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ATRA increases iodine uptake and inhibits the proliferation and invasiveness of human anaplastic thyroid carcinoma SW1736 cells: Involvement of β-catenin phosphorylation inhibition

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Abstract. All-trans-retinoic acid (ATRA) can enhance iodine uptake capability of thyroid tumors, but the mechanisms remain poorly understood. The aim of the present study was to investigate the effects of ATRA on isotope susceptibility, proliferation and invasion of anaplastic thyroid carcinoma (ATC) and potential mechanisms. SW1736 cells were treated with 1 μmol/l ATRA or 1% ethanol for 5 days. A cell line stably expressing β-catenin-shRNA was established. An iodine uptake assay was performed using ^{125}I. Proliferation and invasiveness were tested using MTT and Transwell assays, respectively. Western blotting was used to assess the expression of β-catenin, glycogen synthase kinase-3β (GSK-3β), sodium/iodine symporter (NIS) and proteins involved in epithelial-mesenchymal transition. Cells pretreated with ATRA were injected subcutaneously into SCID mice. Mice were intraperitoneally injected with ^{131}I once on the first day of treatment, and tumor growth was then assessed. After 35 days of ^{131}I treatment, ATRA-pretreated tumor volume and weight were decreased compared with the ^{131}I alone group (163.32±19.57 vs. 332.06±21.37 mm^3; 0.35±0.14 vs. 0.67±0.23 g, both P<0.05). Similar results were observed in the β-catenin shRNA-pretreated tumors. ATRA also increased the uptake of iodine by SW1736 cells (P<0.01), and similar results were observed in β-catenin shRNA cells. ATRA treatment decreased the cell proliferation and invasion compared with control cells (all P<0.05), similar to β-catenin shRNA. ATRA treatment decreased the expression of phosphorylated (p-)β-catenin, p-GSK-3β, vimentin, and fibronectin, and increased the expression of NIS and E-cadherin, compared with the control. ATRA increased the iodine uptake and inhibited the proliferation and invasion of SW1736 cells, involving β-catenin phosphorylation. In conclusion, ATRA could be used to improve the isotope sensitivity of ATC.

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Introduction

Thyroid cancer is an endocrine malignancy with a high morbidity rate (1). Anaplastic thyroid cancer (ATC) is the least common, but most lethal, form of thyroid cancer. It accounts for 1.3-9.8% of thyroid in worldwide, with a 1-year survival rate of 20% and a disease-specific mortality rate of nearly 100% (2). ATC has an annual incidence of between 1 and 2 cases/million people, with a female to male ratio of 3:2 (3). Although the majority of well-differentiated thyroid tumors are effectively treated by isotope therapy, relapse and poorly differentiated thyroid cancer, such as anaplastic and medullary thyroid cancers, show tolerance to isotope treatment (1). Therefore, finding new ways to improve the effectiveness of isotope-based therapies for this type of cancer is important.

All-trans-retinoic acid (ATRA) is naturally produced by humans. ATRA is a natural derivative of vitamin A. ATRA exerts its functions by triggering G1 phase arrest, affecting DNA synthesis and enhancing immune cell killing efficiency (4,5). ATRA has been shown to decrease the proliferation and invasiveness of different types of cancer cells, including pancreatic cancer (6), leukemia (7), breast cancer (8) and thyroid cancer cells (9). Particularly, ATRA can significantly enhance the iodine uptake capability of thyroid tumors and other cancers (4,5,10,11). However, the mechanisms of the

enhancement of iodine uptake in thyroid cancer induced by ATRA remain poorly understood.

The sodium/iodine symporter (NIS) is a transmembrane protein responsible for the transport of iodine into the thyroid follicular cells, which is a crucial step for the synthesis of hormones, including T_3 and T_4 (12). This property of NIS to concentrate iodine ions within thyroid cells constitutes the basis for thyroid scintigraphy and iodine isotope treatment of hyperthyroidism and thyroid cancer (12). Loss of thyroid-specific functions in poorly differentiated thyroid cancer leads to the lack of efficacy of isotope therapy in these cancers (13). Identifying methods to restore the NIS function may be one way to improve the treatment efficacy in anaplastic thyroid carcinoma.

