# Long noncoding RNA PLAC2 regulates PTEN in retinoblastoma and participates in the regulation of cancer cell apoptosis

LING SONG[1], YUEQIN QI[1] and MING LIN[2]

[1]Department of Ophthalmology, Binzhou Hubin Aier Eye Hospital Limited, Binzhou, Shandong 256613; [2]Department of Ophthalmology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, Shanghai 200011, P.R. China

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**Abstract.** Long noncoding RNA placenta-specific 2 (PLAC2) blocks the cancer cell cycle in glioma, suggesting its tumor-suppressive role. The present study aimed to investigate the role of PLAC2 in retinoblastoma (Rb). It was found that PLAC2 was downregulated in Rb tissues and was not affected by the development of Rb. PTEN was also downregulated in Rb and positively correlated with PLAC2. In Rb cells, PLAC2 over-expression resulted in the upregulated expression of PTEN, while PTEN over-expression did not affect PLAC2 expression. PLAC2 and PTEN over-expression caused an increased apoptotic rate of Rb cells. PTEN small interfering RNA silencing led to a decreased apoptotic rate and attenuated the effects of PLAC2 over-expression. Therefore, PLAC2 regulates PTEN in Rb and participates in the regulation of cancer cell apoptosis.

## Introduction

As the most common type of cancer that is developed from the nuclear layer of the retina, retinoblastoma (Rb) mainly affects children before the age of 5-years-old (1). In numerous regions of the world, especially developing countries, most Rb cases are diagnosed at advanced stages and the mortality rate is high (2). It has been estimated that even more Rb patients will die due to this disease in developing countries after treatment (2,3). At present, external-beam radiotherapy and enucleation are the main therapeutic approaches for Rb (4). In addition, chemotherapy has also been developed to treat extraocular and metastatic Rb (5,6). However, treatment outcomes are generally poor.

PTEN signaling is a well-known tumor-suppressive pathway in different types of cancer (7). The PTEN pathway is usually inactivated during cancer progression and the re-activation of this signaling is considered to be a promising approach for cancer therapies (8). In cancer biology, PTEN suppresses cancer progression mainly by inhibiting the phosphatidyl inositide 3 kinase (PI3K)-protein kinase B (Akt) pathway, which is a major activated survival pathway in cancer cells (9). However, the upstream regulator of PTEN has not been well studied. In a recent study, Hu *et al* (10) reported a novel long noncoding (lnc)RNA named placenta-specific 2 (PLAC2) as a novel inhibitor of cell cycle progression in glioma. PLAC2 participates in glioma by interacting with signal transducer and activator of transcription 1 (STAT1), which has crosstalk with PTEN (11). However, the interaction between PLAC2 and PTEN has not been reported. Therefore, this study was carried out to investigate the involvement of PLAC2 in Rb, as well as its possible interaction with PTEN.

*Correspondence to:* Dr Ming Lin, Department of Ophthalmology, Shanghai Ninth People's Hospital, Shanghai Jiao Tong University School of Medicine, 639 Zhi Zao Ju Road, Shanghai 200011, P.R. China
E-mail: linming03@yeah.net

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## Materials and methods

*Study subjects.* A total of 89 Rb patients were admitted by Shanghai Ninth People's Hospital between June 2016 and December 2018. The present study selected 60 Rb cases (sex: 33 males and 27 females; age: 11 months to 4.2 years, 2.2±0.4 years) based on strict criteria. Inclusion criteria: i) Newly diagnosed Rb cases; ii) no initiated therapies were observed. Exclusion criteria: i) Therapies were carried out before this study; ii) recurrent Rb; iii) other clinical disorders were diagnosed; iv) histories of previous malignancies. Based on clinical findings, there were 12, 11, 15, 10 and 12 cases at group A-E (International Classification for Intraocular Retinoblastoma), respectively. Group A, tumors within the retina <3 mm; Group B, tumors within the retina >3 mm; Group C, minor tumor spread within the back of the eye; Group D, tumor spread throughout the back of the eye; Group E, tumor spread to lens, or causes increased eye pressure, or causes bleeding from the eye. All patients' guardians were informed with the experimental details and they all signed informed consent. The aforementioned hospital Ethics Committee approved this study.

*Tissue specimens and cells.* Non-tumor (within 2 cm around the tumor site) and Rb tissues were obtained from each patient by biopsy. All the tissues were checked by at least 3 pathologists

to make sure all the specimens were correct (cancer cell percentage in non-tumor tissues should be below 1%).

