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Original Article

PINX1 promotes malignant transformation of thyroid cancer through the activation of the AKT/MAPK/β-catenin signaling pathway

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Abstract: Although thyroid cancer is the most prevalent endocrine malignancy, overall patients with thyroid cancer have a good long-term survival. However, a small percentage of patients with progressive thyroid cancer have poor outcomes, and the genetic drivers playing a key role thyroid cancer progression are mostly unknown. Here, we investigated the role of the PINX1 in thyroid cancer progression. Interestingly, PINX1 expression was significantly higher in ATC than in PTC in both patients and cell lines. When PINX1 was knockdown in ATC cells, cell proliferation rates, colony formation capacity, and cell cycle progression were significantly reduced. Furthermore, cell motility and the expression of EMT drivers were reduced by PINX1 downregulation. In contrast, the overexpression of PINX1 in PTC cells significantly increased those phenotypes of tumor progression, which demonstrates that PINX1 could promote tumor proliferation and malignant transformation in both PTC and ATC cells. To further understand whether PINX1 is also involved in the progression of PTC to ATC, we examined PI3K/AKT, MAPK, and β-catenin signaling activation after PINX1 modulation. Decreased PINX1 expression reduced the levels of p-AKT, p-ERK, p-p38, and β-catenin in ATC cells, but the increase of PINX1 expression upregulated the phosphorylation of AKT, ERK, and p38 and the levels of β-catenin in PTC cells. These results were all confirmed in xenograft mouse tumors. Our findings suggest that PINX1 regulates thyroid cancer progression by promoting cell proliferation, EMT, and signaling activation, and support the hypothesis that PINX1 could be a prognostic marker and a therapeutic target of thyroid cancer.

Keywords: ATC (Anaplastic Thyroid Carcinoma), PTC (Papillary Thyroid Carcinoma), PINX1 (PIN2/TERF1 interacting telomerase inhibitor 1), EMT, PTC-to-ATC progression

Introduction

Thyroid cancer is the most frequent malignancy of the human endocrine system, and its incidence has been increasing rapidly compared to other cancer types [1]. Although surgery and radioiodine remnant ablation have shown evident efficiency in thyroid cancer, their application is limited to non-metastasized and well-differentiated thyroid carcinoma [2, 3]. Aggressive thyroid cancer, being metastatic and relapsed, is challenging, and investigation of the molecular mechanisms of thyroid cancer progression could help identify new diagnostic and therapeutic approaches. WHO classification subdivides thyroid cancer by the cell of origin and histopathological characteristics into well differentiated thyroid cancers such as papillary thyroid carcinoma (PTC), follicular thyroid carcinoma (FTC) and undifferentiated carcinoma like anaplastic thyroid carcinoma (ATC) as well as poorly differentiated thyroid carcinoma (PDTC) representing intermediate disease entity between well differentiated carcinoma and undifferentiated carcinoma. Differentiated thyroid carcinoma, accounts for 70-80% of thyroid cancers and has a favorable patient survival.

On the other hand, ATC is highly proliferative and invades the surrounding tissues readily; it is less responsive to treatments and represents less than 5% of all thyroid cancers [4]. In addition, previous reports have supported that aggressive ATC could arise from preexisting

PTC less frequently, and it is associated with a poor prognosis [5, 6]. The reasons that a small portion of well-differentiated thyroid cancer, especially PTC, shows more aggressive clinical behavior and which factors are involved in PTC-to-ATC progression, are not fully understood.

Pin2/TRF1 interacting protein X1 (PINX1) was reported at first as a potential telomerase inhibitor that closely interacts with telomerase reverse transcriptase (TERT) and telomerase RNA component [7, 8]. PINX1 is located in the chromosome region 8p23, where deletions are frequent in human malignancies; downregulation of PINX1 is correlated with poor clinical outcomes in several cancer types, including non-small cell lung cancer, colorectal cancer, breast cancer, prostate cancer, and ovarian cancer [9-12]. In contrast, in some malignant tumors such as gliomas, cervical squamous cell carcinomas, and esophageal squamous cell carcinomas, PINX1 was suggested as a possible tumor-promoting factor associated with poor outcome in cancer patients [13-15]. We have also previously reported that the expression of PINX1 is correlated with poor prognostic factors in patients with PTC [16].

This study confirmed that PINX1 expression is associated with poor prognostic factors, including tumor size, lymph node metastasis, and recurrence; therefore, PINX1 could be used as a useful prognostic marker in PTC patients. Although there have been many reports on the prognostic importance of PINX1 in malignancies, its functional role in the tumor progression of thyroid cancer has not been uncovered yet.

In this study, we investigated whether PINX1 can promote thyroid cancer progression. Interestingly, we found that PINX1 expression was higher in ATC patients than in PTC in patient samples and cell lines. PINX1 has been shown to affect cell proliferation, cell cycle progression, migration, and epithelial-mesenchymal transition (EMT) *in vitro* and *in vivo*. Furthermore, PINX1 expression activated some signaling pathways involved in PTC-to-ATC progression. Our results revealed that PINX1 might accelerate the tumor progression of thyroid cancer and could be considered a potential therapeutic target for the treatment of aggressive thyroid cancer.

Materials and methods

Patient

We selected 28 cases of ATC and 175 cases of PTC for this study, based on the patients' electronic medical records who had samples of paraffin blocks and slides of at the Department of Pathology at the Korea Cancer Center Hospital (Seoul, Korea). This study was approved by the Institutional Review Board of the Korea Cancer Center Hospital. Tissue microarrays (TMAs, 2 mm and 4 mm diameter) were extracted from donor blocks and placed in a new recipient paraffin block using a trephine apparatus (SuperBioChips Laboratories, Seoul, Korea). The TMAs consisted of 28 cases of ATC, 175 cases of PTC, and two normal thyroid tissues. We also selected 3 samples with concomitant ATC and PTC component in same mass among the 28 cases of ATC cases to compare the difference of PINX1 protein and mRNA expression according to the histological component.

Cell lines, cell culture, and transfection

Two human anaplastic thyroid cancer cell lines (8505C and SNU-80) and two human papillary thyroid cancer cell lines (BCPAP and SNU-79) were cultivated in RPMI-1640 (Lonza) medium with 10% fetal bovine serum (FBS; Corning) and gentamycin (50 μg/mL; Lonza) at 37°C and 95% air/5% CO_2. 8505C and SNU-80 cell lines were transfected with siRNA specific for PINX1 (SMARTpool siRNA from Dharmacon) using Lipofectamine RNAi MAX (Life Technologies) system. After 24 h, cells were treated with fresh medium and used for further studies. BCPAP and SNU-790 cells were transfected with the PINX1 expression vector obtained from Origene, using Lipofectamine 2000 (Life Technologies). After 6 h, the cells were treated with fresh medium and processed for further investigation.

