Article

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Puerarin Inhibits the Development of Thyroid Cancer Through KLF2/NOTCH1 Signaling

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Background: Puerarin is the major bioactive ingredient extracted from Pueraria lobata. Puerarin has an antitumor effect on many kinds of cancer. Accordingly, the aim of this study was to investigate the effect and mechanism of puerarin on thyroid cancer (TC) proliferation and apoptosis.

Methods: TC and normal thyroid cells experienced exposure to puerarin at 0, 10, 50, or 100 µg/mL. Impacts of short hairpin RNA of Kruppel-like factor 2 (sh*KLF2*), *KLF2* overexpression, and notch receptor 1 (*NOTCH1*) overexpression on the malignant biological phenotypes of TC cells were gauged by cell function experiments. Quantification of KLF2, NOTCH1, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), and Cleaved caspase 3 was completed using quantitative real-time polymerase chain reaction (qRT-PCR) and Western blot analyses. *KLF2* and *NOTCH1* levels in TC were analyzed using the Encyclopedia of RNA Interactomes (ENCORI) project database. Through rescue experiments, whether the anti-tumor effect of puerarin on TC is realized via *KLF2*/*NOTCH1* axis was dissected.

Results: Puerarin inhibited the malignant growth of TC cells by up-regulating *KLF2*, which was reversed by sh*KLF2*. *KLF2* was lowly expressed in TC. Notably, *NOTCH1* was negatively regulated by *KLF2* and was highly expressed in TC. *NOTCH1* overexpression abrogated *KLF2* overexpression-inhibited malignant growth of TC cells, which was manifested as increased cell proliferation and Bcl-2 as well as decreased cell apoptosis, Bax and Cleaved caspase 3.

Conclusion: Puerarin suppresses TC cell proliferation and apoptosis via the *KLF2*/*NOTCH1* axis.

Keywords: thyroid cancer; puerarin; Kruppel-like factor 2; notch receptor 1; malignant growth

Introduction

Thyroid cancer (TC) is a tumor highly prevalent in the endocrine system [1]. Recent years have witnessed the rapidly increasing incidence of TC worldwide, making it one of the fastest-growing malignant tumors [2]. TC is classified pathologically as differentiated types, including papillary and follicular TC, and undifferentiated types, containing medullary and interstitial TC, of which 90% are papillary TC (PTC) [3]. Although surgery, radioactive iodine (RAI), and thyroid-stimulating hormone suppression therapy display a curative power on the majority of TC patients, their therapeutic potential is weak in progressive PTC and locally advanced or metastatic RAI-refractory TC [4]. Given this, the mechanism of TC and promising intervention drugs to effectively mitigate TC necessitate in-depth explorations.

Along with the advancement of medical science, recent reports have presented that using natural products is one of the underlying therapies for tumor disease, for instance, puerarin [5–7]. Puerarin, an isoflavonoid compound, is one of the leading effective constituents extracted from the dried roots of the Chinese medicinal herb *Puerariae radix* [8]. Puerarin possesses significant effects in boosting immunity, protecting cardiomyocytes, and enhancing myocardial contractility, as well as lowering blood pressure and combating platelet aggregation [9]. In addition, numerous studies over decades have demonstrated that high concentrations of puerarin can be involved in proliferation suppression and apoptosis induction in some tumor cells, such as epithelial ovarian, lung, and bladder cancers, through several pathways, including blocking the cell cycle and affecting the mitogen-activated protein kinases (MAPKs) and the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) signaling pathways [10–12]. Nevertheless, the mechanism of puerarin in TC has been incompletely known. Hence, we aim to find a target gene of puerarin in treating TC cells.

Reportedly, puerarin can up-regulate the level of Kruppel-like factor 2 (*KLF2*) [13]. *KLF2*, a Kruppel-like transcription factor family member, participates in cell proliferation, differentiation, embryo development, and other biological processes [14,15]. At present, the existing studies have unraveled that *KLF2* can inhibit TC cell proliferation [16] and suppress the signaling of notch receptor 1 (*NOTCH1*) [17], a promoter of TC malignant progression

[18]. These findings indicated that puerarin may suppress the malignant progression of TC by up-regulating *KLF2* to inhibit *NOTCH1* signaling.

Herein, we are dedicated to investigating the function of puerarin on TC cell growth and uncovering the underlying molecular mechanism, namely whether puerarin suppresses the proliferation and apoptosis of TC cells via the *KLF2*/*NOTCH1* axis.

Materials and Methods

Cell Culture and Drug Treatment

Human TC cell lines TPC-1 and HTH83 (AW-CELLS-H0371, AW-CELLS-H0449) and normal human thyroid cell line Nthy-ori 3-1 (AW-CELLS-H0282) were acquired from AnWei Biotechnology (Shanghai, China). The basal medium (E600028-0500) and supplement reagents (E600001-0500, B540732-0010) were procured from Sangon Biotech (Shanghai, China). Cell culture in RPMI 1640 medium replete with 10% Fetal Bovine Serum (FBS) and 1% Penicillin-Streptomycin Solution was conducted in an incubator (51023126, Thermo Fisher Scientific, Waltham, MA, USA) (5% CO_2, 37 °C). All used cells were tested for mycoplasma contamination and authenticated using the short tandem repeat (STR) method.

