A Role for the Substance P/NK-1 Receptor Complex in Cell Proliferation in Oral Squamous Cell Carcinoma

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Abstract. *Objective: To investigate the presence and distribution of substance P (SP) and neurokinin 1 receptor (NK-1R) in oral squamous cell carcinoma (OSCC) and their relationship with proliferation. Patients and Methods: Ninety OSCCs from 73 patients were immunohistochemically analyzed using monoclonal antibodies against SP, NK-1R and Ki-67 in a case and control study. Results: Seventy-one percent (n=49) of cases expressed SP on tumour cell membrane, 81.3% (n=69) in cytoplasm, 39.4% (n=28) in nucleus, 81.6% (n=71) in infiltrating lymphocytes, and 58.1% (n=43) in peritumoural or intratumoural blood vessels; 14% (n=12) of cases expressed NK-1R on tumour cell membrane, 50% (n=43) in cytoplasm, 48.3% (n=42) in infiltrating lymphocytes and 22.5% (n=18) in tumour blood vessels. All cases expressed Ki-67, which was expressed in >25% of tumour cells in 79.8% of cases (n=63). Direct significant associations were observed in SP expression between different tissue levels ($p<0.01$), between SP and NK-1R tumour cell membrane expression ($p<0.01$), and between joint SP and NK-1R expression in tumour cell cytoplasm and a higher expression of Ki-67 ($p<0.05$). Conclusion: The ubiquitous presence of SP strongly suggests a role for SP/NK-1R complex in tumour development and progression and possibly for NK-1R antagonists, such as L-773060, in the management of patients with oral cancer.*

Tumours have been linked with inflammation since 1863, when Rudolf Virchow discovered leukocytes in neoplastic tissues and made the first connection between inflammation and cancer (1). Since then, chronic inflammation has been identified as a risk factor for cancer, with findings of an association between human papillomavirus and cancer of cervix, oesophagus and larynx, among others (2-5); between *Helicobacter pylori* bacterial infection and gastric adenocarcinoma (6); between hepatitis B virus, cirrhosis and hepatocellular carcinoma (7); between *Schistosoma haematobium* and bladder cancer (8); and between asbestos-induced inflammation and bronchogenic carcinoma or mesothelioma (9).

Substance P (SP) is an endecapeptide that belongs to the tachykinin family of peptides. SP regulates numerous biological functions by binding to the neurokinin 1 receptor (NK-1R) (10). SP has been implicated in the regulation of the cardiovascular system, in neuronal survival and degeneration, the regulation of respiratory mechanisms, sensory perception, movement control, gastric motility, salivation and micturition (11). This neuropeptide has also been implicated in inflammation, pain and depression (12-14). Over the past 20 years, SP has also been identified as an important mediator in the development and progress of mucosal inflammation. This peptide, released from mucosal nerves, sensory neurons and inflammatory cells of the lamina propria during mucosal inflammation, participates in inflammation by interacting, directly or indirectly, with NK-1R expressed on nerves, epithelial cells, and immune and inflammatory cells, such as mast cells, macrophages and T-cells. SP-dependent activation of these cells leads to the release of cytokines and chemokines and other neuropeptides that modulate inflammation (15).

It has been demonstrated that SP acts *via* NK-1R as a mitogen on several human cancer cell lines (glioma, retinoblastoma, neuroblastoma, melanoma, laryngeal carcinoma) (16-21). Therefore, the SP/NK-1R system may play a role in the development of cancer, since SP might be a universal mitogen in NK-1R-expressing tumour cell types. However, despite their extensive investigations Henning *et al.* (22) did not study squamous cell carcinoma in their

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pioneering work on SP receptors in human primary neoplasms, and no report on SP and NK-1R expression in oral squamous cell carcinoma (OSCC) has been published to date. Therefore, the aim of the present study was to investigate the presence and distribution of NK-1R and SP in OSCC and their relationship with proliferative activity, using monoclonal antibodies and immunohistochemistry.

