Original paper

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Immunolocalization of neurokinin 1 receptor in WHO grade 4 astrocytomas, oral squamous cell and urothelial carcinoma

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Abstract

Introduction: *Neurokinin-1 receptor (NK-1R) induces inflammatory reactions in peripheral tissues but its regulatory effects in target tissues is dependent on receptor signalling. Substance P (SP) has a high affinity for the NK-1R, to which it binds preferentially. We aimed to investigate the expression of NK-1R in World Health Organization (WHO) grade 4 astrocytomas as well as in oral squamous cell carcinoma (OSCC) and urothelial carcinoma, and its association with disease progression.*

Material and methods: *The study included tissue samples from 19 brain astrocytomas, 40 OSCCs and 10 urothelial carcinomas. NK-1R expression was quantitatively assessed in the tumour cells using immunohistochemistry. The relationship between NK-1R expression in astrocytomas and recurrence-free interval has been explored.*

Results: *The results showed that the NK-1R was intensely expressed in patients with WHO grade 4 astrocytoma, OSCC and urothelial carcinoma. However, cases clinically diagnosed as a low-grade cancer showed reduced NK-1R expression.*

Conclusions*: NK-1R is overexpressed in all cases of WHO grade 4 astrocytoma, OSCC and urothelial carcinoma. The ubiquitous presence of SP/NK-1R complex during tumour development and progression suggests a possible therapeutic key strategy to use NK-1R antagonist as an adjuvant therapy in the future.*

Key words: *neurokinin-1 receptor, immunohistochemistry, substance P, immunolocalization, glioblastoma, oral squamous cell carcinoma, urothelial carcinoma.*

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Introduction

Neuropeptides such as substance P (SP), neurokinin A (NK-A), calcitonin gene related peptides (CGRP), neuropeptide Y (NP-Y) and vasoactive intestinal polypeptide (VIP), have a major role in inflammation and pain processes in the peripheral tissues [46]. SP preferentially binds to neurokinin-1 receptor (NK-1R), a G-protein-coupled receptor (GPCR) encoded by the *TACR1* gene, which is located on different immune cells such as macrophages, lymphocytes and granulocytes, human dental pulp [31], inflammatory cells, other connective-tissue cells [28], epithelial cells, fibroblasts, endothelium and other oral tissues [5,14]. It has seven transmembrane domains [14], involved in signal transduction, and associated with many physiological and pathological processes [5].

Substance P binds and stimulates the NK-1R on target cells forming SP/NK-1 complex that leads to phosphoinositide hydrolysis, calcium mobilization and mitogen-activated protein kinase (MAPK) activation. This stimulation process is linked to various processes involved in carcinogenesis such as angiogenesis [17], metastasis and movement of cells [16]. Hence, SP/NK-1R is considered dominant in the tumour microenvironment. SP is also secreted from the Vth cranial nerve [34] and activates the orofacial sensations [9], regulation of the mastication and facial musculature [26]. It also regulates a variety of biological processes after binding to NK-1R [16] such as cardiac conduction system [26], respiratory physiology [47], immune system regulation [33], sensory perceptions [24], pain processing [25] and inflammation [42]. Enhanced expression of SP/NK-1R has been reported in several pathological entities such as breast cancers [30], ovarian cancers [45], oral squamous cell [15,32], pancreatic cancers [12] and thyroid cancers [20].

The association between SP/NK-1R and high-grade brain tumours, mainly high-grade astrocytomas, are rarely explored in the literature. Despite surgical resection followed by palliative treatment strategies (combined chemo-radiotherapies), high-grade astrocytoma is considered the most aggressive and deadly cancer of all the human tumours [6,11,27,41,44,48,49]. According to the recent 5th edition of 2021 World Health Organization (WHO) classification of central nervous system (CNS) tumours and European Association of Neuro-Oncology (EANO) guidelines, WHO grade 4 astrocytoma is recently classified into *IDH*-mutant and *IDH*-wild-type, however, *IDH*-wildtype astrocytoma is isolated for glioblastoma [27,48]. Because glioblastoma microenvironment contains different types of cell lines, identifying new dominant cells in this microenvironment may explore new strategic target key therapies [6].

Head and neck squamous cell carcinoma (HNSCC) is the sixth most frequent cancer globally with an average incidence of 0.89 million and annual mortality as 0.45 million in 2018 [11]. Males are at 2-4-fold higher risk of getting HNSCC than females [11]. Oral squamous cell carcinoma (OSCC) is the most common cancer of HNSCC [11]. It includes the cancer of tongue, floor of mouth, oropharyngeal cavity and palate [11]. Global cancer statistics of 2018 suggest an annual incidence of OSCC to be 0.35 million [11]. The common etiological factors include smoking, consumption of alcohol, human papillomavirus (HPV), substance abuse such as pan chewing [44,49]. Previously, we investigated the immunolocalization of SP in OSCCs [32], breast carcinomas [30], products of conception (POCs) [4], dental pulp inflammation [31] and COVID-19 patients [29,33].

Bladder carcinoma (BC) is the fourth most common cancer in men and the ninth most common cancer in women in the United States [3,43]. The incidence of BC has been increasing for the last few years. Incidence of BC is approximately 0.073 million patients and the morality rate is 0.014 patients per year in the United States [3]. Most of the BCs comprise of urothelial carcinoma which is further categorized in low-grade (LGC) and high-grade carcinoma (HGC) depending on the pathological behaviour [3]. LGC usually do not invade the bladder wall, but they recur in a localized area very frequently. HGCs are very invasive in the bladder wall, have high metastasis and poor prognosis [19]. Among the urothelial carcinoma patients, most of them are elderly with an average age range of 69-71 years and males [8].