Previous studies have suggested that the transcription activity of β-catenin is closely associated with the transcription of genes involved in thyroid cell growth and differentiation, as well as NIS gene transcription and translation in thyroid cancer (14,15). Therefore, it may be hypothesized that the transcription activity of β-catenin can directly affect the isotope sensitivity of thyroid cancer cells. The aim of the present study was to investigate whether the transcription activity of β-catenin plays a role in ATRA-mediated iodine uptake in thyroid cancer.

Materials and methods

Cells. Undifferentiated human anaplastic thyroid carcinoma SW1736 cells were provided by Professor Michael Derwahl (Institute of Endocrinology, Hedwig Hospital, Humboldt University, Berlin, Germany); this cell line has a tolerance to isotope treatment (16). The cells were maintained in DMEM (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) with 10% fetal bovine serum (FBS) (Gibco; Thermo Fisher Scientific, Inc.) in an incubator at 37˚C with 5% CO_2.

ATRA (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was dissolved in ethanol to 10 μmol/l as stock solution and stored in the dark. SW1736 cells were seeded at a density of $5x10^4$ cells/well for 48 h, followed by ATRA treatment (1 μmol/l) for 5 days. The negative control cells were treated with 1% ethanol. All cells were kept at 37˚C in the dark using aluminum foil. SW1736 cells stably transfected with β-catenin shRNA were used as positive controls.

β-catenin RNA interference and stable cell lines. The recombinant plasmid pSUPER-β-catenin-shRNA was previously constructed (17). It was transfected into SW1736 cells using Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). SW1736 cells transfected with pSUPER-β-catenin-shRNA were screened by 400 μg/ml puromycin for 4 weeks to select positive clones. Finally, cells were screened and cultured in medium with 200 μg/ml of puromycin to establish the stable transfected cell line.

Proliferation assay. SW1736 cells treated with 1 μmol/l of ATRA or 1% ethanol for 5 days were seeded onto a 96-well culture plate. Each well was loaded with 180 μl of cell suspension containing $1x10^4$ cells. After 24, 48 and 72 h, the supernatant was discarded and cells were treated with 150 μl DMEM and 50 μl MTT solution (2.5 mg/ml; Sigma-Aldrich; Merck KGaA). The MTT solution was discarded following 4 h of treatment, and the cells were mixed with 150 μl dimethyl sulfoxide at low-speed vortex for 10 min. The absorbance of each well was measured at 570 nm using DNM-9602 ELISA spectrometer (Perlong Medical Equipment Co., Ltd., Beijing, China).

In vitro invasion assay. Transwell chambers (Merck KGaA) coated with Matrigel (Sigma-Aldrich; Merck KGaA) were placed into 6-well plates and air-dried under a sterile laminar flux hood. DMEM medium (700 μl) containing 10% FBS was added to the lower chamber. Cell suspension (300 μl) containing $5.0x10^4$ SW1736 cells treated with 1 μmol/l ATRA or 1% ethanol, but without FBS, for 5 days was added to the upper chamber. After 24, 48 and 72 h, the Transwell chambers were washed with phosphate-buffered saline (PBS). The upper microporous membrane was cleaned with cotton swab. Invading cells attached to the lower microporous membrane were fixed with 2% paraformaldehyde for 30 min and stained with hematoxylin. Cell counting was performed by two independent investigators blind to grouping.

In vitro iodine uptake assay. The *in vitro* iodine uptake assay was performed as previously described (18). Briefly, SW1736 cells treated with 1 μmol/l ATRA or 1% ethanol for 5 days were seeded onto 6-well plates ($5.0x10^4$ cells/well). After the cells had attached to the bottom surface, the supernatant was discarded. Cells were washed with Hank's balanced salt solution (HBSS) and incubated with 3.7 kBq of ^{125}I (Thermo Fisher Scientific, Inc.) for 20 min followed by three washes with ice-cold HBSS. Cells were then incubated with 1 ml of ethanol for 20 min. The ethanol solution was collected into test tubes and the radioactivity per minute was measured using a WIZARD γ counter (Perkin-Elmer Life Sciences, Waltham, MA, USA).

