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Original Article

RhoBTB2 (DBC2) functions as a multifunctional tumor suppressor in thyroid cancer cells via mitochondrial apoptotic pathway

Chuan-Jiang Wang, Dong Yang, Ying-Wei Luo

Department of Vascular and Thyroid Surgery, The First Affiliated Hospital of China Medical University, Nanjing North Street 155, Heping District, Shenyang City 110001, Liaoning Province, P. R. China

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Abstract: Thyroid cancer is the most common endocrine malignancy worldwide. Tumor suppressor gene RhoBTB2 (also known as Deleted in Breast Cancer 2, DBC2) was observed in various carcinomas, however, no reports showed the effects of RhoBTB2 on thyroid cancer. In our study, we found that RhoBTB2 decreases proliferation, increases apoptosis, inhibits mobility, and induces mitochondria damage in SW579 cells through increased Bax and decreased Bcl-2 and Bcl-xL protein expression. The effects of RhoBTB2 on SW579 cells were inversed by using butin (an inhibitor of the mitochondrial apoptosis pathway). Our results suggest that RhoBTB2 suppresses the growth of SW579 cells through a mitochondrial apoptosis pathway.

Keywords: RhoBTB2, thyroid cancer, apoptosis, mobility, mitochondrial apoptotic pathway

Introduction

Thyroid cancer is the most common endocrine malignancy, with an estimated age-standardized rate per 100,000 of 2.9 in males and 9.1 in females worldwide in 2008 [1]. There has been a marked increase in the incidence of thyroid carcinoma cases worldwide in the last 30 years [2]. Multiple genetic and epigenetic alterations have been described in thyroid cancers in recent decades [3].

Tumor suppressor gene RhoBTB2 (also known as Deleted in Breast Cancer 2, DBC2) was isolated from human chromosome 8p21 where frequent deletions were observed in various carcinomas including breast [4], lung [5], stomach [6], and bone [7]. RhoBTB2 belongs to the RhoBTB subfamily of Rho GTPases and encodes an 83 kDa, atypical Rho GTPase, comprising a conserved Nterminal GTPase domain followed by two BTB/POZ domains [8]. Alteration of RhoBTB2 levels influences pathways responsible for cell cycle, apoptosis, cytoskeleton, and membrane-trafficking [9].

To our knowledge, the roles of RhoBTB2 in thyroid cancer remain unclear. In the present study, we performed global analysis of antitumor effects of RhoBTB2 by *in vitro* cell functional study. We demonstrated that the exogenous RhoBTB2 protein could inhibit growth and mobility, induce apoptosis and mitochondrial damage in thyroid cancer cells. The implications of these results were also briefly discussed.

Materials and methods

Cell culture

The human thyroid cancer cell line SW579 was purchased from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Shanghai, China) and maintained in DMEM (Life Technologies, Gaithersburg, MD, USA) supplemented with 10% FCS and antibiotics (100 μM penicillin and 100 μM streptomycin). Cells were maintained in a humidified cell incubator with 5% CO_2 at 37°C.

Cell growth inhibition assays (MTT assay)

Cells were plated in 96-well plates at a concentration of 1,500 cells/well and allowed to attach

Table 1. The antibodies used in the western blot analysis

Protein	Producer	Catalog number	Dilution
Bcl-xL	Santa Cruz Biotechnology	sc-8392	1:200
Bcl-2	Santa Cruz Biotechnology	sc-783	1:200
Bad	Santa Cruz Biotechnology	sc-8044	1:200
Bak	Santa Cruz Biotechnology	sc-7873	1:200
Bax	Santa Cruz Biotechnology	sc-7480	1:200
β-actin	Santa Cruz Biotechnology	sc-103656	1:1000

overnight. Cells were then treated with various concentrations of recombinant RhoBTB2 protein (gifted by Yao-Yang Zhang, China Medical University) (0, 10, 50, 100, or 200 nM) for 24 h under 5% CO_2 at 37°C. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (Sigma-Aldrich, Carlsbad, CA, USA) was then added at a final concentration of 0.5 mg/ml for 4 h and absorbance was measured at 550-560 nm. The IC_{50} value for recombinant RhoBTB2 protein was also determined. Butin (7, 3',4'-Trihydroxydihydroflavone) was purchased from Wako Pure Chemical Industries, Ltd. (Tokyo, Japan) and dissolved in dimethylsulfoxide (DMSO).

