FOXP2 regulates thyroid cancer cell proliferation and apoptosis via transcriptional activation of RPS6KA6

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Received October 19, 2021; Accepted January 4, 2022

DOI: 10.3892/etm.2022.11361

Abstract. The transcription factor, forkhead box P2 (FOXP2) has tumor-suppressive effects in several types of cancer. However, the regulatory role and underlying mechanism of FOXP2 in thyroid cancer (THCA) is not completely understood. In the present study, the mRNA expression levels of FOXP2 and ribosomal protein S6 kinase A6 (RPS6KA6) were evaluated using the GEPIA database and THCA cell lines. The association between FOXP2 and RPS6KA6 was analyzed using the LinkedOmics, and GEPIA databases. Then, the binding sites of FOXP2 and the RPS6KA6 promoter was predicted using the JASPAR database, and verified using a dual-luciferase reporter assay and chromatin immunoprecipitation. In addition, functional assays investigating FOXP2 and RPS6KA6 were conducted in the TPC-1 cell line. The data showed that FOXP2 and RPS6KA6 mRNA expression levels were decreased in the THCA tissues, and cell lines. Overexpression of FOXP2 inhibited cell proliferation and promoted apoptosis in the THCA cell lines. Furthermore, RPS6KA6 mRNA expression levels were reduced in THCA and were correlated with FOXP2 expression level. Mechanistic studies revealed that FOXP2 binds directly to the promoter region of RPS6KA6 and modulated the expression level of RPS6KA6 transcriptionally. In addition, rescue experiments showed that knockdown of RPS6KA6 expression reversed the effects of FOXP2 overexpression on THCA cell proliferation and apoptosis, and the regulation of FOXP2/RPS6KA6 may be associated with the PI3K/AKT pathway. In summary, FOXP2 was associated with the proliferation and apoptosis of human THCA cells via the transcriptional activation of RPS6KA6. The FOXP2/RPS6KA6 axis could be a promising target for the treatment of THCA.

Introduction

Thyroid cancer (THCA) is one of the most common malignant tumors in the head and neck, and endocrine system (1). The incidence rate of THCA is increasing rapidly and this disease is projected to become the fourth major type of cancer worldwide (2). It has been reported that the incidence rate of THCA in females is higher compared with that in males (2-4 times), and thyroid nodules often occur in young individuals (3,4). The vast majority of THCA cases are considered to be indolent tumors; however, a small number of patients have a poor prognosis (5). To date, the knowledge of the molecular mechanism underlying the development of THCA has primarily focused on the roles of various genes and oncogenes (6,7). However, the detailed mechanism of the initiation and progression of THCA remains poorly understood, and there only a few available biomarkers used to diagnose and treat patients with THCA (8-10).

The fox transcription factor family have a C-terminal winged-helix/Forkhead DNA binding domain, which is involved in cell proliferation and differentiation, and organism development (11). Forkhead box P2 (FOXP2) is one member of the fox transcription factor family, located on chromosome 7q31 and is involved in embryonic development, the cell cycle and organ development, including the heart, the lungs and the central nervous system (12-14). In addition, FOXP2 expression levels were reported to be decreased in various types of cancer, such as gastric and lung cancer (15,16). Sun et al (17) revealed that FOXP2 expression levels were decreased in THCA samples using integrated microarray and bioinformatics analysis. However, the role and specific mechanism of FOXP2 in THCA remains unclear. In the current study, the biological roles and mechanisms of FOXP2 in THCA cell growth, and apoptosis was investigated. The results revealed that the FOXP2/ribosomal protein S6 kinase A6 (RPS6KA6) axis could be a novel therapeutic target for the treatment THCA.

Materials and methods

Bioinformatic analysis. The GEPIA database (http://gepia.cancer-pku.cn) was used to analyze the mRNA expression levels of FOXP2 and RPS6KA6 in THCA tissues and normal tissues (The original image downloaded from GEPIA database displayed in the form of log transformation). The association
between FOXP2 and RPS6KA6 was characterized using the LinkedOmics (http://www.linkedomics.org) and GEPIA databases. The JASPAR (http://jaspar.genereg.net) database was used to predict the binding site of FOXP2 with the RPS6KA6 promotor.

Cell culture. The normal human Nthy-ori3-1 thyroid cell line and the SW579, CGTH-W3 and TPC-1 THCA cell lines were purchased from the Cell Bank of Shanghai Institute of Biological Sciences, cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.), supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 µg/ml streptomycin, and 100 U/ml penicillin at 37˚C in a humidified incubator with 5% CO2.

