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Overexpression of ING5 inhibits HGF-induced proliferation, invasion and EMT in thyroid cancer cells *via* regulation of the c-Met/PI3K/Akt signaling pathway

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ABSTRACT

The inhibitor of growth 5 (ING5), a novel member of the ING family, is involved in diverse biological processes such as cell growth, apoptosis and DNA repair. Recently, ING5 has been reported to be associated with cancer development. However, its specific role in thyroid cancer has yet to be elucidated. In this study, we found that the expression of ING5 was significantly down-regulated in human thyroid cancer tissues and cell lines. In addition, overexpression of ING5 markedly inhibited hepatocyte growth factor (HGF)-induced proliferation, invasion and epithelial-mesenchymal transition (EMT) of thyroid cancer cells as well as suppressed the tumor growth and metastasis *in vivo*. Furthermore, our data showed that the c-Met/PI3K/Akt signaling pathway was responsible for the inhibitory effect of ING5 on the thyroid cancer. Taken together, these findings provided an essential basis for the tumor-suppression role of ING5 in thyroid cancer.

1. Introduction

Thyroid cancer, a common type of malignant tumors in endocrine system, frequently originates from follicular cells [1]. According to histological features, thyroid cancer is divided into four categories: papillary thyroid cancer (PTC), follicular thyroid cancer (FTC), medullary thyroid cancer (MTC) and anaplastic thyroid cancer (ATC) [2,3]. As the most common type, PTC accounts for almost 85% of all thyroid cancers [4]. In addition, PTC is generally benign and curable and thus patients with PTC often have a high survival rate which reaches up to 95% [5]. But occasionally, PTC may dedifferentiate into more invasive types, resulting in recurrence and distant metastasis in the patients [6,7]. Therefore, it is imperative that we explore novel invasion-specific biomarkers and better understand the mechanisms underlying the progression of thyroid cancer.

The inhibitor of growth (ING) family includes a group of proteins (ING1 to ING5) which share a highly conserved carboxy-terminal plant homeodomain (PHD) [8,9]. These proteins are composed of a series of elements which differ in structures and functions, such as novel conserved regions, leucine zipper like fingers and nuclear localization sequence motifs [10]. All the family members have been followed with interest for their involvement in diverse biological processes including cell growth, apoptosis, senescence, DNA repair and chromatin remodeling [11–13]. ING5, a novel member of the ING family, has recently been investigated more frequently because of its association with cancer development. A growing number of studies have reported that ING5 shows a decreased expression in many cancers and it functions as a tumor suppressor *via* inhibiting cell growth, invasion and EMT [14–20]. However, the specific role of ING5 in thyroid cancer has yet to be elucidated.

The aim of this study was to explore the effect of ING5 on the progression of thyroid cancer. Our data suggested that the expression of ING5 was decreased in thyroid cancer tissues and cell lines. Overexpression of ING5 inhibited HGF-induced proliferation, invasion and EMT of thyroid cancer and this effect was accompanied by the suppression of c-Met/PI3K/Akt signaling pathway.

2. Materials and methods

2.1. Patients and tissue samples

128 patients participated in the study and received no adjuvant therapies before surgical resection. Thyroid cancer tissues and matching normal thyroid tissues were collected from these patients with their written consent. The study was approved by the Huaihe Hospital of Henan University (Kaifeng, China). All the tissue samples

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were frozen in liquid nitrogen and stored at −80 °C until use.

2.2. Cell lines and cell culture

Human thyroid cancer cell lines (TPC1 and FTC133) and the normal thyroid epithelial cell line (HTori-3) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). All cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% fetal bovine serum (FBS; Sigma), 100 mg/mL streptomycin (Sigma) and 100 U/mL penicillin (Sigma), followed by incubation at 37 °C in a humidified atmosphere with 5% CO_2.

2.3. Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was isolated from tissues or cells by TRIzol reagent (Takara Biotechnology, Dalian, China) and then reversely transcribed into cDNA using M-MLV Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). RT-PCR was carried out on the ABI7900 system (Applied Biosystems, Foster City, CA, USA) under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 10 s, 55 °C for 10 s, 72 °C for 20 s and 72 °C for 10 min. The primers were ING5, 5′-TCCAGAACGCCTACAGC AAG-3′ (forward) and 5′-TGCCCTCCATCTTGTCCTTC-3′ (reverse); HGF, 5′-CTGCTCCCCATCGCCATCCCCTATG-3′ (forward) and 5′-TAGGGTAG TCTTTGCTGATTTTTG-3′ (reverse); β-actin, 5′-TTAGTTGCGTTACACC CTTTC-3′ (forward) and 5′-ACCTTCACCGTTCCAGTTT-3′ (reverse). β-actin was used as a control. The $2^{-\Delta\Delta Ct}$ method was used for calculation of fold change.