The preparation of 0, 10, 50, and 100 µg/mL puerarin (ab142939, Abcam, Cambridge, UK) was made by dissolution with dimethyl sulfoxide (DMSO, D8418, Sigma-Aldrich, St. Louis, MO, USA). Cells undergoing 24-hour treatment of puerarin were harvested for the 3-[4, 5-Dimethylthylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide (MTT) assay and quantitative real-time polymerase chain reaction (qRT-PCR) analysis.

Cell Transfection

TPC-1 and HTH83 cells in 24-well plates reaching 70% to 80% confluence experienced transfection utilizing transfection reagent (XTG360-RO, Roche, Basel, Switzerland). According to the manual of transfection reagent, the mixtures of transfection reagent, short hairpin RNA-negative control (shNC)/short hairpin RNA of Kruppel-like factor 2 (sh*KLF2*)/pDONR223 vector/*NOTCH1* plasmid/*KLF2* plasmid/*KLF2* plasmid + *NOTCH1* plasmid and reduced-serum medium (31985070, Thermo Fisher Scientific, Waltham, MA, USA) were added into TC cells for 48 hours. Next, the transfected TC cells were applied for other assays. The sh*KLF2* (sc-35818-SH) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), shNC as control (sc-44230, Santa Cruz, CA, USA). Both *KLF2* plasmid (G126694) and *NOTCH1* plasmid (G152985) were bought from YouBio Biology (pDONR223 vector as NC, Changsha, China), and the *NOTCH1* and *KLF2* plasmids were produced by amplifying the CDS region (**Supplementary Files 1, 2**).

Cell Viability Evaluation

MTT assay (C0009M, Beyotime, Shanghai, China) was performed to measure cell viability after indicated treatments. Briefly, the puerarin-treated (0, 10, 50, and 100 µg/mL)/transfected cells (5×10^3 cells/well) were rest in 10 µL MTT solution (5 mg/mL) for 4 hours, and 100 µL of Formazan solvent for about 3 to 4 hours at 37 °C. Finally, absorbance at 570 nm was read using a microplate reader (MR-96A, Mindray, Guangzhou, China).

qRT-PCR

Total RNA extraction from cells and cDNA synthesis were accomplished by TRI Reagent (T9424, Sigma-Aldrich, St. Louis, MO, USA) and First Strand cDNA Synthesis Kit (D7170M, Beyotime, Shanghai, China), respectively. QPCR reactions were performed in a real-time PCR system (7500, Thermo Fisher Scientific, Waltham, MA, USA) with SYBR GreenER qPCR SuperMix (11762500, Invitrogen, Waltham, MA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. The $2^{-\Delta\Delta CT}$ method was introduced to quantify relative gene expressions [19]. All the primers are displayed in Table 1.

Cell Proliferation Detection

A colony formation assay was performed to determine puerarin's mechanism in the progression of TC. TC cells in a 24-well plate (5×10^3 cells/well) underwent 24-h incubation with RPMI 1640 medium containing 10% FBS. Fixation (4% paraformaldehyde, P0099, Beyotime, Shanghai, China) and color development (0.5% crystal violet, C0121, Beyotime, Shanghai, China) of colonies were separately performed for 20 min and 10 min at room temperature. A microscope (CX23, Olympus, Tokyo, Japan) was employed to tally proliferative cells.

Cell Apoptosis Detection

Annexin V-FITC/Propidium Iodide (PI) detection kit (BB-4101, BestBio, Shanghai, China) was employed to stain apoptotic cells. In short, the cells (1×10^6 cells/mL) were subjected to suspension in a Binding Buffer and 15-min treatment of 5 µL Annexin V-FITC at 4 °C in darkness. Afterward, 10 µL PI solution was used to stain cells for 5 min at the same culture condition. Apoptotic cells were then assessed using a flow cytometer (CytoFLEX, Beckman, Brea, CA, USA) and statistically analyzed with its matching software (version 2.0, Beckman, Brea, CA, USA).

Bioinformatics Analysis

KLF2 and NOTCH1 levels in 510 cancer and 58 normal samples of thyroid carcinoma (THCA) were analyzed using the Encyclopedia of RNA Interactomes (ENCORI) project database (https://rnasysu.com/encori/panGeneDiffExp.php).

Table 1. The primer sequences of related genes.

Gene	Forward primer (5′-3′)	Reverse primer (5′-3′)
KLF2 (human)	CTCCCACCGGGTCTACACTA	CCAATGCACACAACAGGTGG
NOTCH1 (human)	GCAGAGGCGTGGCAGACTAT	CAGTAGAAGGAGGCCACACG
GAPDH (human)	GGATTTGGTCGTATTGGGCG	TCCCGTTCTCAGCCATGTAG

KLF2, transcription factor 2; *NOTCH1*, notch receptor 1; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase.

Table 2. Antibodies used in this study.

