microRNA-144-3p suppresses human neuroblastoma cell proliferation by targeting HOXA7

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Abstract. – OBJECTIVE: Dysregulation of microRNAs (miRNAs) expression often resulted in abnormal cell behaviors. It has been demonstrated that miRs may serve as oncogenic or tumor suppressive functions in tumor. We investigated whether or not miR-144-3p has a role in the progression of human neuroblastoma (NB).

PATIENTS AND METHODS: 46 NB patients were enrolled in this study. miR-144-3p expression in NB tissues and cell lines was detected by reverse transcription-quantitative polymerase chain reaction. The biological functions of miR-144-3p in NB were detected by cell counting kit-8 assay, flow cytometry assay, and wound-healing assay. Luciferase activity assay and Western blot assay were performed to validate the direct targets of miR-144-3p.

RESULTS: We found miR-144-3p expression was reduced in NB tissues and cell lines and resulted in the stimulation of cell proliferation, cell cycle progression, and cell migration *in vitro*. Furthermore, we validated homeobox protein A7 (HOXA7) as a direct target of miR-144-3p.

CONCLUSIONS: Taken together, these results demonstrated the tumor suppressive role of miR-144-3p in NB and may advance the understanding of the underlying mechanisms of miR-144-3p and HOXA7 in NB.

Key Words:
   miR-144-3p, HOXA7, Neuroblastoma, Tumor suppressor.

Introduction

Neuroblastoma (NB) accounts for approximately 15% of all the childhood cancer deaths [1]. Due to the lack of efficient screening measures, NB patients are often diagnosed at advanced stages that resulted in undesired treatment outcomes of the conventional therapeutic approaches such as surgery, chemoradiotherapy, and stem cell transplantation[2,3]. Thus, for a better understanding of the mechanisms underlying the tumorigenesis and aggressiveness of NB, is essential to improve the treatment efficiency. Extensive studies[4,5] have highlighted the importance of microRNAs (miRNAs) in regulating cell behaviors. miRNAs are a class of endogenous RNAs with the length of 22 nt to 25 nt[5]. It has been well established that miRNAs are able to regulate gene expression through interfering the 3'-untranslated region (3'-UTR) or 5'-UTR of their targets to function as tumor suppressor or oncogene in human cancers[6,7]. Previous researches[8,9] have validated several abnormal expressed miRNAs that can regulate almost all of the aspects of tumor biology of NB, suggesting the important of miRNAs in NB. For example, miR-506 directly downregulates the Rho-associated, coiled-coil containing protein kinase 1 (ROCK1) expression through the transforming growth factor (TGF)-β non-canonical pathway to inhibit NB cell invasion and migration[10]. miR-558 was up-regulated in NB tissues to active heparanase transcriptional and eventually facilitate the tumorigenesis and aggressiveness of NB[11]. Deregulated miR-144-3p expression has been found in multiple human cancers including gastric cancer, glioblastoma, and hepatocellular carcinoma[12-14]. However, miR-144-3p expression pattern in NB remains unclear. In this study, we firstly evaluated the expression of miR-144-3p in NB tissues and cell lines. Then, the biological value of miR-144-3p expression on the tumorigenesis of NB was examined.

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Patients and Methods

Patients and Tissue Samples

46 pairs NB tissues and adjacent nontumor tissues used to measure miR-144-3p expression were collected from patients who underwent treatment at The First Affiliated Hospital of Xinxiang Medical University between January 2015 and March 2016. All tissues were immediately frozen in liquid nitrogen. These patients did not receive any anti-cancer treatments ahead of surgery. All the enrolled patients have signed written informed consent, and this study was approved by the Ethic Committee of The First Affiliated Hospital of Xinxiang Medical University.

Cell Culture and Cell Transfection

Human NB cell lines (SK-N-SH and SH-SY5Y) were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The control cell line, human endothelial cell line HUVEC, was purchased from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China). These cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium supplemented with 10% fetal bovine serum (FBS) and 100 U/ml penicillin/100 μg/ml streptomycin (Thermo Fisher Scientific, Inc., Waltham, MA, USA) in a humidified 5% CO_2 incubator at 37°C. The miR-144-3p mimic, miR-144-3p inhibitor and negative control (NC) miRNA were purchased from GenePharma Co., Ltd. (Shanghai, China). The small-interfering RNA (siRNA) targeting HOXA7 and NC siRNA were also purchased from GenePharma Co., Ltd. (Shanghai, China). Cells were seeded at a density of 5 x 10^4 cells/well and transfection was performed using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions.

