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Knockdown of lncRNA HOTTIP Inhibits Retinoblastoma Progression by Modulating the miR-101-3p/STC1 Axis

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Abstract

Objective: Retinoblastoma (RB) is a frequent eye cancer in children. Long non-coding RNA (LncRNA) HOXA transcript at the distal tip (HOTTIP) is aberrantly expressed in cancer tissues. This study explores the underlying mechanism of lncRNA HOTTIP in RB. **Methods:** HOTTIP expression in normal retinal cells and RB cell lines was detected using qRT-PCR. The proliferation of RB cells was measured using CCK-8 and EdU assays, and apoptosis was detected using flow cytometry and Western blotting after the transfection of si-HOTTIP into Y79 cells and pc-HOTTIP into HXO-RB-44 cells. The target relationships between HOTTIP and miR-101-3p, and miR-101-3p and STC1 were predicted by bioinformatics website and verified using dual-luciferase reporter gene assay. The binding of HOTTIP and miR-101-3p was verified using RNA pull-down assay. STC1 mRNA and protein in RB cells were measured using qRT-PCR and Western blotting. Moreover, si-HOTTIP and in-miR-101-3p/in-NC, and si-HOTTIP and pc-STC1/pcDNA were co-transfected into Y79 cells respectively to evaluate cell proliferation and apoptosis. Xenograft study was conducted, and Ki67-positive expression was detected using immunohistochemical staining. **Results:** HOTTIP expression was promoted in RB tissues and cells. Downregulation of HOTTIP inhibited proliferation and promoted apoptosis of Y79 cells, while upregulation of HOTTIP promoted proliferation and inhibited apoptosis of HXO-RB-44 cells. There were target relationships between HOTTIP and miR-101-3p, and miR-101-3p and STC1. Inhibition of miR-101-3p or overexpression of STC1 reversed the effect of si-HOTTIP on the proliferation and apoptosis of RB cells. Xenograft study showed that knockdown of HOTTIP suppressed the growth of RB *in vitro*. **Conclusion:** It could be concluded that HOTTIP sponged miR-101-3p to upregulate STC1 expression, thereby promoting RB cell proliferation and inhibiting apoptosis.

Keywords

LncRNA HOTTIP, microRNA-101-3p, STC1, retinoblastoma, proliferation, apoptosis

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Introduction

Retinoblastoma (RB) is a kind of malignant tumor caused by immature cells in the retina of one or both eyes, which usually occurs in children under 5 years old.[1] The clinical symptoms of RB are marked by pupil abnormality, vision decline, leukocoria, red and irritated eyes, growth retardation or retardation.[2] Currently, the major treatments for RB include enucleation, radiotherapy, chemotherapy and focal therapies.[3] The survival rate of RB in developing countries is still lower where many children die of this disease.[4] In short, RB remains a considerable threat to children' health and quality of life. Further elucidating the molecular mechanism of RB is an urgent issue to be solved in oncology to open up a novel therapy for RB.

Long non-coding RNAs (LncRNAs), a class of non-coding RNA transcripts, take part in the cancer progression

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widely.[5] The dysregulation of lncRNAs is concerned with the clinicopathological characteristics and progression of RB.[6] For example, Dong *et al*. find that lncRNA HOTAIR is markedly increased in RB cells and knockdown of HOTAIR inhibits RB cell proliferation and invasion.[7] Qi *et al*. also reveal that lncRNA H19 silencing has suppressive influences on RB.[8] The HOXA transcript at the distal tip (HOTTIP) has obtained increasing attention in cancer-associated processes.[9] HOTTIP exerts crucial effects on the pathogenesis of cancers and is believed to have a bearing on the poor prognosis of cancers.[10] For example, Wang *et al*. demonstrate that HOTTIP can advance the course of renal carcinoma.[11] HOTTIP expression is promoted in breast cancer cells, which was negatively related to the breast cancer prognosis.[12] However, relatively little is known about the role of HOTTIP in RB so far.

The existing researches indicate that HOTTIP works as an oncogene in human cancers mostly via sponging microRNAs (miRs).[13-15] miRs are endogenous non-coding RNAs that regulate protein-coding genes post-transcriptionally.[16] miRs regulate important physiological processes and pathological conditions including carcinogenesis.[17] For instance, Wan *et al*. reveal that miR-25-3p promotes malignant transformation of RB cells.[18] We speculate that HOTTIP may play a regulatory role in RB via the competitive endogenous RNAs (ceRNA) mechanism. This study herein investigates the effect of lncRNA HOTTIP on RB, along with its underlying miR and downstream target gene, which shall provide the impetus for the determination of new therapeutic targets of RB.

Materials and Methods

Sample Collection and Cell Culture

The RB tissue samples were obtained from 31 patients with RB (15 males and 16 females) in Affiliated Hospital of Qingdao University, ranged from 1 to 5 years old (average 1.41 $\pm$ 1.56 years old). Normal tissue samples came from 11 patients (6 males and 5 females) who had their eyeballs removed from trauma, ranged from 1 to 6 years old (average 1.14 $\pm$ 1.40 years old). No significant difference was observed in age and gender between patients who provided RB tissues and normal tissues ($p > 0.05$), and those receiving chemotherapy, radiotherapy or targeted drug treatment before surgery were excluded from the study. The severity of RB was assessed by International Intraocular Retinoblastoma Classification (IIRC).[19] The tissue samples were stored in liquid nitrogen immediately after operation. RB cell lines (ARPE-19, HXO-RB-44, HXO-RB-44 and SO-RB-50) and Y79 cells were provided by Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were incubated in Roswell Park Memorial Institute-1640 medium (Gibco, Grand Island, NY, USA) added with 10% inactivated fetal bovine serum (HyClone, Logan, UT, USA), 100 U/mL penicillin, and 0.1 mg/mL streptomycin at 37°C with 5% CO_2.

Table 1. Primer Sequences for qRT-PCR.

