

## Substance P and NK-1R expression in oral precancerous epithelium

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**Abstract.** The objectives of this study were to investigate the presence and distribution of substance P and neurokinin 1 receptor in oral premalignant epithelium and their relation with the presence of dysplasia, and to analyze whether the expression of substance P can be considered an early oncogenic event in oral carcinogenesis. Substance P and neurokinin 1 receptor expression was immunohistochemically studied in 83 oral carcinomas and adjacent non-tumor epithelia. The presence and degree of epithelial dysplasia was assessed according to WHO criteria. The nuclear, cytoplasmic, and membrane expression of substance P and the cytoplasmic and membrane expression of neurokinin 1 receptor were assessed in tumor and adjacent non-tumor epithelium. Nuclear and cytoplasmic expression of substance P in non-tumor epithelium was significantly associated with the presence of epithelial dysplasia ( $p < 0.001$ ) and carcinoma *in situ* ( $p = 0.021$ ). Nuclear, cytoplasmic, and membrane expressions of substance P in non-tumor epithelium were significantly ( $p < 0.001$ ) associated with its expression in the corresponding tumor. These findings suggest that substance P plays a role in early oral carcinogenesis by promoting the proliferation and growth of premalignant fields.

### Introduction

The development of oral carcinoma is a multistep process involving multiple oncogenic alterations (1). Some of these

are considered early oncogenic events, including changes in chromosomal regions 3p, 9p, and 17p (2,3). Although tumor suppressor genes and putative oncogenes in these regions have yet to be identified (4), these chromosomal areas are known to be carriers of important cell-cycle and apoptosis-regulating genes. Thus, CDKN2A locus, which encodes for p16 protein, is located in chromosome 9p21, and p53 gene is in chromosome 17p13 (5). These early oncogenic events have been demonstrated in precancerous fields (4) and endow cells with an increased proliferation rate that affords them a growth advantage over epithelial cells of the adjacent healthy oral mucosa. This phenomenon considerably increases the number of target cells in which new oncogenic events can take place, which would ultimately lead to the development of one or more invasive carcinomas (4).

There has been increasing interest over the past few years in the role of substance P (SP) in the development and progression of certain types of tumor (6). SP, an undecapeptide of the tachykinin peptide family, regulates multiple biological functions by binding to the NK-1 receptor (NK-1R), including cardiovascular and respiratory mechanisms, sensory perception, and response to stress, among others (7-10). However, one of its most important functions is related to its action as a potent mitogen on some precancerous epithelial lesions and some human cancer cell lines, e.g., glioma, retinoblastoma, neuroblastoma, melanoma, and laryngeal carcinoma as previously reported by our group (6,11-17). These observations suggest that SP may play a role in cancer development by acting as a promoter of cell growth. No information has been published to date on SP expression in oral cancers or in the precancerous epithelia that frequently accompany them. Given that an important characteristic of these epithelia is their increased proliferation rate, the present study was designed to investigate the presence and distribution of SP and NK-1R in oral premalignant epithelium and their relationship with the presence of dysplasia, and to determine whether SP expression can be considered an early oncogenic event in oral carcinogenesis.

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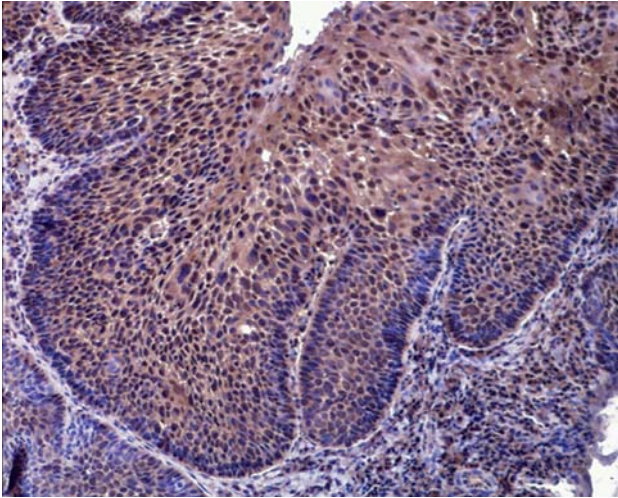


Figure 1. Cytoplasmic and nuclear expression of SP in non-tumor epithelium adjacent to an invasive carcinoma with severe dysplasia/carcinoma *in situ* (immunohistochemical technique; x20).

