Pre-operative incisional biopsy of oral squamous cell carcinoma: high podoplanin expression is related to perineural invasion and may be a useful predictor of disease progression

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Objective. Immunohistochemical analysis of podoplanin expression as a pre-operative molecular marker for perineural invasion (PNI) may represent an attractive strategy for surgical management of oral squamous cell cancer (OSCC). We evaluated the relationship between podoplanin expression and PNI in pre-operative incisional biopsies of OSCC.

Study Design. After performing pathological staging and histologic and immunohistochemical evaluation of 83 surgical specimens, we performed multivariable logistic regression analysis to examine the relationship between PNI and independent variables. To evaluate the utility of podoplanin immunopositivity for discrimination of PNI status pre-operatively, we calculated the sensitivity, specificity, positive predictive value, and negative predictive value. We performed receiver operating characteristic curve analysis to evaluate the diagnostic accuracy of podoplanin immunopositivity for predicting PNI alone and in combination with age, T stage, N stage, and index site.

Results. We observed podoplanin expression in 42 (50.6%) of all the 83 pre-operative incisional biopsies and 29 of the pre-operative biopsies of the 31 (93.5%) postoperative specimens with PNI. The rate of podoplanin expression was significantly higher in patients with pT3 to pT4 stage and pN+ stage disease. Podoplanin positivity in the pre-operative biopsy showed high sensitivity in predicting PNI in the surgical specimen.

Conclusion. Podoplanin expression appears to be an independent pre-operative variable significantly related to PNI and a possibly valuable prognostic marker for therapeutical planning and surgical treatment of OSCC. (Oral Surg Oral Med Oral Pathol Oral Radiol 2024;137:53−60)

Oral squamous cell carcinoma (OSCC) is an aggressive tumor characterized by a high risk of recurrence and a low survival rate, with 30% of patients treated by surgical resection experiencing a second neoplastic event.[1] Oral squamous cell carcinoma prognosis primarily depends on the size of surgical margins and extent of depth of invasion, the pattern of invasion, perineural spread, and nodal metastasis.[2] Recent evidence has shown that OSCC is a neurotropic tumor with neoplastic cells that can spread through the nerve fibers into surrounding tissues and thus escape local disease control.[3] Perineural invasion (PNI) is an emerging factor related to unfavorable outcomes.[3,4] Many recent studies have shown that PNI is related to advanced tumor (T) and node (N) tumor stage, extranodal extension, poor tumor differentiation, lymphovascular invasion, and increased depth of invasion,[5] making it one of the most important negative prognostic factors in OSCC.[6-10]

Unfortunately, PNI expression and other histopathologic parameters related to OSCC prognosis can only be determined from analysis of postoperative samples, making OSCC diagnosed by PNI expression unamenable to therapeutic planning and pre-operative patient

Statement of Clinical Relevance

High podoplanin immunohistochemical expression in pre-operative biopsies of patients with oral squamous cell cancer is associated with the presence of perineural invasion. Podoplanin expression may be a valuable pre-operative marker for therapeutical planning and surgical treatment in these patients.

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management. Pre-operative analysis of PNI in incisional biopsies is of limited prognostic utility because the amount of neoplastic tissue that can be taken is small and may not represent the entire tumor.[11,12] Therefore, identification of a marker strongly associated with prognosis that can be measured even in pre-operative incisional biopsies is highly desired.

