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Original Article

MiRNA-7 targets cathepsin K to inhibit invasion and metastasis of neuroblastoma cells

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Abstract: Background: We aimed to investigate the function of miR-7 in the invasion and metastasis of neuroblastoma cells and to explore its mechanism. Methods: QRT-PCR or western blot was used to validate the expression level of miR-7 and candidate gene or protein in the neuroblastoma tissues or neuroblastoma cells. The cell adhesion assay, scratch migration assay and Matrigel invasion assay were used to compare cell invasion and metastasis ability, respectively. Bioinformatics and luciferase reporter gene Assay were conducted to validate the regulation of miR-7 on the cathepsin K (CatK) expression. Xenografts assay was used to test the inhibiting effect of miR-7 or miR-7 inhibitor on the neuroblastoma cells in vivo. Results: MiR-7 was over-expressed in metastatic neuroblastoma tissues compared to primary tumors. over-expression or inhibition of miR-7 inhibits or promotes cell invasion and metastasis of neuroblastoma cells in vitro and in vivo. Besides, catK was confirmed as a direct target of miR-7 by using bioinformatics analysis and validation at both the gene and protein levels. Furthermore, the knock down of CatK by using siRNA had a similar effect on the neuroblastoma cell invasion and metastasis to the over-expression of miR-7 in SH-N-SH or SH-SY5Y cells. Finally, the down-regulated ability of cell invasion and metastasis following the over-expression of miR-7 could be reversed by adding CatK in vitro. Conclusion: This newly identified function of miR-7 by targeting CatK provides novel insights into neuroblastoma and may foster therapeutic applications.

Keywords: miR-7, cathepsin K, invasion and metastasis, neuroblastoma cells

Introduction

Neuroblastoma (NB), a malignant embryonal tumor of sympathetic nervous system, is the most common solid extracranial malignancy of childhood and accounts for 15% of all childhood cancer deaths [1]. The biological behavior of NB is extensively heterogeneous, ranging from spontaneous regression to rapid progression despite multimodal aggressive therapy [2]. Because metastasis is the leading cause of death in this disease, better elucidation of the underlying mechanisms is important for improving the therapeutic efficiency [2].

Emerging evidence showed that microRNAs (miRNAs), a new class of small noncoding RNAs, participated in regulating gene expression at the post-transcriptional level by targeting the 3'-untranslated region (3'-UTR) of messenger RNAs (mRNAs) [3]. Dysregulation of certain miRNAs has been implicated in the pathogenesis of NB [3]. For example, miR-34a and miR-362-5p were significantly low-expressed in NB tissues [4, 5]. miR-34a exerted the tumor-suppressive function by targeting MYCN [4], while ectopic upregulation of miR-362-5p inhibited proliferation and migration of NB cells by targeting PI3K-C2β [5]. Recently, researchers identified that miR-421 could promote tumorigenesis and aggressiveness of NB cells through the down-regulation of tumor suppressor menin [6].

In our previous study, we showed a widespread alteration of 54 miRNAs in metastatic NB compared with primary NB [7]. Among of them, miR-7 was significantly down-regulated in metastatic NB compared to primary NB in animal models, implying the potential roles of miR-7 in regulating NB metastasis [7]. CatK plays a critical role in facilitating the tumor cells to remodel and penetrate extracellular matrix (ECM) [8]. It has been established that CatK promotes tumor invasion by functioning as a pericellular collagenase and an activator of other protease, and CatK is directly linked to tumorigenesis,

metastasis, and angiogenesis [8-10]. However, the expression and function of CatK in NB still remained largely unknown. In this study, we showed, for the first time, that miR-7 regulated the expression of CatK through directly targeting the 3'-UTR, which suppressed the invasion and metastasis of NB cells in vitro and in vivo.

Materials and methods

Human tissue samples

Fifteen paired NB tissue sections from patients with metastasis and primary tissues were obtained from the Tumor Bank Facility of the Affiliated Hospital of Medical College, Qingdao University, China. Detailed pathologic and clinical data were collected for all samples including Edmondson tumor grade, invasion and metastasis. The diagnoses of these samples were verified by pathologists. The collection of human tissue samples was approved and supervised by the Ethics Committee of Qingdao University.

Cell culture and transfection

Human NB cell lines SH-SY5Y (CRL-2266), and SK-N-SH (HTB-11) were purchased from American Type Culture Collection. Cells were grown in RPMI-1640 medium (Life Technologies, Inc.) supplemented with 10% FBS (Life Technologies, Inc.), penicillin (100 U/mL) and streptomycin (100 μm/mL). Cells were maintained at 37°C in a humidified atmosphere of 5% CO_2. MiR-7 mimics, miR-7 inhibitors and negative control (RiboBio Co. Ltd.) were transfected into confluent cells with Lipofectamine 2000 (Life Technologies, Inc.).

RNA preparation and quantitative PCR

RNA was extracted from cells or tissue samples using the mirVana miRNA Isolation Kit (Ambion, USA) according to the manufacturer's instructions. Small RNA fraction (smaller than 200 nt) were separated and purified according to this procedure. The levels of mature miR-7 were determined using Bulge-Loop miRNAs qPCR Primer Set (RiboBio Co. Ltd.). After cDNA was synthesized with a miRNA-specific stem-loop primer, the quantitative PCR was conducted with the specific primers. The miR-7 levels were normalized to those of U6 snRNA. Quantitative RT-PCR was performed on an ABI 7500 thermocycler (Applied Biosystems). The primers used are listed as follows: miR-7 F: 5'-UGGAAGACUAG-3', miR-7 R: 5'-UGAUUUUGUUG-3'. U6 was used as internal control in QRT-PCR: U6 F: 5'-GCTTCGGCAGCACATATACTAAAAT-3', U6 R: 5'-CGCTTCACGAATTTGCGTGTCAT-3'. CatK F: 5'-GCAAATCTCTGCCCATAACC-3', CatK R: 5'-CCCGTCATCTTCTGAACCAC-3', GAPDH F: 5'-TCACCACCATGGAGAAGGC-3', GAPDH R: 5'-GCTAAGCAGTTGGTGGTGCA-3'. Experiment was performed independently for three times.

