

Inhibition of thyroid carcinoma cells with YAP1 protein interference and its mechanism

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Abstract. – OBJECTIVE: To investigate the effects and mechanism of yes-associated protein 1 (YAP1) on thyroid carcinoma cells.

MATERIALS AND METHODS: Quantitative Real-time PCR (qRT-PCR) and Western blot assay were used to detect the expression of YAP1 in normal thyroid cells (HT-ori3) and four types of thyroid carcinoma cells: FTC-133, IHH-4, TPC-1 and NPA. The cell lines with the highest expression of YAP1 were selected as the experimental materials. qRT-PCR and Western blot assay were used to detect the interference effect of si-YAP1. The cell proliferation and the effect on the PI3K-Akt signal pathway were examined by MTT and Western blot.

RESULTS: The expression of YAP1 significantly increased in the thyroid carcinoma cell line compared with normal thyroid cells, among which the expression of YAP1 in TPC-1 was the highest. Quantitative PCR and Western blot results showed significant interference effects. The MTT assay indicated that YAP1 interference suppressed the proliferation of cells and the expression of p-Akt.

CONCLUSIONS: The interference of YAP1 can inhibit the growth of thyroid cancer cells, and its mechanism may be associated with the PI3K-Akt signaling pathway.

Key Words:

YAP1, Thyroid cancer cells, Proliferation, PI3K-Akt.

recent years². The growth of malignant tumors is closely related to the aggressive proliferation of cells. The Hippo signaling pathway inhibits growth and it is involved in cell proliferation, cell death, and cell differentiation³. Its core components are YAP1/TAZ transcriptional regulators, which are closely related to the occurrence and development of cancer^{4,5}. Researches⁶ have found that persistently high YAP1 expression can lead to the occurrence of malignant tumors. The high expression of YAP1 in thyroid cancer and its role in thyroid cancer cells have rarely been reported⁷. In this work, YAP1 gene silencing was used to observe the effect of YAP1 on proliferation and the related signaling pathway to provide a theoretical basis for YAP1 gene therapy in thyroid carcinoma.

Materials and Methods

Cell Lines

Thyroid cancer cell lines FTC-133, IHH-4, TPC-1 and NPA, as well as normal cell line HT-ori3, were purchased from Shanghai ATCC (Shanghai, China). Cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) (containing 100 µg/mL of streptomycin) at 37°C in a 5% CO₂ incubator.

Reagents

DMEM basal medium, FBS (HyClone Laboratories, South-Logan, UT, USA) and thiazolyl blue (Sigma-Aldrich, St. Louis, MO, USA) were used in this work. The MTT was made into a 5 mg/ml solution, filtered and stored at -20°C. An RT-PCR kit (TaKaRa, Otsu, Shiga, Japan) was used for DNA synthesis. YAP1, GAPDH, p-Akt and Akt antibodies were purchased from Cell Signaling

Introduction

In recent years, the incidence of thyroid cancer has trended upward. This kind of malignancy has increased faster than all others in the past two decades. The cancer cells typically takes over 5% of the thyroid nodules and 1% of the thyroid tumor¹. It has become the most common malignant tumor in the endocrine system and a hotspot of cancer research in

Technology (Danvers, MA, USA). YAP1 primers were used in DNA synthesis (Thermo Fisher Scientific, Waltham, MA, USA) (Table I). Si-YAP1 was purchased from Shanghai Gemma Medical Technology (Shanghai, China).

