Original Research Article

Upregulation of SMAD4 inhibits thyroid cancer cell growth via MAPK/JNK pathway repression

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Abstract

**Purpose:** To investigate whether the effect of mothers against decapentaplegic homolog 4 (SMAD4) on thyroid cancer cell survival was via the MAPK/JNK pathway.

**Methods:** Papillary thyroid cancer (TPC)-1 cells were cultured and transfected with SMAD4 overexpression plasmid or siRNA to achieve SMAD4 overexpression or knockdown, respectively. In TPC-1 cells, the mRNA and protein expression levels of SMAD4, mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK) were quantified using reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. Cell viability and apoptosis were measured using MTT assay and flow cytometry, respectively. MAPK and JNK inhibitors (U0126 and SP600125) were used for rescue experiments. The sensitivity of TPC-1 cells to chemotherapeutic drugs, cisplatin and doxorubicin, was also assessed.

**Results:** A reduction in viability and an enhancement in apoptosis (p < 0.01) were found when SMAD4 was overexpressed in TPC-1 cells. Knockdown of SMAD4 elicited opposite results (p < 0.01). Overexpression of SMAD4 caused a decrease in the activation of MAPK and JNK, as evidenced by lower levels of phosphorylated MAPK and phosphorylated JNK (p < 0.05). Results from rescue experiments indicate that the increase in cell viability after SMAD4 knockdown was reversed by MAPK/JNK inhibitors (p < 0.05 and p < 0.01). Finally, overexpression of SMAD4 increased cytotoxic susceptibility of thyroid cancer cells to cisplatin/doxorubicin.

**Conclusion:** These results indicate that SMAD4 inhibits thyroid cancer cell growth via inactivation of MAPK/JNK pathway. Overexpression of SMAD4 also increased thyroid cancer cell sensitivity to cisplatin/doxorubicin.

**Keywords:** Thyroid cancer, SMAD4, TGF-β signaling pathway, MAPK/JNK, Cell survival, Drug sensitivity

INTRODUCTION

Thyroid cancer is a malignancy that develops from the cells of the thyroid gland, which participates in the regulation of blood pressure, heart rate, body weight, and body temperature [1]. Most people with thyroid cancer undergo surgery to remove all or most of the thyroid.
Besides, patients with metastatic or inoperable thyroid cancer are often treated with chemotherapeutic drugs which have limited efficacy [2-4]. Therefore, for patients with thyroid cancer, it is of scientific significance and clinical value to identify new targets. The TGF-β pathway is reported to be a negative regulator of thyroid cell growth [5]. In normal tissues, TGF-β triggers the formation of the SMAD heterooligomeric complex, which then translocates to the nucleus to regulate target gene transcription, leading to the regulation of cellular proliferation [6-9].

Mothers against decapentaplegic homolog 4 (SMAD4) is a core regulatory protein in the TGF-β-SMAD signaling pathway, which is involved in tumor cell proliferation, invasion, differentiation and metastasis [10]. The expression levels of SMAD4 was downregulated in various cancers, such as colorectal cancer, gastric cancer, hepatic carcinoma, breast cancer [11,12], and papillary thyroid carcinoma [13] and so on. The deletion of SMAD4 could activate MAPK/JNK and AKT pathways, causing chemoresistance of head and neck cancers [14], and colorectal cancer [15]. Previous study also found that, in thyroid papillary carcinoma cells, SMAD4 level was downregulated, and overexpression of SMAD4 was able to restore the TGF-β signaling pathway and reduce cell metastasis [13]. However, the function of SMAD4 in thyroid cancer requires further research.

This study was aimed to investigate whether the effects of SMAD4 on the growth of papillary thyroid carcinoma cells were associated with the MAPK/JNK signaling pathway. Indeed, results verified our hypothesis and found that overexpression of SMAD4 increased thyroid cancer cell sensitivity to the chemotherapeutic drugs, cisplatin and doxorubicin. The present study suggested that TGF-β-SMAD pathway may serve as a potential new target for the treatment of thyroid cancer.

**EXPERIMENTAL**

**TPC-1 cell culture and transfection**

The TPC-1 cell lines were obtained from the ATCC (American Type Culture Collection, Manassas, VA, USA), and maintained in RPMI 1640 medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), which contained heat-inactivated fetal bovine serum (10 %, FBS, Invitrogen; Thermo Fisher Scientific, Inc.), and incubated(37 °C, in an atmosphere with 5 % CO2) [16]. MAPK inhibitor, U0126 was purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA) and incubated with cells at a concentration of 10 μM for 30 min. JNK inhibitor SP600125, doxorubicin, and cisplatin were purchased from Sigma-Aldrich. 20 μM of SP600125 was used to incubate TPC-1 cells for 10 min.