Western blot analysis. SW1736 cells treated with 1 μmol/l ATRA or 1% ethanol for 5 days were lysed with 1% NP-40 lysis buffer (Sigma-Aldrich; Merck KGaA) at 4˚C for 30 min. Total proteins were collected after centrifugation at 25,000 x g for 10 min at 4˚C. Proteins were quantified using Coomassie brilliant blue G-250. Samples of 20-50 μg per well were separated on 8-12% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk for 2 h followed by incubation with primary antibodies against β-catenin (dilution: 1:4,000; cat. no. ab16051; Abcam, Cambridge, MA, USA), phosphorylated (p-)β-catenin Ser45 (dilution, 1:1,000; cat. no. PA5-17685; Invitrogen; thermo Fisher Scientific, Inc.), p-β-catenin Y654 (dilution, 1:1,000; cat. no. ab59430, Abcam), glycogen synthase kinase-3β (GSK-3β) (dilution, 1:10,000; cat. no. ab75814, Abcam), p-GSK-3β Ser9 (dilution, 1:10,000; cat. no. ab75814; Abcam), E-cadherin (dilution, 1:1,000; cat. no. sc-7870; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cytokeratin 18 (CK18) (dilution: 1:1,000; cat. no. sc-28264; Santa Cruz Biotechnology, Inc.), vimentin (dilution: 1:1,000; cat. no. sc-5565; Santa Cruz Biotechnology, Inc.), urokinase-type plasminogen activator (uPA) (dilution: 1:1,000; cat. no. sc-14019; Santa Cruz Biotechnology, Inc.) and its receptor uPAR (dilution: 1:1,000; cat. no. sc-10815; Santa Cruz Biotechnology, Inc.), and fibronectin (dilution, 1:1,000;

cat. no. sc-9068; Santa Cruz Biotechnology, Inc.) at 4˚C overnight. The next day, the membranes were extensively washed with Tris-buffered saline and incubated with goat anti-rabbit secondary antibody (dilution: 1:30,000; cat. no. A9919; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) for 2 h at 4˚C. The bands were visualized by electrochemiluminescence (Pierce; Thermo Fisher Scientific, Inc.). Densitometry images were analyzed using ImageJ software (Rawak Software, Inc., the Tomancak Laboratory, Dresden, Germany).

Heterotopic xenograft mouse model. SW1736 cells ($2x10^6$) were treated with 1 μmol/l ATRA or 1% ethanol for 5 days, and SW1736 cells stably transfected with β-catenin shRNA were suspended in 200 μl of culture medium and implanted subcutaneously into the left forelimb of 4-6-week-old male SCID mice (22-25 g body weight). Intervention was applied after the subcutaneous tumor size reached 50 mm^3. The experimental mice (alcohol, β-catenin shRNA and ATRA groups) were intraperitoneal injected with 37 MBq of ^{131}I (Thermo Fisher Scientific, Inc.) once on the first day of treatment. The control mice (ATRA and alcohol groups) were intraperitoneally injected with 0.1 ml of normal saline (NS) instead (ATRA+NS and Alcohol+NS). There were 10 mice in each group (supplied by Beijing Huafukang Biotechnology Co., Ltd., Beijing, China). The ^{131}I intervention was commenced on the first day of the experiment. Subcutaneous tumor size was measured every 5 days using a caliper (volume=long diameter x short diameter^2 + 2). Mice were sacrificed by CO_2 inhalation after 35 days of observation. Tumors were collected and weighed. All procedures and animal experiments were approved by the Animal Care and Use Committee of Beijing Jishuitan Hospital (Beijing, China).

Statistical analysis. All statistical analyses were conducted using SPSS 13.0 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation (SD) from three independent experiments performed in triplicate. Statistical significance was evaluated by one-way analysis of variance, with the least significant difference test for post-hoc analysis. P<0.05 was considered to indicate a statistically significant difference.

