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The expression of podoplanin is associated with poor outcome in cutaneous squamous cell carcinoma

Background: Cutaneous squamous cell carcinoma (CSCC) is the second most frequent cancer in humans and can be both locally invasive and metastatic at distant sites. While research efforts have been made to predict poor outcome of CSCC, there is a lack of knowledge regarding molecular markers. Podoplanin has been associated with poor outcome in several types of cancer including CSCC, but this is controversial and only a few studies have evaluated the prognostic implications of podoplanin in the development of this tumor.
Methods: We evaluated podoplanin expression in a series of 94 CSCCs, and searched for associations between podoplanin expression and histopathological characteristics and with events of poor clinical evolution of the disease.
Results: Podoplanin expression was observed in 48.9% of the cases and the expression was considered moderate to intense in 19 of the cases. Moderate/intense podoplanin was associated with infiltrative growth pattern, desmoplasia, lymphovascular invasion, higher risk of nodal progression (NP) and short disease-free survival, specifically with a short latency to NP.
Conclusions: This article provides evidence supporting the implication of podoplanin expression as a marker of bad prognosis of CSCC.

Keywords: cutaneous squamous cell carcinoma, D2-40, podoplanin, prognosis

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Cutaneous squamous cell carcinoma (CSCC) represents the most common form of skin cancer after basal cell carcinoma and can be both locally invasive and metastatic at distant sites.[1,2] Around 700,000 CSCCs new cases are diagnosed annually in the United States, comprising a proportion of 20% of all non-melanoma skin cancer (NMSC).[3] While most CSCC cases have an excellent prognosis, there is a subset with a high risk of developing local recurrence (LR) and metastases. The risk of nodal metastases ranges from 3.7 to 5.2%, and disease-specific death ranges

from 1.5 to 2.1% in CSCC patients.[4] Given the high frequency of CSCC and its increasing incidence, the clinical implications of CSCC are of paramount importance and its associated costs are high.[5] Several clinical and histopathological risk factors have been used to predict bad clinical evolution in patients diagnosed with CSCC,[6–8] but there is an evident lack of knowledge concerning molecular and protein markers that could be used to predict outcome of this disease.

Podoplanin is essential in lymphatic vessel development[9] and also participates in tumor invasion and progression.[10] It was originally identified as a cell-surface antigen expressed in mouse epidermal keratinocytes during skin remodeling and carcinogenesis.[11] Podoplanin expression was also linked to epidermal to mesenchymal transition (EMT), cell migration[12] and to an alternative pathway for tumor cell invasion in the absence of EMT.[13] Podoplanin is also involved in the actin remodeling of the cytoskeleton of tumor cells and may promote tumor cell invasion by increasing cell motility.[13]

Podoplanin expression has been associated with poor outcome in some types of cancer. Podoplanin is upregulated in several human cancers such as squamous cell carcinoma (SCC) of the oral cavity,[14–16] skin adnexal carcinomas,[17] esophageal SCC[18] and SCC of the penis.[19] Some papers have showed that podoplanin expression is associated with a higher risk of nodal metastasis in CSCC,[20,21] but there is still debate regarding its real relevance as a prognostic marker. This study provides further evidence regarding the prognostic implications of podoplanin expression in primary CSCCs, and highlights its relevance in predicting disease-free survival.

Patients, material and methods

Clinical, epidemiological and histopathological features of CSCCs

We evaluated a series of 94 CSCCs. We selected some tumors in a prospective way, and other tumors were retrospectively collected in order to obtain a higher proportion of CSCC with poor outcome and high risk features. Patients were included following the Helsinki Declaration and the requirements set out by the Local Bioethical Committee of the University Hospital of Salamanca. We considered different tumor and epidemiological variables of CSCCs, clinical-epidemiological and histopathological features and their distribution along the cohort are described on Table S1, Supporting Information. Specifically, we considered the following values:

Clinical and epidemiological variables

(a) Age and sex of patients; (b) Previous history of dermatological diseases, including actinic keratoses, NMSC and CSCC; (c) Tumor location was categorized as: (i) head and neck high-risk areas (ear, lower lip, temple, nose, eyelid and preauricular region); (ii) head and neck low-risk areas (rest of head and neck locations) and (iii) trunk and extremities; (d) Presence of immunosuppression; (e) History of chronic sun exposure and (f) American Joint Committee on Cancer (AJCC) stage at diagnosis and at the end of the follow-up period.

