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Effects of lncRNA MALAT1 and lncRNA NKILA on proliferation, invasion and apoptosis of retinoblastoma

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Abstract. – OBJECTIVE: To assess the expression of LncRNA NKILA and MALAT1 in retinoblastoma and its related mechanisms.

PATIENTS AND METHODS: Tixty-eight cases of retinoblastoma patients admitted to our hospital from February 2017 to February 2019 were collected as a research group, while 70 healthy people who came to our hospital for checkup at the same time were chosen as a control group. Both retinoblastoma and human colorectal mucosa cells were purchased, expression and clinical value of NKILA and MALAT1 in serum of Rb patients were tested, and sh-NKILA, si-NKILA, NC, sh-MALAT1 and si-MALAT1 were transfected into Weri-Rb1 and Y79 cells. qRT-PCR was adopted to detect the NKILA and MALAT1 levels in samples, and WB was adopted to detect the cle-caspase-3, cle-caspase-9, Bax, Cyclin B1, CDC2 and p-CDC2 protein levels in cells. Cell proliferation was conducted *via* MTT assay, invasion was carried out through transwell assay, and apoptosis was confirmed by flow cytometry assay.

RESULTS: NKILA and MALAT1 were low expressed in retinoblastoma, and AUC of LncRNA NKILA and MALAT1 was over 0.8. LncRNA NKILA and MALAT1 were associated with tumor size, classification and clinical grading in children with retinoblastoma. Over-expression of NKILA and MALAT1 could promote apoptosis, inhibit cell growth and Bcl-2 protein, and promote upregulation of the expression levels of clecaspase-3, clecaspase-9 and Bax.

CONCLUSIONS: By regulating MALAT1 and NKILA, we controlled the growth and apoptosis of Rb cells, which was expected to be a potential clinical therapeutic target for Rb.

Key Words:
LncRNA MALAT1, LncRNA NKILA, Retinoblastoma, Proliferation, Invasion, Apoptosis.

Introduction

Retinoblastoma (Rb) is a kind of familial predisposition intraocular malignant tumor derived from photoreceptor precursor cells, and it is common in infants and rare in adults[1-3]. Rb is a key threat leading to blindness in infants, its morbidity and mortality are extremely high, and the mortality of children who are not treated in time is as high as 100%[4]. The clinical manifestations of Rb are complex, and intracranial and distant metastases are easy to occur, often endangering the lives of children. At present, the clinical treatment for Rb includes intravenous chemotherapy, ophthalmic artery interventional chemotherapy and local laser, using refrigeration or radiotherapy to preserve the useful vision of patients to the greatest extent[5,6]. However, when the volume or metastatic area of Rb tumor exceeds half of the eyeball, only enucleation can be adopted. Therefore, early detection, diagnosis and treatment are the keys to improve its cure rate and reduce its mortality[7,8]. With the continuous expansion of Rb-related molecular biology research for the past few years, molecular targeted therapy is one of the most popular research areas[9]. Molecular targeting acts crucially on Rb's diagnosis, staging and comprehensive therapy. To better improve children's quality of life and avoid enucleation, furthermore, it's particularly important to determine specific biological markers that lead to the development and progression of Rb, and to affect the biological functions of Rb cells through effective targeted therapy.

Studies have found that long-chain non-coding RNA (LncRNA) participates in various normal life

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activities of cells, affects their proliferation, apoptosis and differentiation, and is related to Rb metastasis. It has been reported that the condition monitoring of Rb can be monitored by detecting LncRNA in tissues of patients[10]. Recent researches report suggest that LncRNA MALAT1 is strongly linked to the development and progression of tumors[11-13]. Among them, the abnormal expression of lncRNA NKILA and lncRNA MALAT1 is tied to the proliferation and invasion of lung cancer and colorectal cancer cells, but its effect on Rb is rarely reported[14,15]. Notwithstanding, MALAT1 and NKILA's expression and forecast value in Rb is short of research. Hence, this study was expected to provide a new theoretical basis for the diagnosis and treatment of Rb in molecular biology and to find suitable Rb specific molecular markers. The expression characteristics of MALAT1 and NKILA in Rb and influence on related cell biological functions were studied experimentally.

Patients and Methods

Data Collection

From February 2017 to February 2019, 30 cases of Rb children admitted to our hospital were collected as the research group, while the control group selected healthy people who came to our hospital for physical examination at the same time. Gender and age between both groups signified no conspicuous difference (p>0.05), which was comparable. Inclusion criteria were as follows: all the collected children met the World Health Organization diagnostic criteria for retinoblastoma[16]; Rb was confirmed by pathology, cytology and imaging; those confirmed by pathology, cytology and imaging as RB[16]; Rb patients who did not receive relevant preoperative chemotherapy, immunotherapy, radiotherapy and other anti-tumor treatment. Exclusion criteria were as follows: those comorbid with liver cirrhosis and coagulation dysfunction; those with incomplete general clinical data and did not cooperate with follow-up; those with expected survival time less than 1 month; those lost to be followed up. This study has been reported to the clinical trial management department of the clinical Trial Institution and approved by the Clinical Trial Ethics Committee, and patients all have signed an informed consent form in advance.

Main Instruments and Reagents

Rb cell lines Weri-Rb1, Y79 and normal retinal pigment epithelial cell lines hTERT RPE-1 (BNCC100322, BNCC341293, BNCC338048) were purchased from BeNa Culture Collection. ABI StepOne Plus fluorescence quantitative Real-Time PCR, Lipofectamine™ 2000 transfection kit (Invitrogen, Carlsbad, CA, USA); TRIzol extraction kit (Invitrogen, Carlsbad, CA, USA); Annexin V/PI apoptosis detection kit (Invitrogen, Carlsbad, CA, USA); SYBR Green PCR Master Mix (Applied Biosystems, Waltham, MA, USA); MTT kit (Beyotime Biotechnology Co., Ltd., Product Number: C0009); 10% fetal bovine serum, penicillin-streptomycin mixed solution (100×double antibody), DMEM, transwell kit (Gibco™ BRL, Gaithersburg, MD, USA), β-catenin polyclonal goat IgG, cyclin D1 polyclonal goat IgG, c-myc polyclonal goat IgG, β-actin, horseradish peroxidase (HRP) labeled goat anti-mouse secondary antibody (R&D Systems, Minneapolis, MN, USA); ECL luminescence kit, BCA protein kit, Multiskan™ GO Spectrum (Thermo Fisher Scientific, Shanghai, China); FACSCanto flow cytometry (Becton Dickinson, Franklin Lakes, NJ, USA), DR5000 ultraviolet-visible spectrophotometer (BioRad, Hercules, USA). All primer sequences were synthesized by Sangon Bioengineering Co., Ltd (Shanghai, China).

