polyvinylidene fluoride (PVDF) membranes using Bio-Rad blot apparatus. Following transfer, the membranes were washed with Tris buffered saline-Tween 20 (0.1% by volume, TBS-T) 2 times, blocked with 5% milk for 4 h at room temperature and incubated with the appropriate primary antibody overnight at 4°C. After washing with TBS-T 3 times, secondary antibody conjugated to horseradish peroxidase at a 1:1500 dilution was added and incubated for 1 h at room temperature. Finally, the blot was incubated in detection reagent (Western Lightning Plus-ECL, NEL105001EA, Perkin Elmer, Waltham, USA) and exposed to a Hyperfilm ECL. β-actin (Beyotime Institute of Biotechnology, Jiangsu, China) served as a loading control and protein bands were quantified using Image J Software.

Tumor cell xenograft

All experimental procedures involving animals were approved by the Institutional Ethics Committee of Zhujiang Hospital of Southern Medical University, China. A genomic sequence encoding miR-483-3p and the negative control were amplified, and then cloned into pLenti6.3/V5-DEST Gateway Vector (Invitrogen, USA). Ten male nude mice (4-6 weeks of age, 18-20 g) were purchased from the Laboratory Animal Center of Sun Yet-Sen University. Cell suspensions of SH-SY5Y (either infected with miR-483-3p or the negative control) were injected subcutaneously at a concentration of 5×10^6 cells in 200 μl. Mice were observed daily to ensure that the injection site was healthy. Animals were sacrificed 28 days after injection and the relative expression of miR-483-3p was assayed by qRT-PCR. The expression level of PUMA was tested by Western blot, and the tumor length and width was measured every four days. The tumor volume was calculated using the formula: tumor volume =1/2 (length × width^2).

Statistical analysis

All the data were expressed as mean±standard error of the mean (SEM). Each assay was performed at least three times. Statistical analysis was performed with SPSS version 19.0 software (Chicago, IL, USA) or GraphPad Prism 5 software (San Diego, CL, USA). The difference between groups was analyzed using a Student's t-test when only two groups were present, or assessed by one-way analysis of variance (ANOVA) when more than two groups were compared. Pearson's coefficient correlation was applied for analysis of the relationship between gene expression and protein level, in neuroblastoma tissues. Statistical significance is shown as * (P<0.05) or ** (P<0.01).

Results

miR-483-3p is overexpressed in neuroblastoma tissues compared to adjacent normal tissues

To investigate the potential involvement of miR-483-3p in the process of human neuroblastoma development, expression was investigated in 20 neuroblastoma tissues and 20 matched adjacent normal tissues, by quantitative real-time PCR (qRT-PCR). We found that miR-483-3p expression was upregulated in neuroblastoma tissues compared to adjacent normal tissues (P<0.01) (**Figure 1A**). Compared with stage 1, 2, 3, or 4 s neuroblastoma tissue, stage 4 neuroblastoma tissues had higher miR-483-3p expression (P<0.01) (**Figure 1B**). We then investigated the relative miR-483-3p expression in several neuroblastoma cell lines (SH-SY5Y, SK-N-BE, IMR-32) and found that it was higher than the expression found in HEK-

