



The stem cell markers expression CD44v6 and podoplanin in lip cancer: clinical significance

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Abstract

This study aimed to analyze the immunoexpression of cancer stem cell markers, CD44v6, and podoplanin in 91 patients with lip squamous cell carcinomas (LSCC). The immunostaining of podoplanin and CD44v6 was evaluated in ten high-power fields ($\times 400$ magnification) at the invasive front of LSCC, using a semi-quantitative score method. Chi-square test or Fisher's exact test was used to verify the association of podoplanin and CD44v6 expressions with clinicopathologic variables. Spearman's correlation test was used to analyze the correlation between the two antibodies in lip cancer. Disease-free survival probabilities in 5 and 10 years were estimated according to the Kaplan-Meier method and compared using the log-rank test. The independent effects of the significant variables were analyzed by Cox proportional hazards regression model. A strong podoplanin expression was observed in the membrane and cytoplasm of most lip tumor cells, and this was inversely associated with locoregional recurrence ($p = 0.028$) and with histopathological grade of malignancy ($p = 0.026$). Additionally, CD44v6 immunostaining was strongly expressed in the membrane of tumor cells in 95.4% of the LSCC. Patients with strong membranous ($p = 0.016$) or strong cytoplasmic ($p = 0.030$) podoplanin-positive tumors resulted in significantly better disease-free survival than those who had podoplanin weak/negative tumors, confirming podoplanin expression as a favorable independent prognostic factor. Podoplanin and CD44v6 were strongly expressed by tumor cells and podoplanin immunoexpression can help to determine lip cancer patients with lower risk for disease recurrence.

Keywords Podoplanin · cd44 antigens · Lip · Squamous cell carcinoma

Introduction

In the last few years, the involvement of cancer stem cell, a very specialized cell with unique abilities of self-renewal,

proliferative potential, and metastatic phenotype, in oral tumorigenesis has been extensively studied [1–7]. In addition to previously identified molecules, other proteins such as podoplanin have been described as a candidate cancer stem cell marker in oral squamous cell carcinoma [4, 8–10].

Our research group has previously investigated the participation of podoplanin in oral tumor invasion [11–17], and its overexpression in the invasive front of lip squamous cell carcinoma was regarded as being a predictive biomarker of locoregional recurrences [15]. These evidences are interesting because, although the lip cancer is an indolent tumor with favorable prognosis, the locoregional recurrence continues to be a potential risk factor for poor clinical outcome and patients' survival [18].

Additionally, we verified the correlation between podoplanin and cytoskeleton linkers such as ezrin and moesin in oral carcinogenesis [13, 15, 16]. Initial studies suggested that the cytoplasmic tail of podoplanin connects to the membrane cytoskeleton linkers, ezrin or moesin, through Rho-A

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phosphorylation, activating transductional signals to the cell membrane, leading to loss of adhesion, cell motility, and cellular proliferation [19–21]. Based on the positive correlation of podoplanin, ezrin, and Rho-A immunorexpression in lip squamous cell carcinoma, Assao et al. (2016) suggested a cooperative participation of these proteins in cell movement and invasion of these tumors.

The interaction of podoplanin with CD44 driving cell migration in squamous cell carcinoma was demonstrated *in vitro* [22] and the two proteins were considered coordinately involved in hyaluroanmediated cell adhesion in oral squamous cell carcinoma cell lines [23]. Studies have speculated that the coexpression of podoplanin and CD44 could identify a cancer stem cell subpopulation with high potential to disseminate and metastasize [22].

CD44 has been widely used as a stem cell marker in oral squamous cell carcinomas [5–7], and deregulated expression of CD44 isoforms was positively correlated with invasion, metastasis, and local recurrences in head and neck cancer [1, 3, 4, 24, 25]. Additionally, the high expression of CD44v6 isoform in cancer stem cells derived from head and neck cancer was considered a potential prognostic marker of tumor progression [1–4, 10, 24].

The purpose of analyzing the coordinated expression of podoplanin and CD44v6 in lip squamous cell carcinoma for the first time was to explore any potential association between these molecules in lip carcinogenesis and the clinical importance of these stem cell biomarkers in the patients' prognosis.