2.4. Western blot analysis

Tissues or cells were lysed in RIPA buffer (Beyotime, Shanghai, China). The protein concentration was measured using a BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). Protein was subjected to 10% SDS-PAGE and then transferred onto PVDF membranes. After blocking in 5% skim milk, the membranes were incubated overnight at 4 °C with primary antibodies against ING5, E-cadherin, N-cadherin, c-Met, p-c-Met, Akt, p-Akt and β-actin. Subsequently, the membranes were washed for three times and then incubated with HRP-conjugated secondary antibodies. All the antibodies in the study were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein bands were visualized using an ECL detection system (Pierce, Rockford, IL, USA) and their density was analyzed using the Image-J software (National Institutes of Health, Bethesda, MD, USA).

2.5. Cell transfection

The empty vector pcDNA3.1 and ING5 expression vector pcDNA3.1-ING5 were purchased from GenePharma (Shanghai, China). TPC1 cells were transfected with pcDNA3.1-ING5 or pcDNA3.1 using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 48 h later, overexpression of ING5 was confirmed by western blot analysis.

2.6. Cell proliferation assay

Cell proliferation was assessed using the MTT assay. Cells were seeded in 96-well plates at a density of 5×10^3 cells/well and cultured for different hours. MTT reagent (Sigma) was added to each well and the cells were incubated for another 4 h. Then the culture medium was removed and DMSO (Sigma) was added. The absorbance was measured at 450 nm using a micro-plate reader (Bio-Rad, Hercules, CA, USA).

2.7. Cell invasion assay

Transwell chambers with Matrigel-coated filters were used to analyze cell invasion. 5×10^4 cells were suspended in serum-free medium and added to the upper chamber. The lower chamber was filled with complete medium containing 10% FBS. After incubation for 24 h, cells on the upper surface of the filter were removed with a cotton swab while cells invading to the lower surface of the filter were fixed and stained with Giemsa. The number of invading cells was counted under a microscope (200×; Nikon, Tokyo, Japan).

2.8. In vivo xenograft tumor assay

Male BALB/c nude mice (4–5 weeks) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China). All mice were housed under a specific pathogen-free condition and used for the study with the approval of the Animal Care and Use Committee in Henan University. Each experimental group contained 8 mice. For the tumor growth assay, 2×10^5 transfected TPC1 cells or control cells were injected subcutaneously into the left flank of each mouse (n = 8). Tumors were measured at different time points and the tumor volume was calculated by the following formula: length × width^2/2. After 5 weeks, mice were sacrificed and tumors were stripped and weighed. For the tumor metastasis assay, 2×10^5 transfected TPC1 cells or control cells were injected intravenously injected into the tail vein of each mouse (n = 8). 5 weeks later, mice were sacrificed to check for tumor metastasis in lungs. The number of metastatic nodules was counted under a microscope.

2.9. Statistical analysis

The data were expressed as means ± standard deviation (SD). The statistical analysis was performed using SPSS 17.0 software. The significance of differences between different groups was determined by the Student's *t*-tests. Each experiment was performed at least three times. Results were considered statistically significant if $p < 0.05$.

3. Results

3.1. The expression of ING5 is down-regulated in thyroid cancer tissues and cell lines

To evaluate the clinical relevance of ING5 in the major types of thyroid cancer, we first examined ING5 expression in 128 pairs of thyroid cancer tissues (81 cases FTC and 47 cases ATC) using RT-PCR and western blot assays. As shown in Fig. 1A and B, the mRNA and protein expression levels of ING5 were remarkably decreased in thyroid cancer tissues compared with the normal thyroid tissues. However, we found no significant difference in the mRNA and protein levels of ING5 in FTC and ATC, indicating that down-regulation of ING5 in thyroid cancers was uncorrelated with the grade of malignancy. To further explore the role of ING5 in thyroid cancer progression, we analyzed the correlation between the expression level of ING5 and the clinical features of thyroid cancer patients. As shown in Table 1, the expression level of ING5 was significantly correlated with the TNM stage ($p = 0.007$) but did not vary with age ($p = 0.141$) and gender ($p = 0.269$) .We also investigated the expression of ING5 in thyroid cancer cell lines (TPC1 and FTC133) and the normal thyroid epithelial cell line (HTori-3). Similar to the previous results, ING5 expression was much lower in TPC1 and FTC133 cell lines than that in HTori-3 cell line at both the mRNA (Fig. 1C) and protein (Fig. 1D) levels. Taken together, these data implied that ING5 may play an important role in the process of thyroid cancer progression.

3.2. Up-regulation of ING5 inhibits HGF-induced proliferation and invasion of thyroid cancer cells

HGF has been reported to be frequently overexpressed in various cancers and has the capability of enhancing cell proliferation and

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