Doxorubicin and cisplatin were incubated with cells at various doses (1, 5, 10, 20 and 50μM) for 1 h. Smad4 siRNA (CGA AUA CAC CAA GUAG ATT) and negative control (NC) siRNA (scramble, UUC UCC GAA CGU GUC ACG UTT) were obtained from Shanghai GenePharma Co., Ltd. (Shanghai, China). TPC-1 cells were transfected with Smad4 siRNA, negative control (NC) siRNA, Smad4 overexpression plasmid and control plasmid by using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was extracted from TPC-1 cells and2 μg of RNA was reverse-transcribed into cDNA (Transcriptor first-strand cDNA synthesis kit, Roche Diagnostics, Basel, Switzerland). The experiment was performed using an ABI Prism 7900HT (Applied Biosystems; Thermo Fisher Scientific, Inc.). The qPCR primers were listed in Table 1. For calculating the fold-change for each gene, the 2^-ΔΔCq method[17] was used, and normalized to GADPH.

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward</th>
<th>Reverse</th>
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<tbody>
<tr>
<td>SMAD4</td>
<td>5'-TGTAAGACGAGGCCAGT-3'</td>
<td>5'-CAGGAAACAGCATGACC-3'</td>
</tr>
<tr>
<td>GAPDH</td>
<td>5'-GGTGAAGGTCGGTGTAAC-3'</td>
<td>5'-CTCGCTTCTTGGAAATCG-3'</td>
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**Western blotting**

TPC-1 cells were harvested by centrifugation and solubilized in standard SDS lysis buffer Roche, Indianapolis, IN). 20 μg Cell lysates () were separated using electrophoresis on 8–10 % SDS polyacrylamide gels, transferred to PVDF membranes (Millipore, Billerica, MA), and incubated with antibodies to SMAD4 (Cat# 38454S), MAPK (Cat# 4699S), phosphorylated MAPK (p-MAPK) (Cat# 4370S), JNK (Cat # 9258S), phosphorylated JNK (p-JNK) (Cat# 4671S), cleaved caspase-3 (Cat# 9661S), cleaved caspase-9 (Cat# 52873S), and GAPDH (Cat# 5174), which were purchased from Cell Signaling Technology (Danvers, MA). All antibodies were used at a 1:1,000 dilution.
except for p-MAPK, which was used at a 1:2,000 dilution.

**Determination of cell viability**

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich (St. Louis, MO) was dissolved in distilled H₂O (5 mg/mL) and sterilized. TPC-1 cells were seeded in a 96-well plate (5,000 cells/well) overnight. During the experiment, 10 µL of MTT stock solution was added and incubated (37 °C, 1.5 h). Absorbance (at 490 nm) was measured by a μQuant microplate reader (BioTek Instruments, Winooski, VT).

**Apoptosis and flow cytometry analysis**

The TPC-1 cells were stained using propidium iodide (PI) and an annexin V-FITC kit (Beyotime Institute of Biotechnology, Jiangsu, China) [6], and performed on flow cytometry (FACS Canto II; BD Biosciences). The results were analyzed using FlowJo software v.7.6 (Tree Star, Inc., Ashland, OR, USA).

**Statistical analysis**

All experiments were repeated at least three times. Results are presented as mean ± standard error (SE). Analysis of statistical significance for the indicated datasets was performed using the Student’s t-test for non-paired replicates. A p value less than 0.05 was considered as significant.

**RESULTS**

**SMAD4 overexpression inhibits viability and enhances apoptosis of thyroid cancer cell**

To investigate the role of SMAD4 in thyroid cancer cell survival, TPC-1 cells were transfected with either SMAD4 overexpression plasmid or siRNA. As shown in Figure 1 A, transfection of SMAD4 overexpression plasmid resulted in high SMAD4 mRNA and protein levels. However, transfection of SMAD4 siRNA caused decreases in SMAD4 mRNA and protein levels, as shown by qPCR and western blot results, respectively (Figure 1 B). MTT results showed that the cell viability was significantly decreased in the SMAD4-overexpressing group and significantly increased in the SMAD4 knockdown group as compared to the corresponding control groups (Figure 1 C). In addition, cells were labeled with annexin V-FITC and PI, and analyzed using flow cytometry to detect apoptosis. The percentage of cells in SMAD4-overexpressing group was increased when compared with control group (Figure 1 C). The percentage of cells in si-SMAD4 group was decreased when compared with NC group. (Figure 1 D). Furthermore, the levels of the activated forms of caspase-3 and caspase-9 in cells overexpressing SMAD4 was increased when compared with control group, but decreased when SMAD4 was knocked down (Figure 1 E).