Patients and methods

Patients and tumor samples

The study cohort included patients who underwent surgical treatment for lower-lip squamous cell carcinoma, at the Department of Head and Neck Surgery and Otorhinolaryngology of the A.C. Camargo Cancer Center, São Paulo, Brazil, from 1970 to 2009. This tumor sample was previously selected by Assao et al. (2016) using the following inclusion criteria: diagnosis of lip squamous cell carcinoma, confirmed by biopsy; patients submitted to surgery as initial treatment; no other primary tumors occurring simultaneously; complete clinical data and follow-up; and tumor tissue available for microscopic analysis. Thus, 91 patients were eligible for this study, and their median follow-up period was 10 years. All clinical data (age, ethnic group, gender, tobacco and alcohol consumption, TNM stage-UICC, 2004, treatment and clinical follow-up) or pathological data (primary anatomic localization, lymph node status, tumor size, perineural infiltration, glandular infiltration, muscular infiltration) were obtained from the medical files of the A.C. Camargo Cancer Center.

Hematoxylin & Eosin (HE) staining of each tumor sample was performed in order to verify or exclude the presence of surgical margins and to select the most representative lip tumor area. Afterwards, the histopathological grade of malignancy of lower-lip squamous cell carcinoma was determined, according to Brandwein-Gensler et al. (2005).

The Research Ethics Committee of the A.C. Camargo Cancer Center, São Paulo, Brazil, approved this study (process #1778/13 - CAAE 01594912.0.3001.5432).

Immunohistochemistry

Paraffin-embedded lip cancer blocks were cut into 3- μ m sections and mounted on silane-coated slides. Immunohistochemistry was performed on tumor sections according to the protocol of the Department of Pathology of the A.C. Camargo Cancer Hospital, São Paulo, Brazil, previously described in the study of Assao et al. (2017). Individual slides containing lip cancer or control sections were incubated overnight with primary anti-podoplanin antibody (D2-40 clone, Dako North America, CA, USA) diluted at 1:200 and primary anti-CD44v6 antibody (Abcam ab7890, Cambridge, UK) diluted at 1:1000. The reaction products of both antibodies (anti-podoplanin and anti-CD44v6) were detected using 3,3'-diaminobenzidine tetrahydrochloride (DAB/SIGMA, ref D-5637, St. Louis, MO, USA), counterstained with Mayer hematoxylin and coverslipped. Normal palatine tonsil was used as a positive control for each staining. For negative control, sections were treated as described above, but without the primary antibody.

Immunohistochemistry evaluation

Approximately ten high-power fields ($\times 400$ magnification) were randomly selected for microscopic evaluation of podoplanin and CD44v6 immunorexpressions, at the invasive front of the lip squamous cell carcinomas. The tumor images were captured with a digital camera (AxioCam MRc, Zeiss, Jena, Germany) attached to a microscope (Axioskop 2 Plus, Zeiss, Jena, Germany) and sent to an image software (Axiovision 4.9, Zeiss, Jena, Germany) for analysis.

The immunohistochemical expression of podoplanin and CD44v6 of lip cancer was evaluated by two independent, experienced pathologists, blinded to the clinical and pathological characteristics, according to a semi-quantitative score method, previously described by Faustino et al. (2008). Discordant cases were reviewed by these examiners to achieve a consensus. The colocalization of podoplanin expression by malignant cells (membranous or cytoplasmic) was registered separately.

Statistical analysis

The association between podoplanin and CD44v6 expressions and the clinicopathologic data was verified by the chi-square test (χ^2) or Fisher's exact test. Spearman's correlation test was used to analyze the correlation between the two antibodies in lip cancer.

The probability of disease-free survival in 5 and 10 years of follow-up was measured from the date of surgery to date of the last information about recurrence of the tumor. Survival curves were estimated according to the Kaplan-Meier method and compared using the log-rank test. The independent effects of the significant variables were analyzed using the Cox proportional hazards regression model.

All statistical analyses were carried out using the statistical software IBM SPSS Statistics for Windows version 21.0 (SPSS Inc., Chicago, IL, USA) and *p* values lower than 0.05 were considered statistically significant.

Results

Patients' characteristics

Lip squamous cell carcinoma samples were obtained mainly from male patients (82.4%), older than 45 years, with a mean age of 58.33 years, standard deviation of 15.71. The majority of patients with lip cancer reported alcohol and/or tobacco consumption (Table 1).

Clinically, the tumors were smaller than 3 cm, with clinical time of evolution ranging from 1 to 120 months (average of 12 months). According to UICC criteria, most lip squamous cell carcinomas were classified as T1 (42.7%) and T2 (38.2%), and only 14 patients presented positive lymph nodes (N+) at the time of clinical diagnosis, as described in Table 1. All patients underwent surgical resection of the primary tumor, and 21 of them were submitted to adjuvant postoperative radiotherapy. The clinical follow-up of lip cancer patients revealed that 24 of them presented local and/or regional tumor recurrence.