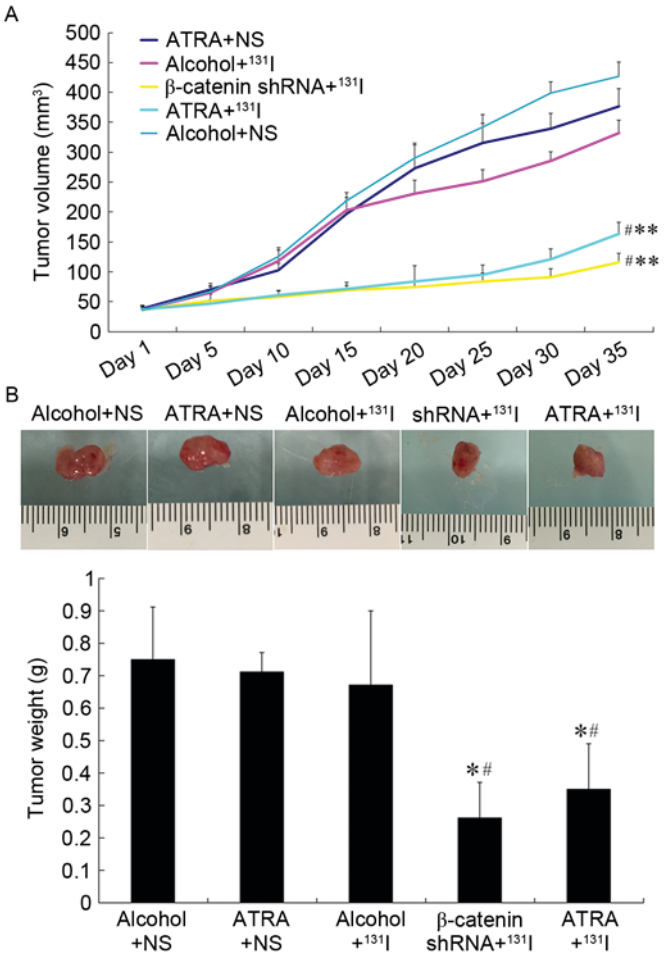

Figure 1. Effect of ATRA pretreatment in thyroid cancer cells on tumor growth in a heterotopic xenograft mouse model treated with radioactive iodine. Undifferentiated human thyroid cancer SW1736 cells were pre-treated with alcohol, β-catenin shRNA or ATRA for 5 days, and were implanted subcutaneously into the left forelimb of SCID mice. When the tumor size reached 50 mm^3, mice were intraperitoneally injected with 37 MBq of ^{131}I once on the first day of treatment. Control mice were injected with 0.1 ml NS instead of ^{131}I (ATRA+NS and Alcohol+NS). Tumor size was measured every 5 days using calipers. Mice were sacrificed after 35 days. (A) Tumor volume. (B) Tumor weight. Data are shown as the mean ± standard deviation (n=10 each group). **P<0.01 vs. the Alcohol+NS group; $^{\#}$P<0.05 vs. the alcohol+^{131}I group. ATRA, all-trans-retinoic acid; NS, normal saline; shRNA, short hairpin RNA.

Results

ATRA pretreatment improved ^{131}I sensitivity and inhibited tumor growth in the heterotopic xenograft mouse model. After 35 days of ^{131}I treatment, ATRA-pretreated tumor volume and weight were decreased compared with the alcohol+^{131}I group (163.32±19.57 vs. 332.06±21.37 mm^3; 0.35±0.14 vs. 0.67±0.23 g; both P<0.05) (Fig. 1). Similar results were observed in the β-catenin-shRNA+^{131}I group (116.3±14.4 mm^3 and 0.26±0.11 g, both P<0.05 vs. the alcohol+^{131}I group). There were no differences in tumor volume and weight among the tumors treated with ATRA+NS, Alcohol+NS, and Alcohol+^{131}I groups (all P>0.05). Furthermore, there were no differences in the tumor volume and weight between the tumors treated with ATRA+^{131}I and β-catenin-shRNA+^{131}I (both P>0.05) (Fig. 1).

Effect of ATRA on iodine uptake in the SW1736 cells. The iodine uptake assay revealed that ATRA increased the uptake of iodine by SW1736 cells compared with the alcohol control group (1.4±0.2 vs. 0.4±0.2x10^3/min; P<0.01) (Fig. 2A). Similar results were observed in the β-catenin-shRNA cells compared with the no transfection group (1.7±0.3 vs. 0.6±0.2x10^3/min; P<0.01).

ATRA inhibited the invasion and proliferation of SW1736 cells. MTT and Transwell invasion assays indicated that ATRA treatment decreased the cell proliferation (Fig. 2B) and invasion (Fig. 2C) compared with untreated and alcohol control cells (P<0.05). β-catenin-shRNA cells showed results that were similar to the ATRA-treated cells.

