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The long noncoding RNA MALAT1/microRNA-598-3p axis regulates the proliferation and apoptosis of retinoblastoma cells through the PI3K/AKT pathway

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Purpose: This study was designed to dissect the role of long noncoding RNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) in retinoblastoma (RB) and its underlying mechanism.
Methods: Gain- and loss-of-function experiments were adopted to explore the effects of MALAT1 and microRNA (miR)-598-3p on the biologic behaviors of RB cells. Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was used to assess the expression of MALAT1 and miR-598-3p in Y79 and HXO-RB44 cells. The proliferation of RB cells was determined with the cell counting kit-8 (CCK-8) assay and 5-ethynyl-2'-deoxyuridine (EdU) staining. Flow cytometry was employed for the measurement of the apoptotic rate, western blotting for examination of the expression of apoptosis-related proteins (Bax and Bcl-2) and phosphoinositide 3-kinase/protein kinase-B (PI3K/AKT) pathway-related factors (PI3K, AKT, p-PI3K, and p-AKT), and the luciferase reporter assay for assessment of the interaction between MALAT1 and miR-598-3p.
Results: High expression of MALAT1 and low expression of miR-598-3p were noticed in Y79 and HXO-RB44 cells. MALAT1 upregulation or miR-598-3p downregulation facilitated RB cell proliferation and inhibited cell apoptosis, as evidenced by the increased proliferation rate and Bcl-2 expression, as well as diminished Bax expression and apoptotic rate, in the RB cells after transfection with pcDNA3.1-MALAT1 or miR-598-3p inhibitor. MALAT1 bound to and negatively regulated miR-598-3p. The PI3K/AKT pathway activation occurred with MALAT1 overexpression. MALAT1 promoted RB cell proliferation and repressed cell apoptosis by repressing miR-598-3p to activate the PI3K/AKT pathway.
Conclusions: MALAT1 repressed miR-598-3p to activate the PI3K/AKT pathway, thus facilitating cell proliferation and inhibiting cell apoptosis in RB.

Retinoblastoma (RB) is a disease of infants and toddlers, with almost two-thirds of cases occurring before the age of 2 years and 95% of cases diagnosed by age 5 [1]. The most common presentation of RB is leukocoria, followed by strabismus [2]. Due to the close connection between clinical care and genetic predispositions, RB is considered the prototype of hereditary cancer [3]. Recent advances in the genetics of RB and the multimodal treatment for RB, including chemotherapy, focal treatment, radiation therapy, and surgery, have helped improve the overall clinical management of this malignancy [4]. However, further exploration is needed for detailed information regarding cellular and molecular pathways and the underlying mechanism of RB.

Among various biomarkers, long noncoding RNAs (lncRNAs) have emerged as attractive tools for the detection of RB [5,6]. LncRNAs, with more than 200 nucleotides, are transcripts that lack any protein-coding capacity but are involved in numerous kinds of human tumors [7,8]. LncRNA metastasis-associated lung adenocarcinoma transcript 1 (MALAT1), also known as noncoding nuclear-enriched abundant transcript 2, is ubiquitously expressed in almost all human tissues [9]. In recent years, MALAT1 has been reported to be involved in the dysregulation of cell signaling and is closely relevant to cancer development through the regulation of cell growth. For instance, MALAT1 contributes to the progression of non-small cell lung cancer (NSCLC) by inducing proliferation and repressing apoptosis through the microRNA (miRNA/miR)-374b-5p/serine- and arginine-rich splicing factor 7 axis [10]. Additionally, a previous study revealed that MALAT1 may increase cell viability and suppress cell apoptosis in RB [11]. Thus, MALAT1 may be used as a new candidate for the detection of RB.

miRNAs are known as a class of small noncoding RNAs that act as cellular and molecular regulators [12]. Accumulating evidence has suggested that miR-598-3p may function

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as a tumor suppressor in cancers by affecting proliferation, invasion, and apoptosis. In osteosarcoma, cell invasion, proliferation, apoptosis, and migration can be affected by miR-598 [13]. miR-598, as a tumor suppressor, can negatively regulate DERL1 and epithelial–mesenchymal transition to suppress the invasion and migration in NSCLC. More importantly, miR-598 was found to inhibit RB cell viability and metastasis by downregulating E2F1 and inactivating the AKT pathway [14]. Although it has been reported that MALAT1 or miR-598-3p has a close correlation with the biologic activity of cancer cells, such as RB cells, there is still a lack of specific evidence for the precise mechanism underlying RB.

MALAT1 is involved in the regulation of several molecular pathways, such as phosphoinositide 3-kinase (PI3K)/AKT, leading to an alteration in cell proliferation, death, invasion, and tumorigenicity [15]. The present study was performed to explore the expression pattern of MALAT1 in RB cells and to investigate its effects on the occurrence and development of RB, as well as to investigate whether MALAT1 can interact with miR-598-3p.

