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ORIGINAL RESEARCH

CUX2 functions as an oncogene in papillary thyroid cancer

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Background: In recent years, the incidence of thyroid cancer (TC), the most common endocrine malignancy, has been increasing. Emerging evidence indicates that the CUT/CUX/CDP family of proteins can play an important role in tumor development and progression by regulating many cancer-related functions. However, the molecular functions of *CUX2* in TC remain unknown.
Methods: In this study, we used a series of loss-of-function experiments and Western blot analysis to investigate the function of *CUX2* in TC and the mechanisms involved.
Results: Our data revealed that *CUX2* expression levels were upregulated in papillary thyroid cancer (PTC). Functionally, *CUX2* silencing significantly inhibited PTC cell line (KTC-1 and BCPAP) proliferation, colony formation, migration, invasion, and apoptosis. Furthermore, *CUX2* induced epithelial–mesenchymal transition (EMT) and influenced the phosphorylation of AKT and mTOR in the PI3K–AKT–mTOR pathways.
Conclusion: In summary, *CUX2* may function as a tumor promoter in TC.
Keywords: papillary thyroid carcinoma, *CUX2*, oncogene

Introduction

Thyroid cancer (TC) is the most frequent endocrine malignancy, with 53,990 estimated diagnosed cases and 2,060 estimated deaths in the United States in 2018. In recent years, its incidence has shown a significant upward trend worldwide. However, with the changes in clinical practice guidelines, TC incidence may appear to be decreasing, particularly among Whites.[1,2] Papillary thyroid cancer (PTC) accounts for 80%–85% of all TCs.[3] RAS mutations represent the second most identified genetic alteration in TC, and RAS-mutated PTC appears to be more aggressive and is associated with poor prognosis compared with other types of cancers.[4,5] The PI3K/AKT pathway plays an extensive role in thyroid tumorigenesis, and its inhibitor can be a therapeutic target in TC.[6]

The CUT/CUX/CDP family of nuclear proteins was first reported in *Drosophila*; it plays an important role in mediating the dendrite branching pattern.[7,8] Since then, a human version of CUT, named CUT-like homeobox 1 and 2 (*CUX1* and *CUX2*), was subsequently identified as the mammalian orthologue of the *Drosophila CUT* gene. The full-length CUX proteins are characterized by four conserved DNA-binding domains, including the CUT homeodomain and three CUT repeat DNA-binding sequences (CR1, CR2, and CR3), each composed of 60–80 highly similar amino acids.[9–11] Depending on the different cellular contexts, *CUX* genes can express various isoforms and exhibit regulated expression levels, which might contribute to functional diversity.[12,13] *CUX* genes are known to be associated with the initiation and progression of multiple diseases, such as brain diseases involved in synapse, dendrite, and axon development and various cancers.[13–16] Paradoxically, *CUX* has been implicated in cancer, both as a tumor

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suppressor and an oncogene, depending on distinct protein isoforms and perhaps on the dosage of gene expression. *CUX1* expression is upregulated in many advanced cancers, such as glioblastomas, colon rectal cancer, and breast cancer.[17–19] However, some genetic and functional evidence also point that many cancers (uterine leiomyomas, breast cancer, acute myeloid leukemias, and myelo proliferative diseases) commonly exhibit loss or inactivation of one *CUX1* allele, resulting in decreased expression and activity and promoting tumorigenesis.[20–24] Increasing evidence implicate the notion that *CUX1*, a haploinsufficient tumor suppressor gene, might be associated with tumor initiation, whereas increased copy number and expression promote tumor progression.

Unlike *CUX1*, which is broadly expressed in many tissues, *CUX2* shows a more restricted expression pattern and is primarily expressed in the nervous system.[25,26] Recently, Klampfl et al[27] found that a mutation in *CUX2* is also linked with myeloproliferative neoplasms. *CUX1* and *CUX2* have a 48% amino acid identity. *CUX2* has several reported transcript variants.[11] It exhibits similar DNA-binding specificities and binds to the same sequences as *CUX1*, although its kinetics appear to be much more transient and rapid.[28] CUX2 proteins have been found to bind not only to CCAAT-containing sites but also to other promoter sequences, acting as a repressor or an activator to regulate transcriptional activity in different contexts.[29,30] Interestingly, in the liver, *CUX2* functions as a female-specific transcription activator and inhibits male-biased genes.[31,32] In addition, *CUX2* is involved in various biological processes, including accelerating the repair of oxidative DNA damage, cell cycle progression, apoptotic signals, and other pathways.[30,33–35]

To date, few studies have focused on the relationship between *CUX2* and TC. Hence, in this study, we investigated the role of the *CUX2* gene in TC.