Podoplanin appears to mediate single-cell invasion by inducing epithelial−mesenchymal transition through the downregulation of E-cadherin, which is responsible for cell−cell adhesion in epithelial tissue.[13] The increase in cell motility driven by podoplanin could explain why several studies have found high levels of podoplanin in aggressive OSCC. Specifically, podoplanin expression has been observed to have a significant association with cervical lymphatic dissemination.[13] Hypothetically, increased cell motility related to podoplanin expression may impact lymph node dissemination and nerve invasion of malignant cells in OSCC. Indeed, the ability to invade tissues as well as perform epithelial−mesenchymal transition are both crucial abilities that cancer cells must acquire during the PNI process.[14]

Having been identified in several types of tumors in the central nervous system, podoplanin overexpression is a potential indicator of malignant progression and poor prognosis in gliomas and glioblastomas.[15] Measurement of podoplanin expression as a pre-operative molecular marker for PNI may represent an attractive strategy for surgical management of OSCC patients. However, few studies have investigated the association between PNI and podoplanin overexpression in OSCC and those that have examined it in postoperative specimens. Among them, the Almeida et al. retrospective study showed that patients with strong podoplanin expression in postoperative OSCC samples tended to have a higher frequency of PNI. However, the relationship between podoplanin expression and PNI was not statistically significant.[16] In contrast, Faustino et al. observed a significant association between peritumoral vessel density and PNI in their investigation of immunohistochemically determined podoplanin expression as a selective marker of intratumoral and peritumoral lymphatic vessel density in postoperative OSCC samples.[17]

Based on these findings, podoplanin expression, as measured by immunohistochemical (IHC) analysis, a simple and inexpensive method, has been proposed as a prognostic marker in oral oncology. To our knowledge, no previous investigations have examined the relationship between podoplanin expression in pre-operative biopsy specimens and PNI in OSCC. To fill this research gap, we investigated whether podoplanin expression in pre-operative OSCC biopsies could be an effective indicator for identifying PNI in patients with OSCC.

MATERIALS AND METHODS

Ethics statement

We designed this prospective study in accordance with the principles of the Declaration of Helsinki. We obtained approval to conduct it from the Institutional Ethics Committee of Bologna, Italy (Comitato Etico Area Vasta Emilia Centro, CE AVEC study number: 237/2016/O/Tess) and obtained informed consent for participation from all patients.

Population study

Our study population consisted of 83 consecutive patients with histologically confirmed OSCC who had been referred to the Maxillofacial Surgery Unit, University of Bologna, Italy, between January 2017 and October 2019. The inclusion criteria were (1) diagnosis of histologically confirmed OSCC based on pre-operative incisional biopsy, (2) availability of the results of IHC analysis of podoplanin expression before surgical resection, (3) availability of the results of radiologic locoregional staging (TNM staging) from head and neck computed tomography and/or magnetic resonance imaging before surgical OSCC resection, and (4) age over 18 years at the time of presentation. We followed up with the patients from the date of diagnosis to recurrence or metastasis or the last follow-up visit and collected clinical data regarding age, sex, smoking status, and location of the primary tumor.

Treatment modality

After performing pre-operative staging according to the eighth edition of the American Joint Committee on Cancer criteria,[2] conducting diagnostic work-ups, and engaging in a multidisciplinary team discussion, we surgically treated all patients in our sample. Surgical treatment consisted of composite resection, including excision of the primary oral cancer associated with ipsilateral or bilateral neck dissection, according to disease stage. We performed selective supraomohyoid neck dissection simultaneously with tumor resection in all cases diagnosed as cN0 and performed radical or modified radical neck dissection for patients diagnosed as cN+. We performed reconstruction using pedicled or re-vascularized flaps according to the size of the defect and administered adjuvant radiation or chemoradiation therapy according to National Comprehensive Cancer Network criteria.[18]

Histology and *IHC*

We fixed all tissues in 10% buffered formalin and routinely processed them for embedding in paraffin. We performed hematoxylin-eosin staining and immunostaining on 4-μm-thick serial sections from paraffin-embedded, formalin-fixed tissue blocks of pre-operative incisional biopsy specimens. Using an automatic

stainer (Ventana Benchmark Ultra; Ventana Medical Systems, Inc.) at 36°C for 32 minutes, we subjected sections mounted on positively charged glass slides to immunostaining with a monoclonal anti-podoplanin antibody (clone D240, product no. 760-4395 RTU; Cell Marque).

