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Perineural Invasion Assessed by Galanin and Protein Gene Product 9.5 in Oral Squamous Cell Carcinoma

Farah Sabah Rasheed* and Bashar Hamid Abdullah

College of Dentistry, University of Baghdad, Baghdad, Iraq

**Corresponding e-mail: farahsabah80@yahoo.com*

ABSTRACT

Objective: *Perineural invasion in oral squamous cell carcinoma is an ominous process enabling tumor cells spread through and along nerves. This study was performed to highlight the frequency of perineural invasion in oral squamous cell carcinoma using immunohistochemical aid in addition to the evaluation of the role of galanin in this process.* ***Methods:*** *Total 54 paraffin-embedded tissue blocks of radical resections of oral squamous cell carcinoma were evaluated for perineural invasion both histopathologically and immunohistochemically using Protein Gene Product 9.5 with the assessment of the role of galanin in this process.* ***Results:*** *About 22 cases showed perineural invasion in histopathological sections that were elevated to 41 upon staining with Protein Gene Product 9.5. In histopathological sections, the largest nerve diameter showed perineural invasion was significantly correlated with a tumor depth of invasion (p=0.025), however, this correlation was non-significant in Protein Gene Product 9.5 immunostained sections (p=0.203). Perineural invasion status in both hitopathological and Protein Gene Product 9.5 immunostained sections showed no significant association in regard to tumor grade and stage (p=0.848, p=0.520) for histopathological, and (p=0.238, p=0.216) for Protein Gene Product 9.5 immunostained sections, respectively. Galanin expression showed no significant association with perineural invasion status in histopathological sections, however, this association was significant in Protein Gene Product 9.5 sections (p=0.180, p=0.027) respectively.* ***Conclusions:*** *Perineural invasion can dramatically be elevated using immunohistochemical aid, by which it may accentuate the role of galanin as a neuropeptide involved in perineural invasion in oral squamous cell carcinoma.*

Keywords: Oral squamous cell carcinoma, Perineural invasion, Protein Gene Product 9.5, Galanin

INTRODUCTION

Perineural invasion (PNI) or so-called neurotropism, is regarded as a tumor marker reflecting an offensive biological behavior ending with a poor prognosis [1,2]. The exact PNI mechanisms are till yet not well comprehend despite the presence of many studies that had gone through defining its biological features [3]. Recent experiments suggest PNI as an ongoing process comprising mutual harmony between tumor cells and nerves [4,5]. Tumor cells can motivate neurite outgrowth in the direction of the tumor under the influence of neurotrophic factors and axonal guidance molecules [6]. Other studies highlighted the role of neuropeptides in cellular migration and invasion [7].

Since PNI detection can be a sort of challenge to most pathologists considering vigorous and subjective definitions produced in the literature, the enhancement of detection of neural invasion by immunohistochemical stains added a lot for a better description of a neural affinity of tumor cells [8,9]. Protein Gene Product 9.5 (PGP 9.5) is a cytoplasmic protein that was originally identified in normal human brain, being specific for nerves and neuroendocrine system, axons and neuronal cells are highly identified by this antibody, therefore, it was used for better delineation of fine nerves, oral, and dental innervations in addition to higher neural density in neurotropic cancers [10,11].

It was proposed that the greater ability of the tumor to invade deeper into the tissues, the more aggressive is the behavior and poorer the outcome, a tumor depth of invasion (DOI) of 4 mm may predict tumor metastasis into regional lymph nodes in patients with oral squamous cell carcinoma (OSCC) of the tongue [12].

Neuropeptides are molecular messengers that can organize multiple actions in the central and peripheral nervous system [13]. Galanin (GAL) is one of those neuropeptides that upon conjugation with GAL receptors will trigger the

multiple regulatory actions in neuronal cells [14]. Scanlon, et al., proved by using a special experiment that GAL may have a pivotal role in PNI by binding to Galanin receptor 2 (GALR2), that eventually promotes neurite outgrowth in both autocrine and paracrine fashion [4].

This study was conducted to detect the frequency of PNI in histopathological hematoxylin and eosin (H and E) and immunostained sections of OSCC using PGP 9.5, moreover detecting the role of GAL as a neuropeptide was involved in this invasion.

PATIENTS AND METHODS

Total 54 formalin fixed paraffin embedded tissue blocks of OSCC radical resections were retrieved from the archives of the Department of Oral and Maxillofacial Pathology, College of Dentistry, Baghdad University, Iraq. The research was done with the understanding of the ethical principles and was approved by the Institutional Review Board (IRB) of the College of Dentistry, Baghdad University, Iraq. Tumor grading was done according to Broders classification, and staging based on the 8th edition of the American Joint Committee on Cancer (AJCC) staging manual for lip and oral cavity cancer was used [15,16]. In this study, image J was used for measuring tumor depth of invasion (DOI) in addition to the diameter of nerves that showed PNI in H and E and PGP 9.5 immunostained sections. The photos were taken using a special camera and a light microscope.

