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Evaluation of Serum Substance P Level and Tissue Distribution of NK-1 Receptor in Papillary Thyroid Cancer

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Abstract

Background: Papillary thyroid carcinoma (PTC) is the most prevalent malignancy of the endocrine system. This study was aimed at evaluating the serum substance P (SP) levels, the tissue distribution of Neurokinin-1 receptors (NK1-R), and their possible diagnostic value in PTC.

Method: The present case-control study included 31 healthy volunteers and 31 cases (age range: 25-64, 40.26 ±12.77) who were primarily diagnosed with PTC and were candidates for total thyroidectomy. Pre-operative serum level of SP was measured using a commercial ELISA kit. The tissue distribution of NK1-R was assessed immunohistochemically.

Results: The serum level of SP in the patient group was higher than the healthy volunteers (*P*-value =.005). Besides, the expression of NK1-R was higher in tumoral tissues compared with their normal surroundings (*P*-value =.005). However, we observed no significant correlation between either SP level or NK1-R expression and the disease stage or lymph node involvement.

Conclusion: SP level and NK1-R expression were upregulated in PTC patients, showing the involvement of SP/NK1R complex in PTC pathophysiology. Nonetheless, proposing SP/NK1R as a diagnostic factor requires further studies because we found no correlation between SP/NK1R and clinical stage or lymph node involvement.

Keywords: Thyroid carcinoma, Substance P (SP), NK1R, Tachykinin, Cancer

Introduction

Papillary thyroid carcinoma (PTC) is the most prevalent type (more than 80%) of thyroid cancer in newly diagnosed patients.[1] Although thyroid cancer is relatively rare,[2] it is predicted that it will turn into the fourth most common malignancy in the United State until 2030.[3] PTC typically presents as nodules in thyroid or lymph nodes due to its common invasion into the adjacent lymph nodes. The primary treatment for PTC patients includes total or partial thyroidectomy.[4] The diagnosis is usually made by fine-needle aspiration (FNA) biopsy[5] and cytological examination.[6]

Similar to other cancers, PTC occurs when genetic and epigenetic rearrangements give rise to uncontrolled cellular proliferation. Inflammation is one of the factors contributing to such genetic rearrangements .[7] Inflammatory cells and mediators are inseparable components of the tumor microenvironment.[8] The mechanisms that link inflammation and carcinogenesis were reviewed by Ohshima and colleagues.[9] They suggested three main mechanisms: the direct effect of infectious agents on host cells, immunosuppression, and the production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) caused by inflammatory processes.

Substance P (SP), neurokinin A (NKA), and neurokinin B (NKB) are pro-inflammatory members of the tachykinin neuropeptide family.[10] These peptides exert their biological function through interacting with G-protein coupled neurokinin receptors including neurokinin-1 receptor (NK1-R), neurokinin-2 receptor, and neurokinin-3 receptor. Among these neuropeptides, SP was shown to play an important role in regulating peripheral inflammation via binding to NK1-R as its preferred receptor. Our knowledge of SP participation in inflammation in based on the elevated levels of SP and the increased expression of its main receptor (NK1-R) in inflamed tissues. It was also shown that using NK-1R antagonists or knocking down the NK-1 receptor in animals might be beneficial for treating inflammatory diseases.[11] As a pro-inflammatory modulator, the involvement of SP/NK1R was reported in various types of cancer such as esophageal cancer,[12] colorectal cancer,[13] melanoma,[10] laryngeal cancer,[14] pancreatic cancer,[15] breast cancer,[16] lung cancer,[17] glioblastoma,[18] and endometrial cancer.[19]

SP/NK1-R complex was shown to trigger many biological processes contributing to cancer progression, including mitogenesis, angiogenesis, cell migration, and metastasis.[20, 21] In addition to the involvement of SP/NK1-R complex in initiating and promoting malignancies, it was proposed to have a diagnostic value in distinguishing the cancer stages in certain cancer types.[22, 23] In this regard, an increase was reported in both the plasma level of SP and its expression in tumoral cells of medullary thyroid carcinoma (MTC),[24] which is in line with the findings reported by Bessho.T.[25] and Cremins.J et al.[26] regarding MTC. Most of the emphasis in this field has been placed on MTC despite the higher incidence rate of PTC. The involvement of SP/NK-1R complex in PTC or its diagnostic value is rather ill-defined; therefore, in the present study, we evaluated the serum SP levels and NK1-R expression in FNA-tissue biopsy specimens of PTC patients to elucidate the role of tachykinins in PTC. We also assessed the correlation of SP/NK1-R with lymph node involvement and disease stage.

