

## Original Research Article

# Baicalein inhibits cell development in papillary thyroid cancer by regulating miR-206/RAP1B pathway

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### Abstract

**Purpose:** To investigate the therapeutic effect of baicalein on papillary thyroid cancer (PTC) cells *in vitro* and its underlying molecular mechanism.

**Methods:** The human PTC cell line TPC-1 was divided into five groups and treated with distilled water or baicalein at 10, 20, 50, or 100  $\mu$ M. Next, miR-206, miR-206 inhibitor, the respective negative controls of miR-206 and miR-206 inhibitor, RAP1B small interfering RNA (siRNA), and control vector siRNA were synthesized and transfected into TPC-1 cells. Cell viability, migration, and invasion were measured using the 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) and Transwell assays. miR-206 expression and Ras-related protein (RAP1B) levels were assessed by quantitative real-time reverse transcription-polymerase chain reaction and western blotting, respectively.

**Results:** Baicalein inhibited TPC-1 cell viability, migration and invasion, upregulated miR-206 expression, and reduced the RAP1B level in a concentration-dependent manner ( $p < 0.01$ ). miR-206 negatively regulated RAP1B expression and increased the baicalein-induced reduction of RAP1B expression. Moreover, RAP1B overexpression relieved the suppression of cell viability, migration, and invasion caused by baicalein ( $p < 0.01$ ).

**Conclusion:** Baicalein suppresses cell growth in PTC cells by regulating the miR-206/RAP1B pathway, providing a new therapeutic strategy for PTC treatment.

**Keywords:** Baicalein, Papillary thyroid cancer (PTC), miR-206, RAP1B, Cell viability, Cell invasion

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## INTRODUCTION

Thyroid cancer is a malignant tumor that originates in the follicular epithelium of the thyroid, and it usually presents as a painless neck mass or nodule [1]. Based on differences in tumor origin and differentiation, thyroid cancer can be classified as papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary

thyroid carcinoma (MTC), and anaplastic thyroid carcinoma (ATC) [2]. PTC is the most common type of thyroid cancer with slow growth, low malignancy, and a good prognosis [3]. PTC is mainly treated by surgery, supplemented with endocrine therapy and molecular-targeted therapy. However, the treatment time of PTC is longer, and the drugs taken by patients have more side effects, including hyperthyroidism [3].

Therefore, identifying a natural drug substitute for PTC is very important.

Baicalein (also known as 5, 6, 7-trihydroxyflavone) is a flavonoid compound extracted from the dried roots of *Scutellaria baicalensis* [4]. Baicalein has multiple pharmacological effects, such as antibacterial, anti-inflammatory, anti-infection, and anti-oxidation effects [5,6]. In addition, baicalein has anti-cancer effects. For example, baicalein inhibits the growth, metastasis, and invasion of prostate cancer and gastric cancer cells [6,7]. Baicalein also regulates the mitogen-activated protein kinase (MAPK) pathway to induce apoptosis in ATC cells [8]. Therefore, baicalein may be an ideal therapeutic compound for thyroid cancer.

MicroRNAs (miRNAs) are closely related to tumor development [9]. Studies have found that multiple miRNAs are linked to the occurrence, development, and prognosis of PTC. For example, Chou *et al* reported that the expression levels of miR-146b, miR-221, and miR-222 are correlated with extra-glandular invasion and tumor stage of PTC [10].

The earliest study on miR-206 is related to breast cancer [11]. Since then, miR-206 has been gradually found to affect apoptosis and metastasis in various tumor cells. For example, miR-206 promotes apoptosis in cervical cancer cells by targeting the NOTCH3 signaling pathway [11]. Additionally, miR-206 inhibits thyroid cancer proliferation and invasion by targeting Ras-related protein (RAP1B) [12].

This study systematically investigated the therapeutic effect of baicalein on the PTC cell line TPC-1 and its molecular mechanism *in vitro* and provides a new strategy for PTC treatment.