Patients and Methods

Patients. Ninety consecutive cases of OSCC were identified from 73 patients at Jaen Hospital Complex (Spain) and their clinical records were reviewed with permission of the institutional review board of the hospital. The patient sample comprised 17 (23.3%) females and 56 (76.7%) males, with a mean age of 59.8 years (range, 27-91 years). Clinicopathological data gathered for all patients included tumour site, clinical presentation (ulcer, tumour, leukoplakia, erythroplasia, erythroleukoplakia), size, presence of cervical and distant metastases, and tumour stage. Data collection was in accordance with the American Joint Committee for Cancer Staging and End Results Reporting, and tumour differentiation was recorded following WHO criteria (well-differentiated, moderately well-differentiated, and poorly differentiated squamous cell carcinoma). The emergence of other tumours was also recorded. Secondary tumours were distinguished from recurrences by the absence of surgical margins with neoplastic cells in the surgical specimen of the index tumour and, following WHO criteria, recurrence was diagnosed when the tumour was in deeper tissue and not associated with the epithelial surface (23).

Immunohistochemistry. For the immunohistochemical staining, 4-μm sections were cut from the paraffin blocks. The peroxidase–antiperoxidase technique was used, performing immunohistochemical analysis by means of the avidin–biotin method. Slides were deparaffinized in xylene, hydrated and incubated with 0.5% (v/v) H_2O_2 in methanol for 20 min to block endogenous peroxidase activity. Slides were then washed with Tris-buffered saline and heated for 15 min at 100˚C in 10 mM sodium citrate buffer (pH 6.0) for antigen retrieval. Non-specific binding was blocked by incubation with 1% BSA for 1 h. Sections were incubated with primary antibodies overnight at 4˚C. The colour was developed using diaminobenzidine as chromogen. Slides were extensively washed with TBS after each step. Finally, they were counter-stained with Mayer's hematoxylin and mounted with DPX mountant. For the negative control, the primary antibody was replaced with phosphate-buffered saline. For the positive control, tissue was used from a laryngeal carcinoma known to intensively express the proteins under study. The expression of SP in peripheral nerves was considered as a positive internal control for this protein (Figure 1A). The following primary antibodies were used: anti-Ki-67 (Mib-1 clone; Dako, Carpinteria, CA, USA), anti-SP (Sigma, St Louis, MO, USA) and anti-NK-1R (Sigma-Aldrich, Madrid, Spain).

Protein expression was studied by cell count in four randomized high-magnification fields of tumour tissue (×40). Counts were made of total and labelled cells in each field, calculating mean percentages of expression for each case. Brown-stained cells were considered positive, and no account was taken of the intensity of the staining. The immunohistochemical expression of Ki-67 was detected as brown staining in the nucleus of epithelial cells, and SP expression as brown staining in the corium, in the membrane, cytoplasm and nucleus of epithelial cells and in infiltrating lymphocytes and tumour blood vessels. NK-1R expression was

Table I. *Clinical and histopathological variables of the studied patients*.

Variable	n	%
Degree of differentiation		
Well-differentiated	34	42.5
Moderately differentiated	27	33.8
Poorly differentiated	19	23.8
Missing data	10	
Tumour site		
Tongue	48	53.3
Floor of mouth	13	14.4
Floor + tongue	11	12.2
Retromolar triangle	04	4.4
Oral mucosa + triangle	01	1.1
Oral mucosa	04	4.4
Soft palate + oral mucosa	03	3.3
Lower lip	02	2.2
Vestibular floor	01	1.1
Gingiva	03	3.3
Clinical presentation		
Ulcer	35	45.5
Leukoplakia + tumour	08	10.4
Lichen planus + ulcer	02	2.6
Tumour	24	31.2
Erythroleukoplakia	04	5.2
Leukoplakia + ulcer	03	3.9
Erythroleukoplakia + ulcer	01	1.3
Missing data	13	
Tumour size		
T1	28	33.7
T2	30	36.1
T3	10	12.0
T4	15	18.1
Missing data	07	
Node metastasis		
N0	53	3.9
N1	20	24.1
N2a	05	6.0
N2b	03	3.6
N2c	01	1.2
N3	00	0.0
Nx	01	1.2
Missing data	07	
Distant metastasis		
M0	66	79.5
M1	01	1.2
Mx	16	19.3
Missing data	07	
Stage		
I	23	27.7
II	20	24.1
III	19	22.9
IVa	20	24.1
IVb	00	0
IVc	01	1.2
Missing data	07	