Tumor characteristics

Based on the histopathological features, 42.9% of lip squamous cell carcinomas were described as being well-differentiated tumors, and the histological factors that identified a greater aggressiveness, such as lymphatic embolization, perineural, muscular, and glandular infiltrations were observed in a small number of tumors. The free surgical margins were detected in 97.7% of the lip cancers. Twenty-seven patients underwent to elective neck dissection, and 15 of them presented infiltration of positive lymph node (pN+) cancer cells (Table 1).

Morphologically, the majority of lip squamous cell carcinoma presented a pattern of invasion in broad islets and solid

Table 1 Clinicopathological characteristics of 91 patients with lip squamous cell carcinoma. A.C. Camargo Cancer Center, São Paulo, Brazil, 1970 to 2009

Clinical and microscopical variables	Number of patients (%)
Gender	
Male	75 (82.4)
Female	16 (17.6)
Smoke*	
Yes	65 (81.2)
No	15 (18.8)
Alcohol*	
Yes	42 (53.8)
No	36 (46.2)
Tumor diameter*	
≤ 1 cm	28 (32.2)
> 1 cm and < 3 cm	32 (36.8)
≥ 3 cm	27 (31.0)
T stage*	
T1/T2	72 (80.9)
T3/T4	17 (19.1)
N stage*	
N0	54 (79.4)
N1/N2	14 (20.6)
Recurrence	
Yes	24 (26.4)
No	67 (73.6)
Lymphatic embolization*	
Yes	09 (28.1)
No	23 (71.9)
Perineural infiltration*	
Yes	11 (32.3)
No	23 (67.7)
Lymph node (pN)	
No dissection	64 (70.3)
pN negative	12 (13.2)
pN positive	15 (16.5)
Compromised margins*	
Yes	02 (2.30)
No	85 (97.7)
Histologic malignancy risk*	
Low risk	42 (46.1)
Intermediate/high risk	49 (53.9)
Total	91 (100%)

*Excluded patients without records

cords, an intense chronic inflammatory infiltrate, without perineural invasion. Based on these characteristics, the lip cancers were classified as low or intermediate (medium) and high malignancy risk patients for local recurrences and lower overall survival, according to the Brandwein-Gensler histopathological malignancy grade system (Table 2).

Table 2 Distribution of histopathological malignancy grading of 91 lower-lip squamous cell carcinoma. A.C. Camargo Cancer Hospital, São Paulo, Brazil, 1970 to 2009

Brandwein-Gensler grading	<i>N</i>	%
Low risk (score 0)	42	46.1
Intermediate risk (scores 1 to 2)	40	43.9
High risk (scores 3 to 9)	09	09.0
Total	91	100

Immunohistochemical findings

The results of immunostaining analysis of podoplanin and CD44v6 in lip squamous cell carcinomas are illustrated in Fig. 1.

Podoplanin and CD44v6 were markedly overexpressed in the invasive front of the lip cancer. Podoplanin-positive tumors

showed a predominantly strong membranous (61.5%) and cytoplasmic (60.4%) expression by the neoplastic cells, mainly located at the periphery of the tumor islands (Fig. 1a, b).

The CD44v6 distribution pattern was predominantly strong and located in the membrane of malignant cells in the invasive front of the tumor (95.4%). In addition, a slight reduction in CD44v6 immunorexpression in lip cancer was observed when compared with the oral mucosa, as illustrated in Fig. 1.

Association of clinicopathologic characteristics with cancer stem cell markers (podoplanin and CD44v6)

The association of podoplanin and CD44v6 with clinicopathologic characteristics in lip squamous cell carcinomas is summarized in Table 3.

Fig. 1 The microscopical features of lip squamous cell carcinomas are illustrated in hematoxylin and eosin stain (a and b). In a, hyperplastic oral mucosa near to the neoplastic area and well-differentiated squamous cell carcinoma. In b, the pattern of invasion of well-differentiated squamous cell carcinoma showing island and cords of neoplastic cells in connective tissues. Strong immunorexpression of podoplanin (c and d) and CD44v6 (e and f) by tumor cells of the lip squamous cell carcinoma. In b and c, details of membranous/cytoplasmic podoplanin expression and CD44v6 membranous expression in the invasive front tumor, respectively. The reduction of CD44v6 immunorexpression by neoplastic cells when compared to oral mucosa is observed in d. (a, b, c, and f = 100×; d and e = 400×)