Patients and methods

Patients and samples

We selected 20 paired PTC tissues and matched noncancerous thyroid tissues from patients who underwent thyroid resection at the First Affiliated Hospital of Wenzhou Medical University. These tissues were flash frozen in liquid nitrogen immediately after surgery and stored at −80°C before RNA isolation and quantitative real-time PCR (qRT-PCR) analysis. Further clinicopathological data were available. The use of all tissue samples in this study was approved by the ethics committee of the First Affiliated Hospital of Wenzhou Medical University, and written informed consent was obtained from each patient.

Cell culture

Human TC cell lines, KCT-1, TPC-1, BCPAP, FTC-133, and Htori-3, were provided by the Stem Cell Bank, Chinese Academy of Sciences. These cells were cultivated in RPMI 1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% FBS (Thermo Fisher Scientific), 1× Minimum Essential Medium nonessential amino acids (Thermo Fisher Scientific), and 1× sodium pyruvate (Thermo Fisher Scientific) and incubated in a humidified atmosphere containing 5% CO_2 at 37°C.

RNA extraction and qRT-PCR

The total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific) according to the manufacturer's instructions and then reverse transcribed into cDNA using a kit from Toyobo (Tokyo, Japan). qRT-PCR was performed in triplicate by using the THUNDERBIRD SYBR qPCR Mix (Toyobo) according to the manufacturer's instructions. The following gene-specific primers were used: CUX2 (F: 5′-TGAACCATAGGCACAACC-3′; R: 5′-AAACACCA AGAGGGGAAG-3′) and GAPDH (F: 5′-GGTCGGAGTC AACGGATTTG-3′; R: 5′-ATGAGCCCCAGCCTT CTCCAT-3′).

RNA interference

For knockdown studies, siRNA for *CUX2* was purchased from Shanghai Gene Pharma (Shanghai, China). Cell transfection was performed using RNAiMAX (Thermo Fisher Scientific) according to the manufacturer's protocol. The knockdown efficiency was confirmed by qRT-PCR and Western blot analyses.

Cell proliferation and colony formation assays

We utilized the colony formation and Cell Counting Kit-8 (CCK-8; Sigma-Aldrich Co., St Louis, MO, USA) assays to determine the proliferative ability. For the colony formation assay, the transfected KTC-1 and BCPAP cells (1.5×10^3/well) were seeded in six-well plates. After 7 days, the cells were fixated with 4% paraformaldehyde (PFA) for 30 minutes and stained with 0.1% crystal violet for 30 minutes. The colonies were counted only if they included at least 50 cells. For the proliferation assay, the transfected cells (1.5×10^3) were plated in 96-well plates and measured every 24 hours using the CCK-8 reagent following the manufacturer's instruction. The absorption was measured at 450 nm after adding the reagent and incubating for 2 hours in a 37°C incubator. All experiments were performed in triplicate.

Cell migration and invasion ability analyses

Cellular migration and invasion assays were performed in a Boyden chamber system with a pore size of 8 mm. For invasion assays, the inserts were coated with Matrigel matrix before cell seeding. The transfected cells (4×10^5 cells for KTC-1 and 5×10^5 cells for BCPAP, double amount for invasion) were seeded in the upper chamber, and the chamber was placed into a 24-well plate filled with a medium containing 20% FBS. The cells were incubated for 24 (KTC-1 cells) or 26 hours (BCPAP cells) at 37°C. Then, the cells adhering to the lower surface of the membrane were fixed with 4% PFA for 30 minutes, stained with 0.01% crystal violet for 30 minutes, and photographed using a light microscope. All experiments were performed at least three times.

Cell apoptosis assay

Two days after infection, the cells were harvested and double stained with Annexin V conjugated to phycoerythrin and 7-aminoactinomycin (7-AAD) (Apoptosis Detection Kit-1; BD Pharmingen, San Diego, CA, USA). Apoptotic events were analyzed using FlowJo software. All these experiments were repeated in triplicate.