Reverse Transcription-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA was isolated from the tissues and cells with TRIzol Reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. RNA concentration was measured by NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA). RNA was reverse transcribed to cDNA using reverse transcription kit (TaKaRa, Dalian, Liaoning, China) according to the provided protocol. RT-qPCR was performed using SYBR Premix Ex Taq™ kit (TaKaRa, Dalian, Liaoning, China) at ABI PRISM 7500 Quantitative PCR System (Applied Biosystems, Foster City, CA, USA) in line with the manufacturer's protocol. The following PCR conditions were used in this study: 30 s denaturation at 94°C, 30 s annealing at 58°C and 30 s extension at 72°C (30 cycles). The primers used were as follows: forward, 5'-ATCCAGTGCGTGTCGTG-3' and reverse, 5'-TGCTTATACAGTATAGATG-3' for miR-144-3p; forward, 5'-AGAGCCTGTG-GTGTCCG-3' and reverse 5'-CATCTTCAAA-GCACTTCCCT-3' for U6 snRNA.

Western Blot

Total proteins were extracted using RIPA protein lysis buffer with protease inhibitor (Beyotime, Haimen, Jiangsu, China). Samples were separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF, Beyotime, Shanghai, China) membranes. Following, the membranes were blocked with non-fat milk for 1 hand incubated with mouse monoclonal antibody to HOXA7 (ab51235, Abcam, Cambridge, MA, USA) or GAPDH (ab9482, Abcam, Cambridge, MA, USA), and the horseradish peroxidase (HRP) conjugated goat anti-mouse secondary antibody (ab205719, Abcam, Cambridge, MA, USA). Finally, protein signals were detected by ECL Kit (Beyotime, Shanghai, China) following the manufacturer's instructions.

Cell Proliferation Assay

Cell counting kit-8 (CCK-8) assay was conducted to measure the effects of miR-144-3p or HOXA7 on cell viability. Briefly, cells were seeded at a density of 1 x 10^3 cells/well. Cell proliferation rates were detected at 0, 24, 48 and 72 h following incubation. 10 μl CCK-8 solution (Beyotime, Shanghai, China) were added into each well and incubated for 3 h at the aforementioned conditions. Optical density was measured at 450 nm to calculate the cell proliferation rate.

Flow Cytometry Assay

Flow cytometry assay was conducted to measure the effects of miR-144-3p or HOXA7 on cell cycle progression. Cultured cells were trypsinized and fixed with 70% ethanol on ice for overnight. Subsequently, 0.05 mg/ml propidium iodide (PI) and 0.1 mg/ml RNAse A (Beyotime, Shanghai, China) were added to the samples at darkness and incubated at 4°C for 30 min. Cell cycle distribution was examined using a BD FACSCalibur flow cytometer (BD Biosciences, Franklin lakes, NJ,

USA) and analyzed using Modfit 5.0 software (Verity Software House, Topsham, ME, USA).

Cell Migration Assay

Wound-healing assay was conducted to measure the effects of miR-144-3p or HOXA7 on cell migration. Cell surface were scraped with 200 µl pipette tips. Phosphate-buffered saline (PBS) buffer was used to remove the detached cells or cell debris. Cell images were photographed at 0 and 24 h post wound creation. The percentage of wound closure was calculated using Image 1.42 software (NIH, Bethesda, MD, USA).

Luciferase Reporter Assay

The targets of miR-144-3p were predicted using Targetscan (www.targetscan.org). The wild-type (WT) and mutant (Mut) 3'-UTR sequences of HOXA7 were cloned into pRL-TK vector (Promega, Madison, WI, USA). For luciferase activity assay, the cells cultured at a density of 1 x 10^5 cells/well and were co-transfected with WT / Mut HOXA7 3'-UTR and miR-144-3p mimic/NC miRNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h of transfection, dual-luciferase reporter assay kit (Promega, Madison, WI, USA) was used to determine luciferase intensity. The results were expressed as the relative luciferase activity normalized to Renilla luciferase activity.

