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# Low expression of lncRNA TUBA4B promotes proliferation and inhibits apoptosis of colorectal cancer cells *via* regulating P15 and P16 expressions

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**Abstract. – OBJECTIVE: To explore the expression, function, and regulation mechanism of the long non-coding ribonucleic acid (lncRNA) tubulin alpha 4b (TUBA4B) in colorectal cancer (CRC) tissues and cells.**

**PATIENTS AND METHODS: Cancer and adjacent tissues were collected from 60 CRC patients. CRC cell lines SW480, SW620, HCT116, Caco-2, DLD-1 and HT29, and colonic epithelial cell line CCD841 were also enrolled. Then, the expression of TUBA4B in CRC tissues and cell lines was detected *via* quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR). *In vitro* assays [cell counting kit-8 (CCK-8) assay, clone formation assay, and flow cytometry] were performed to study the biological function of TUBA4B in CRC. Additionally, the downstream regulatory targets of TUBA4B were investigated through Western blotting analysis and qRT-PCR assay.**

**RESULTS: The results of qRT-PCR revealed that compared with adjacent tissues, the expression of TUBA4B was down-regulated in 47/60 CRC tissues (47/60, 78.3%). According to *in vitro* assays (CCK-8 assay, clone formation assay, and flow cytometry), over-expression of TUBA4B inhibited the proliferation and promoted the apoptosis of CRC cells. TUBA4B remarkably regulated mRNA and protein levels of p15 and p16.**

**CONCLUSIONS: LncRNA TUBA4B is down-regulated expression in CRC tissues and cells, which facilitates CRC cell proliferation and suppresses apoptosis by regulating the expressions of p15 and p16.**

*Key Words:*
LncRNA TUBA4B, CRC, P15, P16, Biological function.

## Introduction

Colorectal cancer (CRC), one of the most common malignancies, ranks third in the most prevalent tumors among males and females[1]. The early diagnosis and standard treatment of CRC greatly prolong the survival time and improve life quality. Searching for biomarkers with high sensitivity and specificity for CRC provides new technical support for the radiotherapy, chemotherapy, and targeted therapy of tumors[2].

Long noncoding ribonucleic acids (lncRNAs), new aspects in the research field of tumor pathogenesis in recent years[3], are products of non-coding genes and widely involved in the physiological and pathological processes of cells. LncRNAs are able to regulate cell cycle, apoptosis, and signaling pathways, which are dysregulated in many malignant tumors like lung cancer, breast cancer, and gastric cancer[4-6]. It is reported that the abnormal expression of lncRNAs is closely correlated with the development and progression of CRC. Ye et al[7] pointed out that colon cancer associated transcript 1 (CCAT1) is highly expressed in colorectal adenocarcinoma and CRC tissues. Its expression level is associated with clinical tumor stage, lymph node metastasis, and CA199 level in CRC patients. They demonstrated that c-MYC directly binds to its promoter region to promote the transcription of CCAT1. Yin et al[8] found that maternally expressed gene 3 (MEG3) is lowly expressed in tumor tissues and cells ($p<0.01$). Overexpression of MEG3 influences the development and metastasis of CRC by regulating levels of matrix metalloproteinase-2 (MMP2) and

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MMP9. However, the expression and function of the lncRNA tubulin alpha 4b (TUBA4B) in the colorectum have not been reported.

LncRNA TUBA4B is down-regulated in many tumors and exerts anti-tumor functions. Guo et al[9] discovered that in gastric cancer, lowly expressed TUBA4B adsorbs micro RNA-124 (miR-214) and miR-216a/b to directly regulate PTEN, thereby repressing the development and progression of gastric cancer. In non-small cell lung cancer, the expression of TUBA4B is down-regulated, and its low level predicts a poor prognosis[10]. This study found that TUBA4B was lowly expressed in CRC tissues and cells. Overexpression of TUBA4B suppressed the proliferation and promoted the apoptosis of CRC cells, indicating that TUBA4B can act as a potential therapeutic target for CRC.