Two pathologists (A.S. and V.P.F.) performed IHC to assess podoplanin status without knowing each case's clinical parameters or outcomes. They resolved discrepancies in IHC results by discussion with a senior pathologist (M.P.F.) while viewing the sections with a multi-headed microscope. In accordance with criteria previously defined for IHC evaluation,[19] they counted a minimum of 400 cells and reported the podoplanin expression as positive when >20% of the neoplastic cells showed immunostaining of the cytoplasmic membrane. They used the endothelial cells of lymphatic vessels as internal controls and the cut-off value of 20% positivity in accordance with a previously published work.[19]

Pathological features

We performed pathological classification and staging of surgical specimens according to the World Health Organization 2022 Classification of Head and Neck Tumors[20] and the eighth edition of the American Joint Committee on Cancer criteria.[2] We performed tumor sampling at the time of diagnosis according to current guidelines.[21] Specifically, we completely embedded pT1 to T2 tumors in paraffin and embedded 60% to 80% of the neoplastic mass of pT3 to T4 tumors in paraffin for histological evaluation, taking care to histologically evaluate the deep portion of the tumor. We determined grade, primary T status, regional lymph N status, PNI, resection margins, and tumor stage. Based on the evaluation of hematoxylin-and-eosin-stained slides, we considered a patient PNI positive if we could observe neoplastic cells within peripheral nerves and/or invasion of the neoplastic cells into the perineural space.[22]

Statistical analysis

We performed all analyses using Stata version 15.1, employing the user-written code for the STPM2 package for Royston−Parmar survival estimation,[23] and considered $P < .05$ an indication of statistical significance. We performed multivariable logistic regression analysis to determine the relationship between PNI in surgical specimens and the following independent variables: podoplanin expression in pre-operative incisional biopsy specimens (positive vs negative), age (<65 vs ≥65 years), smoking status (negative vs positive), T status (T1-T2 vs T3-T4), N status (N0 vs N+), grade (G1 vs G2 vs G3), and surgical margin (clear vs close/involved). To evaluate the utility of podoplanin immunopositivity for discrimination of PNI status preoperatively, we calculated the sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV).

We performed survival analysis to identify predictors of time to locoregional control, defined as local recurrence (LR), second primary tumor, or lymph node metastasis. To evaluate clinical and pathologic variables and podoplanin expression, we used Kaplan−Meier curves and the log-rank test. To confirm the associations of PNI and podoplanin expression with time to LR, we selected the best-fitting multivariable survival model that included PNI and podoplanin expression as major risk factors in a Cox proportional hazards model and among a range of Royston−Parmar models (i.e., the model with the lowest Akaike information criterion and Bayesian information criterion values).[24] We also assessed podoplanin expression as a predictor of time to LR in models that did not include PNI. Finally, we performed receiver operating characteristic curve analysis to evaluate the diagnostic accuracy of podoplanin immunopositivity for predicting PNI alone and in combination with age, T stage, N stage, and site.

RESULTS

Our case series consisted of 83 patients with OSCC (43 males and 40 females) with a mean age of 63.0 ± 10.7 years (range = 41-91 years) and a mean incisional biopsy size of 6 ± 1 mm (range = 4-9 mm). Tumor sites were the tongue and/or floor of the mouth in 31 (37.3%), cheek (right or left) in 15 (18.1%), and gingiva (superior and inferior) in 37 (44.6%) of the 83 patients. Although all the pre-operative incisional biopsies were diagnostic of OSCC, none of the 83 OSCC biopsies showed histologic evidence of PNI.

Based on TNM staging of tumors, we classified 45 (54.2%) cases as pT1-T2 and 38 (45.8%) as pT3-T4; 56 (67.5%) as pN0 and (32.5%) as pN1 or higher (i.e., with single or multiple neck metastases; and 23 (27.7%) with well-differentiated (grade 1 [G1]) lesions, 48 (57.8%) with moderately differentiated (G2) lesions, and 12 (14.5%) with poorly differentiated (G3) lesions. A secondary neoplastic event had occurred in 26 patients (31.3%), who had a median survival of 12.5 months (range = 3-36 months) and among whom 10 (38.5%) died of OSCC during the follow-up period.