Immunohistochemistry

Immunohistochemical staining was performed with the automated immunostainer. (Lab Vision™ Auto Stainer 480S, Fremont, CA, USA) following manufacturer's instruction. Antigen retrieval after deparaffinization by heating to 98°C for 20 min using module buffer 1 (Thermo Scientific, Fremont, CA, USA) was performed

and we used the UltraVision LP Detection System (Thermo Scientific, Fremont, CA, USA) for specific detection of IgG antibodies. PINX1 antibody (12368-1-AP; Proteintech, Rosemont, IL, USA, 1:100) was treated and incubated for 60 min at RT. HRP polymer (Thermo Scientific, Fremont, CA, USA) and DAB chromogen-substrate were used after treatment of the primary antibody enhancer (Thermo Scientific, Fremont, CA, USA). Counterstaining was performed by applying Gill2-hematoxylin for 1 s and the slides were mounted. Staining with skin tissue and without PINX1 antibody was used as a positive and negative controls.

Real-time qRT-PCR

Total RNAs from FFPE (Formalin Fixed Paraffin Embedded) thyroid cancer specimens were isolated using an RNeasy FFPE kit (QIAGEN, Hilden, Germany), which were proceeded to reverse transcription for synthesizing cDNA by the SuperScript® III First-Strand Synthesis System (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. All the reaction components and specific primers were mixed and distributed into a real-time PCR plate (LightCycler® 480 Multiwell Plates 96; 04729692001, Loche, Mannheim, Germany). All PCR reagents were from FastStart Essential DNA Green Master (06402712001, Roche, Mannheim, Germany). mRNA levels were measured in triplicated reaction. Quantitative RT-PCR was performed through the LightCycler® 96 Real-Time PCR System (Roche, Mannheim, Germany). PCR was performed for 40 cycles of 10 s at 95°C, 10 s at 60°C, and 10 s at 72°C, followed by thermal denaturation. Expression levels of PINX1 and GAPDH were determined using the $2^{-\Delta\Delta CT}$ method. To simplify the data presentation, the relative expression values were multiplied by 10^2. The primers used for the real-time qRT-PCR: PINX1, forward, 5'-CCA GAG AAC GAA ACC ACG-3', reverse, 5'-ACC TGC GTC TCA GAA ATG TCA-3'; GAPDH, forward, 5'-GGA CTC ATG ACC ACA GTC CAT GCC-3', reverse, 5'-TCA GGG ATG ACC TTG CCC ACA G-3'.

Western blot assay

Cells and tumor tissues were lysed by the radioimmunoprecipitation assay lysis buffer (50 mM Tris, pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM dithiothreitol (DTT), 20 mM EGTA, 25 mM NaF, 1 mM Na_3VO_4, 5 U/mL aprotinin, and 0.3 mM phenylmethanesulfonyl fluoride (PMSF)); protein concentration in the lysates were determined using a BioRad protein assay kit (BioRad Laboratories). Protein samples were denatured with SDS-PAGE, transferred to a nitrocellulose membrane (Amersham), and then blocked with 5% skim milk in TBST (10 mM Tris, 100 mM NaCl, and 0.1% Tween 20) for 1 h by rocking at RT. Next, blots were incubated with primary antibodies overnight at 4°C, washed with TBST for 3 times, and subsequently applied with the specific HRP-conjugated anti-mouse IgG or anti-rabbit IgG (Cell Signaling Technology). Bands were visualized with ECL Plus Western Blotting Detection Reagents (GE Healthcare). Antibodies specific for the following proteins were used for western blotting: PINX1 (12368-1-AP, Proteintech); phosphor-p38 (4631, Cell Signaling Technology), phosphor-ERK (4370, Cell Signaling Technology), β-catenin (8480, Cell Signaling Technology); phospho-AKT (7985R, Santa Cruz Biotechnology), E-cadherin (71008, Santa Cruz Biotechnology), β-actin (47778, Santa Cruz Biotechnology); phosphor-mTOR (ab109268, Abcam), snail (ab180714, Abcam), twist (ab49254, Abcam), vimentin (ab8978, Abcam), Fibronectin (ab2413, Abcam); N-cadherin (610921, BD Bioscience); and Zeb1 (HPA027524, Sigma).

Cell proliferation assay

The effect of PINX1 regulation on cell proliferation was tested in PINX1 knockdown or overexpressing cells; 1×10^4 cells of each cell line were seeded. Survived cells were counted and analyzed by trypan blue exclusion every day.

Colony formation assay

5×10^2 cells were seeded into 35-mm dishes and cultivated for 24 h. The cells were treated with the PINX1 siRNA or PINX1 overexpression vector for 24 h and then allowed to grow for 7-14 days. After fixing with 10% methanol and 10% acetic acid, the cells were incubated with 0.5% crystal violet for 10 min at RT for staining, washed with water, and dried up overnight.

Cell cycle assay

8505C and SNU-80 cells were treated with control siRNA or PINX1 siRNA for 48 h. BCPAP and SNU-790 cells were transfected with an empty vector (control) or PINX1 expressing vector for 48 h. After each treatment and incubation, cells were trypsinized, fixed with ice-cold

Table 1. PINX1 expression and correlation between papillary and anaplastic thyroid carcinoma

PINX1 expression	Histological type		*P*-value
	PTC (%)	ATC (%)	
Negative	144 (82.2)	17 (60.7)	0.009
Positive	31 (17.8)	11 (39.3)	
Total	175 (100)	28 (100)	

70% ethanol. Following the washing with PBS, cells were incubated with 10 mg/mL propidium iodide (Sigma) with 100 mg/mL RNase (Sigma) for 30 min at 37°C. BD FACS CANTO II flow cytometer (BD Biosciences) was used for analyzing the cells population stained with propidium iodide. At least 10,000 cells were measured for each sample, and cell cycle distributions were investigated through the BD FACSuite software.

Transwell migration assay

A migration assay was performed to check the effect of PINX1 regulation on cell motility *in vitro*. The cells were transfected with siRNA or expression vector for PINX1 modulation, subsequently seeded into the insert chambers of Transwells (Corning) in serum-free medium and the outer plate was filled with the medium supplemented with 10% FBS as attractant. After 72 h, to visualize and analyze the cells migrating into the counterpart, the Transwell membranes were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet (Sigma-Aldrich). The stained cells were counted under a light microscope.