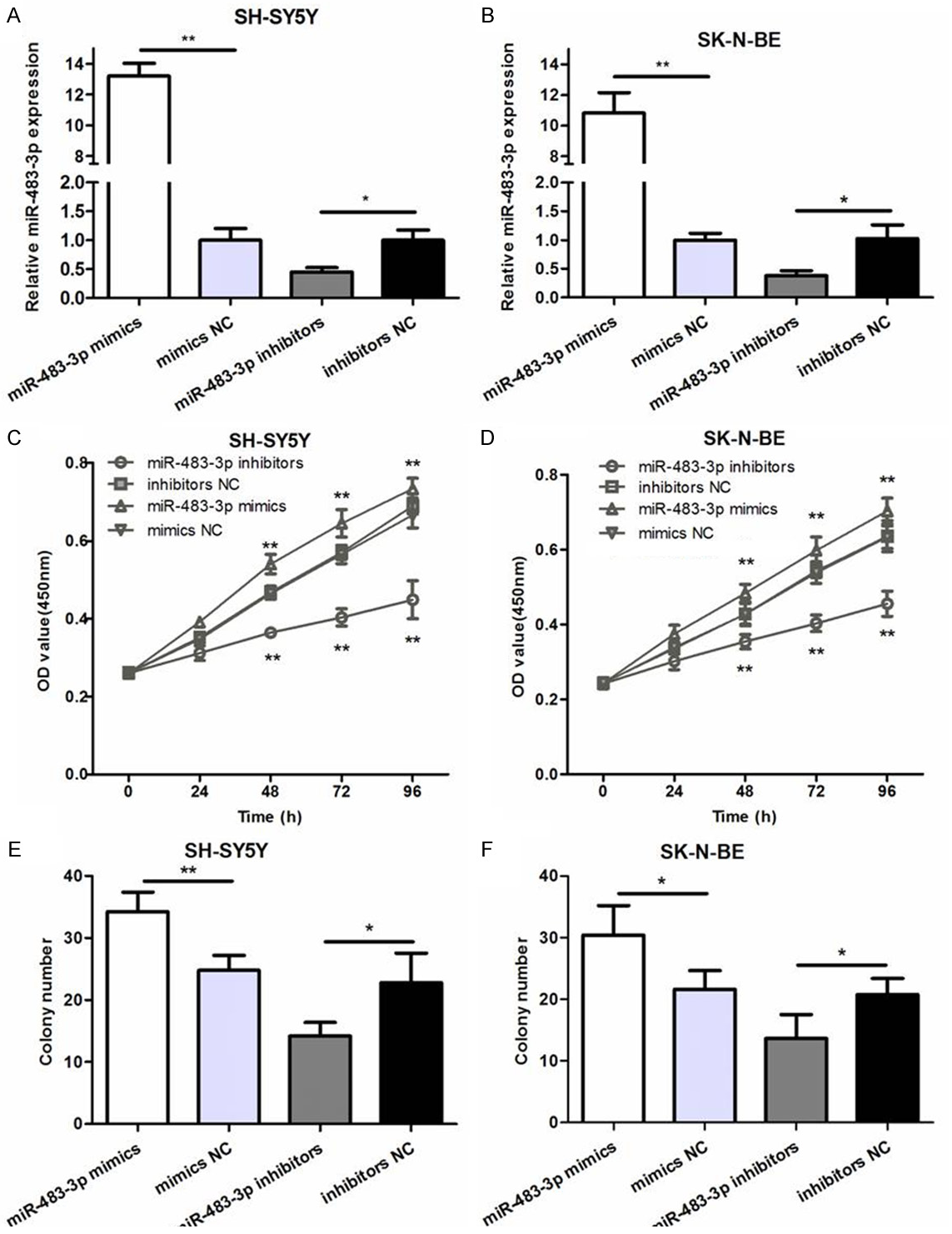

Figure 2. Overexpression of miR-483-3p induced proliferation of SH-SY5Y and SK-N-BE cells *in vitro*. OD, optical density; NC, negative control. A, B. SH-SY5Y and SK-N-BE cells were transfected with miR-483-3p mimics, miR-483-3p inhibitors or the relevant negative control. The expressionlevel of miR-483-3p was measured by qRT-PCR. C, D. Cell counting kit (CCK-8) assay was performed to investigate the effect of miR-483-3p on the proliferation of SH-SY5Y and SK-N-BE cells at different time points. E, F. Up-regulation of miR-483-3p increased colony formation of SH-SY5Y and SK-N-BE cells. **P*<0.05, ***P*<0.01.

293 cells (**Figure 1C**). However, the high-risk neuroblastoma cell lines (SH-SY5Y, SK-N-BE), formed by metastatic tumor and had a higher level of miR-483-3p expression than the low-

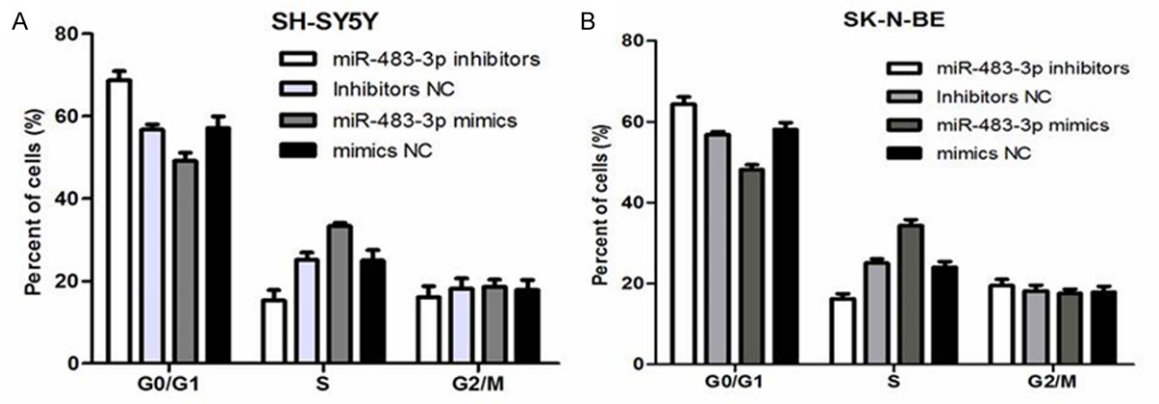

Figure 3. Overexpression of miR-483-3p induced an accumulation of SH-SY5Y (A) and SK-N-BE (B) cells in the G0/G1 phase with a corresponding decrease in the number of cells in S phase and the G2/M phase, compared with the negative control group. **P*<0.05.