Methods

To measure the amplification of DNA using fluorescent dyes, qRT-PCR and Western blot techniques were used. This showed the expression of YAP1 in four thyroid cancer cell lines and a normal thyroid cell line. Thyroid cancer cells and normal thyroid cells were inoculated into six-well plates. After the cells were adhered, 1 ml of TRIzol was added. Total RNA was extracted from the cells using TRIzol and purified with a TaKaRa reagent kit (Otsu, Shiga, Japan). The qRT-PCR was performed using a TaKaRa reagent kit (Otsu, Shiga, Japan). The GAPDH gene was used as an internal reference. The conditions were as follows: preheating at 95°C for 2 min, denaturation at 95°C for 30 s, annealing at 60°C for 60 s and extension at 72°C for 1 min 30 s for 40 cycles. The experiment was repeated three times. The results are expressed as $2^{-\Delta\Delta Ct}$. Thyroid cancer cells and normal thyroid cells were inoculated into the culture dish. The cells were rinsed three times with phosphate-buffered saline (PBS), and 100 μ l radio immunoprecipitation assay (RIPA) lysis buffer were added into each well on ice for 30 min. The cells were centrifuged at 12,000 g at 4°C for 15 min. The supernatant was collected and stored at -80°C. Protein concentration was determined according to the bicinchoninic acid (BCA) protein method, and the protein was adjusted to the same concentration. The protein was separated by 10% Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), then transferred to a polyvinylidene fluoride (PVDF) membrane and blocked with 3% bovine serum albumin (BSA) solution for 2 h. Next, YAP1 and GAPDH were added at the ratio of 1:1000. It was incubated overnight at 4°C and washed three times with PBS for 5 min each. The secondary antibody was then added and incubated for 2 h. The protein was washed three times with PBS

for 5 min each and placed on the electrochemiluminescence (ECL) for exposure and development. To detect si-YAP1 interference, qRT-PCR and Western blot methods were used. The cells were divided into three groups: NC group, si-NC group, and si-YAP1 group. The total RNA and protein were extracted (si-YAP1 was added with small YAP1 interference fragments; si-YAP1 as the positive control; and NC as the negative control). The method was the same as detailed previously. The interference efficiency was calculated. If the efficiency was achieved, it could be used for follow-up experiments

Effect of si-YAP1 on TPC-1 Cell Proliferation

First, 2×10^3 cells were seeded in 96-well plates, with 3 duplicates and blank controls. Transient transfection of si-YAP1 was then completed according to instructions, and the cells were incubated for 5 days. The supernatant was discarded, and 20 ml of (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) solution was added to each well. After 4 h of incubation, the supernatant was discarded, and 150 μ l of dimethyl sulfoxide (DMSO) were added to each well. The optical density (OD) of each well at 570 nm was measured using a microplate reader (Bio-Rad, Hercules, CA, USA). The growth curve was plotted.

Effect of si-YAP1 on p-Akt

The experiment was divided into three groups: NC group, si-NC group, and si-YAP1 group, to detect the expression of p-Akt and Akt proteins. The Western blot method was used as stated previously.

Statistical Analysis

Statistical analysis was performed using Graph-Pad Prism 5.0 (La Jolla, CA, USA) and SPSS19.0 (SPSS Inc., Chicago, IL, USA). The quantitative data were expressed using ($\bar{x} \pm s$). The variance homogeneity test followed by Least Significant Difference (LSD) was carried out when multiple groups were compared. A variance of $p < 0.05$ was considered statistically significant.

Table I. Primers of YAP1 and GAPDH in quantitative Real-time PCR.

Gene	Forward primers	Reverse primers
YAP1	5'-CCTAGTATATAGTAGCTTCACTTATAGGTACTATAAAC-3'	5'-GTTTATAGTACCTATAAGTGAAGCTACTATATACTAGG-3'
GAPDH	5'-AGAAGGCTGGGGCTCATTTG-3'	5'-AGGGGCCATCCACAGTCTTC-3'

