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Expression of sodium/iodide transporters and thyroid stimulating hormone receptors in thyroid cancer patients and its correlation with iodine nutrition status and pathology

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Abstract. – OBJECTIVE: To investigate the relationship between the expression of sodium/iodide transporter (NIS) and thyroid stimulating hormone receptor (TSHR) and iodine nutritional status in patients with thyroid carcinoma.

PATIENTS AND METHODS: 146 cases of thyroid cancer in Shanghai Gongli Hospital, the Second Military Medical University between February and December 2014 were selected as thyroid cancer group, 120 cases of normal thyroid morphology examined by thyroid ultrasound at the same period were selected as normal group. General information and thyroid function of two groups were recorded and analyzed. H&E staining was used to perform histopathological study on both normal group and thyroid cancer group, and immunohistochemistry was used to detect NIS and TSHR protein expression and position. Reverse transcriptase polymerase chain reaction (RT-PCR) was used for quantitative detection of NIS and TSHR mRNA in the two groups, and the relationship between iodine nutrition and NIS and TSHR expression in thyroid cancer patients was studied. The expression of NIS and TSHR in each group was detected by Western blotting, and the difference in NIS and TSHR expression was analyzed by SPSS 17.0 statistical software.

RESULTS: The difference of serum total triiodothyronine (TT3), total thyroxine (TT4), and thyroid stimulating hormone (TSH) levels between the normal group and the thyroid cancer group was statistically significant ($p < 0.05$). H&E staining showed that the histopathology of the thyroid cancer group was significantly different from that of the normal group. Immunohistochemistry showed the positive expression of NIS and TSHR in thyroid cancer group. The expression of NIS and TSHR mRNA and protein in thyroid cancer patients was significantly lower than that in normal group detected by RT-PCR and Western blotting. Analysis of variance showed that the difference of NIS and TSHR expression between the two groups was statistically significant ($p < 0.05$).

CONCLUSIONS: These findings indicated that the expression of NIS and TSHR in thyroid cancer is closely related to iodine nutritional status, which has important research value.

Key Words:

Thyroid cancer, Iodine, sodium/iodide transporters, Thyroid stimulating hormone receptors.

Introduction

Thyroid cancer is the most common type of thyroid malignant tumor in recent years. Incidence of thyroid cancer is related to the region, race, and gender. In recent years, the incidence of thyroid cancer has been significantly increased, it has become one of the malignant tumors with highest increase in incidence[1]. Pathogenesis of thyroid malignancies is not clear. Occurrence of this disease is related to various factors including oncogenes, growth factors, ionizing radiation, genetic factors, iodine and estrogen[2]. Shanghai Pudong area is one of the coastal non-iodine-deficient areas, seafood products are preferred in daily life. However, the incidence of thyroid cancer increased significantly year by year, and the specific reasons remain unknown[3,4]. According to the health examination data from our hospital, the detection rate of thyroid nodules by B ultrasound for adults can be as high as 60%, and the incidence of thyroid cancer for male was significantly increased. For now, no clear data of the iodine nutritional status of the local residents have been obtained, especially the data of thyroid cancer

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patients. The relationship between increased incidence of thyroid cancer and iodine nutritional status is still not clear. Therefore, there is urgent need to access the survey data of local iodine nutrition status of thyroid cancer patients and analyze the relationship with the increased incidence of thyroid cancer. In this study, we investigated the relationship between the expression of NIS and TSHR and the status of iodine nutrition in thyroid cancer patients in Shanghai Pudong area, so as to provide guidance for the prevention and treatment of thyroid disease.

Patients and Methods

Patients

A total of 146 patients with thyroid cancer underwent surgical treatment and pathologically confirmed thyroid cancer in Shanghai Gongli Hospital, the Second Military Medical University from February to December 2014 were selected as the thyroid cancer group, 120 cases of normal thyroid morphology examined by thyroid ultrasound at the same period were selected as normal group. Inclusion criteria: living in Shanghai Pudong area for more than 10 years, first admission for surgical treatment for the thyroid cancer group, all the specimens underwent histopathological examination after operation. The study was approved by the Ethics Committee of Shanghai Gongli Hospital, the Second Military Medical University and informed consents were signed by the patients and/or guardians.

