Angiogenesis and lymphangiogenesis in early-stage laryngeal carcinoma: Prognostic implications

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ABSTRACT: *Background.* Many studies have recently emphasized the role of tumor angiogenesis and lymphangiogenesis in regional and distant spread of disease. Although early laryngeal cancer has a favorable oncologic outcome after conservative surgery or radiation therapy, we observed few cases with poor prognosis in terms of locoregional relapse, organ preservation, and survival. The aim of our study was to evaluate the immunohistochemical expression of CD31 and podoplanin to define angiogenic and lymphangiogenic patterns and their possible prognostic implications in previously untreated T1–T2 glottic squamous cell carcinoma.

Methods. Four hundred twenty-eight patients with previously untreated early-stage laryngeal cancer underwent a laser surgical resection in the period between January 1994 and December 2007. Twenty-seven cases with poor outcome were identified and compared with a selected sample of 28 patients. All specimens were negative for the presence of high-risk human papillomavirus genotypes. Patients were followed up until death or for at least 24 months after treatment. Three-micrometer sections were obtained from formalin-fixed and paraffin-embedded tumoral tissues, and an immunohistochemical evaluation was performed. Monoclonal antibodies against CD31 and podoplanin were used for the detection of blood and lymphatic vessels, respectively. A morphometric measurement was used for the analysis of angiogenesis whereas lymphangiogenesis was studied with a semiquantitative technique. The data were analyzed by use of chi-square and Mann-Whitney tests as appropriate.

Results. An increased tumor angiogenesis correlated with local relapse ($p = .01$), locoregional relapse ($p = .01$), and death of disease ($p = .03$). The presence of lymphatic vessels in peritumoral fields had an impact on local ($p = .004$) and locoregional recurrence ($p = .01$).

Conclusions. Evaluation of angiogenesis and lymphangiogenesis in early-stage laryngeal cancer could be useful to identify patients at higher risk of recurrence and consequently to modulate treatment planning and follow-up strategy. © 2012 Wiley Periodicals, Inc. *Head Neck* **00**: 000–000, 2012

KEY WORDS: angiogenesis, lymphangiogenesis, podoplanin, laryngeal cancer

INTRODUCTION

The study of biologic mechanisms underlying tumor progression continues to be a challenging area of research. Several studies have evaluated the possible clinical and prognostic relevance of angiogenesis and lymphangiogenesis in head and neck squamous cell carcinoma (SCC), but their role is still a matter of debate.[1–4] Angiogenic and lymphangiogenic patterns in laryngeal SCC were investigated in few studies with contrasting results,[3,5,6] which may reflect different methodologic strategies adopted.

Early-stage glottic cancer shows a favorable good oncologic outcome either after transoral CO_2 laser surgery (TLS) or radiation therapy.[7,8] The 5-year disease-specific survival rate of patients affected by T1–T2 glottic cancer treated with TLS is 99% and 98%, respectively.[7]

Nevertheless, it is common to find in this cluster of patients a few cases with poor prognosis in terms of locoregional control, organ preservation, and survival, probably in view of their biologic profile. The variability in the patterns of expression of angiogenesis and lymphangiogenesis could be one of the factors able to influence the prognosis.

The aim of our study was to evaluate the angiogenic and lymphangiogenic patterns in a selected group of T1–T2 glottic SCC treated by TLS by looking with immunohistochemical (IHC) studies at the expression of CD31 and podoplanin.

PATIENTS AND METHODS

Patient selection

Between January 1994 and December 2007, 428 patients affected by previously untreated early-stage (T1–T2) laryngeal SCC underwent TLS at the Department of Otorhinolaryngology of the University of Brescia and were monitored until death or for at least 24 months. Their clinical history was negative for other benign or malignant head and neck lesions. A group of 27/428 (6.3%) patients with poor prognosis in terms of local and regional control, organ preservation, and determinate survival was identified. Fourteen (52%) patients had a single

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TABLE 1. Clinical and histopathologic features of the 2 groups of patients (group 1: patients with poor prognosis; group 2: patients with favorable prognosis;).

	Group 1 ($n = 27$)	Group 2 ($n = 28$)
Clinical features		
Mean age	62 y	62 y
Smoking habits	26 (96%)	26 (93%)
Supraglottic extension	4 (15%)	5 (18%)
Subglottic extension	2 (7%)	2 (7%)
Ventricular extension	3 (11%)	1 (4%)
Anterior commissure involvement	3 (11%)	9 (32%)
Histopathologic features		
High-risk HPV positivity (ISH)	—	—
Grading		
G1	3	5
G2	14	13
G3	10	10
pT classification		
pT1	8	9
pT2	19	19
VM infiltration	10	11
Close margins (< 1 mm)	15	16

Abbreviations: AC: anterior commissure; VM: vocal muscle; ISH: in situ hybridization; HPV: human papillomavirus.

local recurrence (6 treated by a second CO_2 laser excision and 8 by total laryngectomy); 7 (26%) had more than 1 local recurrence (4 of them ultimately underwent total laryngectomy); 5 (18%) had a locoregional recurrence (one of which required total laryngectomy); 1 (4%) had a nodal recurrence treated by neck dissection. Four (14%) of 27 patients in the first group died of disease. The 5-year determinate survival, local recurrence, regional recurrence, and organ-preservation rates were 87.3%, 96.3%, 19.7%, and 61.4%, respectively. A second group of 28 patients with favorable prognosis (no recurrence, no death from the disease) was identified from our files and compared with the previous one in a case-matched fashion. Patients included in the second group were carefully selected to avoid any statistically significant difference in clinical and histopathologic findings (Table 1). All patients considered were negative for high-risk human papillomavirus (HPV) infection excluded by HPV in situ hybridization assay, with the automated Bond-Max system (Leica Microsystems, Buffalo Grove, IL).