METHODS

Cell culture and transfection: The human RB cell lines (Y79, WERI-RB-1, SO-RB50, SO-RB70, and HXO-RB44), as well as the human RPE cell line ARPE-19, were provided by the American Type Culture Collection (Rockville, MD) in Appendix 1. Cells were grown in Roswell Park Memorial Institute 1640 medium (72,400,120, Gibco, Carlsbad, CA) containing 15% fetal bovine serum (16,140,071, Gibco) at 37 °C with 5% CO_2.

pcDNA3.1-MALAT1, short hairpin RNA (sh)-MALAT1, miR-598–3p mimic, miR-598-3p inhibitor, and their negative controls (NCs; pcDNA3.1-NC, sh-NC, mimic NC, and inhibitor-NC) were supplied by Genechem (Shanghai, China). The vectors (100 ng/well) were incubated with Lipofectamine 2000 reagents (11,668,019, Invitrogen, Carlsbad, CA) and Opti-MEM I (31,985,062, Gibco), followed by exposure to 8 ng/ml polybrene (TR-1003, Sigma-Aldrich, St. Louis, MO). The Y79 and HXO-RB44 cells were soaked in a culture medium with vectors for 48-h cell transfection. The RB cells transfected with the NC vectors were in the control group.

CCK-8 assay: Transfected cells were cultured in a 96-well plate (3,000 cells/well), with the fresh medium replaced every day. The viability of the cells was measured with the Cell Counting Kit (CCK)-8 (Beyotime, Shanghai, China) every 24 h for 5 consecutive days. Briefly, 10 µl CCK-8 solutions were added to each well for 2-h incubation at 37 °C. The absorbance (optical density) was examined using a SpectraMax M5 microplate reader (Molecular Devices, San Jose, CA) at 450 nm.

EdU assay: The proliferation of RB cells was assessed using a 5-ethynyl-2’-deoxyuridine (EdU) staining proliferation kit (C0075L, Beyotime). Specifically, cells in the 96-well plate were subjected to 2-h incubation with 0.1 ml of 2X EdU solution, and then the medium was removed. The cells were fixed in 0.1 ml of 4% paraformaldehyde for 15 min at room temperature before 15 min of permeabilization with 0.1 ml PBS (1X; 120 mM NaCl, 20 mM KCl, 10 mM $NaPO_4$, 5 mM KPO_4, pH 7.4; Gibco) containing 0.3% Triton X-100 and 20 min of incubation with endogenous peroxidase blocking solution. After the blocking solution was discarded, the cells in each well underwent 30-min incubation with 50 µl Click reaction buffer in the dark and 10-min nuclear staining with 1 ml Hoechst 33342 avoiding light. The number of positive cells was observed, and pictures were captured under a fluorescence microscope.

Flow cytometry: The cells were counted when cell confluence reached 80%. They (1×10^6) were washed twice with precooled PBS, suspended in 1X Annexin buffer, and exposed to 5 µl Annexin V-fluorescein isothiocyanate (Becton, Dickinson and Company, Franklin Lakes, NJ) for 10 min in the dark. Following one wash with precooled PBS, the cells were suspended in 1X Annexin buffer (300 µl). The apoptotic rate was measured using a flow cytometer (Guava easyCyte HT, EMD Millipore, Billerica, MA).

RT-qPCR: The cells were dissolved in 1 ml TRIzol reagent (Thermo Fisher Scientific, Waltham, MA) for the extraction of total RNA according to the specification. The M-MLV reverse transcriptase and random primers were applied to harvest the cDNA template through reverse transcription of total RNA. The reaction system was prepared in light of the manuals of the Premix Ex Taq™ II kit (Takara, Dalian, China). Then, reverse transcription quantitative PCR (RT-qPCR) was performed on the ABI7500 quantitative PCR instrument (Applied Biosystems, Foster City, CA). The quantification of the lncRNA was normalized to glyceraldehyde-3-phosphate dehydrogenase, while that of the miRNA was normalized with U6. The fold changes were calculated with the $2^{-\Delta\Delta Ct}$ method [16]: $\Delta\Delta Ct=[Ct_{(target\ gene)} - Ct_{(reference\ gene)}]_{experimental\ group} - [Ct_{(target\ gene)} - Ct_{(reference\ gene)}]_{control\ group}$. All primers are listed in Table 1.

Western blotting: The cells were lysed with lysis buffer, and the total protein concentration was determined using a bicinchoninic acid kit (23,227, Thermo Fisher Scientific). After being diluted with 5X sample buffer, proteins underwent 90 min of electrophoresis in a 12% separation gel and 1 h of inactivation in PBS with 5% (w/v) nonfat dried milk at

Table 1. Primer sequence information.

Name of primer	Sequences (5′-3′)
MALAT1-F	ATGCGAGTTGTTCTCCGTCT
MALAT1-R	TATCTGCGGTTTCCTCAAGC
miR-598–3p-F	ACTGCTACTGTTGCTA
miR-383–5p-F	TCGGTGTTAGTGGAAG
miR-4524b-5p-F	CTCTGTCCGAATACG
miR-2115–3p-F	GATCGGAGGTACTTAA
miR-503–5p-F	GACGTCTTGACAAGGGC
miRNA-R	TTGGGTAGGCAGCATCTCTT
U6-F	CTCGCTTCGGCAGCACA
U6-R	AACGCTTCACGAATTTGCGT
GAPDH-F	GTGGCTGGCTCAGAAAAAGG
GAPDH-R	GGGGAGATTCAGTGTGGTGG

Notes: F: forward primer; R: reverse primer.

room temperature. The membranes were incubated with the primary antibodies (1:500, Abcam, Cambridge, England) against B cell lymphoma-2 (Bcl-2)-associated X (Bax; ab32503), Bcl-2 (ab32124), PI3K (ab32089), phosphorylation (p)-PI3K (ab182651), AKT (ab38449), or p-AKT (ab8805) overnight at 4 °C. The membranes were washed before being incubated with secondary antibodies (1:500, ab150077, Abcam) at room temperature for 1 h. The BioSpectrum Imaging System (UVP, Upland, CA) was used for picture capture and observation.