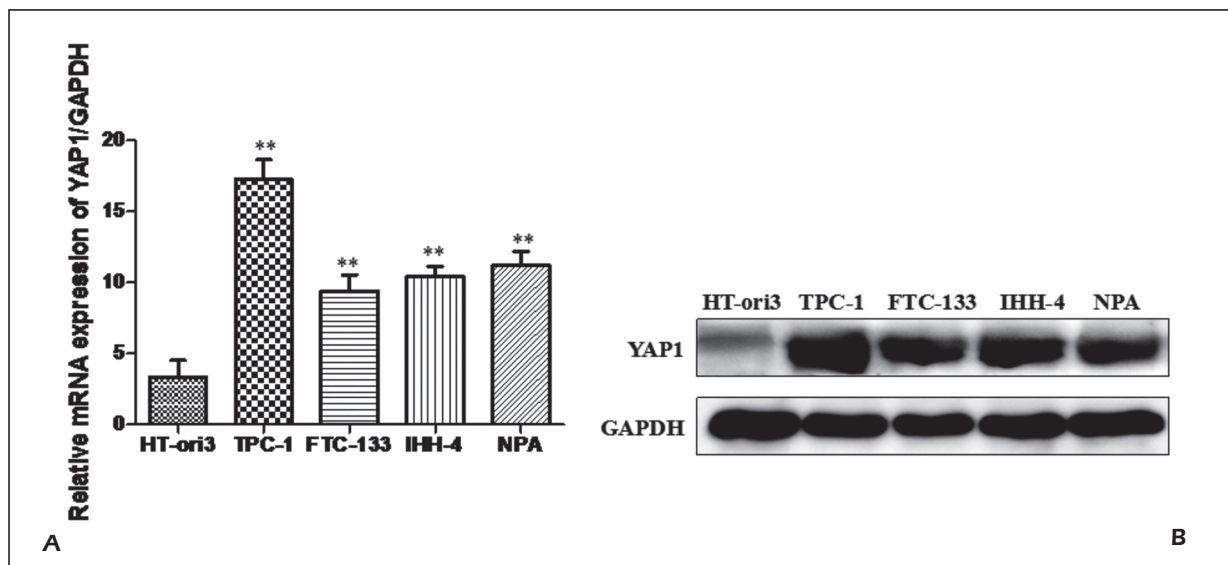


Figure 1. (A) qRT-PCR of the expression of YAP1 in four thyroid cancer cell lines and a normal thyroid cell line. $**p < 0.001$ compared with HT-ori3. (B) Western blot detection of the expression of YAP1 in four thyroid cancer cell lines and a normal thyroid cell line

Results

With qRT-PCR and the Western blot method, the expression of YAP1 in four-thyroid cancer cell lines and a normal thyroid cell line was detected. The expressions of YAP1 mRNA and protein in four thyroid carcinomas were significantly higher than those in normal thyroid cells ($F = 59.979$, $p < 0.001$) (Figure 1 and Table II).

Detection of si-YAP1 Interference

The mRNA and protein expression of si-YAP1 were significantly lower than that shown in the NC group and si-NC group ($F = 58.047$, $p < 0.001$) (Figure 2). The interference efficiency was 55-65%, which suggests possible applications in future experiments (Table III).

Table II. The Ct value of YAP1 in four thyroid cancer cell lines (FTC-133, IHH-4, TPC-1, and NPA) and one thyroid cell line (HT-ori3) were detected by qRT-PCR.

Cell lines	$2^{-\Delta\Delta C_t} (\bar{x} \pm s)$	p -value
HT-ori3	3.365 ± 1.163	
TPC-1	17.257 ± 1.41	$< 0.001^{**}$
FTC-133	9.402 ± 1.132	$< 0.001^{**}$
IHH-4	10.408 ± 0.752	$< 0.001^{**}$
NPA	11.228 ± 0.974	$< 0.001^{**}$

$**$ Differences in FTC-133, IHH-4, TPC-1 and NPA compared with HT-ori3.

Effect of si-YAP1 on TPC-1 Cell Proliferation

Compared with the NC group and siNC group, the proliferative capacity of the si-YAP1 group began to differ significantly at 72 h ($p < 0.05$). The difference was the most significant at 96 h and 120 h ($p < 0.001$) (Figure 3).

