Recombinant ING4 suppresses the migration of SW579 thyroid cancer cells via epithelial to mesenchymal transition

CHUAN-JIANG WANG, DONG YANG and YING-WEI LUO

Department of Vascular and Thyroid Surgery, The First Affiliated Hospital of China Medical University, Shenyang, Liaoning 110001, P.R. China

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Abstract. Thyroid cancer is a common endocrine malignancy that has rapidly increased in global incidence. Inhibitor of growth 4 (ING4) has been identified in various types of carcinoma; however, to the best of our knowledge, no previous studies have investigated the effects of ING4 on thyroid cancer. In the present study, SW579 thyroid cancer cells were treated with recombinant ING4 protein, and the results confirmed that recombinant ING4 protein was able to reduce the rate of proliferation, increase the rate of apoptosis and inhibit the mobility of SW579 cells. These results were obtained using a colony formation, fluoroscein isothiocyanate/propidium iodide double staining and Transwell assays, respectively. Furthermore, in the western blot analysis assays, ING4 was demonstrated to inhibit the Wnt/β catenin signaling pathway and epithelial to mesenchymal transition (EMT). Therefore, the present study demonstrated the antitumor activities of recombinant ING4 and identified ING4 could inhibit EMT in thyroid cancer cell. However, additional studies are required to confirm these results in other cell types.

Introduction

Inhibitor of growth 4 (ING4) is located on chromosome 12p13 and encodes a 249-amino acid protein containing a novel conserved region in the N terminus, a nuclear localization signal in the central region and a highly conserved plant homeodomain in the C terminus (1). Downregulation of ING4 has been reported in various types of cancer, including hepatocellular and gastric carcinomas, and breast, colon and lung cancers (2-6). Previous studies have also demonstrated that ING4 is involved in a variety of cellular processes, including cell proliferation, apoptosis, migration, angiogenesis and the DNA damage response (7-10).

In epithelial to mesenchymal transition (EMT), epithelial cells transform into a mesenchymal phenotype, which involves an increase in fibroid morphology, invasiveness and resistance to apoptosis, as well as an increase in extracellular matrix components (11). Increasing evidence indicates that cancer cells acquire invasive properties by EMT (12-15).

Thyroid cancer is a common endocrine malignancy that has exhibited a rapid increase in global incidence in recent decades (16). However, the roles of recombinant ING4 protein in thyroid cancer remain unclear. Therefore, in the present study, the effect of recombinant ING4 on the growth, mobility and apoptosis of thyroid cancer cells was investigated, as well as the association between ING4 and EMT.

Materials and methods

Cell culture. SW579, a human thyroid cancer cell line, was purchased from the Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China). The cells were cultured at 37˚C with 5% (v/v) CO_2 in Dulbecco's modified Eagle's medium (DMEM; Life Technologies, Gaithersburg, MD, USA), which was supplemented with 10% (v/v) fetal calf serum (FCS; Life Technologies) and antibiotics (100 μM penicillin and 100 μM streptomycin; Sigma-Aldrich, Carlsbad, CA, USA).

Colony formation assay. Cells were seeded at 200 cells per well in 24-well tissue culture plates for 24 h under 5% CO_2 at 37˚C. Subsequently, the cells were treated with various concentrations (0, 50, 100, 150, 200 or 250 ng/ml) of recombinant ING4 protein (Sino Biological, Inc., Beijing, China), and the plates were incubated for 1 week in a humidified incubator at 37˚C. Colonies were stained with 0.05% crystal violet (Beyotime Institute of Biotechnology, Shanghai, China) containing 50% methanol, and counted. The colonies were counted in four or five random fields of vision for each of the duplicate samples using a CX71 microscope (Olympus Corporation, Tokyo, Japan) at a magnification of x100. The IC_{50} value of the recombinant ING4 protein was determined and for use in later experiments.

Flow cytometric analysis of the apoptosis rate. To detect the rate of apoptosis, an annexin V-fluorescein isothiocyanate

Correspondence to: Dr Chuan-Jiang Wang, Department of Vascular and Thyroid Surgery, The First Affiliated Hospital of China Medical University, 155 Nanjing North Street, Shenyang, Liaoning 110001, P.R. China
E-mail: cjwangcmu@126.com

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Table I. Antibodies used in western blot analysis.

