EFFECTS OF BRAF ON GROWTH AND PROLIFERATION OF THYROID CANCER CELLS BY REGULATING THE RAS-BRAF-MEK-ERK SIGNALING PATHWAY

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ABSTRACT

Objective: *To analyze the effects of BRAF on growth and proliferation of thyroid cancer cells by regulating the RAS-BRAF-MEK-ERK signaling pathway.*

Methods: *Thyroid cancer SW579 cell line and thyroid tumor cells were cultured in vitro, and mRNA expression of BRAF in thyroid cancer cells was detected by real-time quantitative PCR assay. Thyroid cancer SW579 cells were randomly divided into control group, down-regulated BRAF 1 group and down-regulated BRAF 2 group, and transfected with control interference fragment, BRAF interference fragment 1 and BRAF interference fragment 2, respectively. An MTT assay was used to detect the effect of down-regulated BRAF expression on the proliferation of thyroid cancer cells, and a western blot was used to measure the effect of down-regulated BRAF expression on the protein expressions of RAS, BRAF, p-MEK, MEK, p-ERK and ERK.*

Results: *The expression level of BRAF mRNA in thyroid cancer cells was significantly higher than that in thyroid tumors, and the difference was statistically significant ($P < 0.05$). After 25 nmol siRNA was used to interfere with BRAF, the cell growth ability was detected. Compared with the control group, the down-regulated BRAF 1 group and the down-regulated BRAF 2 group could significantly inhibit the growth of thyroid cancer cells after 7 days of interference. The cell proliferation rate was detected at 12, 24 and 36 h and it was found that compared with the control group, the proliferation of thyroid cancer cells in the down-regulated BRAF 1 group and the down-regulated BRAF 2 group was remarkably inhibited at each time point ($P<0.05$). Compared with the control group, BRAF and RAS protein levels and MEK and ERK phosphorylation levels were obviously reduced in the down-regulated BRAF 1 group and the down-regulated BRAF 2 group, with statistically significant differences ($P<0.05$).*

Conclusion: *The BRAF gene is highly expressed in thyroid cancer cells, and down-regulation of its level can significantly inhibit the growth and proliferation of thyroid cancer cells, and its mechanism may be related to the inhibition of the expression of the RAS-BRAF-MEK-ERK signaling pathway.*

Keywords: *BRAF, RAS-BRAF-MEK-ERK signaling pathway, thyroid cancer cell, growth; proliferation.*

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Introduction

Thyroid cancer is the most common malignant thyroid tumor, accounting for about 1% to 2% of all human tumors. Papillary carcinoma is the most common with a low malignant degree and good prognosis. Its typical clinical manifestations are mass found in the thyroid, accompanied by invasion of surrounding tissues. It is more common in young women and has a good prognosis(1). The specific etiology of thyroid cancer is not clear, and it is considered at present to be closely related to factors such as iodine intake, radiation and hormone effects. In recent years, with the progress of neck ultrasound examination and puncture biopsy techniques, the detection rate of thyroid cancer has significantly increased. Due to the high recurrence rate of thyroid cancer, its clinical incurability and mortality have increased. Relevant data show that the risk of recurrence of breast cancer in patients with a history

of thyroid problems is significantly higher than that in the normal population[2].

Different histological types of thyroid cancer have different cell sources, some of which are derived from parafollicular C cells, and the main pathogenesis is the abnormal activation of RET signal caused by mutation of the RET gene[3]. BRAF belongs to the tryptophan/serine kinase RAF family, which has been confirmed to show high frequency BRAF gene mutation in thyroid papillary carcinoma, and it is believed that BRAF may play a biological role through the RAS-BRAF-MEK-ERK signaling pathway. Activation of the RAS-BRAF-MEK-ERK pathway can be detected in several gene-altered thyroid cancers, which can regulate various biological behaviors such as cell growth and proliferation, but its specific role is still unclear[4-5]. Therefore, this study aimed to analyze the effect of BRAF on the growth and proliferation of thyroid cancer cells by regulating the RAS-BRAF-MEK-ERK signaling pathway.