## Patients and Methods

### Tissue Specimens

Tissue samples were taken from 60 CRC patients treated in the General Surgery Department of The First People's Hospital of Yueyang from January 2015 to January 2018. They were diagnosed as colorectal adenocarcinoma by the Pathology Department of the Hospital. Resected tissues were immediately frozen in liquid nitrogen. None of the patients received chemotherapy, radiotherapy, targeted therapy, or immunotherapy. Subjects were informed signed the informed consent before surgery. The study protocol was approved by the Ethics Committee of the Hospital.

### Cell Culture

CRC cell lines SW480, SW620, HCT116, Caco-2, LD-1, and HT29, and colonic epithelial cell line CCD841 were provided by American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) or Dulbecco's Modified Eagle's Medium (DMEM; Gibco, Rockville, MD, USA) containing 10% fetal bovine serum (Gibco; Rockville, MD, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% $CO_2$ incubator at 37°C. Cell passage was conducted at regular intervals.

### Extraction and Reverse Transcription of RNAs

Total RNAs were extracted from CRC tissues and cells using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). Then, the purity and concentration of RNAs were determined. Qualified RNAs were reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using a reverse transcription kit (Thermo Fisher Scientific; Waltham, MA, USA) under the following reaction conditions: at 40°C for 60 min, at 25°C for 5 min, and at 75°C for 5 min.

### Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

A 20 μL system of PCR mixture was prepared in accordance with the instructions of SYBR PCR Master Mix (Thermo Fisher Scientific; Waltham, MA, USA) for PCR amplification. The reaction conditions were pre-denaturation at 95°C for 10 min, followed by 30 cycles of denaturation at 95°C for 15 s, annealing at 58°C for 30 s, and extension at 72°C for 30 s, and then extension at 72°C for 7 min. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal reference. Relative expression level was calculated by $2^{-\Delta\Delta Ct}$. QRT-PCR was repeated three times. Primer sequences were as follows: P15: F 5′-CAGTGGGGAACTCTGACTCG-3′, R 5′-GTGCCTGGTGCTCTCTTACC-3′; P16: R 5'-UUCUCCGAACGUGUCACGUTT-3', F: 5'-GCCAGAAUGUUCCUAUUUATT-3'; GAPDH: F 5′-ACAGTCAGCCGCATCTTCTT-3′; R: 5'-TGCTACTGCCTGCAAAGACT-3; TUBA4B: F 5'-GCTTCTCACACTCTGGGTCTTA-3', R 5'-CCTCATTCTCTGCCTCTTCTAC-3'. LncRNA TUBA4B overexpression plasmids were synthesized by Shanghai Genechem Co., Ltd. (Shanghai, China).

### Cell Counting Kit-8 (CCK-8) Assay

At 24 h before the assay, the well-grown CRC cells were inoculated into a 96-well plate at a density of $3\times10^3$ cells/well. 10 μL of CCK-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added at 0 h, 24 h, 48 h, 72 h, and 96 h, respectively, followed by incubation at 37°C for 2 h. Lastly, the optical density (OD) at 450 nm was measured using a microplate reader. The assay was repeated three times.

### Clone Formation Assay

The cells in the logarithmic growth phase were collected from experimental group and control group, re-suspended in 1× phosphate-buffered saline (PBS) and seeded in a 6-well plate at a density of $8\times10^2$ cells/well. After 14-day cell

culture, visible colonies were fixed with 0.5 mL of methanol for 30 min and stained with 1% crystal violet solution for 15 min. Next, an optical microscope (×100) was utilized to count the number of colonies. The assay was repeated three times.