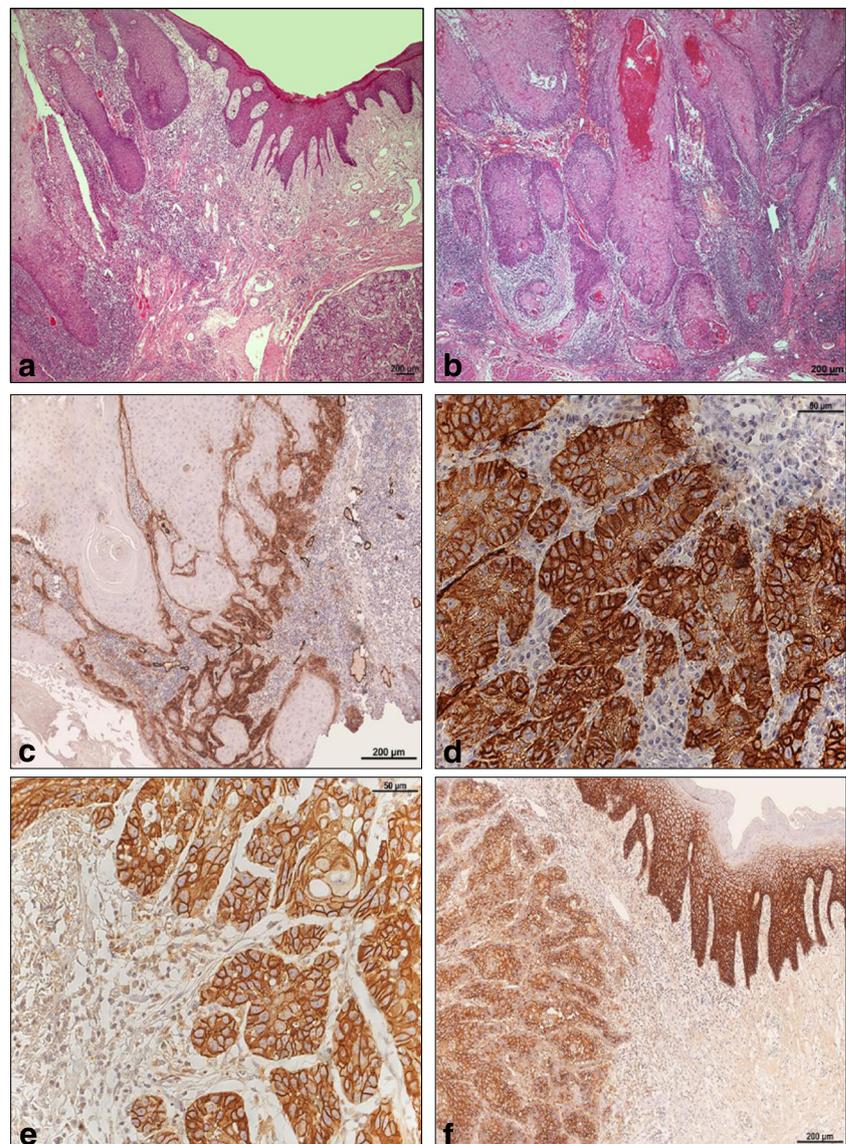


Table 3 Clinical and pathological features of the 91 patients with lip cancer and their associations with membranous podoplanin, cytoplasmic podoplanin, and CD44v6 expression. A.C. Camargo Cancer Center, São Paulo, Brazil, 1970 to 2009