Genes	Sequences
HOTTIG	F: 5′-GTGGGGCCCAGACCCGC-3′
	R: 5′-AATGATAGGGACACATCGGGGAACT-3′
STC1	F: 5′-GCAGGAAGAGTGCTACAGCAAG-3-3′
	R: 5′-CA TTCCAGCAGGCTTCGGACAA-3′
GAPDH	F: 5′-ACCCACTCCTCCACCTTTGAC-3′
	R: 5′-TGTTGCTGTAGCCAAATTCGTT-3′
miR-101-3p	F: 5′-TCCGAAAGTCAATAGTGTC-3′
	R: 5′-GTGCAGGGTCCGAGGT-3′
U6	F: 5′-CTCGCTTCGGCAGCACA-3′
	R: 5′-AACGCTTCACGAATTTGCGT-3′

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Total RNA of cells was extracted using the RNA extraction kit (Takara, Dalian, China) and the concentration of extracted RNA was detected. Next, miR-101-3p and U6 were reversely transcribed into cDNA using the One Step PrimeScript miRNA cDNA Synthesis kit (Takara). HOTTIP, STC1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were reversely transcribed into cDNA using the PrimeScript RT reagent kit with gDNA Eraser (Takara). The expressions of HOTTIP, STC1, miR-101-3p were detected on the instructions of SYBR Premier Ex Taq II (Takara) using qRT-PCR, with cDNA acting as the template. The relative expression of miR and mRNAs was calculated by $2^{-\Delta\Delta Ct}$ method, with U6 and GAPDH acting as the internal reference. Primer sequences are illustrated in Table 1.

Cell Transfection

Empty vector of pcDNA3.1 (pcDNA), overexpressed vector of pc-HOTTIP and pc-STC1 were provided by OriGene Technologies, Inc. (Beijing, China). Small interfering RNA (siRNA)-HOTTIP (si-HOTTIP), miR-101-3p mimic (mi-miR-101-3p), miR-101-3p inhibitor (in-miR-101-3p) and their negative controls (si-NC, mi-NC and in-NC) were provided by Gene Pharm (Shanghai, China). Then, siRNAs (100 pmol), mimic/inhibitor (100 nM) and vectors (4 μg) were transfected using the Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA). The subsequent experiments were performed after 48 hours of transfection.

Dual-Luciferase Reporter Gene Assay

The binding sites of HOTTIP and miR-101-3p, and miR-101-3p and STC1 were predicted using the Starbase v2.0 (http://starbase.sysu.edu.cn/index.php). The binding sequence or mutant sequence of HOTTIP containing miR-101-3p was synthesized and then cloned to the downstream of pmirGLO promoter vector (Promega, Madison, WI, USA). Subsequently, the wild type (WT) vectors (HOTTIP-WT/STC1-WT) and the mutant type (MUT) vectors (HOTTIP-MUT/STC1-MUT) were

constructed. Then the constructed vectors were co-transfected with mi-miR-101-3p or mi-NC into HEK293 T cells (Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China). The luciferase activity was evaluated after 48 hours of transfection.

RNA Pull Down Assay

Y79 and HXO-RB-44 cells were lysed by RIPA lysis buffer (Invitrogen) and incubated for 1 hour at 4°C with biotin-labeled NC, HOTTIP-MUT and HOTTIP-WT (Shanghai GenePharma Co, Ltd, Shanghai, China). After that, the lysate and Dynabeads M-280 Streptavidin (Invitrogen) were incubated at 4°C for 3 hours. TRIzol reagent (Invitrogen) was applied to extract RNA on the beads, and HOTTIP expression was detected using qRT-PCR.

Cell Counting Kit-8 (CCK-8) Assay

Cells were seeded in the 96-well plate (1×10^5 cells/well) and supplemented with 200 μL 0.5 mg/mL MTT solution (Solarbio Science & Technology Co., Ltd, Beijing, China) at 0, 24, 48 and 72 hours. After 4 hours of incubation, each well was supplemented with 100 μL dimethylsulfoxide (Solarbio) followed by gently shaking for 10 minutes. Thereafter, the optical density (OD) of each well at a wavelength of 450 nm was measured.

5-Ethynyl-2?-Deoxyuridine (EdU) Labeling Assay

The cell proliferation was determined using an EdU kit (Guangzhou RiboBio Co., Ltd, Guangdong, China). Cells were seeded in the 96-well plate (1×10^5 cells/well) and incubated in the medium containing 50 μmol/L EdU solution for 2 hours. Then, cells were fixed with 4% paraformaldehyde (pH = 7.4) for 30 minutes and treated with 0.5% Triton X-100 for 20 minutes. After phosphate buffer saline (PBS) washing, cells were stained with anti-EdU working solution and incubated with 100 μL Hoechst (5 μg/mL) for 30 minutes. Thereafter, 5 fields were selected for observation under the fluorescence microscope (Olympus, Tokyo, Japan), and the ratio of EdU-positive cells to total cells was calculated.

Apoptosis Assay

Cells were seeded into the 24-well plate (5×10^5 cells/well) and then stained with Annexin V-FITC and propidium iodide (PI) in the light of the instructions of FITC Annexin V apoptosis detection kits (Beyotime Biotechnology Co., Ltd, Shanghai, China) for 10 minutes. The stained cells were measured using flow cytometry (BD, San Jose, CA, USA) and the apoptosis rate was analyzed.

Western Blotting

Total protein was extracted with RIPA lysate (strong) (Beyotime Biotechnology Co., Ltd, Shanghai, China). Then the concentration of proteins was tested by the bicinchoninic acid assay (Beyotime). Next, the proteins were separated by electrophpresis and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% skimmed milk and washed by tris buffered saline tween (TBST). After that, the membranes were cultured with the primary antibodies at 4°C overnight: P21 activated kinase 2 (1:5000, ab76293, Abcam, Cambridge, MA, USA), B-cell lymphoma 2 (Bcl-2) (1:1000, ab32124, Abcam), Bcl-2-associated X protein (Bax) (1:2000, ab32503, Abcam), cleaved caspase-9 (1:2000, ab2324, Abcam), cleaved caspase-3 (1:2000, ab2302, Abcam) and β-actin (1:5000, ab179467, Abcam). After being washed by TBST (3 times/10 minutes), the membranes were cultured with secondary antibody horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) H&L (1:2000, ab205718, Abcam) for 1 hour and then washed by TBST (3 times/10 minutes) before chemiluminescence developing and visualization. The image of protein blotting was analyzed by Image J2x v2.1.4.7 software (Rawak Software, Inc. Germany).