Apoptosis assay

For the apoptosis assay, equal numbers of cells (3×10^5) were seeded in 6-cm plates. Following the manufacturer's instructions (Apoptosis Detection Kit, KeyGEN, Nanjing, China), cells were trypsinized, washed twice with cold PBS, then resuspended in 200 μl binding buffer. AnnexinV-FITC was added to a final concentration of 0.5 μg/ml. And samples were incubated at room temperature in the dark. After 20 min, 300 μl binding buffer containing 0.5 μg/ml PI was added and samples were immediately analyzed on a FACSCalibur flow cytometer (Becton Dickinson Medical Devices; Shanghai, China).

Determination of mitochondrial membrane potential

The mitochondrial membrane potential (MMP) was analyzed using the fluorescent dye 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolycarbocyanine iodide (JC-1) following the manufacturer's protocol (KeyGEN).

Transwell assay

The migration assay was performed using the Boyden chamber (8 μM pore size polycarbonate membrane; Cell Biolabs; San Diego, CA, USA). Cells were resuspended in FBS-free DMEM to a concentration of 3×10^5 cells/ml. The upper chamber was loaded with 100 μl of cell suspensions and the lower chamber was loaded with 600 μl of DMEM containing 10% FBS. After incubation for 12 h in normal culture conditions, we did not see any cells floating in the upper chamber, indicating that the cells had not undergone apoptosis at this time point. The filter was fixed in 4% paraformaldehyde (Sigma) and stained with crystal violet (Beyotime, Shanghai, China). The cells on the upper side of the filter were wiped off using a cotton swab. The cells that migrated to the undersurface of the membrane were counted using a light microscope. Ten microscopic fields (400 ×) were randomly selected to count cells. For invasive assay, the procedures were the same as above, excluding the matrigel-coated insert (BD Bioscience).

TRAP telomerase activity assay

Cells were lysed in lysis buffer (10 mM Tris HCl pH=7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM PMSF, 5 mM 2-mercaptoethanol, 0.5% CHAPS, 10% glycerol) on ice for 30 min and then centrifuged at high speed for 30 min, the suspension containing telomerase was used for TRAP assay. Telomerase activity was measured with the TRAPeze telomerase detection kit (Chemicon, Temecula, CA, USA) according to the instruction of the manufacturer [10].

Immunoblotting

Protein extracts were analyzed by Western blot analysis using the antibodies listed in **Table 1**. Cell extracts obtained in Laemmli buffer were resolved on SDS-PAGE, followed by electrotransfer to nitrocellulose membranes. Following a blocking step in 5% milk in Tween-TBS, membranes were incubated with primary and secondary antibodies. Membranes were then developed and visualized with ECL (Pierce, Thermo Fisher Scientific Inc., Waltham, MA, USA). Densitometric quantification for western-blot was performed using Scion Image software (Bethesda, MD, USA).

Statistical analysis

All numerical data were expressed as means ± SD. Differences among the mean values were

