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Prognostic Significance of Substance P and Neurokinin-1 Receptor in Bladder Cancer

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Abstract

Background: Bladder cancer is one of the most common genitourinary cancers with significant mortality. Finding reliable tumor markers and potential drug targets can improve early diagnosis, prognosis, and more effective therapeutic protocols. Previous studies have reported the involvement of the substance P (SP)/neurokinin-1 receptor (NK-1R) system in cancers. The potential prognostic role and the interaction of SP and NK-1R in bladder tumor are yet to be elucidated.

Methods: Serum samples from 22 primarily diagnosed patients with bladder cancer as well as 22 healthy controls were examined for SP level using ELISA method. Tissue distribution of NK-1R in tumor samples and their adjacent normal tissues was evaluated through immunohistochemistry.

Results: Serum SP levels in patients with bladder cancer were higher than the healthy group ($p< 0.001$) and had a significant correlation with NK-1R staining intensity ($p< 0.001$), percentage of stained cells ($p< 0.001$), and NK-1R tissue distribution. Also, the immunoreactivity of NK-1R in cancer samples increased significantly without correlation with tumor characteristics. However, no significant association was found between SP and NK-1R levels with clinical characteristics including tumor size ($p= 0.33$), tumor stage ($p= 0.29$), grade ($p= 0.93$), NK-1R staining intensity ($p= 0.53$), and percentage of stained cells ($p= 0.32$).

Conclusions: According to our findings, despite the lack of association between SP and NK-1R with clinical characteristics of bladder cancer, their serum levels were higher in patients with bladder cancer. Further studies are needed to confirm the potential prognostic role of SP and NK-1R in bladder cancer.

Keywords: Biomarker, Bladder cancer, Neurokinin-1 receptor, Substance P, Prognosis.

Introduction

Increased expression of neuropeptides (e.g., neuropeptide Y, substance P (SP), hemokinin-1, etc.) and their specific receptors has been reported to be associated with cancer initiation and progression (1-3). These peptides are known to be implicated in modulating various biological processes in cancer cells, such as proliferation, angiogenesis, and metastasis (4, 5). Consequently, their cognitive receptors were deemed potential targets for drug development and early cancer diagnosis (6, 7).

The tachykinins (TKs) are an evolutionarily conserved group of peptides expressed not only in the nervous system (as

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neurotransmitters) but also in other tissues of different species (8). The TK family members are characterized by a common carboxyl-terminal domain, FXGLM-NH_2, where X can be either an aliphatic or aromatic amino acid. The genes producing TKs encode pre-pro-tachykinin precursor proteins, which are consequently modified into smaller peptides by post-translational modifications (8, 9). Multiple alternative splice variants have been observed in mRNA molecules that convey genetic information from three pre-pro-tachykinin genes to ribosomes (8, 9). Neurokinin A (NKA), neurokinin B (NKB), and SP are the first well-characterized molecules in the TK family, which exert their biological activities through three neurokinin receptors (NK-1R, NK-2R, and NK-3R) (8). SP is an undecapeptide first found in equine brain and intestinal tissue extracts by Gaddum and von Euler in 1931 (10). This intensively studied neuropeptide displays the greatest affinity and selectivity for NK-1R (10, 11). SP and NK-1R appear to be crucial factors capable of regulating several biological and pathological processes such as immunomodulation, vasodilation, pain transmission, inflammation, wound healing, mood disorders, anxiety, stress, vomiting, and cystitis (12-19). Also, their expression seems to have a close connection to the development of several cancers including pancreatic, esophageal, glioma, and melanoma (2, 8). It is suggested that tumor cells exploit the SP/NK-1R system to enhance tumor progression and metastasis while NK-1R antagonists (NK-1RAs) might be effective in treating different cancer types (2, 4, 7).

Bladder cancer is principally a disease of aging which remains underrecognized as a significant burden on the health care system despite its high prevalence, morbidity, and mortality rates, (20). According to the national cancer institute (NCI) bladder cancer is currently regarded as the sixth common cancer type worldwide. Research has shown that bladder cancer arises from accumulating different risk factors such as occupational exposure, Schistosoma haematobium infection, cigarette smoking, genetic variations, and some medications. Furthermore, it has been reported that the incidence of bladder cancer is higher in men (21).