Statistical Analysis

Statistical analysis was performed on SPSS 17.0 software (SPSS Inc., Chicago, IL, USA). For comparison of two groups, Student's *t*-test was performed. For comparison of multiple groups, one-way analysis of variance followed by a Tukey's post-hoc test was performed. Correlation between miR-144-3p expression and clinicopathological features was analyzed by x^2-test. Correlation between miR-144-3p expression and HOXA7 expression was analyzed by Spearman's correlation test. Results were expressed as the mean ± SD. $p < 0.05$ was considered to indicate a statistically significant difference

Results

miR-144-3p Expression Was Decreased in NB Tissues and Cell Lines

We firstly examine miR-144-3p expression in NB tissues by RT-qPCR. As presented in Figure 1A, the results indicated that miR-144-3p expression was significantly reduced in NB tissues compared with the adjacent noncancerous tissues. Further, we investigated miR-144-3p expression in NB cell lines and HUVEC. We found miR-144-3p expression was also remarkedly reduced in NB cell lines compared to HUVEC (Figure 1B).

Correlation Between miR-144-3p Expression and Clinicopathological Features

According to the median expression level of miR-144-3p, we classified the enrolled patients into high or low miR-144-3p expression group. The correlation between miR-144-3p expression and clinicopathological featured was analyzed and summarized in Table I. We found low miR-144-3p expression was closely correlated with unfavorable clinicopathological features inclu-
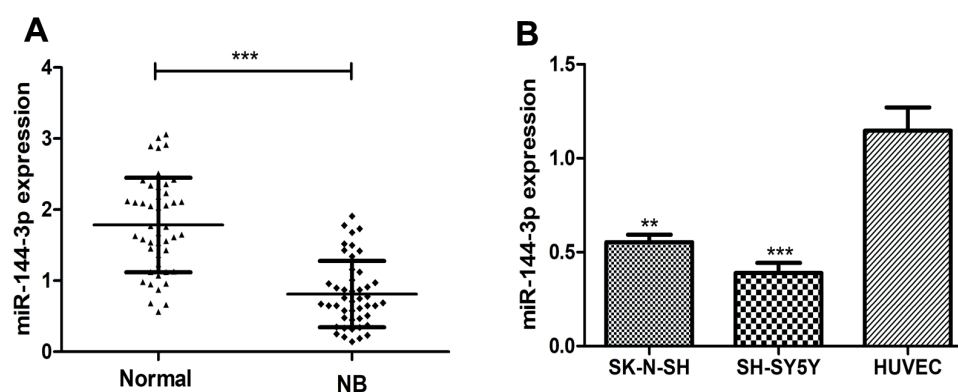