Materials and Methods

Materials and chemicals

SP enzyme-linked immunosorbent assay (ELISA) kit was obtained from Abcam (kit number ab133029, USA). The Immunohistochemical staining was

performed using the antibodies from Abcam Co, USA (ab219600 and ab205718). All other reagents were purchased from Sigma-Aldrich, Germany.

Study population

In the present case-control study, 31 patients (23woman and 8 men, age range: 25-64, 40.26±12.77) who were primarily diagnosed with PTC were recruited. The control group for serum SP assessment consisted of 31 age- and sex-matched healthy volunteers. For the tissue distribution of NK1R, the healthy margin of tumor samples was used as the control group.

The exclusion criteria were patients having any other types of cancer, those with a history of radioactive iodine therapy, and head and neck surgery or radiotherapy, and patients diagnosed with another disease that might distort the results of this study. Lymphatic node invasion was done based on the information related to sonographic imaging and the findings obtained during the surgery. For disease staging, we used the data on the presence or absence of metastasis, the tumors size, and lymph node involvement.

Ethical statement

All the procedures carried out in this study on human participants were exactly consistent with the ethical considerations of Helsinki's declaration. The Ethics Committee of the Research Council in Mashhad University of Medical Sciences approved this study (Ethics code: 910817). Each participant provided written informed consent.

SP measurement

To measure the SP levels, the ELISA was performed using a high sensitivity ELISA kit from Abcam (ab133029) according to the manufacturer's instructions. The assay is based on the competitive binding of a monoclonal antibody to SP or SP conjugated alkaline phosphatase molecules. Briefly, 5 ml blood sample was taken from each patient prior to any medical procedures. The blood samples were centrifuged (2000 g for 15 minutes) and the serums were separated and kept at -80°C until laboratory analysis. Before performing the ELISA test, all solutions were allowed to reach 25°C. A set of standard solutions with different concentrations were prepared according to the manufacturer's instructions. A standard curve was plotted to calculate the accurate SP concentration in the serum samples.

NK-1R immunohistochemichal analysis

Tumoral tissues and their healthy margins were obtained during the thyroid lobectomy surgery and paraffinized according to the standard procedure. In brief, four micron tissue sections were provided; they were primarily deparaffinized with xylene followed by dehydration through descending proportions of ethanol to deionized water. After antigen retrieval with the appropriate buffer (Tris–EDTA buffer) for 30 minutes and water bath (95 °C), the sections were allowed to reach 25°C. To inactivate the endogenous peroxidase activity, the sections were placed into 5% H_2O_2 for 10 minutes. Afterwards, the desired sections were incubated with a primary antibody, rabbit anti-human NK-1R (ab219600), at 4°C overnight. Next, the Goat Anti-Rabbit IgG H&L (HRP) (ab205718), as the secondary antibody, was added to the sections for 20 minutes at 25 °C. Finally, to visualize with the light microscope, the tissue samples were stained with 3-3 ′-diaminobenzidine (DAB) and counterstained with Mayer's hematoxylin. After that, the tissue samples underwent alcohol-mediated dehydration and cleaning with xylene and then mounted on the slides. The stained slides were assessed by two experienced pathologists blinded to the clinical data. The specimens were scored and classified through the semi-quantitative scale (Table 1).

Statistical analysis

We analyzed the data using SPSS, version

18. The quantitative results were presented as Mean ± SD and assessed using the student t-test. A *P*-value ≤0.05 was considered as statistically significant.

Results

Clinicopathological and surgical data

Table 2 illustrates the patients' clinicopathological and surgical characteristics. Among all the patients, 10 (32.3 %) had a family history of thyroid cancer while the others mentioned no family history of cancer. Twelve patients (38.7%) presented with unilateral thyroid nodule and the rest (61.3%) presented bilateral thyroid nodule. Only one patient presented with capsular invasion.