### Determination of Cell Apoptosis

The cells transfected for 48 h were collected, digested with ethylenediaminetetraacetic acid (EDTA)-free trypsin, re-suspended in 500 μL of pre-cooled phosphate-buffered saline (PBS), and transferred to a flow tube. Next, 5 μL of Annexin V fluorescein isothiocyanate (FITC)-specific antibody and 5 μL of propidium iodide (PI) were added, followed by incubation in a dark place for 15-20 min. Lastly, the apoptosis was detected using a flow cytometer (FACSCalibur; BD Biosciences, Detroit, MI, USA). The experiment was repeated three times.

### Determination of Cell Cycle

After 48 h of transfection, the cells were digested and collected into a 5 mL centrifuge tube. Then, the cell pellet was washed once with pre-cooled PBS at 4°C, and fixed with 70% alcohol for at least 1 h. Next, the pellet was washed with PBS once again, incubated with PI staining solution, and fully re-suspended. After that, the distribution of cell cycle in each group was detected using the flow cytometer.

### Western Blotting

Total proteins were extracted with radioimmunoprecipitation assay (RIPA; Beyotime, Shanghai, China), and protein concentration was detected using a bicinchoninic acid (BCA) kit (Pierce, Rockford, IL, USA). Then, the proteins were separated with 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA) by wet transfer method. After blockage with 5% skim milk powder at room temperature for 2 h, membranes were incubated with primary antibodies (p15 and p16: 1:2 000, GAPDH: 1:1 000) (CST; Danvers, MA, USA) at 4°C overnight. Next day, membranes were washed with tris-buffered saline with Tween®20 (TBST) for 3 times, and incubated with corresponding secondary antibody (diluted at 1:500) at room temperature on a shaker for 90 min. After washing with Tris-Buffered Saline and Tween-20 (TBST) for 3 times, enhanced chemiluminescence (ECL) solution was added, and the resulting product was loaded in a gel imaging system to collect data, with GAPDH as the internal reference.

### Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 software (SPSS Inc., Chicago, IL, USA) was employed to process all data. Measurement data were expressed as ($\chi \pm s$). The *t*-test was applied for comparison between groups. One-way analysis of variance (ANOVA) was conducted for comparison among groups, followed by Post-Hoc Test (Least Significant Difference). $p<0.05$ or $p<0.01$ suggested that the difference was statistically significant.

## Results

### Expression of TUBA4B Declined in CRC Tissues and Cells

Total RNAs were extracted from cancer and adjacent tissues of 60 CRC patients. The expression level of TUBA4B was measured by qRT-PCR, and the results revealed that 47/60 cases of CRC tissues displayed down-regulatedTUBA4B compared with adjacent tissues (Figure 1A). Next, qRT-PCR assay was carried out to detect the relative expression level of TUBA4B in CRC cells. It was found that the TUBA4B expression was down-regulated in CRC cells as well (Figure 1B). To investigate the biological function of TUBA4B in CRC cells, TUBA4B overexpression plasmids were constructed in this study and transiently transfected into CRC cells for 48 h. Transfection efficacy was detected (Figure 1C and 1D).

### TUBA4B Inhibited Proliferation of CRC Cells

CCK-8 assay was performed to explore the effect of overexpressed TUBA4B on the proliferation of CRC cells, and the results uncovered that overexpression of TUBA4B suppressed the proliferation of CRC cells compared with control group (Figure 2A and 2B). Colony formation assay yielded similar results to those of CCK-8 assay (Figure 2C and 2D). Lastly, the effect of TUBA4B on CRC cell cycle was determined *via* flow cytometry, and it was discovered that after overexpression of TUBA4B arrested CRC cells in G1 phase (Figure 2 E and 2F).