Detection Methods

Sample collection: each 5 mL elbow venous blood of Rb children and healthy children was collected, centrifuged 10 min at 3000×g, and the serum was stored for later use.

Cell Culture and Transfection Experiments

Rb cell lines were transfected into DMEM medium containing 10% fetal bovine serum as well as penicillin-streptomycin mixed solution, and cultured in a 5% CO_2, 37°C constant temperature saturated humidity cell incubator. NKILA-siRNA (si-NKILA), NKILA-shRNA (sh-NKILA) and no-load plasmid (siRNA-NC) were transfected respectively according to the instruction manual of Lipofectamine™ 2000 transfection kit. The primers were transfected into the cells with the greatest difference in NKILA expression. Six hours after transfection, they were substituted by a medium containing 10% fetal bovine serum for further culture. qRT-PCR was used to verify the transfection efficiency of cells.

QRT-PCR Detection

SiRNA expression in serum and cells was observed by qRT-PCR. Total RNA in serum was extracted and dissolved in 20 μL DEPC water based on TRIzol reagent operation instructions. It was then reverse transcribed by a reverse transcription

kit. The reaction system was as below: M-MLV 1 μl, Olig (dT) 1 μl, RNA enzyme inhibitor 0.5 μl, d NTPs 1 μl, RNase free water added to 15 μl. Then, it was incubated for 60 min at 38°C. We took 1 μL c DNA at 85°C for 5 s. The synthesized cDNA was taken as a templet for qRT-PCR amplification. The PCR reaction system was prepared as follows: 10×PCR buffer 2.5 μl, d NTPs 1 μl, upstream and downstream primers 1 μl each, Taq DNA Polymerase 0.25 μl, dd H_2O supplemented to 25 μl. The reaction conditions were as follows: pre-denaturation at 95°C for 15 min, denaturation at 95°C for 15 s, annealing at 58°C for 30 s, a total of 35 cycles. Eventually, 72°C was extended for 15 min, and 3 multiple wells were supplied in each sample for 3 replicated tests. NKILA used β-actin as internal reference and MALAT1 regarded GAPDH as internal reference. After completing the reaction, we confirmed the amplification and melting curve of Real-Time PCR and calculated the relative amount of the target gene on the basis of the result parameters. At last, we calculated the relative quantification of target gene *via* $2^{-\Delta CT}$ (Table I).

Western Blot Test

The lysed cells were collected and transferred to a centrifuge tube, they were centrifuged for 10 min at 12000×g at 4°C, while the supernatant was collected as a protein sample, and its protein concentration was observed through BCA method. Lysis buffer was added to dilute the protein sample to prepare 20 mg/ml protein, 8.00% separating gel and 5.00% stacking gel. SDS-PAGE electrophoresis and polyvinylidene difluoride (PVDF) membrane transfer were performed. β-catenin, cyclin D1, c-myc (1:1000) primary antibody were added, β-actin (1:3000) was considered as internal reference, and they were sealed at 4°C all night. HRP labeled sheep anti-mouse secondary antibody (1: 5000) was added, incubated 1 h at 37°C, and rinsed 3 times with TBST for 5 min each time. The proteins on the membrane were developed in a dark room using the enhanced chemiluminescence (ECL) reagent, and the excess liquid was absorbed with a filter paper. The protein bands were scanned, and the gray values were analyzed using Quantity One software (Molecular Devices Corp., The Bay Area, CA, USA).

Cell Proliferation Experiment

Cell viability was determined by MTT assay, and cells harvested 24 h after transfection were collected. Cell density was adjusted to 5×10^3 cells/well, and the cells were inoculated on 96-well plates and incubated 24, 48, and 72 h respectively at 37°C. A total of 20 μL MTT solution (5 μmg/ml) was added at each time point, culture was continued for 4 h at 37°C, and 200 μL dimethyl sulfoxide was supplemented to each well, and the OD value of cells in each group was verified by spectrophotometer at 570 nm wavelength.

Transwell Invasion Experiment

Matrigel glue was coated in transwell chamber and was allowed to stand 30 min at 37°C. The cells were resuspended in serum-free DMEM culture with a cell density of 4×10^5 cells/mL, and 200 μL of cell suspension was supplemented to the upper chamber, as well as 800 μL of DMEM medium (10% fetal bovine serum) was supplemented to the lower chamber. Altogether, 24-48 h after continuous culture, the chamber was taken out and fixed with 4% paraformaldehyde, and then, 0.1% crystal violet staining was adopted to wipe the cells in the upper chamber. Ten high power fields were randomly selected under an optical microscope to count the cells passing through the basement membrane of the small chamber, indicating their invasion.

Apoptosis Assay

Cells transfected for 48 h were digested with 0.25% trypsin, washed twice with PBS, resuspended with AnnexinV-binding buffer 100 μL, configured as 1×10^6 cells/mL suspension, added with Annexin-V/FITC solution 5 μL, and incubated 15 min at 4°C. A total of 5 μl PI staining solution was added and then they were incubated 5 min at 4°C. We used Flow cytometry for detection, repeated the experiment 3 times, and then, we took the average.

Table I. MALAT1, NKILA and their internal reference primer sequences.