SP/NK-1R relevance in cancer signalling has made it an interesting target for cancer therapeutics [18]. However, there is not much evidence of the behaviour of NK-1R expression in brain tumours, OSCC and urothelial carcinoma. This study was thus aimed to investigate the expression of NK-1R in brain tumours, OSCC, and urothelial carcinoma and its association with cancer progression.

Material and methods

Tissue collection

A total of 19 formalin-fixed paraffin embedded (FFPE) tissue samples of WHO grade 4 astrocytomas were collected from the King Abdulaziz University (Saudi Arabia) after taking ethical approval. Out of these samples, 10 cases were *IDH*-wildtype glioblastomas, and 9 cases were *IDH*-mutant grade 4 astrocytomas. Another 50 FFPE tissue samples of non-brain tumours (40 cases of OSCC and 10 cases of urothelial carcinoma) were also collected from the University of Lahore, Pakistan after obtaining ethical approval. Detailed clinic-pathological data including age, gender, and tumour location, staging and grading were revisited. All the cases of OSCC were categorized as: well differentiated (WD), moderately differentiated (MD) or poorly differentiated (PD), based on the cellular morphology. Experimental procedure of haematoxylin-eosin (HE) staining and NK-1R immunohistochemistry were performed on all studied cases at the Research Unit, Faculty of Allied Health Sciences, University of Lahore, and have been blindly interpreted by two histopathologists.

Immunohistochemistry protocol

3 to 5 mm sections of FFPE tissue blocks of the 59 samples were prepared for the immunohistochemistry (IHC) with NK-1 receptor (ab219600, Anti-NK-1R antibody, Rabbit polyclonal, 1 : 100). After the overnight incubation at 4°C NK-1R (Abcam, 1 : 100) staining was performed manually. Sections were incubated in peroxidase-blocking solution (Dako Cytomation A/S) for 5 min and heated at 100°C for 60 min. This step was followed by incubation with a protein block serum-free reagent (Dako Cytomation A/S). After incubation for 32 min at 37°C, the tissue sections were incubated with a universal secondary antibody (Roche Diagnostics KK) for 20 min at 37°C and then visualized by the DAB Map detection kit (Roche Diagnostics KK).

Immunohistochemistry assessment for NK-1R

The presence or absence of staining and the intensity of the immunoreactivity were noted, as well as the number and type of cells showing a brown staining and whether the staining was localized in the nucleus, cytoplasm cells and/or in the plasma membrane. The results were recorded as positive (expressed) when they showed cellular and/or plasma membrane staining ranging from moderate to strong in more than 10% of the cells [29,42]. The specimens were examined and photographed utilizing a digital microscope camera (Olympus AX80 DP21; Olympus, Tokyo, Japan) interfaced with a computer. All protein levels were evaluated using the nuclear labelling index (%), recorded as the percentage of positively stained nuclei in 100 cells in the hot spot (Table I).

Statistical analysis

Data were analysed by using SPSS 25.0. All the quantitative variables were presented as mean ± SD and qualitative variables as frequencies and percentages. For brain tumours, Kaplan Meier curve (KMC) and log rank test was applied to find out the significant difference between the type of chemotherapy and recurrence. *P*-value less than 0.05 was considered as significant. The correlation between the nuclear labelling indexes was assessed by Spearman's rank correlation.

Results

WHO grade 4 astrocytomas

The mean age of patients was 55.74 years (±20.36). There were 14 males and 5 females. Most of the tumours were in the frontal lobe (n = 9, 47.36%) followed by temporal lobe (n = 5, 26.31%). Other locations included parietal lobe (n = 3), occipital lobe (n = 1), and lateral ventricle (n = 1). Main clinical presentations among patients were seizure (n = 6, 31.6%), headache (n = 6, 31.57%), and focal neurological deficit (n = 5, 26.3%). All patients underwent radical resection followed by radiotherapy except 4 patients who did not receive any kind of adjuvants. Chemotherapies were given to 12 patients (63.15%).

Table I. Quantitative and qualitative grading assessment of NK-1R expression using immunohistochemistry technique and digital microscopy

Cellular staining intensity	Expression grading
0-10%	Negative
10-30%	+1 (weak staining)
30-60%	+2 (moderate staining)
60-100%	+3 (strong staining)

Table II. Demographic data of patients in this study (n = 19)

Factor	Frequency	%
Age (years)	55.74 ±20.36	
Gender		
Males	14	73.7
Females	5	26.3
IDH-wildtype glioblastomas	13	68.42
IDH-mutant grade 4 astrocytoma	6	31.57
Tumour location		
Frontal	9	47.36
Temporal	5	26.31
Parietal	3	15.78
Lateral ventricle	1	5.26
Occipital	1	5.26
Main clinical presentation		
Seizure	6	31.57
Headache	6	31.57
Focal neurological deficit	5	26.31
Others	2	10.52
Adjuvant therapy		
Combined chemoradiotherapy	12	63.15
Radiotherapy	3	15.78
None	4	21.05
NK-1R expression		
Moderate positive (+2)	6	31.57
Strong positive (+3)	13	68.48
Recurrence-free interval		
≤ 1 year	8	42.12
> 1 year	6	31.57
≥ 2 years	5	26.31