Histopathological variables

(a) Tumor size and thickness (in millimeters), both measured using the OV100 software (Olympus™, Tokyo, Japan); (b) grade of differentiation, which was classified as good, moderate or poor, according to previous reports[22]; (c) growth pattern was classified as expansive, infiltrative or mixed. Infiltrative growth pattern was considered when the tumor exhibited small nests, rows of cells and/or isolated tumor cells in the periphery of the tumor, an expansive pattern was assigned when the tumor exhibited a compact growth, with no-disaggregated cells in the front of invasion and mixed growth pattern was assigned when the tumor displayed an expansive growth pattern and tended to infiltrate in any part of the front of invasion; (d) perineural invasion; (e) lymphovascular invasion; (f) desmoplasia was defined by the thickening of collagen bundles around and inside the tumor involving at least 30% of the stroma[6]; (g) solar elastosis and (h) actinic keratosis associated with tumor (in flanking epithelium).

Time to events of poor clinical evolution during follow up (in months)

(a) LR; (b) NP; (c) distant progression, which was considered when the tumor developed metastases in solid organs during the follow-up period; (d) death from CSCC and (e) any of the aforementioned events.[23]

Tissue microarray and immunohistochemistry

Tissue samples, formalin-fixed paraffin-embedded, were used to prepare tissue microarrays using a tissue arrayer device (Beecher

Instruments, Sun Prairie, WI, USA). Three 1-mm-diameter cylinders from each tumor were included to ensure reproducibility and homogeneous staining of the slides. Three different areas of each tumor were selected to reproduce the heterogeneity of the samples.

Immunohistochemical studies were performed using the *AutostainerLink 48* (Dako, Santa Clara, CA, USA). Antibody anti-human against podoplanin, clone D2-40 (Dako, Santa Clara, CA, USA) of mouse origin, was used (prediluted). The detection system used the kit *EnVision/HRP Flex Plus* (Dako®, Santa Clara, CA, USA) and positive controls were included in each slide.

A semiquantitative analysis of podoplanin expression level was performed by three independent observers (JC, CR-C and EC-A). The intensity of podoplanin expression and the percentage of positive cells in each tumor were calculated as carried out by other groups.[20,21] Podoplanin intensity of expression was calculated as 0: null; 1: weak; 2: moderate and 3: intense; and the percentage of positive cells was considered as 0: null (<25% of stained cells); 1: weak (25–50%); 2: moderate (50–75%) and 3: intense (>75%). A mean with podoplanin intensity of expression and podoplanin percentage of stained cells were calculated to determine the amount of podoplanin expression in each tumor, as previously described.[21] Podoplanin expression was later transformed into a dichotomous variable with category 0 (absent and weak podoplanin expression: −/+) and category 1 (moderate and intense podoplanin expression: ++/+++). We only considered podoplanin expression in epithelial tumor cells in order to classify CSCC.[20,21]

Lymphovascular invasion was evaluated and considered positive when tumor cells (either isolated or organized in clusters) were surrounded by podoplanin-positive lymphatic vessels.

Statistical analysis

Statistical analysis was performed using the IBM SPSS v.21 software. Categorical variables were compared using the Chi-square or the Fisher exact test when appropriate. The Kolmogorov-Smirnov test showed that continuous variables did not follow a normal distribution. When two independent samples were analyzed, the Mann-Whitney *U* test was used. To evaluate which variables predicted events of poor clinical evolution, we developed logistic regression models and used the Wald test. We considered p values <0.05 as significant, and confidence intervals at 95%.