**Overexpression of SMAD4 inhibits MAPK/JNK pathway activation in thyroid cancer cells**

To investigate the mechanism underlying, the protein levels of MAPK/JNK pathway, which is important for the regulation of cell survival, were examined. The overexpression of SMAD4 caused a decrease in p-MAPK, which is the activated form of MAPK, compared with the control group (Figure 2 A). In addition, p-JNK protein levels also decreased in cells overexpressing SMAD4 compared with control cells. Similar results were obtained in cells treated with U0126, widely used as MAPK inhibitor as previously [18], and SP600125, a JNK inhibitor.
SMAD4 inhibits thyroid cancer cell viability via MAPK/JNK pathway

As shown in Figure 3 A, in TPC-1 cells transfected with SMAD4 siRNA, both p-MAPK and p-JNK levels were upregulated without affecting the MAPK and JNK levels. However, MAPK inhibitor U0126 or JNK inhibitor SP600125 suppressed the high expression levels of p-MAPK and p-JNK induced by SMAD4 knockdown. The cell viability and apoptosis were subsequently assessed using MTT assay and flow cytometry, respectively. The increased cell viability caused by SMAD4 knockdown was reversed by MAPK/JNK inhibitors (Figure 3 B). Furthermore, SMAD4 knockdown reduced apoptosis compared with control cells. However, additional treatment with U0126 or SP600125 reversed this effect (Figure 3 C).

Overexpression of SMAD4 increases cytotoxic susceptibility of thyroid cancer cells to cisplatin/doxorubicin

Further study was performed to study the role of SMAD4 in the sensitivity of TPC-1 cells to cisplatin and doxorubicin. The viability of TPC-1 cells was decreased with increasing concentration of cisplatin or doxorubicin (Figure 4 A and B). Importantly, overexpression of SMAD4 aggravated the inhibitory effects of cisplatin or doxorubicin as compared to control group (Figure 4 A), whereas SMAD4 knockdown alleviated the decreased cell viability caused by cisplatin or doxorubicin (Figure 4 B).
DISCUSSION

Endocrine cancers include thyroid cancer, pancreatic cancer, pituitary gland tumors, parathyroid gland tumors, adrenal gland tumors, and multiple endocrine neoplasia, among others. The most common types of endocrine cancers are thyroid cancer and pancreatic cancer; the others are very rare, and information about them is limited. Thyroid cancer has been analyzed historically using different methods [19]. In particular, mothers against decapentaplegic homolog 4 (SMAD4) was firstly identified as a cancer-related gene in pancreatic cancer by Ham et al in 1996 [20]. In the present study, its role as a tumor suppressor was demonstrated in thyroid cancer cells.

Here, SMAD4 overexpression caused a reduction in cell survival and decrease in the activation of p-MAPK and p-JNK. In contrast, cells in which SMAD4 was knocked down showed significant increases in viability and the activation of MAPK and JNK. Consistently, Ozawa et al reported that, in head and neck cancer cells, a loss of SMAD4 was closely related to the induction of MAPK/JNK activation [14]. Thus, it seems that methods to increase the susceptibility of thyroid cancer cells to a chemotherapeutic agent is worthy of investigation. Here, results illustrated that SMAD4 overexpression increased the susceptibility of thyroid cancer cell to doxorubicin or cisplatin. This association may shed light on future research on the TGF-β-SMAD signaling pathway as a new therapeutic target for thyroid cancer.

The role of chemotherapy as a treatment for surgically unresectable thyroid cancer is unclear. The most commonly used chemotherapeutic agent for thyroid cancer is doxorubicin (adriamycin), which has a partial response rate of 30 to 45%. The combination therapy of doxorubicin and cisplatin has been disappointing and has not demonstrated improved outcomes compared to single-agent trials [1]. Thus, it seems that methods to increase the susceptibility of thyroid cancer cells to a chemotherapeutic agent is worthy of investigation. Here, results illustrated that SMAD4 overexpression increased the susceptibility of thyroid cancer cell to doxorubicin or cisplatin. This association may shed light on future research on the TGF-β-SMAD signaling pathway as a new therapeutic target for thyroid cancer.

DECLARATIONS

Competing interests

The authors declare that they have no competing interests with regard to 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. HYC and XNY conceived and designed the experiments, ZRJ and BL analyzed and interpreted the results of the experiments, QYC and HBH performed the experiments.

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REFERENCES