For *in vitro* experiments, human Rb cell lines Y79 and WERI-Rb-1 (American Type Culture Collection) were used. Cells culture conditions were 5% $CO_2$ and 37˚C. The cell culture medium was RPMI-1640 Medium (20% fetal bovine serum).

*Transient transfections*. PLAC2 and PTEN expression vectors were constructed using pcDNA3 (Sangon Biotech Co., Ltd.). PTEN small interfering (si)RNA (5'-UAGCAG AAACAAAAGGAGAUAUC-3') and negative control siRNA (5'-GUCGUCAAAGUCAGGUACACCGA-3') were from Shanghai GenePharma Co., Ltd. Y79 and WERI-Rb-1 cells were collected at the confluence of 70-80%. Nucleofector™ Technology (Lonza Group, Ltd.) was used to transfect 10 nM PLAC2 and PTEN expression vector, 10 empty pcDNA3 vectors negative control (NC), 35 nM PTEN siRNA, or 35 nM NC siRNA were transfected into $10^5$ cells. The control group included cells with no transfections. Subsequent experiments were performed at 24 h post-transfections.

*Reverse transcription-quantitative (RT-q)PCR*. Ribozol (Thermo Fisher Scientific., lnc.) was mixed with Y79 and WERI-Rb-1 cells (1 ml per $10^5$ cells) and tissues (0.5 ml per 0.02 g tissue) to extract total RNAs. All RNA samples were digested with DNase I. AMV Reverse Transcriptase XL (Clontech Laboratories, Inc.) was used to perform reverse transcription by incubating at 25˚C for 10 min, 55˚C for 20 min and 80˚C for 10 min. SYBR Green Master Mix (Bio-Rad Laboratories, Inc.) was used to prepare qPCR reaction mixtures. The expression of PLAC2 and PTEN was detected using 18S rRNA or GAPDH as endogenous control, respectively. Reaction conditions were: 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec and 60˚C for the 50 sec. It is worth noting that multiple primers were used and similar results were obtained. Primer sequences were: 5'-CGGCTACTAGCGGTT TTAC-3' (forward) and 5'-AAGAAGATGCGGCTGACTG-3' (reverse) for GAPDH; 5'-TGTGGCCCAAACTCAGGGATA CA-3' (forward) and 5'-GATGACAGTGGCTGGAGTTGT C-3' for PLAC2 (reverse); 5'-GAGTTCCCTCAGCCGTTA CCT-3' (forward) and 5'-AGGTTTCCTCTGGTCCTGGTA-3' for (reverse) PTEN mRNA; 5'-GCTTAATTTGACTCAACA CGGG-3' (forward) and 5'-GCTATCAATCTGTCAATCCTG TC-3' for (reverse) 18S rRNA. All experiments were repeated 3 times and data were processed using the $2^{-\Delta\Delta Cq}$ method (12). The sample with the highest ΔCq value was set to '1', all other samples were normalized to this sample.

*Western blotting*. Y79 and WERI-Rb-1 cells were collected at 24 h post-transfections and 1 ml RIPA solution (Thermo Fisher Scientific, Inc.) was used to mix with $10^5$ cells to extract total proteins. BCA assay (Thermo Fisher Scientific, Inc.) was used to measure protein concentration. Protein samples were incubated at 100˚C for 10 min and electrophoresis was performed using 10% SDS-PAGE gel with 30 $\mu$g protein per well. Following protein transfer to PVDF membranes, blocking was performed in non-fat milk (5%) for 2 h at room temperature. Primary antibodies of rabbit polyclonal PTEN (cat. no. ab31392; 1:900; Abcam) and rabbit polyclonal GAPDH (cat. no. ab9485; 1:900; Abcam) for at least 12 h at 4˚C. IgG-horseradish peroxidase secondary antibody (1:800; goat anti-rabbit; cat. no. MBS435036; MyBioSource, Inc.) was used to further incubate with PVDF membranes at room temperature for 2 h. Signals were developed using Immobilon® Western Chemiluminescent HRP Substrate (cat. no. WBKLS0050; Sigma-Aldrich; Merck KGaA) and signals were processed using Image J v1.46 software (National Institute of Health). The gray value of the control group was set to 1, all other groups were normalized to this group.