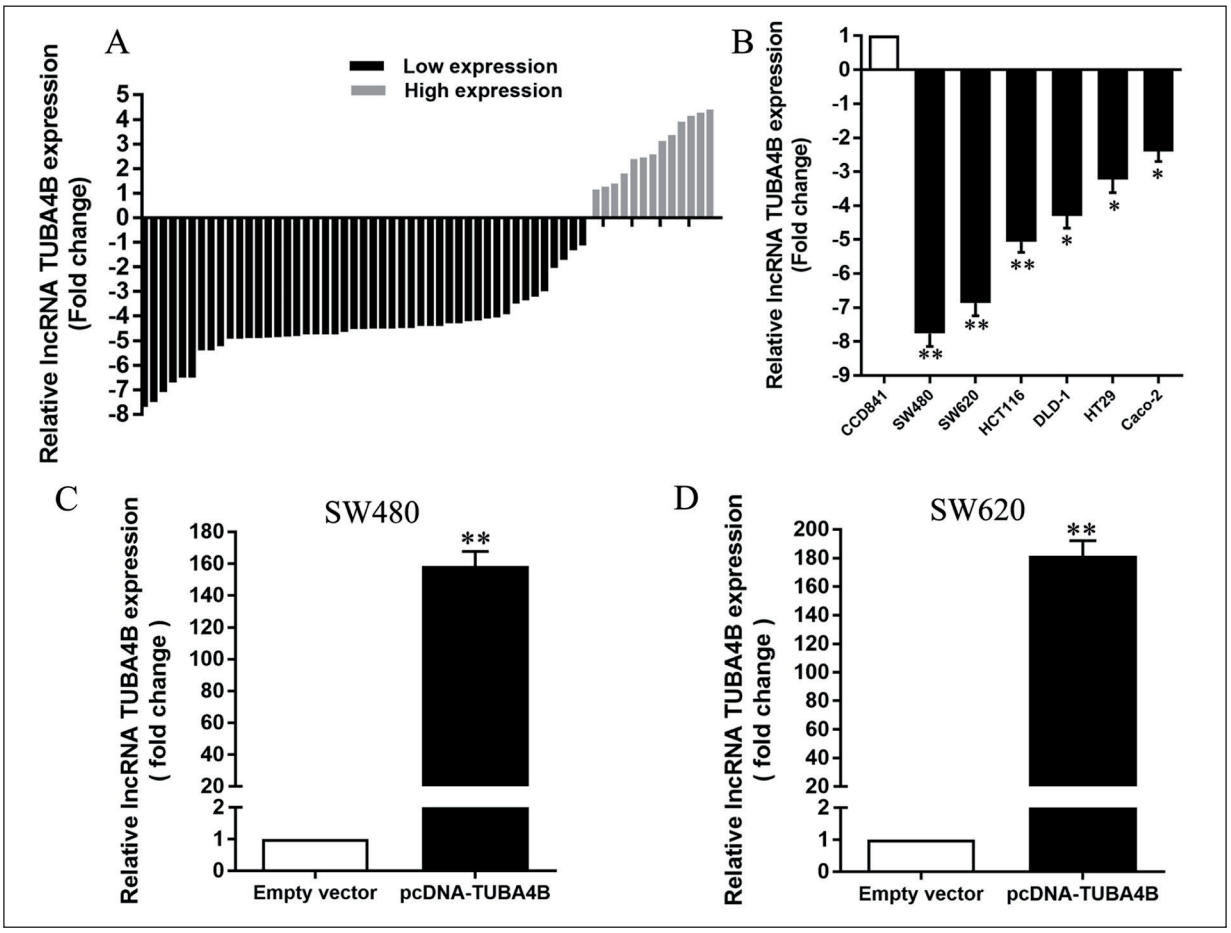


**Figure 1.** Expression of lncRNA TUBA4B reduces in CRC. **A,** Expression level of TUBA4B in 60 cases of CRC tissues measured through qRT-PCR assay. The results reveal that the expression of TUBA4B declines in 47 specimens, with GAPDH as the internal reference. **B,** Relative expression level of TUBA4B in CRC cells determined *via* qRT-PCR assay. It is found that its expression reduces. **C, D,** Overexpression efficiency detected *via* qRT-PCR assay.