Cell transfection. A pcDNA3.1 expression vector containing full-length FOXP2 [overexpression (Ov)-FOXP2] and a negative control (Ov-NC) were constructed by Shanghai GenePharma Co., Ltd. The small interfering (si)RNA oligonucleotides targeting RPS6KA6 (5'-RPS6KA6-1/2) and corresponding control (si-NC) were purchased from GeneCopoeia, Inc. Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used for transfection. The following siRNA sequences were used: si-RPS6KA6-1, 5'-GGAAUGAGAAAGAUAUAAUG-3'; si-RPS6KA6-2, 5'-GCUACUCUGCUACUCACU-3'; si-NC, 5'-UUCUCCCAGCGUGUCAAGU-3'. After 48 h, the cells were harvested for subsequent experiments.

Cell Counting Kit (CCK)-8 assay. After transfection, the cells were seeded into a 96-well plate at the density of 5x10³ cells/well and cultured at 37˚C. After 24, 48 and 72 h, 10 µl CCK-8 solution (Beyotime Institute of Biotechnology) was added to each well. The plates were incubated for 2 h and the absorbance was measured using a microplate reader (Thermo Fisher Scientific, Inc.) at 450 nm.

Colony formation assay. TPC-1 cells were seeded in triplicate in 6-well plates at 500 cells/well and cultured at 37˚C, with 10% FBS at 37˚C. The cells were fixed in 4% paraformaldehyde at room temperature for 15 min and stained with 0.1% crystal violet for 30 min at room temperature 2 weeks later. The number of visible colonies (defined as >50 cells/colony) were counted using a light microscope (Olympus Corporation).

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from THCA tissues and cells using TRIzol® (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. Then, 0.5 µg RNA was converted into cDNA at 37˚C for 1 h using PrimeScript RT MasterMix (Takara Bio, Inc.). qPCR was performed using SYBR® qPCR MasterMix (Vazyme Biotech Co., Ltd.). The following primer sequences were used: FOXP2 forward, 5'-AGTGCAAGACGGACAGCTC-3' and reverse, 5'-GCCGTATTTTCATCACACTCA-3'; RPS6KA6 forward, 5'-CTCCTGTTTGAAGTGCTCTGA-3' and reverse, 5'-GGGAAACCTGTTGGGTAG-3' and reverse, 5'-GAGTGGGTTGTCGCTGTGA-3'. The following thermocycling conditions were used: Initial denaturation at 95˚C for 5 min, followed by 35 cycles at 95˚C for 30 sec, 60˚C for 30 sec and 72˚C for 1 min, and 72˚C final extension for 7 min. GAPDH was used as an internal reference and the 2^(-ΔΔCq) method (18) was used to calculate the relative quantification.

TUNEL assay. A TUNEL assay was used to analyze cell apoptosis using an apoptosis detection kit (Roche Diagnostics, GmbH). Fluorescein isothiocyanate (FITC; green) and 4', 6-diamidino-2-phenylindole (DAPI; blue) were used to stain the apoptotic cells and the nuclei for 10 min at room temperature in the dark, respectively. The labeled cells were washed with PBS and visualized using a fluorescence microscope (Olympus BX53; Olympus Corporation) and at least 10 fields of view for each sample were examined.

Dual-luciferase reporter assay. The wild-type (WT) and corresponding mutant (MUT) RPS6KA6 promoter fragments, including the putative FOXP2 sites, were cloned into the pGL3-basic vector (Promega Corporation). The TPC-1 cell line was co-transfected with the constructed luciferase reporter vectors and Ov-FOXP2/Ov-NC. Luciferase activity was then detected using a Dual-Luciferase Reporter Assay kit (Promega Corporation) after transfection for 48 h using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Luciferase activities were normalized against Renilla luciferase.