Genes	Forward primer	Reverse primer
NKILA	5'-CAGCAGGCAAAAATAACCAG-3'	5'-GACAGAATCAACTTCGGAAC -3'
β-actin	5'-GGGAAATCGTGCGTGACATTAAG-3'	5'-TGTGTTGGCGTACAGGTCTTTG-3'
MALAT1	5'-AAA GCAAGG TCT CCC CACAAG-3′	5'-GGT CTG TGC TAG ATC-3'
GAPDH	5'-CAAAGGTGGATCAGATTCAAG-3'	5'-GGTGAGCATTATCACCCAGAA-3'

Statistical Analysis

The Statistical Product and Service Solution (SPSS) 20.0 (IBM Corp., Armonk, NY, USA) was applied in statistical analysis. Normal distribution data were expressed in mean±standard deviation (meas±SD), and independent-samples *t*-test was carried out to compare the measurement data among groups. Data of multiple time points were compared by repeated measures analysis of variance (ANOVA), and Bonferroni method was adopted in back testing. One-way ANOVA was applied in comparison among varied groups of mean values, and LSD-t test was used afterwards. The diagnostic value was evaluated by ROC curve, and Pearson test was applied to correlation. $p<0.05$ had statistically significance.

Results

Expression and Clinical Value of NKILA and MALAT1 in Serum of Rb Patients

By detecting the expression levels of NKILA and MALAT1 in serum of the subjects, we found that those in serum of the research group were lower than those in the control group ($p<0.001$). Pearson test analysis found that the NKILA and MALAT1 levels in serum of Rb patients were positively correlated ($p<0.001$). AUC of NKILA and MALAT1 were 0.883 and 0.850 respectively by plotting ROC curves. By further analyzing the relationship between the two indicators and the pathological data of the patients, we found that NKILA and MALAT1 were closely related to tumor size, classification and clinical grading ($p<0.05$). More details were shown in Table II, III, and Figure 1.

Expression of NKILA and MALAT1 in Cells and Their Effects on Cell Biological Functions

NKILA expression of cell lines in each group was tested by qRT-PCR. It was found that the NKILA expression in Weri-Rb1 and Y79 cells was lower than that in hTERT RPE-1 normal retinal pigment epithelial cells ($p<0.05$). We selected Weri-Rb1 and Y79 cells with the greatest expression difference for transfection; compared with hTERT RPE-1 normal retinal pigment epithelial cells, the NKILA levels in Weri-Rb1 and Y79 cells were significantly downregulated ($p<0.05$); meanwhile, the NKILA expression in siRNA-NKILA group was lower than that in siRNA-NC group ($p<0.01$), and that in shRNA-NKILA group was higher than that in siRNA-NC group ($p<0.01$).

Table II. Relationship between serum NKILA and Rb pathological parameters (mean±SD).

Clinicopathological parameters		NKILA	*t*	*p*
Gender			0.271	0.788
Male	13	0.46±0.20		
Female	17	0.44±0.20		
Onset age (years)			0.266	0.792
<2	16	0.43±0.21		
≥2	14	0.45±0.20		
Tumor size (mm)			3.514	<0.001
<3	21	0.58±0.20		
≥3	9	0.30±0.20		
Average course of disease (days)			0.140	0.889
<158	15	0.46±0.19		
≥158	15	0.45±0.20		
Eyes			0.444	0.661
Left	16	0.40±0.18		
Right	14	0.43±0.19		
Typing			0.122	0.904
Genotype	15	0.41±0.23		
Non-hereditary type	15	0.42±0.22		
Clinical classification			2.422	0.022
A-C	22	0.55±0.2		
D-E	8	0.35±0.2		
Clinical staging			3.801	<0.001
Intraocular/glaucomatous stage	20	0.60±0.21		
Extraocular/systemic metastasis	10	0.30±0.19		

Table III. Relationship between MALAT1 and pathological data of Rb patients (mean±SD).

Clinicopathological parameters		NKILA	*t*	*p*
Gender			0.271	0.788
Male	13	0.30±0.10		
Female	17	0.29±0.10		
Age (years)			0.259	0.798
<2	16	0.29±0.11		
≥2	14	0.30±0.10		
Tumor size (mm)			3.012	0.006
<3	21	0.35±0.10		
≥3	9	0.23±0.10		
Average course of disease (days)			0.248	0.806
<158	15	0.31±0.12		
≥158	15	0.30±0.10		
Eyes			0.521	0.607
Left	16	0.25±0.09		
Right	14	0.27±0.12		
Typing			0.219	0.828
Genotype	15	0.29±0.13		
Non-hereditary type	15	0.28±0.12		
Clinical classification			2.422	0.022
A-C	22	0.30±0.10		
D-E	8	0.20±0.10		
Clinical staging			6.233	<0.001
Intraocular/glaucoma phase	20	0.45±0.11		
Extraocular/systemic metastasis	10	0.15±0.15		