*Cell apoptosis assay*. Y79 and WERI-Rb-1 cells were collected at 24 h post-transfection and $5x10^4$ cells were mixed with 1 ml RPMI-1640 medium (no serum) to prepare single-cell suspensions. A 6-well plate was used to cultivate cells (2 ml per well) under conditions of 5% $CO_2$ and 37˚C for 48 h. Following digestion using 0.25% trypsin, cells were stained with propidium iodide and Annexin V-FITC (Dojindo Molecular Technologies, Inc.) for 30 min in dark at 4˚C. Finally, apoptotic cells were separated by flow cytometry using NovoCyte Benchtop Flow Cytometer. Data were processed using FCSalyzer v.0.9.12 software (free available from: https://sourceforge.net/projects/fcsalyzer/).

*Statistical analysis*. All experiments were performed with at least 3 biological replicates. Mean ± SEM values were calculated and were used for all comparisons GraphPad Prism 6 (GraphPad Software, Inc.). Differences between non-tumor and Rb tissues were analyzed by performing a paired t-test. Differences among different cell transfection groups were explored by performing analysis of variance (ANOVA; one-way) and Tukey test. Correlations were analyzed by Pearson's correlation coefficient. $P<0.05$ was considered to indicate statistically significant.

## Results

*PLAC2 is downregulated in Rb tissues but not affected by cancer development*. PLAC2 in two types of tissues of Rb patients (n=60) was detected by performing RT-qPCR. Expression data were compared between two types of tissues by performing a paired t-test. It was observed that expression levels of PLAC2 were significantly decreased in Rb tissues compared with non-tumor tissues ($P<0.05$; Fig. 1A). Based on clinical findings, there were 12, 11, 15, 10 and 12 cases at group A-E (International Classification for Intraocular Retinoblastoma), respectively. Expression levels of PLAC2 in Rb tissues were compared among 5 groups of patients by performing an ANOVA (one-way) and Tukey test. It was observed that expression levels of PLAC2 in Rb tissues were not significantly different among the 5 groups (Fig. 1B).

*PTEN was positively correlated with PLAC2*. PTEN mRNA in two types of tissues of Rb patients (n=60) was detected by performing RT-qPCR. Paired t-test analysis showed that expression levels of PTEN mRNA were significantly decreased in Rb tissues compared with non-tumor tissues ($P<0.05$; Fig. 2A). Correlations between PTEN mRNA and PLAC2 were analyzed by performing Pearson's correlation coefficient. It was observed that PTEN mRNA and PLAC2

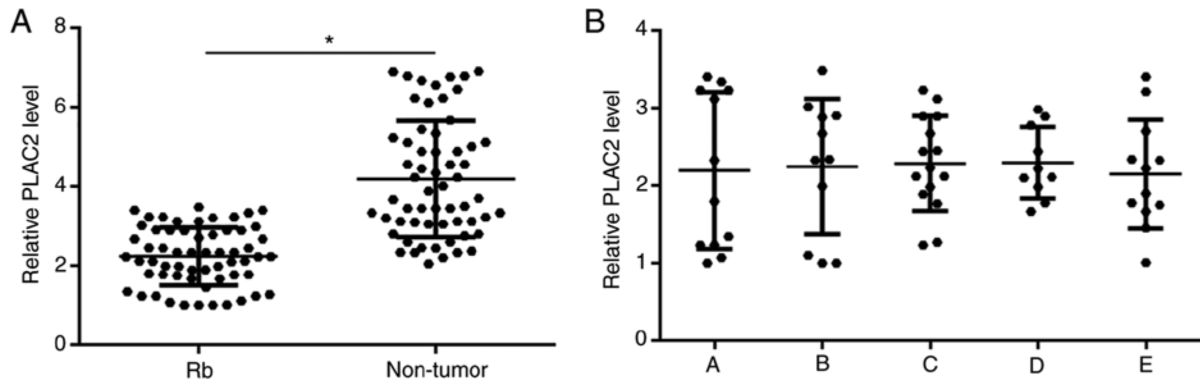


Figure 1. PLAC2 is downregulated in Rb tissues but not affected by cancer development. (A) PLAC2 levels measured by reverse transcription-quantitative PCR and compared by paired t-test showed that expression levels of PLAC2 were significantly lower in Rb tissues compared with non-tumor tissues. (B) Analysis of variance (one-way) and Tukey test analysis showed that expression levels of PLAC2 in Rb tissues were not significantly different among 5 groups. *P<0.05. Rb, retinoblastoma; PLAC2, placenta-specific 2.