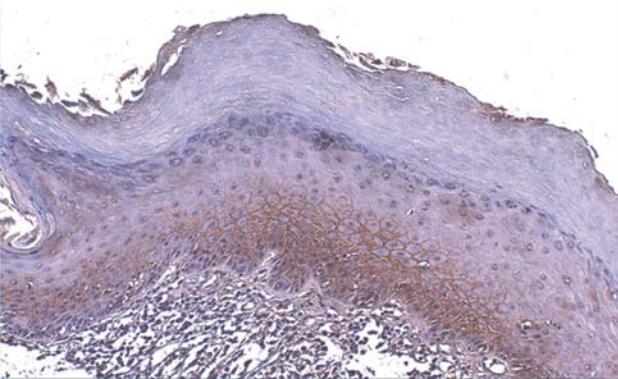


Figure 2. Cytoplasmic and membrane expression of NK-1R in non-tumor epithelium that is adjacent to an invasive carcinoma and shows squamous hyperplasia (immunohistochemical technique; x20).

## Patients and methods

**Patients.** We studied 83 oral squamous cell carcinomas (OSCC) that had non-tumor epithelium associated with the invasive tumor. They derived from 67 patients aged between 27 and 91 years ( $60.0 \pm 11.6$ ) under treatment at the Jaen Hospital Complex (Spain); 51 patients (76.1%) were male, and 38 (73%) were smokers or ex-smokers. The hospital medical records of the patients were reviewed, and data were gathered on the clinicopathological characteristics of lesions. Hematoxylin and eosin staining was used to assess the histological grade of non-tumor epithelium adjacent to the OSCC according to WHO classifications (18): squamous hyperplasia, mild dysplasia, moderate dysplasia, severe dysplasia, or carcinoma *in situ*. WHO assessment criteria (18) were used for the diagnosis of epithelial dysplasia. Clinicopathological variables were considered missing when they were not included in the clinical records or, in the histological grade assessment of non-tumor epithelium, when there was inadequate amount of non-tumor epithelium for a reliable assessment of dysplasia.

**Immunohistochemistry.** For the immunohistochemical staining, 4- $\mu$ m sections were cut from paraffin blocks of the tissue samples. 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 color was developed using diaminobenzidine as chromogen. Slides were extensively washed with TBS after each step. Finally, they were counterstained 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 this protein in peripheral nerves was considered as positive internal control. Anti-SP (Sigma, St. Louis, MO), and anti-NK-1R (Sigma-Aldrich, Madrid, Spain) primary antibodies were used, evaluating SP expression in nucleus, cytoplasm, and membrane (Fig. 1) and NK-1R expression in cytoplasm and membrane (Fig. 2). Markers were considered positive when the color brown appeared in some of these localizations. Expression of these markers in premalignant epithelium was assessed in four randomized high-power fields (x40), dividing the epithelium thickness into thirds. In each of the fields analyzed and for each epithelial compartment considered, the total cell number and the number of positive cells were counted, obtaining a mean expression percentage for each case in each epithelial compartment. The markers were also assessed in the tumor tissue accompanying the precancerous epithelium. Counts were performed in four randomized high-power fields (x40) to obtain the mean expression percentage for each tumor.

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 (++++). The histological and immunohistochemical analyses were always performed by the same examiner (MAGM), who was blinded to the clinical stage, treatment, and course of the disease. Some cases of adjacent epithelium or tumor were considered non-assessable when the immunohistochemical technique did not yield reliable results despite repetition.

**Statistical analysis.** Statistical methods are reported in Table footnotes. To calculate means and standard deviations of SP and NK-1R, we considered the mean value of each interval: negative (0%), mean of 0%; + (1%-25%), 13%; ++ (26%-50%), 38%; +++ (51%-75%), 63%; and ++++ (76%-100%), mean of 88%. SPSS-Windows v.15.0 (SPSS Inc., Chicago, IL) was used for the descriptive analysis. For the calculation of p-values, we applied SUDAAN v.7.0 (Research Triangle Institute, RTP, NC), using WR (with-replacement) design to account for clustering (multiple oral cancers in patients).