Name	Catalog	Molecular weight (kDa)	Dilution	Manufacturer
NOTCH1	ab280898	272	1/1000	Abcam, Cambridge, UK
Bcl-2	ab59348	26	1/1000	Abcam, Cambridge, UK
Bax	ab32503	21	1/1000	Abcam, Cambridge, UK
Cleaved caspase 3	ab32042	17	1/500	Abcam, Cambridge, UK
GAPDH	ab8245	36	1/5000	Abcam, Cambridge, UK
Goat anti-rabbit	ab97051	—	1/10,000	Abcam, Cambridge, UK
Goat anti-mouse	ab96879	—	1/10,000	Abcam, Cambridge, UK

NOTCH1, notch receptor 1; Bcl-2, B-cell lymphoma-2; Bax, Bcl-2-associated X; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Western Blot

Total protein isolation from TPC-1 and HTH83 cells was performed with RIPA Lysis Buffer (P0013B, Beyotime, Shanghai, China), and the lysates were quantified by Bicinchoninic Acid assay (BCA) Protein Assay Kit (PC0020, Solarbio, Beijing, China). After separation by 10% sodium dodecyl sulfate (SDS) polyacrylamide gels (P0690, Beyotime, Shanghai, China), the extracts underwent transference onto polyvinylidene difluoride membranes (IPVH00010, Millipore, USA), which were first treated with 5% blocking buffer (C510053-0020, Sangon Biotech, Shanghai, China) and then incubated with primary at 4 °C overnight and secondary antibodies at room temperature for 1 hour. Related antibodies are displayed in Table 2. NOTCH1, B-cell lymphoma-2 (Bcl-2), Bcl-2-associated X (Bax), and Cleaved caspase 3 protein levels were normalized by GAPDH. Visualization of bands was carried out by an enhanced chemiluminescence (ECL) system (17-373BP, Millipore, Billerica, MA, USA) with ECL Substrate (32106, Thermo Fisher Scientific, Waltham, MA, USA). Quantity One software (vision 4.6.6, Bio-Rad, Hercules, CA, USA) finally evaluated relative protein levels.

Statistical Analysis

All experiments were carried out thrice or above. Analysis of statistics was performed by Statistical Product and Service Solutions (SPSS, vision 20.0, International Business Machines Corporation, Armonk, NY, USA) or GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), and data were expressed as the mean ± standard. Multi-group contrast was completed by one-way analysis of variance (ANOVA), followed by the post hoc Tukey's or Dunnett test. When $p < 0.05$, the statistics were deemed significant.

Results

ShKLF2 Reversed the Effects of Puerarin on Dampening TC Cell Viability and Boosting KLF2 Expression

Fig. 1A presents the chemical structure of puerarin. TC cell lines (TPC-1 and HTH83) and normal thyroid cell line (Nthy-ori 3-1) underwent treatment with puerarin (0, 10, 50, and 100 µg/mL) to explore how puerarin impacts cell viability. The results indicated puerarin dose-dependently inhibited cell viability, with slight toxicity at 10 µg/mL and remarkable toxicity at 50, 100 µg/mL (Fig. 1B, $p < 0.01$). Puerarin (0, 10, 50, and 100 µg/mL) did not impact Nthy-ori 3-1 cell viability (Fig. 1B). qRT-PCR results determined that puerarin concentration-dependently elevated *KLF2* level (Fig. 1C, $p < 0.01$). To prevent the toxic effect of puerarin on cells, 50 µg/mL puerarin was selected for subsequent experiments.

Next, we performed *KLF2* knockdown in cells to reveal the mechanism of puerarin in TC progression. The down-regulated *KLF2* verified the successful transfection, which later was reversed by puerarin (Fig. 1D, $p < 0.01$). Besides, it was discerned that *KLF2* knockdown promoted TC cell viability, which was also offset by puerarin (Fig. 1E, $p < 0.05$). The effects of puerarin on augmenting *KLF2* level (Fig. 1D, $p < 0.001$) and weakening TC cell viability (Fig. 1E, $p < 0.001$) were counteracted by sh*KLF2*.

ShKLF2 Offset the Role of Puerarin in Blocking TC Cell Proliferation and Enhancing Apoptosis

In light of the results from the colony formation assay, proliferation was inhibited in puerarin-treated cells (Fig. 2A,B, $p < 0.001$), which was reversed by sh*KLF2* (Fig. 2A,B, $p < 0.001$). Similarly, puerarin-induced in-