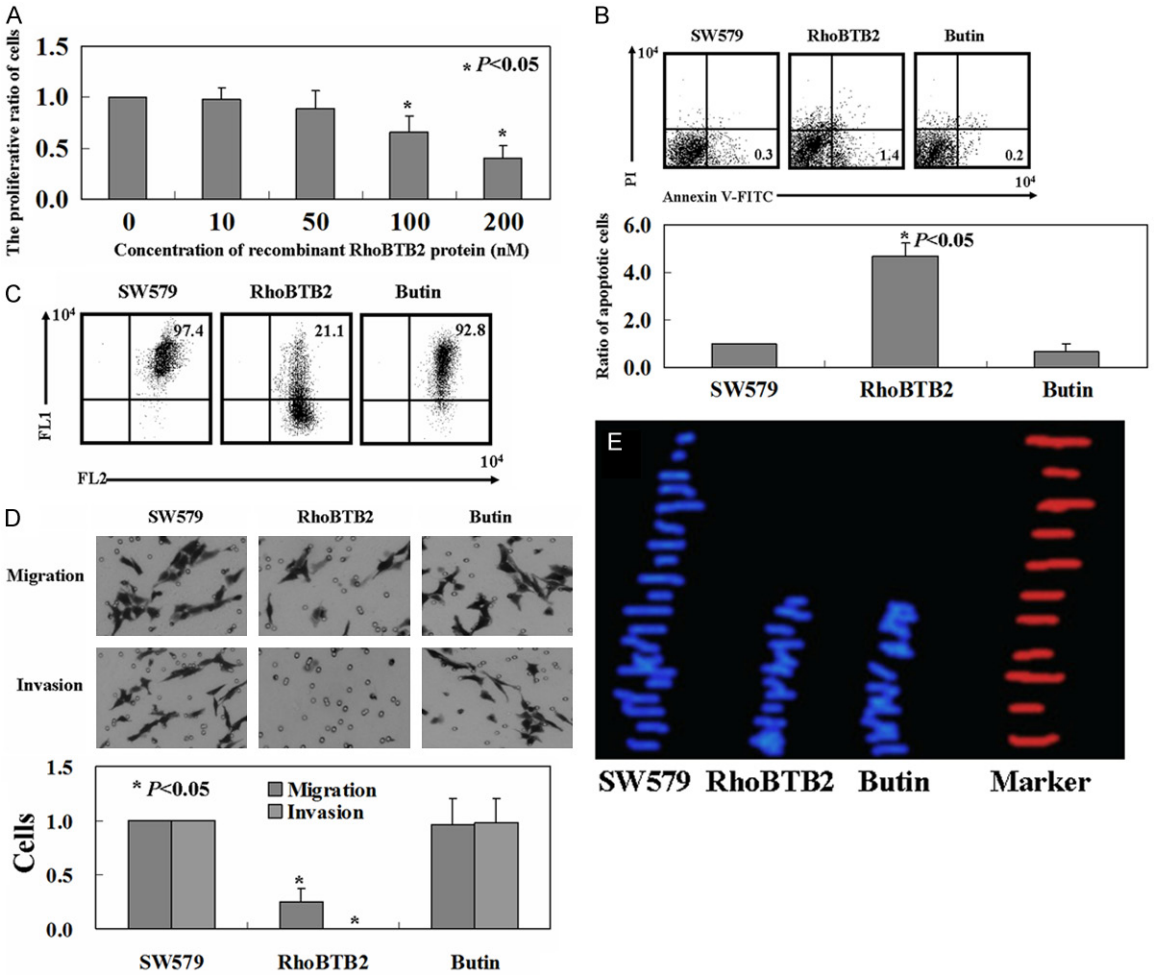

Figure 1. Anti-tumor activities of RhoBTB2 in SW579 cells. A. Proliferation ratio of SW579 cells treated with recombinant RhoBTB2 protein was measured using MTT assay. B. Apoptotic ratio of cells was analyzed by double stained with Annexin-V/propidium iodide (PI). C. Mitochondrial-Membrane Potential was analyzed by Flow cytometry. D. Transwell assays were performed. Cells that migrated to the bottom side of the membrane were stained and counted. E. RhoBTB2 treated SW579 cells showed reduced telomerase activity. These experiments were repeated three times. SW579: untreated SW579 cells; RhoBTB2: RhoBTB2 treated SW579 cells; Butin: both butin and RhoBTB2 treated SW579 cells.

evaluated using Student's t-test. All statistical analyses were conducted by SPSS 11.0 (SPSS Inc., USA). *P*-values <0.05 were considered to statistically significant.

Results

The effects of recombinant RhoBTB2 protein on SW579 cells

Cell viability was monitored using a MTT assay, and **Figure 1A** showed recombinant RhoBTB2 protein could inhibit the growth of SW579 cells. IC_{50} value of recombinant RhoBTB2 protein was 153.2 nM. Then we utilized the Annexin V-FITC and PI double staining to detect the apoptotic cells. Our results showed that the apoptotic ratio of the cells after treated with recombinant RhoBTB2 protein was increased significantly (P<0.05, **Figure 1B**). Additionally, changes in MMP were detected in recombinant RhoBTB2 protein treated cells by using flow cytometry (**Figure 1C**). Motility was significantly decreased in recombinant RhoBTB2 protein treated cells compared to untreated ones (P<0.05, **Figure 1D**). Interestingly, recombinant RhoBTB2 protein treated cells showed reduced telomerase activity, while untreated cells displayed robust telomerase activity (**Figure 1E**). Based on these results, we hypothesized that RhoBTB2 induced apoptosis in SW579 cells was associated with the mitochondrial apoptotic signaling pathway.