Protein extraction and Western blot analysis

The total cellular protein was extracted using a RIPA protein lysis buffer (Beyotime, Shanghai, China). Equal amounts of protein (20 μg) were loaded and separated by SDS-PAGE and transferred onto the polyvinylidene fluoride membrane. After blocking with 5% skimmed milk, the membrane was incubated with a relative antibody (Abcam, Cambridge, UK) overnight at 4°C. Then, the membrane was washed and incubated with horseradish peroxidase-linked secondary anti-goat immunoglobulin G antibody (Abcam) at room temperature for 1 hour. GAPDH was used as internal control. All experiments were performed at least three times.

Statistical analyses

The data are expressed as mean ± SD. The different gene expression levels of *CUX2* in tumor tissues were analyzed using paired sample *t*-test. We used the *t*-test or one-way ANOVA test for continuous variables. All *P*-values were two sided, and a *P*-value of <0.05 was considered statistically significant. Statistical analysis was performed with SPSS software version 19.0 (IBM Corporation, Armonk, NY, USA). GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA) was used for the graphs.

Results

CUX2 is overexpressed in TC

To evaluate the expression level of *CUX2* in PTC, qRT-PCR analysis was performed on 20 paired PTC tissues and adjacent noncancerous thyroid tissues. Results showed that *CUX2* was significantly higher than the corresponding normal tissues ($P<0.05$; Figure 1A). Meanwhile, we also assessed the mRNA expression levels of *CUX2* in TC cell lines and found that *CUX2* was consistently upregulated in KCT-1, TPC-1, BCPAP, and FTC-133 cells compared with the normal thyroid cell line, Htori-3 (Figure 1B).

CUX2 knockdown decreases proliferation and colony formation

To investigate the functional role of *CUX2* in papillary thyroid cell lines, we sequentially performed loss-of-function experiments to explore the biological effect of *CUX2*. Considering that the TPC-1 cell viability worsened after siRNA treatment, the KTC-1 and BCPAP cells were finally selected to be transfected with siRNA targeting *CUX2*. The results of qRT-PCR and Western blot analyses showed that the expression of *CUX2* was successfully decreased (>30%) both at the mRNA and protein levels (Figures 1C and 3C). Then, we performed colony formation and CCK-8 assays to investigate the biological role of *CUX2*. The colony formation assay indicated that *CUX2* knockdown inhibited the proliferation in KTC-1 and BCPAP cells compared with the control group (Figure 2A and D). Furthermore, the CCK-8 assay (Figure 2G and H) showed a consistent result with the colony formation assay. The proliferative capacity of the cell lines transfected with siRNA-*CUX2* was significantly attenuated. These data indicated that downregulation of *CUX2* can suppress the proliferation and growth abilities of KTC-1 and BCPAP cells.

CUX2 knockdown impairs the migration and invasion

Given that cell migration and invasion are critical steps for cancer metastasis, we carried out migration and invasion assays to further identify whether the knockdown of *CUX2* expression can regulate TC metastasis abilities. We performed cell migration assay using the Transwell chamber migration assay. The cancer cells with *CUX2* knockdown displayed fewer cells that migrated through the membrane after 24 hours than cells transfected with the negative control. We then examined the number of cells that penetrated through the Matrigel in a Transwell chamber to investigate the invasiveness. Consistently, the invasion assays showed the same tendency,