Xenograft Tumor in Nude Mice

Ten BALB/c nude mice (aged 4 weeks and weighed 18-20 g) purchased from Guangdong Medical Laboratory Animal Center (Guangdong, China) [SYXK (Guangdong) 2018-0002] were assigned into si-HOTTIP group (subcutaneous injection of 5×10^6 Y79 cells transfected with si-HOTTIP) and si-NC group (subcutaneous injection of Y79 cells transfected with equal si-NC). The nude mice were placed in a sterile environment at 25°C with 50% humidity and provided ad libitum access to the water and feed. The length (L) and width (W) of tumors were measured weekly and the tumor volume (V) was measured with a caliper according to the formula [$V = (L \times W^2)/2$]. The nude mice were euthanized by injection of benzobarbital 4 weeks later. Thereafter, the tumors were dissected, weighed and embedded in paraffin.

Immunohistochemistry

The paraffin-embedded tumors were sectioned at 5 μm. After deparaffination and rehydration, the sections were cultured with the primary antibody Ki67 (1:200, ab16667, Abcam) at 4°C overnight and the secondary antibody anti-IgG (1:1000, ab6721, Abcam) for 30 minutes, following the development with 2,4-diaminobutyric acid (Solarbio). Thereafter, 5 visual fields were selected to analyze the OD of Ki67-positive cells using ImageJ2x (Rawak).

Statistical Analysis

Data analysis was introduced utilizing the SPSS 21.0 (IBM Corp., Armonk, NY, USA). Kolmogorov-Smirnov method checked the data were in normal distribution. Data are expressed as mean ± standard deviation. The *t* test was adopted for analysis of comparisons between 2 groups. One-way analysis of variance (ANOVA) or two-way ANOVA was

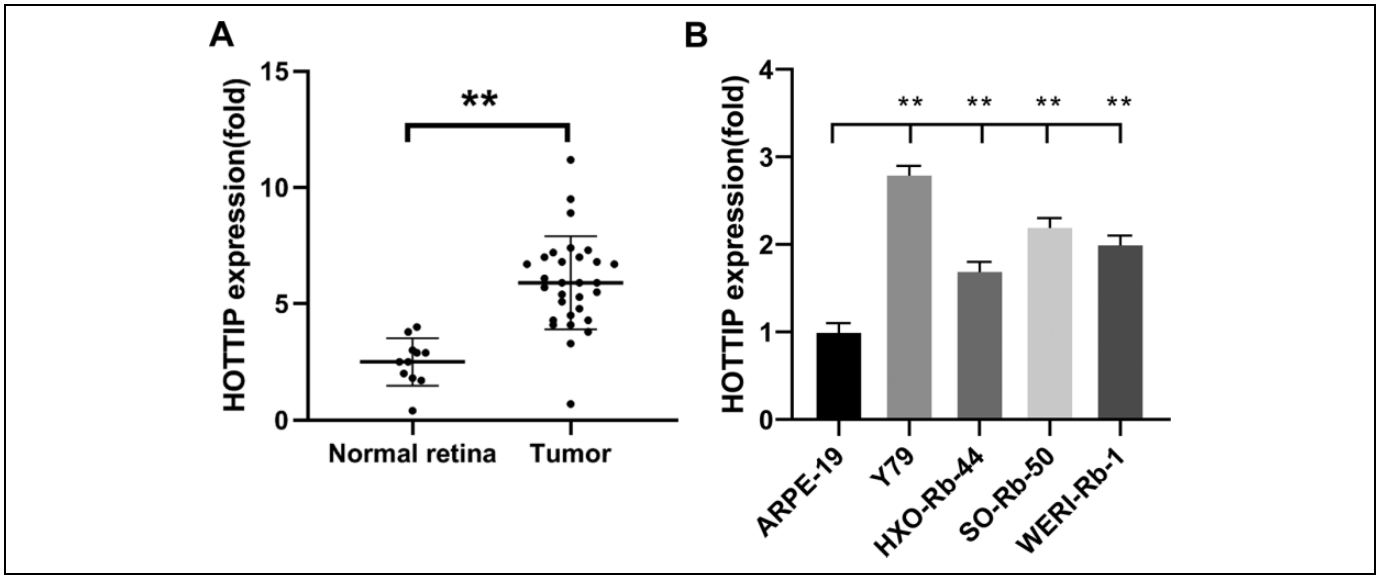

Figure 1. HOTTIG was upregulated in RB tissues and cells. (A) HOTTIP expression in RB tissues (n = 31) and normal retina tissues (n = 11) was detected using qRT-PCR; (B) HOTTIP expression in normal retina cells (ARPE-19) and RB cell line (HXO-RB-44, WERI-Rb-1, SO-RB-50 and Y79) was detected using qRT-PCR. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panel A were statistically analyzed by the t test, and data in panel B were analyzed using one-way ANOVA and Tukey's multiple comparisons test, **p < 0.01 vs normal retina or ARPE-19.

employed for the comparisons among multiple groups, and Tukey's multiple comparison test was applied for the post hoc test after ANOVA. The *p* value was obtained from a two-tailed test, and *chi* square test was utilized for analysis of HOTTIG expression and clinical parameters in RB patients. And $p < 0.05$ meant a statistically significance.

Results

HOTTIG Was Upregulated in RB Tissues and Cells

HOTTIG is reported to affect diabetic retinopathy.[20] Therefore, we speculated that HOTTIG might exert influences on RB. qRT-PCR demonstrated that HOTTIG expression in human RB tissues was notably higher than that in normal retinal tissues (Figure 1A) ($p < 0.01$). HOTTIG expression in RB cell lines (HXO-RB-44, WERI-Rb-1, SO-RB-50 and Y79) significantly increased compared with that in normal retinal cells (ARPE-19) (Figure 1B) ($p < 0.01$). The median value of HOTTIP expression was set as a critical value, with which the RB patients were divided into high-expression group (n = 16) and low-expression group (n = 15). As shown in Table 2, HOTTIP expression was related to IIRC stage, optical nerve invasion and differentiation grade ($p < 0.01$), implying that HOTTIP expression might affect the development of RB.