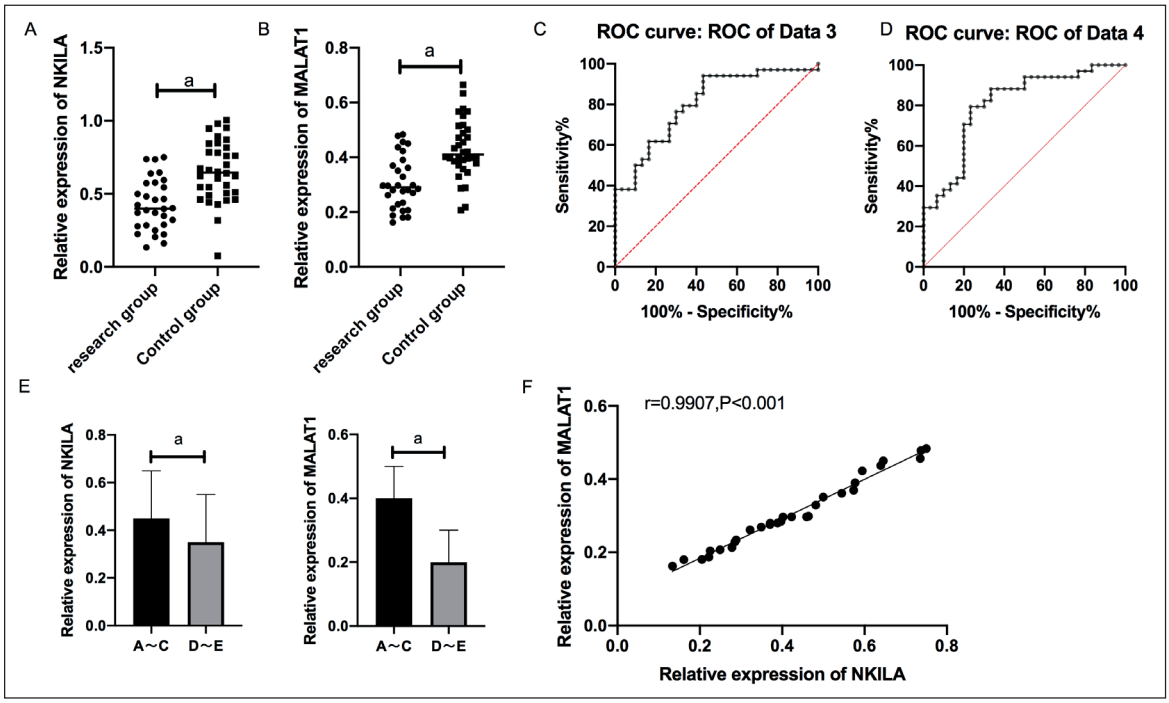

Figure 1. Expression and clinical value of NKILA and MALAT1 in serum of Rb patients. **A**, The expression levels of NKILA in the research group were significantly higher than those in the control group, and NKILA was low expressed in serum of Rb patients; a indicates p<0.001. **B**, The expression levels of MALAT1 in the research group were significantly lower than those in the control group, and MALAT1 was low expressed in Rb patients; a indicates *p*<0.001. **C**, AUC of NKILA was 0.8069. **D**, AUC of MALAT1 was 0.8059. **E**, The expression levels of NKILA and MALAT1 in patients of Figures **D-E** in research group were significantly lower than those in those of Figures **A-C**; a indicates *p*<0.001. **F**, The expression levels of NKILA and MALAT1 in serum of Rb patients was positively correlated (r=0.9907, *p*<0.001).

MTT results suggested that the proliferation from siRNA-NKILA group was significantly higher than that from siRNA-NC group (p<0.05), while that from siRNA-NKILA group was lower than that from siRNA-NC group (p<0.05). Flow cytometry revealed that the apoptosis rate of siRNA-NKILA group was lower than that from siRNA-NC group (p<0.001), while that from shRNA-NKILA group was remarkably higher than that from siRNA-NC group (p<0.001). Transwell experiment verified that the cell invasion from siRNA-NKILA group was notably higher than that from siRNA-NC group (p<0.001), and that from shRNA-NKILA group was significantly lower than that from siRNA-NC group (p<0.001) (Figure 2).

Expression of MALAT1 in Each Group of Cell Lines Through qRT-PCR

It was found that the MALAT1 expression in Weri-Rb1 and Y79 cells was significantly downregulated compared with hTERT RPE-1 normal retinal pigment epithelial cells (p<0.05). We selected Weri-Rb1 and Y79 cells with the greatest expression difference for transfection; compared with hTERT RPE-1 normal retinal pigment epithelial cells, the MALAT1 expression in Weri-Rb1 and Y79 cells was significantly downregulated (p<0.05); moreover, the MALAT1 expression from siRNA-MALAT1 group was significantly lower than that from siRNA-NC group (p<0.01), and that from shRNA-MALAT1 group was remarkably higher than that from siRNA-NC group (p<0.01). MTT results showed that the proliferation of siRNA-MALAT1 group was significantly higher than that of siRNA-NC group (p<0.05), while that of shRNA-MALAT1 group was downregulated than that of siRNA-NC group (p<0.05). Flow cytometry showed that the apoptosis rate from siRNA-MALAT1 group was significantly lower than that from siRNA-NC group (p<0.001), while that from shRNA-MALAT1 group was remarkably higher than that from siRNA-NC group (p<0.001). Transwell experiment results showed that the cell invasion from siRNA-MALAT1 group was notably higher than that from siRNA-NC group (p<0.001), while that from shRNA-MALAT1 group was significantly lower than that from siRNA-NC group (p<0.001) (Figure 3).