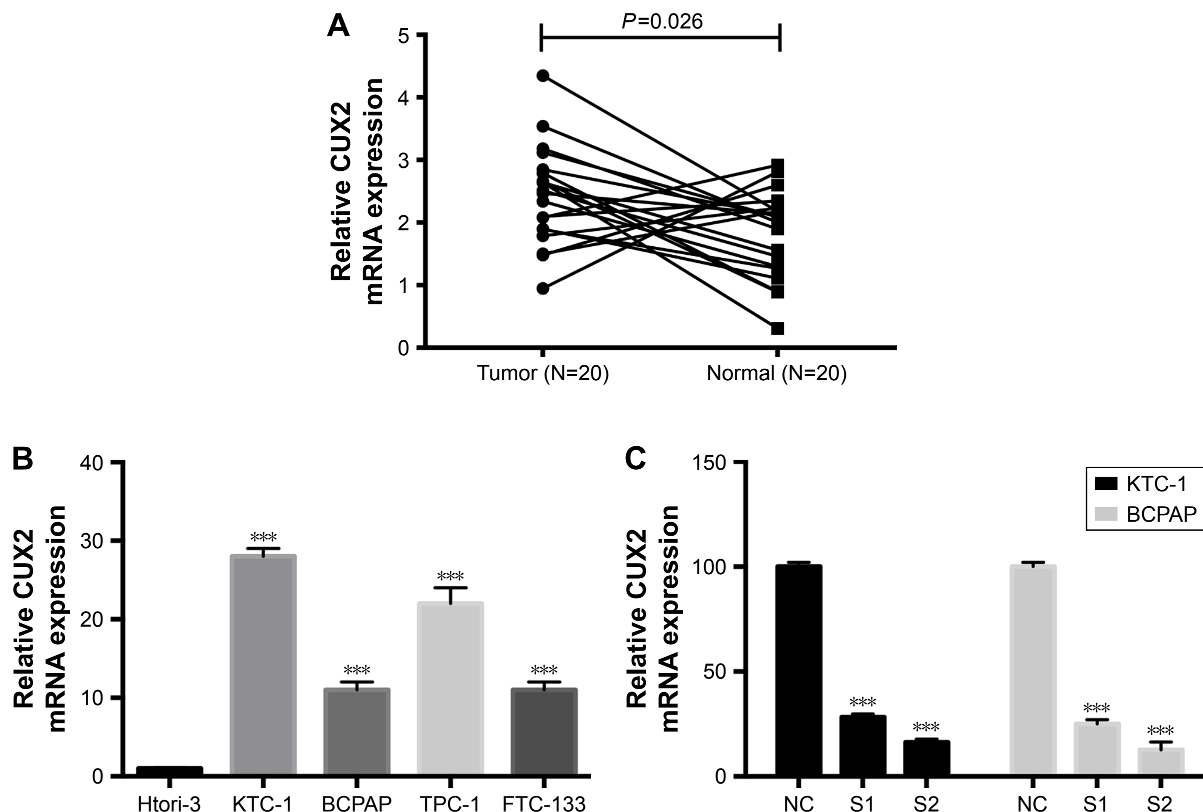

Figure 1 *CUX2* gene is overexpressed in thyroid cancer and knockdown *CUX2* gene in thyroid cancer cell lines.
Notes: (**A**) mRNA expression of *CUX2* in our clinical PTC tissues (n=20). (**B**) mRNA expression levels of *CUX2* in TC cell lines and a control cell line. (**C**) qRT-PCR analysis of the knockdown efficiency of *CUX2* in KTC-1 and BCPAP cells treated with two specific siRNAs. Data represent mean ± SD from three independent experiments. ***$P<0.001$ compared with siRNA-*CUX2* and siRNA-negative control.
Abbreviations: qRT-PCR, quantitative real-time PCR; PTC, papillary thyroid cancer; TC, thyroid cancer.

that is, *CUX2* knockdown dramatically attenuated the invasion capacity compared with vector-transfected cells (Figure 2B, C, E, and F). Therefore, *CUX2* knockdown has a significant role in inhibiting tumor metastasis in TC cell lines.

CUX2 knockdown induces apoptosis

To further explore the possible mechanism of the abovementioned observations, we used 7-AAD and Annexin V staining in KTC-1 and BCPAP cells after different treatments to analyze cell death and apoptosis by flow cytometry. We found that the percentage of apoptotic cells markedly increased in both cell lines compared with that of the control group (Figure 3A and B). These findings suggested that *CUX2* knockdown caused proliferation, and metastasis arrest might trigger cell apoptosis.

CUX2 facilitates cell migration and invasion by regulating the expression of E-cadherin and vimentin

Emerging evidence has verified that the epithelial–mesenchymal transition (EMT) is an integral process that involves cancer invasion, metastasis, and other tumor progression behaviors.[36,37] Meanwhile, the deregulation or loss of the expression of E-cadherin plays a fundamental role in the EMT process. Inversely, upregulation of the expression of vimentin, a mesenchymal associated marker, contributes to cell metastasis.[38–40] On the basis of our previous observation that *CUX2* promotes PTC cell invasion, we next investigated whether *CUX2* can mediate the EMT process of PTC cell lines. Through Western blot analysis, we observed that the expression levels of E-cadherin were increased, whereas those of vimentin were obviously downregulated in *CUX2* knockdown cells compared with the vector-transfected cells (Figure 3C). These results indicate that the expression of *CUX2* might regulate the expression of E-cadherin and vimentin, thereby affecting the EMT process.