Materials and methods

Experimental reagents and instruments

Thyroid cancer SW579 cell line (Shanghai Guandao Bioengineering Co., Ltd.); siRNA sequence (Biomics Biotechnology Co., Ltd.); paraformaldehyde (Beijing Biolab Technology Co., Ltd.); Reverse transcriptome kit (GeneCopoeia, USA); Trizol and PVDF membrane (Beijing Kairiji Biotechnology Co., Ltd.); BCA protein concentration kit (Shanghai Kanglang Biotechnology Co., Ltd.); ECL chemiluminescence kit (Shanghai Yiji Industrial Co., Ltd.); Anti- RAS, BRAF, p-MEK, MEK, p-ERK, ERK antibodies (Sigma, USA).

Super-clean worktable (Guangzhou Haohan Instruments Co., Ltd.); Electrothermal thermostatic water bath (Hepeng (Shanghai) Biotechnology Co., Ltd); Low temperature high speed centrifuge (Wuhan Yipu Biotechnology Co., Ltd.); Optical microscope (Guangzhou Dahui Biotechnology Co., Ltd.); -80oC Cryogenic Refrigerators (Sensi Technology Co., Ltd.); Vertical electrophoresis tank (Shanghai Zhennuo Biotechnology Co., Ltd.); Transmembrane instrument (Shanghai Aiyan Biotechnology Co., Ltd.); Automatic gel imaging system (Bio-Rad Laboratories, Shanghai).

Cell culture, transfection and grouping

Thyroid carcinoma SW579 cell line and thyroid tumor cells were placed in the medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 g/ml streptomycin. The cells were cultured in an incubator at 37°C with 5% CO_2. When the cells covered about 80% of the bottom area of the culture flask, the cells were subcultured, and the cells at logarithmic growth phase were used in the further experiment. Thyroid cancer SW579 cells were randomly divided into control group, down-regulated BRAF 1 group and down-regulated BRAF 2 group, and transfected with control interference fragment, BRAF interference fragment 1 and BRAF interference fragment 2, respectively.

The transfection procedure was as follows: the inoculated cells were cultured overnight, siRNA-liposome complex was added to each well, and the transfection effect was observed under a fluorescence microscope 48h after transfection.

Detection method

• A real-time quantitative PCR method was used to detect BRAF mRNA expression in thyroid cancer cells. Total RNA was extracted according to the instructions for Trizol Reagent. Agarose gel electrophoresis was used to detect RNA integrity. RNA concentration and purity were determined by nucleic acid protein analyzer. According to the instructions of the reverse transcription kit, the total RNA extracted was synthesized into cRNA, reverse transcription was performed to synthesize cDNA reaction system, and PCR amplification was conducted. Reaction conditions: pre-denaturation at 94°C for 3 min, 94°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 50 s, and 72°C for 7 min, with a total of 30 cycles. BRAF mRNA expression was analyzed by formula $2^{-\triangle\triangle CT}$.

• Effect of down-regulated BRAF expression on thyroid cancer cell growth. Thyroid cancer cells at the logarithmic growth phase were inoculated into 96-well plates with a density of 1×10^4 per well, and cell growth was observed and photographed every day. At the same time, 3 cells in each group were counted every day and the average value was calculated. After continuous counting for 7 days, the cell growth curve was drawn to analyze the effect of down-regulated BRAF expression on the growth and proliferation of thyroid cancer cells.

• Thyroid cancer cells at the logarithmic growth phase were inoculated into 24-well plates, and modified RPMI-1640 medium with 20 μL and 180 μL of 5 mg/ml MTT solution was added to each well. After 4 hours of culture, the supernatant was discarded and 150 μL of DMSO was added. The

supernatant was shaken in a constant temperature oscillating chamber for 10 min, and the absorbance value of each group at the wavelength of 492 m was determined by enzyme-linked immunosorbent assay. Cell proliferation inhibition rate = (1-OD value of the experimental group/OD value of the control group).