Radiotherapy treatment alone was given to three patients (15.78%) (Table II). There were 6 grade 4 astrocytoma patients with *IDH*-mutation (31.57%) and 13 *IDH*-wild type astrocytoma (glioblastoma) patients (68.42%). Normal brain tissue showed minimal per neuronal NK-1R receptor expression (Fig. 1). The expression was not seen in normal glial cells. All cases of WHO grade 4 astrocytoma showed NK-1R expression with different intensities (Fig. 2). NK-1R was moderately expressed (+2) in 6 cases (31.57%) and strongly expressed (+3) in 13 cases (68.48%) (Fig. 2, Table II). The five cases with moderate NK-1R expression were *IDH*-wild type (Table III). There was single case with *IDH*-mutation.

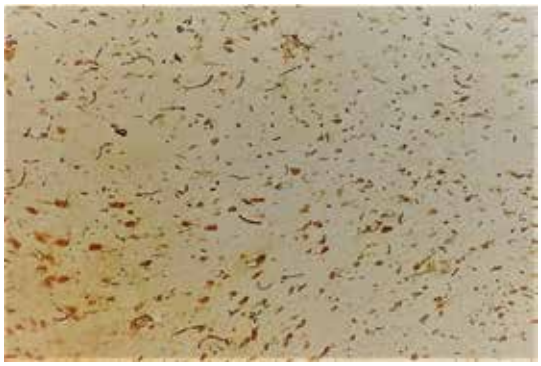

Fig. 1. NK-1R immunohistochemistry of brain tissue as a control.

On the other hand, NK-1R was strongly expressed in 8 cases of *IDH*-wildtype glioblastoma compared to 5 cases with *IDH*-mutation (Table III) (p = 0.342, insignificant). All grade-4 astrocytoma cases have recurred after surgical resection and adjuvant treatment. Around 42% (n = 8) of cases have recurred within 1 year of the treatment, 31% (n = 6) after 1 year and before 2 years of treatment, and 26% of cases recurred (n = 5) after two years of the treatment (Table I). Cases with NK-1R moderate (+2) staining showed mean recurrence-free interval (RFI) of 667 ±434.0 days and strong positive (+3) cases had mean RFI of 300 ±160.0 days (Fig. 3). RFI was higher in the *IDH*-wildtype case and cases with strongly positive NK-1R (+3) (Fig. 3).

Oral squamous cell carcinoma

Forty cases of OSCC were included in this study (Table IV). There were 29 (72.4%) cases of males and 11 females (27.5%). Tongue was the most common site (10, 25%), followed by cheek (7, 17.5%) and floor of mouth (6, 15%). There were 14 cases of WD-OSCC, 14 cases with MD-OSCC and 12 cases of PD-OSCC (Table IV). WD-OSCC cases had clear cell boundaries, fine cytoplasm, and well-differentiated nucleus (Fig. 4A-C). Cells of moderately differentiated cases were also recognizable but with little distorted morphology (Fig. 4D-F). However, cells of PD cases were more irregular, had disturbed morphology and were not clearly distinguishable (Fig. 4G-I). In WD-OSCC, NK-1R expression was weak positive, +1 intensity at 10×, 20× and 40× (Fig. 5A-C). MD-OSCC had intermediate positivity for NK-1R, +2 intensity (Fig. 5D-F). PD-OSCC cases had NK-1R strongly positive, intensity