Cell adhesion assay

Homogeneous single-cell suspensions (2×10^4 tumor cells) were inoculated into each well of 96-well plates that were precoated with 100 μL of 20 μg/mL Matrigel (BD Biosciences) or 50 μL of 10 mg/L fibronectin (BD Biosciences), and incubated at 37°C in serum-free complete medium (pH 7.2) for 1 hour. After incubation, the wells were washed 3 times with PBS and the remaining cells were fixed in 4% paraformaldehyde for 20 minutes at room temperature. The cells were stained with 0.1% crystal violet and washed 3 times with PBS to remove free dye. After extraction with 10% acetic acid, absorbance of the samples was measured at 570 nm.

Scratch migration assay

Tumor cells were cultured in 24-well plates and scraped with the fine end of 1-mL pipette tips (time 0). Plates were washed twice with PBS to remove detached cells, and incubated with the complete growth medium. Cell migration was photographed using 10 high-power fields, at 0 and 24 hours postinduction of injury.

Matrigel invasion assay

A total of 1×10^5 cells/well SK-N-SH and SH-SY5Y single-cell suspensions (in 0.2 ml 160 RPMI 1640 with 5% FBS) were added to the upper Transwell chambers (Corning, USA), which was pre-coated with 1 mg/ml Matrigel (Growth Factor Reduced BD Matrigel TM Matrix) for 2 h. In the lower part of the chamber, 0.6 ml RPMI 1640 with 20% FBS was added and allowed to invade for 24 hours at 37°C in a CO_2 incubator, chambers were disassembled and the membranes were stained with 2% crystal violet for 10 min and placed on a glass slide. Then cells invading across the membrane were counted in

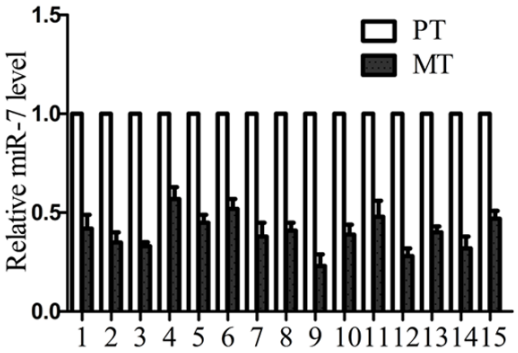

Figure 1. The expression levels of miR-7 in fifteen pairs of human tissues (metastatic NB and primary NB tissues). The miR-7 expression level had an average of 40% reduction in metastatic tumor tissues compared with paired primary tumor tissues. Results were representative of three separate experiments and bars indicated the SD. Data were expressed as the mean ± standard deviation.

5 random visual fields under a light microscope. All assays were performed in triplicate and independently performed three times.

Xenografts assays in vivo

The animal study was carried out in accordance with the guidelines approved by the Animal Experimentation Ethics Committee of Qingdao University. The protocol was approved by the committee, all surgery was performed under sodium pentobarbital anesthesia, and all efforts were made to minimize suffering. Athymic Balb/c nude mice (aged 6 weeks) were provided by Slac Laboratory Animal Co. Ltd. (Shanghai, China), and the mice were housed in a pathogen-free animal facility and randomly assigned to the control or experimental group (8 mice per group). A genomic sequence encoding miRNA-7 and negative control were amplified and then cloned into pLenti6.3/V5-DEST Gateway Vector (Invitrogen, USA). After confirmed by gene sequencing, the lentiviruses infected to SH-SY5Y cells. The experimental metastasis (0.4×10^6 tumor cells per mouse) studies were conducted with 2-month-old male nude mice as previously described [12, 16, 17].

miRNA target prediction

miRNA targets were predicted using the algorithms, including miRanda Human miRNA Targets, miRDB, RNA22, and TargetScan. To identify the genes commonly predicted by these 4 different algorithms, results of predicted targets were intersected using miRWalk [11].

Luciferase reporter assay

The human CatK 3'UTR containing the putative binding site of miR-7 was synthesized by GenPharm (Shanghai, China). Luciferase constructs were made by ligating the synthesized 3'UTR as well as the seed-sequence mutated version after the luc ORF in the pMIR-REPORT luciferase vector (Ambion). For the fluorescent reporter assay, cells were seeded in a 48-well plate the day before transfection. The cells were co-transfected with miR-7 mimics or inhibitors as well as the controls and CatK-3'UTR or CatK-3'UTR mut. The cells were lyzed 48 h later and the intensity of luciferase was detected.

Western blot

Cellular protein was extracted with 1× cell lysis buffer (Promega) according to the manufacturer's instructions. Western blotting was done with antibodies specific for CatK and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Santa Cruz Biotechnology) to determine the protein expression of CatK. The GAPDH was regarded as the endogenous normalizer. Enhanced chemiluminescence substrate kit (Amersham) was used for the detection of signals with autoradiography film (Amersham).

CatK over-expression and knockdown assay

The over-expression vector pcDNA3/CatK (without CatK 3'-UTR) tagged by GFP was synthesized by GenePharma (Shanghai, China) and the empty vector (pcDNA3) was served as negative control. To restore miR-7-induced downregulation of CatK, tumor cells were cotransfected with the recombinant vector. The siRNA targeting CatK (region 153-177, GTGCATTCTTA) was chemically synthesized (RiboBio Co. Ltd.) and transfected with Genesilencer Transfection Reagent (Genlantis). The scramble siRNA (si-Scb) was applied as controls.