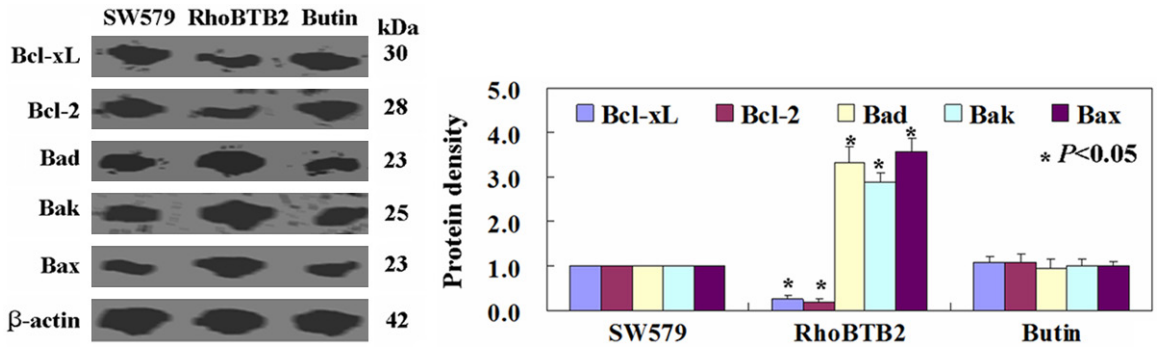

Figure 2. Effects of RhoBTB2 on mitochondrial apoptosis related proteins. Western blot analysis was performed. Cell lysates were electrophoresed and Bax, Bcl-2, Bcl-xL, Bad, Bak proteins were detected by their specific antibodies.

Cells were treated with butin as a positive control. After treatment, recombinant RhoBTB2 protein failed to play its anti-tumor activities in SW579 cells (**Figure 1**). However, butin showed no effects of recombinant RhoBTB2 protein on telomerase activity (**Figure 1E**).

The mechanism(s) of recombinant RhoBTB2 protein induced apoptosis in SW579 cells

To identify the mechanism of recombinant RhoBTB2 protein in SW579 cells, we detected the protein expression of Bad, Bak, Bax, Bcl-2, and Bcl-xL by Western blot analysis. We found a decrease in Bcl-2 and Bcl-xL protein as well as an increase in Bad, Bak, and Bax protein levels in SW579 cells after RhoBTB2 treatment compared to untreated cells ($P<0.05$, **Figure 2**). These changes could be reversed by using butin. Taken together, these results suggest that recombinant RhoBTB2 protein affects SW579 cells through the mitochondrial apoptotic signaling pathway.

Discussion

RhoBTB2 (DBC2) functions as a tumor suppressor via inhibiting proliferation and inducing apoptosis in many cancer cells, such as breast cancer [11], osteosarcoma [7], and head and neck squamous cell carcinoma [12]. Consistent with previous studies, we also confirmed RhoBTB2 could inhibit proliferation and mobility, induce apoptosis, and cause mitochondrial injury in thyroid cancer cells. This is the first report on the role of RhoBTB2 in thyroid cancer cells.

Interestingly, we found RhoBTB2 could reduce telomerase activity. Telomerase is the enzyme responsible for the maintenance of telomeres, which cap and protect the ends of chromosomes. In cells that lack telomerase, telomeres shorten with each round of cell division and this attrition eventually limits cellular lifespan [13]. One of the capacities of cancer cells is replication without limits, which is achieved by telomerase-mediated telomere maintenance [14]. Thus, telomerase inhibitors have the potential to be used as a selective anti-cancer therapy which disrupts the replicative capacity of telomerase-positive cancer cells [15]. However, we didn't find the mechanism of RhoBTB2-reduced telomerase activity. We only confirmed RhoBTB2-induced apoptosis in SW579 cells through the mitochondrial apoptotic signaling pathway. The balance of pro-apoptotic (Bax, Bad and Bid) and anti-apoptotic members (Bcl-2, Bcl-xL) controls the sensitivity of cells to apoptosis activators [16-18]. Butin is known to provide protective effects against H_2O_2-induced apoptosis by scavenging ROS and activating antioxidant enzymes [19]. In our study, we confirmed RhoBTB2 showed no effects on SW579 cells after butin treatment.

In summary, our results confirmed the antitumor activities and mechanism(s) of RhoBTB2-induced apoptosis in SW579 cells. However, the mechanism of RhoBTB2-reduced telomerase activity remains unclear. These results provide initial evidence suggesting that RhoBTB2 might be a useful tool for targeting thyroid cancer cells.

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Disclosure of conflict of interest

None.

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

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