Previous studies have investigated the effect of tachykinins on the urinary tract system; however, to the best of our knowledge, there is no data regarding the correlation between TKs and bladder cancer. TK proteins have also been shown to be involved in central and peripheral regulation of urinary tract functions through neurokinin receptor activation (22-24). The primary sources of TK peptides in urinary system are the primary afferent neurons expressing capsaicin receptors called transient receptor potential vanilloid-1 (TRPV1) (22). The recognized sources of SP as the prototypic TK protein in bladder are a major population of unmyelinated group C (C-fibers) and lightly A-delta (Aδ) afferent nerve fibers (17). Besides the TK peptides, the expression of substance P receptors (SPR or NK-1R) has been reported in bladder, renal pelvis, ureter, and urethra (25).

Overexpression of NK-1R has also been found in bladder inflammation (17, 26). The TK system has important functions in the urinary tract (22, 27-34). They can regulate smooth muscle tone, bladder contractions, ureteral peristalsis, and mucus secretion (30-34). Initiation of neurogenic inflammation and immune cell recruitment are other reported effects mediated by TK system (22, 27-29, 34).

Despite the discussed TKs roles in urinary system, the association between the TK system and bladder cancer is yet to be elucidated. This study was designed to evaluate serum SP concentrations in patients with bladder cancer and to assess the tissue distribution of NK-1R in bladder tumoral tissues compared to their adjacent healthy tissues.

Materials and Methods

Compliance with ethical standards.

This study was ethically approved by the Ethical Committee, Mashhad University of Medical Sciences (IR.MUMS.fm.REC.1397.229), and informed consent was provided by each volunteer who participated in this study.

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Study population and samples

A total of 22 patients with primarily diagnosed bladder cancer referred to Imam Reza hospital, Mashhad, Iran, were recruited for this case-control study. Detailed data of the patients consisting of age, tumor size, TNM staging, and cancer grade was recorded. Tumor tissue as well as the adjacent healthy tissues specimens were collected during cystectomy after informed consent was signed by the patients. Serum samples were also taken from each patient before the surgery. Serum samples were also taken from 22 healthy age and gender matched volunteers as controls. Unwilling patients to participate, patients suffering from other tumor types or inflammatory and infectious conditions, and those receiving anticancer drugs were excluded from the study. The approval to conduct this study was provided by the ethics committee, Mashhad University of Medical Sciences, Mashhad, Iran (IR.MUMS.fm.REC.1397.229).

SP concentration measurement

Five ml blood samples from the brachial vein of each patient as well as the control group members were collected preoperatively, centrifuged, and stored at -80 ℃ until further assessment. SP quantification was performed using an enzyme-linked immunosorbent assay (ELISA) kit (ZellBio GmbH, Germany) after thawing the serum samples to 25 ℃.

Immunohistochemistry

Five µm slices of formalin-fixed paraffin embedded tissue samples were mounted on glass slides and deparaffinized at 56-60 °C for 15 min, followed by xylene bath. After draining the excess liquid, the tissue sections were rehydrated in serial alcohol dilutions (100, 96, 80, and 70%). The slides were washed in phosphate-buffered saline (PBS) at room temperature for 30 min for further rehydration. The rehydrated tissue sections were immersed in Tris-ethylene diamine tetra acetic acid (Tris-EDTA; TE) buffer for 30 min to retrieve antigens masked by formalin fixation. To stain the samples with horseradish peroxidase (HRP), the sections were treated with 5% hydrogen peroxide (H_2O_2) in PBS for 10 min to block endogenous peroxidase activity. Subsequently, rabbit anti-NK-1R antibody (ZellBio GmbH, Germany) was applied for 24 hr at 4 °C. After washing with Tris buffer, the sections were incubated with a goat anti-rabbit IgG secondary antibody (ZellBio GmbH, Germany) for 1 h.