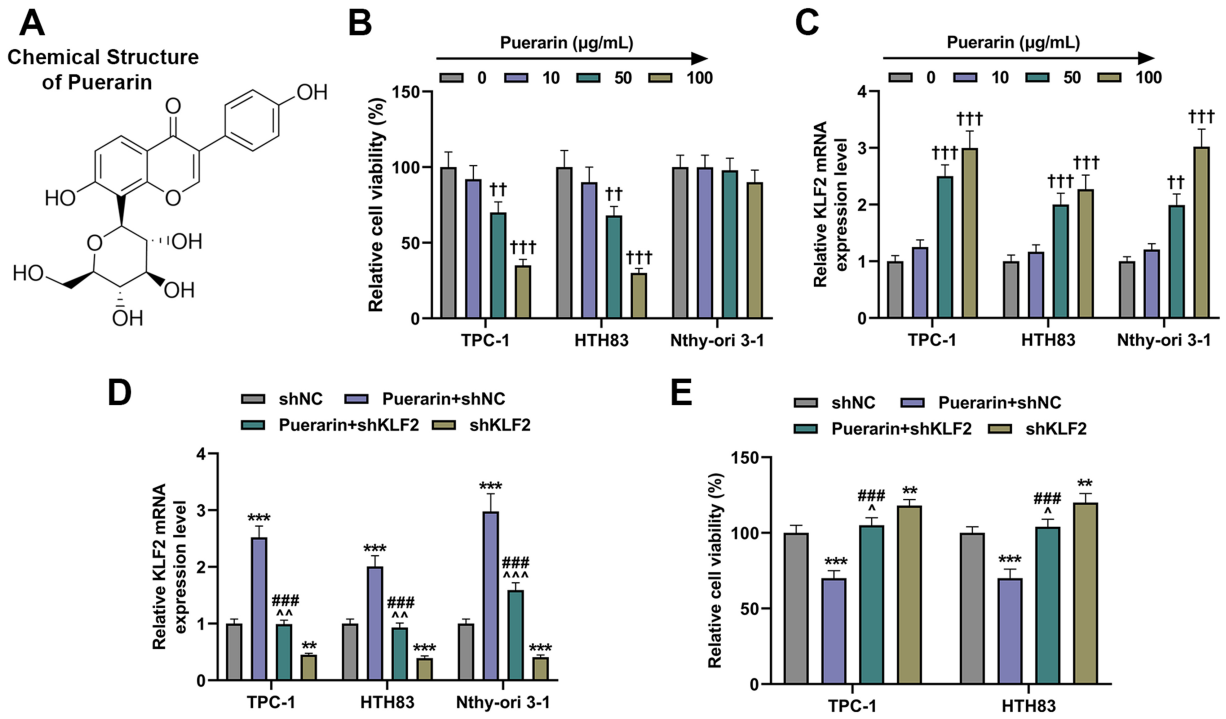

Fig. 1. Puerarin suppressed TC cell viability and boosted *KLF2* expression, partially reversed by sh*KLF2*. (A) The chemical structure of puerarin. (B) Cell viability after puerarin treatment at 0, 10, 50, and 100 μg/mL (MTT assays). (C) *KLF2* expression in cells after puerarin treatment at 0, 10, 50, and 100 μg/mL (qRT-PCR, *GAPDH* as a loading control). (D) *KLF2* expression in cells after transfection of shNC/sh*KLF2* and treatment with/without 50 μg/mL puerarin (qRT-PCR, *GAPDH* as a loading control). (E) Cell viability was detected after transfection of shNC/sh*KLF2* and treatment with/without 50 μg/mL puerarin (MTT assays). All experiments were repeated three times. $^{\dagger\dagger}p < 0.01$, $^{\dagger\dagger\dagger}p < 0.001$ vs. 0; $^{**}p < 0.01$, $^{***}p < 0.001$ vs. shNC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. shKLF2; $^{\#\#\#}p < 0.001$ vs. Puerarin + shNC. TC, thyroid cancer; *KLF2*, Kruppel-like factor 2; sh, short hairpin; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase; MTT, 3-[4, 5-Dimethylthylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide.

creases in apoptosis rates of TPC-1 and HTH83 cells (Fig. 2C,D, $p < 0.001$) were abrogated in the presence of sh*KLF2* (Fig. 2C,D, $p < 0.001$). Additionally, puerarin attenuated the effects of *KLF2* knockdown by reducing cell proliferation and enhancing apoptosis (Fig. 2A–D, $p < 0.05$).

NOTCH1 was Negatively Regulated by KLF2 and Highly Expressed in TC

Further, *KLF2* and *NOTCH1* levels in TC were assessed using the ENCORI project database. Our data exhibited that *KLF2* was lowly expressed (Fig. 3A, $p = 6.1 \times 10^{-6}$) while *NOTCH1* was highly (Fig. 3A, $p = 0.0078$) in patients with THCA. Their plasmids were co-transfected into TC cells to explore the regulatory relationship between *KLF2* and *NOTCH1*. In contrast to the NC group, *KLF2* (Fig. 3B, $p < 0.001$) and *NOTCH1* were successfully overexpressed in TC cells (Fig. 3C–E, $p < 0.001$). Of note, our outcomes revealed that *KLF2* overexpression obviously reduced the mRNA and protein levels of NOTCH1 (Fig. 3C–E, $p < 0.05$), and *NOTCH1* overexpression also reversed the role of *KLF2* in *NOTCH1* (Fig. 3C–E, $p < 0.01$).

NOTCH1 Overexpression Reversed the Influences of KLF2 Overexpression on Blocking Malignant Growth of TC Cells

The role of the *KLF2/NOTCH1* axis in the malignant phenotypes of TC cells was further investigated using rescue experiments. As shown in Fig. 4A–D, *KLF2* overexpression reduced cell proliferation and elevated cell apoptosis ($p < 0.01$), while *NOTCH1* overexpression increased cell proliferation ($p < 0.001$). These effects were offset after the co-transfection of *KLF2* and *NOTCH1* plasmids (Fig. 4A–D, $p < 0.01$). Moreover, at the protein level, *NOTCH1* overexpression up-regulated Bcl-2 and down-regulated Bax and Cleaved caspase 3 in TC cells (Fig. 5A–D, $p < 0.05$), but *KLF2* overexpression oppositely impacted these apoptosis-related proteins (Fig. 5A–D, $p < 0.01$). *KLF2* and *NOTCH1* overexpression influences on these apoptosis-related proteins were neutralized by co-transfection of *KLF2* and *NOTCH1* plasmids (Fig. 5A–D, $p < 0.05$).