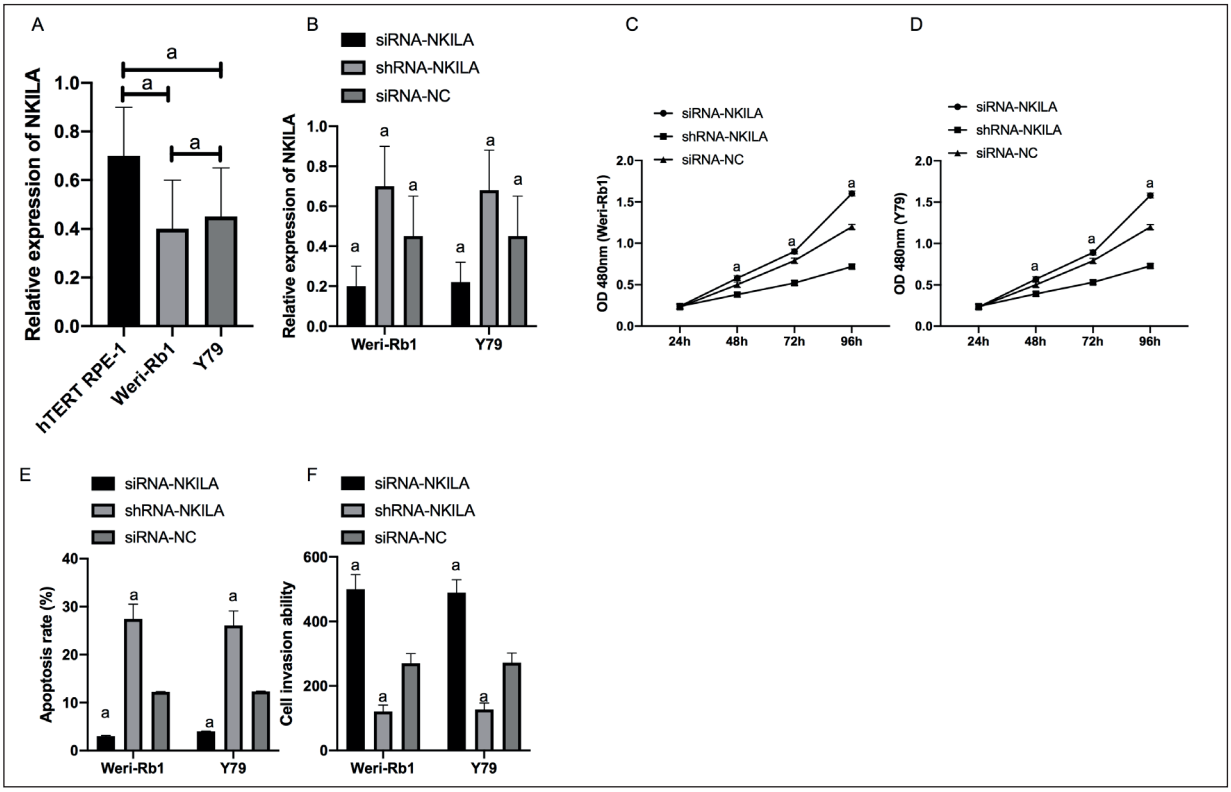

Figure 2. NKILA expression in cells and its effect on cell biological function. **A**, Expression of NKILA in each cell line. **B**, Expression of NKILA after transfection of Weri-Rb1 and Y79 cells . Proliferation of Weri-Rb1 (**C**) and Y79 (**D**) cells after transfection. **E**, Apoptosis of Weri-Rb1 and Y79 cells after transfection. **F**, Invasion of Weri-Rb1 and Y79 cells after transfection. Note: *p<0.05, **p<0.01, ***p<0.001.

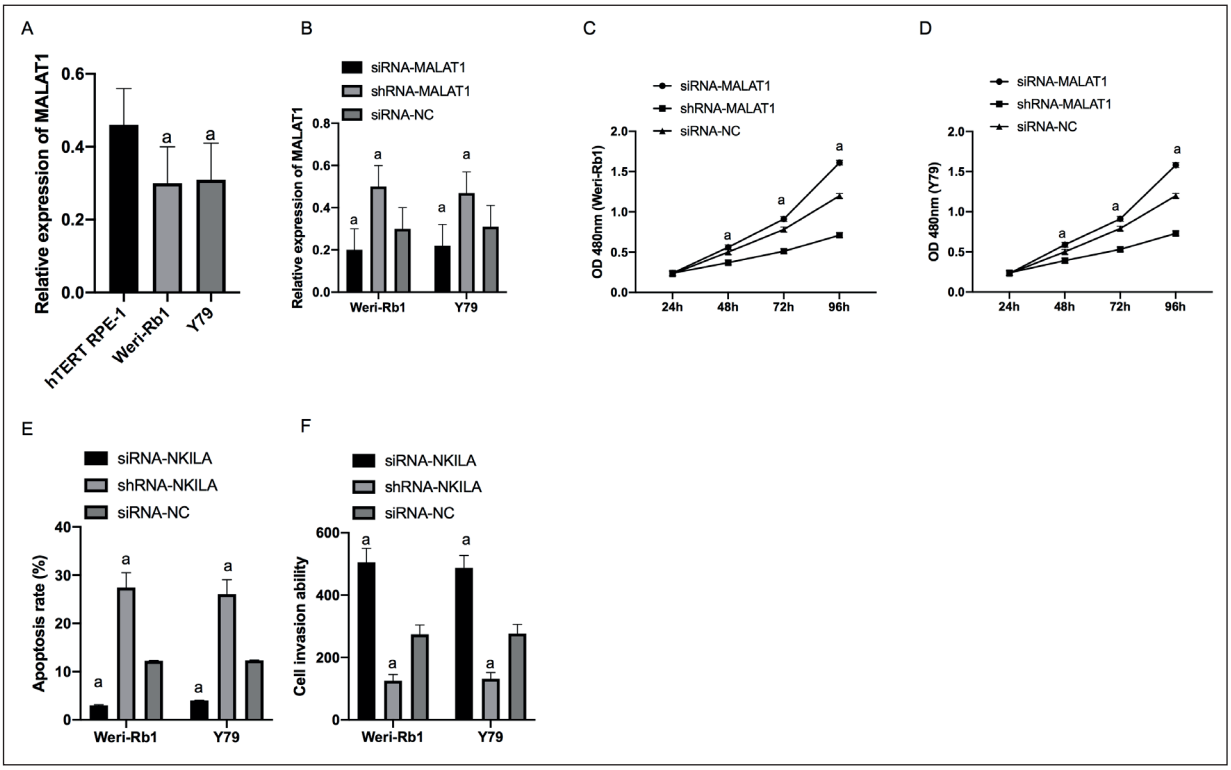

Figure 3. MALAT1 expression in cells and its effect on cell biological function. **A**, Expression of MALAT1 in various cell lines. **B**, Expression of MALAT1 after transfection of Weri-Rb1 and Y79 cells. Proliferation of Weri-Rb1 (**C**) and Y79 (**D**) cells after transfection. **E**, Apoptosis of Weri-Rb1 and Y79 cells after transfection. **F**, Invasion of Weri-Rb1 and Y79 cells after transfection. Note: *p<0.05, **p<0.01, ***p<0.001.