### *TUBA4B Regulated the Expressions of p15 and p16 to Repress the Proliferation of CRC Cells*

Flow cytometry was adopted to detect the regulatory effect of TUBA4B on CRC apoptosis. The results revealed that overexpression of TUBA4B promoted apoptosis of CRC (Figure 3A and 3B). P15 and p16, known as cyclin-dependent protein kinase inhibitors (CDKIs), are tumor-suppressor genes that directly bind to and inhibit Cyclin/CDK compounds[11]. After overexpressing TUBA4B in CRC cells, the mRNA expressions of p15 and p16 were determined through qRT-PCR. It was demonstrated that the expressions of p15 and p16 were upregulated (Figure 3C). Next, the Western blotting analysis revealed that protein levels of p15 and p16 were up-regulated after overexpression of TUBA4B (Figure 3D).

## Discussion

CRC is one of the most prevalent tumors with a relatively high mortality rate around the world, and malignant proliferation of tumors is the major cause of death from CRC[12]. Recently, there are great advances in the treatment methods for CRC[13,14], including targeted therapy and immunotherapy. However, the prognosis of CRC patients is unsatisfactory. Moreover, the malignant proliferation of tumors is an extremely complex process, in which the key event is the accumulation of multiple genetic and epigenetic changes, leading to the activation or inactivation of different genes[15]. Given this, it is urgent to investigate the molecular mechanism of the progression of CRC.

LncRNAs are abundantly expressed in different diseases and involved in regulating the