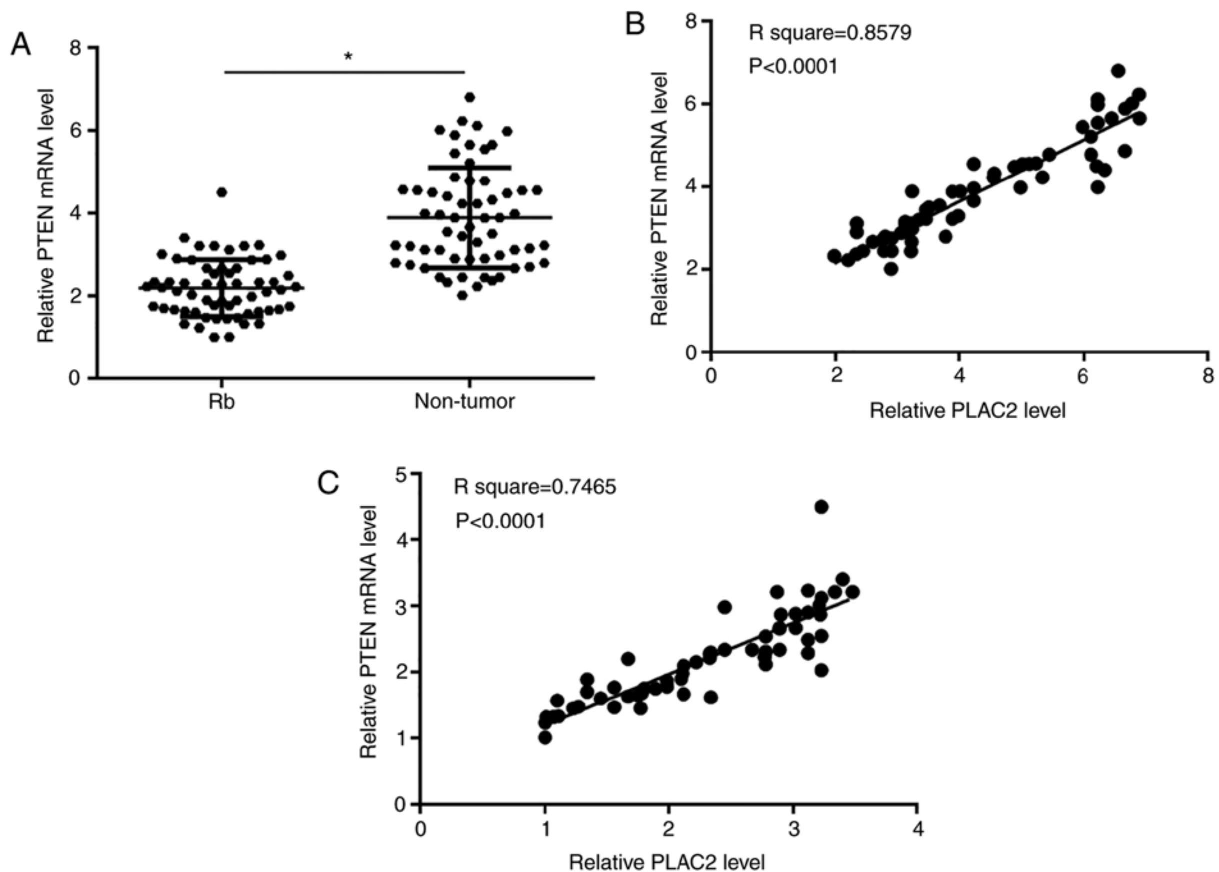


Figure 2. PTEN is positively correlated with PLAC2. (A) PTEN mRNA expression levels measured by reverse transcription-quantitative PCR and analysis by paired t-test analysis showed that expression levels of PTEN mRNA were significantly decreased in Rb tissues compared with non-tumor tissues *P<0.05. Pearson's correlation coefficient showed that PTEN mRNA and PLAC2 were positively correlated both in (B) non-tumor tissues and (C) Rb tissues. Rb, retinoblastoma; PLAC2, placenta-specific 2.

were positively correlated both in non-tumor tissues (Fig. 2B) and Rb tissues (Fig. 2C).