Histopathological DOI was identified by measuring the depth of a vertical line drawn from the level of the basement membrane of the neighboring intact mucosa to the deepest point of tumor invasion under 4X magnification [16]. For measuring nerve diameter, a photo was taken at 40X for each foci that showed PNI, by which the latter was identified according to Liebig's definition when "tumor cells were seen within any of the 3 layers of the nerve sheath and/or tumor cells were seen in close proximity to the nerve and encircling one-third or more of its circumference" [3].

Other PNI criteria for each case were also assessed including the density of PNI foci which were identified as unifocal or multifocal, with total foci number estimation. The diameter, pattern of invasion, and location of the largest nerve bundle showing PNI was also assessed. The location of the foci showed PNI which was categorized into intratumoral (for those foci located within the tumor), extratumoral (if the foci were located outside the tumor), and peripheral (for those foci located arbitrary at 0-0.2 mm from the tumor edge) [17].

The immunohistochemical procedure was done according to the manufacturer's data sheet provided with Abcam, immunohistochemistry detection kit, ab80436-EXPOSE Mouse and Rabbit Specific HRP/DAB, USA [18]. Primary antibodies were added for each slide as follows: Anti-PGP 9.5 (ab53057, rabbit polyclonal, Abcam, USA) and Anti-GAL (ab216399, mouse polyclonal Abcam, USA) both at a dilution 1:300. DAB solution was prepared and added to expose the reaction.

Only the number of cells that were positive for GAL showing cytoplasmic/secreted immunoreactivity was quantified by counting at least 1000 cells in 5 representative fields at 40X objective in each case, and then the average of the fields was counted. Cases were assigned to one of the following scores: negative=0% positive cells, (+)=1-25% positive cells, (++)=26-50% positive cells, (+++)=51-75% positive cells, or (++++)= more than 75% positive cells [19].

SPSS statistical package for social sciences (version 20.0 for Windows, SPSS, Chicago, IL, USA) was employed for this study. Students t-test was used for measuring differences of quantitative data between groups, for the relation between variables Pearson's correlation was used; while qualitative relations were evaluated using the Chi-square test. A p-value<0.05 was considered statistically significant.

RESULTS

The average tumor DOI in mms was 6.4 ± 3.2 (ranging from 1.1-14.5 mm). Total 22 cases (41%) of the study sample revealed the presence of PNI in H and E sections, the largest nerve diameter showing PNI was between (0.026-0.379) mm with a mean of 0.135 mm, 15 of them were of intratumoral location, and most of them were of the partial encirclement pattern (19 cases) (Figure 1).

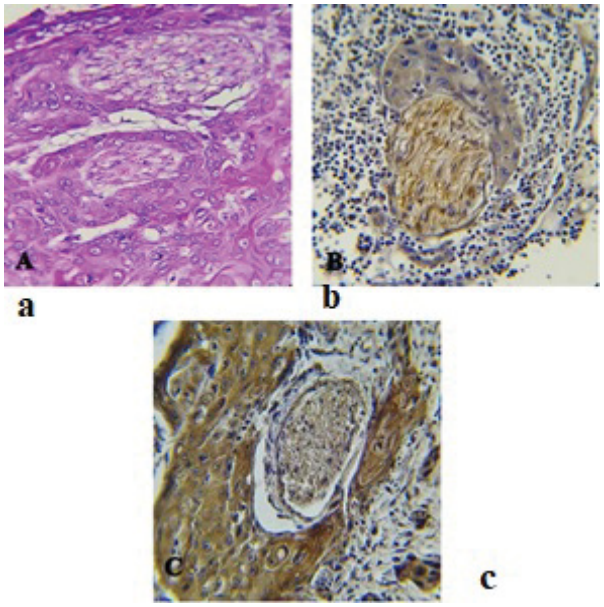

Figure 1 Perineural invasion (PNI) in OSCC (original magnification x400) in, A: H and E sections involving 2 intratumoral nerves with complete and partial encirclement of tumor cells; B: PGP9.5 immunostained sections involving inrtratumoral nerve with partial encirclement of tumor cells; C: Galanin immunostained section with high immunoreactivity of tumor cells

The total number of foci was ranging from 1-10 for each case, where 15 of the cases were of multifocal involvement and the rest were unifocal (Table 1).