Chromatin immunoprecipitation (ChIP). ChIP experiments were performed according to the method previously described (19). The cells were cross-linked with 1% formaldehyde for 10 min at 37˚C and quenched with 2.5 M glycine for 5 min at room temperature. After being immunoprecipitated from the cell lysates using a FOXP2 antibody (1:200; cat. no. #5337; Cell Signaling Technology, Inc.) for incubation at 4˚C overnight, the precipitated DNA was obtained via phenol/chloroform extraction and ethanol precipitation, and PCR was performed as aforementioned to amplify the FOXP2 binding site. The data obtained were normalized to the corresponding DNA precipitated by IgG. The sequences used for PCR were as follows: FOXP2 forward, 5'-AGTGCAAGACGGACAGCTC-3' and reverse, 5'-GCCGTATTTTCATCACACTCA-3'; RPS6KA6 forward, 5'-CTCCTGTTTGAAGTGCTCTGA-3' and reverse, 5'-ACTGGATGATACGCAGTCG-3'.

Western blot analysis. Total protein was extracted from THCA tissues and cells using RIPA buffer (Changsha Auragene Biological Technology Co., Ltd.) and quantified using a BCA Protein Assay kit (Beijing DinggGuoChangsheng Biotechnology Co., Ltd.). The lysates were incubated at 95˚C for 5 min, separated using 10% SDS-PAGE (Bio-Rad Laboratories) and transferred onto PVDF membranes (MilliporeSigma). After being blocked with 5% skimmed milk, primary antibodies targeting FOXP2 (1:1,000; cat. no. ab16046; Abcam), Ki67 (1:1,000; cat. no. ab92742; Abcam), PCNA (1:1,000; cat. no. ab29; Abcam), Bcl-2 (1:1,000; cat. no. ab32124; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), cleaved (C)-caspase 3 (1:500; cat. no. ab32042; Abcam), RPS6KA6 (1:1,000; cat. no. ab76117; Abcam), phosphorylated (p)-PI3K (1:1,000; cat. no. ab182651; Abcam), PI3K (1:1,000; cat. no. ab86714; Abcam), p-Akt (1:1,000; cat. no. ab38449; Abcam), Akt...
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(1:500; cat. no. ab8805; Abcam) or GAPDH (1:1,000; cat. no. ab8245; Abcam) were added and incubated overnight at 4˚C. Subsequently, the blots were incubated with horseradish peroxidase-conjugated secondary antibody (1:2,000; cat. no. ab6721; Abcam) at room temperature for 1 h. The protein bands were visualized using an ECL detection system and analyzed using ImageJ software (version 1.46; National Institutes of Health).

**Statistical analysis.** All the data are presented as the mean ± SD. Statistical analysis was performed using SPSS v13.0 statistical software (SPSS, Inc.) or GraphPad Prism v5.0 (GraphPad Software, Inc.). Significant differences between groups were analyzed using an unpaired Student’s t-test or one-way ANOVA followed by a Bonferroni’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**FOXP2 expression levels are decreased in THCA tissues and cells.** To investigate the role of FOXP2 in THCA progression, the mRNA and protein expression levels of FOXP2 in THCA was analyzed. As shown in Fig. 1A, FOXP2 mRNA expression levels were significantly decreased in patients with THCA compared with that in the normal tissues from healthy individuals, based on data from the GEPIA database. In addition, the results from RT-qPCR and western blot analysis revealed that the mRNA and protein expression levels of FOXP2 in THCA cells were markedly decreased compared with that in the normal thyroid cell line (Fig. 1B and C). Among the THCA cell lines, TPC-1 showed the lowest mRNA and protein expression levels of FOXP2; therefore, this was selected for the subsequent experiments.

Overexpression of FOXP2 inhibits cell proliferation and promotes apoptosis in the TPC-1 cell line. Subsequently, FOXP2 overexpression vectors were designed and transfected into the TPC-1 cell line to overexpress FOXP2. RT-qPCR and western blot analysis was used to determine the transfection efficiency (Fig. 2A and B). Subsequently, cell proliferation was evaluated using CCK-8 and colony formation assays, as well as western blot analysis. The CCK-8 results showed that the optical density values at three time points were reduced in the Ov-FOXP2 group compared with that in the NC group (Fig. 2C). Consistently, the number of colonies in the Ov-FOXP2 group was markedly lower compared with that in the NC group (Fig. 2D). In addition, the results from the TUNEL assay revealed that the apoptosis rate in the TPC-1 cell line was significantly increased following FOXP2 overexpression (Fig. 2E). Furthermore, the western blot results revealed that the protein expression levels of Ki67, PCNA and Bcl-2 were decreased, while the protein expression levels of Bax and C-caspase 3 were increased in the cells transfected with Ov-FOXP2 (Fig. 2F).

