

Original Research Article

FOXP2 regulates the proliferation, migration, and apoptosis of thyroid carcinoma cells via Wnt/ β -catenin signaling pathway

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Abstract

Purpose: To determine the effect of Forkhead box P2 (FOXP2) on thyroid carcinoma cell growth and metastasis.

Methods: Expression of FOXP2 in thyroid carcinoma cells was determined using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) and western blot. The 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) was applied to evaluate cell viability, while cell migration and invasion were assessed by wound healing and Transwell assays, respectively. Flow cytometry and western blot were conducted to investigate cell apoptosis. The underlying mechanisms were investigated using western blot assay.

Results: Expression of FOXP2 was lower in primary thyroid carcinoma tissues than in normal tissues based on data from the TCGA database. Similarly, FOXP2 was lower in thyroid carcinoma cells at the mRNA and protein levels. Ectopic FOXP2 expression decreased cell viability, and retarded the migration and invasion of thyroid carcinoma cells. FOXP2 overexpression in thyroid carcinoma cells led to down-regulated expression of matrix metalloproteinase (MMP) 2, MMP 9, and proliferating cell nuclear antigen (PCNA), as well as induction of cell apoptosis. Moreover, FOXP2 overexpression resulted in enhanced Bax expression while Bcl-2 was reduced. Ectopic expression of FOXP2 decreased β -catenin, c-myc, and cyclin D1 in thyroid carcinoma cells.

Conclusion: FOXP2 suppresses the proliferation and metastasis of thyroid carcinoma cells, but promotes apoptosis through suppression of the Wnt/ β -catenin signaling pathway. These results provide an insight that may lead to the development of a novel potential therapeutic strategy for treating thyroid carcinoma.

Keywords: FOXP2, Cell proliferation, Metastasis, Apoptosis, Thyroid carcinoma, Wnt/ β -catenin

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INTRODUCTION

Thyroid cancer is one of the most malignant endocrine tumors, and its incidence has been

increasing globally in recent decades [1,2]. The mortality rate of thyroid cancer is higher than other endocrine tumors [3]. Therefore, it is necessary to search for key genes that play

important roles in the occurrence and development of thyroid cancer because they may potentially contribute to the development of effective and promising therapies.

Forkhead box P2 (FOXP2) functions as a transcription factor to regulate genes involved in facial movement [4]. FOXP2 is generally reduced in highly metastatic tumor cells [5], and knockdown of FOXP2 contributes to tumor cell metastasis [6]. The migration and invasion of breast cancer cells were promoted by down-regulation of FOXP2 through regulation of the transforming growth factor beta (TGF β)/Smad signaling pathway [7]. Therefore, FOXP2 is an attractive anticancer target. However, the effect of FOXP2 on the biological function of thyroid cancer cells is unknown.

The Wnt/ β -catenin signaling pathway, which is conserved, plays a key role in tumorigenesis [8]. β -Catenin is overexpressed in thyroid cancer tissues, and abnormal activation of Wnt/ β -catenin signaling contributes to the progression of thyroid cancer [9]. The Wnt/ β -catenin signaling pathway has been considered a potential therapeutic target for thyroid cancer. Because the transcriptional activity of FOXP2 is reportedly mediated by the Wnt/ β -catenin pathway [10], we investigated whether FOXP2 modulates the progression of thyroid cancer by regulating Wnt/ β -catenin signaling.

EXPERIMENTAL

Cell culture

Human thyroid cancer cell lines (TPC-1, SW579, BCPAP) and a normal human primary thyroid follicular epithelial cell line (Nthy-ori 3-1) were purchased from ATCC (Manassas, VA, USA). The cells were cultured at 37°C in DMEM (Hyclone, South Logan, UT, USA) containing 10% fetal bovine serum (Hyclone).

Cell transfection

TPC-1 thyroid cancer cells were placed in a 96-well plate (5×10^3 cells/well) and transfected with pcDNA3.1-FOXP2 that was constructed by RiboBio (Guangzhou, China) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA).

Quantitative reverse transcription polymerase chain reaction (qRT-PCR)

Total RNA was isolated from thyroid cancer cells using TRizol (Thermo Fisher Scientific) and transcribed into cDNAs with the Reverse

Transcription System (Thermo Fisher Scientific). Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) was used for qRT-PCR analysis of FOXP2 mRNA expression. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous control. The primers used are shown in Table 1.

Table 1: Primers used in qRT-PCR

ID	Sequence (5'-3')
GAPDH Forward	AGGTCGGTGTGAACGGATTG
GAPDH Reverse	TGTAGACCATGTAGTTGAGGTC
FOXP2 Forward	AACAACAGCAGGCTCTCCAG
FOXP2 Reverse	GGCACCTGCAGTGGTCTC

Determination of cell viability

Transfected TPC-1 thyroid cancer cells were plated in a 96-well plate (5×10^3 cells/well) for 24, 48, or 72 hours. Cells were incubated with MTT solution (0.5 mg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 3 hours and then lysed with 10% SDS in 0.01 M HCl buffer. Absorbance at 450 nm of each well was measured using a microplate reader (Thermo Fisher Scientific) to determine cell viability.