• A western blot assay was used to detect the effect of down-regulated BRAF expression on the protein expression of RAS, BRAF, P-MEK, MEK, P-ERk and ERK. Thyroid cancer cells at the logarithmic growth phase were taken and total protein was extracted by adding RIPA protein lysis buffer according to the protein extraction kit. The protein content was detected according to the instructions of BCA-200 Protein Assay Kit. 20 μg protein was used for SDS-PAGE electrophoresis, and a western blot was carried out. The separation gel and stacking gel were prepared. After electrophoresis, the slides were transferred to PVDF membrane and sealed at room temperature for 1h with blocking buffer. The diluted RAS, BRAF, p-MEK, MEK, p-ERK and ERK primary antibodies were added and rotated on the drum for 2 h or in the cold chamber overnight. The corresponding secondary antibodies were added and rotated on the drum for 2 h. The ECL chemiluminescence method was used for color development. The imaging scanning analysis system was used for scanning, development and quantification of the protein bands, and the exposure time and development time was appropriately adjusted according to the depth of the bands to make the band clearer.

Statistical methods

In this study, all measurement data were represented by ($\bar{x}\pm s$). An independent sample t-test was used to compare the mean between two groups, and analysis of variance was used to compare the mean among multiple groups. $P<0.05$ indicated the difference was statistically significant. The data in this study were analyzed by SPSS20.0 software package.

Results

Changes of BRAF mRNA expression in thyroid cancer cells

Real-time quantitative PCR results showed that the expression level of BRAF mRNA in thyroid cancer cells was significantly higher than that in thyroid tumors, and the difference was statistically significant ($P<0.05$). The results are shown in Figure 1.

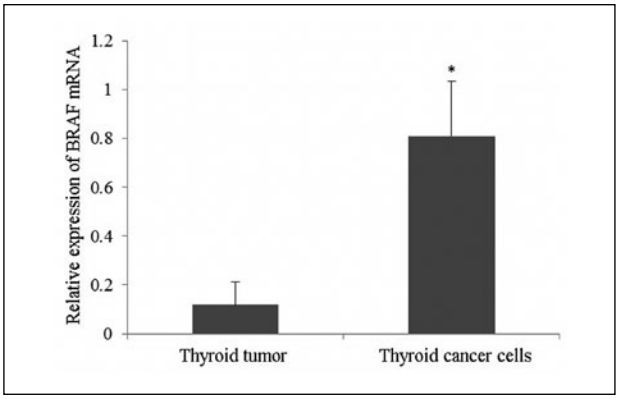

Figure 1: Changes of BRAF mRNA expression in thyroid cancer cells.

Effect of down-regulated BRAF expression on thyroid cancer cell growth

After 25 nmol siRNA was used to interfere with BRAF, the cell growth ability was detected. Compared with the control group, the down-regulated BRAF 1 group and the down-regulated BRAF 2 group could significantly inhibit the growth of thyroid cancer cells after 7 days of interference. The results are shown in Figure 2.

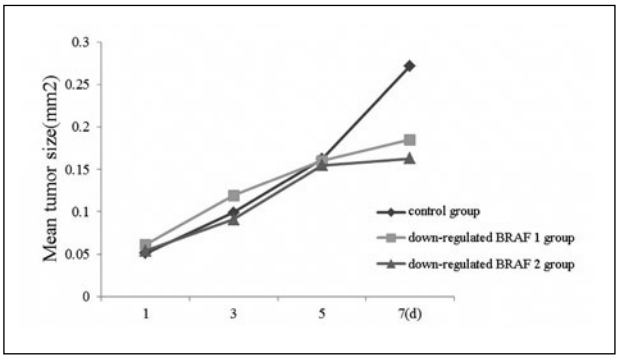

Figure 2: Effect of down-regulated BRAF expression on thyroid cancer cell growth.