Statistical analysis

Unless otherwise stated, all data were shown as mean ± standard deviation. The differences between groups were analyzed using Student's t test when only two groups were present, or

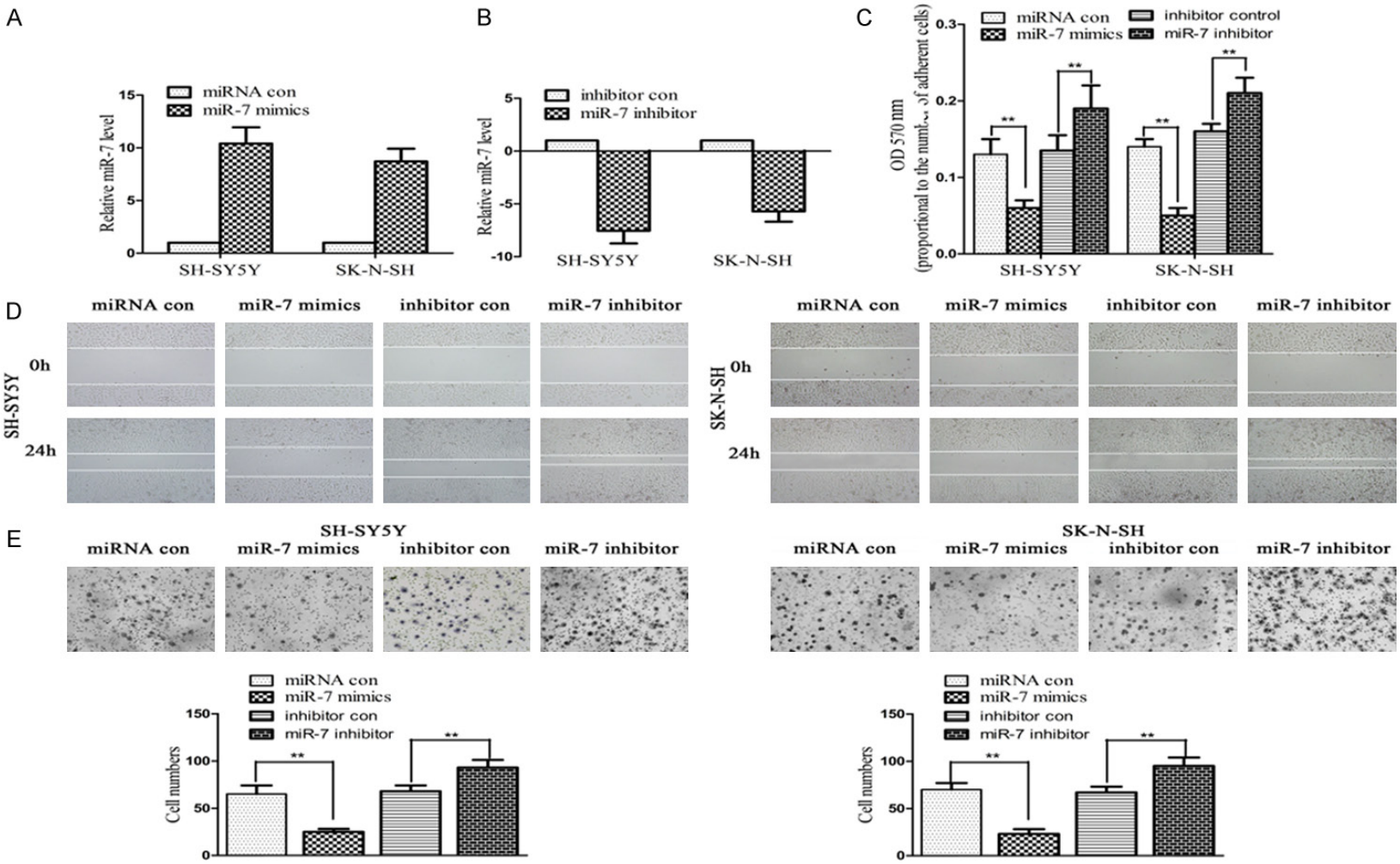

Figure 2. The function of miR-7 on the adhesion, migration and invasion of NB cells in vitro. A. QRT-PCR results of miR-7 mimics transfection efficiency. The level of miR-7 in the miR-7 mimics group was significantly higher than that in the miRNA control group ($P<0.05$, respectively in SH-SY5Y or SK-N-SH cells). B. QRT-PCR results of miR-7 inhibitor transfection efficiency. The level of miR-7 in the miR-7 inhibitor group was significantly lower than that in the inhibitor control group ($P<0.05$, respectively in SH-SY5Y or SK-N-SH cells). C. In adhesion assay, the tumor cells transfected with miR-7 mimics or miR-7 inhibitors possessed the decreased or increased ability in adhesion to the precoated matrigel or fibronectin, when compared with those transfected with negative control, respectively in the SH-SY5Y or SK-N-SH cells ($P<0.01$). D. In scratch migration assay, the over-expression (mimics) or loss (inhibitor) of miR-7 decreased or increased the migration capabilities in the SH-SY5Y or SK-N-SH cells, respectively. The width in the miR-7 mimics or inhibitor group was bigger or smaller than that in the control groupin the SH-SY5Y or SK-N-SH cells, respectively. E. Transwell analysis showed that NB cells transfected with miR-7 mimics or inhibitor presented a weak or strong invasion capacity compared to the negative control cells in the SH-SY5Y or SK-N-SH cells ($P<0.05$). Data were from three experimental determinations and bars indicated the SD. Data were expressed as the mean ± standard deviation (**$P<0.01$).