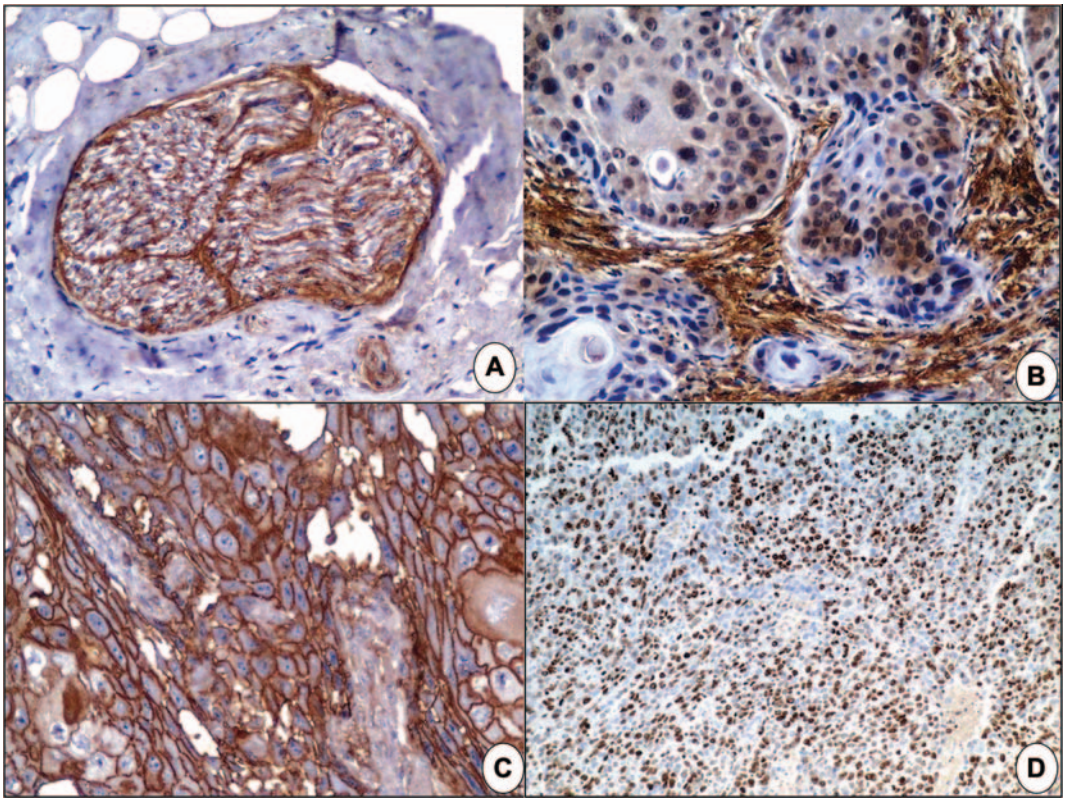

Figure 1. *A) SP expression in a peritumoral nerve. Positive internal control (×40). B) SP expression in corium, lymphocytes, cytoplasm and nucleus of some tumour cells. The image also shows some negative cells (×40). C) NK-1R expression in membrane, with some cells also showing cytoplasmic expression (×40). D) Extensive Ki-67 expression in tumour (×10).*

detected as brown staining in membrane and cytoplasm of epithelial cells, in the inflammatory infiltrate, and in tumour blood vessels.