Variable	n (%)
<b>Localization</b>	
Tongue	45 (54.2)
Mouth floor	13 (15.7)
Floor + tongue	10 (12.0)
Trigone	4 (4.8)
Other <sup>b</sup>	11 (3.3)
<b>Clinical presentation</b>	
Ulcer	33 (46.5)
Tumor	22 (31.0)
Leukoplakia + tumor	8 (11.3)
Other <sup>c</sup>	8 (11.3)
Missing	12
<b>Size</b>	
T1	25 (32.5)
T2	29 (37.7)
T3	10 (13.0)
T4	13 (16.9)
Missing	6
<b>Adenopathies</b>	
N0	49 (63.6)
N1	19 (24.7)
N2a	5 (6.5)
N2b	2 (2.6)
N2c	1 (1.3)
N3	0 (0.0)
NX	1 (1.3)
Missing	6
<b>Metastasis</b>	
M0	61 (79.2)
M1	1 (1.3)
MX	15 (19.5)
Missing	6
<b>Stage</b>	
I	20 (26.0)
II	19 (24.7)
III	19 (24.7)
IVa	18 (23.4)
IVb	0 (0.0)
IVc	1 (1.3)
Missing	6
<b>Degree of differentiation</b>	
BD	31 (41.9)
MD	26 (35.1)
PD	17 (23.0)
Missing	9

<sup>a</sup>Corresponding to 67 patients. <sup>b</sup>Oral mucosa + trigone (n=1), buccal mucosa (n=3), soft palate + oral mucosa (n=2), lower lip (n=2), and gingiva (n=3). <sup>c</sup>Lichen planus + ulcer (n=2), Erythroleukoplakia (n=3) and Leukoplakia + ulcer (n=3).

## Results

Table I lists the clinicopathological data of the tumors. The most frequent localization was the tongue (45 cases; 54.2%), and the most frequent clinical presentation was ulceration of malignant appearance (33 cases; 46.5%). In this series, 25 cases (32.5%) were T1, 29 cases (37.7%) T2, 49 cases (63.6%) N0, and 61 cases (79.2%) M0. Table II includes data on the multiple tumors in 10 of the patients (15%). Regarding the histological grade of non-tumor epithelium adjacent to the OSCC, 20 epithelia (28.2%) had squamous hyperplasia, 11 epithelia (15.5%) mild epithelial dysplasia, 24 (33.8%) moderate dysplasia, and 16 (22.5%) severe dysplasia/carcinoma *in situ*. In the remaining 12 cases, the examiner did not report the presence or severity of epithelial dysplasia. Table III shows the percentage expression of SP and NK-1R in non-tumor epithelia adjacent to OSCC and Table IV the percentage expression of SP and NK-1R in the tumors. Table V shows the expression of SP and NK-1R in adjacent non-tumor epithelia as a function of the presence of severe epithelial dysplasia/carcinoma *in situ*. A significant association was found between SP expression in nucleus/cytoplasm at all epithelial levels studied and the presence of severe epithelial dysplasia carcinoma *in situ*. Table VI shows the correlation between SP expression in adjacent non-tumor epithelium and in the invasive tumor. A significant direct correlation was found between tumor and epithelial expression of SP, regardless of the presence or not of dysplasia.

## Discussion

This study revealed an elevated SP expression and a less marked expression of NK-1R in the membrane, cytoplasm and nucleus of epithelial cells in non-tumor epithelia adjacent to invasive carcinomas, providing the first demonstration of SP and NK-1R expression in oral precancerous epithelium.