Effect of down-regulated BRAF expression on proliferation of thyroid cancer cells

The results of the MTT assay showed that, after 25 nmol siRNA was used to interfere with BRAF, the cell proliferation rate was detected at 12, 24 and 36 h and it was found that compared with the control group, the proliferation of thyroid cancer cells in the down-regulated BRAF 1 group and the down-regulated BRAF 2 group were remarkably inhibited at each time point ($P<0.05$). The results are shown in Table 1.

The western blot results showed that compared with the control group, BRAF and RAS protein levels, as well as MEK and ERK phosphorylation levels were obviously reduced in the down-regulated BRAF 1 group and the down-regulated BRAF

2 group, with statistically significant differences (P<0.05). The results are shown in Table 2 and Figure 3.

Groups	Sample size	Control group	Down-regulated BRAF 1 group	Down-regulated BRAF 2 group
12h	8	5.63±1.87	3.64±1.52	2.05±0.75
24h	8	9.10±1.45	5.08±1.34	4.41±1.16
36h	8	15.26±2.70	7.41±1.27	6.30±1.36

Table 1: Effect of down-regulated BRAF expression on proliferation of thyroid cancer cells.

Groups	BRAF	RAS	pMEK	MEK	pERK	ERK
Control group	1.44±0.11	0.22±0.03	2.94±0.84	5.11±1.03	1.37±0.45	3.02±0.85
Down-regulated BRAF 1	0.10±0.03	0.08±0.02	1.97±0.73	4.87±0.82	0.56±0.12	3.37±0.95
Down-regulated BRAF 2	0.32±0.05	0.04±0.01	2.04±0.68	5.23±1.11	0.67±0.34	3.01±0.79

Table 2: Effect of down-regulated BRAF expression on the RAS-BRAF-MEK-ERK signaling pathway in thyroid cancer cells.

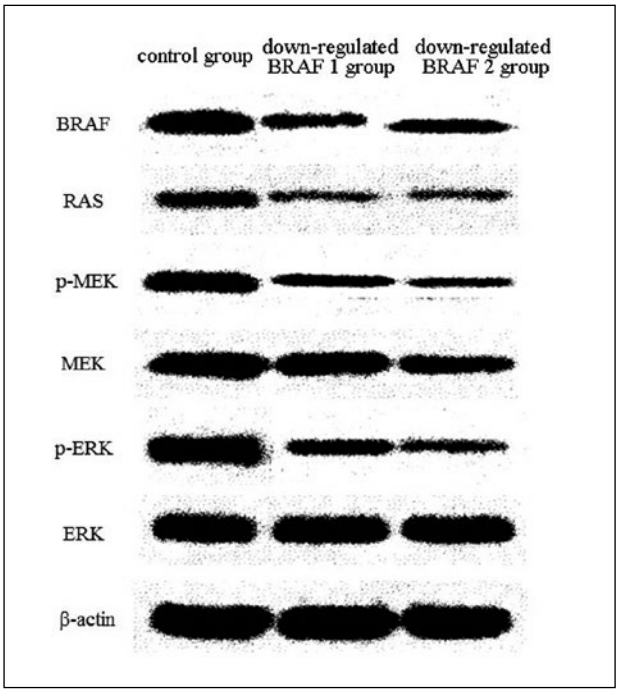

Figure 3: Comparison of BRAF and RAS protein levels, as well as MEK and ERK phosphorylation levels in each group.

Discussion

The thyroid gland is a very important endocrine trachea of the human body. Thyroid lesions can lead to the occurrence of a variety of diseases, including thyroid cancer, which is one of the top 10 cancers that endanger the health of patients. Thyroid cancer can be divided into papillary carcinoma and follicular adenocarcinoma according to pathological types, about 90% of which are well-differentiated thyroid carcinoma. Papillary carcinoma is the most common histological type of thyroid tumor, accounting for about 70% to 80% of cases. Most thyroid cancers have low malignancy and a good prognosis, and early diagnosis and treatment are of great clinical significance[6-7]. The typical clinical symptoms of thyroid cancer are masses found in the thyroid gland, with hoarseness and dysphagia in the late stage, which seriously affects the quality of life of patients. The common clinical treatment for thyroid cancer is total thyroidectomy or subtotal thyroidectomy, but it is prone to recurrence and metastasis. At present, the pathogenesis of thyroid cancer is not fully understood, and it is generally believed that the occurrence and development of thyroid cancer are regulated by multiple genes including oncogenes, tumor suppressor genes and anti-apoptotic genes[8-9].