Luciferase reporter assay: The binding site of MALAT1 to miR-598-3p was predicted by StarBase. Then, mutant-type and wild-type sequences (MT-MALAT1 and WT-MALAT1) were designed and cloned into pGL3-Basic. The vectors were cotransfected with miR-598-3p mimic or mimic NC (30 nM), respectively, into HEK293T cells. Following cell cotransfection, firefly and *Renilla* luciferase activities were determined. *Renilla* luciferase activity was deemed the internal control, and the ratio of the activities of firefly luciferase and *Renilla* luciferase was calculated as the relative activity.

Statistical analysis: All experiments were repeated three times, unless otherwise specified. The statistical analysis of the data was implemented using SPSS 18.0 (IBM Corp., Armonk, NY) and GraphPad Prism 6.0 (GraphPad Software Inc., San Diego, CA). Data were displayed as mean ± standard deviation. The *t* test was applied for the comparison between the two groups. Comparisons among multiple groups were conducted using one-way analysis of variance (ANOVA) and confirmed with Dunnett's multiple comparisons test. A p value of less than 0.05 was regarded as a statistically significant difference.

RESULTS

MALAT1 contributed to the increased proliferation ability of RB cells: MALAT1 performs as an oncogene to promote the proliferation and invasion of cancer cells. MALAT1 expression in RB cell lines (Y79, SO-RB50, HXO-RB44, WERI-RB-1, and SO-RB70) and ARPE-19 cells was examined to address the role of MALAT1 in RB. RT-qPCR showed high expression of MALAT1 in Y79, SO-RB50, SO-RB70, HXO-RB44, and WERI-RB-1 cells and low expression of MALAT1 in ARPE-19 cells (Figure 1A, $p<0.05$, $p<0.01$). The most striking difference in MALAT1 expression was observed in HXO-RB44 and Y79 cells, which were selected for the subsequent experiments. As depicted in Figure 1B, transfection with pcDNA3.1-MALAT1 increased MALAT1 expression, and transfection with sh-MALAT1 decreased MALAT1 expression in HXO-RB44 and Y79 cells ($p<0.01$), suggesting the favorable transfection efficiencies of sh-MALAT1 and pcDNA3.1-MALAT1. The rise in the proliferation rate in the pcDNA3.1-MALAT1 group (versus the pcDNA3.1-NC group) and the decline in the sh-MALAT1 group (versus the sh-NC group) were noted after the detection of cell proliferation ability with the CCK-8 assay and EdU staining (Figure 1C, D, $p<0.05$, $p<0.01$). Additionally, flow cytometry and western blotting results revealed that MALAT1 upregulation elevated the Bcl-2 protein level and reduced the cell apoptotic rate and Bax expression (Figure 1E, F, $p<0.01$, versus the pcDNA3.1-NC group), whereas reverse trends were observed in cells with downregulated MALAT1 (Figure 1E, F, $p<0.01$, versus the sh-NC group). These data indicated that MALAT1 might confer a carcinogenesis effect on RB by facilitating RB cell proliferation and inhibiting cell apoptosis.