Experimental Methods

Determination of Thyroid Function

The general condition of patients was recorded, then thyroid function was detected by radioimmunoassay reagents provided by Beijing North Institute of Biological Technology (Beijing, China), serum total triiodothyronine (TT_3), total thyroxine (TT_4), and thyroid stimulating hormone (TSH) levels were measured by chemiluminescence assay.

Histopathological Examination

The tissue of normal group and thyroid cancer group was embedded in paraffin wax to prepare 5 μm wax pieces, followed by H&E staining. Tissue sections were soaked in xylene (10 min/time, 2 times). Then, they were soaked in gradient ethanol concentration (5 min for each concentration), stained by hematoxylin-eosin for 5 min and washed for 10 min. Then, sections were soaked in 1% hydrochloric acid for 30s, followed by 0.5% eosin staining for 30s, and washing with distilled water for 30s. Tissue sections were soaked in gradient ethanol concentration, 5 min for each concentration. Then sections were soaked in xylene and sealed with gum. Stained slices were checked under a 200-fold light microscope (Nikon Eclipse TE2000-U, Nikon, Japan) for histopathological analysis.

Immunohistochemical Staining

Tissue sections of normal and thyroid cancer groups were immersed in xylene (10 min/time, 2 times), then, were immersed in gradient ethanol for 5 min. After antigen repair, samples were washed with PBS (3 min/time, 3 times). After stained by SP method, the slices were washed with PBS (3 min/time, 3 times). Samples were incubated with normal goat serum at room temperature for 15 min after the PBS was dried. NIS or TSHR monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added dropwise, then the samples were incubated overnight at 4°C in a wet box. After washing with PBS (5 min/time, 3 times), secondary antibody was added and samples were incubated at 37°C for 15 min. After washed with PBS (5 min/time, 3 times), the working solution was added, incubated at 37°C for 15 min and washed with PBS (5 min/time, 3 times), and then the freshly prepared DAB solution was added dropwise, observed under the microscope to control the degree of color, and then re-stained with hematoxylin, 1% hydrochloric acid alcohol differentiation for 20s, then the solution returned to blue. Finally, after dehydrated with a gradient of ethanol, the slices were sealed with neutral gum. The stained sample sections were observed and analyzed on a 200x optical microscope (Nikon Eclipse TE2000-U, Nikon, Japan).

Real-Time PCR analysis

The tissue of the normal group and the thyroid cancer group were transferred to 1 mL Trizol reagent for proper grinding into homogenate. The cells were allowed to stand for 5 min at room temperature so that the samples were completely cleaved, then were centrifuged at 12000 g and 4°C for 5 min, and the supernatant was carefully removed. Chloroform was added to the supernatant, mixed well and stood at room temperature for 5 min, centrifuged at 12000 g and 4°C for 15

min, then the supernatant was carefully removed. The same volume of isopropanol was then added and allowed to stand at room temperature for 10 min, centrifuged at 12000 g, 4°C for 10 min, then the precipitation was reserved, 75% ethanol was added and well-mixed to wash the RNA precipitation. Finally, RNase-free water was added to completely dissolve the precipitation. The OD_{260} and OD_{280} values were determined and the RNA concentration was calculated from the OD_{260}/OD_{280} ratio. Then, gradual amplification was performed according to the primer templates shown in Table I (synthesized by Shanghai Sangon Biotech Co., Ltd., Shanghai, China), the reaction product underwent RT-PCR.

Table I. Primers used in the study.