Figure 1. miR-144-3p was frequently decreased in NB tissues and cell lines. (A) Expression level of miR-144-3p was determined in NB tumor tissues and matched normal tissues by RT-qPCR. (B). Expression level of miR-144-3p was examined in HUVEC and NB cell lines including SK-N-SH and SH-SY5Y by RT-qPCR. (**$p < 0.01$, ***$p < 0.001$.) miR-144-3p: microRNA-144-3p, NB: neuroblastoma, RT-qPCR: reverse transcription-quantitative polymerase chain reaction.
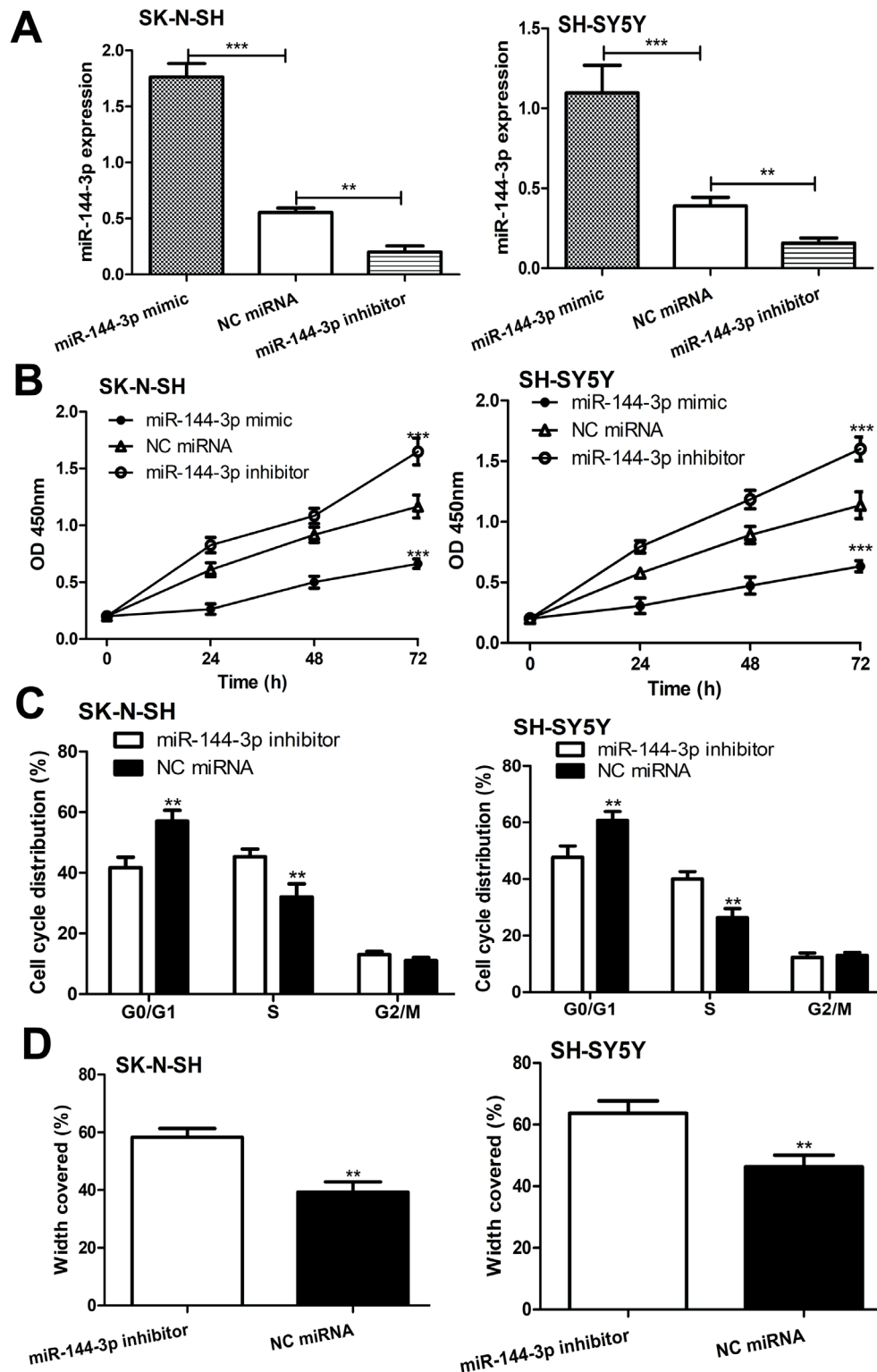
Figure 2. Effects of miR-144-3p expression on NB cell proliferation, cell cycle and cell migration. *(A)* The expression of miR-144-3p in SK-N-SH and SH-SY5Y after miRNAs transfected. *(B)* CKK-8 assay was conducted to examine the proliferation ability, *(C)* Flow cytometry assay was conducted to examine the cell cycle distribution, and *(D)* Wound-healing assay was conducted to examine the migration ability of SK-N-SH and SH-SY5Y after miRNAs transfected. (**$p < 0.01$, ***$p < 0.001$.) miR-144-3p: microRNA-144-3p, NB: neuroblastoma, CCK-8: cell counting kit-8, NC: negative control.

Table I. Correlations between the expression of miR-144-3p and clinicopathological features.

Features	No.	miR-144-3p		p value
		High	Low	
Gender				
Male	25	12	13	0.298
Female	21	9	12	
Age				
> 5	24	10	14	0.340
< 5	22	11	11	
Size of carcinoma				
> 3	29	14	15	0.023
< 3	17	7	10	
Lymph node metastasis				
Negative	17	8	9	0.026
Positive	29	13	16	
Tumor stage				
I-II	17	6	11	0.014
III	29	15	14	

miR-144-3p: microRNA-144-3p.

ding tumor stage (p = 0.023), carcinoma size (p = 0.026), and lymph node metastasis (p = 0.014). No significant correlation was observed between miR-144-3p expression and age and gender.