Table 2. Relationship Between HOTTIP Expression and Clinicopathological Features of RB patients.

	HOTTIP expression		
Parameters	Low expression (n = 16)	High expression (n = 15)	*p*
Age			0.458
≤ 3 years	12	9	
> 3 years	4	6	
Sex			0.724
Male	7	8	
Female	9	7	
IIRC stage			0.002
Early (A, B, C)	1	9	
Advanced (D, E)	15	6	
Optic nerve invasion			0.001
Negative	4	13	
Positive	12	2	
Differentiation grade			0.009
Well	6	13	
Poorly	10	2	

Low Expression of HOTTIP Suppressed RB Cell Proliferation and Promoted Apoptosis

si-HOTTIP was transfected into Y79 cells to decrease HOTTIP expression and pc-HOTTIP was transfected into HXO-RB-44 cells to increase HOTTIP expression. qRT-PCR verified the successful transfection ($p < 0.01$) (Figure 2A). Subsequently, CCK-8 and EdU assays were conducted to evaluated cell proliferation. The proliferation of Y79 cells transfected with si-HOTTIP reduced significantly while that of HXO-RB-44 cells transfected with pc-HOTTIP promoted markedly (all $p < 0.01$) (Figure 2B/C). The results of flow cytometry indicated that the number of apoptotic Y79 cells transfected with si-HOTTIP significantly increased while that of HXO-RB-44 cells transfected with pc-HOTTIP decreased (Figure 2D) ($p < 0.01$). Moreover, levels of Bax, cleaved caspase-9, cleaved caspase-3 and Bcl-2

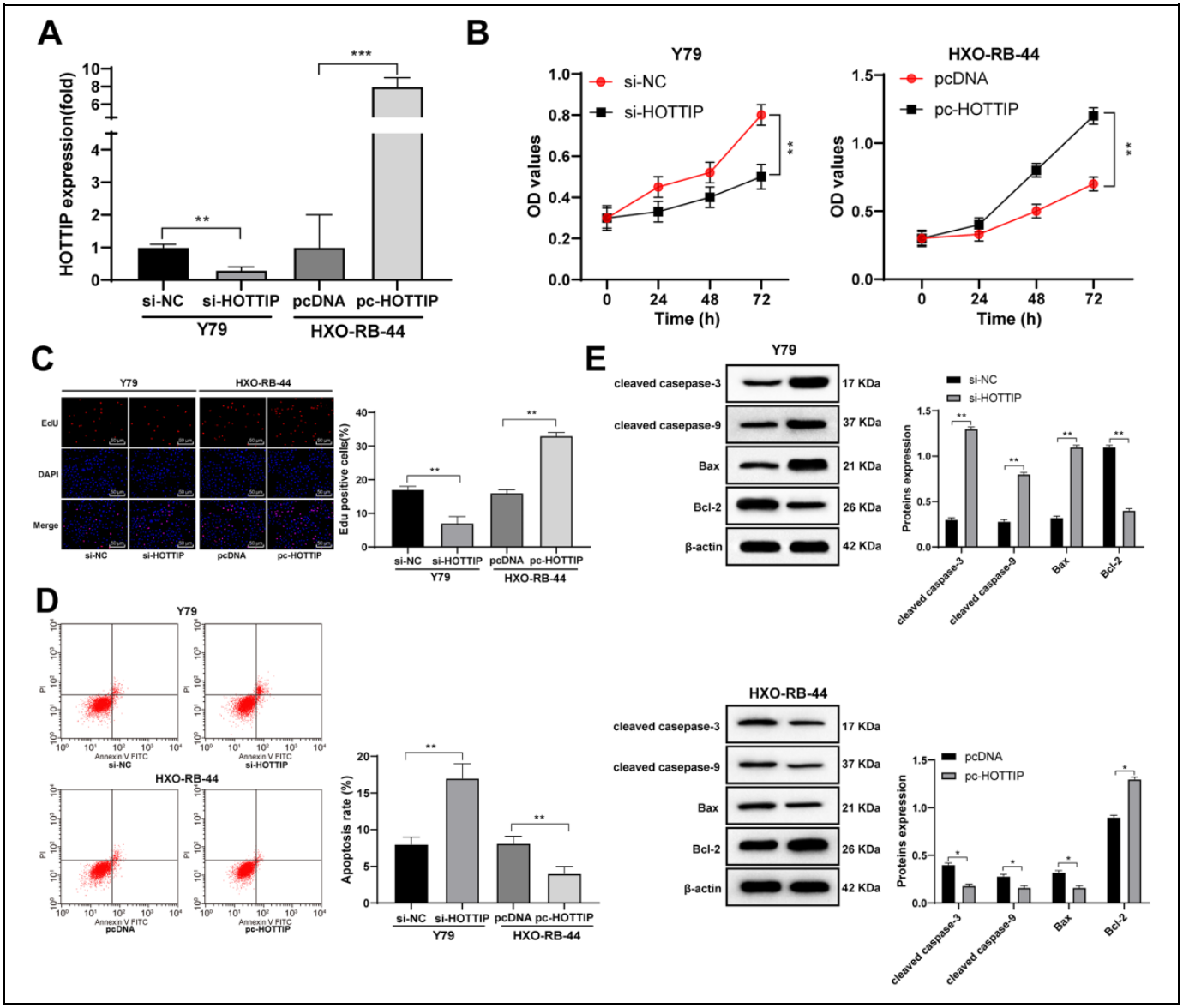

Figure 2. Low expression of HOTTIP suppressed RB cell proliferation and promoted apoptosis. (A) HOTTIP expression in Y79 cells transfected with si-HOTTIP and in HXO-RB-44 cells transfected with pc-HOTTIP was detected using qRT-PCR; (B) proliferation of Y79 and HXO-RB-44 cells was detected using CCK-8 assay; (C) EdU-positive rate of Y79 and HXO-RB-44 cells was detected using EdU assay; (D) apoptosis of Y79 and HXO-RB-44 cells was detected by flow cytometry; (E) expression of apoptosis-related proteins in Y79 and HXO-RB-44 cells was detected by Western blot. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panels A-E were analyzed using two-way ANOVA and Tukey's multiple comparisons test, **p < 0.01.

in Y79 cells transfected with si-HOTTIP increased significantly while HXO-RB-44 cells transfected with pc-HOTTIP showed the opposite trends (Figure 2E) (all $p < 0.01$). In brief, low-expressing HOTTIP inhibited RB cell proliferation and promoted apoptosis, while overexpressing HOTTIP had the opposite effect on RB cells.