SP can activate 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 (19). *In vitro* studies have revealed the importance of MAPK activation in the proliferative activity of keratinocytes in squamous epithelium. Thus, epithelial stem cells in suspension acquired characteristics of transient amplifying cells, which developed terminal differentiation after two or three proliferative cycles (20). However, some situations involving activation of the MAPK cascade (e.g., interaction of keratinocytes in suspension with  $\beta$ 1 integrin or introduction of active MAPK mutant into culture) promote persistence of a hyperproliferative state of stem cells in which they do not develop terminal differentiation (21), and this state is characteristic of precancerous epithelium (2). Moreover, SP can also induce cell proliferation via transactivation of the epidermal growth factor receptor (EGFR) (6). Hence, our results suggest that SP may play an oncogenic role by stimulating the cell proliferation of oral precancerous epithelium. The less marked expression of NK-1R suggests that SP proliferation pathways in oral precancerous epithelium may, at least in part, differ from those mediated by receptor NK-1. Therefore, the nuclear expression of SP may also

Table II. Clinicopathological characteristics of patients with multiple tumors (n=10 patients and 27 tumors).

Patient	Age <sup>a</sup>	Gender	Tumor	Localization	T	N	M	Grade
1	54	M	1	R Mobile tongue	T1	N1	Mx	WD
			2	R Base of tongue	T1	N1	Mx	WD
2	45	M	1	R Tongue + Floor	T4	N1	M0	MD
			2	L Tongue + Floor	T4	N1	M0	MD
3	61	M	1	R Mouth floor	T4	N0	M0	WD
			2	L Mouth floor	T4	N0	M0	MD
4	49	M	1	L Base of tongue	T1	N1	Mx	MD
			2	L Mobile tongue	T1	N1	Mx	MD
5	62	F	1	L Mobile tongue	T2	N0	M0	MD
			2	Anterior mouth floor	T2	N0	M0	MD
6	70	F	1	L Base of tongue	T2	N0	M0	PD
			2	L Mobile tongue	T2	N0	M0	MD
			3	R Mobile tongue	T2	N0	M0	MD
7	68	M	1	L Mobile tongue	T2	N1	M0	MD
			2	L Base of tongue	T2	N1	M0	MD
			3	Anterior mouth floor	T2	N1	M0	MD
8	52	F	1	L Mobile tongue	T4	N0	M0	WD
			2	R Base of tongue	T4	N0	M0	WD
			3	R Oral mucosa	T3	N0	M0	WD
9	49	M	1	L Anterior mouth floor	T1	N0	M0	MD
			2	R Anterior mouth floor	T1	N0	M0	MD
			3	R Base of tongue	T1	N0	M0	WD
10	63	F	1	Anterior lower gingiva	T3	N0	M0	MD
			2	R Lower gingiva	T2	N0	M0	WD
			3	Lower lip	T2	N0	M0	WD
			4	R Trigone	T2	N0	M0	WD
			5	L Upper gingiva	T1	N0	M0	WD

<sup>a</sup>Mean ± sd = 57.3±8.6 years. R, right; L, left; WD, well differentiated; MD, moderately differentiated; PD, poorly differentiated.

indicate the presence of nuclear receptors for this protein other than NK-1R, as reported for other proteins (e.g., melatonin, that has nuclear and membrane receptors) (22), which may be responsible for the proliferative stimulus. Our findings also suggest that SP may contribute to the growth of precancerous fields in the same way as early oncogenic alterations of these fields, affecting chromosomal loci (3p, 9p, 17p) that host cell cycle-regulating genes (2). Loss of the corresponding functions allows field cells to acquire proliferative advantages and progressively replace normal oral mucosa.

Unlike the molecular identification of precancerous fields, it would be feasible to routinely test for SP over-expression as a potential indicator of precancerous fields, although prospective studies are required to test this hypothesis. The present results also demonstrate a significant association between SP expression and the presence of severe epithelial dysplasia/carcinoma *in situ*, which indicates that

SP-mediated cell mechanisms operate at stages close to malignant transformation. Furthermore, there was a significant and direct correlation between the expression of SP in adjacent non-tumor epithelium and the expression of SP in the OSCC, suggesting that cell clones with an early proliferative stimulation maintain this SP-mediated advantage even after acquiring the capacity to invade. In this context, a previous report by our group on SP and oral lichen planus (17) proposed an SP diffusion pathway of lymphocyte origin via the corium towards membrane, cytoplasm, and nucleus of epithelial cells, where it exerts its proliferation-promoting action. Therefore, the immunological surveillance system developed by lymphocytes adjacent to a precancerous and cancerous epithelium may represent a source of SP that is to some extent responsible for tumor progression and growth. This would be another example of how the inflammatory infiltrate itself may constitute a risk factor for tumor development (23).