Genes	Primers
NIS	5'-3' CCATCCTGGATGACAACTTGG 3'-5' AAAAACAGATCCTCATTG
TSHR	5'-3' CCATCAGAGGAGGAGGACTTCA 3'-5' ATTGGGCAGATTAGAAAATG
β-actin	5'-3' GAGCCGGGAAATCGTGCGT 3'-5' GGAAGGAAGGCTGGAAGATG

Western Blotting Analysis

The tissue of normal and thyroid cancer group was washed with ice saline and the protein concentration was measured by BCA protein concentration assay kit (Beyotime Biotechnology Co., Ltd., Shanghai, China), and stored at -80°C. According to the instructions of the Whole Protein Extraction Kit, IP lysates (containing PMSF and protease inhibitors) were added, the sample was placed on ice and grinded thoroughly. Then, the homogenate was centrifuged at 4°C and 12,000 g for 10 min, the supernatant was obtained and centrifuged at 4°C and 12,000 g for 20 min, the supernatant was obtained again. The protein was quantified according to the instructions of the Whole Protein Extraction Kit. The protein samples containing the same amount of total protein were loaded into the sample wells and then electrophoresed at 220 V constant pressure until the bromophenol blue reached the bottom of the gel. According to the molecular weight of the target protein, the gel was cut and placed into transfer buffer, PVDF film was cut according to the size of the gel and immersed into methanol for 10 seconds, the PVDF film and the filter paper was placed into the transfer buffer. The proteins on the gel were transferred to the PVDF membrane at the appropriate time at 110 V constant pressure in the order of the positive electrode – filter paper – PVDF film – gel – filter paper – negative electrode. The protein-containing PVDF membrane was incubated in 5% skim milk powder at room temperature for 3 h on a shaker and incubated at 4°C overnight with NIS and TSHR primary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA). (10 min/time, 3 times). On the second day, after the film was thoroughly washed with TBST (10 min/time, 3 times), secondary antibody Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added and incubated at room temperature for 1h, washed with TBST (10 min/time, 3 times), then ECL coloring liquid (Beyotime Biotechnology Co., Ltd., Beijing, China) was added for color reaction and photography.

Statistical Analysis

The experimental data was expressed as mean ± standard deviation (mean ± SD). The experimental results were analyzed by SPSS 17.0 statistical software (SPSS Inc., Chicago, IL, USA). Analysis of variance or *t*-test was used for data analysis and the post hoc test was SNK test, $p < 0.05$ for the difference was considered as statistically significant.

Results

Thyroid Function

As shown in Table II, there was no significant difference in gender, age, and race between nor-

Table II. General information comparison between two groups.

Group	Cases	Gender (M/F)	Age	Ethnic (Han/ minority)	Lymph node metastasis (N_0/N_1)	Recurrence risk (Low risk/high risk)
Normal	120	63/57	31-45	102/18	/	/
Thyroid cancer	146	41/105	36-52	134/14	76/70	62/84
p-value		0.7250	0.687	0.8661		

mal group and thyroid cancer group. In 146 thyroid glands, the degree of lymph node metastasis was 76/70 for N0/N1 and 62/84 for low-risk/high-risk recurrence. At the same time, as shown in Figure 1, significant differences in serum levels of TT3, TT4, and TSH were found between two groups ($p < 0.05$).

H&E Staining Method to Observe the Pathological Situation

Histopathological differences were observed by H&E staining of the tissue sections of the normal group and the tissues of the thyroid cancer group. Normal thyroid tissue is composed of follicles. Follicles are intact and filled with glial. Follicles are formed by single cubic follicular epithelial cells, and size is uniform. Follicles also contain elastic fibers. Compared with normal group, size of thyroid follicles in the thyroid cancer group was not uniform, and follicular epithelium was flattened and degenerated. Follicles formed by fibrovascular papilla and tumor cells have typical characteristics of the nucleus: big nuclear, sparse chromatin, glass-like changes, nuclear inclusions and nuclear ditch can be observed. As shown in Figure 2, the tissues of the normal group and the tissues of the thyroid cancer group were significantly different in histopathology.

Immunohistochemistry Detection of the Expression of NIS and TSHR in Thyroid Carcinoma

From the histological sections of thyroid cancer group, immunohistochemical staining of NIS, and TSHR showed positive expression of NIS and TSHR in the thyroid cancer group, as shown in Figure 3. It is clear that NIS and TSHR positive products are located in the cell membrane and/or cytoplasm, showing brown and dark brown.

RT-PCR Results of NIS and TSHR in Normal and Thyroid Cancer Groups

Total RNA was extracted from the tissue of normal group and thyroid cancer group. The expression of NIS and TSHR in the thyroid cancer group was significantly lower than that in the normal group after RT-PCR (Figure 4).