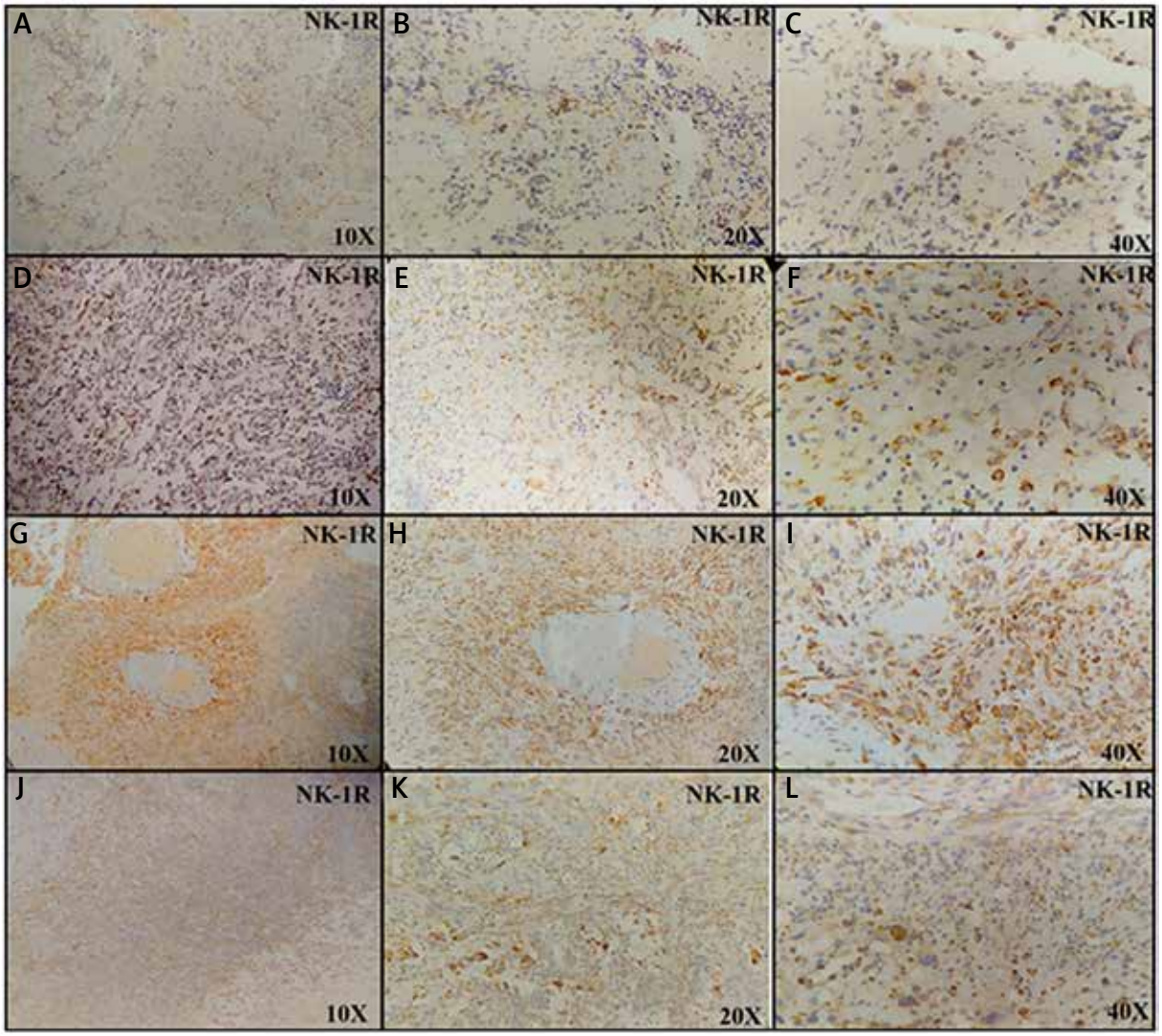

Fig. 2. A-F) Photomicrograph of NK-1R immunohistochemistry in grade 4 astrocytomas at 10, 20 and 40×, showing +2 intensity of staining and expression grading as +2, **G-L)** NK-1R immunohistochemistry in grade 4 astrocytoma at 10, 20 and 40×, showing +3 intensity of staining and expression grading as +3.

+3 and bundles of malignant cells at 10×, 20× and 40× (Fig. G-I).

Urothelial carcinoma

Patients had mean age of 64.8 ±5.73 years. There were 7 males (70%) and 3 females (30%) (Table V). There were 4 (40%) patients with low-grade carcinoma (LGC) (Table V, Fig. 6A-C) and 6 cases (60%) were high grade carcinoma (Table V, Fig. 6D-F). Only one patient had no NK-1R staining, 3 (30%) cases showed staining, and 6 (60%) cases had strong NK-1R staining intensity and expression. Intensity of stain and expression was +1 in LGC (Fig. 7A-C) and +3 in HGC (Fig. 7D-F). A positive correlation was observed between the cancer grade and expression of NK-1R. Intensity and expression of NK-1R was higher in HGC (0.976**, p = 0.000) (Table VI, Fig. 7A-F).

Table III. NK-1R expression in IDH-mutant or wild-type WHO grade 4 astrocytomas

WHO grade 4 astrocytomas	NK-1R expression		
	Moderate +	Strong +	Total
IDH-wildtype	5	8	13
IDH-mutant	1	5	6
Total	6	13	19

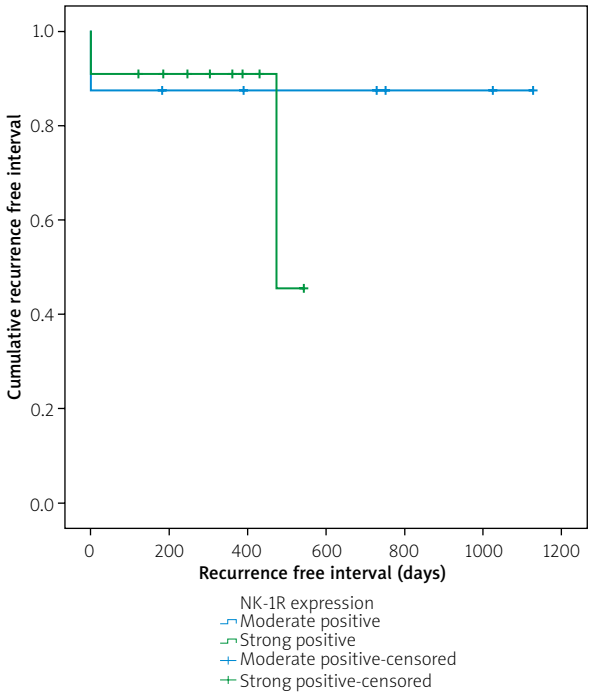

Fig. 3. The relationship between NK-1R expression in IDH-mutant or wildtype grade 4 astrocytomas and RFI.