Effects of ATRA on phosphorylation of β-catenin and GSK-3β in SW1736 cells. Western blot analysis revealed that ATRA

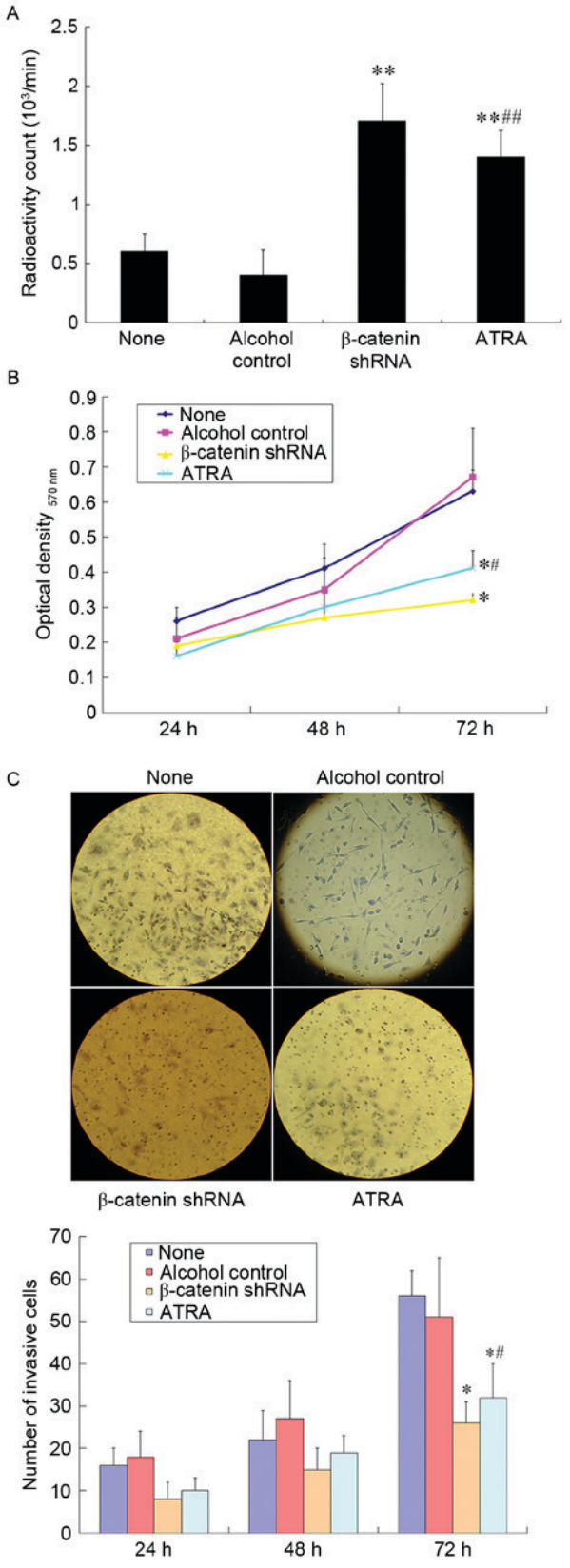

Figure 2. Effects of ATRA on iodine uptake, cell proliferation, and invasion of thyroid cancer SW1736 cells. SW1736 cells were treated with ATRA 1 μmol/l or 1% alcohol (as negative control) for 5 days. SW1736 cells stably transfected with β-catenin shRNA were considered as a positive control. (A) Iodine uptake. (B) Cell proliferation determined by MTT. (C) Cell invasion determined by Transwell assay. Data are shown as mean ± standard deviation from three independent experiments performed in triplicate. *P<0.05, **P<0.01 vs. None; $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. alcohol control. None, SW1736 cells without any treatment; ATRA, all-trans-retinoic acid; shRNA, short hairpin RNA.

treatment decreased the expression of t-β-catenin, p-β-catenin Ser45, p-β-catenin Y654 and p-GSK-3β, and increased the expression of t-GSK-3β compared with untreated and alcohol control cells (Fig. 3A). Similar results were observed in β-catenin-shRNA cells.

Western blot analysis also revealed that ATRA treatment increased the expression of NIS, E-cadherin and CK18, and decreased the expression of vimentin, uPA, uPAR and fibronectin compared with untreated and alcohol control cells (Fig. 3B). Similar results were observed in β-catenin-shRNA cells.

Discussion

Loss of thyroid-specific functions in undifferentiated thyroid cancers leads to a lack of efficacy of isotope therapy (1). ATRA may restore the NIS function to enhance iodine uptake capability in thyroid carcinoma (4,5,10,11), but the exact mechanisms remain poorly understood. In the present study, ATRA treatment has been shown to increase iodine uptake by SW1736 cells, both *in vitro* and *in vivo*. This is consistent with the study by Muhlbauer *et al* (10), which revealed that ATRA could induce iodine accumulation in rats via the NIS. Similar results were also observed in medullary thyroid cancer stem cells and in mice (11).