Results

Podoplanin expression in CSCC

To determine the utility of podoplanin expression as a marker of prognosis in CSCC, we evaluated podoplanin expression by immunohistochemistry in a cohort of 94 CSCCs (Fig. 1 and Table 1). A total of 48 tumors (51.1%) displayed no expression, 27 tumors (28.8%) showed weak expression, 15 tumors (16%) showed moderate expression and 4 tumors (4.3%) displayed intense expression of podoplanin (Table 1). Podoplanin expression was located mainly in lymphatic vessels, but in some cases also in tumor cells in which podoplanin exhibited a cytoplasmic staining, sometimes with cell membrane enhancement. Podoplanin also tended to be expressed along the front of invasion of the tumors, especially in those cases with an infiltrative growth pattern (Fig. 1).

Podoplanin expression is associated with histopathological tumor traits and with events of poor outcome in CSCC

Following on, we studied the existence of associations between podoplanin expression and histopathological tumor traits. Moderate/intense podoplanin expression was associated with the presence of desmoplasia ($p = 0.031$), with an infiltrative growth pattern ($p = 0.013$) and with the presence of lymphovascular invasion ($p = 0.011$). Moderate/intense podoplanin expression was also found more frequently in those tumors that exhibited ulceration ($p = 0.002$) and a poor grade of differentiation with a statistical trend ($p = 0.057$) although, expression was not associated with perineural invasion (Fig. 2 and Table 1).

Moderate/intense podoplanin expression was not associated with AJCC stage at diagnosis but it correlated with AJCC stage at the end of follow up. Thus, podoplanin expression was more common in tumors at the higher stages ($p = 0.032$) and with progression of the AJCC stage ($p = 0.022$). More interestingly, moderate/intense podoplanin expression in the primary tumor correlated with higher risk of nodal metastasis during follow up ($p = 0.022$) (Table 1), which was particularly evident for tumors at stage 2 ($p = 0.01$) (Fig. 2C).