Western Blotting Results of NIS and TSHR in Normal and Thyroid Cancer Groups

Western blotting results showed the expression of NIS and TSHR in normal and thyroid cancer group. As shown in Figure 5, the expression of NIS and TSHR protein was significantly reduced in the thyroid cancer group compared with the normal group.

Discussion

Thyroid cancer is one of the fastest growing malignancies in recent years, with an increase of 14.51% per year[5]. Compared with the data from the Chinese incidence of cancer in 2007 and 2009, the incidence of thyroid cancer has increased significantly, especially in women[6,7]. According to the statistics of the resident in Pudong New Area from 2002 to 2006, the incidence of thyroid cancer was significantly higher than the total incidence in China of the same period[8]. At present, the cause of the increase in

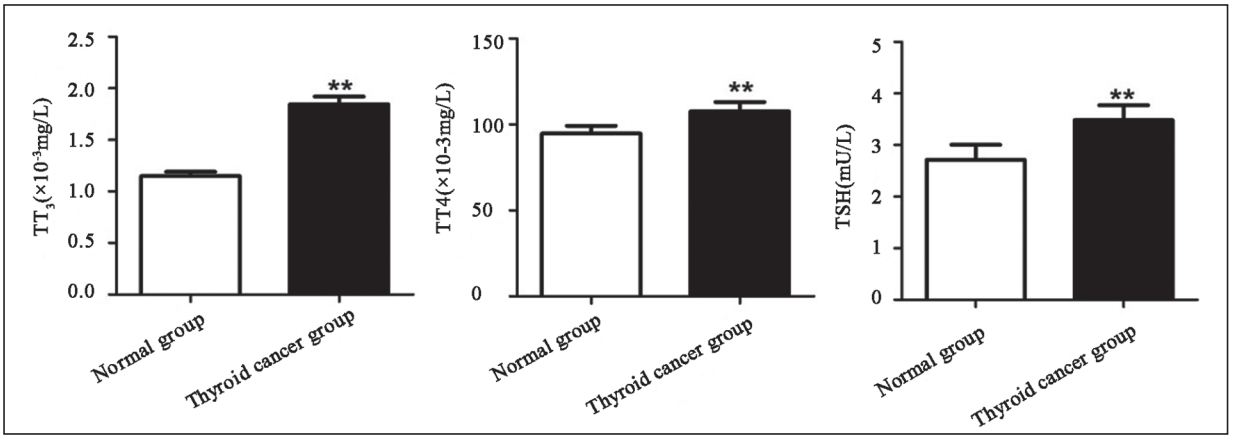

Figure 1. Comparison of thyroid function and thyroid autoantibodies expression in normal and thyroid cancer group. Compared with the normal group, $^{**}p < 0.01$.