Table IV. Clinicopathological data of the patients with oral squamous cell carcinoma (OSCC). It clarifies the distribution of the cases based on grading and locations (N = 40)

Factor	*n* (%)
Gender	
Males	29 (72.5)
Females	11 (27.5)
Mean age	53.5
WD cases	14 (35.0)
MD cases	14 (35.0)
PD cases	12 (30.0)
Tumour site	
Tongue	10 (25.0)
Cheek	7 (17.5)
Floor of mouth	6 (15.0)
Vocal cord	4 (10.0)
Buccal area	3 (7.5)
Larynx	3 (7.5)
Posterior cricoid region	2 (5.0)
Supra glottis region	1 (2.5)
Marjolin	1 (2.5)
Scalp	1 (2.5)
Maxillary sinus	1 (2.5)

Discussion

This study evaluated the immunohistochemical expression of NK-1R in WHO grade 4 astrocytoma, OSCC and urothelial carcinoma as well as its association with disease progression. NK-1R expression was intense in high-grade cases of all three types of cancers. A significant association of NK-1R expression was observed among different tumour tissues in this study as well as in other studies [30,32,39] supporting the hypothesis that overexpression of NK-1R is associated with progression of different cancers. Hence, SP potentiates the mechanisms and pathways triggering the translation of proteins and transcription factors involved in the switching (off and on) of the genes responsible for the cancer progression pathways. NK-1R inhibition by specific antagonists may be a novel strategy to control and manage the cancers [23,39]. SP binds to NK-1R and induces the activation of tumour pathways such as proliferation, invasion, angiogenesis, and migration of tumour cells to metastasize/metastasis. All the activities are blocked by NK-1R antagonists by activation of apoptotic pathways [21].

Recurrence in WHO grade 4 is present approximately in ninety percent of the patients which is quite high. Muñoz and Coveñas proposed in 2019 that glioma cells overexpress NK-1Rs and its antagonists may block this pathway and may be used as a new therapeutic approach [35]. Afshari *et al.* in 2021 agreed to Muñoz and Coveñas [1]. In the present study, we have also observed recurrence in majority of the WHO grade 4 astrocytomas. RFI in most of the cases was approximately 1 year (Table I). There was no statistically significant difference in RFI among cases with *IDH*-mutation or *IDH*-wildtype cases regardless of the NK-1R expression status ($p > 0.05$) (Table III).

In a clinical trial conducted by Cordier *et al.*, in 2014, the expression of NK-1R in gliomas was exploited by intratumoral injection of radiolabelled SP, which is also a ligand for NK-1R. The response of the patients to therapy was not homogenous, some were good responders as compared to the others, which suggested a personalized therapy in these patients based on NK-1R expression. They also explored the RNA levels of full length or truncated NK-1R expression in 4 different cell lines of glioma.