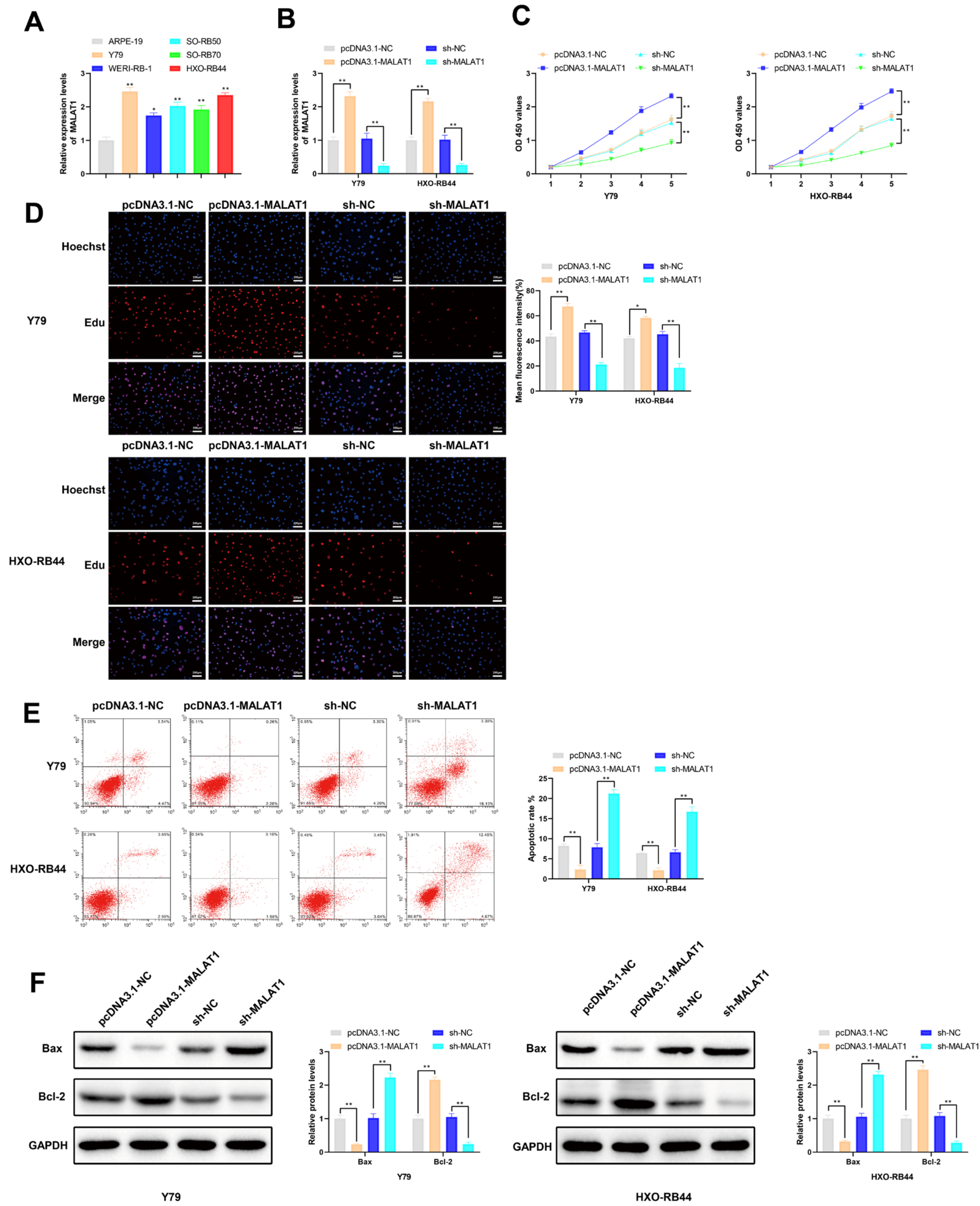

Figure 1. Overexpression of MALAT1 promotes retinoblastoma (RB) cell proliferation. After RB cells were transfected with pcDNA3.1-MALAT1 or sh-MALAT1. **A**: Reverse transcription quantitative polymerase chain reaction (RT-qPCR) was applied to detect the differential expression of MALAT1 in ARPE-19 cells and RB cell lines Y79, SO-RB50, HXO-RB44, WERI-RB-1, and SO-RB70. **B**: Then, the transfection efficiencies of pcDNA3.1-MALAT1 and sh-MALAT1 in Y79 and HXO-RB44 cells were evaluated with (**B**) RT-qPCR. The (**C**) cell counting kit-8 (CCK-8) assay and (**D**) 5-ethynyl-2'-deoxyuridine (EdU) staining (50X) were employed to assess cell proliferation ability, (**E**) flow cytometry for inspection of the cell apoptotic rate, and (**F**) western blotting for measurement of the expression of apoptosis-related proteins Bax and Bcl-2 in Y79 and HXO-RB44 cells. *$p<0.05$, **$p<0.01$.

MALAT1 negatively regulated miR-598–3p: To screen the downstream miRNAs of MALAT1, the online software TargetScan was used to predict the miRNAs that had binding sites to MALAT1. MiR-383-5p, miR-4524b-5p, miR-2115-3p, miR-503-5p, and miR-598-3p were subsequently selected, followed by the measurement of their expression in Y79, HXO-RB44, and ARPE-19 cells. The RT-qPCR results showed that miR-383-5p, miR-503-5p, and miR-598-3p expression in Y79 cells and HXO-RB44 cells were low versus ARPE-19 cells (Figure 2A, p<0.05, p<0.01). Additionally, miR-598-3p had the most noticeably decreased expression in Y79 cells and HXO-RB44 cells, and then miR-598-3p was selected for the following research.

The binding site of MALAT1 to miR-598-3p and the designed mutation site are presented in Figure 2B. The luciferase reporter assay showed that cells cotransfected with WT-MALAT1 and miR-598-3p mimic had diminished luciferase activity compared to cells cotransfected with WT-MALAT1 and mimic NC (Figure 2C, p<0.01). However, no prominent difference was noted between cells cotransfected with MT-MALAT1 and miR-598-3p mimic and cells cotransfected with MT-MALAT1 and mimic NC with regard to relative luciferase activity (Figure 2C, p>0.05), indicating that MALAT1 specifically bound to miR-598-3p.

To determine whether MALAT1 directly regulated miR-598-3p expression in RB cells, Y79, and HXO-RB44 cells were transfected with pcDNA3.1-MALAT1, sh-MALAT1, pcDNA3.1-NC, or sh-NC. The results revealed that overexpression of MALAT1 reduced miR-598-3p expression, while knockdown of MALAT1 enhanced miR-598-3p expression in HXO-RB44 and Y79 cells (Figure 2D, p<0.01). The findings above suggested that MALAT1 bound to and negatively regulated miR-598-3p.

MiR-598-3p upregulation suppressed the proliferation of RB cells: To ascertain whether miR-598-3p affected the proliferation and apoptosis of Y79 cells and HXO-RB44 cells, the cells were transfected with miR-598-3p mimic, miR-598-3p inhibitor, mimic NC, or inhibitor-NC. RT-qPCR showed an increase in miR-598-3p expression in the miR-598-3p mimic group (versus the mimic NC group) and a decrease in miR-598-3p expression in the miR-598-3p inhibitor group (Figure 3A, p<0.01, versus the inhibitor-NC group), suggesting that miR-598-3p was successfully overexpressed or inhibited in Y79 cells and HXO-RB44 cells.

Subsequently, we found that transfection with the miR-598-3p mimic contributed to an enhanced cell apoptotic rate and Bax protein expression and a reduced cell proliferation rate and Bcl-2 protein expression. However, cells transfected with the miR-598-3p inhibitor exhibited opposite trends regarding the factors above (Figure 3B–E, p<0.05, p<0.01). The results showed that miR-598-3p upregulation might repress proliferation and facilitate apoptosis in RB cells.