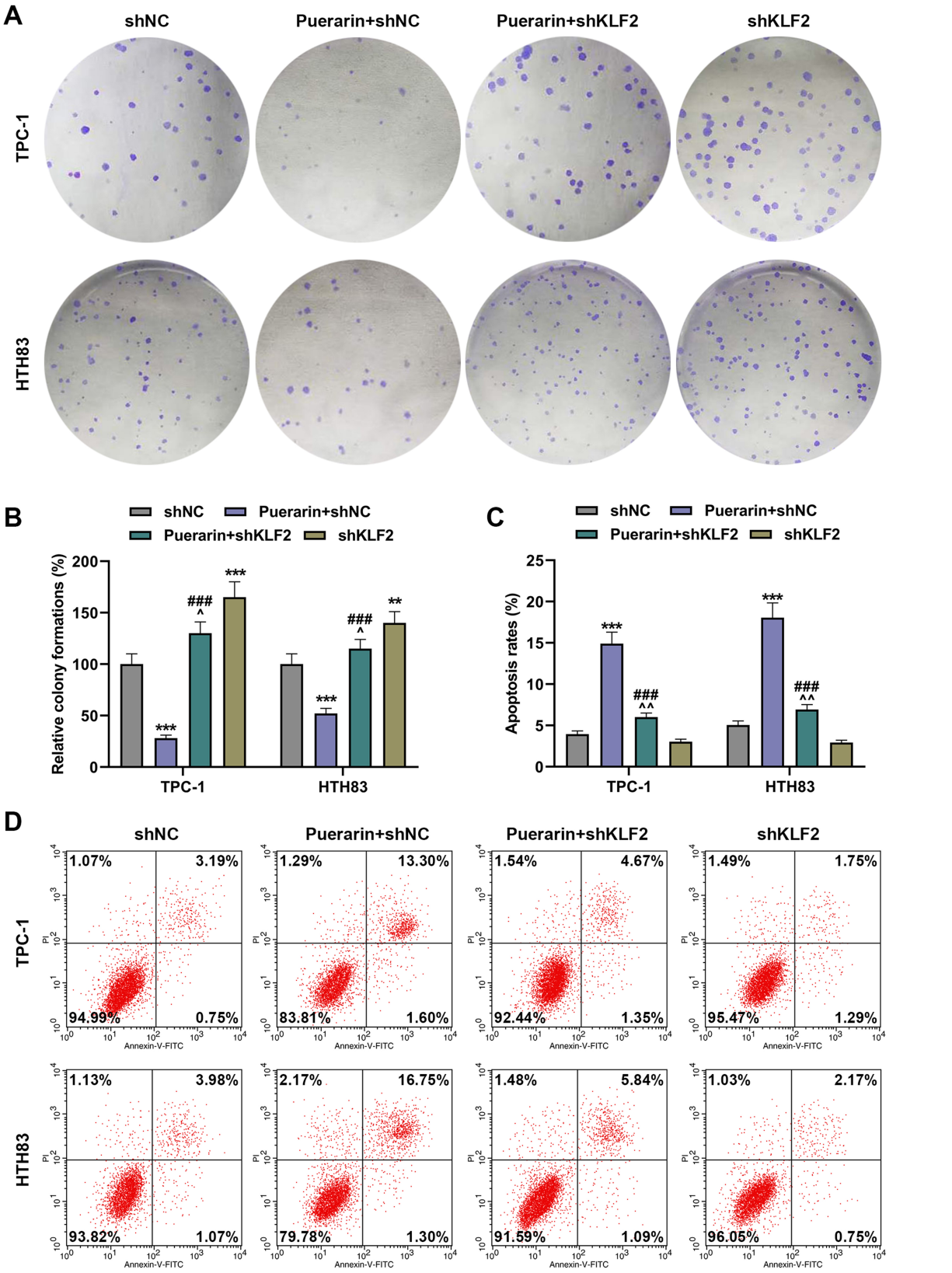

Fig. 2. Puerarin inhibited TC cell proliferation and promoted apoptosis, which was partially counteracted by sh*KLF2*. After transfection of shNC/sh*KLF2*, TC cells were treated with/without 50 μg/mL puerarin. (A,B) Cell proliferation (colony formation assays). (C,D) Cell apoptosis (flow cytometry). All experiments were repeated three times. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. shNC; $^{\wedge}p < 0.05$, $^{\wedge\wedge}p < 0.01$ vs. shKLF2; $^{\#\#\#}p < 0.001$ vs. Puerarin + shNC. TC, thyroid cancer; *KLF2*, Kruppel-like factor 2; sh, short hairpin; NC, negative control.