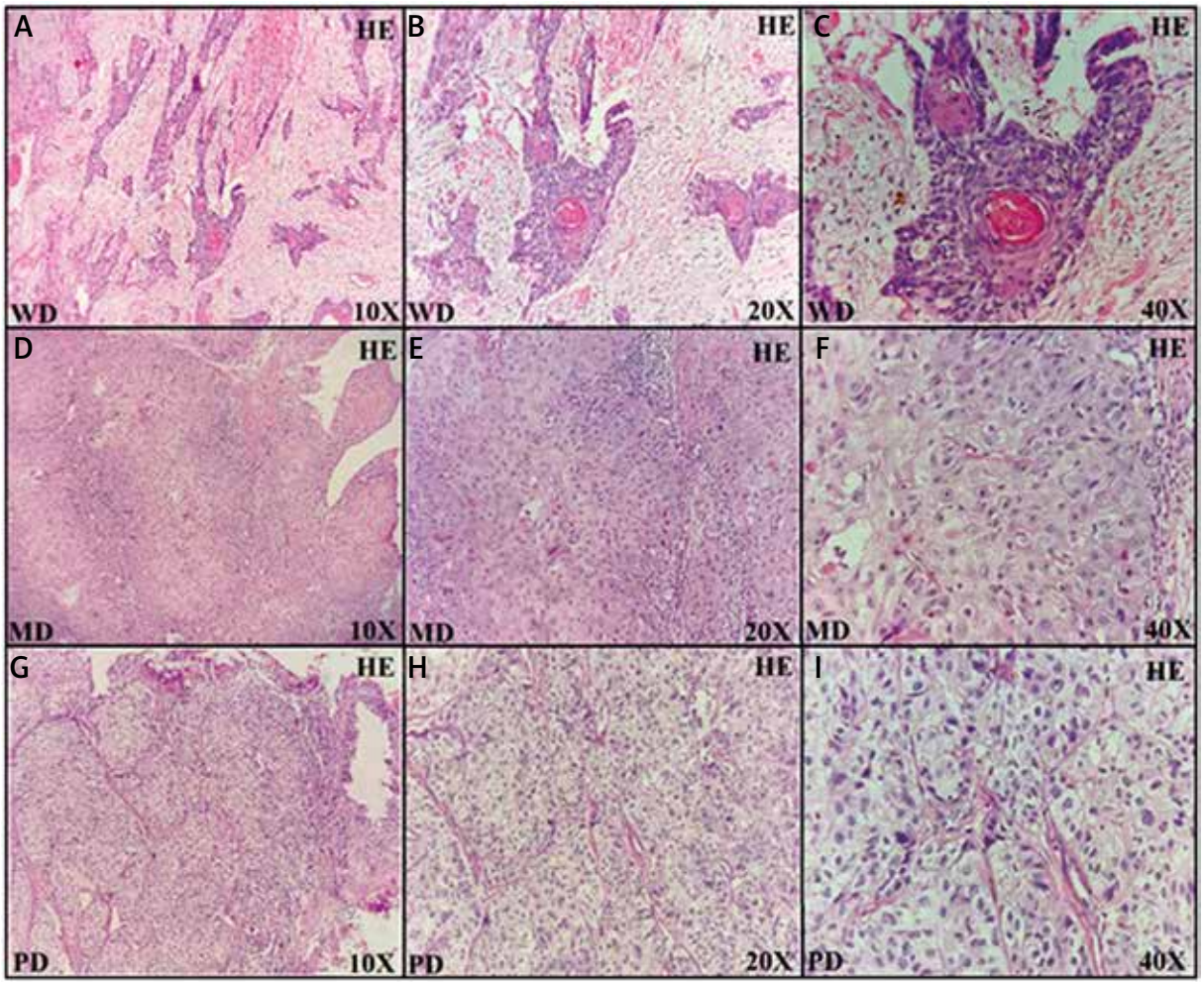

Fig. 4. A-C) Photomicrograph of haematoxylin-eosin (HE) staining in well-differentiated oral squamous cell carcinoma at 10, 20 and 40×, showing clear cell morphology and nucleus, **D-F**) HE staining in moderately differentiated oral squamous cell carcinoma at 10, 20 and 40×, showing slightly disturbed morphology of cells, **G-I**) HE staining in poorly differentiated oral squamous cell carcinoma at 10, 20 and 40× disrupted cellular morphology.

NK-1R was expressed in all the cell lines but with varying expression and LN319 cell line exhibited the highest level of full-length NK-1R RNA [7]. In the current study we have also observed strong NK-1R expression in all the cases of grade 4 astrocytoma (Table III).

In OSCC, the NK-1R expression was the highest in PD cases (Fig. 4G-I) and weak in WD cases (Fig. 5A-C). Moderate expression was observed in MD cases (Fig. 5D-F). The results are in alignment with our previous study on SP expression and immunolocalization in OSCC cases which also showed a strong expression in PD cases, followed by MD and then WD cases [45]. Another study was conducted on eighty three oral carcinoma tissue biopsies and SP/NK-1R expression was evaluated immunohistochemically in these carcinogenic tissues and their adjacent non-tumour epithelia. WHO criteria were used for the assessment of presence or degree of epithelial dysplasia. It was observed that the expression of SP/NK-1R was significantly associated with the adjacent non-tumour epithelium. It was suggested that SP/NK-1R expression plays a significant role in early carcinogenesis by promoting the proliferation and growth of premalignant cells [13].

Regarding the expression of NK-1R in urothelial carcinoma, no study has been reported to the best of our knowledge, however, there was one study