Cases were assigned to one of the following categories: 0% positive cells (–), 1-25% positive cells (+), 26-50% positive cells (++), 51-75% positive cells (+++), or >75% positive cells (++++). SP expression in the corium was considered positive when brown staining appeared in at least two of the four high-power fields studied. Markers were recorded as "not assessed" in three cases due to doubts about the immunohistochemistry result despite repetition of the test, possibly as a result of fixation and tissue preservation problems. In the remaining cases in which the markers were not assessed, doubts were mainly raised by the intense expression of the marker in one tissue compartment that masked or hindered interpretation of the marker in other compartments. Thus, the intense cytoplasmic expression of SP hampered evaluation of SP expression in membrane in cases with poorly defined cell borders and sometimes affected the evaluation of nuclear expression. The histological and immunohistochemical analyses were always performed by the same observer (MAGM), who was blinded to the clinical stage, treatment and course of the disease.

Statistical analysis. For descriptive purposes, SPSS-Windows v.15.0 (SPSS Inc., Chicago, IL, USA) was used. For the correlation analysis with SP, NK-1R and Ki-67, the mean value of each interval was considered: Negative (0%) to 0%, + (1%-25%) to 13%, ++ (26%-50%) to 38%, +++ (51%-75%) to 63%, and ++++ (76%-100%) to 88%. For analytical purposes (calculation of *p*-values), SUDAAN v.7.0 (Research Triangle Institute, RTP, NC, USA), was used with design WR (with-replacement) to account for clustering (multiple oral tumours within patients).

Results

Results of the study of clinicopathological variables are compiled in Table I. Tumours were well-differentiated in 42% of cases and located on the tongue in 53.3% of cases; they presented as ulcers or tumours in 45.5% and 31.2% of cases, respectively, and were classified as T2 in 36.1%, N1 in 24.1% and M0 in 79.5% of cases. Secondary tumours were detected in 13.7% of patients (n=9) (not included in Table). Table II shows the expression of SP, NK-1R and Ki-67 in tumour tissue (Figures 1B, C and D). SP was expressed on tumour cell membrane in 71% (n=49) of cases, in cytoplasm in 81% (n=69), in nucleus in 39.4% (n=28), in infiltrating lymphocytes in 81.6% (n=71) and in peritumoural or intratumoural blood vessels in 58.1% (n=43) of cases. NK-1R was expressed in tumour cell membrane in 14% (n=12) of cases, in cytoplasm in 50% (n=43), in infiltrating lymphocytes in 48.3% (n=42), and in peritumoural or intratumoural blood vessels in 22.5%

Table II. *Expression of SP, NK-1R and Ki-67 in tumour tissue.*

Variable	n	%
SP in tumour corium		
Negative	20	23
Positive	67	77
Not assessed	3	
SP in epithelial cell membrane		
Negative	20	29
1-25%	11	15.9
26-50%	16	23.2
51-75%	15	21.7
76-100%	7	10.1
Not assessed	21	
SP in epithelial cell cytoplasm		
Negative	16	18.8
1-25%	14	16.5
26-50%	14	16.5
51-75%	22	25.9
76-100%	19	22.4
Not assessed	5	
SP in epithelial cell nucleus		
Negative	43	60.6
1-25%	12	16.9
26-50%	5	7.0
51-75%	7	9.9
76-100%	4	5.6
Not assessed	19	
SP in infiltrating lymphocytes		
Negative	16	18.4
1-25%	20	23.0
26-50%	20	23.0
51-75%	21	24.1
76-100%	10	11.5
Not assessed	3	
SP in tumour blood vessels		
Negative	31	41.9
1-25%	10	13.5
26-50%	5	6.8
51-75%	21	28.4
76-100%	7	9.5
Not assessed	16	
NK-1R in epithelial cell membrane		
Negative	74	86.0
1-25%	6	7.0
26-50%	4	4.7
51-75%	1	1.2
76-100%	1	1.2
Not assessed	4	
NK-1R in epithelial cell cytoplasm		
Negative	43	50.0
1-25%	17	19.8
26-50%	15	17.4
51-75%	9	10.5
76-100%	2	2.3
Not assessed	4	
NK-1R in infiltrating lymphocytes		
Negative	45	51.7
1-25%	18	20.7
26-50%	13	14.9
51-75%	9	10.3
76-100%	2	2.3
Not assessed	3	
NK-1R in tumour blood vessels		
Negative	62	77.5
1-25%	1	13.8
26-50%	6	7.5
51-75%	1	1.3
76-100%	0	0.0
Not assessed	10	
Ki-67 nuclear expression		
Negative	0	0.0
1-25%	16	20.3
26-50%	13	16.5
51-75%	27	34.2
76-100%	23	29.1
Not assessed	11	