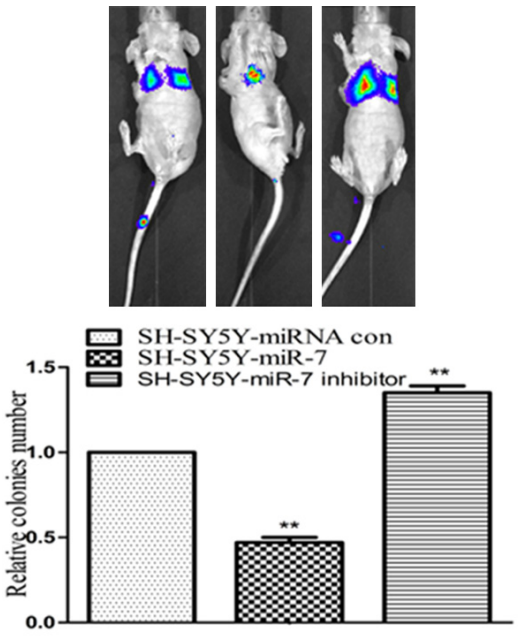

Figure 3. The function on the NB cells in vivo. SH-SY5Y cells stably transfected with miR-7 or miR-7 inhibitor established statistically less or more lung metastatic colonies in the miR-7 or miR-7 inhibitor group than that in the control group (*P*<0.01, respectively). Data are expressed as the mean ± standard deviation. Data from the experimental determinations and bars indicate the SD (***P*<0.01).

assessed by one-way analysis of variance (ANOVA) when more than two groups were compared. All data were analyzed using SPSS (version 13.0; SPSS Inc., Chicago, IL, USA) and Graph Pad Prism 5.0. In this study, P<0.05 is considered statistically significant.

Results

Down-regulation of miR-7 in metastatic NB compared to primary NB

Previously, we established a heterotopic transplant mice model of NB and performed a microarray-based analysis identifying expression of miR-7 was decreased (a 37% reduction) in six metastatic tumor tissues compared with paired primary tumor tissues [6]. To investigate the potential involvement of the miR-7 in the process of human NB metastasis, we measured the expression levels of miR-7 in fifteen pairs of human tissues. As shown in **Figure 1**, we found that miR-7 expression level had an average of 40% reduction in metastatic tumor tissues compared with paired primary tumor tissues.

MiR-7 suppressed the adhesion, migration and invasion of NB cells in vitro

As our results indicated, the expression of miR-7 in metastatic tumor tissues is significant lower than that in primary NB tissues. MiR-7 might play a role in regulating NB metastasis. Gain/loss-of-function experiments are widely used for functional studies of miRNAs. To investigate the role of miR-7 in NB cell metastasis, miR-7 mimics, inhibitors and negative control were synthesized, and the transfection efficiency was confirmed by QRT-PCR. In SH-SY5Y or SK-N-SH lines, we found that the miR-7 expression in the miR-7 mimics group was higher than that in the negative control group (P<0.05, **Figure 2A**), while the miR-7 inhibitors suppressed the miR-7 expression (P<0.05, **Figure 2B**). In adhesion assay, the tumor cells transfected with miR-7 mimics possessed the decreased ability in adhesion to the precoated Matrigel or fibronectin, when compared with those transfected with negative control (P<0.01, respectively, **Figure 2C**). In scratch migration assay, miR-7 over-expression decreased the migration capabilities of SH-SY5Y or SK-N-SH cells, respectively (**Figure 2D**). And the difference between the miR-7 over-expression group and the control group was statistically significant (P< 0.05, respectively). Transwell analysis showed that NB cells transfected with miR-7 mimics presented an weak invasion capacity compared to the negative control cells in the SH-SY5Y or SK-N-SH cells (P<0.05, **Figure 2E**). On the other hand, we examined the effects of miR-7 knockdown on NB cells. Introduction of miR-7 inhibitors into NB cells resulted in enhanced abilities in adhesion, migration and invasion capabilities in the SH-SY5Y or SK-N-SH cells (P<0.01, respectively, **Figure 2C-E**). These results indicated that miR-7 remarkably decreased the adhesion, migration and invasion of NB cells in vitro.

MiR-7 could decrease the metastasis of NB cells in vivo

Our studies in vitro indicated that miR-7 probably acted as a tumor suppressor in NB cells, and then we investigated whether miR-7 could inhibit tumor metastasis of NB in vivo. SH-SY5Y cells stably transfected with miR-7 or miR-7 inhibitor established statistically less or more lung metastatic colonies in the miR-7 or miR-7