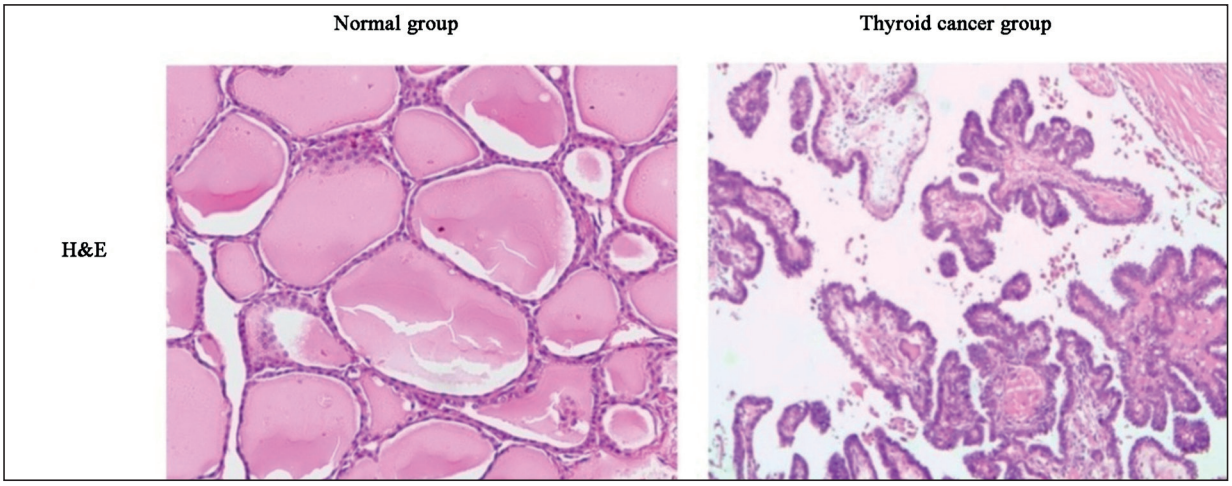

Figure 2. H&E staining results of tissue in the normal group and the thyroid cancer group (× 200). Compared to the normal group, follicles composed of fibrotic artery axis papilla and tumor cells can be observed in the thyroid cancer group, the nuclei were enlarged and the chromatin was sparse. Cells were irregularly arranged with polar disorders.

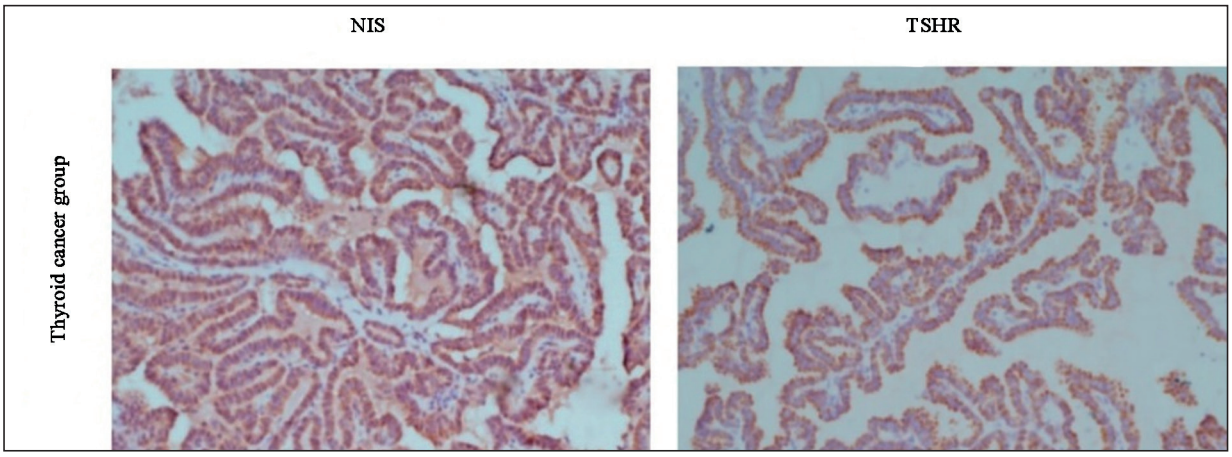

Figure 3. Expression of NIS and TSHR in thyroid cancer group (× 200). NIS and TSHR were positive in the thyroid cancer group and were localized in the cell membrane and/or cytoplasm.