Serum SP concentration

Mean serum levels of SP were 13.2 ± 3.4 ng/ml in the patients and 6.2 ± 2.1 ng/ml in the controls (Figure 1). The t-test showed a significant increase in serum SP levels in the patients compared to the healthy individuals (*P*<.05). However, we found no significant correlation between serum SP levels and the cancer stage. Also, there was no correlation between age, lymphatic invasion, or tumor size and serum SP level.

NK-1R tissue expression

We obtained the tissue distribution of NK-1R by multiplying the staining intensity score and the percentage of the stained cells. There was a significant difference between NK-1R expression in tumoral tissues and their healthy margins (*P*=0.005) (Figure 2). However, we observed no association between NK-1 expression and tumor size, involvement of lymphatic nodes, or tumor stage.

Discussion

In the present study, we evaluated the serum SP levels and NK-1R tissue distribution in PTC patients and compared them with healthy controls. In agreement with previously published findings, we showed that NK-1R expression was higher in tumoral tissues compared with their healthy margins. Of note, NK-1R expression was observed in none of the healthy tissues. Furthermore, serum SP levels were significantly higher in PTC patients in comparison to the healthy controls. Although the data from several studies suggest that SP/NK-1R system is correlated with cancer stage,[22, 23] we found no such association between SP levels or NK1-R tissue distribution and the clinical stage of PTC. To our knowledge, this is the first study to evaluate the SP and NK-1R levels in PTC. Although this lack of correlation between SP levels or NK1-R tissue distribution and the clinical stage of PTC that we observed might be true, this discrepancy with other studies may be due to some limitations in this study like a smaller sample size (31 patients).[22] It is not known whether this lack of correlation is due to the nature of the PTC or the limitations of this study.

It is currently well-established that SP/NK-1R complex is involved in processes related to cancer pathogenesis, such as initiation of tumor cell proliferation, angiogenesis, and metastasis.[27] Likewise, studies have demonstrated that the density of NK-1R on glioma and colorectal cancer cells is correlated with the disease stage[22, 23] and a predictor of prognosis in colorectal cancer.[22] Both SP and NK-1R are derived from different post-translational modifications of Tac1 (preprotachykinin-A) gene. Seemingly, there is a complex and important molecular mechanism behind the expression of this gene. Hence, any disturbance in this equilibrium can entail irreversible consequences.[28] In normal breast cells, repressor element 1-silencing transcription factor (REST) constantly suppresses the expression of Tac1. However, during breast cancer, the absence of the inhibitory effects of REST on Tac1 leads to the appearance of

the oncogenic functions of this gene.[29] The increase in serum SP levels and NK-1R tissue expression were observed in various types of cancers including melanoma,[10] glioblastoma,[18] endometrial,[19] breast,[16] colorectal,[13] laryngeal,[14] pancreatic,[15] and lung cancer.[17] Although PTC is the most common malignancy of the endocrine system, there is not enough information regarding the SP/NK1-R complex in this cancer.

It has been suggested that SP-mediated activation of NK1-R receptors can activate the members of the mitogen-activated protein kinases (MAPKs) pathway, such as p38MAPK and extracellular signal-regulated kinases 1 and 2 (ERK1/2). It also causes ERK1/2 to translocate into the nucleus in order to induce cell proliferation and protect the cell from apoptosis.[30] Some studies have proved the efficiency of blocking this molecular pathway in cancers and promising results are currently being obtained from NK-1R antagonists for cancer treatment.[31] Previous studies established that antitumoral effects could be achieved by targeting SDF-1α (stromal cell-derived factor 1 alpha), a regulator of Tac1 expression, in PTC.[32] Bigioni and colleagues found that tachykinin receptors antagonists exerted cytostatic effects on human breast carcinoma cells.[33] Furthermore, blocked tachykinin receptors were reported to exert antitumoral action in neuroblastoma,[34] lung,[17] colon,[35] and gastrointestinal [36] cancer cells.

There is need for further studies to determine the effect of tachykinins in pathogenesis and possibly treating PTC. Further comprehensive and more extensive studies should be undertaken to investigate the correlation between SP/NK-1R system upregulation and PTC stage. Moreover, we found that SP/NK-1R system was upregulated in PTC patients; therefore, it is only reasonable to perform studies in order to determine the effect of tachykinin receptor antagonists in PTC cells.

Conclusion

We found that serum SP level and NK-1R tissue expression were upregulated in PTC patients; however, this increase did not correlate with the cancer stage, age, tumor size, or lymphatic invasion.