RPS6KA6 is correlated with FOXP2. To further investigate the underlying mechanism of FOXP2 in the development of THCA, bioinformatics analysis was performed to identify the potential targets of FOXP2. Data from the LinkedOmics database revealed that there was an association between RPS6KA6 and FOXP2 (Fig. 3A-C). In addition, data from the GEPIA database also showed that RPS6KA6 expression levels were correlated with FOXP2 expression levels (Fig. 3D and E). Next,
RPS6KA6 mRNA and protein expression levels were found to be decreased in THCA tissues based on data from the GEPIA database and in THCA cells using western blot analysis, respectively (Fig. 3F and G). The effects of FOXP2 overexpression on RPS6KA6 protein expression levels in the TPC-1 cells were subsequently investigated. The results revealed that RPS6KA6 protein expression levels were increased following FOXP2 overexpression, indicating an association between RPS6KA6 and FOXP2 (Fig. 3H).

**RPS6KA6 is a direct transcriptional target of FOXP2.** To investigate how FOXP2 targets RPS6KA6 in the TPC-1 cell line, the consensus sequences between FOXP2 and the promotor region of RPS6KA6 was predicted using the JASPAR database (Fig. 4A). To confirm the direct binding of FOXP2 with the RPS6KA6 promotor region, a dual-luciferase reporter assay was performed. The results showed that the luciferase activity of the WT RPS6KA6 promotor was significantly increased following FOXP2 overexpression, while there were no notable changes in luciferase activity in the other groups (Fig. 4B). To further verify the interaction between FOXP2 and RPS6KA6 promotor, a ChIP assay was performed. The results showed that FOXP2 binds to the predicted binding sites of RPS6KA6 (Fig. 4C).

FOXP2 regulates the proliferation and apoptosis of the TPC-1 cell line by targeting RPS6KA6. Then, si-RPS6KA6-1/2 was transfected into the TPC-1 cell line to knockdown the expression of RPS6KA6. The results from RT-qPCR and
western blot analysis showed that both si-RPS6KA6-1 and si-RPS6KA6-2 decreased the mRNA and protein expression level of RPS6KA6, respectively. In addition, si-RPS6KA6-2 showed more significant interference efficiency; therefore, si-RPS6KA6-2 was selected for subsequent experiments (Fig. 5A and B). Next, it was found that knockdown of RPS6KA6 reversed the effects of FOXP2 overexpression on cell proliferation and colony formation (Fig. 5C and D). Furthermore, the promoted apoptosis due to FOXP2 overexpression was also reduced following knockdown of RPS6KA6.
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**Discussion**

Emerging evidence has indicated the crucial role of FOXP2 in the initiation and progression of numerous types of cancer, including breast cancer, hepatocellular carcinoma, gastric cancer and multiple myeloma (24,25). However, the functional role and underlying molecular mechanisms of FOXP2 in THCA cell growth and apoptosis have not been completely clarified. In the current study, it was found that FOXP2 mRNA and protein expression levels were decreased in THCA cells, and FOXP2 played an inhibitory role in cell proliferation and an promoting role in cell apoptosis. Furthermore, we found that RPS6KA6 mRNA expression levels were correlated with FOXP2 mRNA expression levels and was activated by FOXP2 transcriptionally. Lastly, it was revealed that the PI3K/AKT signaling pathway was associated with FOXP2-mediated transcriptional activation of RPS6KA6 in the THCA cell line. These findings suggested that a FOXP2/RPS6KA6 axis exerts a tumor-suppressing role in THCA.

FOXP2, a transcription factor, is known to be essential for language and memory function, and has been associated with an increased susceptibility to schizophrenia (26-28). Recent studies reported the dysregulation of FOXP2 in...
multiple types of cancers (16,29,30). Chen et al (31) revealed that low expression levels of FOXP2 were associated with poor relapse-free survival times in breast cancer, and FOXP2 inhibited breast cancer cell migration, invasion and epithelial-mesenchymal transition. In the present study, it was found that the FOXP2 expression level was decreased in patients with THCA from the GEPIA database. The in vitro experiments also proved the decreased expression levels of FOXP2 in THCA cell lines, which is consistent with a previous report that FOXP2 mRNA expression levels were decreased in THCA using microarray analysis (17). However, it is controversial that some studies found that FOXP2 was expressed at low levels in several tumors, such as breast cancer, hepatocellular carcinoma and gastric.
cancer biopsies (15,32,33), while FOXP2 was found to be overexpressed in some other types of cancers, including multiple myelomas, several subtypes of lymphomas, osteosarcoma, neuroblastomas, and ERG fusion-negative prostate cancers (34-36). Thus, FOXP2 cannot be defined simply to act as a tumor suppressor or an oncogene. Based on the down-regulation of FOXP2 in THCA tissues and cells, FOXP2 was overexpressed to observe its role in THCA. Functional experiments revealed that FOXP2 overexpression significantly suppressed THCA cell proliferation and induced cell apoptosis, indicating FOXP2 may exert suppressive effects on THCA progression.