(n=18). Table III shows the associations found in SP expression at different tissue levels, and Table IV the associations among the expressions of SP, NK-1R and Ki-67.

Discussion

This study demonstrates for the first time the expression of SP and NK-1R in OSCC, reporting an abundant expression of both proteins, especially SP, in all tissue compartments analyzed (corium, blood vessels, infiltrating lymphocytes, membrane, cytoplasm and nucleus of tumour cells). SP performs a wide range of functions, including regulation of the intensity of noxious stimuli (24), regulation of neurogenic inflammation and immune response (25, 26), and participation in psychological stress pathways (27, 28). By binding to NK-1R, SP activates members of the mitogen-activated protein kinase (MAPK) cascade, including extracellular signal-regulated kinases 1 and 2 (ERK 1/2), which translocate to the nucleus to induce cell proliferation and protect the cell from apoptosis (29). SP can also stimulate cell proliferation *via* transactivation of the epidermal growth factor receptor (EGFR) (30). The link between these functions and the SP/NK-1R complex has led to suggestions that this complex may be associated with tumour development and progression (29). Because SP can be produced by B- and T-cells in an autocrine or paracrine manner, it could be speculated that the expression of SP by tumour-infiltrating lymphocytes, whose function should be to increase the antitumor immune response

Table III. *Linear correlation between different sites of SP expression in oral tumours (n=90).*

	Membrane	Cytoplasm	Nucleus	Lymphocytes	Vessels
Corium	0.53** (n=69)	0.42** (n=85)	0.34** (n=71)	0.55** (n=87)	0.35** (n=74)
Membrane		0.54** (n=69)	0.32** (n=57)	0.53** (n=69)	0.42** (n=60)
Cytoplasm			0.52** (n=71)	0.50** (n=85)	0.50** (n=73)
Nucleus				0.57** (n=71)	0.70** (n=63)
Lymphocytes					0.79** (n=74)

**$p<0.01$, calculated with the REGRESS procedure of SUDAAN, to correct for clustering (90 oral carcinomas from 73 patients).

Table IV. *Linear correlations among SP, NK-1R and Ki-67 expressions in oral tumours (n=90).*

SP	NK-1R				Ki-67	
	Membrane	Cytoplasm	Lymphocytes	Vessels	All cases	Cases expressing SP and NK-1R
Corium	0.18** (n=85)	0.18* (n=85)	0.34** (n=86)	0.09 (n=80)	0.14 (n=79)	-
Membrane	0.33** (n=68)	0.15 (n=68)	0.48** (n=68)	0.21 (n=64)	–0.03 (n=62)	0.13 (n=7)
Cytoplasm	0.11 (n=84)	0.14 (n=84)	0.34** (n=84)	0.08 (n=79)	0.09 (n=78)	0.35* (n=38)
Nucleus	0.01 (n=70)	0.10 (n=71)	0.33* (n=71)	0.11 (n=66)	–0.09 (n=66)	-
Lymphocytes	0.11 (n=85)	0.37** (n=85)	0.50** (n=86)	0.16 (n=80)	–0.01 (n=79)	–0.01 (n=34)
Tumour vessels	0.02 (n=72)	0.26* (n=72)	0.39** (n=73)	0.09 (n=69)	–0.14 (n=67)	0.26 (n=8)

*$p<0.05$, **$p<0.01$. Calculated with the REGRESS procedure of SUDAAN to correct for clustering (90 oral carcinomas from 73 patients).