Wound-healing assay

To investigate whether PINX1 could enhance cell motility, would-healing assay was performed. After culturing the cells to 80% confluency, the cell monolayers were scratched with a 200 μL pipette tip, after which both cell lines were further incubated with fresh medium without treatment for 24 h. Olympus IX71 inverted microscope (Olympus Optical Co. Ltd.) was used for taking photomicrographs in 100x magnification.

Mouse xenograft studies

All animal experiments were performed in accordance with the Korea Institute of Radiological and Medical Science (KIRAMS) Animal Care and Use Committee (Seoul, Korea). For xenograft experiments, 1×10^6 of 8505C cells or 1.5×10^6 BCPAP cells were subcutaneously injected into the right flank of 6- to 8-week-old male athymic nude mice purchased from Orient Bio. Tumor sizes were measured once a week using calipers, and volume was calculated as 1/2 × long diameter × short diameter^2.

Statistical analysis

All numerical data are presented as the mean ± standard deviation (SD) from at least triplicated experiments, which were analyzed by a one-way ANOVA for ranked data with a Tukey's honestly significant difference test, and a two-way ANOVA for ranked data followed by a Bonferroni post-test. Prism 5 software (GraphPad Software) was used for all statistical analyses. Pearson's chi-squared test was performed using SPSS ver. 18 (SPSS Inc., Chicago, IL) to assess the significance of PINX1 expression when comparing ATC and PTC samples. A *P*-value <0.05 was considered statistically significant.

Results

Higher expression of PINX1 in ATC than in PTC

To investigate the expression pattern of PINX1 in ATC and PTC, we assessed the protein and mRNA levels of PINX1 in both ATC and PTC patient tissues. PINX1 immunoreactivity occurs mainly in the cytoplasm but can also be observed in the nucleus. Immunoreactivity was evaluated by both the intensity (0: negative; 1: low intensity; 2: high intensity) and the percentage of immune-reactive cells [1: (0%-25%), 2: (26%-50%), 3: (51%-75%), 4: (76%-100%)]. Immunoreactive score (IRS) multiplying the scores of staining intensity and the percentage of positive cells was used to evaluate level of PINX1 staining. Based on the immunoreactive score (IRS), the PINX1 immunoreactivity was categorized as negative (IRS: 0-4) or positive (IRS: 6 and 8). As shown in **Table 1**, the proportion of patients with positive PINX1 expression was significantly higher in ATC patients than in PTC patients (P=0.009). We confirmed that protein and mRNA expression of PINX1 was higher in ATC samples than in PTC samples (**Figure 1A** and **1B**). To verify the increase of PINX1 in ATC *in vitro*, we assessed the expres-

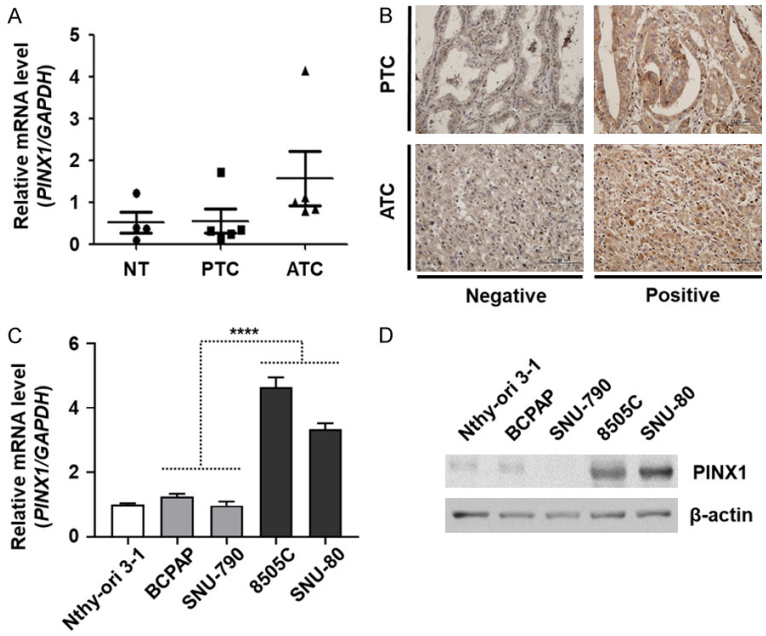

Figure 1. The higher expression of PINX1 in ATC than in PTC. A. Relative mRNA expression levels of PINX1 in patient tissues. Total RNA was isolated from FFPE thyroid cancer tissues, which were proceeded in real-time qRT-PCR. B. Immunohistochemistry (IHC) in papillary thyroid carcinoma (PTC) and anaplastic thyroid carcinoma (ATC) patient tissues. Representative negative and positive IHC staining images of PINX1 are shown. Scale bars represent 100 μm. C. The levels of PINX1 expression in normal thyroid cell line and thyroid cancer cell lines were investigated by real-time qRT-PCR. Nthy-ori 3-1 cell was selected for normal thyroid cell line, BCPAP and SNU-790 cells were used for representative PTC cell lines, and 8505C and SNU-80 cells were selected for ATC cell lines. *P* values were determined by one-way ANOVA. (****: $P<0.0001$). D. PINX1 expression was confirmed in normal thyroid, PTC and ATC cell lines. The level of β-actin is used as internal quantitative control.

sion of PINX1 in PTC cell lines, BCPAP and SNU790, and ATC cell lines, 8505C, and SNU-80. Interestingly, mRNA and protein expression of PINX1 was significantly higher in ATC cell lines than in PTC cell lines (**Figure 1C** and **1D**). On the other hand, PINX1 expression in normal thyroid cell line, Nthy-ori 3-1, was significantly lower than ATC cell lines, but not meaningfully different with PTC cells. These results indicate that PINX1 is increased in ATC compared to PTC in both patient tissues and cell lines.

PINX1 promotes the proliferation of thyroid cancer cells

To understand the effects of PINX1 expression on cell proliferation, we transfected PINX1 siRNA and PINX1-expressing vector to ATC and PTC cell lines, respectively (**Figure 2A** and **2B**). As shown in **Figure 2C** and **2D**, cell proliferation after PINX1 downregulation was significantly decreased in ATC cell lines, whereas it was increased in PINX1 overexpressing PTC cell lines. Colony formation was also reduced after transfection with PINX1 siRNA in ATC cell lines and increased after transfection with the PINX1 expression vector in PTC cell lines (**Figure 2E** and **2F**). We further investigated the effect of PINX1 regulation in thyroid cancer cells proliferation by testing whether cell cycle status could also be altered by PINX1 modulation in vitro, by PI-staining based cell cycle analysis. When the expression of PINX1 was abrogated in ATC cell lines, cell cycle progression was inhibited and arrested at the G1 phase (**Figure 2G**). In contrast, cell cycle progressed in PTC cell lines over expressing PINX1 (**Figure 2H**). These findings indicate that PINX1 can efficiently promote cell proliferation and cell cycle progression in both PTC and ATC cells.