Effect of NKILA and MALAT1 Expression on Apoptosis-Related Proteins

Western Blot results indicated that compared with cells transfected with siRNA-NC, the cle-caspase-3, cle-caspase-9 and Bax protein levels were remarkably upregulated in those transfected with sh-NKILA, and the Bcl-2 expression was downregulated. However, compared with cells transfected with siRNA-NC, the cle-caspase-3, cle-caspase-9 and Bax protein levels were significantly downregulated in those transfected with sh-NKILA, and the Bcl-2 expression significantly increased (p<0.05).

Compared with cells transfected with siRNA-NC, the cle-caspase-3, cle-caspase-9 and Bax protein levels were remarkably up-regulated and the Bcl-2 expression was significantly downregulated in those transfected with sh-MALAT1; compared with cells transfected with siRNA-NC, the cle-caspase-3, cle-caspase-9 and Bax protein levels were notably downregulated in those transfected with sh-MALAT1, and the Bcl-2 expression notably enhanced (p<0.05) (Figure 4).

Effect of NKILA and MALAT1 Expression on Cell Cycle-Related Proteins

Western blot results revealed that the Cyclin B1, CDC2 and p-CDC2 levels were significantly downregulated in cells transfected with sh-NKILA compared with those transfected with siRNA-NC; however, compared with cells transfected with siRNA-NC, their expression levels significantly increased in those transfected with sh-NKILA (p<0.05).

The Cyclin B1, CDC2 and p-CDC2 levels were significantly downregulated in cells transfected with sh-MALAT1 compared with those transfected with siRNA-NC; however, compared with cells transfected with siRNA-NC, those significantly increased in cells transfected with sh-NKILA (p<0.05) (Figure 5).

Discussion

Recent scholars[17] have found that LncRNA is relevant to various tumors, LncRNA plays a key part in carcinogenesis as well as tumor progression,