Variable	Membranous podoplanin			Cytoplasmic podoplanin			CD44v6		
	<i>N</i>	Strong expression <i>N</i> (%)	<i>p</i>	<i>N</i>	Strong expression <i>N</i> (%)	<i>p</i>	<i>N</i>	Strong expression <i>N</i> (%)	<i>p</i>
Gender									
Male	75	46 (61.3)	0.931	75	45 (60.0)	0.853	73	70 (95.9)	0.533
Female	16	10 (62.5)		16	10 (62.5)		15	14 (93.3)	
Age*									
≤ 45	24	16 (66.6)	0.174	24	14 (58.3)	0.342	23	21 (91.3)	0.560
> 45 and ≤ 70	44	30 (68.2)		44	30 (68.2)		43	42 (97.7)	
> 70	22	10 (45.4)		22	11 (50.0)		21	20 (95.2)	
Smoke*									
Yes	65	43 (66.1)	0.160	65	41 (63.1)	0.824	62	59 (95.2)	1.000
No	15	07 (46.6)		15	09 (60.0)		15	14 (93.3)	
Alcohol*									
Yes	42	27 (64.3)	0.772	42	21 (50.0)	0.024	42	39 (92.8)	0.626
No	36	22 (61.1)		36	27 (75.0)		33	32 (97.0)	
Diameter*									
≤ 1 cm	28	18 (64.3)	0.837	28	18 (64.3)	0.253	26	26 (100)	0.190
> 1 cm and < 3 cm	32	19 (59.4)		32	16 (50.0)		32	31 (96.9)	
≥ 3 cm	27	18 (66.6)		27	19 (70.4)		26	23 (88.5)	
T stage*									
T1	27	19 (70.3)	0.489	27	14 (51.8)	0.405	36	35 (97.2)	0.321
T2	24	13 (54.1)		24	16 (66.6)		33	32 (97.0)	
T3/T4	13	08 (61.5)		13	06 (46.1)		17	15 (88.2)	
N stage*									
N0	54	32 (59.2)	0.182	54	30 (55.5)	0.710	52	51 (98.0)	0.111
N1/N2	14	11 (78.6)		14	07 (50.0)		14	12 (85.7)	
Radiotherapy*									
Yes	21	13 (61.9)	1.000	21	11 (52.4)	0.400	21	19 (90.5)	0.254
No	67	40 (59.7)		67	42 (62.7)		64	62 (96.9)	
Recurrence									
Yes	24	11 (45.8)	0.065	24	10 (41.7)	0.028	24	22 (91.7)	0.299
No	67	45 (67.2)		67	45 (67.2)		64	62 (96.9)	
Lymphatic embolization*									
Yes	09	07 (77.8)	0.642	09	04 (44.4)	0.282	09	07 (77.8)	0.195
No	23	16 (69.6)		23	15 (65.2)		22	21 (95.4)	
Perineural infiltration*									
Yes	11	08 (72.7)	0.850	11	07 (63.6)	0.877	11	09 (81.8)	0.252
No	23	16 (69.6)		23	14 (60.9)		22	21 (95.4)	
Compromised margins*									
Yes	02	01 (50.0)	1.000	02	01 (50.0)	1.000	02	02 (100)	1.000
No	85	52 (61.2)		85	53 (62.3)		82	78 (95.1)	
Lymph node (pN)									
No neck dissection	64	38 (59.4)	0.227	64	39 (60.9)	0.323	61	60 (98.4)	0.084
pN negative	12	10 (83.3)		12	09 (75.0)		12	11 (91.7)	
pN positive	15	08 (53.3)		15	07 (46.7)		15	13 (86.7)	
Histopathological malignant grade									
Low	42	31 (73.8)	0.026	42	26 (61.9)	0.791	41	40 (97.6)	0.620
Medium/high	49	25 (51.0)		49	29 (59.2)		47	44 (93.6)	
Total	91	56 (61.5)		91	55 (60.4)		88	84 (95.4)	

N, number of tumors. *p* value obtained by chi-square test or Fischer's exact test

p < 0.05 was considered statistically significant (in italic)

*Excluded patients without records

Statistical analysis revealed a significant association between cytoplasmic podoplanin expression, and alcohol consumption ($p = 0.024$) and locoregional recurrence ($p = 0.028$). In addition, CD44v6 expression was not significantly associated with clinicopathologic characteristics of lip cancer patients.

Spearman's correlation analysis demonstrated that CD44v6 expression was not correlated with membranous

($r = 0.163$, $p = 0.129$) or cytoplasmic podoplanin ($r = 0.157$, $p = 0.144$) in lip squamous cell carcinomas.

Survival data

The mean follow-up period of patients with lip cancer was 98 months (ranging from 0.03 to 394 months). The disease-

free survival test was used to analyze the following variables: gender, age, tobacco consumption, clinical T staging, radiotherapy, lymph node infiltration, histopathological malignancy grade, CD44v6 expression, membranous, and cytoplasmic podoplanin expressions (Table 4).

Multivariate analysis showed that gender, age, tobacco, radiotherapy, lymph node (pN), histopathological grade of malignancy, and CD44v6 had no influence on the disease-free survival rates of patients with lip cancer (Table 4).

During the follow-up, 24 locoregional recurrences occurred, and for lip cancer patients, the disease-free survival rates in 5 and 10 years were 75.7% and 67.1%, respectively. The multivariate analysis showed statistical significance for clinical T staging and cytoplasmic/membranous podoplanin expression. Patients presenting T3/T4 tumor clinical staging were more likely to develop locoregional recurrence when compared with individuals with clinical T1 and T2 ($p = 0.031$) tumors, as described in Table 4.

Strong cytoplasmic ($p = 0.035$) and membranous ($p = 0.014$) podoplanin expressions were associated with higher disease-free survival rates in 5 and 10 years, when compared

with absent/weak podoplanin expressions in lip cancer patients (Figs. 2 and 3).