PINX1 promotes cell migration and EMT in vitro

PINX1 was associated with a poor prognosis and advanced tumor grades in several cancer types [13, 14]. We investigated whether PINX1 expression can augment cell migration and EMT in thyroid cancer cells. As shown in **Figure 3A** and **3B**, the migration of ATC cells was significantly reduced after transfection with PINX1-specific siRNA. In contrast, cell migration was increased after PINX1 overexpression in PTC cell lines (**Figure 3C** and **3D**). Wound closure was slower in ATC cells transfected with PINX1 siRNA (**Figure 3E**). In contrast, wound healing was faster in PTC cells overexpressing PINX1 (**Figure 3F**). To investigate whether PINX1 is responsible for EMT, the expression levels of Twist, Zeb1, and Snail, which are key transcription factors of EMT, were assessed by western blot assay. As shown in **Figure 3F** and **3G**, the expression of Snail was significantly decreased by treatment with PINX1 siRNA in

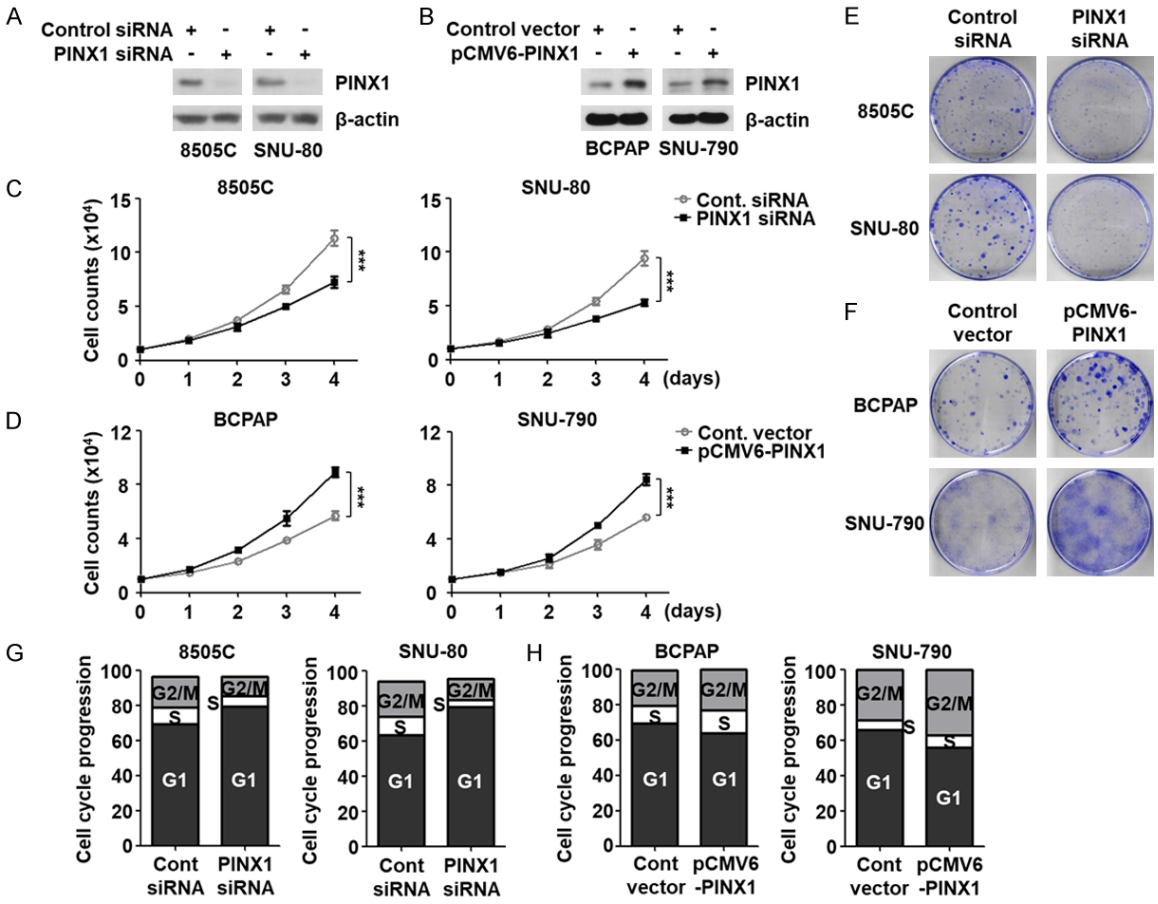

Figure 2. PINX1 promotes the proliferation of thyroid cancer cells. (A and B) The expression levels of PINX1 were assessed after PINX1 gene regulation. The decrease in PINX1 expression after PINX1 knockdown in ATC cell lines (A) and the increase in PINX1 levels after PINX1 overexpression in PTC cell lines (B) were confirmed by western blotting. (C and D) The effect of PINX1 regulation on cell proliferation was analyzed by a proliferation assay. 1.0 × 10^4 cells were seeded after PINX1 downregulation (C) or PINX1 overexpression (D), and the number of cells was checked for every 24 h. *P* values were determined by two-way ANOVA. (***: $P<0.001$). (E and F) The effect of gene regulation of PINX1 on cell proliferation was examined by colony formation assay. (G and H) Effect of PINX1 knockdown or PINX1 overexpression on cell cycle progression was assessed with flow cytometry after PI staining.

ATC cells and increased by PINX1 overexpression in PTC cells. Twist and Zeb1 expression were partially regulated by PINX1 expression in both types of thyroid cancer cells. The levels of E-cadherin, an epithelial marker, increased after PINX1 knockdown in ATC cells and decreased after transfection of the PINX1 expression vector in PTC cells. Moreover, the levels of fibronectin, N-cadherin, and vimentin, which are mesenchymal markers, decreased after transfection of PINX1 siRNA in ATC cells and increased with PINX1 overexpression in PTC cells (**Figure 3F** and **3G**). When we checked the effects of PINX1 knockdown in PTC cell lines to clarify its role in PTC, the expression of Snail was significantly decreased, but cell migration and the other EMT markers did not show meaningful changes (data not shown). These results showed that PINX1 plays a key role in cell motility alteration and EMT induction, which are well-known critical factors of tumor metastasis.