Protein	Manufacturer	Catalog number	Dilution
E-cadherin	Santa Cruz Biotechnology[a]	sc-7870	1:200
Vimentin	Santa Cruz Biotechnology[a]	sc-6260	1:200
N-cadherin	Santa Cruz Biotechnology[a]	sc-7939	1:200
Anti-Wnt5b	Abcam[b]	ab94914	1:200
LRP6	Santa Cruz Biotechnology[a]	sc-17982	1:200
p-LRP6 (Ser1490)	Cell Signaling Technology[c]	3395	1:200
Axin2	Cell Signaling Technology[c]	2151	1:200
GSK-3β	Santa Cruz Biotechnology[a]	sc-81462	1:200
β-catenin	Santa Cruz Biotechnology[a]	sc-1496	1:200
β-actin	Santa Cruz Biotechnology[a]	sc-130656	1:1,000
β-tubulin	Santa Cruz Biotechnology[a]	sc-55526	1:500

[a]Dallas, TX, USA; [b]Hong Kong, P.R. China; [c]Beverly, MA, USA. LRP, low-density lipoprotein receptor-related protein; GSK, glycogen synthase kinase.

(FITC) apoptosis detection kit (KeyGen, Nanjing, China) was used in accordance with the manufacturer's instructions. The samples were immediately analyzed on a FACSCalibur flow cytometer (Becton-Dickinson Medical Devices, Shanghai, China).

Transwell assay. A Transwell migration assay was performed using the Boyden chamber (8 μM pore size; polycarbonate membrane; Cell Biolabs, San Diego, CA, USA). The cells were resuspended in FCS-free DMEM to a concentration of $3x10^5$ cells/ml. The upper chamber was loaded with 100 μl cell suspension, while the lower chamber was loaded with 600 μl DMEM containing 10% FCS. Following incubation for 12 h in normal culture conditions, the filter was fixed in 4% paraformaldehyde (Sigma-Aldrich) and stained with crystal violet. The cells on the upper side of the filter were removed using a cotton swab, and the cells that had migrated to the undersurface of the membrane were counted using a light microscope (6XC; Shanghai Guangmai Instruments Ltd., Shanghai, China). In total, 10 microscopic fields (magnification, x400) were randomly selected for the counting of the cells.

Gelatin zymography. A gelatin zymography assay was performed according to the methods outlined by Song and Zhao (17). Briefly, 50 mg protein was applied to 10% polyacrylamide gels, with 1% gelatin incorporated as a substrate for the gelatinolytic proteases. Subsequent to running the gel, the sodium dodecyl sulfate (SDS) was removed by washing twice in 2.5% Triton X-100 for 30 min. The gels were incubated overnight in a zymography development buffer, which contained 50 mM Tris-HCl (pH 7.4), 2 mM NaN_3 and 5 mM $CaCl_2$. Following development, the gels were stained for 3 h in 45% methanol/10% glacial acetic acid containing 1% (w/v) Coomassie Brilliant Blue R-250 (Beyotime Institute of Biotechnology), and subsequently partially destained with the same solution without dye. The gelatinolytic activity of each matrix metalloproteinase (MMP) was qualitatively evaluated as a clear band against the blue-stained gelatin background.

Chick chorioallantoic membrane (CAM) assay. A CAM assay was conducted according to the methods outlined by Lokman *et al* (18). Chick eggs (Dezhou Food Imp & Exp Co. Ltd., Dezhou, China) were incubated in a MultiQuip incubator (MultiQuip, Austral, NSW, Australia) at 37˚C with 60% humidity. Under aseptic conditions, a small window was made in the shell on day 3 of chick embryo development to observe the underlying vasculature. The window was resealed with adhesive tape and the eggs were returned to the incubator until day 11 of chick embryo development. On day 11, all three groups of SW579 cells were labeled with CellTracker™ Green 5-chloromethylfluorescein diacetate (Invitrogen Life Technologies, Carlsbad, CA, USA) in suspensions ($1x10^6$ cells/well) and mixed with growth factor reduced Matrigel (8.9 mg/ml; BD Biosciences, Franklin Lakes, NJ, USA) to a total volume of 30 μl. Subsequently, the Matrigel grafts were placed on top of the CAM, and the eggs were resealed and returned to the incubator for 72 h until day 14 (n=6 chicken embryos per cell line; SW579, PBS and ING4). Matrigel grafts with the surrounding CAM were harvested from each embryo, fixed with 4% paraformaldehyde for 24 h and embedded in Optimum Cutting Temperature compound (Tissue-Tek; Sakura Finetek USA, Inc., Torrance, CA, USA). The samples were stored at -80˚C, and frozen samples were cut into 5-μm sections using a Cryomicrotome CM 1850 (Leica Microsystems, Bannockburn, IL, USA). Images were acquired using an Olympus fluorescence microscope (Olympus Corporation, Osaka, Japan).