PI3K–AKT–mTOR pathway is involved in the regulatory effects of *CUX2*

The PI3K–AKT–mTOR pathway, an important survival pathway that is activated in many types of cancers, regulates cellular metabolism, tumor proliferation, and apoptosis.[41]

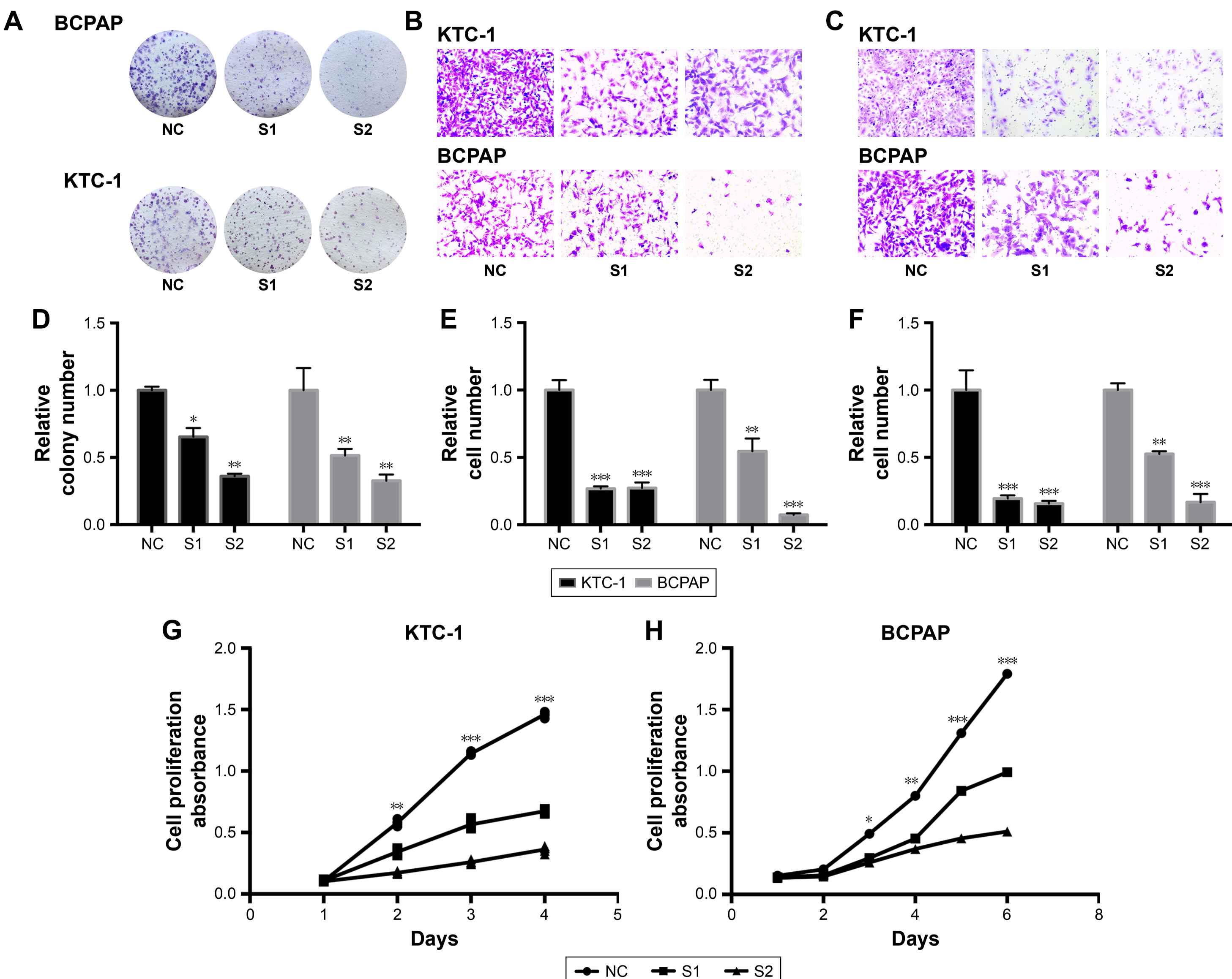

Figure 2 *CUX2* knockdown suppressed PTC cell tumorigenesis.