## EXPERIMENTAL

### Cell culture and treatment

The human PTC cell line TPC-1 was cultured in Roswell Park Memorial Institute (RPMI)-1640 medium (Procell, Wuhan, China) with 10% fetal bovine serum (FBS) and 1 % penicillin-streptomycin solution (Procell) in an incubator with 95 % air and 5 % CO<sub>2</sub> at 37 °C.

The cultured cells were divided into five groups. The cells in one group were treated with distilled water (control or 0 μM baicalein group), and the cells in the other four groups were treated with 10, 20, 50, or 100 μM baicalein (Abmol Bioscience Inc, Houston, TX, USA) for 72 h.

### Cell viability assay

TPC-1 cells in the 50-μM baicalein group were seeded into 96-well plates at a density of  $2 \times 10^3$  cells/well and were cultured for 3 days. Next, 20 μL of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT, 5 mg/mL in phosphate-buffered saline solution) purchased from Beyotime (Shanghai, China) was added directly to each well. The cells were incubated for 2 h at 37 °C and then were added to 5 mL of acidified isopropanol (Sigma-Aldrich, St. Louis, MO, USA). The absorbance of the cell suspension at 570 nm (OD<sub>570</sub>) was measured using a spectrophotometer (Lab-Spectrum Instruments Co. Ltd, Shanghai, China).

### Cell migration and invasion assays

The TPC-1 cells treated with 50 μM baicalein were digested with 0.25% trypsin. The upper membrane of the Transwell chamber was incubated in Matrigel solution (BD Biosciences, San Jose, CA, USA) for 30 min at 37°C and then was co-incubated with digested TPC-1 cells for 24 h at 37°C. At the same time, FBS was added to the lower membrane of the Transwell chamber. The incubated cells were stained with 0.1% hematoxylin (Sigma-Aldrich) for 30 min. The numbers of migrating and invading cells were counted using a microscope (Leica, Tokyo, Japan) and photographed using a digital camera (Canon, Tokyo, Japan).

### Cell transfection

miR-206, miR-206 inhibitor, the negative controls of miR-206 and miR-206 inhibitor (miR-NC and NC inhibitor, respectively), RAP1B small interfering RNA (siRNA) or control vector siRNA were synthesized by Sangon Biotech Co. Ltd (Shanghai, China). According to the manufacturer's protocol, the cells were transfected using Lipofectamine 3000 (Thermo Fisher Scientific, Waltham, MA, USA) for 24 h at 37 °C.

### Quantitative real-time reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA from TPC-1 cells was extracted using the Trizol Extraction Kit (Sigma-Aldrich). The RNA quantity and quality were detected using the NanoDrop 2000c system (Thermo Fisher Scientific). The RNA was reverse transcribed to cDNA using reverse transcriptase. The cycling conditions were as follows: 95°C for 1 min, followed by 40 cycles of amplification (94 °C for 10 s, 55 °C for 40 s, and 72 °C for 20 s), and 72 °C for 8 min. The fold changes were calculated

using the relative quantification ( $2^{-\Delta\Delta Ct}$ ) method. The sequences of the PCR primers used are shown in Table 1.

**Table 1:** Primers and sequences

Primer		Sequence (5'-3')
miR-206	Forward	UGGAAUGUAAGGAAGUGUGU
	Reward	ACACACUCCUACAUUCCAUU
miR-206 inhibitor	Forward	CCACACACUCCUACAUUCC
	Reward	CAGUACUUUUGUGUAGUACAA
RAP1B	Forward	CGTGGCGCCTAGAGTAG
	Reward	AGTACCTCTCAGGCTGTCACC