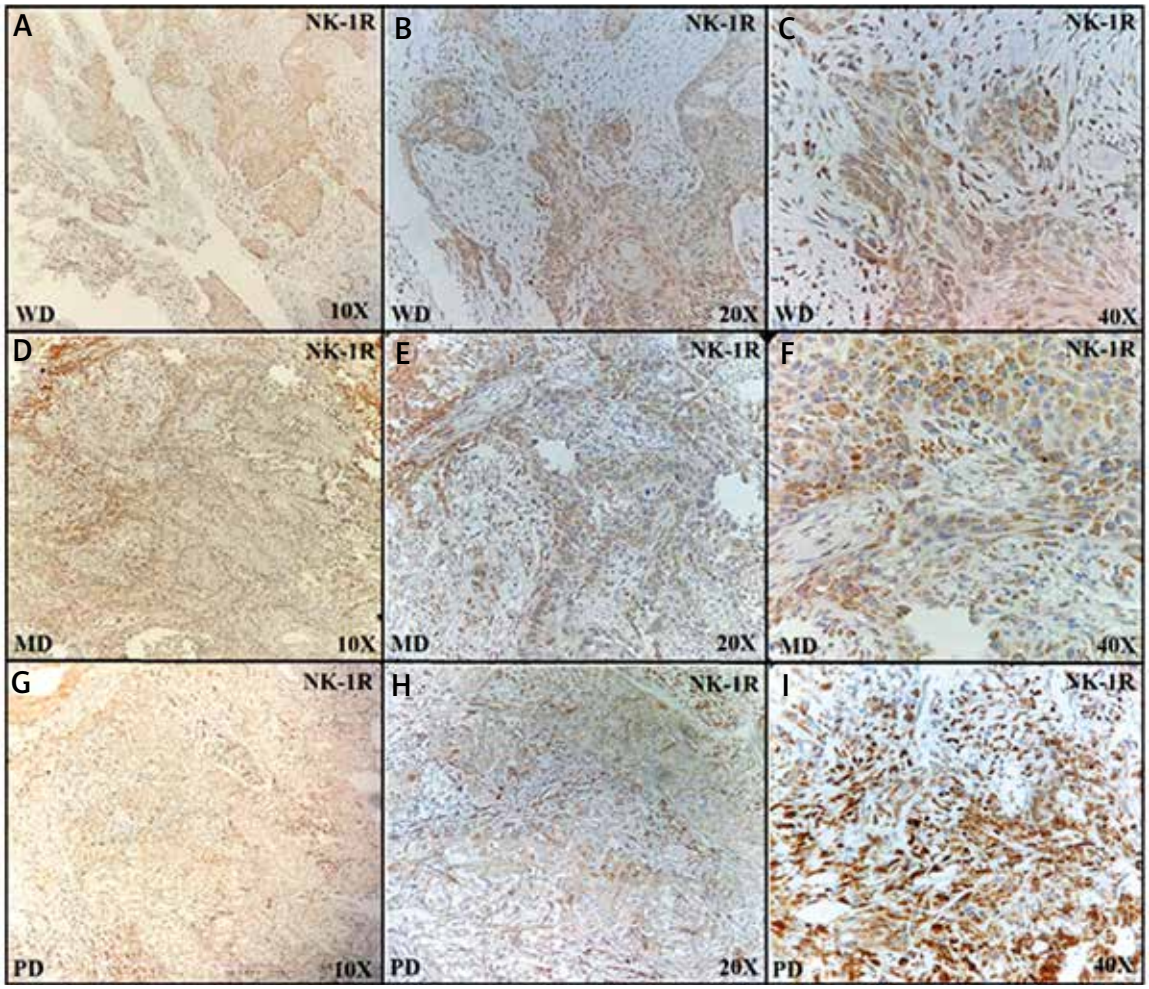

Fig. 5. A-C) Photomicrograph of NK-1R immunohistochemistry in well-differentiated oral squamous cell carcinoma at 10, 20 and 40×, showing +1 intensity of staining and expression grading as +2, **D-F**) NK-1R immunohistochemistry in moderately differentiated oral squamous cell carcinoma at 10, 20 and 40×, showing +2 intensity of staining and expression grading as +3, **G-I**) NK-1R immunohistochemistry in poorly differentiated oral squamous cell carcinoma at 10, 20 and 40×, showing +3 intensity of staining and expression grading as +3.

Table V. Neurokinin-1 receptor expression and grading in urothelial carcinoma

Variables	Frequency	%
Age (mean ±SD)	59.30 ±11.54	
Gender		
Male	7	70.0
Female	3	30.0
Intensity of NK-1R staining		
Negative	1	10
Mild	3	30
Severe	6	60
Grade of cancer		
Low grade cancer	4	40
High grade cancer	6	60

conducted by Ercan *et al.* in 2006, who studied the role of SP during stress-induced mast cell degranulation urothelial injury in rat bladder. It was observed that stress and SP injected intraventricularly, caused bladder injury by activating the mast cell and inflammation. The urothelial damage was prevented by the injection of NK-1R antagonist [10].

The current research is in accordance with the previous researches showing the relationship of the SP/NK-1R system with advancement of carcinogenesis and proliferation [30,36,39]. It has been observed in a study conducted on pancreatic cancer cell lines, by Muñoz and Coveñas in 2014 that NK-1R antagonists inhibit the proliferation of cancer cells in a con-