*PLAC2 upregulates PTEN in Rb cells*. Y79 and WERI-Rb-1 cells were transfected with PTEN and PLAC2 expression vectors. Expression levels of PTEN mRNA and PLAC2 were measured by qPCR at 24 h post-transfection. Compared with NC and C the control groups, expression levels of PTEN and PLAC2 were significantly increased at 24 h post-transfections in cells of both Y79 and WERI-Rb-1 cell lines (P<0.05; Fig. 3A). Moreover, compared with the two controls, PLAC2 over-expression resulted in significantly upregulated PTEN in cells of both Y79 and WERI-Rb-1 cell lines (left, mRNA expression detected by qPCR; middle, representative western blot image; right, normalized western blot data; P<0.05; Fig. 3B), while PLAC2 expression was not significantly

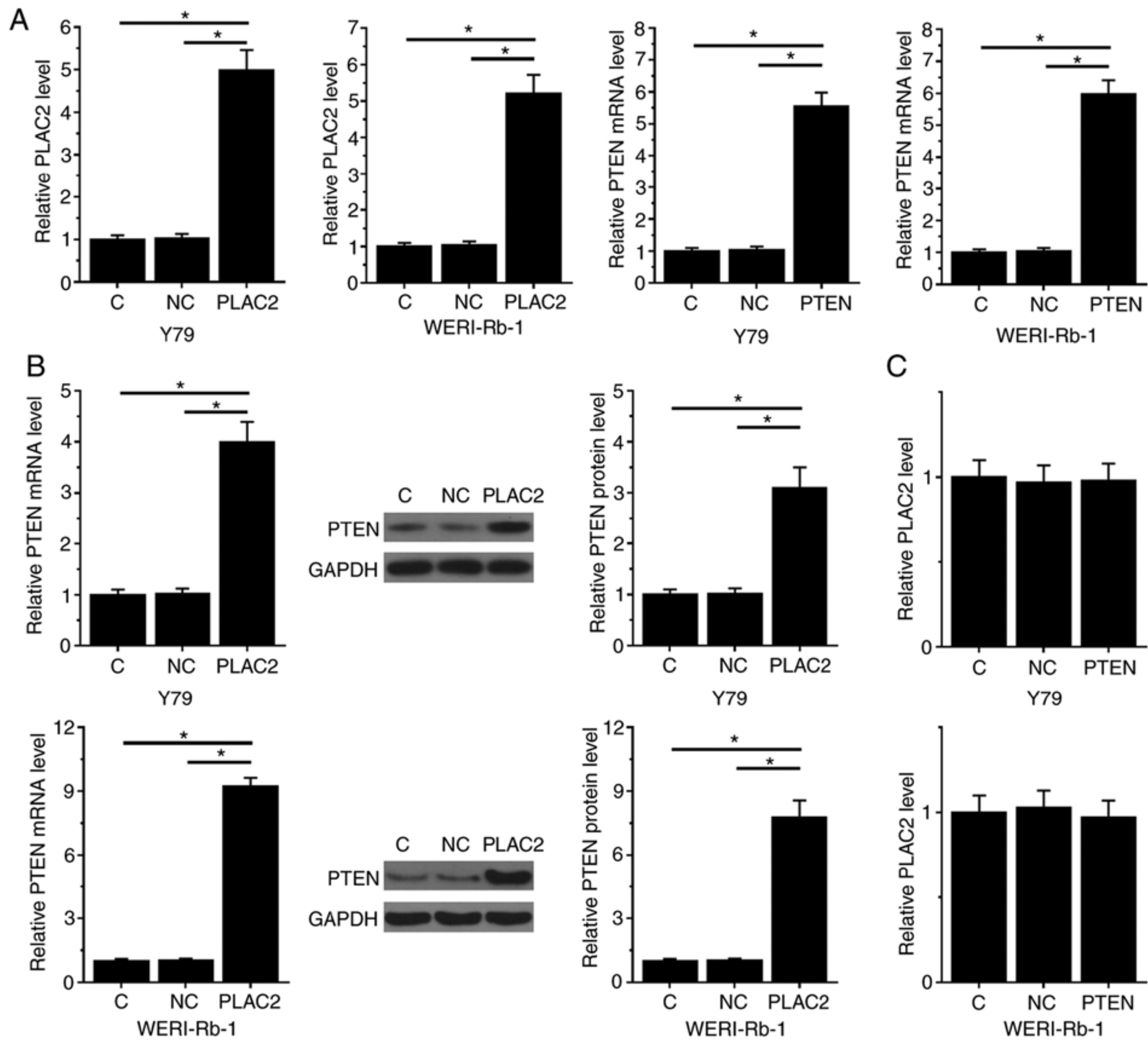


Figure 3. PLAC2 upregulates PTEN in Rb cells. (A) Compared with NC and C two control groups, expression levels of PTEN and PLAC2 were significantly increased in Y79 and WERI-Rb-1 cells at 24 h after the transfections of PTEN and PLAC2 expression vectors. (B) PLAC2 over-expression resulted in the upregulated PTEN ($P<0.05$), while (C) PLAC2 expression was not significantly affected by PTEN over-expression. $^{*}P<0.05$. Rb, retinoblastoma; PLAC2, placenta-specific 2; C, control, untransfected cell; NC, negative control, cells transfected with empty vector.

affected by PTEN over-expression in cells both Y79 and WERI-Rb-1 cell lines (Fig. 3C).