MALAT1 facilitated RB cell proliferation by downregulating miR-598-3p: The results above indicated that MALAT1 and miR-598-3p regulated RB cell proliferation and apoptosis and that MALAT1 negatively regulated miR-598-3p. Therefore, we speculated that MALAT1 might regulate miR-598-3p to affect the proliferation and apoptosis of RB cells. Toward this end, HXO-RB44 and Y79 cells were transfected with pcDNA3.1-MALAT1/sh-MALAT1 or cotransfected with pcDNA3.1-MALAT1 + miR-598-3p mimic/sh-MALAT1 + miR-598-3p inhibitor. It was discovered that transfection with pcDNA3.1-MALAT1 enhanced cell proliferation and Bcl-2 expression, as well as lowered Bax expression and the cell apoptotic rate, which was nullified with further transfection with the miR-598-3p mimic (Figure 4A–D, p<0.05, p<0.01). Furthermore, the sh-MALAT1 + miR-598-3p inhibitor group

Figure 2. MALAT1 targets and negatively regulates miR-598–3p. The lowly expressed miR-383-5p, miR-503-5p, and miR-598-3p in Y79 cells and HXO-RB44 cells were screened with (**A**) reverse transcription quantitative polymerase chain reaction (RT-qPCR). The (**B**) online software TargetScan predicted the binding site of MALAT1 to miR-598-3p, and the mutation site was designed. The interaction between MALAT1 and miR-598-3p was verified with the luciferase (**C**) reporter assay. The regulatory effect of MALAT1 on miR-598-3p in Y79 cells and HXO-RB44 cells was examined with (**D**) RT-qPCR. *p<0.05, **p<0.01.

had elevated Bcl-2 expression and cell proliferation but diminished Bax expression and a decreased cell apoptotic rate compared to the sh-MALAT1 group (Figure 4A–D, p<0.01). The indicators in the pcDNA3.1-MALAT1 + miR-598-3p mimic group and the sh-MALAT1 + miR-598-3p inhibitor group were not considerably different from those in the control group. In conclusion, MALAT1 enhanced cell proliferation and inhibited cell apoptosis by inversely regulating miR-598-3p in Y79 and HXO-RB44 cells.

MALAT1 activated the PI3K/AKT pathway: The PI3K/AKT pathway was identified to explore whether MALAT1 exerted an effect on the biologic behaviors of RB cells via this pathway. Y79 and HXO-RB44 cells were transfected with sh-MALAT1/pcDNA3.1-MALAT1 or cotransfected with sh-MALAT1 + miR-598-3p inhibitor/pcDNA3.1-MALAT1 + miR-598-3p mimic. Analysis of western blotting showed that upregulation of MALAT1 elevated the phosphorylation ratio of the PI3K/AKT pathway-related downstream proteins, which was counterweighed through the following miR-598-3p mimic transfection (Figure 5A, B, p<0.01). Silencing of MALAT1 lowered the phosphorylation ratio, whereas additional miR-598-3p inhibitor transfection abrogated this ratio (Figure 5A, B, p<0.01). The phosphorylation ratio of the PI3K/AKT pathway-related downstream proteins was not obviously different among the pcDNA3.1-MALAT1 + miR-598-3p mimic, sh-MALAT1 + miR-598-3p inhibitor, and control groups. Collectively, MALAT1 performed biologic functions in RB cells by repressing miR-598-3p to activate the PI3K/AKT pathway.

To further validate that the MALAT1/miR-598-3p axis functioned through the PI3K/AKT pathway, the impacts of PI3K/AKT antagonist (LY294002) and agonist (740 Y-P) on Y79 cell functions were evaluated. As presented in Appendix 2, LY294002 eliminated the promotion of cell proliferation induced by upregulation of MALAT1, while 740 Y-P abolished the inhibitory effect of MALAT1 knockdown on cell proliferation and reduced apoptosis. To examine the regulatory effects of MALAT1 on normal retinal epithelial cells, sh-MALAT1 and pcDNA3.1-MALAT1 were transfected into ARPE-19 cells. The results showed that upregulation or knockdown of MALAT1 had no obvious effect on the proliferative capacity of APRE-19 cells (Appendix 3).

DISCUSSION

RB is a type of embryonic malignancy of the eye retina, which occurs in childhood and is triggered by RB gene mutations in the developing retina [17]. Without timely and effective treatment, RB may spread through the optic nerve to the brain or via the blood, particularly to bone marrow, and result in death [3]. Therefore, it is vital to study the molecular mechanism underlying the occurrence and development of RB, aiming to find new effective treatment targets for RB. Y79 and HXO-RB44 cells were selected from human RB cell lines and used for experiments. This study focused on the function of MALAT1 in RB progression and characterized MALAT1 as a molecular sponge for miR-598-3p after identifying the profile of differentially expressed MALAT1 and miR-598-3p in RB cells. The data showed that the biologic activities of RB cells regarding apoptosis and proliferation were affected by the MALAT1/miR-598-3p axis.