It is well-known that transcription factors bind to specific sequences of a gene to regulate protein expression at a specific intensity and time, by repressing or activating transcription of target genes (37,38). Thus, we intend to study the mechanism how FOXP2 affects THCA as a transcription factor. RPS6KA6, also known as X-linked ribosomal S6 kinase 4 (RSK4; one member of RSK family), is a ribosomal protein and associated with 'P53 dependent proliferation arrest', which can; therefore, act as a tumor suppressor (39,40). RPS6KA6 expression levels were found to be decreased in several types of cancer, such as breast and ovarian cancers (41,42). Mei et al (22) revealed that overexpression of RPS6KA6 suppressed migration and invasion, and promoted apoptosis in drug resistant breast cancer cells. In addition, Hu et al (43) reported that knockdown of RPS6KA6 expression inhibited cell apoptosis and promoted cell proliferation, migration, and invasion in gastric cancer. These data indicate that RPS6KA6 may play inhibitory role in tumors. Data from the LinkedOmics database demonstrated that the RPS6KA6 gene was associated with FOXP2. The GEPIA database showed that its mRNA expression levels were decreased in THCA tissues and cells, and RPS6KA6 and FOXP2 expression levels were correlated in THCA. In support of this view that FOXP2 interacts with RPS6KA6, the binding sites between FOXP2 and the RPS6KA6 promoter regions were predicted using the JASPAR database and verified using dual-luciferase reporter and ChIP assays. Rescue experiments also showed that knockdown of RPS6KA6 expression facilitated cell growth and reduced cell apoptosis in the THCA cells by reversing the effects of FOXP2 overexpression.

A previous study has shown that the activation of the PI3K/AKT signaling pathway promotes the transcription of downstream genes, including CDK4, cyclin D1 and Bax, participating in the regulation of cell proliferation, apoptosis and other cellular processes in cancer (44). The PI3K/AKT signaling pathway is also an important regulatory pathway in THCA (45). In addition, a recent study has confirmed that overexpression of RPS6KA6 in breast cancer cells reversed Adriamycin-resistance by inhibiting the PI3K/AKT signaling pathway (22). RPS6KA6 also functions as an endogenous inhibitor of the MAPK pathway and represses the phosphorylation of AKT (46). In the present study, knockdown of RPS6KA6 expression reversed the FOXP2-mediated reduced protein expression levels of p-PI3K and p-AKT, suggesting that RPS6KA6 may be associated with the regulation of FOXP2 in THCA cells by blocking the PI3K/AKT pathway. Notably, RSK has also been reported to be a downstream target of PI3K/AKT in breast cancer (47). We hypothesize that RPS6KA6 and PI3K/AKT may adjust to each other or RPS6KA6 yields different functions on PI3K/AKT in different type of cancers. Moreover, it was preliminarily revealed the inhibitory effects of FOXP2 and RPS6KA6 on the phosphorylation of PI3K/AKT, but the specific mechanism was not investigated. PI3K and AKT phosphorylation are usually activated by receptor tyrosine kinases and G-protein-coupled receptors (48). Based on this, the molecular mechanism by which RPS6KA6 inhibits the PI3K/AKT pathway or interacts with this pathway in THCA will be investigated in a further study.

In conclusion, the data from the present study indicated the essential inhibitory role of the FOXP2/RPS6KA6 axis in THCA and revealed the important role of RPS6KA6 in FOXP2-driven THCA cell proliferation, and apoptosis. This suggests that the FOXP2/RPS6KA6 axis could be an independent prognostic marker and a promising therapeutic strategy for patients with THCA.
Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

FY and ZX designed the study, drafted and revised the manuscript. ZX and SZ analyzed the data and searched the literature. FY, ZX and SZ performed the experiments. All authors read and approved the final manuscript. FY and ZX confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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