PINX1 promotes proliferation and aggressiveness of thyroid cancer cells in mouse xenografts

To investigate the effects of PINX1 on cell proliferation and EMT *in vivo*, we subcutaneously injected 8505C and BCPAP cells with or without regulation of PINX1 expression and checked tumor volume and EMT marker expression in xenografts. Using the lentiviral constructs, 8505C and BCPAP stable knockdowns and PINX1-overexpressing cell lines were estab-

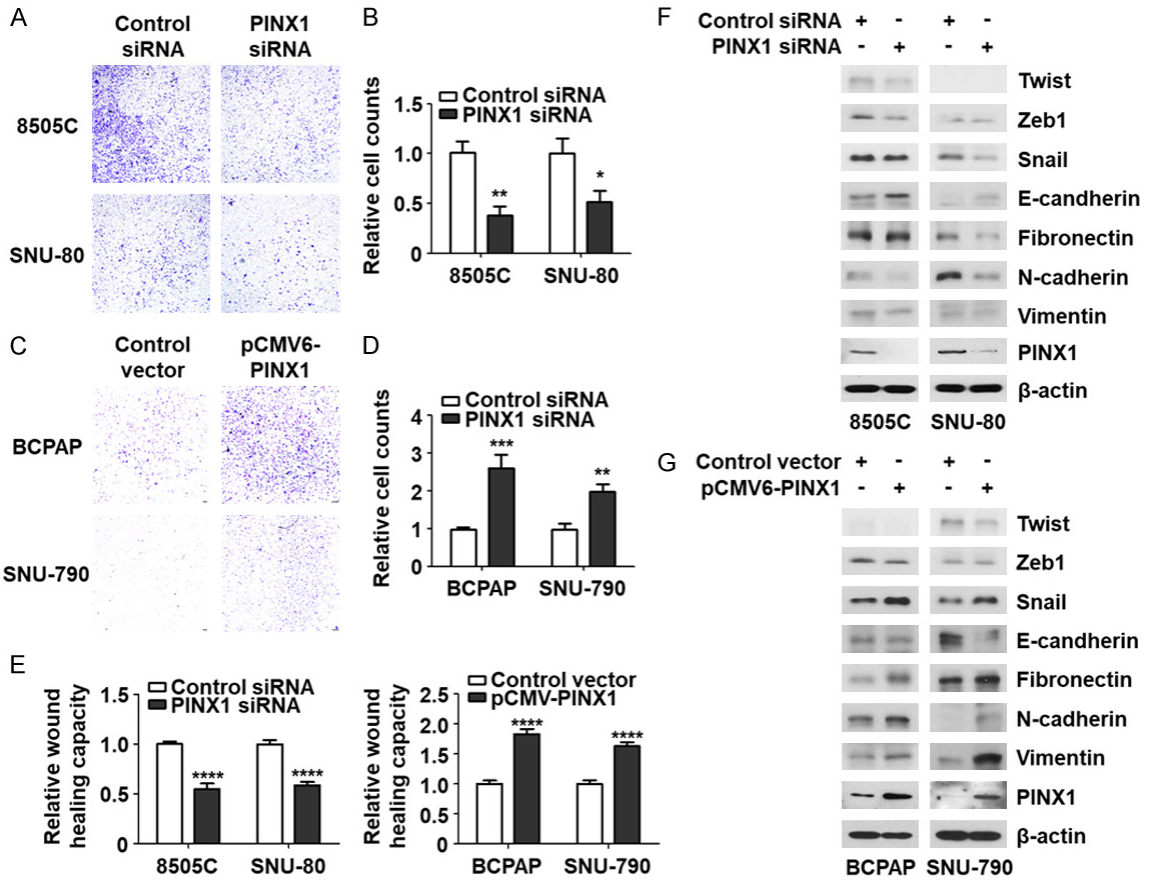

Figure 3. PINX1 promotes cell migration and EMT *in vitro.* (A-D) The effect of PINX1 downregulation and PINX1 overexpression on cell motility was assessed with the transwell migration assay. Representative images of cell migration after PINX1 gene regulation are shown in (A and C). The relative cell counts in the migrated area of transwell are shown in (B and D). (E) Wound healing capacity after PINX1 knockdown was checked in 8505C and SNU-80 cell lines. (F and G) Effect of PINX1 knockdown or PINX1 upregulation on EMT markers expression was analyzed by western blotting. Statistical analyses were performed by unpaired 2-tailed t test for (B-E) (**P*<0.05; ***P*<0.01; ****P*<0.001; *****P*<0.0001).

lished (**Figure 4A** and **4B**). As shown in **Figure 4C** and **4D**, knockdown of PINX1 significantly decreased tumor proliferation in xenograft mice. On the other hand, PINX1 overexpression showed apparent effects of an increase in tumor volume in BCPAP xenografts. We confirmed the effects of PINX1 on tumor proliferation by Ki-67 staining. PINX1 downregulation in 8505C xenografts decreased the levels of Ki-67, but PINX1 induction in BCPAP xenografts increased Ki-67 staining (**Figure 4E**). These results suggest that PINX1 expression is positively correlated with tumor proliferation in an *in vivo* xenograft mouse model. To further confirm the effects of PINX1 expression on EMT *in vivo*, we examined the expression levels of EMT modulators in xenograft tumors. When the expression of PINX1 decreased, the levels of Zeb1, Snail, and Twist decreased in 8505C xenografts (**Figure 4F**). Moreover, the overexpression of PINX1 significantly increased the levels of Zeb1, Snail, and Twist in BCPAP xenograft tumors (**Figure 4G**). Through these findings, we confirmed that PINX1 could regulate cell proliferation and metastatic conversion in an *in vivo* mouse model.

PINX1 activates signaling pathways which promote PTC-to-ATC transition in vitro and in vivo

ATC and PDTC develop as a consequence of step-wise dedifferentiation of preexisting well-differentiated thyroid cancer, including PTC [17-19]. To explore the role of PINX1 in PTC-to-ATC progression, we investigated the expression of PINX1 in the ATC patients having PTC compo-