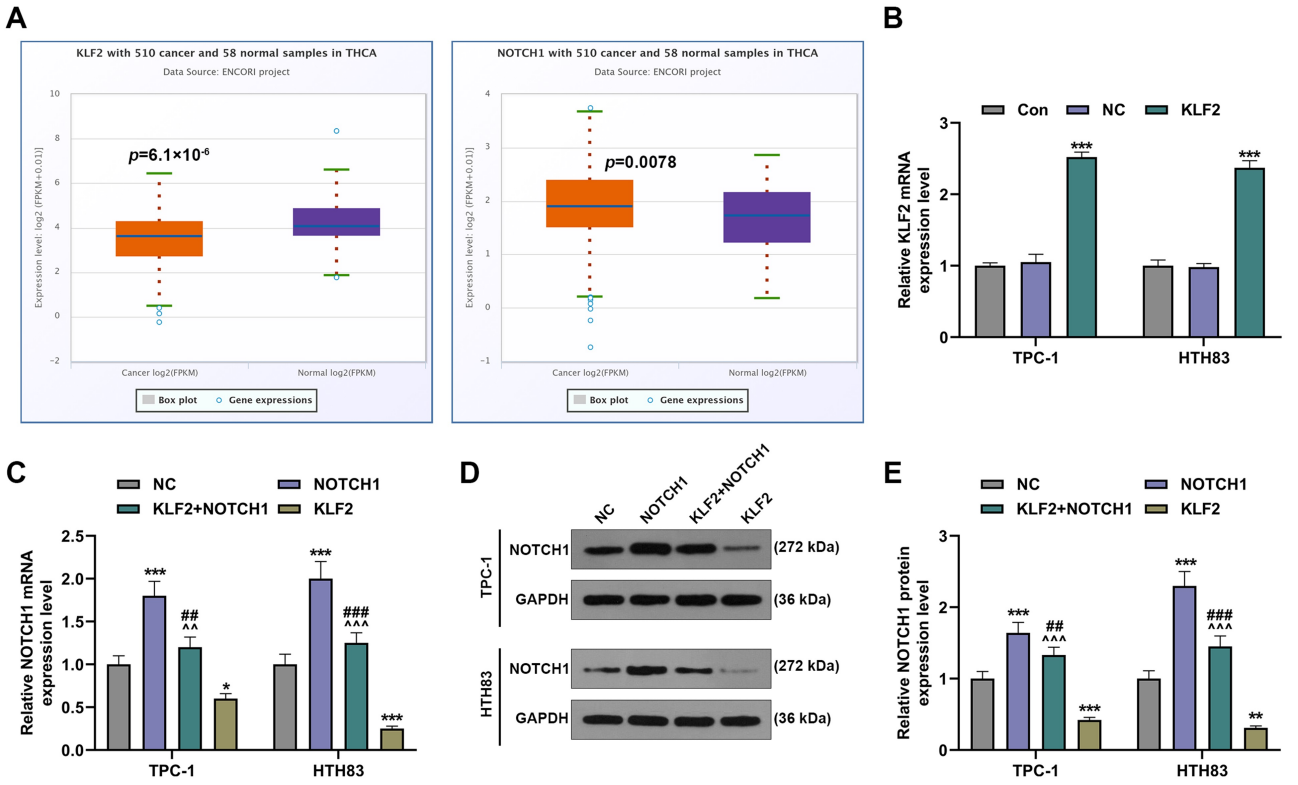

Fig. 3. *NOTCH1* was negatively regulated by *KLF2* and highly expressed in TC. (A) *KLF2* and *NOTCH1* levels in 510 cancer and 58 normal samples of THCA (ENCORI project database (https://rnasysu.com/encori/panGeneDiffExp.php)). TC cells were transfected with pDONR223 vector/*NOTCH1* plasmid/*KLF2* plasmid/*KLF2* plasmid + *NOTCH1* plasmid. (B) *KLF2* expression (qRT-PCR, *GAPDH* as a loading control). (C) *NOTCH1* expression (qRT-PCR, *GAPDH* as a loading control). (D,E) NOTCH1 protein level (Western blot, GAPDH as a loading control). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC; $^{\wedge\wedge}p < 0.01$, $^{\wedge\wedge\wedge}p < 0.001$ vs. KLF2; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOTCH1. TC, thyroid cancer; *KLF2*, Kruppel-like factor 2; *NOTCH1*, notch receptor 1; ENCORI, Encyclopedia of RNA Interactomes; THCA, thyroid carcinoma; NC, negative control; qRT-PCR, quantitative real-time polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Discussion

The therapeutic efficiency of TC has been elevated due to refined diagnosis and treatment modalities, and the emergence of novel molecular-targeted therapies in the past few decades [20]. In the context of the breakthroughs in the mechanisms of tumor development, increasing plant extracts such as paclitaxel, resveratrol, and quercetin have been found to have anti-cancer potential with minimal side effects and widely serve as anti-tumor agents or anti-tumor adjuvants [21–23].

Puerarin, a natural plant extract with a wide range of beneficial biological activities, has been applied clinically to treat inflammation and cardiovascular diseases [24]. Numerous recent studies have pointed out that puerarin exhibits remarkable anti-cancer properties in several cancers [25–28]. Reportedly, puerarin could dose-dependently inhibit the growth of breast cancer cells [29]. Later, Gan *et al.* [30] confirmed that puerarin induces apoptosis of mantle lymphoma cells by blocking the cell cycle in the G1/S phase. Jia *et al.* [31], in their study of cervical cancer, indicated that puerarin inhibits tumor cell growth and migration by regulating the PI3K/mTOR pathway. In this study, we unearthed that puerarin can dose-dependently suppress the malignant growth of TC cells, which supports the anti-cancer effect of puerarin on TC.

As early as it was reported, the protective effect of puerarin on cardiovascular diseases is realized by up-regulating the downstream KLF2 [13]. Yet, it is still unclear whether the anti-cancer effect of puerarin on TC is realized by regulating KLF2. In this study, we discovered firsthand that puerarin can dose-dependently promote the level of KLF2 in TC cells. As a transcription factor, KLF2 is considered as a protective gene in TC [32]. An early study has pointed out that KLF2 silencing can boost the malignant growth of TC cells [16], which is similar to our results. On this basis, it can be concluded that the anti-cancer effect of puerarin on TC is realized by up-regulating KLF2, which was later verified by rescue experiments.