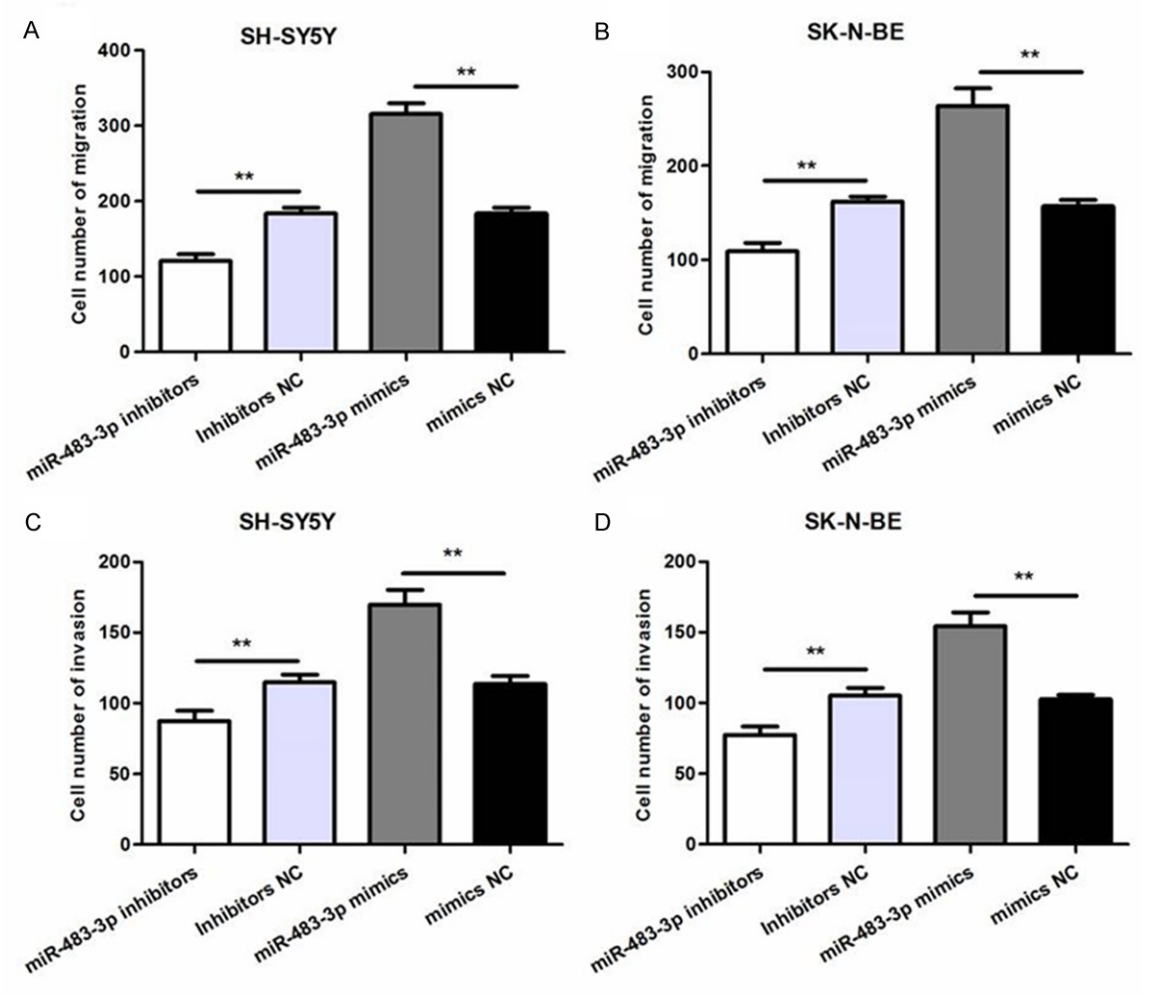

Figure 4. Overexpression of miR-483-3p induced migration and invasion of neuroblastoma cells *in vitro*. A transwell assay was used to evaluate migration and invasion of SH-SY5Y and SK-N-BE cells transfected with miR-483-3p mimics, miR-483-3p inhibitors or the relevant negative control. A, B. Migration; C, D. Invasion. **P*<0.05 ***P*<0.01.