Effects of miR-144-3p Expression on NB Progression in vitro

To manipulate miR-144-3p expression in NB cell lines, the synthesized miR-144-3p mimic, inhibitor, and NC miRNA were used. RT-qPCR results showed that miR-144-3p mimic transfection remarkably elevated miR-144-3p levels in NB cell lines, while miR-144-3p inhibitor transfection significantly reduced miR-144-3p levels (Figure 2A). CCK-8 assays showed that miR-144-3p overexpression inhibits NB cells proliferation, while miR-144-3p inhibitor has the opposite effects on cell proliferation (Figure 2B). Further cell cycle distribution analysis revealed that miR-144-3p deregulation decreased the cells at G0/G1 phase (Figure 2C). Finally, we analyzed the NB cell migration ability post miRNAs transfection. A similar pattern of stimulation on cell migration was observed in NB cells with miR-144-3p inhibitor transfection (Figure 2D). Collectively, these results revealed miR-144-3p inhibits NB tumor progression *in vitro*.

HOXA7 was a Direct Target of miR-144-3p in NB

The prediction results of TargetScan revealed HOXA7 contained a putative binding site for miR-144-3p in its 3'-UTR (Figure 3A). Luciferase reporter assay revealed that the introduction of miR-144-3p mimic in WT HOXA7 3'-UTR transfected NB cells significantly reduced the luciferase activity, while it did not has significant effect on the luciferase activity of Mut HOXA7 3'-UTR transfected cells (Figure 3B). Subsequently, we investigated whether miR-144-3p regulates the protein expression of HOXA7 in NB cells. As shown in Figure 3C, the introduction of miR-144-3p inhibitor enhanced the protein expression of HOXA7 in NB cells. In addition, we found miR-144-3p and HOXA7 expression was inversely correlated in NB tissues (Figure 3D).

Knockdown of HOXA7 Impairs the miR-144-3p Inhibitor Induced NB Progression Stimulation in vitro

To investigate the potential role of HOXA7 in the miR-144-3p induced tumor progression activation, we transfected siRNA-targeting HOXA7 (si/HOXA7) into SH-SY5Y cells. Not surprisingly, we found the protein level of HOXA7 was reduced by siRNA (Figure 4A). CCK-8 assay revealed that co-transfection of si/HOXA7 and miR-144-3p inhibitor partially reversed the promotion effect of miR-144-3p inhibitor on cell proliferation (Figure 4B). We further measured cell cycle distribution and cell migration to determine whether HOXA7 has a role in cell cycle and cell migration regulation. We found HOXA7 knockdown attenuated the cell cycle progression and cell migration capabilities of SH-SY5Y cells (Figure 4C and 4D).