The multiple Cox regression analysis revealed that the clinical T staging and podoplanin expression were independent prognostic factors for disease-free survivals (Table 5). Lip cancer patients presenting tumors with clinical staging T3/T4 showed more than three times the risk of developing locoregional recurrences when compared with patients who had tumors clinically classified as T1 and T2. Likewise, the lip cancer patients showing tumors with strong podoplanin expression presented 0.344 times (membranous) or 0.372 (cytoplasmic) times the risk of developing locoregional recurrences when compared with those patients who showed absent/weak podoplanin expression in the tumors (Table 5).

Discussion

In the last decade, the identification of cancer stem cell markers with the capacity to isolate tumor-initiating cells gave rise to new therapies that effectively targeted resistant cancer cells and

Table 4 Disease-free survival rates in 5 and 10 years of 91 patients with lip cancer according to the clinical, demographic, malignancy risk, podoplanin, and CD44v6 expressions. A.C. Camargo Cancer Center, São Paulo, Brazil, 1970 to 2009

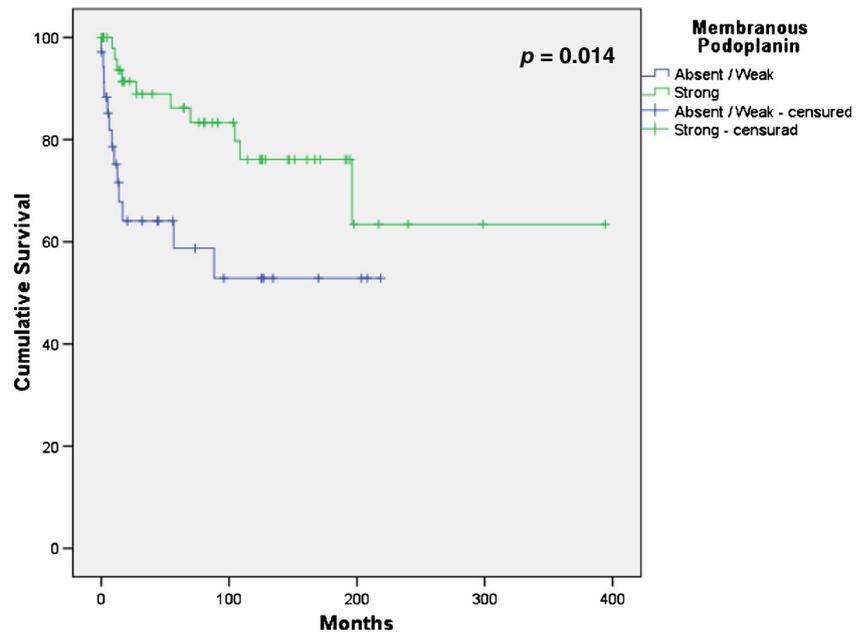
Variable		Disease-free survival		<i>p</i>
		5 years (%)	10 years (%)	
Gender	Male	78.5	68.2	0.602
	Female	64.5	64.5	
Age*	≤ 45	80.5	80.5	0.637
	> 45 and ≤ 70	75.5	59.2	
	> 70	65.0	65.0	
Tobacco*	Yes	78.4	72.3	0.250
	No	71.8	52.4	
T stage*	T1	85.0	75.7	0.031
	T2	81.7	76.3	
	T3/T4	48.8	24.4	
Radiotherapy	Yes	57.0	45.6	0.138
	No	80.9	72.5	
Lymph node (pN)	No dissection	80.0	71.0	0.189
	Negative	78.8	78.8	
	Positive	51.9	49.6	
Histopathological malignancy grade	Low	84.9	70.7	0.335
	Medium/high	68.3	64.5	
Membranous podoplanin	Absent/weak	58.7	52.9	0.014
	Strong	86.2	76.1	
Cytoplasmic podoplanin	Absent/weak	57.8	57.8	0.035
	Strong	86.8	72.2	
CD44v6	Absent/weak	33.3	33.3	0.270
	Strong	76.8	66.9	
Total		75.7	67.1	

p value obtained by log-rank test

$p < 0.05$ was considered statistically significant (in italic)

*Excluded patients without records

Fig. 2 Probability of cumulative disease-free survival in 5 and 10 years of 91 patients with lip squamous cell carcinoma presenting absent/weak and strong membranous podoplanin expression by malignant cells; $p < 0.05$ was considered statistically significant



prevented recurrences [9, 26]. Recent studies involving molecular biology have identified podoplanin and CD44v6 as cancer stem cell surface markers in oral squamous cell carcinomas [1, 24, 27]. However, the immunoprofile and the influence of podoplanin and CD44 on patients' prognosis may differ according to the location of the tumor in the oral cavity [26].