Evaluation of cell migration

TPC-1 cells transfected with the pcDNA vector or pcDNA-FOXP2 were plated in a 6-well plate for 24 hours. A pipette was used to generate a scratch in the middle of each well. The debris were removed, and the wound width was photographed 24 hours later using a microscope (Olympus Corp, Tokyo, Japan).

Assessment of cell invasion

Transfected TPC-1 thyroid cancer cells cultured in 100 μ L serum-free DMEM were plated in the upper chamber of a transwell chamber (Biosciences, San Jose, CA, USA) coated with Matrigel (Clontech; Mountain View, CA, USA). The lower chamber contained 600 μ L DMEM with 15% fetal bovine serum. Two days later, cells in the lower chamber were fixed in 10% formaldehyde and stained with 0.1% crystal violet (Sigma-Aldrich) before measurement of invasive cell numbers by microscopy (Olympus Corp).

Determination of cell apoptosis

Transfected TPC-1 thyroid cancer cells were harvested through centrifugation at 1000 g for 5 min. Fluorescein isothiocyanate (FITC)-labeled Annexin V and propidium iodide (PI) (Sigma-Aldrich) were then used to stain the cells. The cells were analyzed by flow cytometry (Becton

Dickson Immunocytometry-Systems, San Jose, CA, USA).

Western blot analysis

Thyroid cancer cells were lysed in radioimmunoprecipitation assay (RIPA) Lysis and Extraction Buffer (Thermo Fisher Scientific), and the protein concentration of the cellular lysates was determined using a bicinchoninic acid protein kit (Thermo Fisher Scientific). The lysates were separated using SDS-PAGE and then electro-transferred onto polyvinylidene fluoride membrane (Millipore, Bedford, MA, USA). The membranes were blocked in 5% bovine serum albumin and then probed overnight with the following primary antibodies from Cell Signaling Technology (Beverly, MA, USA): anti-FOXP2 (1:2000), anti-PCNA and anti-MMP2 (1:2500), anti-MMP-9 and anti-Bax (1:3000), anti-Bcl-2 and anti- β -catenin (1:3500), and anti-c-myc, anti-cyclin D1, and anti-GAPDH (1:4000). The membranes were incubated with horseradish peroxidase-labeled secondary antibody (1:5000), and the signal was detected using an enhanced chemiluminescence kit (KeyGen, Nanjin, China).

Statistical analysis

Data are expressed as the mean \pm standard error of the mean (SEM), and the statistical analysis between groups was conducted using the one-way analysis of variance or Student's *t*-test. $P < 0.05$ was considered statistically significant.

RESULTS

Reduced FOXP2 in thyroid cancer cells

Analysis based on the TCGA database showed that FOXP2 was down-regulated in thyroid cancer tissues ($n = 505$) compared to normal tissues ($n = 59$, Figure 1 A). Human thyroid cancer cells (TPC-1, SW579, BCPAP) exhibited lower mRNA (Figure 1 B) and protein (Figure 1 C) levels than normal human primary thyroid follicular epithelial cells (Nthy-ori 3-1). The TPC-1 thyroid cancer cell line, which showed the lowest FOXP2 expression among the four thyroid cancer cell lines, was then transfected with pcDNA-FOXP2. Both FOXP2 mRNA (Figure 1 D) and protein (Figure 1 E) expression were higher following pcDNA-FOXP2 transfection relative to the pcDNA vector alone or control.