*PLAC2 promotes Rb cell apoptosis through PTEN.* Compared with NC and C the two control groups PLAC2 and PTEN over-expression caused an increased apoptotic rate of Rb cells. PTEN siRNA silencing led to a significantly decreased apoptotic rate and reduced effects of PLAC2 over-expression on cells of both Y79 (Fig. 4A) and WERI-Rb-1 (Fig. 4B) cell lines. ($P<0.05$). In addition, the cell apoptotic rates of Y79 and WERI-Rb-1 cells were consistent with the expression levels of PTEN mRNA in both Y79 (Fig. S1A) and WERI-Rb-1 (Fig. S1B) cells ($P<0.05$).

## Discussion

To the best of our knowledge, the functionality of PLAC2 has only been reported in glioma (10). The present study investigated the involvement of PLAC2 in Rb. It was found that PLAC2 was downregulated in Rb and may promote cancer cell apoptosis by downregulating PTEN.

PTEN signaling is a well-characterized tumor-suppressive pathway (12). The development and progression of cancer requires a complex network between tumor suppressors and oncogenes, and PTEN is the main player in the inhibitory network by serving as the brake of the PI3K-Akt cancer cell survival pathway (9). The activation of PTEN dephosphorylates PIP3, thereby inhibiting the activity of Akt (13). Therefore, activation of PTEN is a promising approach to induce cancer cell apoptosis, thereby inhibiting tumor growth and progression. Consistently, the present study also observed the downregulation of PTEN in Rb tissues. The present study also observed inhibited Rb cell apoptosis after PTEN silencing and promoted Rb cell apoptosis after PTEN over-expression. The current study further confirmed the tumor-suppressive role of PTEN in Rb.

More and more studies have shown that the expression of PTEN in cancer cells can be regulated by certain