Further, the downstream factor of KLF2 was explored. Reportedly, KLF2 overexpression can mitigate the malignant progression of cancers by suppressing the NOTCH1

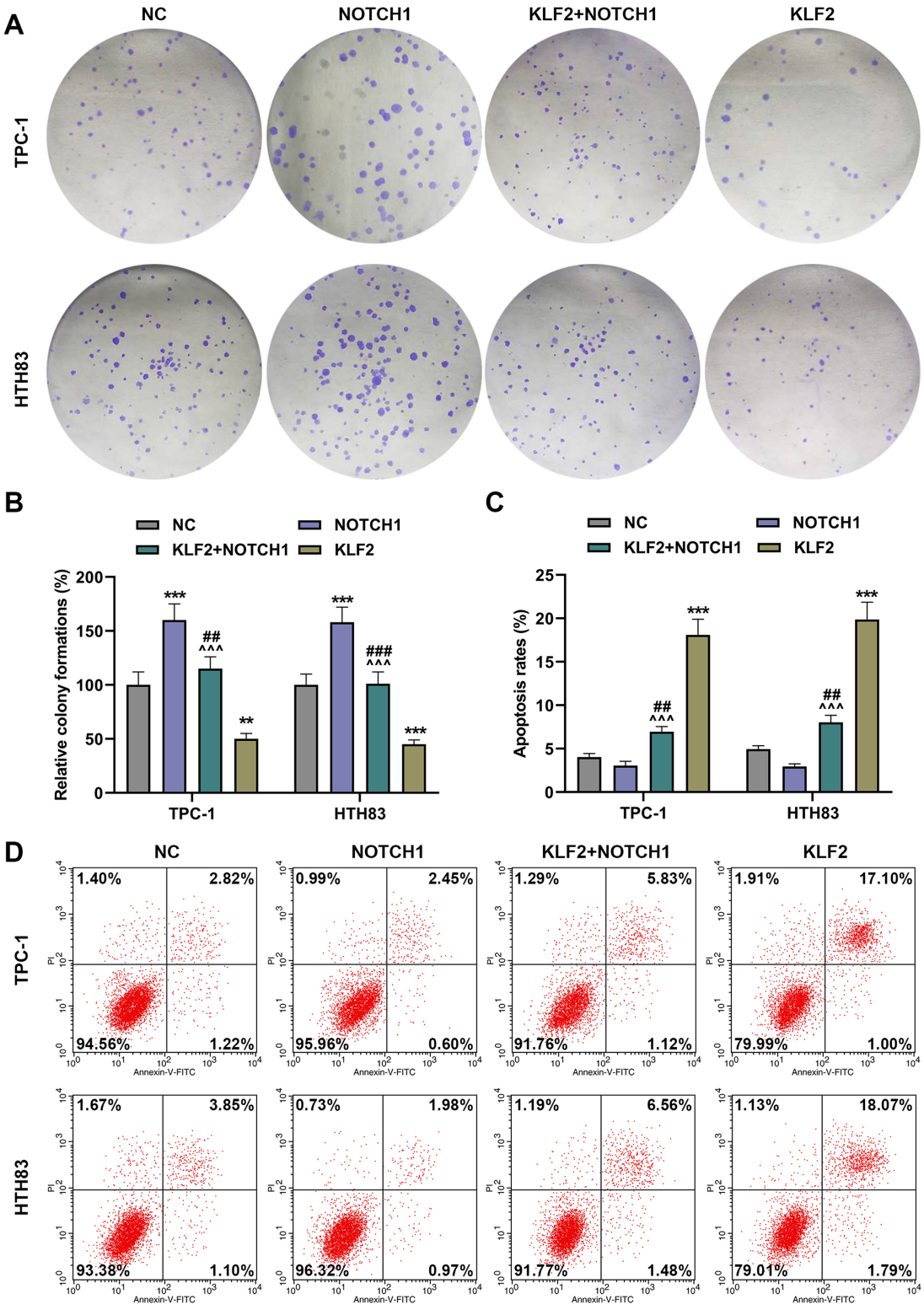

Fig. 4. *NOTCH1* overexpression reversed the diminishing effect of *KLF2* overexpression on TC cell growth. TC cells were transfected with pDONR223 vector/NOTCH1 plasmid/*KLF2* plasmid/*KLF2* plasmid + *NOTCH1* plasmid. (A,B) Cell proliferation (colony formation assay). (C,D) Cell apoptosis (flow cytometry). All experiments were repeated three times. $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC; $^{\wedge\wedge\wedge}p < 0.001$ vs. KLF2; $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOTCH1. TC, thyroid cancer; *KLF2*, Kruppel-like factor 2; *NOTCH1*, notch receptor 1; NC, negative control.