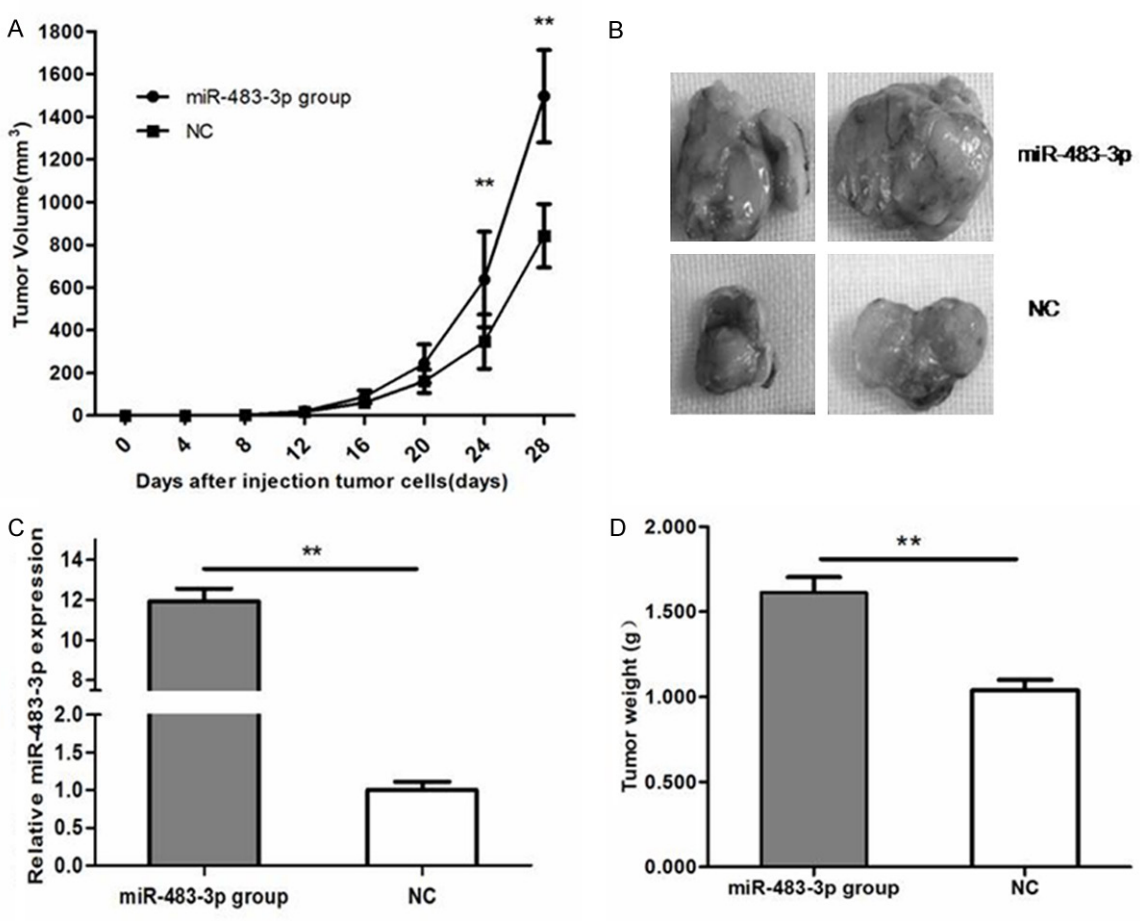

Figure 5. miR-483-3p induces tumor growth of neuroblastoma*in vivo*. A. SH-SY5Y cells stably transfected with miR-483-3p or the negative control miRNA were injected subcutaneously into nude mice. Tumor volumes were measured during the course of the experiment. B. Representative images of xenograft tumors. C. qRT-PCR was used to detect the expression level of miR-483-3p in xenograft tumors. D. Tumor weight formed by miR-483-3p or negative control transfected SH-SY5Y cell lines, were measured at the end of the experiment (28 days after injection). $^{**}P<0.01$,$^{*}P<0.05$ compared with negative control (NC).

risk neuroblastoma cells (IMR-32), which are formed by primary tumor ($P<0.05$) (**Figure 1C**).

Reducing the expression of miR-483-3p inhibits the proliferation of SH-SY5Y and SK-N-BE cells

To investigate the effect of miR-483-3p on neuroblastoma cell growth, SH-SY5Y and SK-N-BE cells were transfected with miR-483-3p mimics, miR-483-3p inhibitors, and the relevant negative controls. The expression of miR-483-3p mimics or miR-483-3p inhibitors were validated by qRT- PCR, and a 10/13-fold increase and a 70/65% reduction was observed using two cell lines (**Figure 2A**, **2B**). The CCK-8 assay showed that miR-483-3p mimics promote proliferation of SH-SY5Y cell lines, and the growth curve showed that the OD values were significantly increased at 48 h, 72 h and 96 h post-transfection (both $P<0.01$) (**Figure 2C**). Conversely, miR-483-3p inhibitors significantly reduced SH-SY5Y cell proliferation. Colony formation assay showed that down-regulation of miR-483-3p led to a 40% reduction in colony number of SH-SY5Y cells. Conversely, up-regulation of miR-483-3p led to the opposite effect (**Figure 2E**). Similar results were also observed in SK-N-BE cells (**Figure 2D**, **2F**).