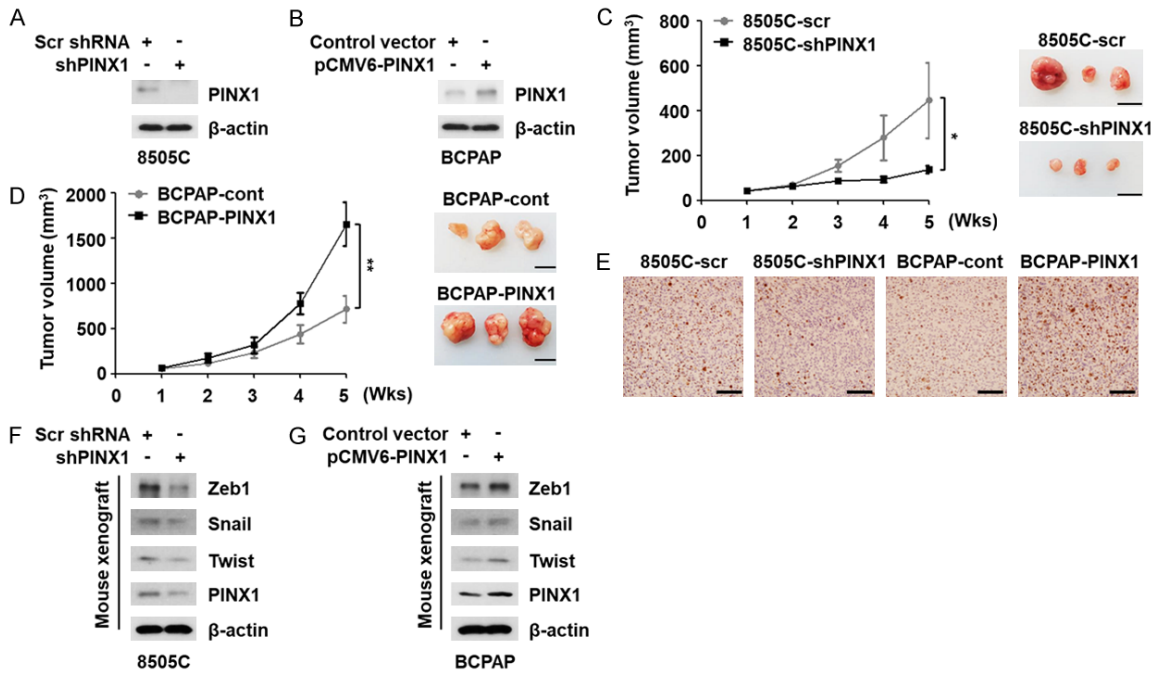

Figure 4. PINX1 promotes proliferation and aggressiveness of thyroid cancer cells in mouse xenografts. (A and B) The levels of PINX1 after PINX1 knockdown or overexpression were assessed with western blotting. 8505C and BCPAP cells were used for the mouse xenografts of ATC or PTC cell lines, respectively. (C and D) Effect of PINX1 expression on tumor growth in mice xenografts. The tumor size is shown in (C and E) The volume of xenograft tumor was measured every week. Error bars represent SEM for tumor volume (n=5). Statistical analysis was performed by 2-way ANOVA (*P<0.05; **P<0.01). (E) The effect of PINX1 downregulation or overexpression on tumor proliferation was analyzed by Ki-67 staining. (F and G) The effect of PINX1 regulation on EMT key modulators was examined by western blotting using xenograft tumors.

nent in the specimens. As indicated in **Figure 5A**, 2 out of 3 patients showed drastically higher expression level of PINX1 in ATC compared to its expression level in PTC. The signaling pathways that are responsible for the malignant conversion of PTC to ATC are the PI3K/AKT pathway, the MAPK pathway, and the Wnt/β-catenin pathway [20, 21]. We investigated whether PINX1 activates these signaling pathways *in vitro* and *in vivo*. Interestingly, the decrease in PINX1 expression dramatically reduced the levels of phospho-AKT, phospho-p38, and phospho-ERK in ATC cell lines (**Figure 5B**). Induction of PINX1 in PTC cell lines also increased the phosphorylation levels of AKT, p38, and ERK (**Figure 5C**). To check whether PINX1 is involved in β-catenin signaling, we assessed the intracellular levels of β-catenin with or without the modulation of PINX1 expression. As shown in **Figure 5D**, β-catenin levels decreased after transfection of ATC cell lines with PINX1 siRNA. In contrast, PINX1 overexpression increased β-catenin levels in PTC cell lines (**Figure 5E**). In xenograft tumor samples, we found that PINX1 downregulation in 8505C slightly decreased the phosphorylated levels of AKT and significantly decreased ERK and p38 phosphorylation. The level of β-catenin was also found to be decreased by PINX1 knockdown (**Figure 5F**). In BCPAP xenografts, phospho-AKT and phospho-p38 levels were significantly increased after PINX1 induction. The β-catenin expression was increased by PINX1 overexpression (**Figure 5G**). In the absence of the exact model of PTC-to-ATC progression, these data suggest that PINX1 is highly expressed in ATC than in PTC, which could activate AKT, MAPK, and β-catenin signaling both *in vitro* and *in vivo*.

Discussion

Although most thyroid cancers are curable, a small number of patients with well-differentiated thyroid carcinoma, as well as most cases of patients with ATC, have a poor prognosis. The mechanistic basis of these aggressive thyroid cancers, which could support the discovery of a