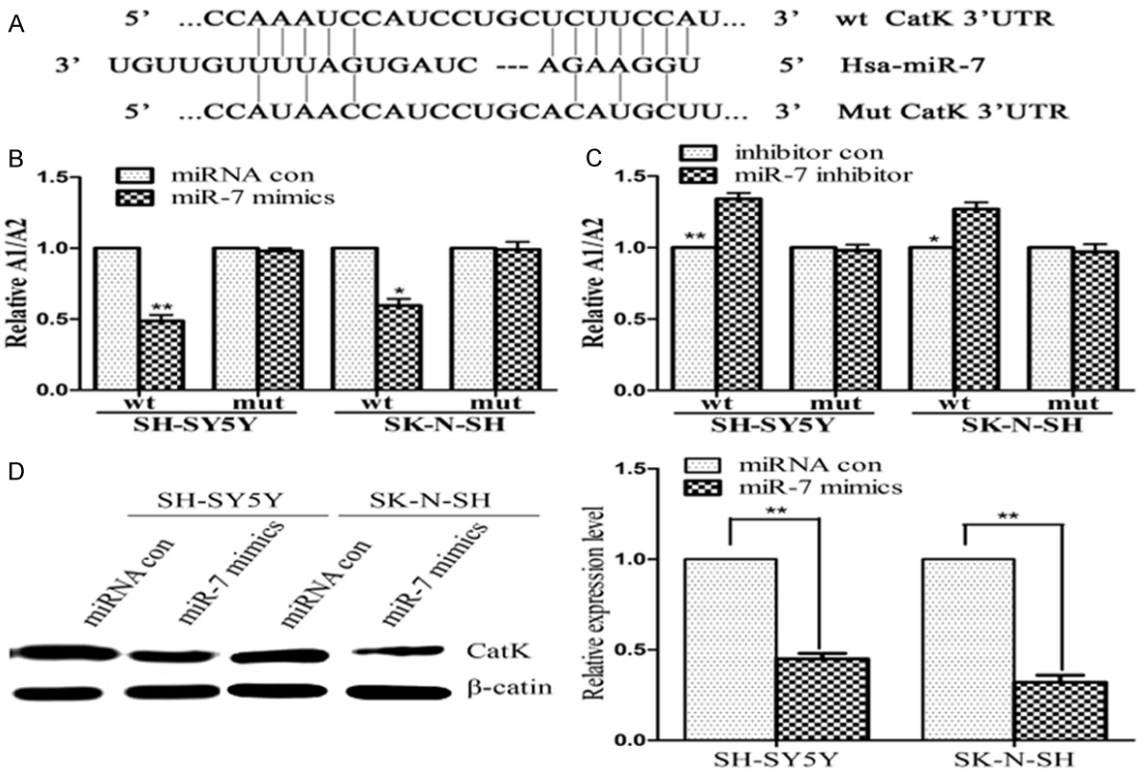

Figure 4. The relationship between miR-7 and CatK in the NB cells. A. Bioinformatics results. The 3'-untranslated region (3'-UTR) of Catk contains a potential miRNA-binding site for miR-7. We inserted the Catk UTR with either a wild-type or mutant miR-7 target sequence downstream of the firefly lucierase gene into the pGL3-control vector (Promega) to create the pGL3-Catk UTR WT or the pGL3-Catk UTR Mut construct, respectively. B. Luciferase reporter assay results. The pGL3-Catk UTR WT, pGL3-Catk UTR Mut and pGL3 constructs were individually transfected into the SH-SY5Y and SK-N-SH cells. MiR-7 mimics significantly decreased the relative luciferase activity of the wild-type Catk 3'UTR compared with the mutant Catk 3'UTR in the SH-SY5Y and SK-N-SH cells (respectively, $P<0.01$ or $P<0.05$). C. Luciferase reporter assay results. The pGL3-Catk UTR WT, pGL3-Catk UTR Mut and pGL3 constructs were individually transfected into the SH-SY5Y and SK-N-SH cells. MiR-7 inhibitor significantly increased the relative luciferase activity of the wild-type Catk 3'UTR compared with the mutant Catk 3'UTR in the SH-SY5Y and SK-N-SH cells (respectively, $P<0.01$ or $P<0.05$). D. The expression of Catk protein in different treated groups. The western blot analysis showed that miR-7 mimics significantly reduced Catk protein levels of SH-SY5Y or SK-N-SH cells compared with that in the negative control (P<0.01, respectively). Results are representative of at least three separate experiments. Data are expressed as the mean ± standard deviation. Data from three experimental determinations and bars indicate the SD (*$P<0.05$, **$P<0.01$).

inhibitor group than that in the negative control group (P<0.01, respectively, **Figure 3**). These results suggested that miR-7 could inhibit the metastasis of NB cells in vivo.

CatK is a target of miR-7 in NB cells

To investigate the mechanisms through which miR-7 suppresses invasion and metastasis, chip analysis was made firstly (unpublished data, not shown in this article) and then computational prediction was done by miRNA databases. In the CatK 3'-UTR, there was one potential binding site of miR-7 with high complementarity, which was coincidentally predicted by at least 3 independent sources. The miR-7-binding site was located at bases 26 to 32 of the CatK 3'-UTR (**Figure 4A**).

A luciferase activity assay was used to determine the potential interaction between miR-7 and CatK. It was found that miR-7 significantly suppressed activity of wild-type 3'-UTR but not the mutant 3'-UTR of CatK in SH-SY5Y and SK-N-SH cells (**Figure 4B**). Moreover, the loss of miR-7 with miR-7 inhibitor increased the luciferase activity in tumor cells (P<0.01 or P< 0.05, **Figure 4C**). In addition, western blot analysis showed that miR-7 mimics significantly reduced Catk protein levels of SH-SY5Y or SK-N-