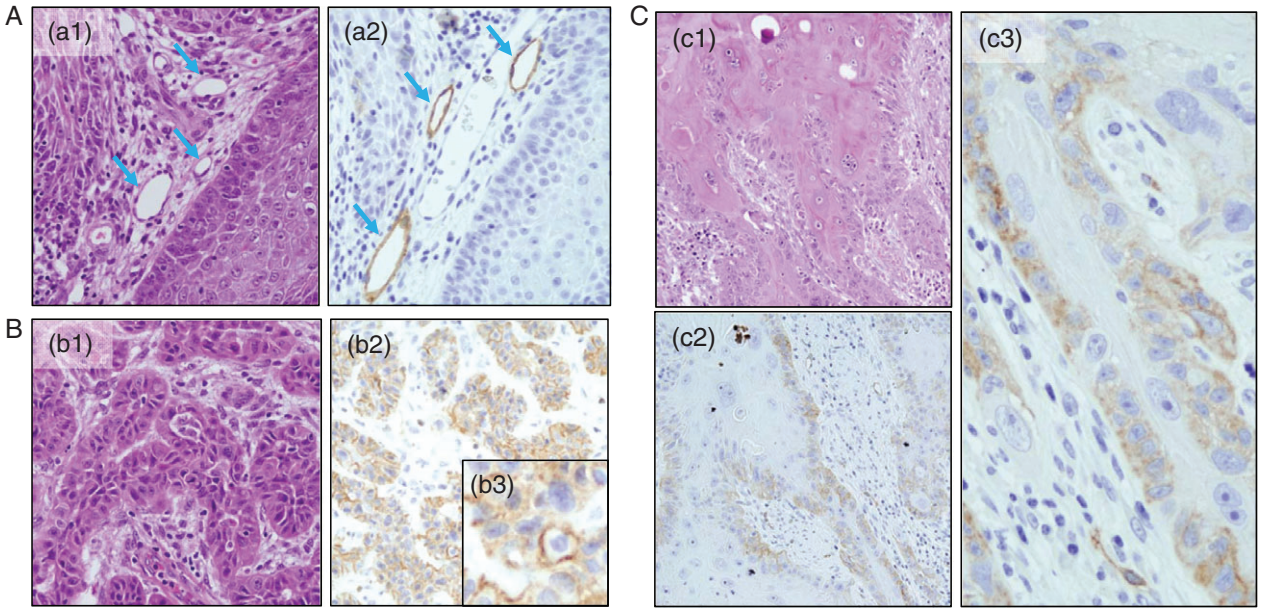

Fig. 1. Evaluation of podoplanin expression by immunohistochemistry in cutaneous squamous cell carcinoma (CSCC). A) Podoplanin expression in lymphatic vessels: (a1) Hematoxilin and eosin (H&E) staining of the CSCC zone with lymphatic vessels (blue arrows) (×400) and (a2) increased magnification (×400) showing more detail. B) Podoplanin expression in an infiltrative CSCC: (b1) H&E of tumor cells (×200); (b2) podoplanin expression in tumor cells showing cytoplasmic podoplanin staining (×200) and (b3) (×400). C) Podoplanin expression along the front of invasion in CSCC: (c1) H&E staining in an infiltrative CSCC (×200). Podoplanin expression along the front of invasion of the tumor (c2) (×200) and (c3) (×400).

Podoplanin predicts short disease-free relapse in CSCC

Later, we assessed whether podoplanin expression could serve as a biomarker of time to disease relapse in CSCC. The median follow-up period of all the patients was 70 months (range 36–90 months). The *latency to any event of poor clinical evolution* was shorter for those patients with tumors that expressed podoplanin at moderate/intense levels ($p = 0.031$) (Fig. 3A). Thus, the median length of time to develop adverse clinical events was 4.5 months [interquartile range (IQR) 6.5] for those patients with tumors that expressed podoplanin at moderate/intense levels, whereas it was 11 months (IQR 21) for those patients with tumors with absent/weak podoplanin expression (Table 1).

Also, moderate/intense podoplanin expression was associated with a *shorter latency to develop lymph nodal metastasis* ($p = 0.008$) (Fig. 3B). Thus, the median length of time to NP was 5 months (IQR 4.88) for those tumors with moderate/intense podoplanin expression, and 13 months (IQR 18) for those with absent/weak podoplanin expression (Table 1).

We carried out a logistic regression model to evaluate the plausibility of podoplanin expression acting as an independent variable able to predict CSCC prognosis. We only found it to be associated with the lymph node metastasis model (Table 2). In addition to podoplanin expression, we introduced tumor traits related to CSCC prognosis and presumed to be predictable variables according to the literature.[6,24,25] These included desmoplasia, growth pattern, perineural infiltration, tumor thickness, tumor size and degree of differentiation, some of which have been associated with poor prognosis by univariate analysis in our cohort (Tables S2 and S3). Only perineural infiltration and podoplanin expression were the variables associated with short disease-free time to relapse in our cohort. AJCC stage did not correlate with differences in the time to events and NP.

Discussion

In this study, we showed that moderate/intense podoplanin expression was associated with histopathological features of poor outcome in CSCC, with NP and a short latency to events of poor clinical evolution generally considered, and in particular, with nodal metastasis.[26]

We found podoplanin expression in lymphatic vessels, and in tumor cells where, in some cases, it exhibited cytoplasmic staining. The specific expression of podoplanin in lymphatic endothelial cells and tumor cells in CSCC is similar to