Podoplanin *IHC* results

We observed podoplanin expression in 42 (50.6%) of the 83 pre-operative incisional biopsies (Table I). The rate of podoplanin expression was significantly higher in patients with pT3-pT4 stage (66.7%, $P < .001$) and pN+ stage (50.0%, $P = .001$) disease. Podoplanin was positive mainly in neoplastic cells located at the

Table I. Characteristics of study population according to podoplanin expression and perineural invasion status

Characteristic	*n (%) of patients*	*Podoplanin expression*			*PNI status*		
		Negative n = 41 (49.4%)	*Positive n = 42 (50.6%)*	*P value**	*Negative n = 52 (62.7)*	*Positive n = 31 (37.3)*	*P value**
Age > 65 y	44 (53.0)	24 (58.5)	20 (47.6)	.319	26 (50.0)	18 (58.1)	.476
Female	40 (48.2)	18 (43.9)	22 (52.4)	.440	22 (42.3)	18 (58.1)	.165
Smoker	40 (48.2)	24 (58.5)	17 (40.5)	.100	31 (59.6)	10 (32.3)	.016
Site				.381			.470
Tongue and/or floor of mouth	31 (37.3)	16 (39.0)	15 (35.7)		22 (42.3)	9 (29.0)	
Cheek	15 (18.1)	5 (12.2)	10 (23.8)		9 (17.3)	6 (19.4)	
Gum	37 (44.6)	20 (48.8)	17 (40.5)		21 (40.4)	16 (51.6)	
T stage				< .001			< .001
T1−T2	45 (54.2)	31 (75.6)	14 (33.3)		39 (75.0)	6 (19.4)	
T3−T4	38 (45.8)	10 (24.4)	28 (66.7)		13 (25.0)	25 (80.6)	
N stage				.001			< .001
N0	56 (67.5)	35 (85.4)	21 (50.0)		45 (86.5)	11 (35.5)	
N+	27 (32.5)	6 (14.6)	21 (50.0)		7 (13.5)	20 (64.5)	
Grade				.074			.004†
G1	23 (27.7)	15 (36.6)	8 (19)		20 (38.5)	3 (9.7)	
G2	48 (57.8)	23 (56.1)	25 (59.5)		28 (53.8)	20 (64.5)	
G3	12 (14.5)	3 (7.3)	9 (21.5)		4 (7.7)	8 (25.8)	
Surgical margin status				.976			.666†
Clear	77 (92.8)	38 (92.6)	39 (92.8)		49 (94.2)	28 (90.3)	
Close or involved	6 (7.2)	3 (7.4)	3 (7.2)		3 (5.8)	3 (9.7)	
Podoplanin expression							< .001
Positive (≥20%)	42 (50.6)				13 (25.0)	29 (93.5)	
Negative (<20%)	41 (49.4)				39 (75.0)	2 (6.5)	

*Log-rank test except where noted overwise.
†Fisher exact test.*PN,* perineural invasion.

periphery of neoplastic nests and in more atypical and less differentiated neoplastic cells (Figures 1A-D and 2A-E).

Podoplanin and PNI status

We observed high levels of podoplanin expression in 29 of the pre-operative biopsies of the 31 (93.5%) post-operative specimens with PNI and 13 of the 52 (25.0%) postoperative specimens without PNI ($P < .001$). Other clinical characteristics frequently and significantly associated with PNI positivity were T3 to T4 stage (80.6%, $P < .001$), N+ stage (64.5%, $P < .001$), non-smoking status (67.7%; $P = .016$), and G2 to G3 grade (64.5%, $P = .004$). We found no significant associations between PNI status and age, sex, site of index OSCC, or surgical margin status (data from penalized maximum likelihood logistic regression are presented in Supplementary . . . S1).