Preparation of nuclear and cytoplasmic protein extracts. Nuclear and cytoplasmic protein fractions were isolated using a CelLytic™ NuCLEAR™ Extraction kit (Sigma-Aldrich), according to the manufacturer's instructions. Protein concentrations were determined using a bicinchoninic acid protein assay, with bovine serum albumin used as a standard (Pierce Biotechnology, Inc., Rockford, IL, USA).

Western blot analysis. Protein extracts were resolved by SDS-PAGE, which was followed by electrotransfer to nitrocellulose membranes (Bio-Rad Laboratories, Inc., Philadelphia,

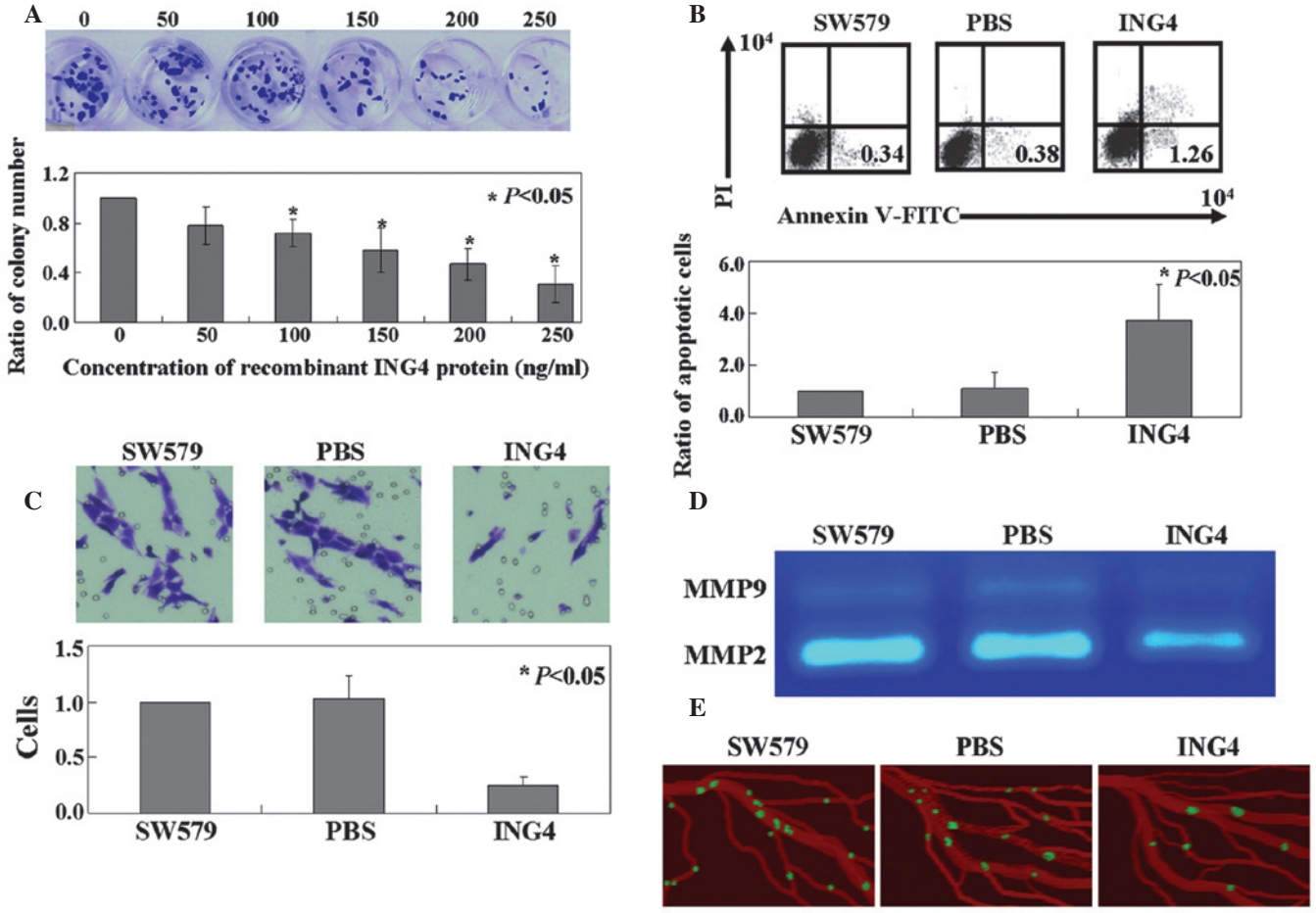

Figure 1. Antitumor activities of ING4 in SW579 cells. (A) Proliferation ratio of SW579 cells treated with recombinant ING4 protein was measured using the colony formation assay. (B) Apoptotic ratio of cells was analyzed by double staining with annexin-V FITC/PI. (C) Transwell assays were performed to detect the mobility of the SW579 cells treated with recombinant ING4 protein. (D) Gelatinolytic activity levels of MMP-2 and -9, secreted from the SW579 cells, were analyzed by zymography. (E) Invasive GFP-labeled SW579 cells were visualized intravascularly. SW579, untreated SW579 cells; PBS, PBS treated SW579 cells; ING4, recombinant ING4 protein treated SW579 cells; ING, inhibitor of growth; FITC, fluorescein isothiocyanate; PI propidium iodide; MMP, matrix metalloproteinase; PBS, phosphate-buffered saline; GFP, green fluorescent protein.

PA, USA). Following a blocking step using 5% milk in Tris-buffered saline with Tween® 20, the membranes were incubated with primary antibodies at room temperature overnight (Table I). Subsequently, the membranes were developed and visualized with enhanced chemiluminescence (Thermo Fisher Scientific, Waltham, MA, USA). Secondary