Effect of si-YAP1 on p-Akt

The expression of p-Akt in the si-YAP1 group was lower than that in the NC group and si-NC group as detected by Western blot (Figure 4).

Discussion

The main members of the Hippo signaling pathway include NF2, Mst1/2, Ww45, Last1/2, Mob1 and YAP1/TAZ. The YAP1 gene is the core member. YAP1 gene overexpression is closely related to pancreatic cancer, lung cancer, gastric cancer, liver cancer, breast cancer and other cancers⁸⁻¹². Upstream NF2, Mst1/2 and Lats1/2 are tumor suppressor genes, which prevent YAP1 from entering the nucleus through phosphorylation inactivation and have an inhibitory effect^{13,14}. The expression of YAP1 in thyroid cancer cells is significantly higher than that in normal thyroid cells. In this research, we used qRT-PCR and Western blot to detect the expression of YAP1 in four thyroid cancer cell lines and normal thyroid cancer cells. The results

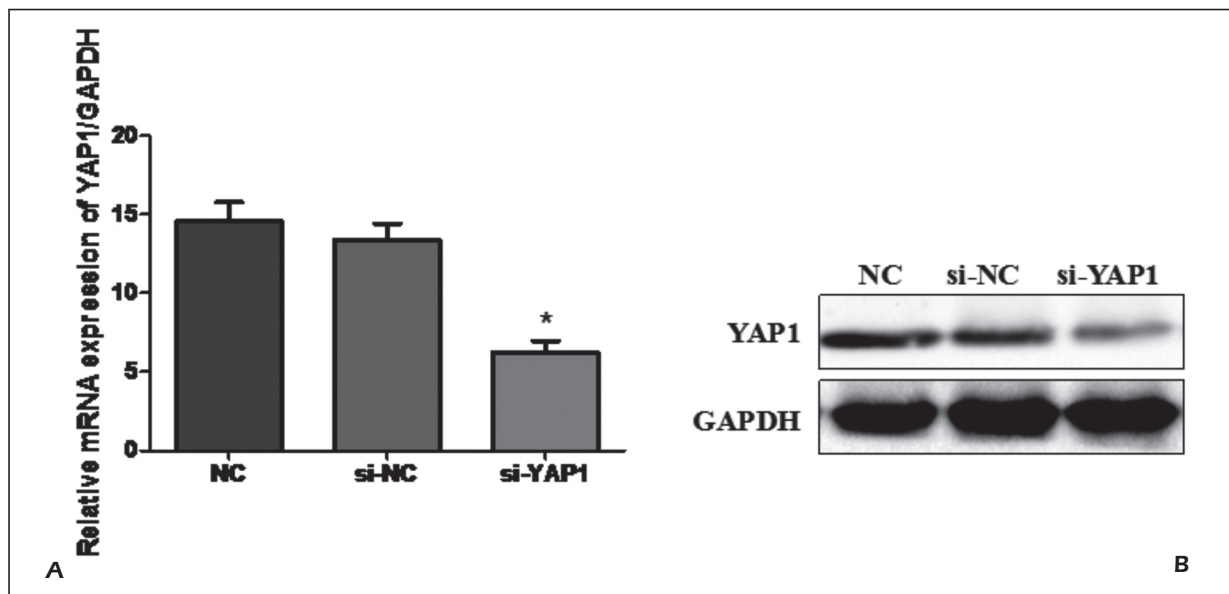


Figure 2. (A) The Ct value of YAP1 in the TPC-1 cell line after transfection with si-YAP1 was measured using qRT-PCR. $**p < 0.001$ compared with the NC group and si-NC group. (B) The YAP1 protein expression in the TPC-1 cell line after transfection with si-YAP1 was detected by Western blot.

Table III. The Ct value of YAP1 was detected by qRT-PCR after transfection with si-YAP1.