Positivity for podoplanin in the pre-operative biopsy showed high sensitivity (93.5%, 95% CI = 78.6-99.2) and NPV (95.1%, 95% CI = 83.5-99.4) for predicting PNI in the surgical specimen (Table II). We observed that 13 of 52 cases showed high podoplanin expression without PNI (specificity 95% CI = 61.1-86.0) and PPV (69.0%, 95% CI = 52.9-82.4). Of the 13 cases with high podoplanin expression but no evidence of PNI, the pTNM classification was pT1N0M0 in 5 patients, pT2N0M0 in 4 patients, pT3N1M0 in 1 patient, pT4N0M0 in 1 patient, pT4N2bM0 in 1 patient, and pT4N2cM0 in 1 patient. The area under the curve of podoplanin alone as an indicator for PNI was 0.843, indicating good discrimination accuracy, and exceeded 0.900 with the addition of other clinical variables (Supplemental Figure S1).

Podoplanin, PNI status, and disease progression

We found that the 31 PNI-positive patients had a greater risk of developing a secondary tumor (LR, second primary tumor, or lymph node metastasis), with 16 (51.6%) developing a secondary tumor, than the 52 PNI-negative patients, with 10 (19.2%) developing a secondary tumor, and developing the tumor within a shorter timespan (median time to recurrence = 8.8 vs 23.1 months, log-rank test: $Chi^2 = 23.07$, $P < .001$; Figure 3). In multivariable analysis, a probit model with one degree of freedom showed the best fit to the data. Perineural invasion positivity was strongly associated with time to locoregional control (hazard ratio [HR] = 5.66, 95% CI = 2.94-10.87, $P < .001$) after removal of all other variables (sex, age >65 years, smoking status, podoplanin expression, site, T and N stage, grade, and margins; Supplementary Figure S2). As we had previously found a strong association between PNI and podoplanin, we examined the