monoclonal antibodies were purchased from the Beyotime Institute of Biotechnology. The secondary monoclonal antibodies included anti-mouse IgG (#A0216), anti-rabbit IgG (#A0239) and anti-goat IgG (#A0181). The membranes were incubated with secondary antibodies for 2 h at room temperature.

Statistical analysis. Numerical data are expressed as the mean ± standard deviation, and differences among the mean values were evaluated using the Student's t-test. Statistical analyses were conducted using SPSS software (version 11.0; SPSS, Inc., Chicago, IL, USA). P<0.05 was considered to indicate a statistically significant difference.

Results

Antitumor effects of recombinant ING4 protein on SW579 cells. Cell viability was assessed using the colony formation assay. As shown in Fig. 1A, the proliferative rate of the cells in the recombinant ING4-treated group was inhibited significantly in a dose-dependent manner (P<0.05). The IC_{50} value of the recombinant ING4 protein was determined to be 192.5 ng/ml. When compared with the untreated SW579 cells or the phosphate-buffered saline-treated cells, the apoptotic ratio of the cells following treatment with recombinant ING4 protein was observed to increase significantly using annexin V-FITC and propidium iodide double staining (P<0.05; Fig. 1B). In addition, the Transwell assay revealed that cell motility was significantly decreased in the recombinant ING4 protein-treated group, as compared with the untreated cells (P<0.05; Fig. 1C). The activity levels of MMP-2 and -9 were shown to be inhibited by ING4 protein in SW579 cells (Fig. 1D). Additionally, using the CAM model, ING4 protein was demonstrated to inhibit SW579 cells from escaping primary tumor sites and scattering among blood vessels (Fig. 1E).