MALAT1, one of the most extensively studied lncRNAs, is highly expressed in several human cancers, such as colon cancer, epithelial ovarian cancer, and cervical cancer [18-20]. In this study, we analyzed the behavior of MALAT1 in RB. Initially, we observed that MALAT1 expression in human RB cell lines was remarkably higher than in the human RPE cell line. To this end, dysregulated MALAT1 was speculated to interfere with RB. Subsequently, MALAT1-promoted development of RB was confirmed by the increases in cell viability and Bcl-2 expression and the decreases in the cell apoptotic rate and Bax expression in RB cells after MALAT1 overexpression. Zhao et al. discovered that MALAT1 expression was upregulated in RB tissues and that silencing of MALAT1 enhanced apoptosis but repressed proliferation in Y79 cells through miR-655-3p [21]. Of note, another study supported the present results that MALAT1 suppressed cell apoptosis and induced cell proliferation to aggravate human RB through the miR-20b-5p/STAT3 axis [22]. In contrast, it was observed in a previous study that upregulation of NKILA and MALAT1 could accelerate cell apoptosis and restrain cell proliferation and invasion in RB [23]. The difference between the present study and the study above may be due to the regulatory network between NKILA and MALAT1, illustrating that MALAT1 may interact with other lncRNAs to regulate RB progression, which warrants further research. It has been discovered that lncRNAs may function as competing endogenous RNA and regulate miRNA expression in various physiologic processes, including cancer progression [24,25]. Thus, we were prompted to further investigate the molecular actions of MALAT1 in regulating RB by investigating its downstream target. Based on the comprehensive analysis by the bioinformatics tool TargetScan and the luciferase reporter assay, we identified miR-598-3p as a direct target of MALAT1. Several studies indicated the regulatory effect of miR-598-3p on tumor development. For instance, miR-598 acts as a tumor suppressor in human gastric cancer by targeting IGF-1R [26]. Low expression of miR-598-3p was noticed in RB cells. After gain- and loss-of-function experiments, MALAT1 was proven to negatively regulate miR-598-3p. Considering the targeting

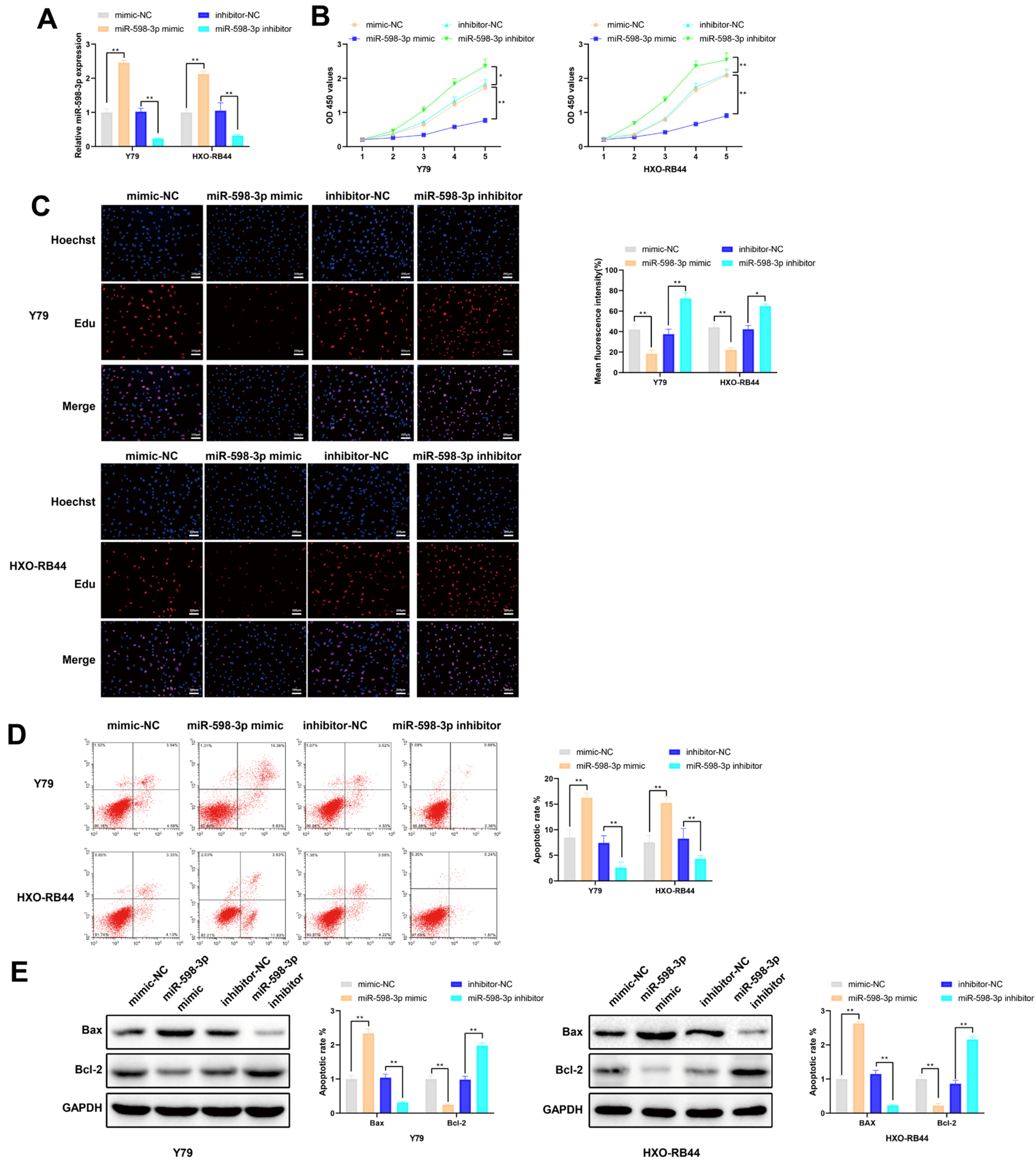

Figure 3. Overexpression of miR-598-3p causes repressed proliferation ability of retinoblastoma (RB) cells. After Y79 and HXO-RB44 cells were transfected with miR-598-3p mimic or miR-598-3p inhibitor, (**A**) reverse transcription quantitative polymerase chain reaction (RT-qPCR) was applied to inspect the transfection effect, (**B**) cell counting kit-8 (CCK-8) assay, and (**C**) 5-ethynyl-2’-deoxyuridine (EdU) staining (50X) to measure cell proliferation ability, (**D**) flow cytometry to examine the apoptotic rate, and (**E**) western blotting to detect the expression of apoptosis-related proteins Bax and Bcl-2 in Y79 and HXO-RB44 cells. *p<0.05, **p<0.01.