### Western blotting

The total protein of TPC-1 cells was extracted by incubating in RIPA lysis buffer containing the protease inhibitor phenylmethylsulfonyl fluoride (PMSF; Beyotime) for 30 min on ice and sampled onto configured gel. Next, the protein extracts were mixed with sodium dodecyl sulfate (SDS) solution (Beyotime), separated by 10 % SDS-PAGE and transferred onto polyvinylidene fluoride (PVDF) membranes soaked in methanol. The membranes were blocked in 5 % milk for 1 h at room temperature, incubated with RAP1B primary antibody (ab154756; 1:1,000; Abcam, Cambridge, UK) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; ab9484; 1:1,000; Abcam) at 4°C overnight, and then incubated with secondary antibody (anti-mouse IgG antibody; ab6728; 1:2,000) for 1 h at room temperature. The immunoblots were visualized and analyzed using ImageJ software (NIH Image, Bethesda, MD, USA).

### Statistical analysis

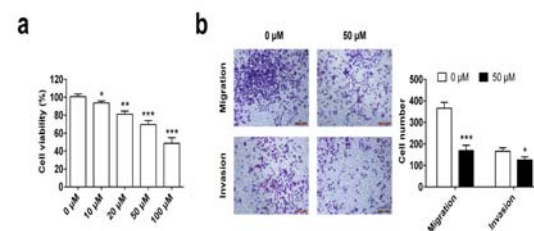
All the experimental data were compared using Student's *t*-test. The results were statistically analyzed using GraphPad Prism v8 software (Graphpad, San Diego, CA, USA) and were represented as means  $\pm$  standard deviation (SD).  $P < 0.05$  indicated statistical significance.

## RESULTS

### Baicalein inhibits TPC-1 cell viability, migration, and invasion

Cell growth was measured to study the therapeutic effect of baicalein on PTC *in vitro*. The MTT assay showed that cell viability was reduced by baicalein in a concentration-

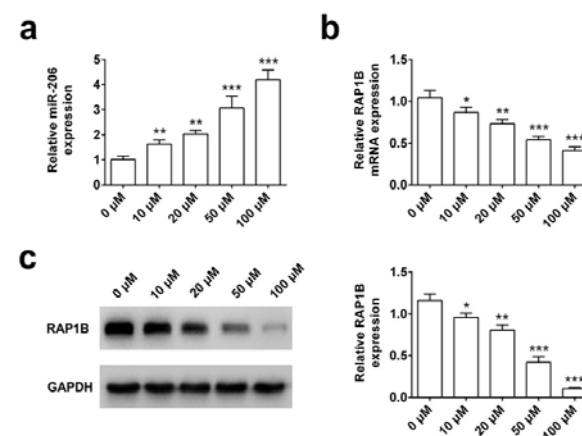
dependent manner in TPC-1 cells (Figure 1 A;  $p < 0.001$ ). The Transwell assay showed that the number of migrating or invading cells in the 50- $\mu$ M baicalein group was less than that in the 0  $\mu$ M baicalein group (Figure 1 B;  $p < 0.001$ ). These data explained that baicalein could suppress PTC cell growth *in vitro*.



**Figure 1:** Baicalein inhibits TPC-1 cell viability, migration and invasion. TPC-1 cells were treated with 0, 10, 20, 50 and 100  $\mu$ M baicalein. (A) Cell viability, (B) migration and invasion were detected using the MTT and Transwell assays. \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