HOTTIP Competitively Bound to miR-101-3p to Upregulate STC1 Expression

Previous studies have shown that lncRNAs work as ceRNAs to reduce miRs expression and thus relieve the inhibition of miRs on their target genes.[21,22] Therefore, bioinformatics website was employed to predict miRs interacting with HOTTIP, in which miR-101-3p can inhibit the proliferation of glioma cells.[23] We chose miR-101-3p for the subsequent analysis to investigate whether miR-101-3p exerts tumor-inhibitory effects on RB. The relative fluorescence enzyme activity of the mi-miR-101-3p group and the HOTTIP-WT group decreased significantly (Figure 3A) ($p < 0.05$), which confirmed the binding relationship between HOTTIP and miR-101-3p. Furthermore, miR-101-3p expression in Y79 cells transfected with si-HOTTIP and HXO-RB-44 cells transfected with pc-HOTTIP was detected, which showed that

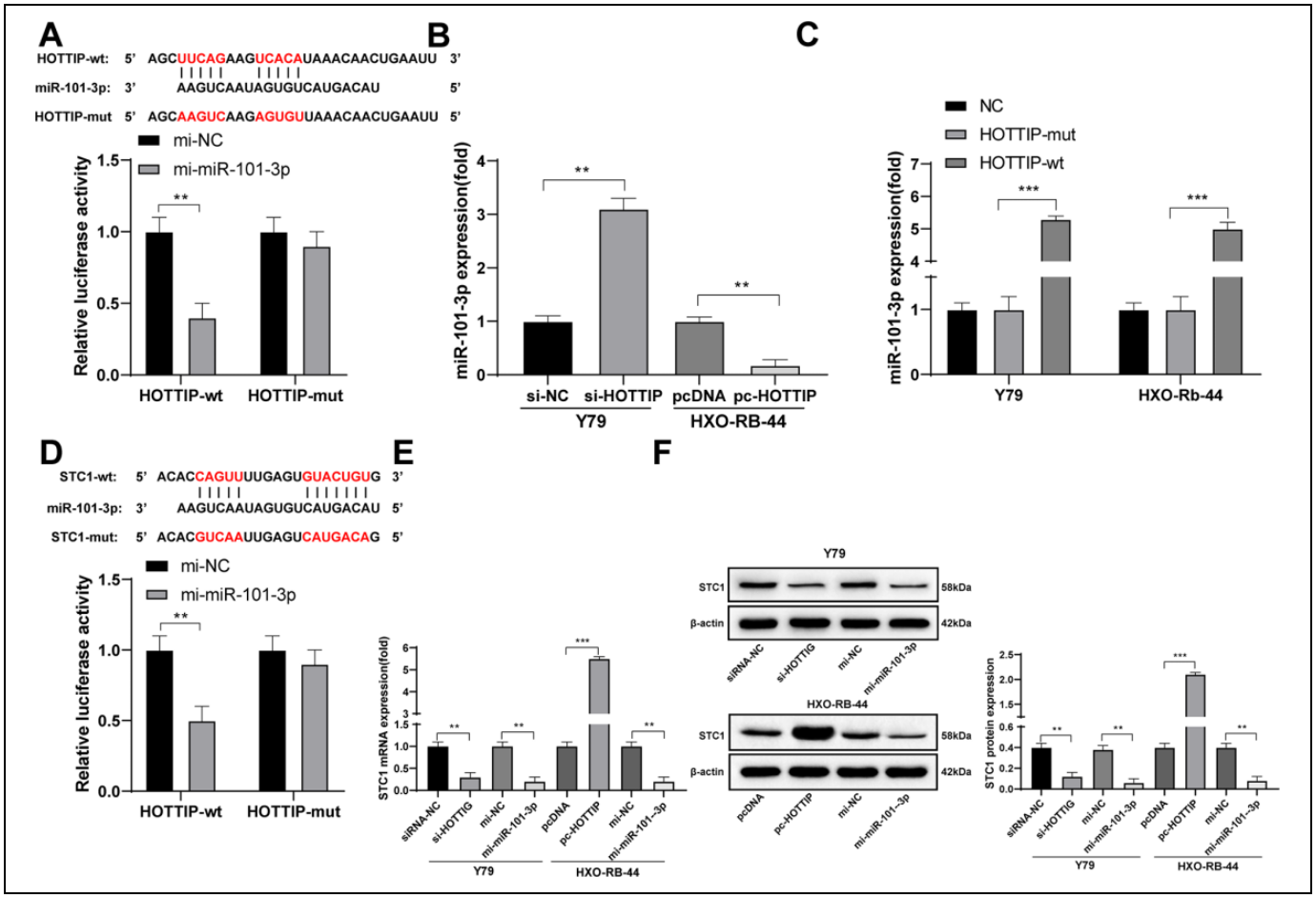

Figure 3. HOTTIP competitively bound to miR-101-3p to upregulate STC1 expression. (A) Binding site of HOTTIP and miR-101-3p was predicted by Starbase and verified by dual luciferase reporter gene assay; (B) expression of miR-101-3p in Y79 cells transfected with si-HOTTIP and in HXO-RB-44 cells transfected with HOTTIP (pcDNA-HOTTIP) was detected by qRT-PCR; (C) binding relationship between HOTTIP and miR-101-3p in RB cells was verified by RNA pull down assay; (D) binding site of STC1 and miR-101-3p was predicted by Starbase and verified by dual luciferase reporter gene assay; (E) expression of miR-101-3p in Y79 cells transfected with si-HOTTIP or mi-miR-101-3p and in HXO-RB-44 cells transfected with pc-HOTTIP or mi-miR-101-3p was detected using qRT-PCR; (F) expression of STC1 protein in Y79 cells transfected with si-HOTTIP or mi-miR-101-3p and in HXO-RB-44 cells transfected with pc-HOTTIP or mi-miR-101-3p was detected by Western bolt. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panels A-F were analyzed using 2-way ANOVA and Tukey's multiple comparisons test, **p < 0.01, ***p < 0.001.