SPANDIDOS PUBLICATIONS Percentage expression of SP and NK-1R in non-tumor epithelia adjacent to oral tumors (n=83).<sup>a</sup>

	Expression (%)					mean ± sd <sup>b</sup>
	Negative (0%)	+(1-25%)	++(26-50%)	+++ (51-75%)	++++ (76-100%)	
Expression of SP (n=69)						
Nucleus						
1/3 lower	73.9	1.4	10.1	13.0	1.4	13.5±24.6
1/3 medium	69.6	2.9	11.6	14.5	1.4	15.2±25.3
1/3 upper	92.8	1.4	2.9	2.9	0.0	3.1±12.3
Maximum <sup>c</sup>	68.1	2.9	13.0	14.5	1.4	15.7±25.3
Cytoplasm						
1/3 lower	52.2	7.2	7.2	29.0	4.3	25.8±30.9
1/3 medium	55.1	5.8	4.3	30.4	4.3	25.4±31.4
1/3 upper	94.2	0.0	1.4	4.3	0.0	3.3±13.6
Maximum	50.7	7.2	5.8	31.9	4.3	27.1±31.3
Membrane						
1/3 lower	49.3	7.2	7.2	36.2	0.0	26.5±29.4
1/3 medium	34.8	15.9	11.6	37.7	0.0	30.2±28.1
1/3 upper	94.2	0.0	0.0	5.8	0.0	3.6±14.8
Maximum	33.3	15.9	11.6	39.1	0.0	31.1±28.1
Expression of NK-1R (n=68)						
Cytoplasm						
1/3 lower	85.3	2.9	7.4	4.4	0.0	6.0±15.9
1/3 medium	77.9	7.4	8.8	5.9	0.0	8.0±17.7
1/3 upper	98.5	1.5	0.0	0.0	0.0	0.2±1.6
Maximum	77.9	7.4	8.8	5.9	0.0	8.0±17.7
Membrane						
1/3 lower	89.7	4.4	4.4	1.5	0.0	3.2±11.0
1/3 medium	86.8	2.9	8.8	1.5	0.0	4.7±13.1
1/3 upper	100.0	0.0	0.0	0.0	0.0	0.0±0.0
Maximum	85.3	4.4	8.8	1.5	0.0	4.8±13.1

<sup>a</sup>Corresponding to 67 patients; <sup>b</sup>mean ± standard deviation of percentage of expressing cells. <sup>c</sup>The highest score out of the three thirds was assigned to each case.

Table IV. Percentage expression of substance P and NK-1R in oral tumors (n=83).<sup>a</sup>

	n	Expression (%)					mean ± sd <sup>b</sup>
		Negative (0%)	+(1-25%)	++(26-50%)	+++ (51-75%)	++++ (76-100%)	
Expression of SP							
Nucleus	65	61.5	15.4	7.7	9.2	6.2	16.2±27.0
Cytoplasm	78	19.2	16.7	14.1	25.6	24.4	45.1±33.4
Membrane	63	28.6	15.9	22.2	23.8	9.5	33.9±29.9
Expression of NK-1R							
Cytoplasm	79	49.4	19.0	17.7	11.4	2.5	18.6±24.4
Membrane	79	86.1	6.3	5.1	1.3	1.3	4.7±14.6

<sup>a</sup>Corresponding to 67 patients; <sup>b</sup>mean ± standard deviation of percentage of expressing cells.