miR-483-3p inhibits apoptosis of SH-SY5Y and SK-N-BE cells

A cell cycle assay was used to investigate the effect of miR-483-3p on neuroblastoma cells apoptosis. Cell-cycle analysis demonstrated

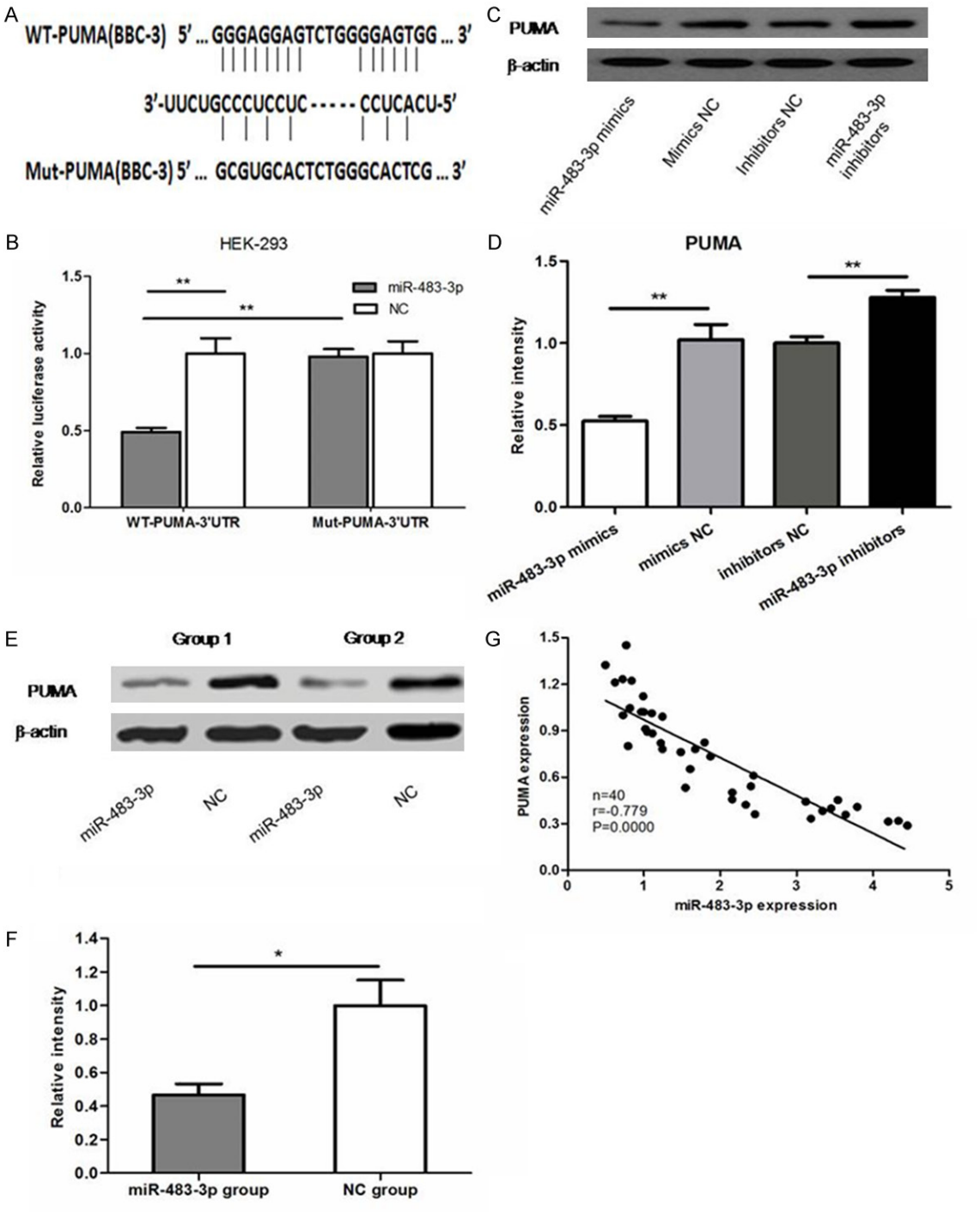

Figure 6. PUMA is a target of miR-483-3p in SH-SY5Y cells. A. A schematic illustration of the predicted seed region in the wild type (WT) PUMA 3'-UTR as well as mutated (Mut) PUMA 3'-UTR used in this study. B. Fluorescent reporter assays: HEK-293 cells were transfected with luciferase reporter constructs together with miR-483-3p or negative control. The relative luciferase activities were normalized against Renilla luciferase. C, D. The effect of miR-483-3p on PUMA expression. PUMA expression was detected by Western blot in SH-SY5Y cells transfected with miR-483-3p mimics or the relevant negative control. β-actin served as a loading control. The results were