HOTTIP negatively regulated miR-101-3p expression (Figure 3B) ($p < 0.01$). RNA pull down assay further confirmed the binding relationship between HOTTIP and miR-101-3p in RB cells (Figure 3C) ($p < 0.01$). These results suggested that HOTTIP functioned as a ceRNA to regulate miR-101-3p expression in RB cells.

STC1 may be related to drug resistance of Y79 cells.[24] We speculated that STC1 was involved in the HOTTIP-miR-101-3p interaction. The binding site of miR-101-3p and STC1 was predicted by bioinformatics website and verified using dual-luciferase reporter gene assay (Figure 3D) ($p < 0.01$). The mRNA and protein levels of STC1 in Y79 or HXO-RB-44 cells were detected. It was found that HOTTIP positively regulated STC1 expression, while miR-101-3p negatively regulated STC1 expression (Figure 3E/F) (all $p < 0.01$). In short, HOTTIP was competitively bound to miR-101-3p to upregulate STC1 expression.

Inhibition of miR-101-3p Counteracted the Effect of si-HOTTIP on RB Cells

Rescue experiments were performed to explore the tumor-promoting effect of HOTTIP on RB cells. Y79 cells were transfected with in-miR-101-3p or in-NC, and it was found that in-miR-101-3p significantly reduced miR-101-3p expression in Y79 cells (Figure 4A) ($p < 0.01$). Then, si-HOTTIP/in-miR-101-3p or si-HOTTIP/in-NC were co-transfected into Y79 cells. The increased miR-101-3p expression caused by HOTTIP knockdown was inhibited by in-miR-101-3p (Figure 4B) (all $p < 0.01$). Subsequently, CCK-8 assay indicated that miR-101-3p silencing promoted Y79 cell proliferation (Figure 4C) and flow cytometry showed that miR-101-3p silencing inhibited Y79 cell apoptosis (Figure 4D) (all $p < 0.01$). It could be concluded that HOTTIP exerted tumor-promoting effects on RB cells by suppressing miR-101-3p expression.

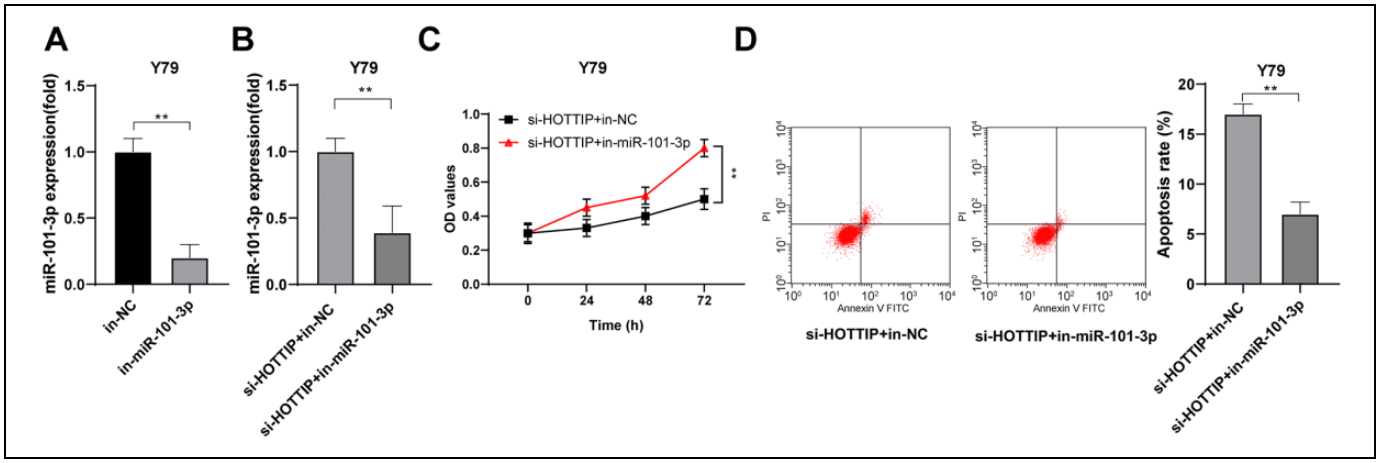

Figure 4. Inhibition of miR-101-3p counteracted the effect of si-HOTTIP on RB cells. (A) Expression of miR-101-3p in Y79 cells transfected with miR-101-3p inhibitor was detected using qRT-PCR; (B) expression of miR-101-3p in Y79 cells co-transfected with si-HOTTIP and mi-miR-101-3p inhibitor or NC-inhibitor was detected using qRT-PCR; (C) proliferation of Y79 cells co-transfected with si-HOTTIP and in-miR-101-3p or in-NC was detected using CCK-8 assay; (D) apoptosis of Y79 cells co-transfected with si-HOTTIP and in-miR-101-3p or in-NC was detected using flow cytometry. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panels A/B/D were statistically analyzed by the *t* test, and data in panel C were analyzed using two-way ANOVA and Tukey's multiple comparisons test, $^{**}p < 0.01$.