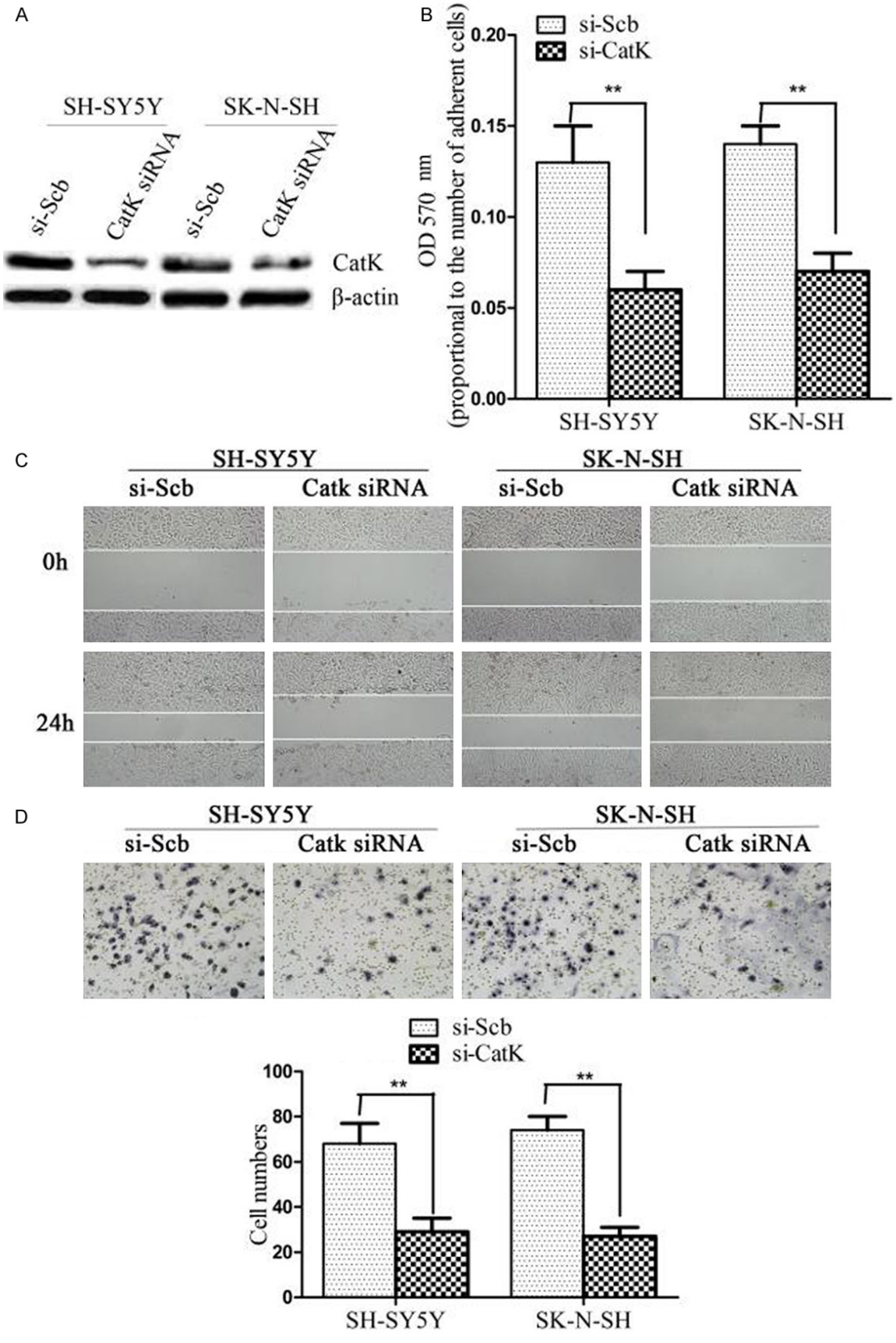
A
SH-SY5Y
SK-N-SH
si-Scb
CatK siRNA
si-Scb
CatK siRNA
CatK
β-actin
B
si-Scb
si-CatK
OD 570 nm
(proportional to the number of adherent cells)
0.20
0.15
0.10
0.05
0.00
**
**
SH-SY5Y
SK-N-SH
C
SH-SY5Y
SK-N-SH
si-Scb
Catk siRNA
si-Scb
Catk siRNA
0h
24h
D
SH-SY5Y
SK-N-SH
si-Scb
Catk siRNA
si-Scb
Catk siRNA
si-Scb
si-CatK
Cell numbers
100
80
60
40
20
0
**
**
SH-SY5Y
SK-N-SH

Figure 5. The function of Catk on the adhesion, migration and invasion of NB cells in vitro. A. Western blot results of Catk protein expression in different treated groups. The level of Catk protein expression was significantly lower in the siRNA group than that in the control group in SH-SY5Y or SK-N-SH cells. B. The adhesion results of different treated groups, The tumor cells transfected with siRNA-CatK possessed the decreased ability in adhesion to the precoated matrigel or fibronectin, when compared with those transfected with the control group, respectively in the SH-SY5Y or SK-N-SH cells ($P<0.01$). C. The scratch migration results of different treated groups, the loss of CatK (siRNA-CatK) decreased the migration capabilities in the SH-SY5Y or SK-N-SH cells, respectively. The width in the siRNA-CatK group was bigger than that in the control groupin the SH-SY5Y or SK-N-SH cells, respectively. D. The results of transwell analysis. The NB cells transfected with siRNA-CatK presented a weak invasion capacity compared to the control cells in the SH-SY5Y or SK-N-SH cells ($P<0.05$, respectively). Results are representative of at least three separate experiments. Data are expressed as the mean ± standard deviation. Data from three experimental determinations and bars indicate the SD (*$P<0.05$, **$P<0.01$).

SH cells compared with that in the negative control (P<0.01, **Figure 4D**).

The loss of CatK suppressed the adhesion, migration and invasion in the NB cells

The above results indicated the negative regulation of CatK expression by miR-7, so we might hypothesize that the loss of CatK could have a similar effect on cultured NB cells. SiRNAs targeting the encoding region of CatK were designed and transfected into SH-SY5Y and SK-N-SH cells. Transfection of si-CatK, but not of si-Scb, resulted in decreased CatK expression in NB cells (P<0.05, **Figure 5A**). The loss of CatK suppressed the adhesion, migration and invasion of SH-SY5Y and SK-N-SH cells (P<0.05, respectively, **Figure 5B-D**). These results were consistent with the findings that over-expression of miR-7 suppressed the adhesion, migration, invasion, and angiogenesis of NB cells in vitro.

