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Original Article

Ginsenoside improves papillary thyroid cancer cell malignancies partially through upregulating connexin 31

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Abstract Connexin 31 (Cx31) is considered a suppressor for many tumors. Ginsenoside (Rg1) is a traditional Chinese herb that is widely acknowledged due to its anti-tumor characteristics. However, limited studies have focused on the role of Rg1 in papillary thyroid cancer (PTC) cells. In the current study, we found that the expression of Cx31 in thyroid cancer tissues and thyroid cancer cell lines was significantly lower than that in normal thyroid epithelial tissues and cell lines. Overexpression of Cx31 reduced thyroid cancer cell proliferation, migration and invasion. Furthermore, we found that Rg1 significantly enhanced the expression of Cx31. Moreover, the proliferation and migration of IHH-4 and BCPAP cells were significantly reduced by Rg1 treatment. In contrast, the silencing of Cx31 enhanced the expression of Ki67 and proliferating cell nuclear antigen (PCNA). Meanwhile, treatment with Rg1 significantly decreased the protein levels of Ki67 and PCNA, but these effects could be abolished by transfection with si-Cx31. In summary, we provide novel evidence that the expression of Cx31 was decreased in thyroid cancer cells, but Rg1 treatment could significantly enhance the expression of Cx31 thereby suppressing thyroid cancer cell proliferation and migration.
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Introduction

Thyroid carcinoma is the most common malignant tumor in the endocrine system [1,2]. The incidence of papillary thyroid cancer (PTC) is the highest among all thyroid malignancies and accounts for more than 80% of thyroid cancers [3]. Thyroid cancer, especially classical PTC, has the characteristics of low mortality and good prognosis, with a five-year survival rate above 90% [4]. However, studies have found that some PTC subtypes are associated with higher mortality rates, and these PTCs are known as high-risk PTCs [5,6]. Thus, it is of great importance to study the mechanism of their occurrence and development.

Gap junctions were first discovered by Karrer in 1960. Gap junction refers to a channel formed between adjacent cells, which constitute an important means of cell communication [7]. The channel allows small molecules and ions to flow through, including cAMP, IP3, and adenosine, thereby transferring intercellular signals and regulating cell proliferation [8–10]. Connexin (Cx) is the basic unit of gap junctions and plays an important role in many physiological processes [11,12]. Cx31 is a member of the connexin family and is mainly expressed in the skin, cornea, testis and other tissues [13,14]. Recently, it has been demonstrated that Cx31 mainly acts as a tumor suppressor in non-small cell lung cancer cells, and ectopic overexpression of Cx31 can inhibit cell proliferation, metastasis and invasion [15]. However, the specific function of Cx31 in thyroid cancer has been poorly understood. Ginsenoside (Rg1) is the main component of ginseng, which has been proven to be effective in the treatment of colon cancer, ovarian cancer and lung cancer [16–18]. Pharmacological studies have demonstrated that Rg1 can promote the apoptosis of tumor cells and enhance sensitization therapy of tumor cells [19,20]. For the first study, we showed the downregulation of Cx31 in PTC cells. Further study indicated Rg1 can inhibit the proliferation of thyroid cancer cells by up-regulating the expression of Cx31, which may shed light on a novel therapy for thyroid cancer patients.

Materials and methods

Patient selection

Twenty PTC patients and ten patients with nodular goiter (NC) who underwent surgery at Rizhao People's Hospital of Shandong Province between January 2016 and December 2016 were selected. Patients treated with neoadjuvant chemotherapy were excluded. The 20 patients with PTC (I:6, II:7; III:6) included 8 males and 12 females with a mean age of 44.01 $\pm$ 11.15 years (24–76 years), while 10 patients with NG included 3 males and 7 females with a mean age of 50.72 $\pm$ 14.57 years (30–74 years). TNM stages were assessed according to the TNM classification of the American Joint Committee on Cancer (AJCC) [21]. For this study, 6 patients were at T1 stage, 7 were at T2 stage, and the rest 6 were at T3 stage. No patient involved in this study received any other cancer treatment preoperatively. This study was approved by the Institutional Review Board (IRB) of Rizhao People's Hospital of Shandong Province.

Histological analysis

For histological analyses, PTC samples were fixed in formalin, embedded in paraffin, and cut into 5 μm sections with a microtome. The sections were de-waxed in xylene for 10 min and then rehydrated by sequential immersion in decreasing ethanol concentrations. The sections were then stained with hematoxylin-eosin and analyzed using an optical microscope (Nikon ECLIPSE Ni, Nikon Instrument Europe B.V. Amsterdam, Netherlands). Two independent observers examined the specimens. To quantify the IHC staining, 10 high-power field images ($\times$ 20) were taken for each slide using a Vectra imaging system (Perkin Elmer) and averaged for each case. Only cells with a whole nucleus were taken into account, and only intratumoral areas were used for quantification. Quantification of IHC staining was performed automatically using inForm software (version 2.0.2) (Perkin Elmer). Positivity was expressed as a percentage of positively stained cells relative to the total number of cells.

Cell culture

Adherent PTC cell lines IHH-4, BCPAP, and thyroid follicular epithelial cell line Nthy-ori 3-1 were purchased from the German Collection of and Cell Cultures (DSMZ, Germany). All cells were cultured in RPMI 1640 medium (HyClone) supplemented with 10% heat-inactivated fetal calf serum (FBS), 100 U/ml penicillin, and streptomycin in 25-cm^2 culture flasks at 37 °C in a humidified atmosphere with 5% CO_2.

Immunohistochemistry (IHC)

In brief, the tissue sections were deparaffinized and rehydrated. Antigen retrieval was performed by heating the sections in 10 mM citrate buffer for 2.5 min. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide for 10 min at room temperature. Next, the sections were washed three times with phosphate-buffered saline (PBS) and incubated overnight at 4 °C in a humidified chamber with Cx31 antibody (Sigma–Aldrich Trading company, China) at a dilution of 1:200. After 24 h, the sections were incubated with mouse secondary antibody (1:200; Zhong Shan Golden Bridge Bio, China) for 30 min at room temperature. Finally, the sections were stained with diaminobenzidine (DAB), and the nuclei were counterstained with Meyer's hematoxylin. A negative control was obtained by replacing the primary antibody with PBS.

Western blot analysis

Proteins isolated from the cultured cells were separated on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). Next, the membranes were blocked in 10% skim milk for 40 min at 37 °C. Subsequently, the membranes were incubated overnight at 4 °C with Cx31, PCNA, Ki67, or GAPDH antibody at a dilution of 1:1000 with PBST. After washing three times with PBST, the membranes were incubated at 37 °C for 40 min with the secondary antibody and then washed 5 times before exposure.

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