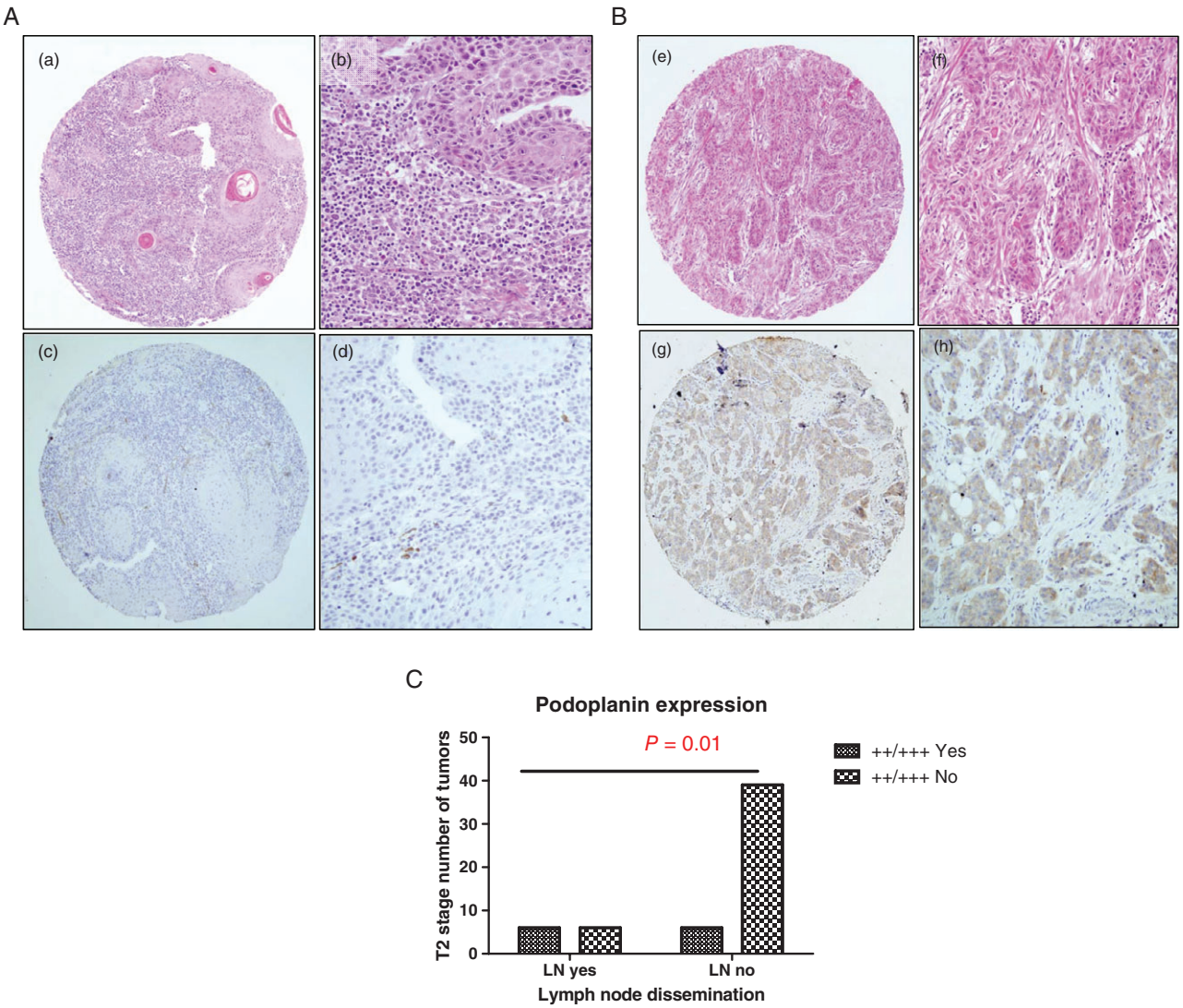

Fig. 2. Podoplanin expression and histopathological tumor traits in cutaneous squamous cell carcinoma (CSCC). A) Well-differentiated CSCCs with an expansive growth pattern of invasion: (a) H&E (×100); (b) H&E (×200); (c) absence of global podoplanin expression in the tumor (×100); (d) note that lymphatic vessels are positively stained (×200). B) Poorly differentiated CSCCs with infiltrative growth pattern of invasion; (e) H&E (×100); (f) H&E (×200); (g) intense podoplanin expression (×100) and (h) (×200). Note the desmoplastic stroma in (f) and (h). C) Association of lymph node metastasis with podoplanin expression in tumors at stage 2.