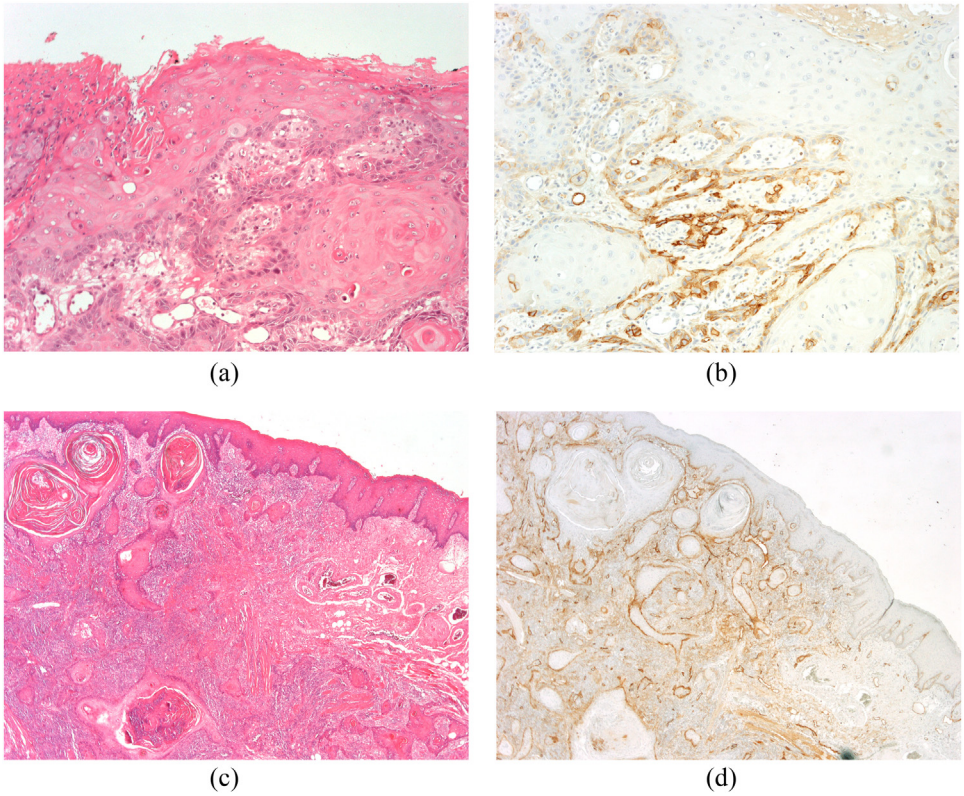

Fig. 1. **(A)** Pre-operative incisional biopsy showing highly keratinized oral squamous cell carcinoma (hematoxylin and eosin staining). **(B)** Thin rim of neoplastic cells at the periphery of the neoplastic nests showing limited podoplanin expression. **(C)** Surgical resection of the same tumor showing no perineural invasion (hematoxylin and eosin staining). **(D)** Podoplanin immunohistochemistry of post-surgical specimen showing staining of <20% of neoplastic cells.

effect of podoplanin on time to LRT in a multivariable model without PNI and the same pre-operative variables, and we found that podoplanin was the only significant predictor (HR = 3.00, 95% CI = 1.65-5.47, $P < .001$).

DISCUSSION

Despite being the optimal treatment strategy for patients with head and neck squamous cell carcinoma, complete surgical resection with free surgical margins often yields clinical outcomes jeopardized by a high rate of LR, resulting in the need for additional salvage surgeries with poor outcomes.[1] Therefore, early identification of patients at risk of local relapse or nodal involvement and prompt implementation of individualized treatment options have become urgent issues in the field of head and neck oncology.

Among the different negative prognostic factors, PNI is emerging as a relevant parameter. Supporting its utility, Miller et al.[25] reported that PNI was related to nodal status and T stage, disease-free survival, and number of metastatic lymph nodes. Likewise, Tai et al.[26] found that PNI was a strong independent predictor of neck metastasis, even in early-stage tumors.[3] Many other studies have also observed that PNI was related to higher treatment failure rates in patients with OSCC.[10,25,26] In accordance, our group previously reported that PNI was an independent predictor of local and regional failure in a retrospective cohort of 236 consecutive patients with oral cancer.[10] Our present prospective study confirmed the role of PNI as an independent predictor of secondary malignancies (HR = 19.2, $P < .001$).

PNI status is commonly assessed in postoperative specimens by histologic analysis of resected specimens.[27] However, determination of PNI status before surgery, which could affect surgical planning, would be much more useful. IHC analysis has been used to determine the associations between biomarkers (analyzed by definitive pathologic examination of OSCCs) and negative prognostic factors. In OSCC, several biomarkers, including nerve growth factor,[28] neural cell adhesion molecule,[29] claudin 1,[30] activin A,[31] p73,[32] p75 nerve growth factor,[33,34] laminin-5,[27] IMP3,[27] and COX-2,[35] have been identified in surgical specimens of cases showing PNI. Although most studies published to date have focused on postoperative surgical