To stain the bladder tissue sections, enough drops of freshly prepared peroxidase substrate solution (1 ml chromogen solution, one drop of diaminobenzidine (DAB)) was added to each glass slide for 10 min and then washed with distilled water. After rinsing the DAB coloring reagent out, hematoxylin staining was used to facilitate bladder cell identification. Subsequently, the stained slides were dehydrated in graded alcohol (70, 80, 90, and 100%) and mounted using aqueous mounting media, such as glycerol gelatin. Finally, the stained-glass slides were observed under an optical microscope (Nikon, Japan) by an experienced pathologist. The percentage of stained cells was divided into four grades, including: ≤ 25% (score: 1), 26-50% (score: 2), 51-75% (score: 3), and≥ 76% (score: 4) (Table 1).

Table 1. The scoring system for analyzing IHC results.

Score	Staining intensity	Receptor-bearing cells percentage
1	Weak	0-25% of the cells
2	Moderate	26-50% of the cells
3	Strong	51-75% of the cells
4	NA	76-100% of the cells

Note: NA: Not applicable

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The staining intensity and percentage of NK-1R positive cells in bladder cancer tissues and their adjacent normal samples were measured to investigate the possible involvement of SP/NK-1R system in the pathogenesis of urinary bladder cancer. The intensity of immunoreactivity was defined in four grades according to a previous report (40) including: weak (score: 1), moderate (score: 2), strong (score: 3), and not applicable (NA). Consequently, the NK-1R tissue distribution was calculated via the multiplication of the staining intensity score by the score for positive cancer cell percentage.

Data analysis

Statistical analysis was performed using SPSS software (version 20.0, SPSS Inc., Chicago, IL, USA). Data were expressed as mean±standard deviation (SD). Significance levels were also set at the 5% level.

Results

A total of 22 bladder cancer patients with an average age of 60.9±12.8 were included in this study. The data were evaluated in terms of age, cancer grade, TNM staging, and tumor size. Majority of the tumors (57.1%) were classified in Grade I, and others (42.9%) were in grade III. Ten patients (47.6%) were in pathologic tumor stage Ta, 7 patients (33.3%) in T1, and 4 (19%) in T2. The tumor size was determined based on their largest dimension and measured between 1 to 9 cm (3.4±1.91 cm).

Evaluation of serum SP levels in the patients and controls

Mann-Whitney analysis revealed significantly higher SP serum levels in patients (16.91±12.19 ng/ml) compared to the controls (1.5±1.75 ng/ml) ($p< 0.001$) (Fig. 1). Spearman test showed significant correlations between patients' serum SP levels and the percentage of stained cells and NK-1R tissue distribution ($p< 0.001$). Furthermore, the Kruskal-Wallis test showed a statistically significant correlation between SP levels and NK-1R staining intensity in cancer patients ($p< 0.001$). Moreover, SP levels appeared to be unaffected by tumor characteristics.

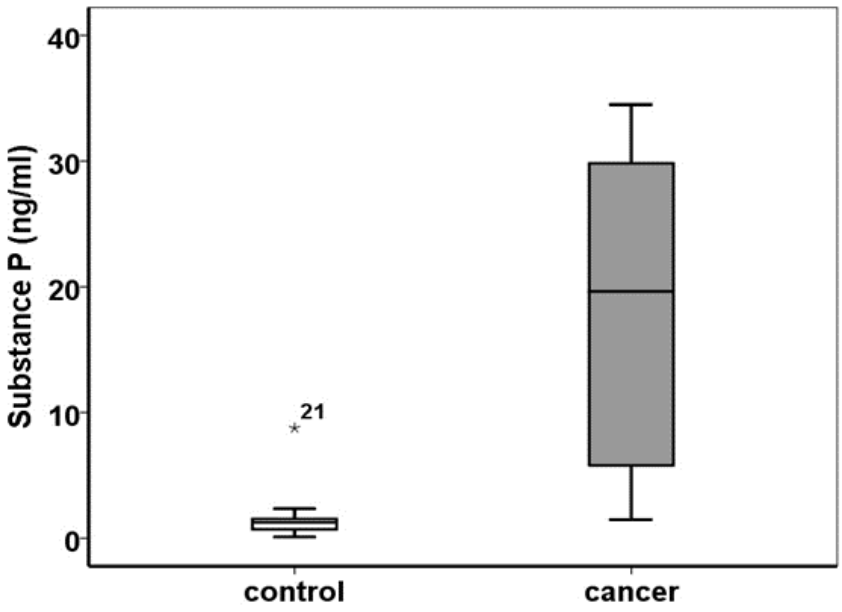

Fig. 1. Comparison between serum SP levels in patients with bladder cancer and healthy controls.