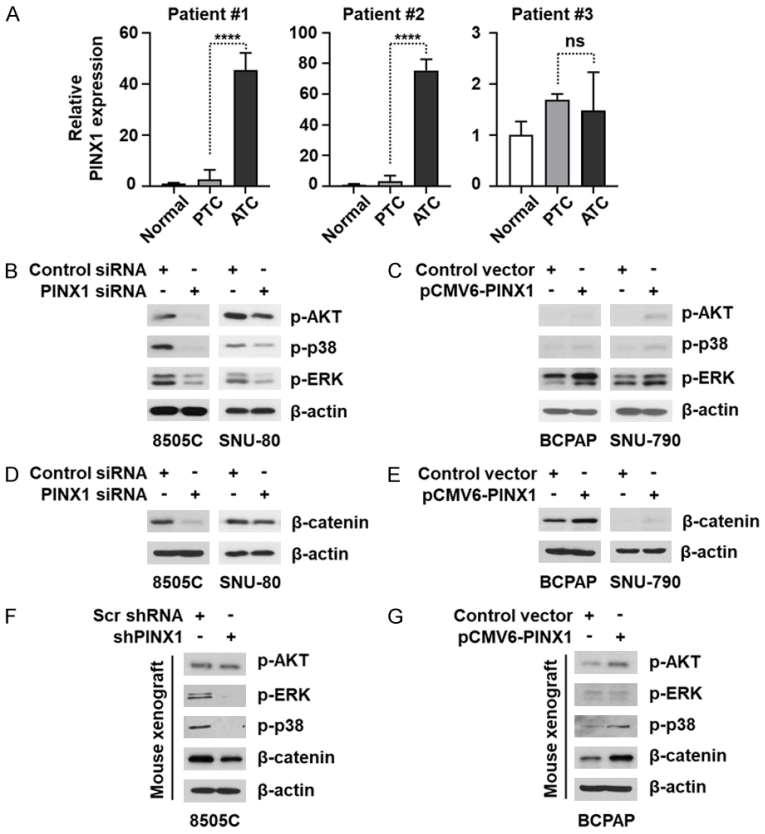

Figure 5. PINX1 activates signaling pathways promoting PTC-to-ATC transition *in vitro* and *in vivo*. (A) The relative expression level of PINX1 in patient specimens was compared between ATC and PTC component within a same case. Total RNA was isolated from FFPE samples, which were used for real-time qRT-PCR. Statistical analysis was performed by one-way ANOVA. (ns: not significant; ****P<0.0001). (B and C) Effect of PINX1 gene regulation on PI3K/AKT and MAPK signaling activation was analyzed by western blotting. The phosphorylation levels of AKT, p38, and ERK were assessed after PINX1 downregulation (A) or PINX1 overexpression (B) *in vitro*. (D and E) Effect of PINX1 knockdown and overexpression on Wnt/β-catenin signaling *in vitro* was confirmed by western blotting. (F and G) Effect of PINX1 gene expression on PI3K/AKT, MAPKs, and Wnt/β-catenin signaling activation in vivo was confirmed by western blotting of mice xenografts.

therapeutic agent for chemo-drugs and radioiodine, is largely unknown. Here, we investigated the roles of PINX1 in PTC and ATC. Our findings indicate that the expression level of PINX1 was significantly higher in ATC patients than in PTC patients. We confirmed this result in an *in vitro* system, suggesting that PINX1 expression was increased in ATC cell lines compared to PTC cell lines (**Figure 1**). The decrease of PINX1 expression in ATC cells significantly reduced cell proliferation and cell cycle progression *in vitro* and disrupted tumor proliferation in mouse xenografts while the induction of PINX1 in PTC cells promoted these characteristics *in vitro* and *in vivo* (**Figures 2** and **4**). These results suggest that the expression of PINX1 plays an important role in making thyroid cancer highly proliferative and aggzressive in both PTC and ATC.

EMT is crucial for tumor cells' resistance to treatment and the first step of tumor metastasis. Recently, it was reported that a small proportion of PTC patients and a high proportion of ATC patients with recurrence and metastases are more resistant to chemo- and radiotherapy [22]. For this reason, there have been many efforts to investigate EMT modulators that are important in thyroid cancer progression. In the same context, our findings revealed that PINX1 regulates cell migration and induces EMT in PTC and ATC cells (**Figures 3** and **4**). When PINX1 expression was disrupted, migration was significantly reduced in ATC cells. In contrast, PTC cells showed increased motility after PINX1 overexpression. Importantly, we also describe that PINX1 also regulates some key EMT inducers, including Snail, Twist, and Zeb1 *in vitro* and *in vivo*. These results mean that PINX1 could promote malignant transformation of thyroid cancer by increasing the motility of tumor cells through the facilitation of EMT expression. This finding is also supported by our previous study, where PINX1 expression was associated with tumor-progressing clinicopathological features of PTC patients, including lymph node metastasis and recurrence [16]. Similarly, we show that PINX1 promotes EMT and tumor cell proliferation, and may be correlated with metastasis and recurrence in PTC patients. Taken together, we suggest that PINX1 is a potential therapeutic target and prognostic marker for thyroid cancer progression.

In thyroid carcinogenesis, BRAF V600E mutations and RET/PTC rearrangements are consid-

ered major oncogenic driving forces [23, 24]. Furthermore, PI3K/AKT signaling, MAPK signaling, and Wnt/β-catenin signaling have recently been proposed as important pathways for malignant progression of thyroid carcinomas [20, 21]. As shown in **Figure 5**, although we could not perform the statistical analysis among the samples because the cases were rare, we found that PINX1 level in ATC was dramatically higher than in PTC component within a same specimen. In addition, we observed that PINX1 regulates the phosphorylation of AKT, MAPKs-including ERK and p38-and intracellular levels of β-catenin in both PTC and ATC cells, although there were slight differences. This is the first time that we have shown that PINX1 could orchestrate signaling pathways involved in the malignant progression of thyroid cancer; however, we did not expand our findings to determine the mechanism of signaling activation by PINX1.

In this study, we investigated in detail the functional roles of PINX1 in thyroid cancer progression; there are limitations regarding how PINX1 could be involved in all those functions, including cell proliferation, EMT, and signaling pathways of aggressive progression. PINX1 consists of two functional domains. The first is the telomerase inhibitory domain (TID) located at its C-terminal region, which interacts with TRF1 and TERT to perform its normal function, which is to stabilize telomerase [25, 26]. The other is the glycine-rich patch (G-patch) domain at the N-terminal region. The G-patch domain in PINX1 was reported to have no effect on telomerase activity [26, 27]. It was also reported that the G-patch domain blocks PINX1 migration to the nuclear region [28]. The G-patch domain and the G-patch domain-containing proteins have been suggested to perform the RNA-binding and processing [29]. Furthermore, their role in miRNA and non-coding RNA biogenesis, which is one of the most important systems of gene expression regulation, has also been reported [19, 30]. We hypothesized that the G-patch domain allows PINX1 to play an extra-telomeric role, which can promote proliferation, EMT, and activate signaling pathways for thyroid cancer progression. Further detailed investigation of the mechanism of the G-patch domain of PINX1 is needed.

In this study, we propose for the first time that PINX1 is an important regulator of thyroid cancer progression. Our findings show that PINX1 enhances thyroid cancer cell proliferation, cell cycle progression, cell motility, and EMT *in vitro* and *in vivo*. Moreover, we evaluated the possible role of PINX1 in the PI3K/AKT, MAPK, and Wnt/β-catenin signaling pathways, which have critical effects on PTC-to-ATC conversion. Taken together, we suggest that PINX1 might be a potential therapeutic target and a useful prognostic marker for aggressive thyroid cancer.

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Disclosure of conflict of interest

None.