MiR-7 over-expression reversed the simulative effect of CatK on NB cells

Finally, we investigated whether over-expression of miR-7 could reverse the effect of CatK to demonstrate the regulation by miR-7 on CatK. First, the transfection efficiency was determined by fluorescence microscopy and the effect of CatK over-expression plasmid on SH-SY5Y and SK-N-SH cells was confirmed by western blot (**Figure 6A** and **6B**). The CatK over-expression plasmid significantly increased the CatK protein level in tumor cells compared to the empty vector (pcDNA3), which was down-regulated by miR-7 (**Figure 6B**). The results of the adhesion, migration and invasion assays showed that over-expression of miR-7 could significantly reverse the suppressive effect of CatK on tumor cells (**Figure 6C-E**). These results provided the further evidence that CatK was involved in miR-7-mediated suppression of NB. Accordingly, identification of CatK as a miR-7 target gene may explain, at least in part, why over-expression of miR-7 suppressed the migration and invasion of NB cells.

Discussion

Over the past several years, it has become clear that alterations in the expression of miRNAs contribute to the pathogenesis of NB, and the miRNAs act as either oncogenes or tumor suppressors [13]. Kristina Althoff reported that miR-542-3p exerted tumor suppressive functions in NB by downregulating survivin, which would induce S-phase arrest and increase apoptosis [14]. Metastasis is a major cause of NB-related death and patients with NB have a tendency to develop malignant lesions outside the primary tumor. Qu reported that miR-558 could promote metastasis of NB cells by activating the transcription of HPSE [15]. Other related miRNAs associated with NB metastasis include miR-9, miR-145, miR-451, miR-21, miR-29a and miR-362-5p [16-21]. In the present study, we investigated the biological role of miR-7 in human NB metastasis.

Human miR-7 was first described by Lim in 2003 [22], and later this miRNA was found to be associated with anti-tumorigenic effects in different types of cancers, including glioma, colorectal cancer and hepatocellular carcinoma cells [23]. Moreover, several lines of evidence indicate that miR-7 might perform novel functions in tumor invasion and metastasis. MiR-7 was reported to be associated with aggressiveness and metastasis in renal cell carcinoma [24] and lung cancer cells [25]. In this study, we demonstrated that miR-7 was down-regulated in metastatic tumor tissues compared to the paired primary tumor tissues. Furthermore, we identified miR-7 could suppress NB cells (SH-SY5Y and SK-N-SH) invasion and

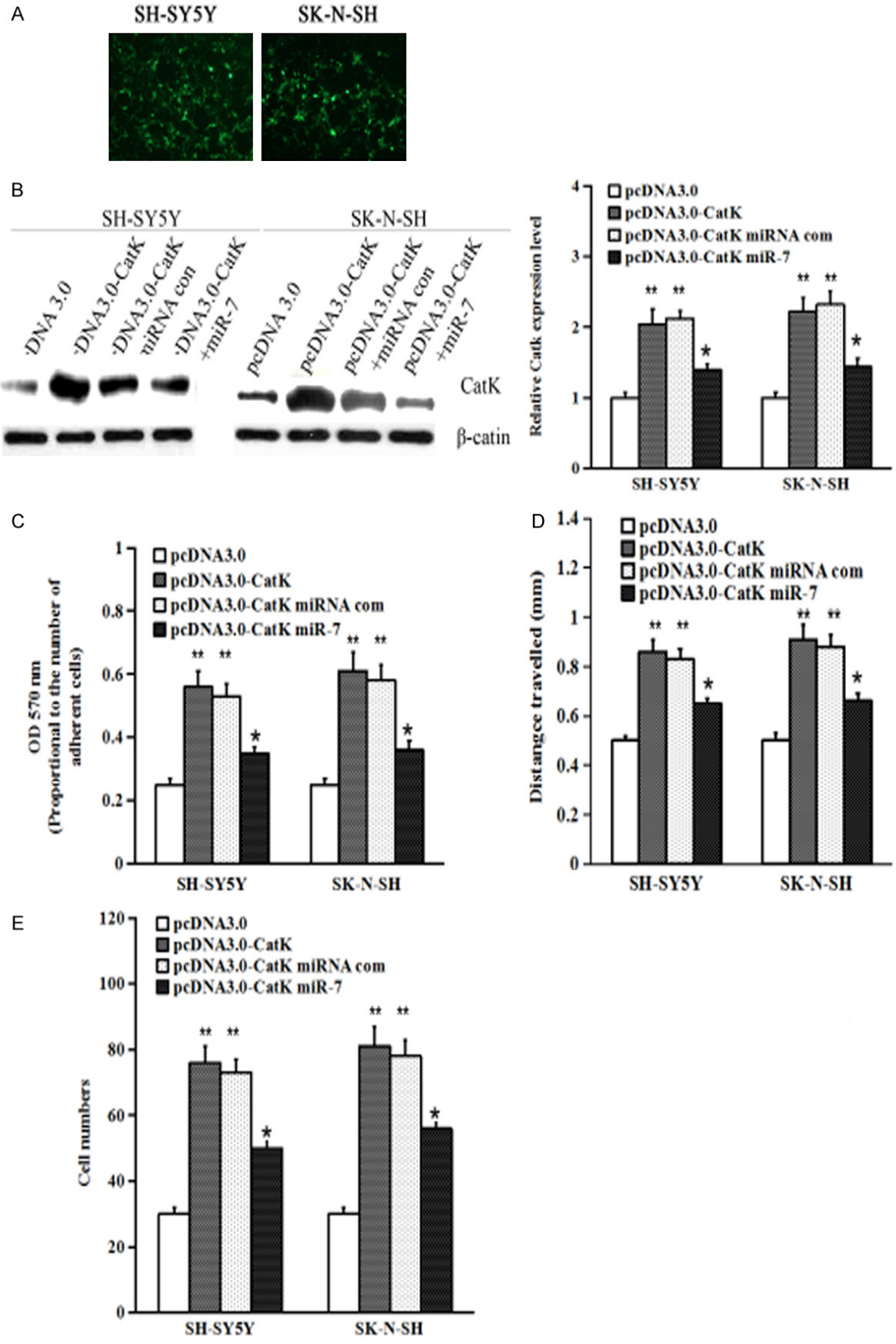
A
SH-SY5Y
SK-N-SH
B
SH-SY5Y
SK-N-SH
pcDNA 3.0
pcDNA3.0-CatK
pcDNA3.0-CatK miRNA con
pcDNA3.0-CatK +miR-7
CatK
β-catin
Relative Catk expression level
pcDNA3.0
pcDNA3.0-CatK
pcDNA3.0-CatK miRNA com
pcDNA3.0-CatK miR-7
C
OD 570 nm (Proportional to the number of adherent cells)
D
Distangce travelled (mm)
E
Cell numbers