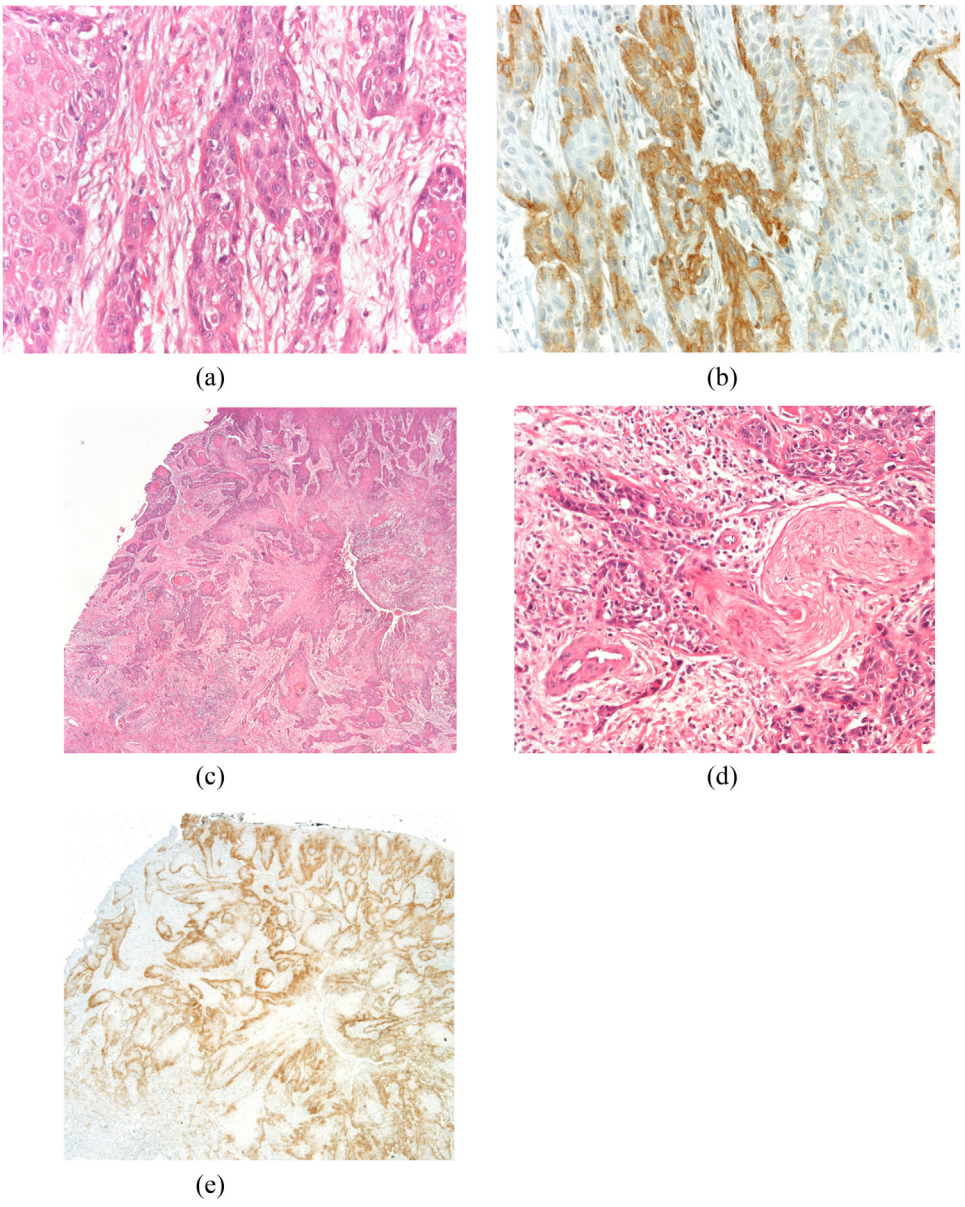

Fig. 2. **(A)** Pre-operative incisional biopsy showing highly keratinizing squamous cell carcinoma (hematoxylin and eosin staining). **(B)** Greater than 20% of neoplastic cells at the periphery of neoplastic nests showing podoplanin expression. **(C)** Surgical resection of the same tumor (hematoxylin and eosin staining). **(D)** Detection of perineural invasion in the deeper part of the tumor. **(E)** Podoplanin immunohistochemistry of post-surgical specimen confirming high expression levels.

specimens, knowledge of the potential for aggressive OSCC is useful pre-operatively.