### Baicalein upregulates RAP1B expression by regulating miR-206 expression

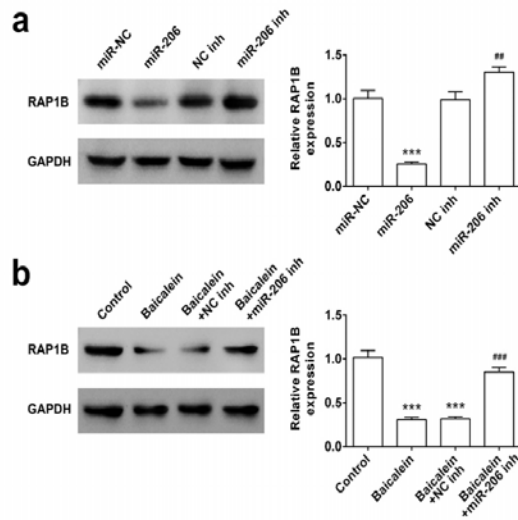
qRT-PCR showed that the miR-206 expression level was elevated by baicalein in a concentration-dependent manner in TPC-1 cells (Figure 2 A;  $p < 0.01$ ). By contrast, qRT-PCR and western blotting results both showed that RAP1B expression was increased when cells were treated with 10, 20, 50, or 100  $\mu$ M baicalein (Figure 2 B and C, respectively;  $p < 0.01$ ). The data suggest that baicalein upregulates miR-206 expression and reduces the RAP1B level.



**Figure 2:** Baicalein upregulates miR-206 expression and reduces RAP1B levels. TPC-1 cells were treated with 10, 20, 50, or 100  $\mu$ M baicalein. The expression levels of (A) miR-206 and (B-C) RAP1B were detected by qRT-PCR and western blotting, respectively; \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$

### Effect of baicalein on RAP1B level

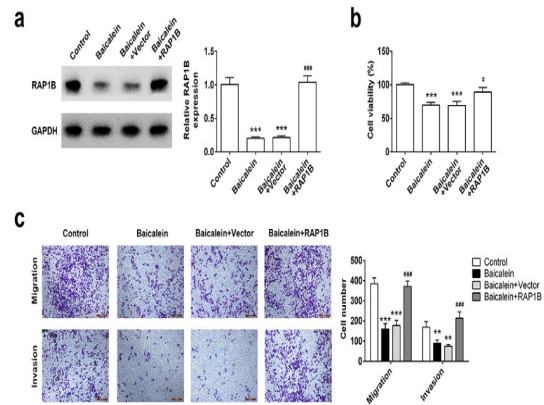
The results in Figure 3 A show that miR-206 overexpression downregulates RAP1B expression, and miR-206 knockdown upregulates RAP1B expression ( $p < 0.01$ ). Moreover, the western blotting data in Figure 3 B showed that RAP1B expression is upregulated in the baicalein + miR-206 inhibitor group compared with that in the baicalein + NC inhibitor group ( $p < 0.01$ ). These results suggest that baicalein can regulate miR-206 expression to increase the RAP1B expression level in TPC-1 cells.



**Figure 3:** Baicalein reduces RAP1B level by regulating miR-206 expression. TPC-1 cells were treated with 50  $\mu$ M baicalein and transfected with miR-206, miR-NC, miR-206 inhibitor, and NC inhibitor. The expression of RAP1B was detected by western blotting. \*\*\* $p < 0.001$ ; ### $p < 0.001$

### Baicalein inhibits cell viability, migration, and invasion by reducing the RAP1B level

RAP1B was transfected into TPC-1 cells to assess the effect of RAP1B on PTC. RAP1B was successfully transfected into TPC-1 cells treated with baicalein (Figure 4 A;  $p < 0.01$ ). Additionally, the MTT assay showed that the cell viability in the baicalein + RAP1B group was more than that in the baicalein + vector group (Figure 4 B;  $p < 0.01$ ). Similarly, the Transwell assay showed that the inhibition of cell migration or invasion was alleviated in TPC-1 cells co-treated with baicalein and RAP1B (Figure 4 C;  $p < 0.01$ ). The data demonstrated that baicalein affects PTC cell viability, migration, and invasion by regulating RAP1B expression.



**Figure 4:** Baicalein inhibits cell viability, migration, and invasion by reducing the RAP1B level. TPC-1 cells were treated with 50  $\mu$ M baicalein and transfected with RAP1B siRNA and control vector siRNA. (A) The expression RAP1B level was measured by western blotting. (B) Cell viability, (C) migration, and invasion were measured using the MTT and Transwell assays; \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ; # $p < 0.05$ , ### $p < 0.001$

## DISCUSSION

Thyroid cancer is a common endocrine tumor in the human head and neck. The incidence rate of thyroid cancer is increasing rapidly worldwide, with the largest increase in the number of patients diagnosed with PTC [2]. Therefore, presently, more studies are emerging concerning the therapeutic methods, natural medicines, and molecular mechanisms of PTC.