Figure 6. The decreased Invasion and metastasis following the overexpressiom of miR-7 could be reversed by CatK in vitro. A. The results of transfection efficiency. The fluorescence microscopy assay showed the successful results in the SH-SY5Y and SK-N-SH cells. B. The western blot results of CatK over-expression plasmid tranfected into SH-SY5Y and SK-N-SH cells in the differently treated groups: The CatK protein expression was increased in the CatK or in the CatK + miRNA control group in the SH-SY5Y and SK-N-SH cells (P<0.01, respectively). Meanwhile, the increased CatK expression was decreased in the CatK + miR-7 group by adding miR-7 compared to the CatK + miRNA control group in SH-SY5Y or SK-N-SH cells (P<0.05, respectively). C. The results of adhesion in the different groups in SH-SY5Y or SK-N-SH cells: The adhesion capacity (OD value) was increased in the CatK group following the expression of CatK in SH-SY5Y or SK-N-SH cells (P<0.01, respectively). However, the increased cells adhesion capacity following the over-expression of CatK could be reversed (not increased) by up-regulation of miR-7 in SH-SY5Y or SK-N-SH cells (P<0.05, respectively). D. The results of migration in the different groups in SH-SY5Y or SK-N-SH cells: The migration capacity (distance travelled) was increased in the CatK group following the over-expression of CatK in SH-SY5Y or SK-N-SH cells (P<0.01, respectively). However, the increased cells migration capacity following the over-expression of CatK could be reversed (not increased) by up-regulation of miR-7 in SH-SY5Y or SK-N-SH cells (P<0.05, respectively). E. The results of invasion in the different groups in SH-SY5Y or SK-N-SH cells: The invasion capacity (cell numbers) was increased in the CatK group following the over-expression of CatK in SH-SY5Y or SK-N-SH cells (P<0.01, respectively). However, the increased cells invasion capacity following the over-expression of CatK could be reversed (not decreased) by up-regulation of miR-7 in SH-SY5Y or SK-N-SH cells (P<0.05, respectively). Results were representative of three separate experiments and bars indicated the SD. Data were expressed as the mean ± standard deviation (*P<0.05, **P<0.01).

metastasis both in vitro and in vivo, which indicated the tumor suppressor role of miR-7 in NB. Our results about miR-7 were consistent with the previous reports.

Similar to classical transcription factors, miRNAs exert their effects via regulating specific target genes. The different roles of miRNA in cancers may mostly depend on the roles of their target genes. To date, the mechanism of miR-7 function in NB has not been investigated, although several targets have been identified including EGFR, Pak1 and Ack1, which were smoothened with the key roles affecting on proliferation, invasion and metastasis [26-28]. Firstly, we found that CatK was a target gene of miR-7 by means of chip analysis and bioinformatics. Moreover, luciferase reporter assays revealed that miR-7 could directly target the 3'-UTR of CatK mRNA. Taken together, we put forward a hypothesis that miR-7 targets CatK to inhibit invasion and metastasis of NB cells. Different from the previous results owing to the different gene and its special function [26-28], our results indicated CatK was targeted by miR-7, yet we could also use the similar method to prove their correlation. Therefore, we proved that exogenous miR-7 down-regulated the expression of CatK protein. Besides, we showed that the loss of CatK performed the similar effects to miR-7 over-expression on NB cells. Furthermore, the over-expression of miR-7 substantially reversed the tumor-active effects of CatK. These results supported that CatK, as a direct target gene of miR-7, could play an important role in regulating biological behaviors of NB tumor such as invasion and metastasis. However, it was also needed to demonstrate the effect in animal models before the clinical trial.

CatK is a member of the lysosomal cysteine cathepsin (LCC) sub group, mainly secreted into the extracellular space where it can degrade fibrillary collagen as found in bone and cartilage [8]. Jon Christensen reported that CatK promoted breast cancer cells bone metastasis by MMP-9 pathway [29]. CatK was found to be up-regulated in chondrosarcoma, where its expression levels correlated with metastatic potential and poor prognosis [10]. Recent studies reported CatK appeared to be over-expressed in various types of cancers especially glioma, such as bone, breast, lung, ovarian carcinoma and prostate cancers, and its expression was closely related to the tumor growth, invasion and metastasis [30]. In the present study, we found that inhibition of CatK remarkably suppressed the growth and motility of NB cells. Our study expanded the function of CatK in NB. Even so, future studies should also determine the CatK feedback effect (the pathways associated with CatK in the above) on miR-7 during the metastasis and invasion in NB cells, and the above mechanism should be further investigated in various kinds of NB cells.

Collectively, our data established a functional link between miR-7 and CatK expression in NB, demonstrating that CatK was directly targeted by miR-7, which was expressed at lower levels in aggressive NB. Restoring miR-7 func-

tion might provide an alternative approach to attenuate aggressive tumor properties by reducing therapeutically CatK expression.

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Informed consent was obtained from all individual participants included in the study. The participants provided their written informed consents to participate in this study, but could not be provided because of too many written informed consents in Chinese or it would not be feasible for me to provide all informed consent because of their number and non-English language.

Disclosure of conflict of interest

None.

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