Table 1 Association of site, pattern, and type of foci of largest nerve showed PNI between H and E and PGP 9.5 sections

Variables		H and E n=22 Count (%)	PGP 9.5 n=41 Count (%)	Test	p-value
Site of largest nerve	Intratumoral	15 (42.9%)	20 (57.1%)	Chi-square	0.145
	Extratumoral	7 (30.4%)	16 (69.6%)		
	Peripheral	0 (0.0%)	5 (100.0%)		
Pattern of largest nerve	Complete encirclement	3 (33.3%)	6 (66.7%)	Chi-square	0.307
	Partial encirclement	19 (38.0%)	31 (61.0%)		
	Intraneural invasion	0 (0.0%)	4 (100.0%)		
Type of foci	Unifocal	7 (58.3%)	5 (41.7%)	Chi-square	0.091
	Multifocal	15 (29.4%)	36 (70.6%)		
p>0.05 was not significant; PNI: Perineural invasion					

There was a significant positive correlation between H and E largest nerve diameter showed PNI and tumor DOI (r=0.477, p=0.025) (Figure 2).

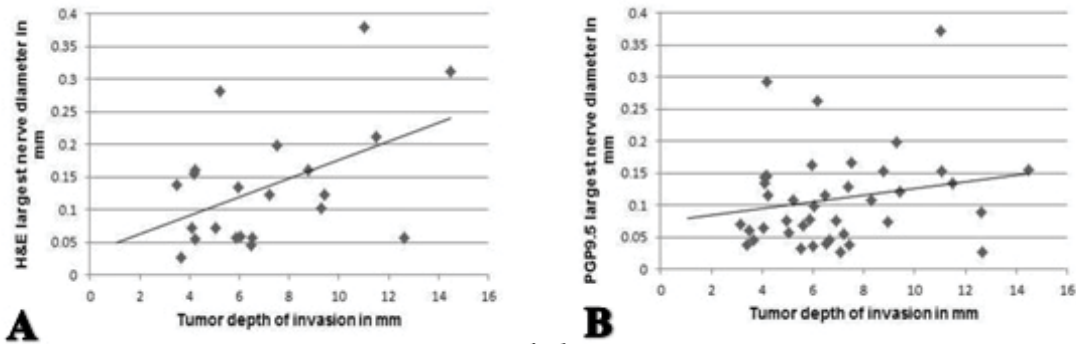

Figure 2 Pearson correlation between the largest nerve diameter showed perineural invasion and tumor depth of invasion in (A) H and E; (B) PGP 9.5 sections

PNI status (present, absent) was non significantly related in regard to tumor grade, stage, and DOI (p=0.848, p=0.520, p=0.181) respectively (Table 2).

Table 2 PNI status in H and E and PGP 9.5 sections in relation to tumor grade, stage, DOI and GAL score

Variables		H and E PNI status		Test+p-value	PGP 9.5 PNI status		Test+p-value
		Present n=22 Count (%)	Absent n=32 Count (%)		Present n=41 Count (%)	Absent n=13 Count (%)	
Grade	Well	14 (63.6%)	18 (56.3%)	Chi-square p=0.848	22 (53.7%)	10 (76.9%)	Chi-square p=0.238
	Moderate	6 (27.3%)	11 (34.45%)		14 (34.1%)	3 (23.1%)	
	Poor	2 (9.1%)	3 (9.4%)		5 (12.2%)	0 (0.0%)	
Stage	1	2 (9.1%)	1 (3.1%)	Chi-square p=0.520	2 (4.9%)	1 (7.7%)	Chi-square p=0.216
	2	4 (18.2%)	7 (21.9%)		11 (26.8%)	0 (0.0%)	
	3	6 (27.3%)	5 (15.6%)		8 (19.5%)	3 (23.1%)	
	4	10 (45.5%)	19 (59.4%)		20 (48.8%)	9 (69.2%)	
DOI	N	22	32	Student t-test p=0.181	41	13	Student t-test p=0.089
	Mean	7.122	5.928		6.833	5.095	
	SD	3.118	3.22		2.836	4.016	
	SE	0.664	0.57		0.442	1.113	
GAL score	+	2 (9.1%)	0 (0.0%)	Chi-square p=0.180	2 (4.9%)	0 (0.0%)	Chi-square p=0.027*
	++	2 (9.1%)	2 (6.3%)		4 (9.8%)	0 (0.0%)	
	+++	5 (22.7%)	14 (43.8%)		10 (24.4%)	9 (69.2%)	
	++++	13 (59.1%)	16 (50.0%)		25 (61.0%)	4 (30.8%)	

*Significant relation; p>0.05 was not significant; DOI: Depth of invasion; GAL: Galanin; PNI: Perineural invasion; SD: Standard deviation; SE: Standard error

In PGP 9.5 sections, the number of cases showing PNI was 41 cases (76%). The largest nerve diameter that showed PNI was between (0.026-0.372) mm with an average of 0.110 mm, about half of them were of intratumoral location, with two-thirds of them of partial encirclement pattern (31 cases), followed by complete encirclement, and intraneural invasion pattern (Figure 1). The total number of foci was ranging from 1-50 for each case in which 36 of them were of multifocal involvement, while only 5 cases were unifocal, (Table 1). No significant correlation was seen between PGP 9.5 largest nerve diameter and tumor DOI (r=0.203, p=0.203) (Figure 2). PGP 9.5 PNI status in term of (present, absent) was non significantly related in regard to tumor grade, stage and DOI (p=0.238, p=0.216, p=0.089) respectively (Table 2).