To our knowledge, our study is the first to provide evidence that podoplanin expression is a reliable marker of PNI, even in pre-operative biopsy specimens. In support of our finding, a recent systematic review highlighted the prognostic role of podoplanin in oral and oropharyngeal squamous cell carcinoma by showing that podoplanin overexpression was associated with lymph node involvement, higher histopathologic grade, advanced clinical stage, and poorer overall survival/disease-free survival.[36] Before this study, our group demonstrated the prognostic role of podoplanin expression in pre-operative biopsy in a study in which we found a significant association between positive podoplanin expression and lymph node metastasis at the time of surgery.[19] However, we did not include PNI in our studied pathologic parameters.

Recently, there has been a movement toward providing personalized medicine based on the evaluation of specific patient data to optimize patient care. Our findings, which support a strong relationship between podoplanin and PNI (sensitivity = 93.5%, NPV = 95.1%, $P < .001$; area under the

Table II. Diagnostic accuracy of podoplanin expression for perineural invasion status

Groups and statistic	*Podoplanin positive (≥20%)*	*Podoplanin negative (<20%)*
PNI-positive status (group 1)	29	2
PNI-negative status (group 2)	13	39
Accuracy	81.9%	
Sensitivity (95% CI)	93.5% (78.6-99.2)	
Specificity (95% CI)	75.0% (61.1-86.0)	
Positive predictive value (95% CI)	69.0% (52.9-82.4)	
Negative predictive value (95% CI)	95.1% (83.5-99.4)	

PNI, perineural invasion.

curve = 0.843), together with the findings of previous studies, suggest that pre-operative determination of podoplanin expression may be useful in tailoring OSCC treatment for individual patients. We believe that using predictive models to tailor treatment will become increasingly important in the future as we attempt to improve outcomes by individualizing therapeutic recommendations.

Our study faced several limitations that should be considered when reviewing our findings. The HRs and corresponding CIs we obtained via survival analysis for PNI were wide as a consequence of the limited number of samples available. However, having found a clear difference between the survival curves of PNI-positive and PNI-negative patients, we are confident that our findings are replicable. In addition, we identified 13 cases with high podoplanin expression without evidence of PNI, which could be interpreted as having tumors at an early stage that have not yet invaded nerves or lymph nodes yet have aggressive and invasive potential. Nevertheless, we cannot rule out the possibility of false-positive high podoplanin results.

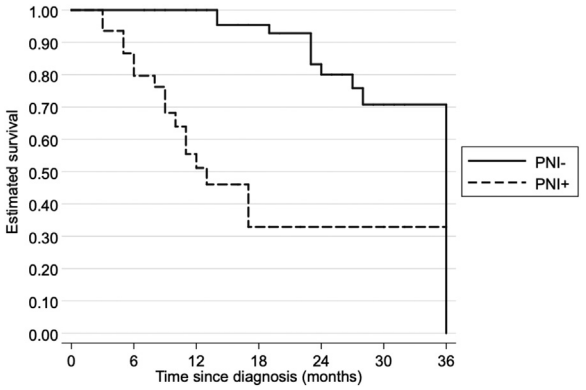

Fig. 3. Kaplan–Meier survival curves of perineural invasion status with loss of locoregional control.

Finally, podoplanin expression in OSCC has commonly been noted at the invasive front and, therefore, may not be routinely detected in incisional biopsies, which provide only a snapshot of the lesion.[36,37]

In the future, the prognostic impact of IHC analysis of pre-operative biopsy should be validated in studies with larger populations conducted at multicenter studies aiming for inter- and intra-observer agreement among pathologists. Likewise, research examining the identification of altered expression of one or more immunohistochemical biomarkers related to PNI infiltration may be important in improving the specificity and PPV of pre-operative incisional biopsy.

Notwithstanding the limitations of this study, IHC analysis of podoplanin expression in pre-operative biopsy specimens appears to be an important prognostic tool for the treatment of patients with OSCC by helping the oncological team determine the most appropriate treatment option according to the patient's risk profile before index tumor resection.

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DECLARATION OF INTEREST

None

SUPPLEMENTARY MATERIALS

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.oooo.2023.08.011.

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