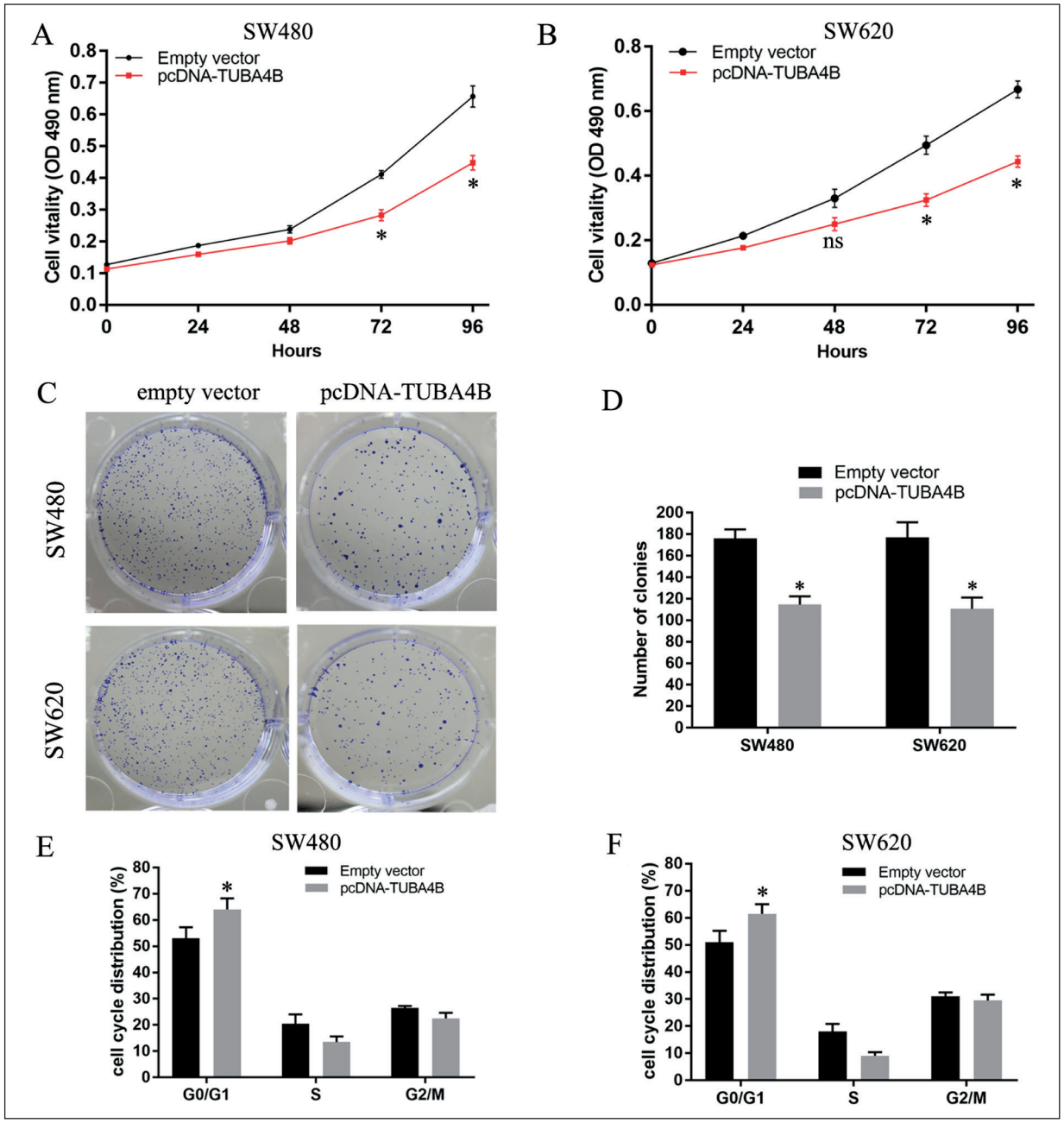


**Figure 2.** LncRNA TUBA4B inhibits cell proliferation. **A, B,** Effect of overexpression of TUBA4B on the proliferation of CRC cells detected by CCK-8 assay. **C, D,** Effect of overexpression of TUBA4B on the proliferation of CRC cells detected *via* clone formation assay (×100). **E, F,** Distribution of CRC cell cycle after overexpressing TUBA4B determined through flow cytometry.

development and progression of tumors, including biological processes like cell proliferation, differentiation, and chromosome inactivation[16]. They can be divided into transcripts, antisense oligonucleotides, circular RNAs, long intergenic ncRNAs, and pseudogenes based on the structure and function, which play different roles in regulating various biological processes[17,18]. LncRNA TUBA4B is lowly expressed in ovarian cancer[19] and other tumors, and plays an anti-tumor role. In our study, we discovered for the first time that TUBA4B was down-regulated in CRC, and overexpression of TUBA4B repressed cell proliferation and facilitated apoptosis.