the expression pattern of podoplanin observed in other types of SCC such as the carcinoma of the oral cavity and the larynx.[27–30] This finding is in agreement with the role of podoplanin in lymphatic vessel generation and remodeling,[9] but there were some differences in the frequency and the intensity of podoplanin expression, being lower in CSCC.[21] Indeed, we did observe that the expression of podoplanin was absent in more than 50% of the tumors in this cohort. As podoplanin expression seems to be linked with lymphatic metastases and with lymphatic tumor spread, these differences in podoplanin expression frequency might reflect the lower nodal metastatic risk in SCC of cutaneous origin.[21] In addition, we observed an association between podoplanin expression in the primary tumor and the presence of lymphovascular invasion, which suggests that podoplanin is associated with lymphatic tumor spread. Podoplanin also tended to be expressed along the front of invasion of tumors, especially in those cases that exhibited an infiltrative growth pattern, and its expression has been associated with an infiltrative growth pattern and desmoplasia in CSCC.[20]

Podoplanin has been previously associated with nodal metastasis[20,21,31] and with short survival rates in CSCC.[32] Thus, podoplanin

Table 1. Associations between podoplanin expression and different clinical and histopathological CSCC variables

CSCC features		Podoplanin expression		p Value
		Absent/weak	Moderate/intense	
Desmoplasia	Yes	13	7	**0.048*****
	No	62	10	
Growth pattern	Infiltrative	37	14	**0.013*****
	Non infiltrative	38	3	
Ulceration	Yes	36	15	**0.007*****
	No	26	1	
Lymphovascular invasion	Yes	2	4	**0.011***
	No	72	13	
Degree of differentiation	Good-moderate	63	12	*0.057**
	Poor	12	7	
Nodal progression	Yes	7	6	**0.022***
	No	68	13	
AJCC stage at diagnosis	I	27	5	N.S.***
	II	48	14	
AJCC stage at the end of follow up	I	26	5	**0.032*****
	II	41	8	
	III	7	3	
	IV	1	3	
Progression AJCC stage	Yes	8	6	**0.033*****
	No	67	13	
Time to events of poor prognosis (months) (Me/IQR)		11 (21)	4.5 (6.5)	**0.031****
Time to nodal progression (months) (Me/IQR)		13 (18)	5 (3.5)	**0.008****

CSCC, cutaneous squamous cell carcinoma; IQR, interquartile range; Me, median; N.S. not significant.
Significant p values are in bold; Statistical trend is indicated by italics.
*Fisher exact test; **Mann-Whitney *U* test; ***Chi-square test was used in those cases with no specification.

seems to be a marker associated with NP in CSCC.[20,21] Here, we also found an association between nodal metastasis and moderate/intense podoplanin expression in CSCC. Although podoplanin expression has been associated with relapse in CSCC, we did not find an association between this marker and LR in our series. Other authors have found correlation between low podoplanin expression and lymphatic spread as well as with nodal metastases in uterine cervical cancer[33] and in lung cancer,[34] and it seems podoplanin may play a different role depending on the cancer type.

The association of podoplanin expression with latency to events of bad clinical evolution has been poorly evaluated in CSCC. In our study, moderate/intense podoplanin expression was associated with a short latency to global events and with a short latency to NP. Indeed, podoplanin expression was the unique variable associated with short disease-free time of to relapse. AJCC stage at diagnosis was inadequate to predict latency to events of bad clinical evolution. Also podoplanin expression, which did not correlate with AJCC stage at diagnosis, did correlate with AJCC stage at the end of follow up and with the progression of AJCC stage. Thus, podoplanin expression could be a useful marker to identity tumors with a high risk of progression and could give additional prognostic information for CSCC AJCC stage at diagnosis, in particular for these tumors at stage 2 (Fig. 2C).