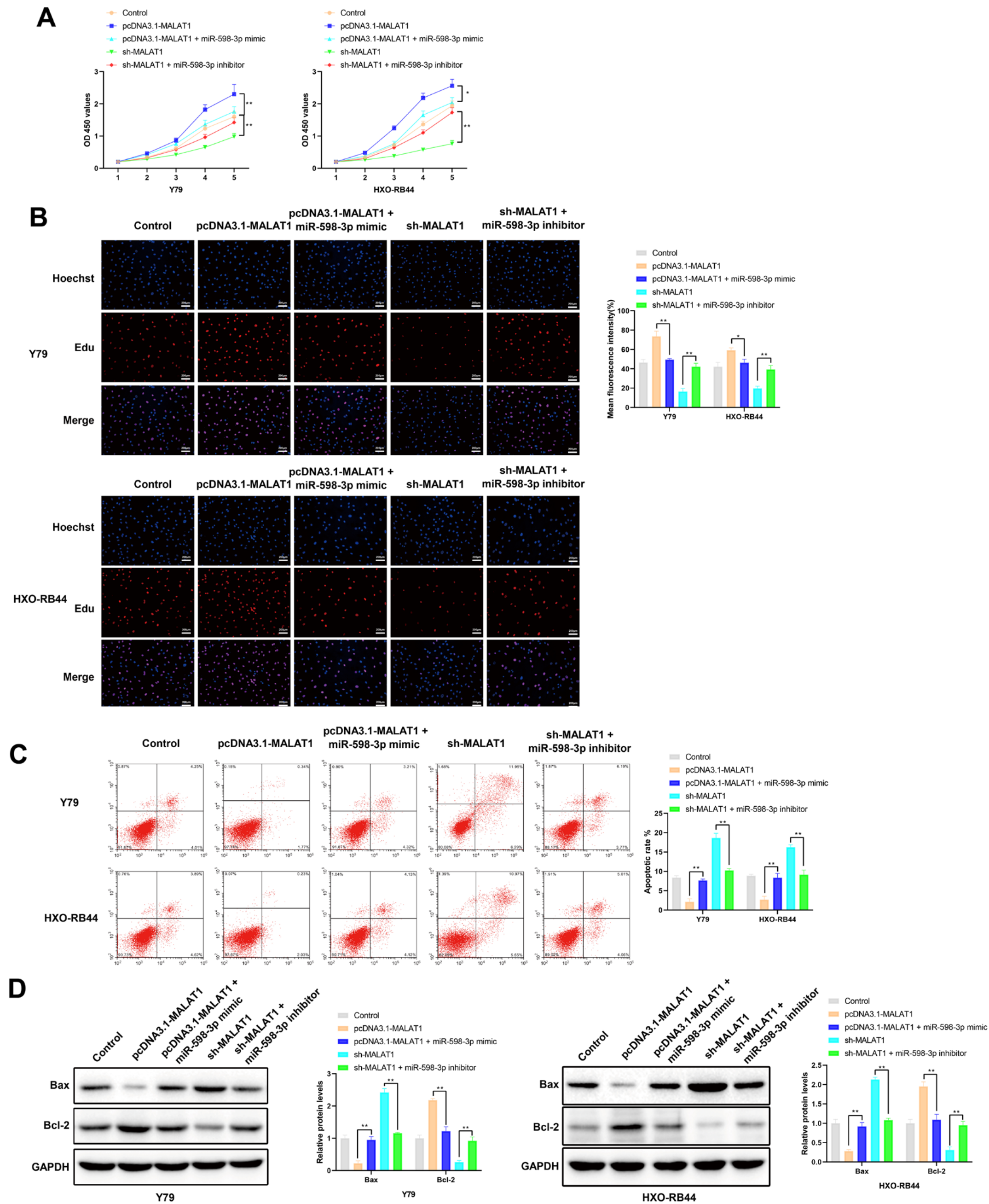

Figure 4. MALAT1 inhibits miR-598–3p to promote the proliferation of retinoblastoma (RB) cells. After the Y79 and HXO-RB44 cells were transfected or cotransfected with pcDNA3.1-MALAT1, miR-598-3p mimic, sh-MALAT1, and miR-598-3p inhibitor, the proliferation ability of the RB cells was determined with (**A**) cell counting kit-8 (CCK-8) assay and (**B**) 5-ethynyl-2'-deoxyuridine (EdU) staining (50X). **C**: Flow cytometry was adopted to assess the apoptotic rate of RB cells.: The expression of apoptosis-related proteins Bax and Bcl-2 in the Y79 and HXO-RB44 cells was detected with (**D**) western blotting. *p<0.05, **p<0.01.