Discussion

The development of NB is a complex process of epigenetic and genetic changes accumulation[15,16]. Recently, miRNAs including miR-10a/b, miR-7 and miR-335, have been identified to be associated with the progression and metastasis of NB[17-20]. However, the precise mechanisms of these miRNAs in the tumor progression process remain to be elucidated. Thus, revealing these mechanisms will surely help us to find novel treatment measures for NB[7,8]. miR-144-3p has been identified to play important roles in the development of several human cancers[12-14]. For example, Cheng et al[13] reported miR-144-3p inhibited the proliferation, invasion and migration of glioblastoma cells by targeting FZD7 and served as independent predictors. Wu et al[14] found miR-144-3p significantly inhibited proliferation, migration and repressed angiogenesis of hepatocellular carcinoma cells by regulating SGK3 activation and acted as a prognostic factor. On the contrary, Xiao et al[21] reported that higher expression of miR-144-3p enhanced the malignancy and resistance to Sunitinib in clear cell renal cell carcinoma through targeting ARID1A. HOXA7 has been reported to be associated with the progression of several human cancers[22,23]. However, HOXA7 expression and its underlying mechanism in NB still remain largely unknown. In this present study, we found miR-144-3p was downregulated but HOXA7 was upregulated in NB. Low miR-144-3p expression was associated with tumor stage, carcinoma size, and lymph node metastasis, implying the tumor suppressive role of miR-144-3p in NB. Following, the *in vitro* cell proliferation, cell cycle, and cell migration assays were conducted to investigate the biological roles of miR-144-3p in NB. We found the cell cycle was arrested in G0/G1 phase, which not surprisingly resulted in cell proliferation inhibition. Also, we found the cell migration was also suppressed by miR-144-3p overexpression.
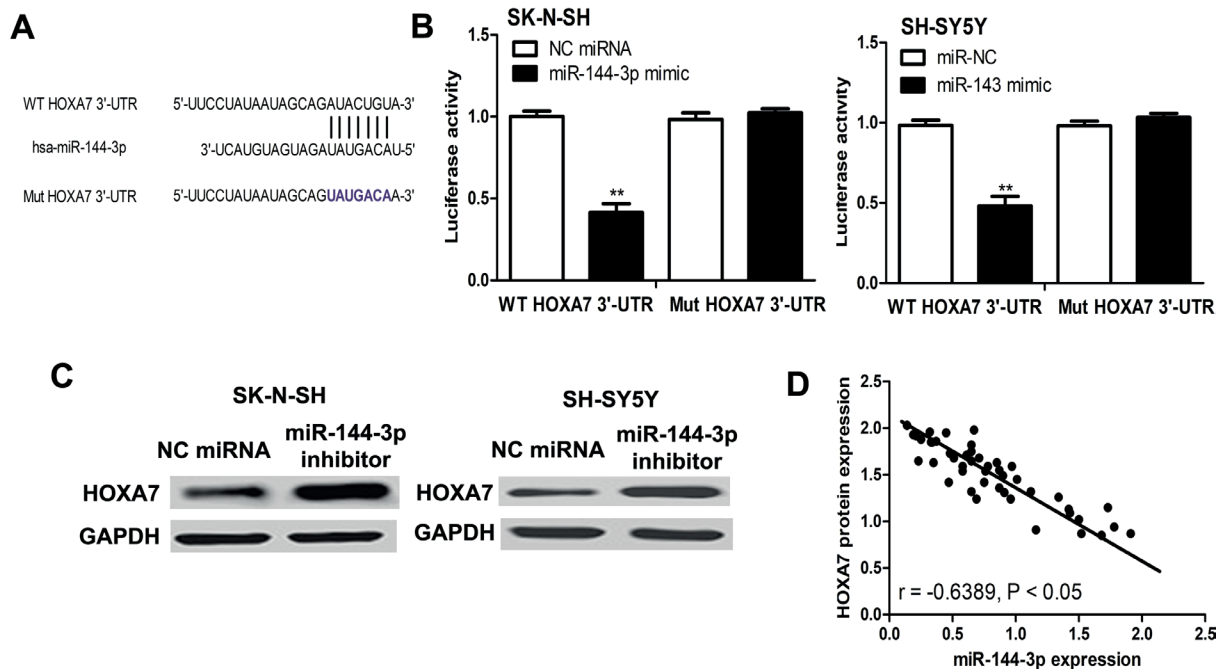