In conclusion, this paper confirms podoplanin as a marker of poor prognosis in CSCC. We show that podoplanin expression predicts a higher risk for the development of nodal metastasis in

Table 2. Identification of podoplanin expression and perineural infiltration as independent variables to predict lymph node metastasis by logistic regression

Logistic regression model	Independent factors	OR	CI	p Value
Lymph node metastasis	PNI	5.75	1.540–21.467	0.009
	Podoplanin (++/+++)	5.165	1.326–20.126	0.018

(++/+++), moderate/intense; CI, confidence interval; OR, odd ratio; PNI, perineural infiltration.

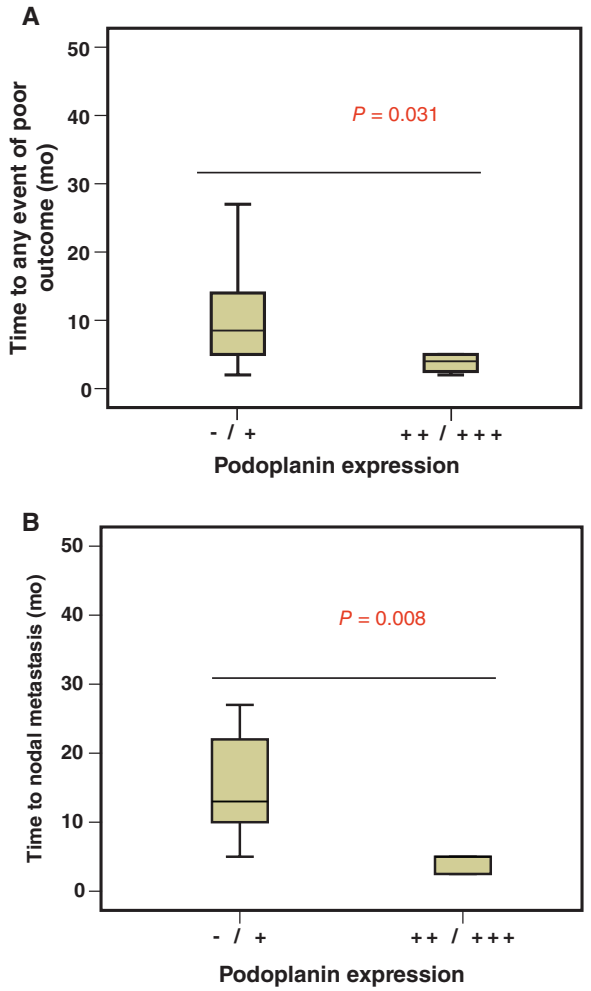

Fig. 3. Differences in disease-free time to relapse associated with podoplanin expression. A) podoplanin expression is associated with short disease-free time of relapse in cutaneous squamous cell carcinoma (CSCC). B) podoplanin expression is associated with a short latency to lymph nodal progression in CSCC.

CSCC, as others have also described,[20,27] and is also a marker of an early appearance of a clinical event. This article provides evidence for the prognostic implications of podoplanin expression, and suggests that CSCCs with moderate/intense podoplanin expression should be closely followed irrespective of other associated risk factors. Moderate/intense expression of podoplanin in the primary tumor, especially in those cases with other pathological traits of aggressiveness, could justify carrying out a sentinel lymph node biopsy for better staging of CSCC. While evidence is growing concerning the prognostic implications of podoplanin expression in CSCC, further studies with larger case series are necessary to confirm these findings.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Clinicoepidemiological and histopathological features of cutaneous squamous cell carcinoma (CSCC) evaluated in our cohort of study.
Table S2. Associations between histopathological tumor traits of cutaneous squamous cell carcinoma (CSCC) with events of poor clinical outcome.
Table S3. Associations of cutaneous squamous cell carcinoma (CSCC) prognosis and podoplanin expression with (A) presence of immunosuppression; (B) location of CSCC in high-risk zones and (C) tumor thickness and size.

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