(29), may be used by tumour cells as a mechanism to increase OSCC proliferation and progression. In this context, a significant association in SP expression was observed among different tumour tissue levels, supporting the proposal of a diffusion pathway for SP from lymphocyte *via* the corium to the malignant epithelium, favouring its cellular action (31, 32). This proposition is also supported by the similar proportion of cases that express SP in infiltrating lymphocytes (81.6%), corium (77%), membrane (71%) and cytoplasm (81%) of epithelial cells.

The nuclear expression of SP was a less frequent finding, as previously observed in keratocystic odontogenic tumours (33), oral lichen planus (34) and neurons (35). The significance of the SP expression in the tumour cell nucleus has not yet been established, although it is tempting to suggest the existence of a second (nuclear) receptor for SP, as found in melatonin. In contrast, the cytoplasmic localization of NK-1R is explained by the internalization of the receptor once it binds with SP, which appears to be essential for the SP/NK-1R complex to exert its actions (15), as observed in other tumour types (36, 37). In this study, a significantly higher expression of SP was observed in membrane and cytoplasm (71% and 81%, respectively) than in the nucleus of tumour cells (39.4%) which *a priori* appears to confer higher importance on SP actions mediated by its binding with NK-1R. This observation is supported by the direct significant association found between tumours with concomitant expression of SP and NK-1R in cytoplasm and a higher cell proliferation index (Ki-67 expression), which appears to indicate the importance of receptor internalization after its binding with SP, permitting its stimulating action on cell proliferation. A direct and significant association between SP expression and NK-1R expression was also found in different compartments of tumour tissue, which suggests that SP overexpression is complemented by an increase in the number of NK-1R able to receive orders mediated by this neurokinin, as demonstrated in other experimental models (29). Thus, it has been demonstrated that malignant cells in certain types of tumour show an increase in the number of NK-1R in comparison to normal cells (38, 39). The acquisition of a higher number of receptors for SP would therefore increase the of mitogenic signals for tumour cells. These proliferative signals would normally lead to the

activation of apoptosis *via* multiple pathways (40), but tumours neutralize these apoptotic signals by mechanisms that are largely unknown. In this regard, SP actions, enhanced by the increased number of NK-1R, may play a role in the neutralization of apoptotic signals in tumour cells (29). The proliferative pathway mediated by the SP/NK-1R complex, acquires special relevance if it is taken into account that actions of SP can be specifically blocked by NK-1R antagonists, such as L-773060. It has been recently demonstrated that SP promotes tumour growth, and that the antagonist L-773060 exerts antitumor activity against different types of malignant cell lines (glioma, retinoblastoma, neuroblastoma, melanoma, laryngeal carcinoma) (16-21, 41). This antitumor activity is dose-dependent and is specifically related to the capacity of antagonists to block the NK-1R expressed by these cell lines (18, 19). These observations open up novel research lines for the treatment of OSCC by using NK-1R antagonists.

Another interesting study finding is related to the expression of SP and NK-1R in tumour blood vessels (58.1% and 22.5% of cases, respectively). NK-1R expression has been reported in the intratumoural and peritumoural blood vessels of most tumour types investigated to date (22). It should be taken into account that a key mechanism for tumour growth, progression and metastasis is angiogenesis stimulus *via* proangiogenic factors. In this regard, it has been demonstrated that NK-1R stimulation by SP is an alternative mechanism by which tumour cells create new blood vessels (42).

In conclusion, this is the first report on SP and NK-1R expression in OSCC. Their ubiquitous presence strongly suggests a role for the SP/NK-1R complex in tumour development and progression and possibly for NK-1 antagonists, such as L-773060, in the management of patients with oral cancer. Further studies are warranted to test these preliminary observations.

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