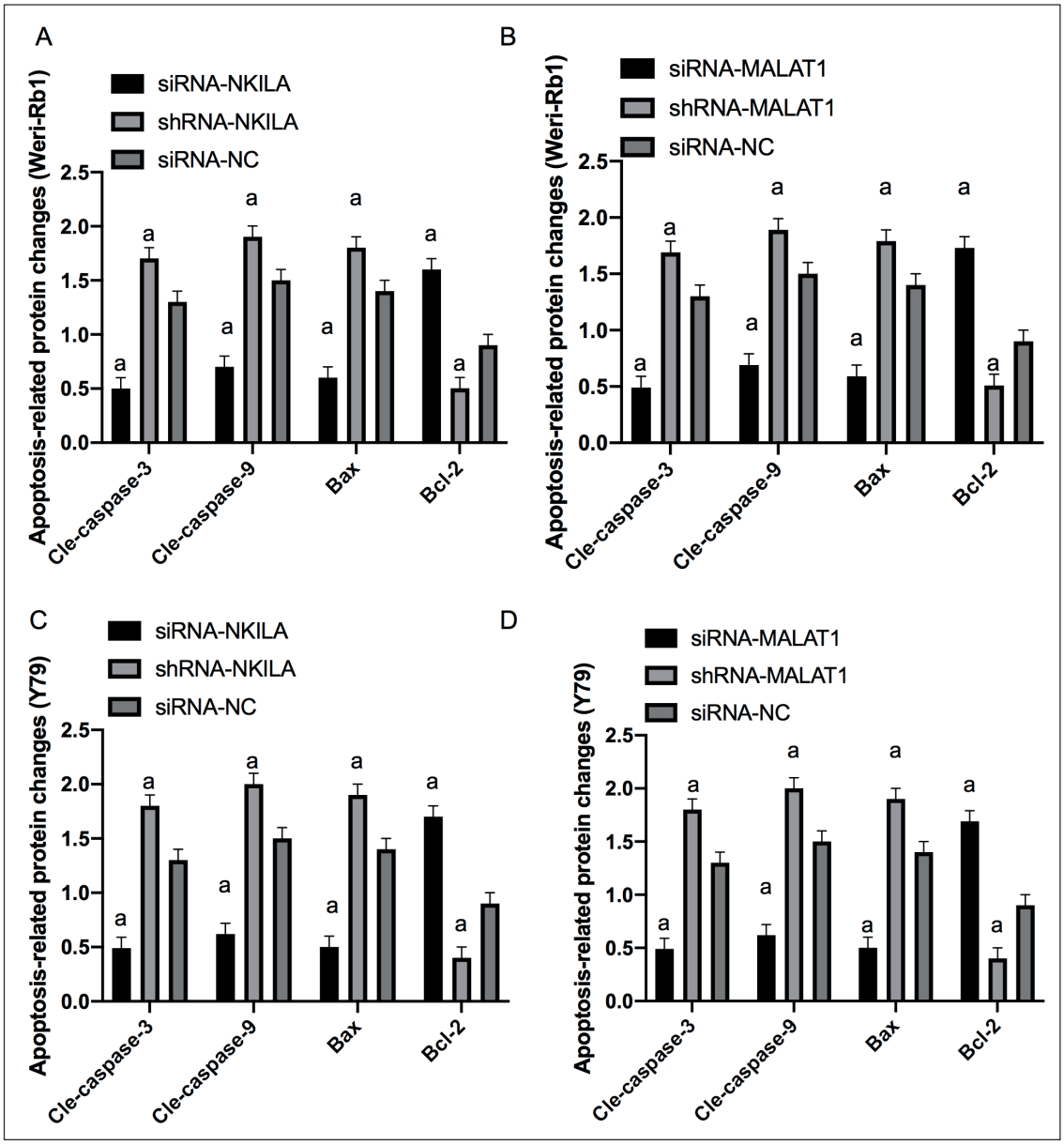

Figure 4. Effect of NKILA and MALAT1 expression on apoptosis-related proteins. **A**, Compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9, and Bax protein were significantly up-regulated and the expression of Bcl-2 in those transfected with sh-NKILA was significantly downregulated. However, compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly down-regulated in those transfected with sh-NKILA, and the expression of Bcl-2 significantly increased. **B**, Compared with the cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly up-regulated and the expression of Bcl-2 was significantly down-regulated in those transfected with sh-MALAT1. However, compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly down-regulated and the expression of Bcl-2 significantly increased in those transfected with sh-MALAT1. **C**, Compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly up-regulated and the expression of Bcl-2 was significantly down-regulated in those transfected with sh-NKILA. However, compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly down-regulated and the expression of Bcl-2 significantly increased in cells transfected with sh-NKILA. **D**, Compared with the cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly up-regulated and the expression of Bcl-2 was significantly down-regulated in those transfected with sh-MALAT1. However, compared with cells transfected with siRNA-NC, the expression levels of cle-caspase-3, cle-caspase-9 and Bax protein were significantly down-regulated and the expression of Bcl-2 significantly increased in those transfected with sh-MALAT1. Note: a indicates comparison with NC group (p<0.001).

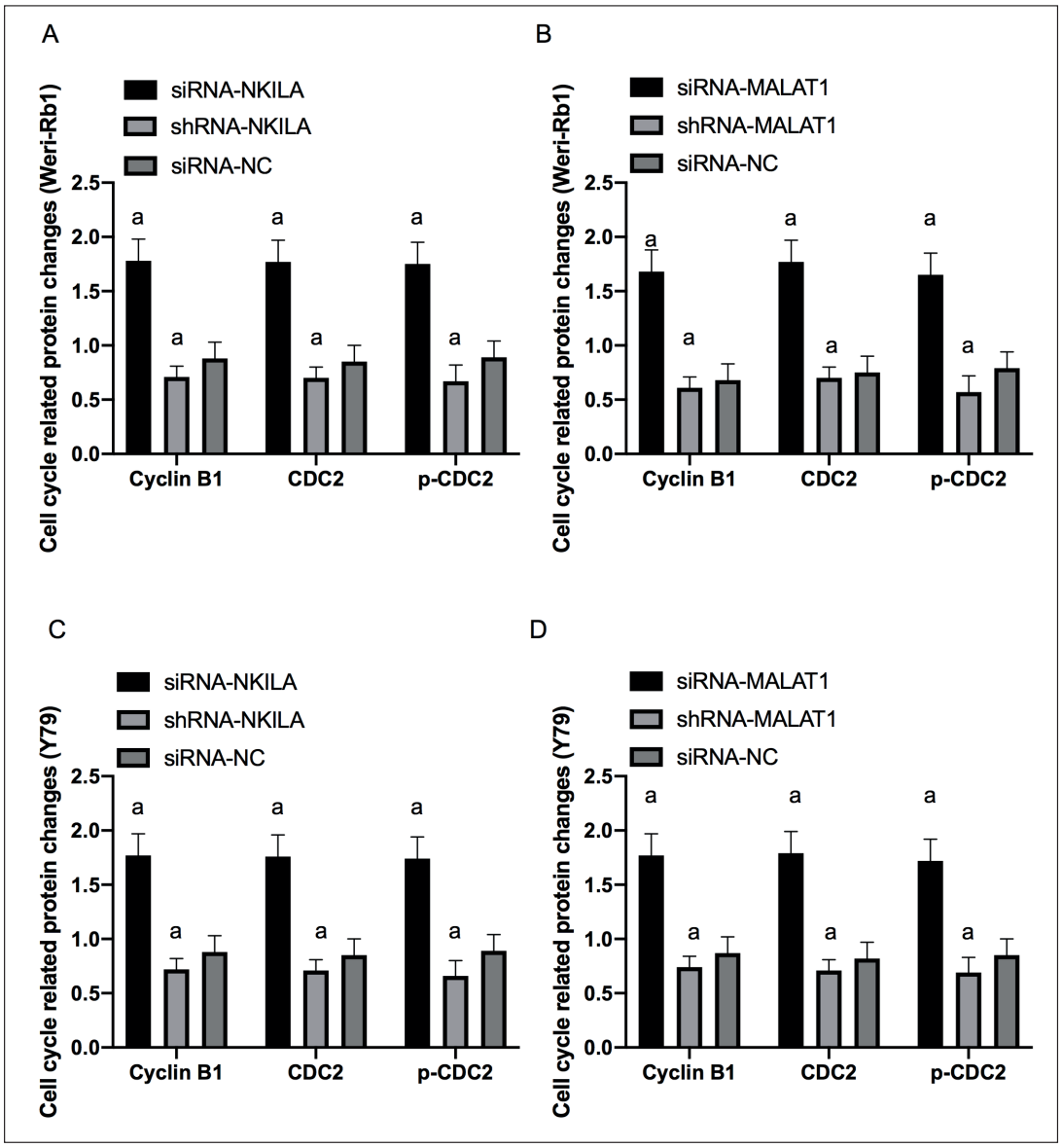

Figure 5. Effect of NKILA and MALAT1 expression on cell cycle-related proteins. **A**, The expression levels of Cyclin B1, CDC2 and p-CDC2 were significantly down-regulated in cells transfected with sh-NKILA compared with those transfected with siRNA-NC; however, the expression levels of Cyclin B1, CDC2 and p-CDC2 significantly increased in cells transfected with sh-NKILA compared with those transfected with siRNA-NC. **B**, The expression levels of Cyclin B1, CDC2 and p-CDC2 were significantly down-regulated in cells transfected with sh-MALAT1 compared with those transfected with siRNA-NC; however, the expression levels of Cyclin B1, CDC2 and p-CDC2 significantly increased in cells transfected with sh-NKILA compared with those transfected with siRNA-NC. **C**, The expression levels of Cyclin B1, CDC2 and p-CDC2 were significantly down-regulated in cells transfected with sh-NKILA compared with those transfected with siRNA-NC; however, the expression levels of Cyclin B1, CDC2 and p-CDC2 significantly increased in cells transfected with sh-NKILA compared with those transfected with siRNA-NC. **D**, The expression levels of Cyclin B1, CDC2 and p-CDC2 were significantly down-regulated in cells transfected with sh-MALAT1 compared with those transfected with siRNA-NC; however, compared with cells transfected with siRNA-NC, the expression levels of Cyclin B1, CDC2 and p-CDC2 significantly increased in cells transfected with si-NKILA. Note: a indicates comparison with NC group (p<0.001).