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The intensity and percentage of staining

Figure 2 shows high and low intensity IHC stained along with non-stained microscopic images of bladder samples.

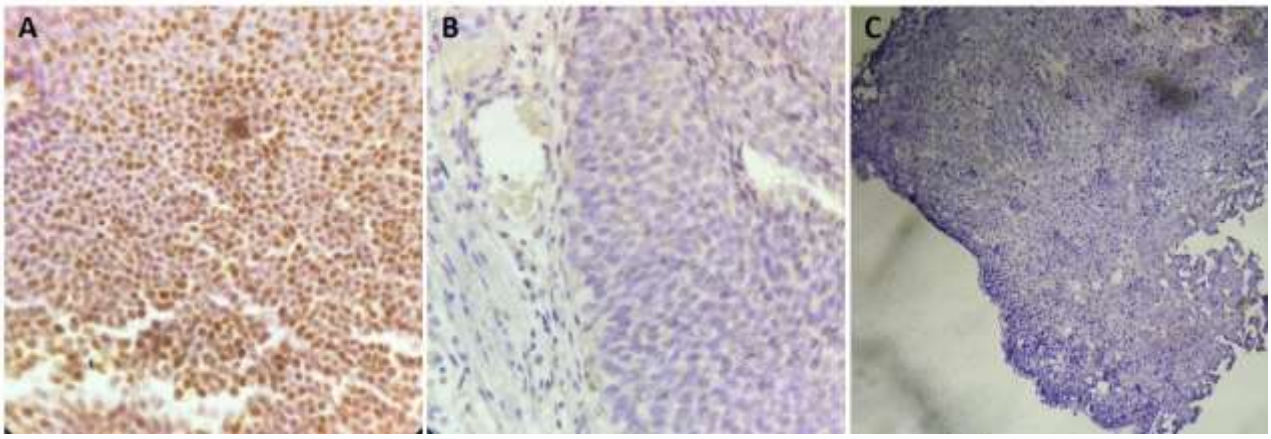

Fig. 2. Bladder cancer and normal tissue samples stained with IHC technique. (A) High-intensity IHC staining in 80% of the tumor cells (×400); (B) Low-intensity IHC staining in 20% of the tumor cells (×400); (C) Non-stained normal urinary bladder tissue (×100).

The intensity and percentage of staining

The staining intensity and percentage of NK-1R positive cells in bladder cancer tissues and their adjacent normal samples were measured to investigate the possible involvement of SP/NK-1R system in the pathogenesis of urinary bladder cancer. In this context, a score was assigned to each bladder sample according to the scoring system presented in Table 1. As shown in Figure 3, the immunostaining results showed that scores for NK-1 receptor-bearing cells percentage were graded 3-4 in tumor tissues and 1-2 in all control samples except one. Tumor sample scores were significantly higher than the control group ($p< 0.001$).