Group	$2^{-\Delta\Delta Ct} (\bar{x} \pm s)$	p -value
NC	14.579 ± 1.202	
si-NC	13.363 ± 1.601	0.197 [#]
si-YAP1	6.211 ± 0.778	$< 0.001^{**}$

[#]There was no significant difference between the si-NC group and NC group. ^{**}There was a significant difference between the si-YAP1 group and NC group.

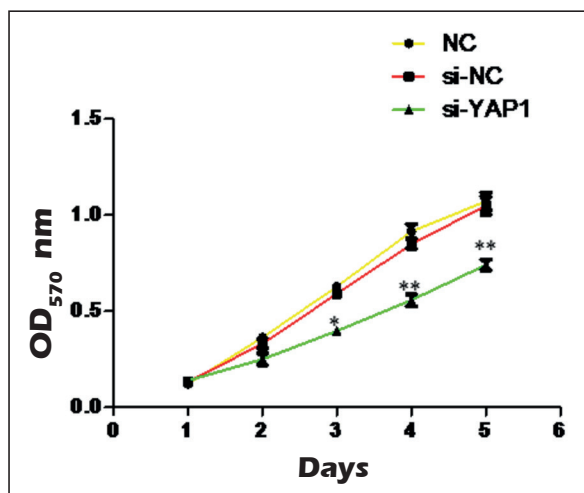


Figure 3. The proliferation of TPC-1 transfected with si-YAP1 at different times as examined by MTT. $*p < 0.05$, $**p < 0.001$ compared to the first day.

showed that the expression of YAP1 in thyroid carcinoma cells was significantly higher than that in normal thyroid cells. The PI3K-Akt signaling pathway is one of the important signal transduction pathways regulating cell growth and apoptosis. It plays an important role in inhibiting apoptosis and promoting proliferation and is closely related to the development and progression of many malignant tumors. The signaling pathway is abnormal in a variety of human tumors, including ovarian cancer, breast cancer, nasopharyngeal carcinoma, malignant glioma, endometrial cancer and medulloblastoma¹⁵⁻¹⁹. Akt is a serine/threonine protein

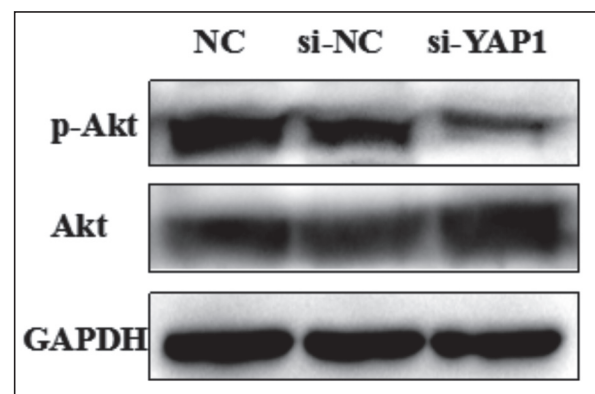


Figure 4. The protein expression of p-Akt in TPC-1 cells transfected with si-YAP1 as detected by Western blot assay.

kinase with a molecular weight of about 57 kD. It can inhibit the downstream target genes BAD, Caspase9 and P21 by phosphorylation, and mediate insulin and various growth factors to control the growth and apoptosis of cells²⁰. In recent years, YAP has been found to be the substrate of Akt. Akt can phosphorylate the Ser 127 site of YAP1. Additionally, phosphorylated YAP can inhibit the transcriptional activity of P73²¹. We suggest that YAP1 can promote the phosphorylation of Akt. Thus, YAP1 interacts with Akt and regulates cell proliferation. Abnormalities of YAP1 in thyroid cancer may serve as a new target for the treatment.

Conclusions

YAP1 is highly expressed in thyroid cancer cells, which can promote the growth of those cells. The mechanism may be related to the PI3K-Akt signaling pathway. The possibility of other signaling pathways involved is worthy of further research and discussion.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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