The natural compound baicalein has demonstrated anti-cancer effects [13]. Baicalein inhibits breast cancer cell migration, adhesion, and invasion and induces liver cancer cell apoptosis and autophagy [14,15]. Moreover, baicalein inhibits cell growth in undifferentiated thyroid cancer through the inhibition of ERK and PI3K/AKT pathways and promotion of apoptosis and autophagy [16]. In this study, baicalein inhibited TPC-1 cell growth, indicating that baicalein has an inhibitory effect on PTC.

In recent years, miRNAs have played an important role in tumor differentiation, growth, and apoptosis [9]. miR-206 was found to be abundantly expressed in skeletal muscle, leading researchers to believe for the first time that miR-206 is related to cell differentiation [17]. Subsequent studies have shown that miR-206, as a tumor suppressor molecule, is poorly expressed in breast cancer, laryngeal cancer, thyroid cancer, and gastric cancer [12,17-19]. Furthermore, baicalein was found to inhibit cell invasion in osteosarcoma via the miR-183/Ezrin pathway [20]. Hence, this study showed that the

mechanism of PTC inhibition by baicalein is related to miRNA and its target genes. Baicalein increased miR-206 expression in a concentration-dependent manner, demonstrating that miR-206 is involved in baicalein inhibition of PTC.

RAP1 is a small Ras-like GTPase (SRG), and RAP1B is a subtype of RAP1. The absence of RAP1B leads to impaired angiogenesis, migration of endothelial cells, and the blockade of the MAPK signaling pathway [21]. RAP1B was reported to be upregulated in colorectal cancer tissues and exerts oncogene functions by promoting cell proliferation and metastasis [22]. Similarly, baicalein inhibited RAP1B expression in a concentration-dependent manner, revealing that baicalein has an inhibitory effect on PTC. Additionally, the higher is the concentration of baicalein, the better is the effect. Zhang *et al* reported that RAP1B is the downstream target gene of miR-518b with a tumor suppressor function [23].

Moreover, a previous study reported a targeting relationship between miR-206 and RAP1B and miR-206 plays a role in inhibiting thyroid cancer by targeting RAP1B [12]. This study confirmed that miR-206 negatively regulates the RAP1B level in PTC cells. Moreover, the baicalein-induced decrease in the RAP1B level was alleviated by miR-206 inhibitor, indicating that miR-206 and RAP1B both play vital roles in the tumor suppressor mechanism of baicalein. We also demonstrated that RAP1B overexpression relieves baicalein-induced inhibition of cell viability, migration, and invasion, indicating that baicalein inhibits PTC cell growth by reducing the RAP1B level.

## CONCLUSION

This study provides evidence that baicalein inhibits cell viability, migration, and invasion via upregulation of miR-206 expression and reduction of RAP1B level in PTC, indicating that baicalein inhibits PTC cell growth by regulating miR-206/RAP1B pathway. This study provides a new drug and molecular target for PTC treatment. However, whether baicalein exerts its cancer-suppressive effects in other ways needs to be investigated more thoroughly.

## DECLARATIONS

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### Competing interest

The authors have no conflicts of interest to disclose.

### Contribution of authors

We declare that this work was performed by the authors named in this article, and all liabilities pertaining to claims related to the content of this article will be borne by the authors. Peng Wang and Jianqiang Zhao designed the study, supervised the data collection, and analyzed the data. Liang Guo interpreted the data and prepared the manuscript for publication. Zhong Liang and Jianlin Lou supervised the data collection, analyzed the data, and reviewed a draft of the manuscript. All the authors read and approved the manuscript.

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