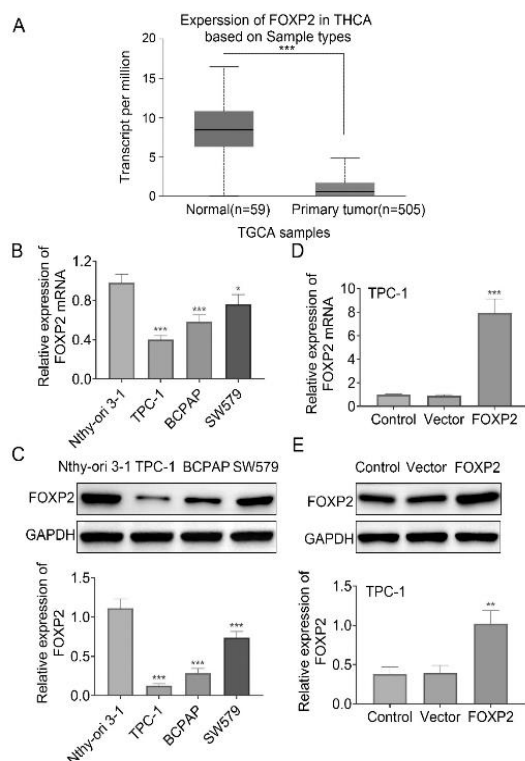


Figure 1: Reduced FOXP2 in thyroid cancer cells. (A) FOXP2 was down-regulated in thyroid cancer tissues ($n = 505$) when compared to normal tissues ($n = 59$) based on data from the TCGA database. (B) Expression of FOXP2 mRNA was down-regulated in thyroid cancer cells (TPC-1, SW579, BCPAP) compared to normal human primary thyroid follicular epithelial cells (Nthy-ori 3-1). (C) The level of FOXP2 protein was down-regulated in thyroid cancer cells (TPC-1, SW579, BCPAP) compared to normal human primary thyroid follicular epithelial cells (Nthy-ori 3-1). (D) Expression of FOXP2 mRNA was higher in TPC-1 cells transfected with pcDNA-FOXP2 than in cells transfected with the pcDNA vector or the control. (E) Level of FOXP2 protein was higher in TPC-1 cells transfected with pcDNA-FOXP2 than in cells transfected with the pcDNA vector or the control. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$

FOXP2 suppressed thyroid cancer cell growth and metastasis

Functional assays were conducted to investigate the regulatory role of FOXP2 on thyroid cancer progression. Ectopic expression of FOXP2 reduced the viability (Figure 2 A), migration (Figure 2 B), and invasion (Figure 2 C) of TPC-1 cells. The level of PCNA, MMP2, and MMP9 protein were decreased in TPC-1 following over-expression of FOXP2 (Figures 2 D and E), thus confirming the anti-proliferative and anti-invasive effects of FOXP2 on thyroid cancer cells.

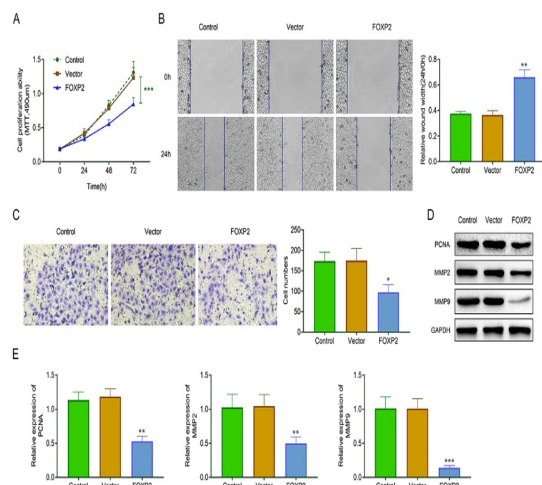


Figure 2: FOXP2 suppressed thyroid cancer cell growth and metastasis. (A) Ectopic expression of FOXP2 reduced TPC-1 cell viability. (B) Ectopic expression of FOXP2 repressed TPC-1 migration. (C) Ectopic expression of FOXP2 repressed TPC-1 invasion. (D) Ectopic expression of FOXP2 reduced the level of PCNA, MMP2, and MMP9 protein in TPC-1. (E) Relative expression levels of PCNA, MMP2, and MMP9 in TPC-1 cells transfected with pcDNA-FOXP2 or pcDNA vector. * $P < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs. pcDNA vector

FOXP2 promotes thyroid cancer cell apoptosis

Flow cytometric analysis demonstrated that apoptosis of TPC-1 cells was enhanced by transfection with pcDNA-FOXP2 (Figure 3 A). Ectopic expression of FOXP2 enhanced the level of Bax and reduced Bcl-2 in TPC-1 (Figure 3 B), indicating a pro-apoptotic role for FOXP2 in thyroid cancer cells.

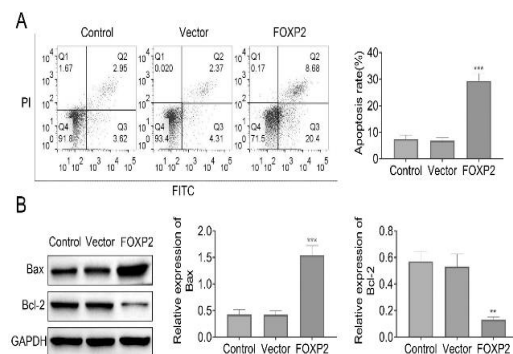


Figure 3: FOXP2 promoted thyroid cancer cell apoptosis. (A) Ectopic expression of FOXP2 promoted apoptosis of TPC-1. (B) Ectopic expression of FOXP2 enhanced the level of Bax and reduced Bcl-2 in TPC-1. ** $P < 0.01$, *** $p < 0.001$ vs. pcDNA vector

FOXP2 mediated β -catenin pathway in thyroid cancer

Over-expression of FOXP2 in TPC-1 decreased the level of β -catenin protein (Figure 4). The target genes of β -catenin, namely c-myc and cyclin D1, were also reduced in TPC-1 following transfection with pcDNA-FOXP2 (Figure 4), revealing that FOXP2 might suppress thyroid cancer progression through inactivation of the β -catenin pathway.