There was no significant association found concerning the site, a pattern of the largest nerve showed PNI in addition to the type of foci between H and E and PGP 9.5 sections (p=0.145, p=0.307, p=0.091) respectively (Table 1). While a significant association was found between H and E and PGP 9.5 PNI status (p=0.005) (Table 3).

Table 3 Association of PNI status between H and E and PGP 9.5 sections

Variables		PGP 9.5 PNI status		Total	Test+ p-value
		Present	Absent		
H and E PNI status	Present	21	1	22	Chi-square
	Absent	20	12	32	p=0.005*
	Total	41	13	54	

*Significant relation; PNI: Perineural invasion

A high cytoplasmic immunoreactivity for GAL was found in tumor cells in almost all of the cases (48 cases were of score 3 and 4) (Figure 1). No significant association was found between GAL expression and PNI status in H and E sections (p=0.180), while it was significant with PGP 9.5 PNI status (p=0.027) (Table 2).

DISCUSSION

The frequency of PNI in the present study was about one-third of the cases upon histological evaluation of H and E sections that were dramatically doubled upon immunohistochemical evaluation using PGP 9.5. This confirms previous studies that showed PNI detection to be greatly enhanced upon the use of immunohistochemical stains

[2,9]. The incidence of PNI in head and neck tumors may reach up to 80% [5], however, a previous study reported 6-30% incidence in head and neck area [1]. The lack of a precise definition of PNI in literature and the subjectivity while examining routine histological slides, together with the use of special neural stains for nerve detection, are contributing factors leading to variations in incidence detection of PNI in head and neck area [20].

By considering the pattern of PNI, previous studies had mentioned that nerve invasion pattern may be variable in different histological sections and actually have no prognostic value [21]. In this study, PNI detection within the bulk of the tumor was more than that detected extratumoral which was in accordance with the study of Rahima, et al., [22]. The 8th edition of AJCC staging manual for lip and oral cavity cancer considered both intra and extratumoral PNI foci as prognostic factors and further recommended the documentation of those extratumoral multifocal foci in pathology reports [16].

The current study revealed that the frequency of PNI detection was almost doubled via the use of PGP 9.5 for delineating nerve fibers especially those small foci that were non-identifiable in routine H and E sections making multifocal and endoneural PNI foci more evident, by which the latter was mentioned to denote a more offensive tumor course [23]. The multifocality may indicate a more neurotropic tumor behavior, advanced tumor stage or the presence of the tumor in the more innervated area (tongue and lip) [20].

The significant correlation between the diameter of the largest nerve showing PNI in H and E sections and tumor DOI can be explained by the more number of cancer cells being in contact with larger nerve calibers as the tumor approaches, which further may enhance more cytokines being released in response to this invasion, however, the ability of the immunostained sections to reveal even those small nerves showing PNI may contribute to the failure of having this significant correlation in PGP 9.5 sections.

In the present study, almost two-thirds of the cases were of high score value (score 3 and 4) for GAL. The presence of a significant association of PNI status with GAL expression in PGP 9.5 sections but H and E sections may indicate that even with the detection of PNI in smaller nerves may figure the role of GAL in PNI and neurite outgrowth. As mentioned before, GAL is released by nerves as well as tumor cells, and that lead to the concept of tumor-cancer reciprocal interaction may take place [4,24]. GAL release was found to be enhanced in response to nerve injury and inflammation [25]. It was found that upon GAL binding to its GALR2 in head and neck SCC may contribute to neurite outgrowth [4]. In the latter study, and by using *in vitro* and *in vivo* special designed model, the experiment showed that nerves release GAL which is taken up by GALR2 on tumor cells, leading to further activation of prostaglandin E2 by cancer cells mediated by cyclooxygenase 2 (COX2) expression, by which the latter was previously found to be related to PNI in pancreatic cancer [26]. As a feedback mechanism, tumor cells were found to release GAL that enhances neuritogenesis [4].

CONCLUSION

Approaching tumor depth may raise the potential for larger nerves being invaded by tumor cells. The use of special stains for neural detection may contribute to multifocal detection of PNI in OSCC; this may indicate a more aggressive tumor behavior that can be manifested as neurite outgrowth in response to this invasion.

DECLARATIONS

Conflict of Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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