Figure 3. HOXA7 was identified as a functional target of miR-144-3p in NB. *(A)* TargetScan software predicted HOXA7 was a potential target of miR-144-3p. *(B)* Luciferase activity was inhibited in NB cells only in the presence of WT HOXA7 3'-UTR and not with Mut HOXA7 3'-UTR. *(C)* miR-144-3p inhibitor transfection increased HOXA7 protein expression in NB cell lines. *(D)* Inversely correlation between miR-144-3p expression and HOXA7 protein expression in NB tissues. (**p < 0.01) miR-144-3p: microRNA-144-3p, NB: neuroblastoma, HOXA7: homeobox protein A7, WT: wild type, Mut: mutant, UTR: untranslated region, NC: negative control.
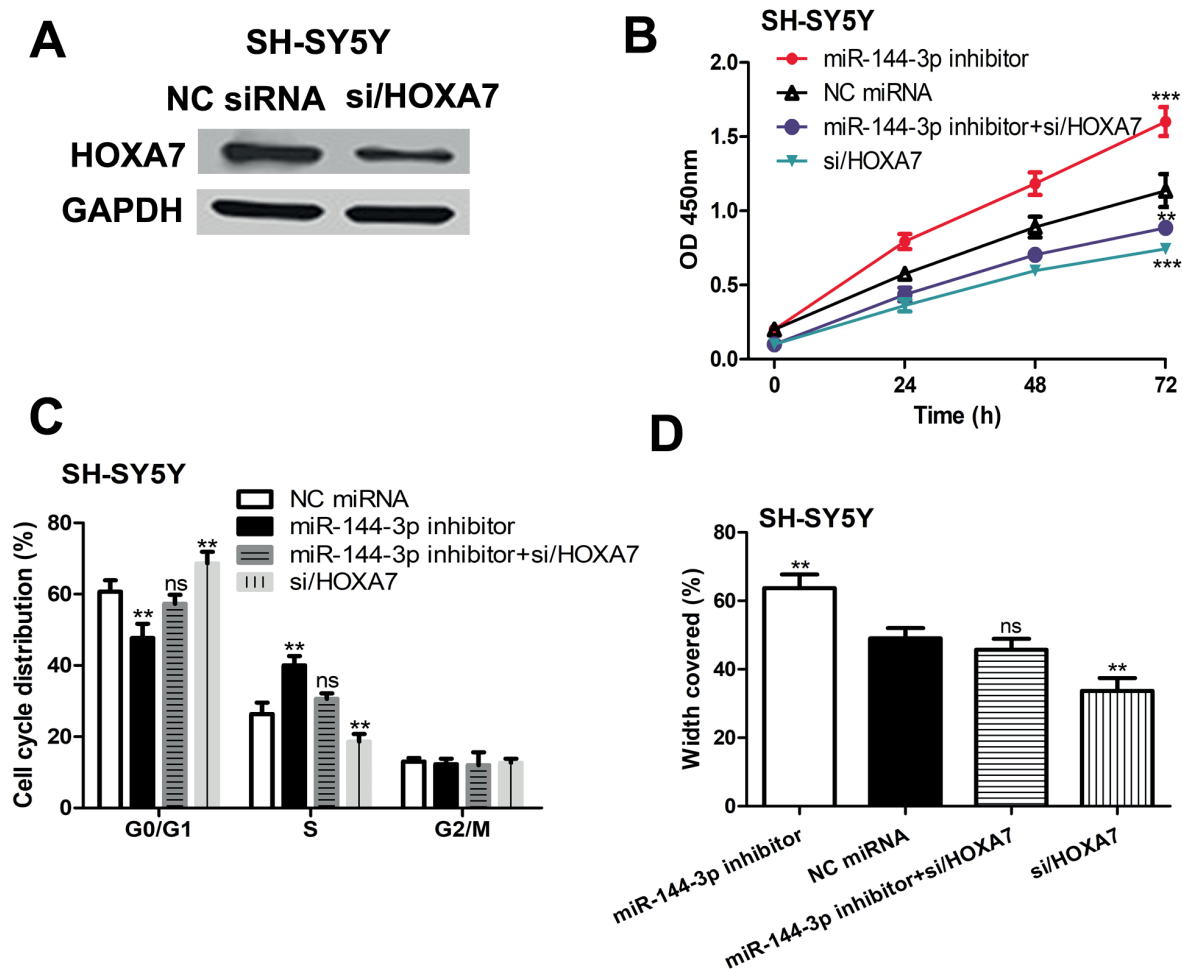
Figure 4. miR-144-3p regulates NB cell proliferation, cell cycle and cell migration by targeting HOXA7. (A) si/HOXA7 transfection decreased HOXA7 protein expression in SH-SY5Y cell line. (B) CKK-8 assay was conducted to examine the proliferation ability, (C) Flow cytometry assay was conducted to examine the cell cycle distribution, and (D) Wound-healing assay was conducted to examine the migration ability of SH-SY5Y after miRNA or siRNA transfected (**$p < 0.01$, ***$p < 0.001$, ns not significant.) miR-144-3p: microRNA-144-3p, NB: neuroblastoma, HOXA7: homeobox protein A7, si/HOXA7: small interfering RNA targeting HOXA7.

Furthermore, we found HOXA7 was a direct target of miR-144-3p through computational algorithm programs analysis and luciferase activity assay. Also, we demonstrated HOXA7 was a mediator for the tumor suppressive role of miR-144-3p by *in vitro* functional assays.

Conclusions

We demonstrated miR-144-3p was frequently downregulated in NB and functioned as a tumor suppressor through targeting HOXA7. Meanwhile, we provide evidence that miR-144-3p might be a potential therapeutic target for NB.

Conflict of Interest

The Authors declare that they have no conflict of interest.

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