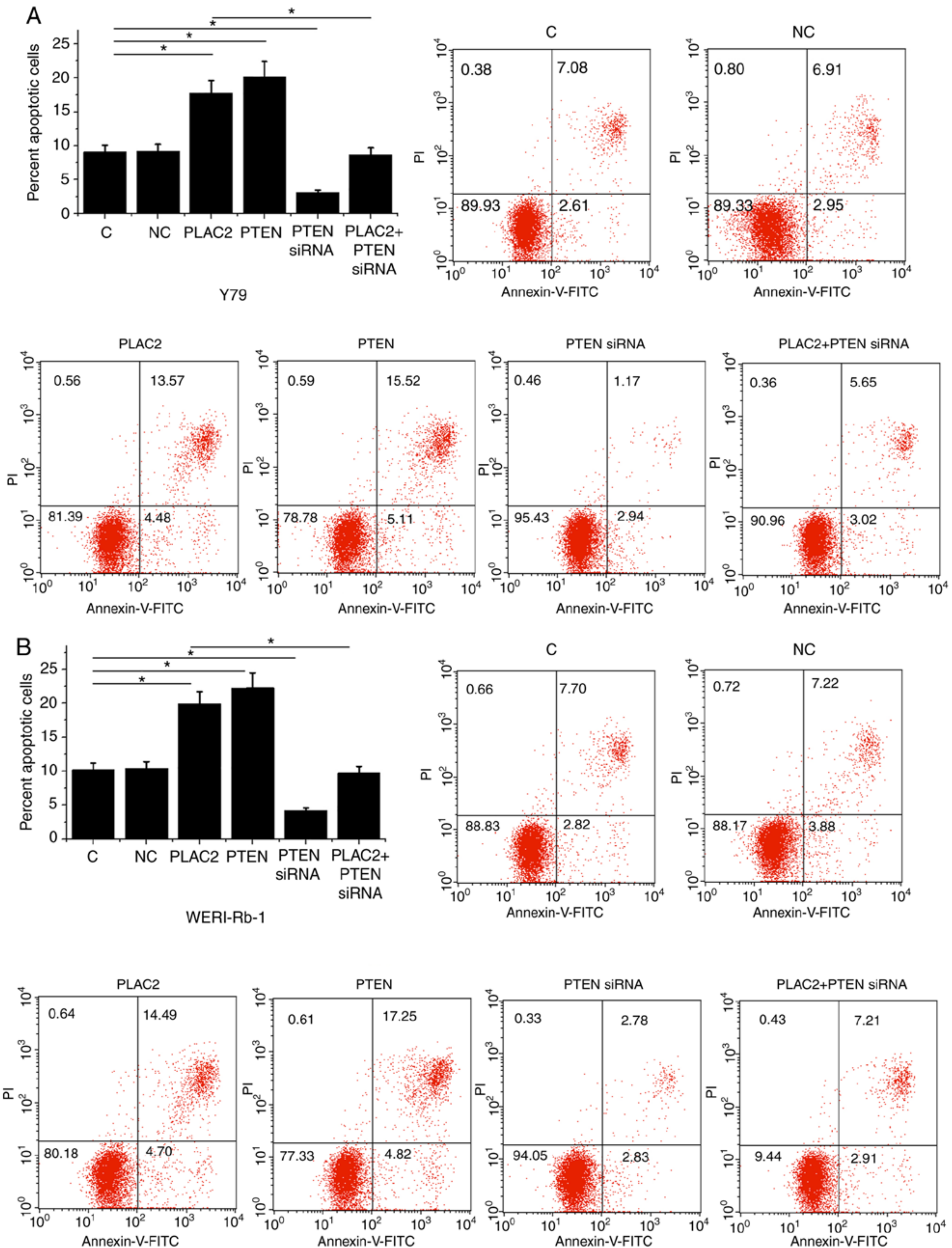


Figure 4. PLAC2 promotes Rb cell apoptosis through PTEN. Compared with NC and C the two control groups, PLAC2, and PTEN over-expression caused an increased apoptotic rate of Rb cells of both (A) Y79 and (B) WERI-Rb-1cell lines. PTEN siRNA silencing led to the decreased apoptotic rate and reduced effects of PLAC2 over-expression. *P<0.05. C, control, untransfected cell; NC, negative control, cells transfected with empty vector; Rb, retinoblastoma; PLAC2, placenta-specific 2; siRNA, small interfering; FITC, fluorescein isothiocyanate; PI, propidium iodide.

lncRNAs (14-16). Guo *et al* (14) reported that GAS5 plays a tumor-suppressive role in endometrial cancer by inducing the expression of PTEN. LncRNA FER1L4 can also upregulate PTEN endometrial cancer to suppresses cancer cell

proliferation and inhibit cell cycle progression (15). In another study, Li *et al* (16) reported that lncRNA UCA1 expression induced by hypoxia inducible factor-1α can inactivate PTEN signaling to accelerate cell proliferation. It has been reported that STAT1 can inhibit the expression of microRNA-18a in colorectal cancer by upregulating PTEN (11). It is also known that in glioma cells, PLAC2 upregulates STAT1 to inhibit cancer cell progression by inhibiting cell cycle progression (10). In the present study, PLAC2 was shown to be a likely upstream activator of PTEN in Rb. Therefore, it is possible that PLAC2 can indirectly upregulate PTEN through the upregulation of STAT1. However, the present study failed to include STAT1, which will be included in the authors' future studies to further verify the present hypothesis.

In conclusion, PLAC2 was downregulated in Rb and PLAC2 over-expression may induce the apoptosis of Rb cells by upregulating PTEN.

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**Availability of data and materials**

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

**Authors' contributions**

LS performed the experimental work, data analysis, clinical research and manuscript writing. YQ performed the data collection and some experimental work. ML performed the research design, literature research and manuscript review. All authors read and approved the final manuscript.

**Ethics approval and informed consent**

Ethical approval was obtained from the Ethics Committee of Shanghai Ninth People's Hospital. Informed consent was obtained from all individual participants included in the study.

**Patient consent for publication**

Not applicable.

**Competing interests**

The authors declare that they have no competing interests and all authors confirm their accuracy.

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