Mechanisms of recombinant ING4 protein-induced apoptosis and inhibited mobility in SW579 cells. To identify the mechanisms underlying the effects of recombinant ING4 protein in SW579 cells, the expression levels of various proteins were

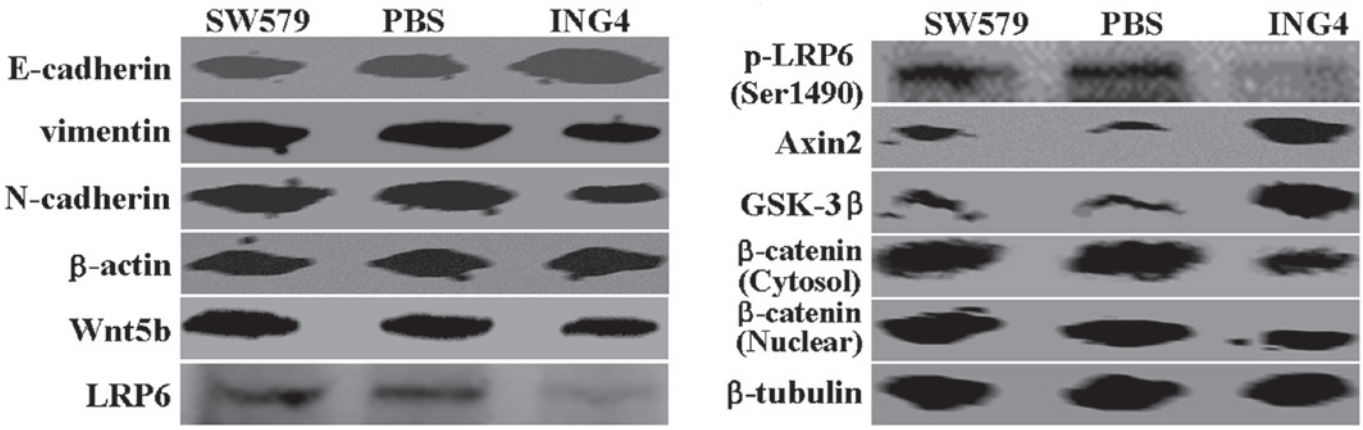

Figure 2. Effects of ING4 protein on the Wnt signaling pathway, as shown by western blot analysis. Cell lysates were electrophoresed and the expression levels of Wnt-associated proteins were detected using their specific antibodies. LRP, low-density lipoprotein receptor-related protein; GSK, glycogen synthase kinase; PBS, phosphate-buffered saline; ING, inhibitor of growth.