quantified and represent data from three independent experiments. *P<0.05, **P<0.01 compared with negative control. E, F. Western blot was used to assess PUMA expression in the xenograft tumors transfected with miR-483-3p. G. The relationship between PUMA and miR-483-3p wasevaluated by Pearson's correlation analysis. PUMA expression was evaluated by Western blot and normalized to b-actin. Expression of miR-483-3p was detected by qRT-PCR and normalized to U6 in neuroblastoma and normal tissue samples.

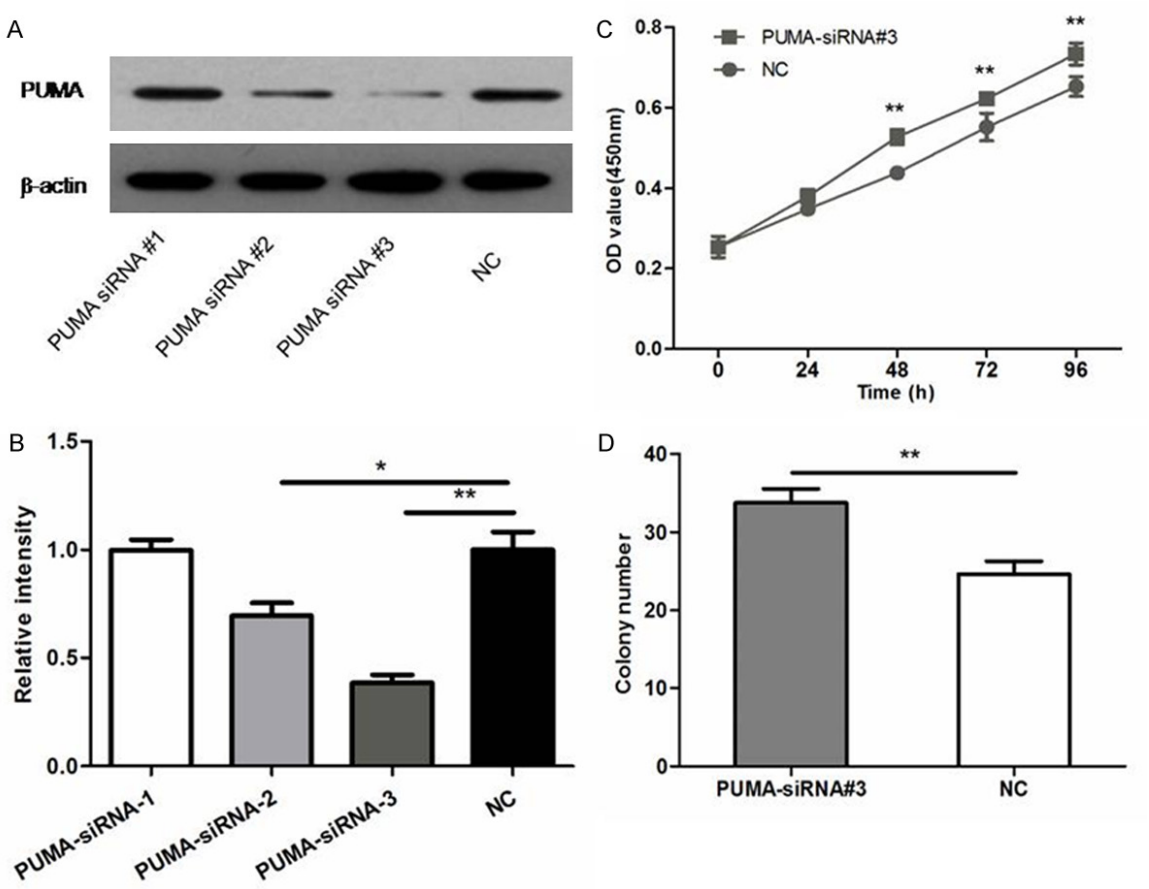

Figure 7. The effect of PUMA on SH-SY5Y cell proliferation. (A, B) PUMA was detected by Western blot in SH-SY5Y cells transfected with PUMA siRNA and negative control siRNA. Each assay was performed three times. CCK-8 assay (C) and colony formation assay (D) were used to detect the proliferation of cells after transfection with PUMA siRNA. **P*<0.05, ***P*<0.01.