Notes: (**A**) Effects of *CUX2* knockdown on colony formation in BCPAP and KTC-1 cell lines. (**D**) Shows the mean colony number from all three independent experiments. (**B** and **C**) Capacity of migration (**B**) and invasion (**C**) of BCPAP and KTC-1 cells transfected with siRNA-*CUX2* and NC. (**E** and **F**) Show the mean value of migrating and invading cells from all three independent experiments of cell migration and invasion. (**G** and **H**) CCK8 assays were performed to demonstrate the proliferation of BCPAP (**G**) and KTC-1 (**H**) transfected with CUX2-siRNA and NC. *$P<0.05$; **$P<0.01$; ***$P<0.001$.

Abbreviations: NC, negative control; PTC, papillary thyroid cancer.

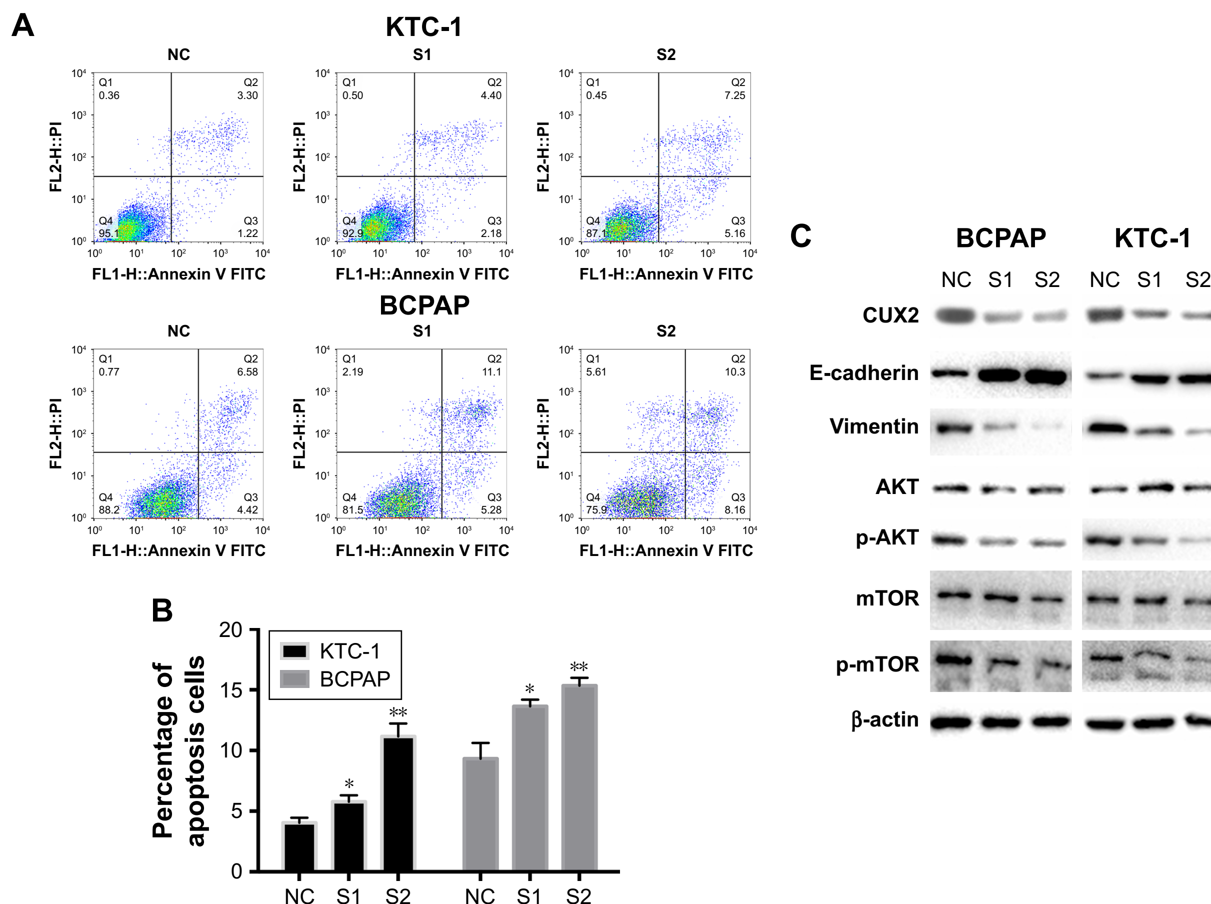

Figure 3 *CUX2* knockdown induced PTC cell line apoptosis, and the EMT and PI3K–AKT–mTOR pathways were involved in *CUX2*'s effect.
Notes: (**A**) Effects of *CUX2* knockdown on cell apoptosis. Cells transfected with siRNA had more apoptosis cells as determined by Annexin V/PI staining and flow cytometry analysis. (**B**) Data represent mean of the percentage of apoptosis cells from all three independent experiments. (**C**) PTC cells were transfected with siRNA-*CUX2* and NC. The expression and phosphorylation of key molecules of the pathways were assessed via Western blot analysis. β-Actin was used as an internal control. **P*<0.05; ***P*<0.01.
Abbreviations: EMT, epithelial–mesenchymal transition; NC, negative control; PI, propidium iodide; PTC, papillary thyroid cancer.

As critical downstream effectors, AKT and mTOR are frequently hyperactivated in human cancers.[42] To determine whether the PI3K–AKT–mTOR pathway is a potential cancer-related pathway of *CUX2* in TC cells, we further investigated the expression of relevant proteins by Western blot analysis. As shown in Figure 3C, the phosphorylation of AKT and mTOR decreased in both PTC cell lines transfected with *CUX2* siRNA, whereas total AKT and mTOR were not significantly altered. According to these results, we can speculate that the PI3K–AKT–mTOR pathway may be associated with the mechanism of *CUX2* in promoting tumorigenesis in PTC.

Discussion

In this study, our experimental evidence supports that the *CUX2* gene may play an oncogenic role in PTC cell lines. The results can be summarized as follows. First, we demonstrated that *CUX2* exhibits higher expression levels in PTC tissues and cell lines compared with the control. Moreover, we also found that *CUX2* knockdown attenuated the ability of proliferation and inhibited the migration and invasion. The suppression of *CUX2* can activate the apoptosis of PTC cell lines. Interestingly, this is reminiscent of the important role of *CUX2* in regulating proliferation and cell cycle length of the nervous system.[30] Pal et al previously found that *CUX2* knockdown reduces proliferation and increases apoptosis in breast cancer cells.[35]