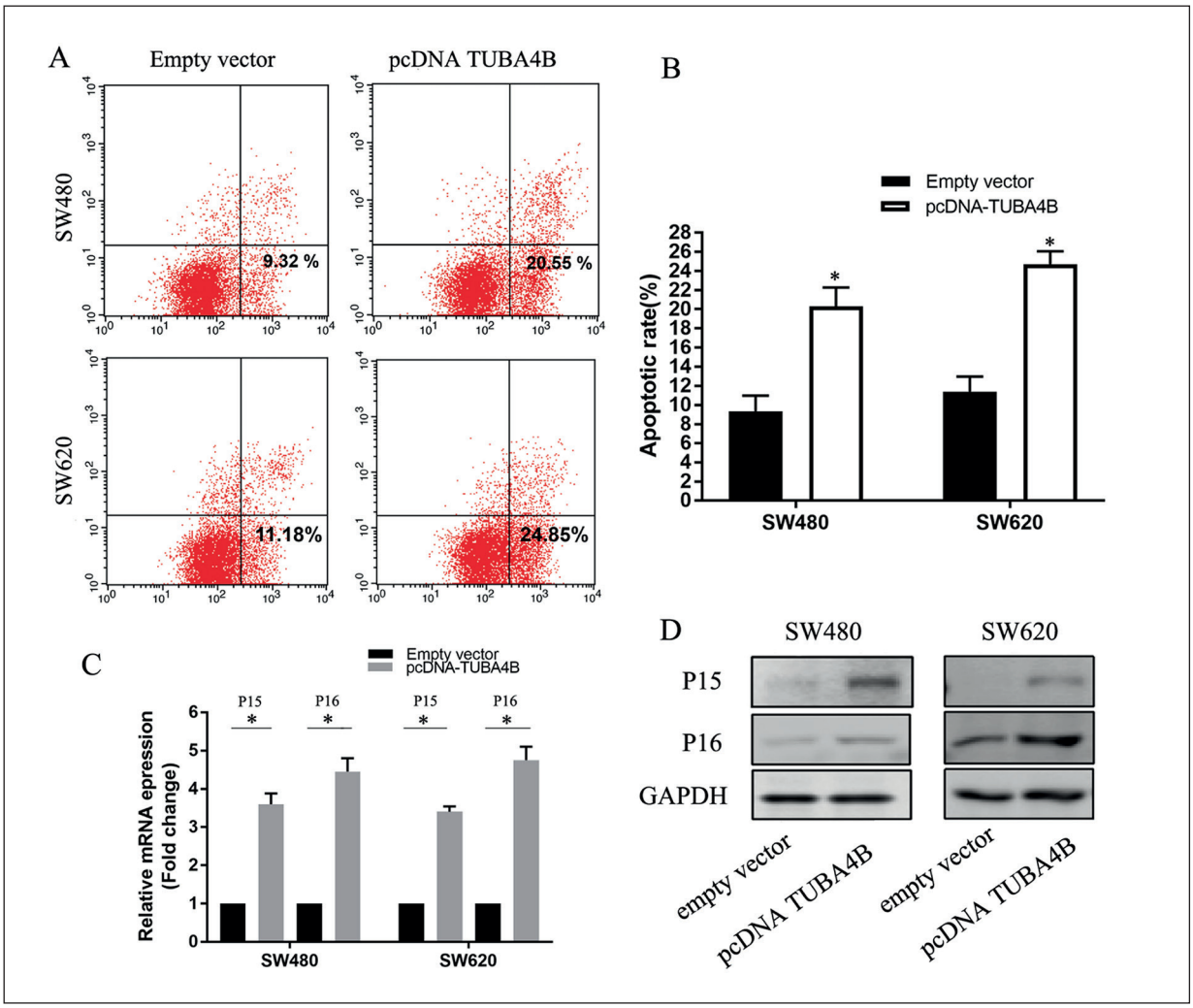


**Figure 3.** TUBA4B regulates expressions of p15 and p16 to repress proliferation of CRC cells. **A B,** Changes in CRC apoptosis after overexpressing TUBA4B detected *via* flow cytometry. **C,** Changes in mRNA expression levels of p15 and p16 after overexpressing TUBA4B determined through qRT-PCR assay. **D,** Changes in protein expression levels of p15 and p16 after overexpressing TUBA4B determined through Western blotting analysis.

P15 and p16, also known as multi-tumor suppressor genes (MTS1 and MTS2), located on human chromosome 9, are negative regulators of the cell cycle. They prevent cells from entering the S phase, thus influencing the development and progression of tumors[20]. It is stated that lncRNAs serve as vital regulators to modulate the expressions of p15 and p16. In gastric cancer, highly expressed lncRNA PVT1 recruits PRC2 complexes to epigenetically suppress the expressions of p15 and p16, thus promoting the development and progression of gastric cancer[21]. In our study, we further confirmed that TUBA4B regulated the expressions of p15 and p16, but the specific molecular mechanism was not further explored.

## Conclusions

This study demonstrated that TUBA4B has a close correlation with the development and progression of CRC, which regulates cell proliferation and apoptosis by modulating the expressions of p15 and p16. It implies that TUBA4B can serve as a potential target for the diagnosis and treatment of CRC.

### Conflict of Interest

The Authors declare that they have no conflict of interests.

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