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References

[1] Xing M. Molecular pathogenesis and mechanisms of thyroid cancer. Nat Rev Cancer 2013; 13: 184-199.
[2] Lebastchi AH and Callender GG. Thyroid cancer. Curr Probl Cancer 2014; 38: 48-74.
[3] Starenki D, Sosonkina N, Hong SK, Lloyd RV and Park JI. Mortalin (GRP75/HSPA9) promotes survival and proliferation of thyroid carcinoma cells. Int J Mol Sci 2019; 20: 2069.
[4] Foote RL, Molina JR, Kasperbauer JL, Lloyd RV, McIver B, Morris JC, Grant CS, Thompson GB, Richards ML, Hay ID, Smallridge RC and Bible KC. Enhanced survival in locoregionally confined anaplastic thyroid carcinoma: a single-institution experience using aggressive multimodal therapy. Thyroid 2011; 21: 25-30.
[5] Smallridge RC, Ain KB, Asa SL, Bible KC, Brierley JD, Burman KD, Kebebew E, Lee NY, Nikiforov YE, Rosenthal MS, Shah MH, Shaha AR and Tuttle RM. American Thyroid Association guidelines for management of patients with anaplastic thyroid cancer. Thyroid 2012; 22: 1104-1139.
[6] McFadden DG, Vernon A, Santiago PM, Martinez-McFaline R, Bhutkar A, Crowley DM, McMahon M, Sadow PM and Jacks T. p53 con-

strains progression to anaplastic thyroid carcinoma in a Braf-mutant mouse model of papillary thyroid cancer. Proc Natl Acad Sci U S A 2014; 111: E1600-1609.
[7] Zhou XZ and Lu KP. The Pin2/TRF1-interacting protein PinX1 is a potent telomerase inhibitor. Cell 2001; 107: 347-359.
[8] Banik SS and Counter CM. Characterization of interactions between PinX1 and human telomerase subunits hTERT and hTR. J Biol Chem 2004; 279: 51745-51748.
[9] Feng YZ, Zhang QY, Fu MT, Zhang ZF, Wei M, Zhou JY and Shi R. Low expression of PinX1 is associated with malignant behavior in basal-like breast cancer. Oncol Rep 2017; 38: 109-119.
[10] Tian XP, Jin XH, Li M, Huang WJ, Xie D and Zhang JX. The depletion of PinX1 involved in the tumorigenesis of non-small cell lung cancer promotes cell proliferation via p15/cyclin D1 pathway. Mol Cancer 2017; 16: 74.
[11] Jiang T, Li H, Jiang R, Li H, Hou P, Chen Y, Bai J and Song J. Pin2/TRF1binding protein X1 inhibits colorectal cancer cell migration and invasion in vitro and metastasis in vivo via the nuclear factorkappaB signaling pathway. Oncol Rep 2018; 40: 1533-1544.
[12] Shi R, Zhao Z, Zhou H, Wei M, Ma WL, Zhou JY and Tan WL. Reduced expression of PinX1 correlates to progressive features in patients with prostate cancer. Cancer Cell Int 2014; 14: 46.
[13] Bai J, Chen YS, Mei PJ, Liu QH, Du Y and Zheng JN. PinX1 is up-regulated and associated with poor patients' survival in gliomas. Int J Clin Exp Pathol 2015; 8: 6952-6959.
[14] Tian XP, Qian D, He LR, Huang H, Mai SJ, Li CP, Huang XX, Cai MY, Liao YJ, Kung HF, Zeng YX and Xie D. The telomere/telomerase binding factor PinX1 regulates paclitaxel sensitivity depending on spindle assembly checkpoint in human cervical squamous cell carcinomas. Cancer Lett 2014; 353: 104-114.
[15] Qian D, Zhang B, He LR, Cai MY, Mai SJ, Liao YJ, Liu YH, Lin MC, Bian XW, Zeng YX, Huang JJ, Kung HF and Xie D. The telomere/telomerase binding factor PinX1 is a new target to improve the radiotherapy effect of oesophageal squamous cell carcinomas. J Pathol 2013; 229: 765-774.
[16] Kang J, Han K, Kim HJ, Park JH, Kong JS, Park S and Myung JK. The clinical significance of PINX1 expression in papillary thyroid carcinoma. Hum Pathol 2018; 81: 176-183.
[17] Lee DY, Won JK, Lee SH, Park DJ, Jung KC, Sung MW, Wu HG, Kim KH, Park YJ and Hah JH. Changes of clinicopathologic characteristics and survival outcomes of anaplastic and poorly differentiated thyroid carcinoma. Thyroid 2016; 26: 404-413.
[18] Spires JR, Schwartz MR and Miller RH. Anaplastic thyroid carcinoma. Association with differentiated thyroid cancer. Arch Otolaryngol Head Neck Surg 1988; 114: 40-44.
[19] Ren G, Xie M, Dou Y, Zhang S, Zhang C and Yu B. Regulation of miRNA abundance by RNA binding protein TOUGH in Arabidopsis. Proc Natl Acad Sci U S A 2012; 109: 12817-12821.
[20] Xing M. Genetic alterations in the phosphatidylinositol-3 kinase/Akt pathway in thyroid cancer. Thyroid 2010; 20: 697-706.
[21] Sastre-Perona A and Santisteban P. Role of the wnt pathway in thyroid cancer. Front Endocrinol (Lausanne) 2012; 3: 31.
[22] Guo Z, Hardin H and Lloyd RV. Cancer stem-like cells and thyroid cancer. Endocr Relat Cancer 2014; 21: T285-300.
[23] Guerra A, Zeppa P, Bifulco M and Vitale M. Concomitant BRAF(V600E) mutation and RET/PTC rearrangement is a frequent occurrence in papillary thyroid carcinoma. Thyroid 2014; 24: 254-259.
[24] Henderson YC, Shellenberger TD, Williams MD, El-Naggar AK, Fredrick MJ, Cieply KM and Clayman GL. High rate of BRAF and RET/PTC dual mutations associated with recurrent papillary thyroid carcinoma. Clin Cancer Res 2009; 15: 485-491.
[25] Chen G, Da L, Xu Y, Xu M, Song L, Li T and Zhao M. C-terminal amino acids 290-328 of LPTS/PinX1 confer telomerase inhibition. Biochem Biophys Res Commun 2010; 398: 683-689.
[26] Li HL, Song J, Yong HM, Hou PF, Chen YS, Song WB, Bai J and Zheng JN. PinX1: structure, regulation and its functions in cancer. Oncotarget 2016; 7: 66267-66275.
[27] Zhang R, Zhao J, Wang X, Wang LL, Xu J and Song C. PinX1 without the G-patch motif suppresses proliferation, induces senescence, but does not inhibit telomerase activity in colorectal cancer SW480 cells. Oncol Rep 2014; 32: 286-292.
[28] Yoo JE, Oh BK and Park YN. Human PinX1 mediates TRF1 accumulation in nucleolus and enhances TRF1 binding to telomeres. J Mol Biol 2009; 388: 928-940.
[29] Aravind L and Koonin EV. G-patch: a new conserved domain in eukaryotic RNA-processing proteins and type D retroviral polyproteins. Trends Biochem Sci 1999; 24: 342-344.
[30] Zhang Y, Cheng YT, Bi D, Palma K and Li X. MOS2, a protein containing G-patch and KOW motifs, is essential for innate immunity in Arabidopsis thaliana. Curr Biol 2005; 15: 1936-1942.