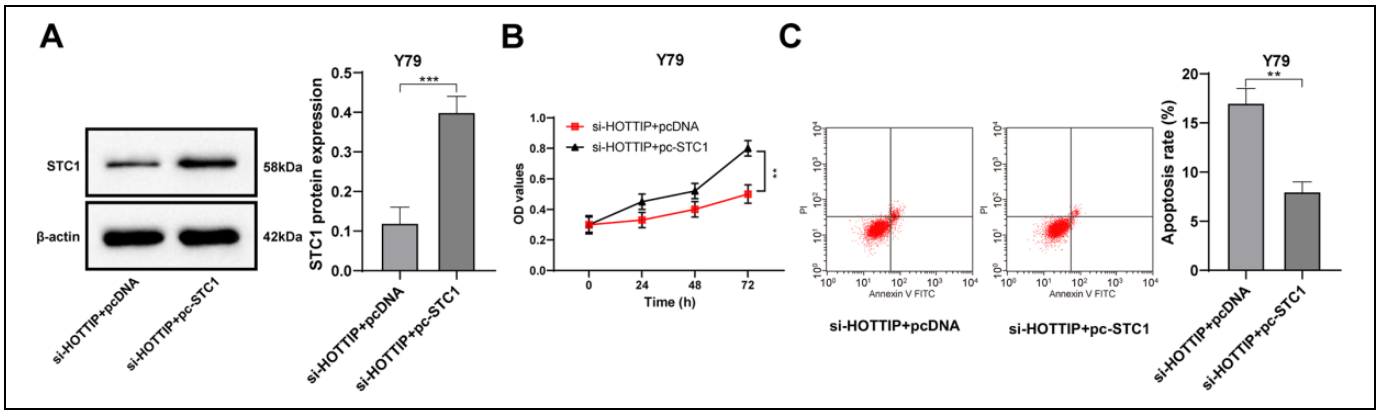

Figure 5. Overexpression of STC1 reversed the effect of si-HOTTIP on RB cells. (A) Expression of STC1 protein in Y79 cells co-transfected with si-HOTTIP and pc-STC1/pcDNA was detected by qRT-PCR; (B) proliferation of Y79 cells co-transfected with si-HOTTIP and pc-STC1/pcDNA was detected by CCK-8 assay; (C) apoptosis of Y79 cells co-transfected with si-HOTTIP and pc-STC1/pcDNA was detected by flow cytometry. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panels A/C were statistically analyzed by the t test, and data in panel B were analyzed using two-way ANOVA and Tukey's multiple comparisons test, $^{**}p < 0.01$, $^{***}p < 0.01$.

Overexpression of STC1 Reversed the Effect of si-HOTTIP on RB Cells

To further determine the role of the HOTTIP/miR-101-3p/STC1 axis in RB cells, we transfected Y79 cells with si-HOTTIP/pcDNA and si-HOTTIP/pc-STC1. STC1 expression in cells was detected using Western blot. Co-transfection of si-HOTTIP and pc-STC1 counteracted the effect of si-HOTTIP on STC1 expression (Figure 5A) (all $p < 0.01$). Moreover, overexpression of STC1 counteracted the effect of si-HOTTIP on proliferation (Figure 5B) and apoptosis (Figure 5C) of Y79 cells (all $p < 0.01$).

Knockdown of HOTTIP Inhibited RB Growth in Nude Mice

Finally, we studied whether HOTTIP could affect the growth of RB *in vivo*. Y79 cells transfected with si-NC/si-HOTTIP were injected into the subcutaneous of nude mice to construct xenograft tumor models. From the second week, the tumor volume of nude mice in the si-HOTTIP group was significantly smaller than that in the si-NC group (Figure 6A) ($p < 0.01$). In the fourth week, the tumor weight of nude mice in the si-HOTTIP group was significantly less than that in the si-NC group (Figure 6B) ($p < 0.01$). The positive rate of Ki67 in the