In this study, we analyzed the immunorexpression of podoplanin and CD44v6 in lip squamous cell carcinomas. Based on the positive expression of podoplanin, tumor cells showing strong membranous (61.5% of tumors) or cytoplasmic (60.4% of tumors) podoplanin expressions were localized mainly at the periphery of tumor islands. The center of the

tumor and well-differentiated cells, such as keratin pearls, demonstrated negative podoplanin expression, an immunoprofile similar to that observed by our research group and by others in oral cancer [11, 15, 16, 28, 29], including some incisional biopsies of lip squamous cell carcinoma [13]. We agree that the subcellular localization of podoplanin in neoplastic cells is of biological importance, and molecular changes of this protein from the membrane to cytoplasm may reflect its active or dormant state, as suggested by Yuan et al. (2006).

According to the cancer stem cell hierarchy, Shimada et al. (2009) and Ohta et al. (2013) suggested that podoplanin expression reflected the most immature status of cancer stem

Fig. 3 Probability of cumulative disease-free survival in 5 and 10 years of 91 patients with lip squamous cell carcinoma presenting absent/weak and strong cytoplasmic podoplanin immunorexpression by malignant cells; $p < 0.05$ was considered statistically significant

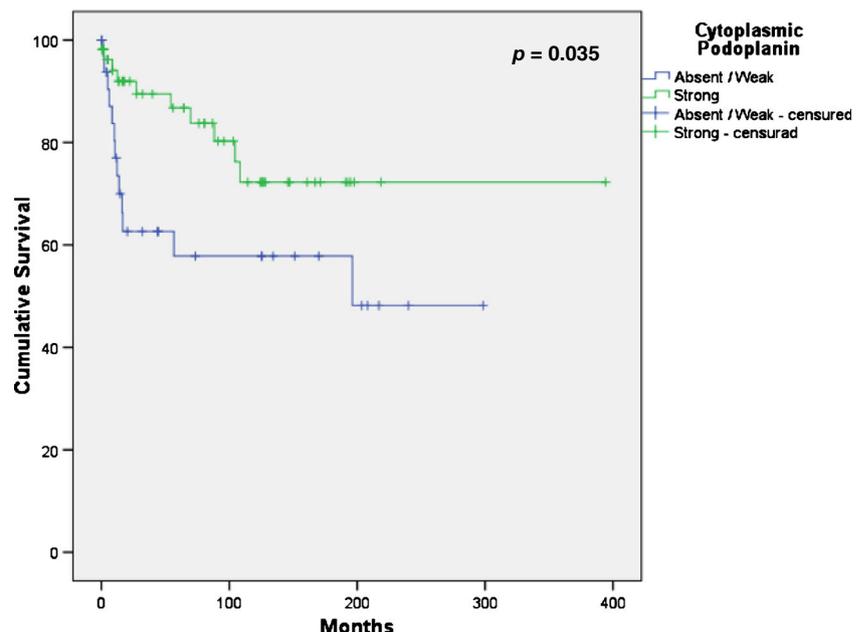


Table 5 Analysis by the Cox proportional hazard regression model of 91 patients with lip cancer according to T stage, membranous and cytoplasmic podoplanin expressions. A.C. Camargo Cancer Center, São Paulo, 1970 to 2009

Variable	Disease-free survival		
	OR	CI (95%)	<i>p</i>
T stage			
T1 (0)	3.01	(1.074; 8.440)	<i>0.036</i>
T3/T4 (1)			
Membranous podoplanin			
Absent/weak (0)	0.344	(0.144; 0.821)	<i>0.016</i>
Strong (1)			
Cytoplasmic podoplanin			
Absent/weak (0)	0.372	(0.152; 0.910)	<i>0.030</i>
Strong (1)			

p value obtained by Cox test; *OR*, odds ratio; *CI*, confidence interval. *p* < 0.05 was considered statistically significant (in italic)

cells in their differentiation process, and that this protein may be a marker of cells with capacity for further maturation in squamous cell carcinoma. Although the precise function of podoplanin controlling the signaling events in carcinogenesis has yet to be further clarified, considering the affirmations of Shimada et al. (2009) in lung cancer, the distribution of podoplanin in the present study and in our previous investigations [13, 15, 16], it was possible to speculate that podoplanin expression seemed to identify mainly immature squamous epithelial cells in oral cancer, including cancer stem cells.