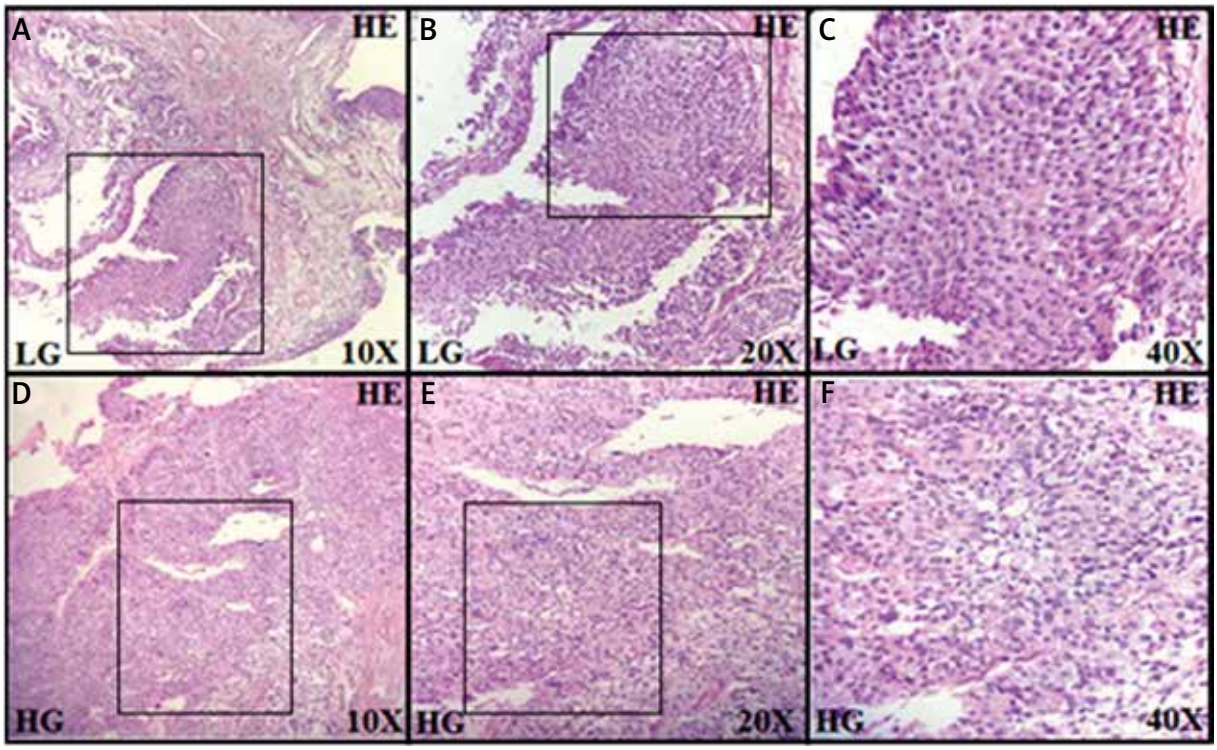

Fig. 6. A-C) Photomicrograph of low grade urothelial carcinoma (transitional cell carcinoma) with more than 10 layers thick neoplastic cells infiltrating the lamina propria, 10, 20 and 40×; HG – high grade, LG – low grade, **D-F)** Photomicrograph of high grade urothelial carcinoma (transitional cell carcinoma) with groups and sheets of neoplastic cells with coarse chromatin open up nuclei, 10, 20 and 40×.

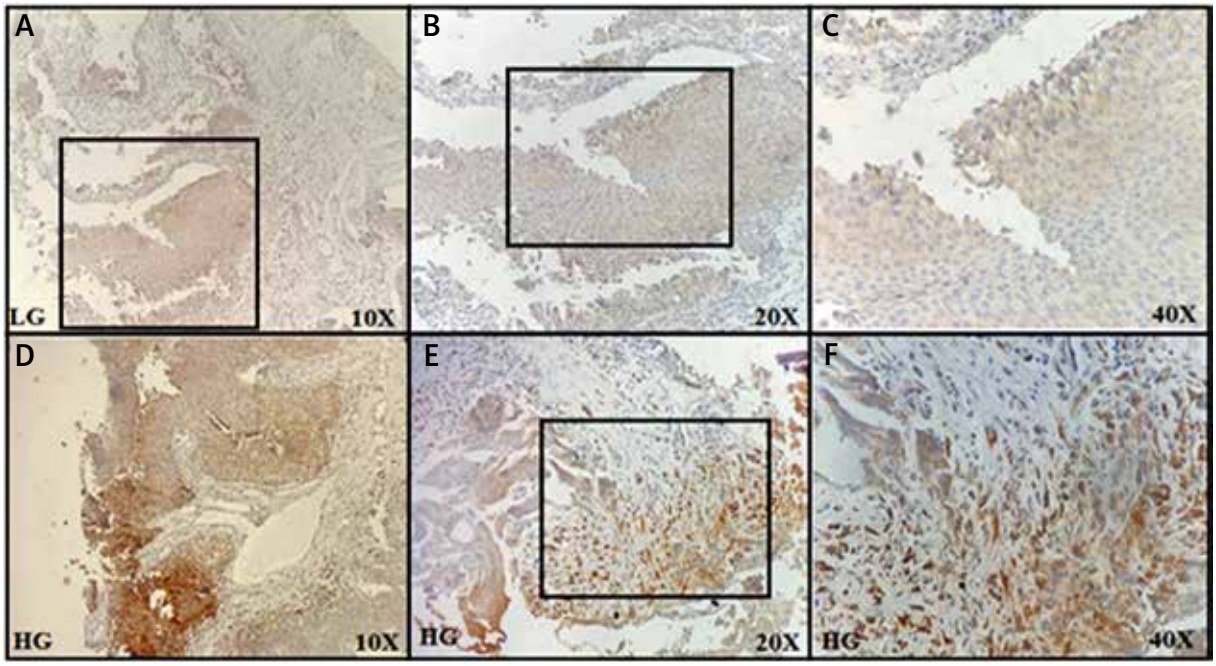

Fig. 7. A-C) Low grade urothelial carcinoma with weak and diffused NK-1R staining at 10, 20 and 40×, **D-F)** High grade urothelial carcinoma with strong positive stain for NK-1R at 10, 20 and 40×.

Table VI. Spearman correlation clarifies the relationship between NK-1R expression and the grading of urothelial carcinoma

Correlations		NK-1R expression	Grades of cancer
NK-1R expression	Correlation coefficient	1.000	0.976**
	p-value	–	0.000
	n	10	10

***Spearman's rho correlation is significant at the 0.01 level (2-tailed)*

centration-dependent manner and cause death of cancer cells by apoptosis. They also suggested SP/NK-1R pathway as a growth driver which may potentiate several cancers and hence it can be an effective target for treating cancers [36].

NK-1R antagonists represent a wide range of potential anticancer drugs. Muñoz *et al.* suggested a therapeutic role of NK-1R in various studies [38,40], but these were mostly based on *in vitro* studies. There is a need for further research in this area and clinical trials. To our knowledge, this is the first study evaluating the expression of NK-1R in WHO grade 4 astrocytoma, OSCC, UC and its relationship with cancer aggression in human samples. The current findings are important clinically, diagnostically as well as from a therapeutic point of view. Identifying the involvement of NK-1R, in nociceptive signalling cascade in these cancers has significance for better understanding of the mechanism of disease progression. It may provide a good therapeutic regimen for the treatment and management of such patients in the future.

Conclusions

NK-1R is strongly expressed in WHO grade 4 astrocytoma, poorly differentiated oral squamous cell carcinoma and high-grade urothelial carcinoma. Considering the association between increased NK-1R expression and advanced grade of disease, NK-1R antagonist may serve as a promising therapeutic key in the treatment.

Data availability statement

All the data are present in this paper and any further information required will also be made available upon request.

Disclosure

The authors declare no conflict of interest.

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