that miR-483-3p mimics led to a significant reduction in the number of cells in the G0/G1 phase and increased the number of cells in the S phase or G2 phase, in both SH-SY5Y and SK-N-BE cells. Interestingly, the miR-483-3p inhibitor led to opposite effect (**Figure 3A**, **3B**). Therefore, up-regulated miR-483-3p expression can promote proliferation of neuroblastoma cells whereas down-regulated miR-483-3p expression increases the number of cells arresting in the G0/G1 phase and reduces the number of cells entering the S and G2 phase (*P*<0.05).

miR-483-3p promote migratory and invasive behavior of SH-SY5Y and SK-N-BE cells

As we have demonstrated, miR-483-3p was significantly overexpressed in high-risk neuroblastoma tumor tissue, compared with low-risk and median-risk neuroblastoma tissue. Therefore, miR-483-3p may play a role in regulating metastasis of neuroblastoma. To investigate this, invasion and migration assays were conducted. We found that the miR-483-3p inhibitor decreased the number of migrated and invaded SH-SY5Y and SK-N-BE cells by 30 and 40%, respectively (both *P*<0.01), while miR-483-3p mimics acted to increase the number of migrated and invaded cells (both *P*<0.01) (**Figure 4A-D**). Overall, these results further confirm that miR-483-3p can promote migration and invasion of neuroblastoma cells.

miR-483-3p promotes growth of neuroblastoma in vivo

Following on from our *in vitro* studies, which suggested that miR-483-3p acts as a tumor 'onco-miR' in neuroblastoma cells, we investigated whether miR-483-3p could promote tumor growth of neuroblastoma *in vivo*. Firstly,

we generated a SH-SY5Y cell line with up-regulated expression of miR-483-3p. The neuroblastoma xenografts formed from SH-SY5Y cells stably transfected with miR-483-3p vectors grew much faster than those derived from SH-SY5Y cells stably transfected with the negative control. Four weeks after injection, the final tumor volume in miR-483-3p tumors was significantly increased compared with negative control tumors ($P<0.05$) (**Figures 5A**, **5B**). The relative expression of miR-483-3p in the miR-483-3p infection group was higher than that in negative control group ($P<0.05$) (**Figure 5C**). The weight of tumors in the miR-483-3p transfection group was also heavier than that in the negative control group ($P<0.05$) (**Figure 5D**). Therefore, this revealed a role for miR-483-3p in promoting neuroblastoma growth in *vivo*.

PUMA is a target of miR-483-3p in neuroblastoma cells

To screen for the functional target of miR-483-3p in neuroblastoma cells, the following software was used: Bioinformatics software miRWalk (http://www.umm.uni-heidelberg.de/apps/zmf/mirwalk/), TargetScan (http://www.targetscan.org/), miRanda (http://www.microrna.org/) and PicTar (pictar.mdc-berlin.de). This revealed several candidate genes in neuroblastoma, and from this selection PUMA (BBC-3) waschosen to test aapossible candidate, sinceit is a known tumor suppressor gene involved in multiple cancer-related pathways.

To determine the potential interaction between miR-483-3p and PUMA, a luciferase activity assay was used. Human wild type PUMA 3'-UTR, harboring one potential miR-483-3p target-binding sequence, and mutant PUMA 3'-UTR plasmid, which was not paired to miR-483-3p, were synthesized (**Figure 6A**). HEK293 cells were used for transfection, as they exhibit low expression of miR-483-3p. HEK-293 cells were transfected with miR-483-3p mimics and a negative control with a scrambled sequence. Using a luciferase activity assay we found that miR-483-3p reduced the PUMA 3'-UTR luciferase activity by about 50% when compared to the negative control (**Figure 6B**). Moreover, repression of luciferase activity was significantly attenuated when wild type PUMA 3'-UTR was replaced by a mutant PUMA 3'-UTR. This result suggests that miR-483-3p may suppress gene expression by binding to the 3'UTR of the PUMA gene.

Subsequently, we used Western blot analysis to assess whether PUMA could be down-regulated by miR-483-3p. SH-SY5Y cells were treated with miR-483-3p mimics, inhibitors or the relative negative control and the cell lysates examined. We found that miR-483-3p mimics reduced the levels of PUMA (BBC-3) ($P<0.05$), while miR-483-3p inhibitors induced further expression of PUMA (**Figure 6C**, **6D**). Since PUMA is one of the targets of miR-483-3p in *vitro*, we chose to evaluate the association between miR-483-3p and PUMA in neuroblastoma and xenograft tumor tissues. PUMA expression in the neuroblastoma xenografts transfected with the miR-483-3p vector was significantly lower than that in the neuroblastoma xenografts transfected with the negative control vector ($P<0.05$) (**Figure 6E**, **6F**). Western blot analysis also revealed a significant reverse correlation between PUMA and miR-483-3p in neuroblastoma and normal tissues (**Figure 6G**, Pearson's correlation coefficient =-0.779; $P=0.000$). PUMA expression and miR-483-3p expression were complementary in both neuroblastoma and xenograft tumor tissues, which could be an indication that miR-483-3p negatively regulates the expression of PUMA in *vivo*.