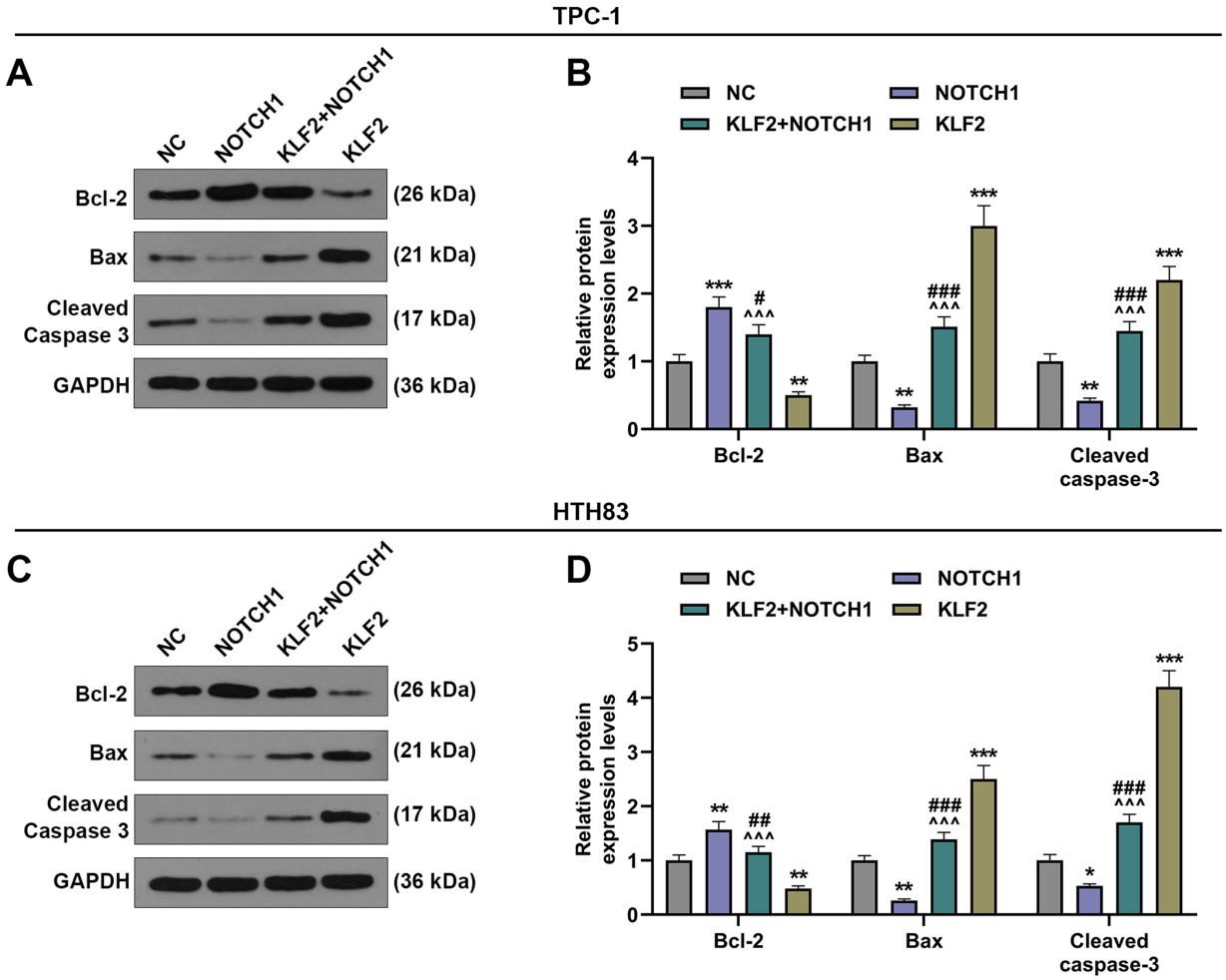

Fig. 5. *NOTCH1* overexpression reversed the promoting effect of *KLF2* overexpression on TC cell apoptosis. TC cells were transfected with pDONR223 vector/*NOTCH1* plasmid/*KLF2* plasmid/*KLF2* plasmid + *NOTCH1* plasmid. (A–D) The expressions of apoptosis-related proteins include Bcl-2, Bax, and Cleaved caspase 3 (Western blot, GAPDH as a loading control). All experiments were repeated three times. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ vs. NC; $^{\wedge\wedge\wedge}p < 0.001$ vs. KLF2; $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ vs. NOTCH1. TC, thyroid cancer; *KLF2*, Kruppel-like factor 2; *NOTCH1*, notch receptor 1; NC, negative control; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

signal pathway [17]. There is a negative relationship between KLF2 and NOTCH1 in prostate cancer [33]. Our data supported the idea that KLF2 was lowly expressed and NOTCH1 was highly expressed in TC patients. Moreover, KLF2 overexpression inhibited NOTCH1 expression, indicating that NOTCH1 was negatively regulated by KLF2. An existing study has revealed that NOTCH1 can promote the malignant progression of TC [18]. Since our data were consistent with these findings, it can be inferred that puerarin improved TC, possibly via the KLF2/NOTCH1 axis, which was testified by rescue experiments.

However, this study has some limitations. (1) No more cell function experiments were designed further to explore the effect of puerarin on TC progression. (2) The involvement of other pathways in the regulation of KLF2/NOTCH1 was not discussed. (3) No *in vivo* experiments were engineered for verification. Based on these, more experiments are necessary to verify puerarin's effect and underlying mechanism on TC progression.

Conclusion

To conclude, our outcomes emphasize the improving effect of puerarin on the malignant growth of TC cells. Mechanistically, we reveal firsthand that puerarin suppresses TC cell proliferation and apoptosis via the KLF2/NOTCH1 axis. Thus, puerarin is supposed to be a novel resource in the development of anti-TC drugs, which, therefore, deserves in-depth investigation to unveil its anti-tumor mechanisms in the future.

Availability of Data and Materials

The analyzed data sets generated during the study are available from the corresponding author on reasonable request.

Author Contributions

YZ designed the research study; WL and HH performed the research; WL and HH collected and analyzed the data. All authors have been involved in drafting the manuscript and all authors have been involved in revising it critically for important intellectual content. All authors have given the final approval of the version to be published. All authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

Acknowledgment

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.24976/Discov.Med.202537196.79.

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