and LncRNA expression affects the growth and decline of tumor cells. LncRNA has a unique expression state in different cancer types, and related cellular biology studies have found that lncRNA can regulate cell signaling function[18]. The NKILA expression in serum or tissue cells of laryngeal cancer patients is downregulated, and it has been found that changes in NKILA can

interfere with the cell cycle of cancer cells, but the specific mechanism has not been elaborated in detail[19,20]. This study was attempted to explore the effects of MALAT1 and NKILA on the biological functions of Rb cells, so as to offer a new academic foundation for Rb's diagnosis and treatment in molecular biology.

In this work, we used qRT-PCR technology to detect MALAT1 expression in serum of Rb patients and healthy people, and found that MALAT1 abnormally decreased in serum of Rb patients. We further found that the low expression of MALAT1 was correlated with the tumor size, typing and clinical grading of Rb patients through correlation analysis of clinical and pathological characteristics, and AUC of MALAT1 was over 0.8 by drawing ROC curve. At present, reports have shown that LncRNA can target downstream gene pairs to regulate cell biological functions[21,22]. We further discovered that there was a targeting relationship between MALAT1 and NKILA through the analysis of the TargetScan database. Clinicopathological features of NKILA and Rb patients were also assessed. It was also found that low expression of NKILA was correlated with tumor size, typing and clinical grading of Rb patients, and AUC>0.8 was found by drawing ROC curve. NKILA participates in the changes of biological functions of various tumor cells and plays an essentially regulatory part in tumor development and differentiation[23]. MALAT1 can regulate Rb cell genes and affect cell cycle[24]. In this study, we tested the TCGA database and the MALAT1 expression in Rb cells, and confirmed that MALAT1 and NKILA were both low expressed, which was consistent with the results of this study, suggesting that the two might both play regulatory roles in Rb.

In vitro, we compared human Rb cell lines Weri-Rb1 and Y79 with hTERT RPE-1 normal retinal pigment epithelial cells, and found that MALAT1 and NKILA were low expressed in Rb cell lines. Subsequently, we treated the MALAT1 and NKILA levels in Weri-Rb1 and Y79 cells by upregulating and downregulating them, and further transfected sh-MALAT1, si-MALAT1, sh-NKILA, si-NKILA, and sequences into Weri-Rb1 and Y79 cells, and observed their biological function. After overexpression of MALAT1 and NKILA, the cell proliferation and invasion were inhibited, and the levels of relevant proteins cle-caspase-3, cle-caspase-9 and Bax protein in the process of apoptosis were increased, and the Bcl-2 expression was lowered. The growth rate of tumor cells was closely tied to the apoptosis environment; as a key enzyme in the process of apoptosis. The changes of Caspase family will lead to changes in the biological functions of tumor cells. Caspase belongs to cysteine protease; as a key enzyme in the process of apoptosis, it is activated along with the activation of signal transduction pathway, then, it undergoes cascade reaction of apoptotic protease, which is responsible for selective cleavage to degrade important proteins in cells and eventually leads to irreversible apoptosis. Bcl-2 has anti-apoptosis effect. When cells are stimulated by outside, Bcl-2 can inactivate caspase through aggregation with them, which can protect them from apoptosis. Combined with the results of this study, it was suggested that the overexpression of NKILA and MALAT1 could inhibit proliferation, invasion and promote apoptosis of Rb cells. The two could be potential targets for Rb therapy, which further suggested the influence of their changes on Rb.

Cell cycle-related proteins greatly promote the growth and proliferation of Rb cells in their apoptosis[25] and lncRNA can promote cancer cell proliferation by regulating the expression of cycle-related proteins[26]. In this study, we observed cell cycle-related proteins by inhibiting or over-expressing MALAT1 and NKILA in Rb cells. The results showed that the Cyclin B1, CDC2 and p-CDC2 levels were significantly downregulated in the cells with high expression of MALAT1 or NKILA, while low expression of NKILA or MALAT1 were opposite, which suggested that the two could both inhibit the activation of Rb cell cycle-related proteins and thus inhibit the activation of cells through regulation. Some studies related to cell cycle also show that silencing LncRNARP1 can inhibit the expression levels of cyclinD1, CDK6, and other cell cycle proteins, thus interfering with the proliferation of cancer cells[27]. Under the action of transcription activator, Cyclin B1, CDC2, p-CDC2 and other cell cycle proteins are overexpressed, which ensures the normal progression of cancer cell cycle and promotes the continuous progress of cancer[28]. Therefore, we believe that overexpression of NKILA and MALAT1 can downregulate the Cyclin B1, CDC2 and p-CDC2 levels, thus affecting the normal cell cycle progression of Rb cells.

In this investigation, we proved that MALAT1 and NKILA were low expressed in Rb, and could overexpress NKILA and MALAT1 to induce cell proliferation. But there are some limitations in this study. For example, we lack the studies on the behavioral differences between lncRNA MALAT1 and lncRNA NKILA in serum and *in vitro*. None-

theless, it is not clear the regulatory network of NKILA and MALAT1; other ways that can affect the development and progression of tumor still need to be clarified. Therefore, we hope to explore the regulatory networks of NKILA and MALAT1 through bioinformatics analysis in the future research to provide more basis for experiments.

Conclusions

To sum up, regulating MALAT1 and NKILA to regulate the growth and apoptosis of Rb cells is expected to be a potential clinical therapeutic target for Rb. MALAT1 and NKILA may be useful markers for diagnosis and prognosis evaluation.

Conflict of Interests

The authors declare that they have no conflict of interest.

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