One of our most relevant clinical results showed that strong membranous ($p = 0.016$) or cytoplasmic ($p = 0.030$) podoplanin expression was a favorable prognostic factor for patients with lip squamous cell carcinomas. The significant impact of podoplanin overexpression on disease-free survival rates in 5 and 10 years, for lip cancer patients (Table 4 and Figs. 2 and 3) confirmed that this protein could help to determine patients who were at lower risk for tumor recurrence. These results corroborated previous suggestions that tumors presenting strong podoplanin immunoexpression indicated a lower level of biological aggressiveness [23, 30] but contrasted with other evidences that associated podoplanin overexpression with more advanced stages of tumors and poor prognosis for oral cancer patients [29, 31–34].

In addition, the majority of lip cancer patients with locoregional recurrence ($n = 24$) showed weak/absent membranous (14 tumors) or cytoplasmic (13 tumors) podoplanin expression. The association of cytoplasmic podoplanin expression with locoregional recurrence was statistically significant ($p = 0.028$), as illustrated in Table 3. Likewise, based on the Brandwein-Gensler histopathological malignancy grade classification, the majority of patients with strong podoplanin expression were classified as being at low risk for local recurrences and having higher overall

survival rates. Confirming these results, Tsuneki et al. (2013) also demonstrated that podoplanin-positive tumors were associated with a lower risk for local recurrence in oral cancer patients. Strong cytoplasmic podoplanin expression was also statistically associated with alcohol consumption in our study ($p = 0.024$), but this association needs to be further investigated.

As regards CD44v6 immunoexpression, strong membranous positivity by malignant cells was observed in 95.4% of the lip cancers with a slight loss of expression when compared with the adjacent normal oral mucosa (Fig. 1d). No association of CD44v6 expression with the clinicopathologic features of lip cancer patients was found (Table 3). As the majority of lip tumors in our study were well-differentiated squamous cell carcinomas, this pattern of CD44v6 distribution, as has been described in other studies of oral cancer [1, 24], was expected. The evidence that membranous and cytoplasmic CD44v6 expression enhances according to tumor progression [3] was not confirmed in our sample of lip cancers that ranged from T1 to T4 tumors.

Moreover, the CD44v6 had no influence on the disease-free survival rates of lip cancer patients (Table 4), in contrast with other studies that verified a significant influence of this protein on tumor behavior and prognosis of oral cancer [1, 3, 4, 24, 25]. However, results similar to those found in our sample reinforced the findings that isolated CD44v6 expression was of no prognostic relevance for patients with lip cancer [35, 36].

We also aimed to investigate the correlation between these cancer stem cell markers, podoplanin, and CD44v6 in lip cancer, based on the hypothesis that podoplanin and CD44v6 are co-localized and activated by a common signaling pathway. However, in our sample, no correlation was found between CD44v6 and membranous ($r = 0.163$, $p = 0.129$) or cytoplasmic ($r = 0.157$, $p = 0.144$) podoplanin expression.

The joint performance of podoplanin with CD44 was demonstrated in aggressive squamous cell carcinomas suggesting that these molecules acted together in the neoplastic cell migration process due to their property of binding to the cellular cytoskeleton through molecules of the ERM (Ezrin, Radixin, Moesin) complex [22]. However, a more recent study [23] revealed that podoplanin and CD44 acted primarily in the neoplastic cell adhesion process of oral squamous cell carcinoma. In our present study, the results showed that podoplanin was a protection factor in lip squamous cell carcinoma, leading us to believe that the primary function of this molecule was in the process of adhesion between malignant cells, and that the loss of podoplanin expression would lead to more aggressive neoplasms with a higher chance of locoregional recurrence. We suggest that additional in vitro analyses must be performed to confirm our previous results and the role of podoplanin and CD44v6 in lip tumor progression.

Conclusions

The stem cell biomarkers, podoplanin and CD44v6, were strongly expressed by lip tumor cells, and podoplanin immunorexpression could help to determine the lip cancer patients at lower risk for disease recurrence.

Contributions Alexandre Simões Garcia contributed to the study concept, design, data collection, histopathological analysis, and writing. Agnes Assao contributed to the study concept, data collection, and writing. André Lopes Carvalho contributed to the statistical analysis. Fernando Augusto Soares contributed to the study concept and providing the infra-structure. Luis Paulo Kowalski contributed to the study concept. Denise Tostes Oliveira contributed to the study concept and design and critically revised the manuscript. All authors approved the publication.

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Compliance with ethical standards

The Research Ethics Committee of the A.C. Camargo Cancer Center, São Paulo, Brazil, approved this study (process #1778/13 - CAAE 01594912.0.3001.5432).

Conflict of interest The authors declare that they have no conflict of interest.

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