In addition, the credible data strongly implied that *CUX1* and *CUX2* displayed a highly conserved sequence and thus exhibited very similar molecular functions.[10,11] Previously, experimental evidence showed the role of *CUX1* in promoting cell migration and invasion in numerous tumor cells by regulating TGF-β signaling and EMT.[18,43] The EMT process has been proven to have a strong association with cancer invasion and migration.[44] Meanwhile, our data revealed that *CUX2* silencing results in E-cadherin upregulation and vimentin downregulation. Therefore, we presumed that *CUX2* may play an important role in PTC cell metastasis via reversing the EMT process. Furthermore, a previous report on *CUX1*

as a downstream effector of the PI3K–AKT pathway can confer resistance to apoptosis in pancreatic cancer,[45] lending credence to the possibility of *CUX2* regulating TPC cells' function via the PI3K–AKT–mTOR pathway. Meanwhile, previous reports demonstrated that the PI3K–AKT–mTOR pathway is one of the cardinal pathways in most malignancies, including TC.[46–48] The PI3K–AKT–mTOR pathways have been demonstrated to regulate cell proliferation, apoptosis, and differentiation. We found that when *CUX2* expression levels changed, phosphorylated AKT and mTOR were altered compared with total AKT and mTOR. Altogether, these results suggest that *CUX2* may affect the protein phosphorylation of the PI3K–AKT–mTOR pathway and promote PTC tumorigenesis.

Moreover, the present observations provide evidence that *CUX2* may function as a tumor-promotive gene in regulating PTC cell proliferation and progression. However, this work has some limitations. First, we analyzed the association between *CUX2* expression and clinicopathological features in our clinical samples but failed to find a statistically significant relationship. This is probably due to the small number of patients. Second, whether *CUX2* expression can influence the tumorigenesis of PTC cells in vivo should be further studied. In addition, previous studies have verified that *CUX* gene knockdown impairs the function of repairing oxidative DNA. In RAS-driven tumor cells, which tend to generate higher levels of ROS, *CUX1* knockdown could exhibit synthetic lethal effect in cancer cells.[35,49] In this regard, exploring the relationship between *CUX2* and RAS mutation in TC may be interesting.

Conclusion

CUX2 may act as a tumor promoter gene in PTC via regulating EMT and influencing the phosphorylation of AKT and mTOR in the PI3K–AKT–mTOR pathways.

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Disclosure

The authors report no conflicts of interest in this work.

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