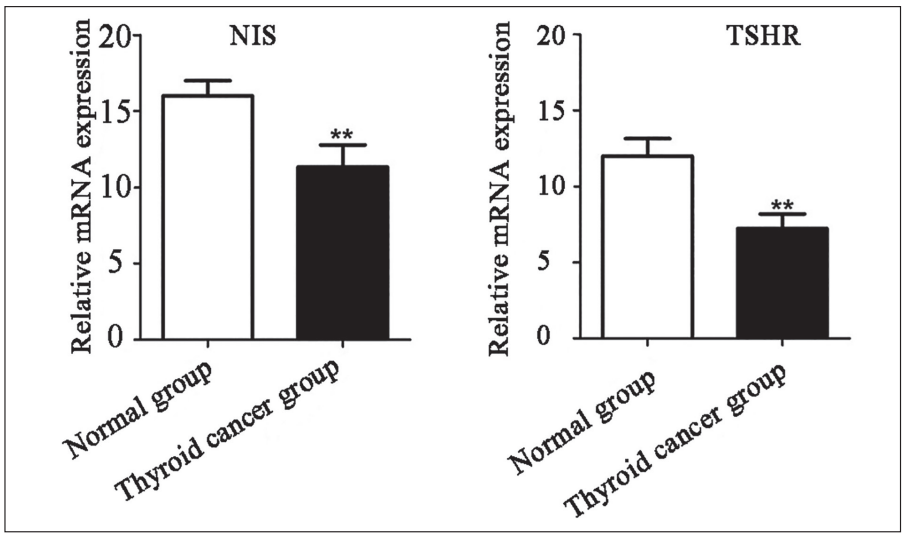

Figure 4. RT-PCR results of NIS and TSHR in normal and thyroid cancer groups. Compared with the normal group, $*p < 0.05$, $**p < 0.01$ (n = 3).

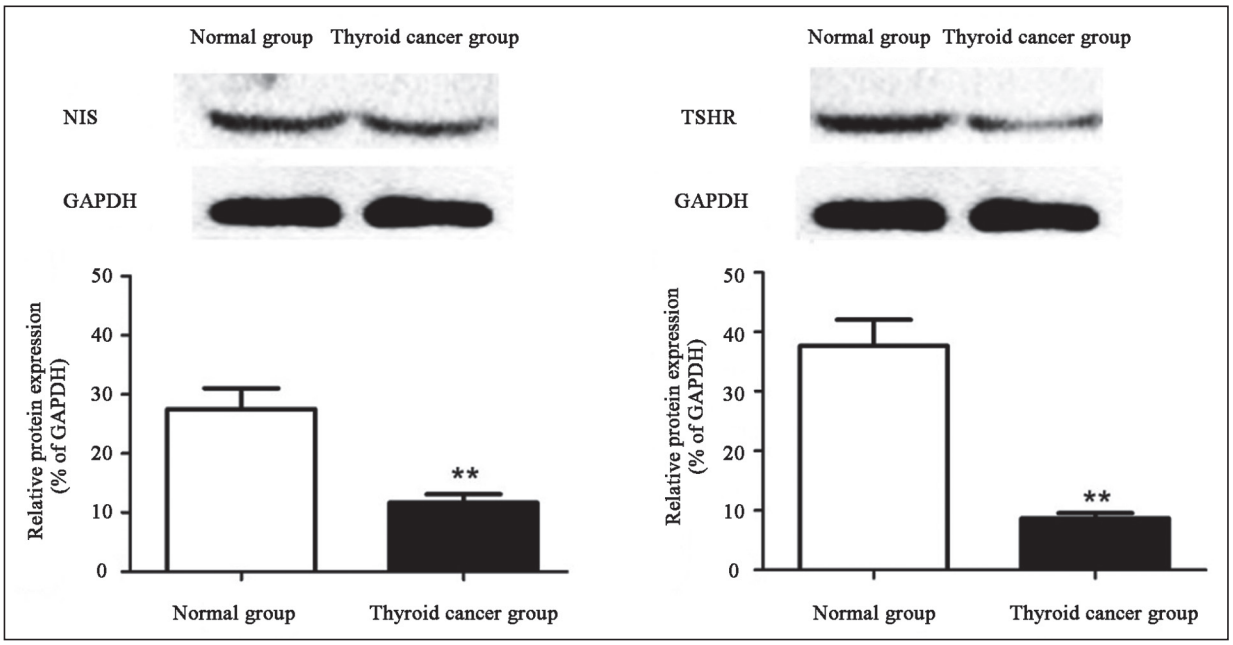

Figure 5. Western blotting results of NIS and TSHR in normal and thyroid cancer groups. Compared with the normal group, the expression of NIS and TSHR protein in thyroid cancer group was significantly decreased. Compared with the normal group, $*p < 0.05$, $**p < 0.01$ (n=3).

the incidence of thyroid cancer is still unknown. There is an urgent need for researches to explore this reason.