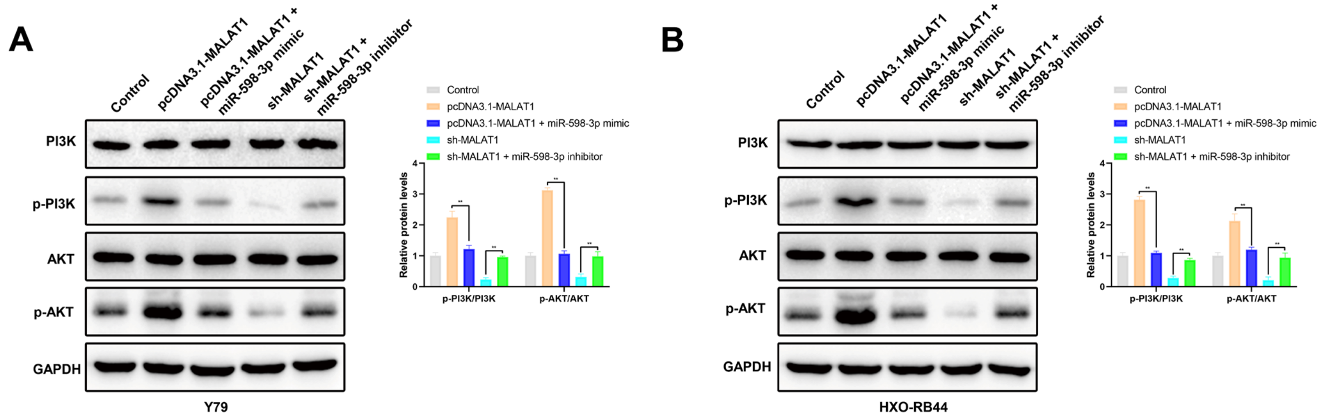

Figure 5. The PI3K/AKT pathway is activated by MALAT1-downregulated miR-598-3p. After the Y79 and HXO-RB44 cells were transfected or cotransfected with pcDNA3.1-MALAT1, miR-598-3p mimic, sh-MALAT1, and miR-598–3p inhibitor, the protein expression of the PI3K/AKT pathway-related factors in the Y79 (**A**) and HXO-RB44 (**B**) cells was measured with western blotting; **p<0.01.

relationship between MALAT1 and miR-598-3p in RB, we boldly speculated that miR-598-3p may participate in RB progression by exerting the synergistic effect of MALAT1 to alter the biologic behaviors of RB cells. The prediction was verified by the present findings that miR-598-3p strikingly increased RB cell apoptosis and reduced their proliferation ability. Additionally, gene cotransfection for rescue experiments revealed that miR-598-3p largely reversed the effect of MALAT1 on the biologic behaviors of RB cells. The miRNA sponge function of MALAT1 in RB was also supported by evidence generated in a previous study [27]. Therefore, the present results proposed that MALAT1 facilitated cell proliferation in addition to inhibiting cell apoptosis by sponging miR-598-3p in RB.

PI3Ks form a family of kinases expressed in virtually all mammalian cells, with essential roles in cell cycle progression, survival, migration, and growth [28]. The effect of PI3K on the biologic activity of cells is exerted by activating downstream AKT [29]. The PI3K/AKT pathway plays a central role in cellular physiology by regulating growth factor signals during organismal growth and critical cellular processes, such as cell proliferation and survival [30]. In the present study, the activation of the PI3K/AKT pathway was associated with the role of MALAT1 in the biologic behaviors of RB cells. Additionally, the gain- and loss-of-function experiments showed that MALAT1 suppressed miR-598-3p expression to activate the PI3K/AKT pathway and that the PI3K/AKT pathway activation promoted cell proliferation and repressed cell apoptosis in RB. Previous research corroborated the present findings that MALAT1 elicited a regulatory role in cell function through the PI3K/AKT pathway [31,32].

In summary, the present findings unraveled the oncogenic role of MALAT1 in Y79 cells and HXO-RB44 cells, and that this effect might be achieved through inverse regulation of miR-598-3p and activation of the PI3K/AKT pathway. These data potentiate the use of the MALAT1/miR-598-3p axis as a diagnostic marker and therapeutic molecule for RB and may provide an experimental basis for clinical prognosis judgment and further targeted intervention therapy for this cancer. However, the absence of an in vivo experiment is a limitation of this study.

APPENDIX 1. STR ANALYSIS.

To access the data, click or select the words "Appendix 1."

APPENDIX 2. MALAT1 ACTS THROUGH THE PI3K/AKT PATHWAY

To access the data, click or select the words "Appendix 2." Notes: Y79 cells were transfected with pcDNA3.1-MALAT1, sh-MALAT1, pcDNA3.1-MALAT1 + LY294002, or sh-MALAT1 + 740 Y-P. CCK-8 assay (A) and EdU staining (B, × 50) were employed to measure the proliferation ability of Y79 cells. Flow cytometry was adopted to assess the apoptotic rate of Y79 cells (C). Western blotting was applied to detect the expression of apoptosis-related proteins Bax and Bcl-2 in Y79 cells (D). Western blotting was used to examine the expression of PI3K/AKT pathway-related proteins in Y79 cells (E). **p<0.01, *p<0.05.

APPENDIX 3. MALAT1 SHOWS NO REGULATORY EFFECT ON ARPE-19 CELLS

To access the data, click or select the words "Appendix 3." Notes: After ARPE-19 cells were transfected with pcDNA3.1-MALAT1 or sh-MALAT1, the CCK-8 assay was adopted to evaluate cell proliferation.

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