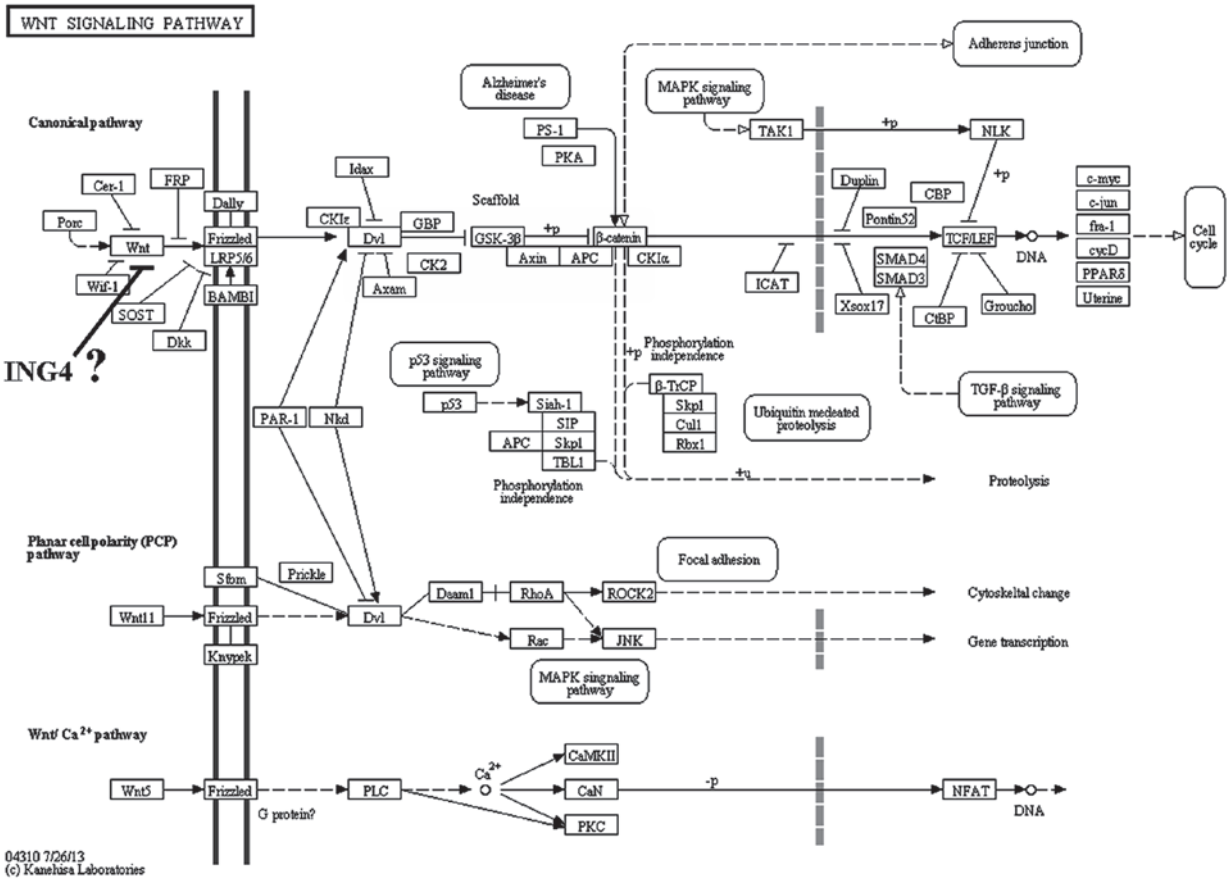

Figure 3. Map of the Wnt signaling pathway, which includes at least three Wnt signaling pathways, namely the canonical pathway, the planar cell polarity pathway and the Wnt/Ca^{2+} pathway.

detected by western blot analysis. When compared with the untreated cells, the ING4-treated SW579 cells exhibited a higher expression level of the epithelial marker, E-cadherin, while lower expression levels of the mesenchymal markers, vimentin and N-cadherin, were detected (Fig. 2). Notably, changes in Wnt5b expression were observed in the SW579 cells following treatment with ING4. Furthermore, the western blot analysis assays detected a significant inhibition of low-density lipoprotein receptor-related protein 6 expression and phosphorylation following treatment with ING4 (Fig. 2). A concomitant increase in Axin2 and glycogen synthase kinase-3β expression was detected in the ING-treated SW579 cells, as compared with the control groups, while decreased expression levels of nuclear and cytosolic β-catenin were observed (Fig. 2). Pathway enrichment analysis was performed in the Kyoto Encyclopedia of Genes and Genomes database (Institute for Chemical Research, Kyoto University, Kyoto, Japan; Institute of Medical Science, University of Tokyo, Tokyo, Japan), and the analysis

results revealed that the Wnt signaling pathway was involved in the antitumor effects of ING4 (Fig. 3).

Discussion

As previously discussed, ING4 is involved in cell proliferation, apoptosis, migration, angiogenesis and the DNA damage response (7-10). Consistent with these previous studies, the present study confirmed the ability of ING4 to inhibit cell proliferation and mobility, and induce apoptosis in SW579 cells. To the best of our knowledge, although ING4 is not observed in a number of cancer types, this is the first study to demonstrate the antitumor roles of ING4 in thyroid cancer.

The primary finding of the present study was the involvement of the Wnt signaling pathway in the mechanism underlying the effects of ING4. Canonical Wnt/β-catenin signaling directly alters gene expression, and has been shown to be a key regulator of cell proliferation, differentiation and apoptosis in a variety of cancer types (19,20). Abnormal activation of the Wnt/β-catenin signaling pathway and the subsequent upregulation of β-catenin has also been associated with the development of breast cancer (21). In the present study, ING4 was demonstrated to suppress Wnt5b expression in SW579 cells. The inhibition of Wnt5b expression results in decreased cytosolic accumulation of β-catenin, followed by reduced β-catenin translocation to the nucleus (22). Furthermore, the present results indicated that targeting the β-catenin pathways may suppress the mobility of SW579 cells, which is associated with EMT. E-cadherin is known to anchor and sequester β-catenin in the membrane to prevent activation; thus, the activation of β-catenin signaling may result from the downregulation of E-cadherin at EMT (22). E-cadherin expression has also been shown to be upregulated following decreased β-catenin expression (22).

In conclusion, the results of the present study have confirmed the antitumor activities and mechanisms of ING4-induced apoptosis in SW579 cells. These results provide initial evidence indicating the potential of ING4 as a therapeutic target for thyroid cancer.

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