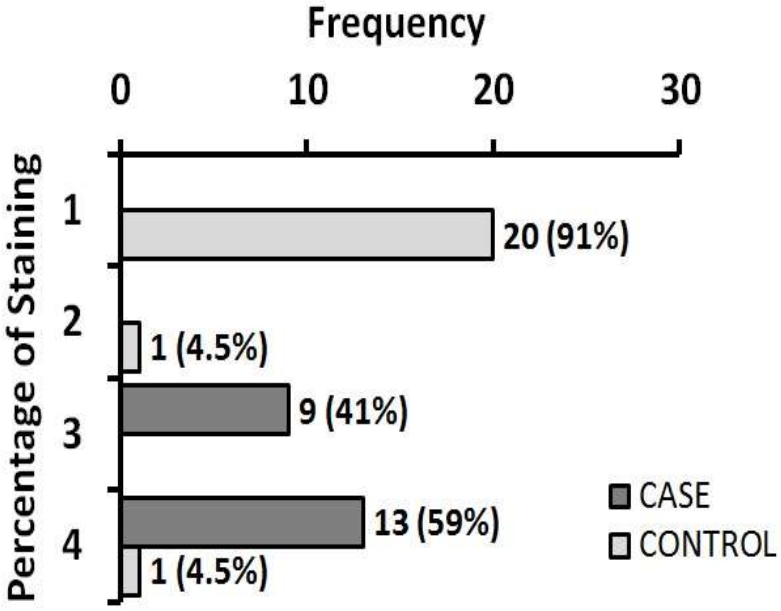

Fig.3. The percentage of NK-1R staining in case and control groups.

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In addition to the mentioned scores for stained cells percentage, it has been shown that the immunostaining intensity scores were in the range of 2-3 in tumor biopsies and 1-2 in all control samples except one.

Generally, our statistical analysis suggests that the staining intensity scores were significantly higher in tumor biopsies as compared with their normal adjacent tissues ($p< 0.001$).

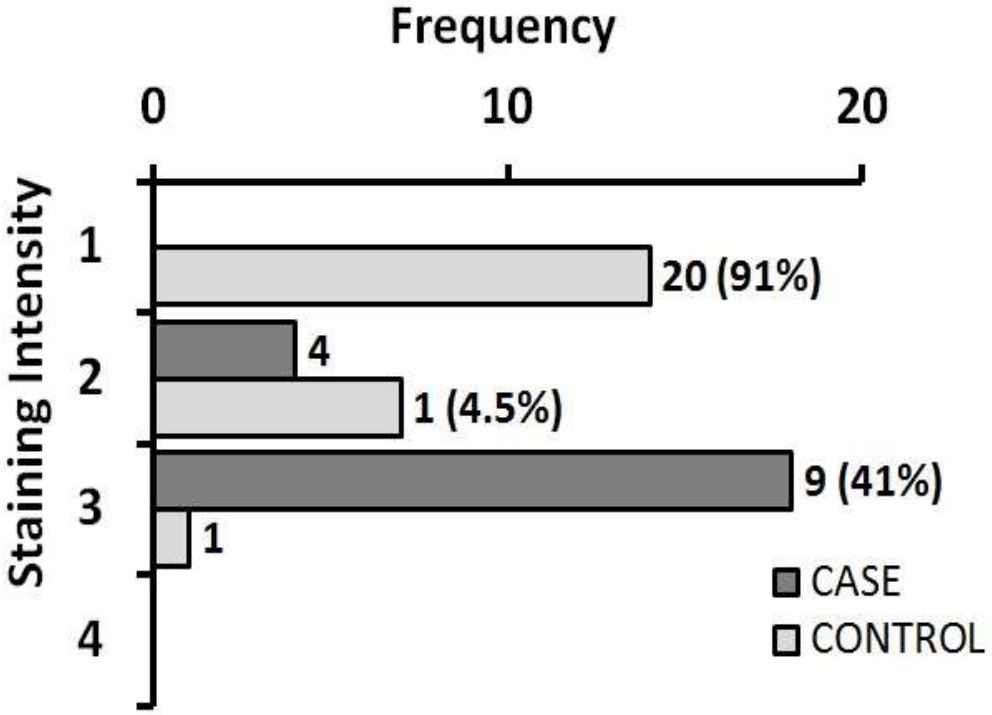

Fig. 4. The staining intensity of NK-1R in case and control groups.

NK-1R tissue distribution

The NK-1R tissue distribution was calculated through multiplying the staining intensity score by the percentage of receptor-bearing cancer cells. Mann-Whitney test showed that the median NK-1R distribution in tumor biopsies was significantly higher than their normal adjacent samples ($p< 0.001$). Also, Spearman's correlation and Kruskal-Wallis test revealed a significant associations between NK-1R tissue distribution and serum SP levels, percentage of receptor-bearing cells, as well as NK-1R staining intensity ($p< 0.001$) in cancer samples without correlations with tumor characteristics ($p> 0.05$).