Development and progression of thyroid cancer is caused by various factors including both internal and external factors. Mental stress, infection, environmental pollution, diet, lifestyle, medicine, and many other factors can induce the occurrence of this disease. Incidence of this disease has increased significantly due to the lack of attention to iodine deficiency. In recent years, with the implementation of the salt iodization program, iodine deficiency has been significantly improved. At the same time, body damage caused by excess iodine intake has gradually attracted people's attention. Studies have shown that high iodine can cause a variety of thyroid diseases, including iodine-induced hypothyroidism, hyperthyroidism, and autoimmune thyroiditis[9]. Whether high iodine causes increased incidence of thyroid cancer or not is still not confirmed by relevant animal experiments or clinical data. Researches have found that high incidence of thyroid cancer occurred in Hawaii and Irish high iodine areas; after the implementation of iodized salt in Australia in 1963, the incidence of thyroid cancer increased to 7.8/10 million per year from 3.07/10 million per year; the incidence of thyroid cancer in Huanghua area, with high iodine in water supply, was 19.37/10 million, which was significantly higher than the international incidence of thyroid cancer, and all cases were papillary thyroid cancer. The above data showed that high iodine intake may be associated with increased incidence of thyroid cancer, but there is no clear experimental evidence to support the conclusion.

Sodium iodide symporter (NIS) is a glycoprotein expressed in the basolateral membrane of the thyroid cells, which plays a key role in the thyroid iodine uptake process, mediates thyroid cells transporting iodine ion into the cells in the inverse concentration, and is involved in the synthesis of thyroid hormones, plays an "iodine pump" function[10-12]. Investigations show that Thyroid Stimulating Hormone (TSH) and iodine are the two most important regulators in the many factors that regulate NIS expression[13]. Under normal physiological conditions, TSH binds to thyroid stimulating hormone receptor (TSHR) located in the basement membrane of the thyroid follicle, playing its role by mediating guanosine triphosphate conjugate protein Gα signaling pathway through adenylate cyclase, causing the cascade of c-AMP signaling pathways, upregulating the expression of NIS and promote its transport to the cell membrane[14]. However, in the case of inflammation or abnormal iodine intake, NIS expression will decline. Animal ex-

periments show that in the case of high iodine, NIS protein is reduced and TSHR mRNA expression is decreased, acute and chronic iodine excess can inhibit the expression of NIS at the gene and protein levels, in which chronic iodine excess can also affect the normal positioning of NIS in the follicular cell basement membrane[15]. It can be inferred that NIS and TSHR are closely related to iodine nutrition.

The expression of NIS protein in thyroid cancer has a certain value in the diagnosis and treatment effect prediction, and the decrease of its expression is one of the main reasons for the poor radiotherapy effect of thyroid cancer. However, the results of the related reports are not consistent. According to the study of Xinjiang Medical University Cancer Hospital[16], NIS is mostly expressed in the cytoplasm of thyroid cells, the expression of patients with small tumor is higher than that of patients with large tumor, the expression of adjacent tissue is higher than that of the cancer tissue, indicating that the closer to normal tissue, the closer to normal expression of NIS. While the work in Gansu province[17] showed that the expression of NIS and TSHR mRNA in thyroid cancer were significantly lower than normal levels, and the protein expression was significantly higher than normal level, suggesting that there was contradictory between the level of transcription and translation, which was consistent with the study in Brazil[18]. The positive rate of TSHR mRNA in the peripheral blood was significantly higher in thyroid cancer patients than that in patients with thyroid benign disease[19,20]. Recent researches in Qingdao found that there was no difference in NIS protein expression in papillary thyroid carcinoma and nodular goiter, but there was difference in adjacent tissue[21]. The reason for the inconsistency of the above study may be the slight difference in the experimental and evaluation methods, the study population coming from different regions, the iodine nutritional status closely related to the expression of the two genes may be a neglected factor.

Conclusions

This paper investigated the iodine nutrition status of thyroid cancer patients underwent surgical treatment in the Shanghai Pudong area, and detected sodium/iodine transporter and thyroid stimulating hormone receptors. The expression and location of protein was detected by immunohistochemistry, the expression of NIS and TSHR mRNA and protein were detected by RT-PCR and Western blotting. The relationship between iodine nutrition status and sodium/iodine transporter and TSHR expression was studied, which provided theoretical basis for exploring the correlation between increased incidence of thyroid cancer and iodine nutrition status, for the prevention and treatment of thyroid cancer in the region, and provided direction for the next step of the research work.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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