Table V. Comparison of expression of SP and NK-1R in epithelium adjacent to oral tumors according to the presence of severe dysplasia/carcinoma *in situ* (n=83).<sup>a</sup>

	Severe dysplasia/carcinoma <i>in situ</i> (mean $\pm$ sd) <sup>b</sup>		p-value <sup>c</sup>
	No	Yes	
Expression of SP	52 <sup>d</sup>	16 <sup>d</sup>	
Nucleus			
1/3 lower	6.1 $\pm$ 16.9	38.6 $\pm$ 30.0	<0.001
1/3 medium	8.3 $\pm$ 19.0	38.6 $\pm$ 30.0	<0.001
1/3 upper	0.3 $\pm$ 1.8	12.6 $\pm$ 23.5	0.034
Maximum <sup>e</sup>	9.0 $\pm$ 19.4	38.6 $\pm$ 30.0	<0.001
Cytoplasm			
1/3 lower	21.4 $\pm$ 29.6	41.8 $\pm$ 31.1	0.023
1/3 medium	20.4 $\pm$ 29.7	43.3 $\pm$ 31.5	0.011
1/3 upper	0.0 $\pm$ 0.0	14.2 $\pm$ 26.0	0.028
Maximum	22.6 $\pm$ 30.0	43.3 $\pm$ 31.5	0.021
Membrane			
1/3 lower	25.3 $\pm$ 29.2	32.3 $\pm$ 30.5	0.414
1/3 medium	29.9 $\pm$ 27.7	33.1 $\pm$ 29.8	0.703
1/3 upper	2.4 $\pm$ 12.2	7.9 $\pm$ 21.5	0.326
Maximum	31.1 $\pm$ 27.8	33.1 $\pm$ 29.8	0.812
Expression of NK-1R	45	11	
Cytoplasm			
1/3 lower	6.2 $\pm$ 14.9	5.7 $\pm$ 19.0	0.937
1/3 medium	8.2 $\pm$ 17.1	10.4 $\pm$ 21.0	0.739
1/3 upper	0.3 $\pm$ 1.9	0.0 $\pm$ 0.0	0.323
Maximum	8.2 $\pm$ 17.1	10.4 $\pm$ 21.0	0.739
Membrane			
1/3 lower	3.9 $\pm$ 13.1	1.2 $\pm$ 3.9	0.248
1/3 medium	5.6 $\pm$ 14.9	4.6 $\pm$ 11.7	0.811
1/3 upper	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	-
Maximum	5.6 $\pm$ 14.9	4.6 $\pm$ 11.7	0.811

<sup>a</sup>Corresponding to 67 patients; <sup>b</sup>mean  $\pm$  standard deviation of percentage of expressing cells. <sup>c</sup>SUDAAN DESCRIPT procedure, to correct for clustering (multiple oral cancers within patients). <sup>d</sup>The difference with the total of 83 tumors corresponds to missing cases for the analyzed variables. <sup>e</sup>The highest score out of the three thirds was assigned to each case.

Table VI. Correlation of the expression of SP between adjacent epithelium and tumor according to the presence of severe dysplasia/carcinoma *in situ* (n=83).<sup>b</sup>

	Severe dysplasia/carcinoma <i>in situ</i>								
	All			No			Yes		
	n <sup>c</sup>	r <sup>d</sup>	p-value <sup>e</sup>	n	r	p-value	n	r	p-value
Nucleus	55	0.71	<0.001	42	0.49	0.002	13	0.71	<0.001
Cytoplasm	64	0.63	<0.001	49	0.56	<0.001	15	0.92	<0.001
Membrane	51	0.61	<0.001	40	0.56	<0.001	11	0.75	<0.001

<sup>a</sup>The highest score out of the three thirds (lower, medium, or upper) was assigned to each case. <sup>b</sup>Corresponding to 67 patients. <sup>c</sup>The difference with the total of 83 tumors corresponds to missing cases for epithelial and/or tumor expression. <sup>d</sup>Linear correlation; <sup>e</sup>calculated with the SUDAAN REGRESS procedure to correct for clustering (multiple oral cancers in patients).



conclusion, the role of SP in the malignant transformation of oral epithelium suggested by our results must be verified in wider studies. Research is also required into the pathogenic pathways by which substance P operates in order to develop novel approaches to prevent the malignant transformation of precancerous epithelium.

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