Discussion

Reliable tumor markers can improve early diagnosis, prognosis, and treatment of bladder cancers. In this study, serum levels of substance P as well as the tissue distribution of neurokinin-1 receptor were evaluated in the bladder cancer. Our results revealed higher serum SP levels in bladder cancer tissue compared to normal counterparts. Also, significant correlations were found between NK-1R staining intensity, percentage of receptor-bearing cells, and bladder cancer. Our results also indicated that NK-1R immunoreactivity was significantly elevated in bladder cancer samples compared to their adjacent non-tumor tissues and correlated with staining intensity and percentage of NK-1R positive cells.

SP protein is well known as the endogenous ligand for NK-1R, which triggers the activation of the SP/NK-1R signaling pathway, and is involved in an extraordinary range of biological processes such as proliferation, apoptosis, metastasis, and

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angiogenesis in malignant tumors (2, 35, 36). Data from several sources have confirmed the SP involvement in the activation of the NFκB pathway under different conditions like inflammation (37, 38). Some studies have demonstrated the overexpression of SP and NK-1R in different types of tumor tissue including pancreatic, glioblastoma, colorectal, breast, and endometrial cancer (2, 39-41). Majority of studies have only focused on the tissue expression of SP in several types of cancer and much less is known about SP alterations in the sera of cancer patients. In this study we evaluated serum levels of SP in patients with bladder cancer. In agreement with our previously published findings (39-41), the results of this study indicated that serum levels of SP are significantly higher in patients with bladder cancer compared to control subjects with no evidence of inflammatory and infectious diseases ($p< 0.001$) (Fig. 2). It can, therefore, be assumed that serum concentrations of SP could be implicated in the diagnosis, pathogenesis, and prediction of bladder cancer. Also, it is possible to hypothesize that this condition is on account of elevated Tac1 gene expression, which has been reported in multiple human tumors (42-44).

Furthermore, the current data highlight the importance of SP activity in developing cancer cells (2). It is interesting to note that serum levels of SP had significant correlations with NK-1R tissue distribution, the percentage of NK-1R positive cells, and staining intensity. Consistent with our previous results, these findings also corroborate the involvement of the SP/NK-1R system in the pathogenesis of bladder cancer in addition to their diagnostic or therapeutic values.

One unanticipated finding was that no significant correlations were found between serum SP levels and bladder cancer characteristics. Several reports have shown that sample description and preparation as well as selection of different analytical methods may contribute to significant variations in SP levels reported in the literature (45, 46).

In this study, we evaluated the tissue distribution of NK-1R in the bladder cancer samples and their adjacent normal tissues. NK-1R expression level has been suggested to be correlated with the degree of malignancy (39-41). Studies have presented evidence that detection of NK-1R by immunohistochemistry may facilitate the identification of cancers overexpressing this SP receptor for either diagnostic or therapeutic purposes (39-41). In line with this, previous research has established that the NK-1R might be a promising drug target, and its antagonists (e.g., aprepitant, L-733,060, etc.) might represent a new strategy for cancer treatment through inhibiting pathophysiological actions mediated by NK-1R agonists (2). Indirect evidence to strengthen these hypotheses in our study was provided by higher percentage of NK-1R positive bladder cancer cells, intensity of staining, and tissue distribution of NK-1R in the tumor tissues compared to the adjacent normal samples. Our findings agree with our previous reports that revealed the increased expression of NK-1R in different malignant tissues (39-41). Combination of these findings supports the conceptual premise that the overexpression of NK-1R in various tumor tissues can extend the possibility of providing specific treatment for cancer using NK-1R antagonists and decreasing the considerable side effects of cancer therapy.

In summary, increased serum concentrations of SP and higher tissue distribution of NK-1R can elucidate the key role of SP/NK-1R system in tumorigenesis processes. These TK family members could be considered as diagnostic and prognostic biomarkers of urinary bladder cancer. NK-1R can be a new therapeutic target in treating urinary bladder cancer, and its antagonists can potentially be used as novel antitumor drugs. Further investigations are suggested to validate the prognostic or diagnostic value of the mentioned biomarkers in urinary bladder cancer.

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