Inhibition of PUMA showed a similar effect to overexpression of miR-483-3p

The above results prompted us to further explore the functional relationship between miR-483-3p and PUMA. To study the effect of PUMA on neuroblastoma cell growth, SH-SY5Y cells were transfected with PUMA siRNA and relative negative control. Successful down-regulation of PUMA was confirmed by Western blot ($P<0.05$) (**Figure 7A**, **7B**). Inhibition of PUMA by siRNA induced growth and colony formation of SH-SY5Y cells, which was similar to the effect observed with miR-483-3p overexpression (**Figure 7C**, **7D**). This result further confirmed that miR-483-3p induced proliferation of neuroblastoma cells and colony formation, at least partly by suppressing PUMA expression.

Discussion

In the last 30 years, numerous genes have been identified relating to neuroblastoma growth, invasion and migration [14]. miRNAs, a class of small regulatory RNAs, have been shown to play important roles in a wide variety of oncogenic activities, such as proliferation, invasion, and angiogenesis [15, 16]. miR-

NAscan act as either activators or suppressors of tumorigenesis in neuroblastoma. One particular miRNA, miR-338-3p, was shown to suppress neuroblastoma proliferation, invasion, and migration and it was shown to affect the PTEN/Akt pathway by down-regulating PREX2a [9]. Other miRNAs, such as miR-329, miR-204, miR-421, and miR-204 have also been found to play important roles in neuroblastoma growth, invasion, and migration [10, 17-19].

In this study, we have shown that miR-483-3p was up-regulated in stage 4 neuroblastoma tissue samples, compared to stage 1, 2, 3, or 4 s neuroblastoma tissuesamples. This was in accordance with previous findings using miRNA chips [20]. The expression level of miR-483-3p has also found to be high in many other tumors, such as Wilms'tumors and pancreatic cancer but is low in squamous cell carcinomas [11-13]. Hence, dysregulation of miR-483-3p might be a novel oncogenic mechanism and therefore it could be a potential therapeutic target or used as a biomarker.

Since the function of miR-483-3p in neuroblastoma had not been previously described, we decided to further study its role in neuroblastoma. This study demonstrated that down-regulation of miR-483-3p inhibited viability, proliferation, migration, and invasion of neuroblastoma cells *in vitro,* whereas overexpression led to the opposite effect. We also discovered that up-regulation of miR-483-3p could induce tumor growth in *vivo*. This finding was in accordance with other studies that also demonstrated that miR-483-3p could act as an 'onco-miR', particularly in pancreatic cancer and Wilms'tumors [12, 13]. In contrast, the opposite function of miR-483-3p was reported in squamous cellcarcinoma [11]. In order to further explain this, it would be necessary to examine the exact gene profile for each tumor type, as the influence of different miRNAs will strongly depend on tumor genetic context.

It has been well described that miRNAs regulate gene expression of multiple targets and perform multiple functions. To investigate the mechanism of miR-483-3p, we identified PUMA (p53-upregulated modulator of apoptosis) as a potential target gene using bioinformatics. This was then validated *in vitro* using Western blot and a luciferase reporter system, to confirm that PUMA is negatively regulated by miR-483-3p. PUMA is a BH3-only protein that was first identified as a transcriptional target of p53 [21]. PUMA is a central regulator of the p53 signaling pathway and contributes to the pathogenesis, maintenance, and chemoresistance of many human cancers [22]. Other studies have also shown that miR-483-3p suppresses the expression of DPC4/Smad4 in pancreatic cancer and inhibited CDC25A in squamous cell carcinoma, suggesting that miR-483-3p might play a different role in different tumors [11, 12]. Taken together, our data indicated that miR-483-3p functions as an 'Onc-miR' in neuroblastoma. However, whether miR-483-3p targets other genes in the regulation of neuroblastoma remains to be elucidated.

In summary, we found that downregulation of miR-483-3p can suppress proliferation, migration, and invasion of neuroblastoma cells *in vitro* and *in vivo* and presents a functional link between miR-483-3p and PUMA expression in neuroblastoma. Although miRNA-based therapeutics are still in the early stages of development, our results suggest that miRNAs may represent a promising therapeutic for neuroblastoma.

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Disclosure of conflict of interest

None.

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