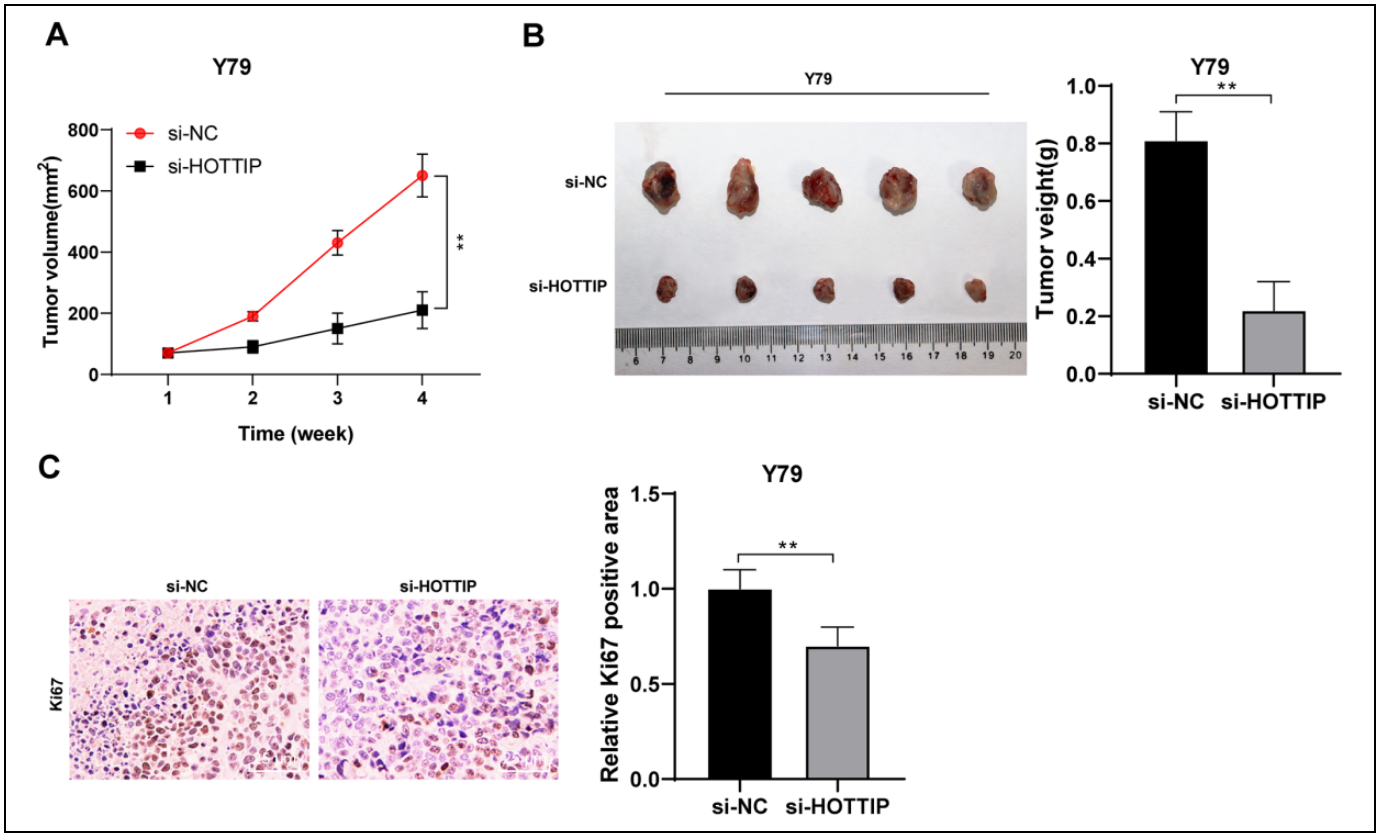

Figure 6. si-HOTTIP inhibited the growth of Y79 cells in nude mice. (A) Change of tumor volume in 1-4 weeks after injection of si-HOTTIP into tumor site of nude mice; (B) tumor weight in nude mice at the 4th week; (C) expression of Ki67 in tumor tissues of nude mice detected by immunohistochemistry. N = 5. Each experiment was repeated for 3 times independently and the data are presented as mean ± standard deviation. Data in panels B/C were statistically analyzed by the t test, and data in panel A were analyzed using two-way ANOVA and Tukey's multiple comparisons test, **p < 0.01.

si-HOTTIP group was also significantly lower than that in the si-NC group (Figure 6C) ($p < 0.01$). It was indicated that knockdown of HOTTIP suppressed RB growth in nude mice.

Discussion

RB represented the most frequent ocular malignancy in children that could be inherited.[2] If left untreated, RB might deteriorate into sporadic or hereditary forms, or even be fatal.[25] The researches on the mechanism of lncRNAs in RB could shed light on the early diagnosis of this malignant tumor.[26] This study herein focused on lncRNA HOTTIP and specifically explored its role in RB cells. This study revealed that knockdown of HOTTIP suppressed proliferation of RB cells and accelerated apoptosis via the miR-101-3p/STC1 axis.

HOTTIP, a long non-coding RNA located in HOXA cluster, played a vital part in the course of cancers.[9,27] For instance, Ge *et al*. reported that HOTTIP was a critical oncogene in hepatocellular carcinoma.[28] Guan *et al*. revealed that HOTTIP was notably promoted in patients with endometrial cancer and enhanced HOTTIP expression boosted the progression of endometrial cancer.[29] Consistently, our study showed that HOTTIP expression was overexpressed in RB tissues and cell line. Knockdown of HOTTIP suppressed RB cell proliferation and promoted apoptosis. Xenograft tumor experiment also showed that knockdown of HOTTIP reduced tumor growth in nude mice. All these results implied that HOTTIP worked as an oncogene in RB. We might unveil a new lncRNA biomarker for RB diagnosis.

Generally, lncRNAs affected the growth of RB cells by regulating miRs and participated in the progression of RB by influencing the target gene expression.[30,31] Emerging evidence showed that miRs extensively regulated RB pathological process.[32] According to Starbase prediction, we found that HOTTIP had a binding relationship with multiple miRs, including miR-101-3p. miR-101-3p had been proven to exert suppressive effects on many cancers including gastric cancer, breast cancer and oral cancer.[33-35] More importantly, miR-101-3p was reported to inhibit proliferation of RB cells.[36] Therefore, we speculated that HOTTIP regulated RB cell proliferation and apoptosis by binding to miR-101-3p. Accordingly, our study showed that miR-101-3p expression was markedly reduced in RB cells. Dual-luciferase reporter gene assay and qRT-PCR confirmed that HOTTIP negatively regulated miR-101-3p. Then we explored the downstream gene of miR-101-3p. STC1 was a kind of secretory glycoprotein involved in various

pathological processes including retinal degeneration.[37] We focused on STC1 among several target genes of miR-101-3p predicted by StarBase. Song *et al*. implied that STC1 was concerned with the mechanism of drug resistance in the treatment of RB.[24] Luan *et al*. also suggested that lncRNA MALAT1 stimulated the course of colon cancer by modulating the miR-101-3p/STC1 axis.[38] The targeting relationship between miR-101-3p and STC1 was verified by dual-luciferase reporter gene assay. Additionally, HOTTIP positively regulated STC1 expression. In brief, HOTTIP is competitively bound to miR-101-3p to upregulate STC1 expression. Subsequently, we performed functional rescue experiments. Inhibition of miR-101-3p or overexpression of STC1 could reverse the effect of si-HOTTIP on RB cells. It was further confirmed that HOTTIP upregulated the STC1 expression via sponging miR-101-3p to exert tumorigenic effect on RB.

Our study clarified that HOTTIP bound to miR-101-3p to upregulate STC1 expression, thus contributing to the malignant development of RB. The current study merely showed that knockdown of HOTTIP could inhibit RB cell proliferation and promoted apoptosis via miR-101-3p/STC1. The in-depth role of HOTTIP in the clinical treatment of RB needs further exploration. There are many target genes of HOTTIP, and we also need to determine whether HOTTIP plays a therapeutic role in RB by regulating multiple signaling pathways. In the future, we shall perform an exhaustive investigation of possible pathways downstream of STC1 in the development of RB from the perspective of epigenetics.

Authors' Note

XY made substantial contributions to the conception of the present study. ZS performed the experiments and wrote the manuscript; CC contributed to the design of the present study and interpreted the data. All authors read and approved the final manuscript. XiangWen Yuan and Zhaoyan Sun contributed equally to this work. The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request. The study got the approval of the Clinical Ethical Committee of Affiliated Hospital of Qingdao University (QYFYYXLL17023) (QYFYWZLL26009). Because the age of the patients was 1-5 years old, the informed consent forms were signed by the patient's guardian. The experiments were carried out with the consent of the patients and their families. All experimental procedures were implemented on the Ethical Guidelines for the Study of Experimental Pain in Conscious Animals.

Declaration of Conflicting Interests

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