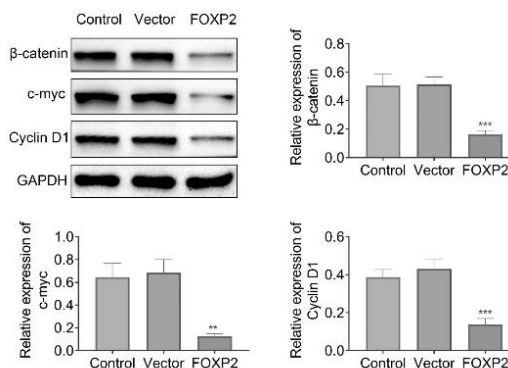


Figure 4: FOXP2 mediated the β -catenin pathway in thyroid cancer cells. Over-expression of FOXP2 in TPC-1 decreased the level of β -catenin, c-myc, and cyclin D1 protein. ** $P < 0.01$, *** $p < 0.001$ vs. pcDNA vector

DISCUSSION

FOXP proteins exert dual biological functions during cancer progression, acting as oncogenes or tumor suppressors through interaction with different targets [11]. It has been reported that triple-negative breast cancer is promoted by FOXP2 through up-regulation of glucose-regulated protein of molecular mass 78 [12]. However, FOXP2 was shown to suppress breast cancer migration and invasion by inactivation of the TGF β /SMAD pathway [7]. Both the oncogenic and tumor suppressive roles of FOXP2 in thyroid cancer were investigated in this study.

Our data demonstrate that FOXP2 was down-regulated in thyroid cancer based on the TCGA database. These observations were confirmed using qRT-PCR and western blot analysis. Functional assays in this study showed that over-expression of FOXP2 promoted thyroid cancer cell proliferation and metastasis. Moreover, PCNA, the key gene involved in thyroid cancer cell proliferation [13], was decreased in thyroid cancer cells by FOXP2 over-expression. Ectopic expression of FOXP2 decreased the level of MMP2 and MMP9 protein in thyroid cancer cells.

MMP2 and MMP9 have been reported to contribute to the epithelial-mesenchymal transition of thyroid cancer [14]. Therefore, FOXP2 might suppress the epithelial-mesenchymal transition of thyroid cancer. In addition to an anti-invasion role in thyroid cancer, apoptosis of thyroid cancer cells was promoted by FOXP2 over-expression, suggesting that FOXP2 exerted anti-invasive and pro-apoptotic effects on thyroid cancer.

FOXP2 regulates various neurogenic signaling pathways, such as the Hedgehog, Wnt, and Notch pathways, and plays a key role in embryonic development and cell cycle progression [15]. Aberrant activation of the Wnt pathway promoted thyroid cancer cell growth and metastasis [16] and inhibition of Wnt/ β -catenin signaling contributed to the repression of thyroid carcinoma cell proliferation and invasion [17]. The results obtained in this study indicate that ectopic expression of FOXP2 decreased the level of β -catenin in thyroid cancer cells, suggesting that FOXP2 might function as a tumor suppressor in thyroid cancer through inactivation of Wnt/ β -catenin signaling. Two target genes of Wnt/ β -catenin signaling, c-Myc and cyclin D1, were enhanced in papillary thyroid carcinoma and functioned as prognostic factors of papillary thyroid carcinoma [18].

The data from the present study demonstrate that c-Myc and cyclin D1 were reduced in thyroid cancer cells transfected with pcDNA-FOXP2, confirming the involvement of Wnt/ β -catenin signaling in FOXP2-mediated thyroid cancer progression. Moreover, FOXP2 has been shown to regulate other pathways, including the Hedgehog and Notch pathways [15]. Further investigation is required to determine whether these pathways are also involved in FOXP2-mediated thyroid cancer progression.

CONCLUSION

This study provides evidence that FOXP2 exerts *in vitro* tumor-suppressive activity in thyroid cancer cells through inactivation of Wnt/ β -catenin signaling. These data suggest the potential of FOXP2 as a therapeutic agent for treating thyroid cancer. Additional investigations in an animal model are needed to understand the suppressive role of FOXP2 in thyroid cancer *in vivo*.

DECLARATIONS

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Hui Zou designed the study and supervised the data collection, Caiyi Tang analyzed and interpreted the data, and Hao Chen prepared the manuscript for publication and reviewed the draft of the manuscript. All authors have read and approved the manuscript.

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