BRAF is a serine/threonine specific kinase, which is called BRAF because of its high homology with C-raf and A-raf. The BRAF gene is located on human chromosome 7q34 and contains seven transcriptional regions. It is involved in cell proliferation, differentiation and apoptosis and is an important factor of the MEK/ERK pathway[10]. Relevant data showed that in malignant tumors such as malignant melanoma, colorectal cancer, thyroid cancer and pancreatic cancer, the proportion of BRAF gene mutation was different, the mutation rate in papillary carcinoma was 29% to 70%, and no mutation was found in benign thyroid lesions and follicular adenocarcinoma[11]. Through the in-depth study of the relationship between BRAF and thyroid cancer, we can not only understand the mechanism of BRAF carcinogenesis, but also help to find new treatment methods. Some scholars found[12] that BRAF presented cytoplasmic distribution in both thyroid tumor and thyroid papillary cancer cells, and the signal in thyroid papillary carcinoma was significantly stronger than that in thyroid tumor tissues. Phosphorylation regulation is the regulatory mechanism of the MEK/ERK pathway, and the signal transduction mechanisms of various information transmission are protein phosphorylation and dephosphorylation.

The MEK/ERK pathway has the most characteristic protein kinases and phosphorylation pathways in cell biological activities[13]. The RAS-BRAF-MEK-ERK signaling pathway is one of the most active pathways studied so far. BRAF is the strongest activator of the downstream signaling

pathway, which can lead to abnormal proliferation and differentiation of cells, leading to the occurrence and development of tumors[14]. Relevant data showed that the proliferation of thyroid papillary cancer cells with BRAFV600E gene mutation could be inhibited by multi-target kinase inhibitor BAY43.9006, which significantly inhibited the growth and proliferation of thyroid papillary cancer cells with rearranged genotype and BRAF mutation[15].

In this study, by interfering with the expression of the BRAF gene in thyroid carcinoma SW579 cell line, the cell growth and proliferation was detected. After 25 nmol siRNA was used to interfere with BRAF, the cell growth ability was detected and it was found that compared with the control group, the down-regulated BRAF 1 group and the down-regulated BRAF 2 group could significantly inhibit the growth of thyroid cancer cells after 7 days of interference. The results of the MTT assay showed that, after interfering with BRAF using 25 nmol siRNA, the cell proliferation rate was detected at 12, 24 and 36 h and it was found that compared with the control group, the proliferation of thyroid cancer cells in the down-regulated BRAF 1 group and the down-regulated BRAF 2 group were remarkably inhibited at each time point ($P<0.05$). It suggested that down-regulation of BRAF expression could significantly inhibit the growth and proliferation of thyroid cancer cells.

The results of the western blot further showed that compared with the control group, BRAF and RAS protein levels, as well as MEK and ERK phosphorylation levels were obviously reduced in the down-regulated BRAF 1 group and the down-regulated BRAF 2 group, with statistically significant differences ($P<0.05$). It indicated that the down-regulation of BRAF expression might inhibit the growth and proliferation of thyroid cancer cells by regulating the RAS-BRAF-MEK-ERK signaling pathway. In conclusion, the BRAF gene is highly expressed in thyroid cancer cells, and down-regulation of its level can significantly inhibit the growth and proliferation of thyroid cancer cells, and its mechanism may be related to the inhibition of the expression of the RAS-BRAF-MEK-ERK signaling pathway.

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