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Conflict of Interest

None declared.

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Table 1. The semi-quantitative scale used to classify NK-1R expression

Score	The intensity of staining	The percentage of positive cells
1	Weak	Staining of ≤25 % of cells
2	Moderate	Staining of 26–50 % of cells
3	Strong	Staining of 51–75 % of cells
4	Not applicable	Staining of ≥76 % of cells

NK-1R: Neurokinin-1 receptor

Table 2. The clinicopathological data and the distribution of samples based on the tumor size and lymphatic invasion and TNM staging

variable	**classification**	**Abundance[%]**
Tumor size	<2 cm	51.6
	2-4cm	9.6
	>4 cm	38.7
Cervical Lymphatic invasion	No invasion	35.4
	supraclavicular and anterior cervical invasion	3.2
	unilateral cervical invasion	25.8
	bilateral cervical invasion	22.6
TNM Staging	Stage I	22.6
	Stage II	6.4
	Stage III	41.9
	Stage IV a	29.0

TNM: Tumor, nodes, and metastases

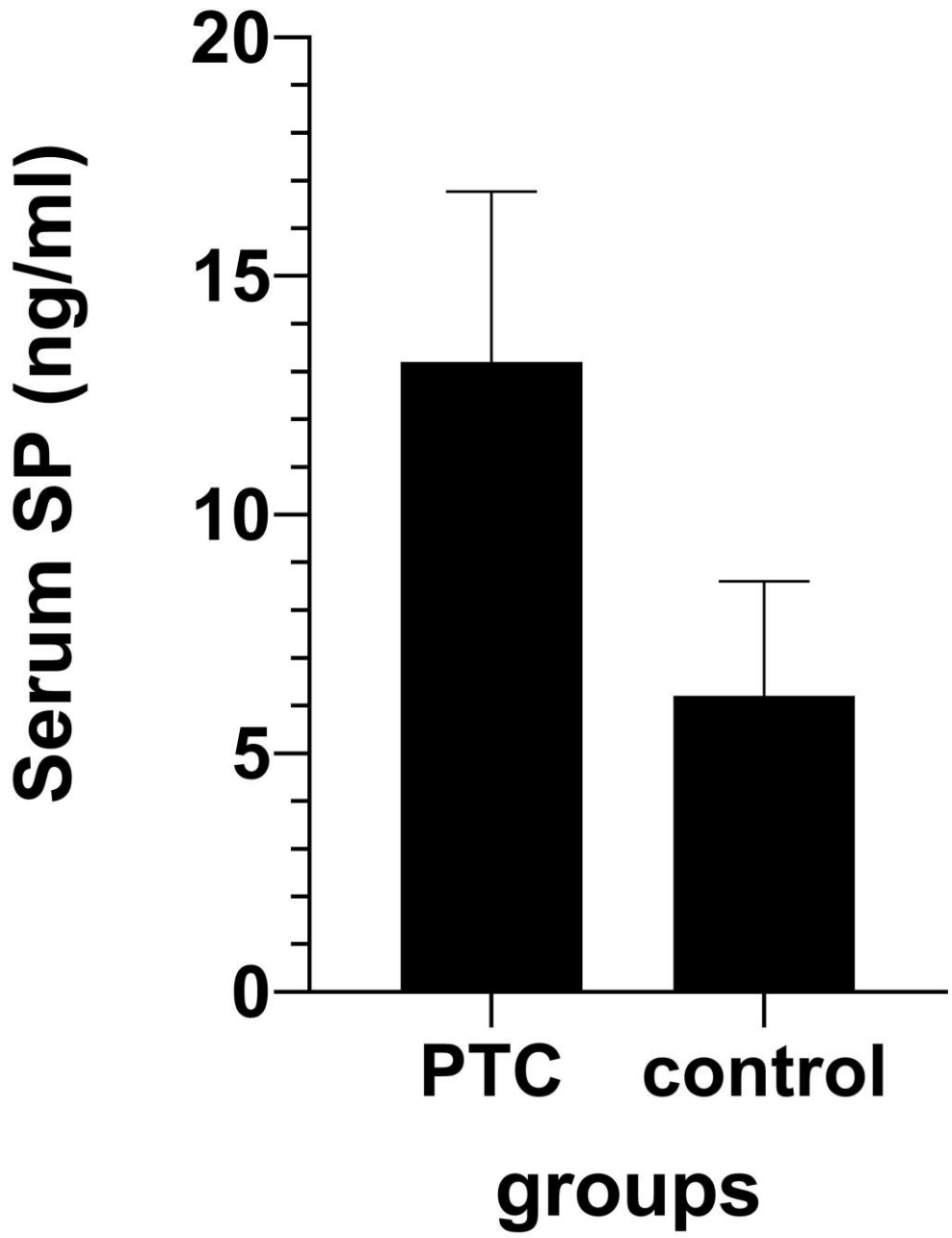

Figure 1. Serum SP (substance P) concentration was significantly higher in PTC (Papillary thyroid carcinoma) patients (13.2 ± 3.4 ng/ml) compared to healthy individuals (6.2 ± 1.7 ng/ml).

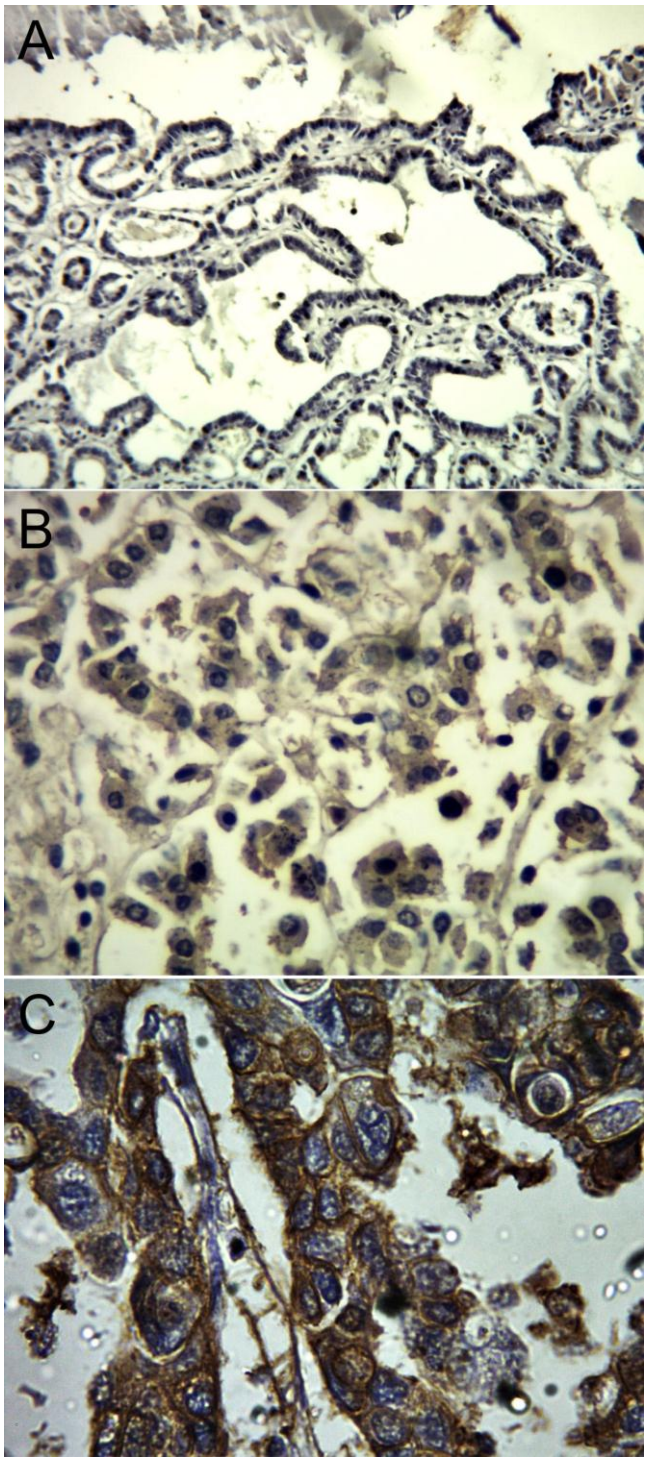

Figure 2. Immunohistochemical staining of the papillary thyroid carcinoma tissues; A) lack of staining (×100); B) weak staining of <10% of cells (×100); C) weak staining of <10% of cells (×400).