The results of the present study indicate that the β-catenin pathway is involved in the process of ATRA-improved iodine uptake by SW1736 cells. ATRA inhibited β-catenin phosphorylation at Ser45 and GSK-3β phosphorylation at Ser9, which should consequently enhance GSK-3β hydrolytic activity and decrease β-catenin levels in the cytoplasm (19). In addition, phosphorylation of β-catenin at Y654 was decreased, which should inhibit the translocation of β-catenin to the nucleus (19), resulting in the restoration of NIS expression and the recovery of cellular iodine uptake capability and susceptibility to radioactive iodine therapy (19).

The restoration of NIS function should improve the sensitivity of undifferentiated thyroid cancers to isotope treatment. A recent study revealed that the Wnt/β-catenin pathway is involved in thyroid function (14), and a previous study showed that β-catenin nuclear translocation suppresses NIS membrane localization in thyroid cancer cells (15). Other studies have shown that a number of pathways could be involved in NIS expression. Ryan *et al* (20) used reverse transcription-quantitative polymerase chain reaction to measure and compare the expression levels of NIS, estrogen receptor α (ERα) and retinoic acid receptor in healthy human breast tissue and breast cancer tissue; NIS mRNA levels were found to be closely associated with ERα and retinoic acid receptor expression. Cheong *et al* (21) further demonstrated that the ERα agonist 17β-estradiol could decrease ATRA-triggered iodine uptake by 50%. Based on these findings, it was suggested that iodine uptake induced by ATRA was associated with ERα-mediated transcription (21). Similarly, Lee *et al* (22) found that in thyroid cancer, ATRA reduced the phosphorylation levels of p38 MAPK and attenuated endoplasmic reticulum stress, subsequently affecting NIS expression and iodine uptake. Furthermore, staurosporine-associated protease inhibitors showed evident interference with ATRA-mediated iodine uptake (23). ATRA

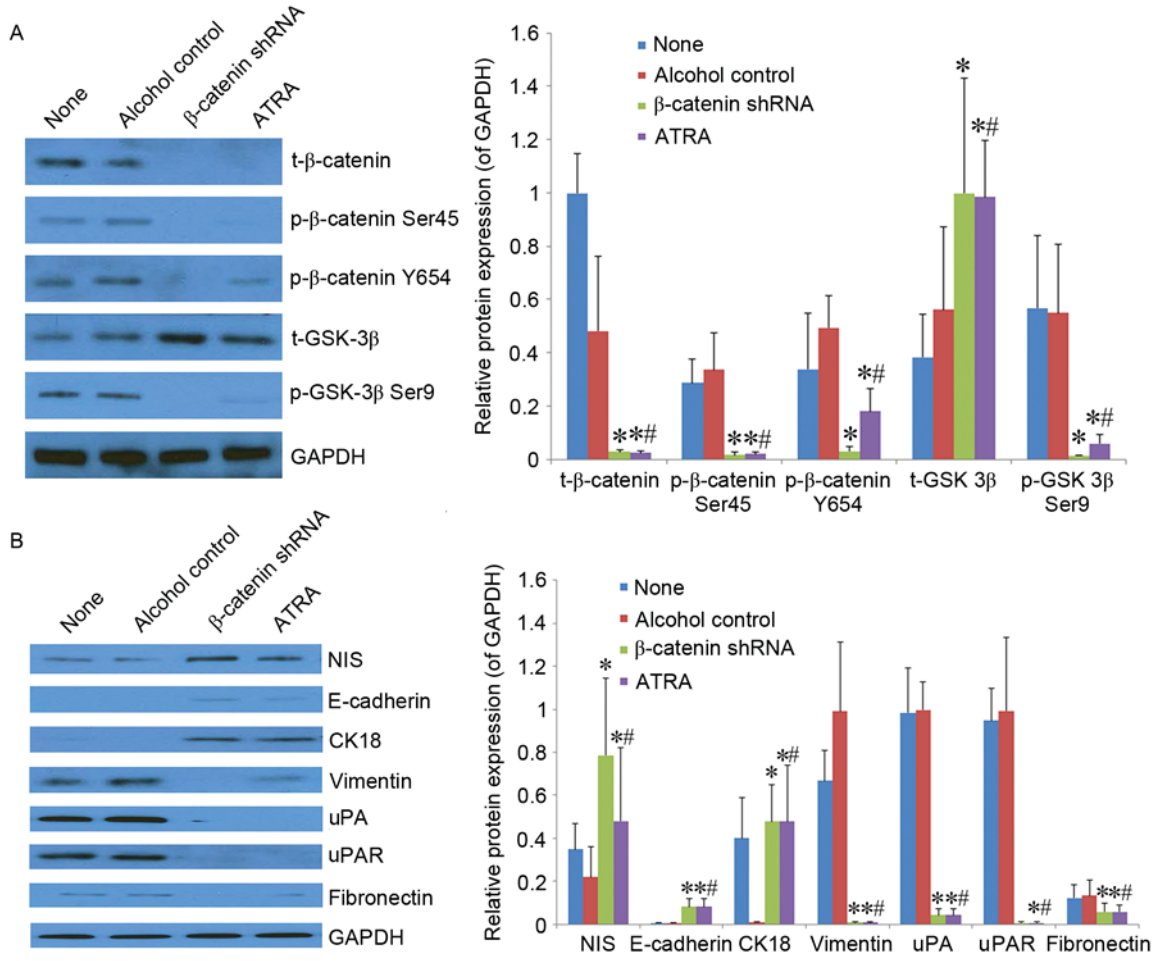

Figure 3. Effects of ATRA on the protein expression of GSK-3β, β-catenin, NIS and proteins involved in epithelial-mesenchymal transition in thyroid cancer SW1736 cells. Protein expression was determined by western blot analysis. GAPDH was used as an internal control. (A) Total β-catenin, total GSK-3β, phosphorylated β-catenin and phosphorylated GSK-3β. (B) NIS, E-cadherin, CK18, vimentin, uPA and its receptor uPAR, and fibronectin. Data are shown as mean ± standard deviation from three independent experiments performed in triplicate. *P<0.05 vs. None; #P<0.05 vs. alcohol control. None, SW1736 cells without any treatment; ATRA, all-trans-retinoic acid; shRNA, short hairpin RNA; t-, total; p-, phosphorylated; GSK-3β, glycogen synthase kinase-3β; CK18, cytokeratin 18; NIS, sodium/iodine symporter; uPA, urokinase-type plasminogen activator.

was reported to bind to its specific receptor and activate the PI3K/AKT signaling pathway, which may be one of the mechanisms involved in improvements of iodine uptake (24). Overall, β-catenin nuclear translocation may be one of the molecular mechanisms by which ATRA promotes iodine uptake, but additional studies remain necessary to confirm these results.

In the present study, ATRA decreased the proliferation of thyroid cancer cells, which has been previously observed in other studies of thyroid cancer cells (9,25,26), as well as in other types of cancer cells, including pancreatic cancer (6), leukemia (7) and breast cancer (8). In addition, the present study showed that ATRA modulates a number of proteins involved in EMT in a way that indicates ATRA should inhibit EMT. Previous studies have shown that ATRA increases the expression of NIS, E-cadherin and CK18, and decreases the expression of vimentin, uPA, uPAR and fibronectin (27-29), as was observed in the present study. These effects should help to decrease EMT in thyroid cancers and maintain NIS potency.

Furthermore, ATRA has been shown to inhibit the proliferation and invasiveness of SW1736 cells by downregulating the transcriptional activity of β-catenin (14,19). β-catenin also participates in the proliferation and invasiveness of cancer cells by modulating the Wnt/β-catenin pathway. Inhibiting the Wnt/β-catenin pathway has been shown to suppress breast cancer metastatic spread (30) and to inhibit gastric cancer stem cells (31). Therefore, the use of ATRA in thyroid cancer could affect three aspects, consisting of NIS upregulation, EMT inhibition and proliferation and invasiveness inhibition. Nevertheless, additional studies remain necessary to obtain a more comprehensive understanding of the mechanisms involved.

In conclusion, ATRA increased iodine uptake and inhibited proliferation and invasion of human anaplastic thyroid carcinoma SW1736 cells. Inhibition of β-catenin phosphorylation may be involved in this process. ATRA could be used to improve the isotope sensitivity of anaplastic thyroid carcinoma.

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