Tissue samples

Fifty-five formalin-fixed, paraffin-embedded tissue blocks obtained from surgical specimens were selected from the archives at the Department of Pathology of the University of Brescia. Approval for the use of human tissue samples was obtained from the Hospital Ethical Committee.

Immunohistochemistry

Angiogenesis and lymphangiogenesis were evaluated by IHC in 3-μm sections from formalin-fixed, paraffin-embedded tissue blocks with monoclonal antibodies against CD31 and podoplanin (Mouse IgG1, clone 1A10, dilution 1:50; Novocastra Laboratories, Newcastle Upon Tyne, United Kingdom; and Mouse IgG1, clone D2-40, dilution 1:40; AbD Serotec, Oxford, United Kingdom, respectively). Two sections were obtained for each block at the level of major depth of neoplastic infiltration. The reactions were revealed with EnVision-HRP Mouse/Rabbit Polymer (Dako, Glostrup, Denmark) followed by diaminobenzidine as chromogen. Sections were then counterstained with Mayer's hematoxylin (Figure 1).

For evaluation of stromal peritumoral podoplanin–positive cells, 4 serial sections of 5 cases were stained (as described above) with anti-CD31, anti-podoplanin, anti-calponin (Mouse IgG1, clone CALP, dilution 1:100; Dako), and anti-cytocheratin B903 (Mouse IgG1, clone 34βE12, dilution 1:30; Thermo Fisher Scientific, Waltham, MA).

Evaluation of staining

A morphometric-quantitative evaluation was carried out for angiogenesis: 2 peritumoral fields at original magnification × 10 showing the highest vascular density and 2 showing the lowest vascular density were selected by the operator and digitally acquired with an Olympus BX-60 microscope equipped with a DP-70 camera (Olympus Optical Corporation LTD, Tokyo, Japan).

For each field, the number, the mean perimeter, and area of CD31-positive hematic vessels were measured by use of CELLF 2.5 software (Olympus Soft Imaging Solutions GMBH, Münster, Germany). For

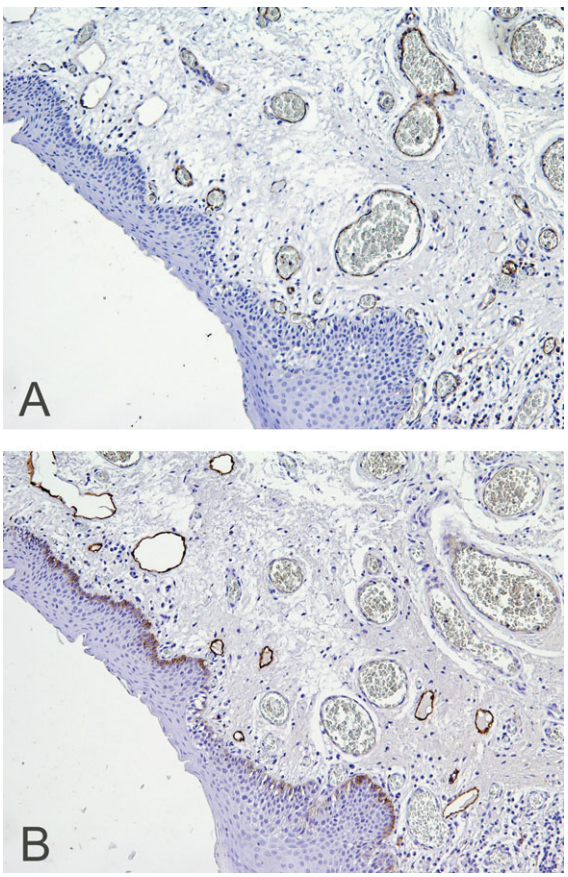

FIGURE 1. Expression of CD31 (A) and podoplanin (B) in hematic and lymphatic vessels comparing 2 serial sections. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE 2. Correlation between vascular surface/mm^2 and outcome.

	Vascular surface/mm^2		
	Patient with poor prognosis	Patient with favorable prognosis	*p* value
Dead of disease	0.12	0.08	.034
Local relapse	0.89	0.78	.329
Regional relapse	0.066	0.085	.244
Need for total laryngectomy	0.86	0.71	.101

lymphangiogenesis we used a semiquantitative evaluation, which distinguished patients with peritumoral lymphatic vessels from those without. Two different assessment approaches in angiogenesis and lymphangiogenesis evaluation were adopted because the density of blood vessels was always significantly higher than that of lymphatic vessels.

Double immunofluorescence staining

Five cases, with stromal peritumoral podoplanin positive cells, were further double stained with anti-podoplanin and anti-smooth muscle actin (Mouse IgG2a, clone 1A4, dilution 1:100, Thermo Scientific) by use of an immunofluorescence technique. Appropriate Texas-Red and FITC-conjugated isotype-specific secondary antibodies (dilution 1:75, 30′ incubation; SouthernBiotech, Birmingham, AL) were used to reveal the reaction. DAPI was used as counterstaining.

Statistical analysis

Statistical analysis was performed with a commercially available computer software package (SPSS for Windows; version 10.0.1, 1999 Chicago, IL). Survival outcomes were calculated by the Kaplan–Meier method, and different subgroups were compared by use of the log-rank test. For univariate comparisons of different IHC patterns and clinicopathologic data, chi-square and Mann-Whitney tests were adopted for categorical and continuous variables, respectively. All *p* values reported were considered significant when less than .05.

RESULTS

Immunohistochemical findings

The comparison between angiogenesis data (vascular surface/mm^2, mean vessel number/mm^2, mean area, and mean perimeter of vessels) and outcomes (death of disease, tumor relapse, and need for salvage total laryngectomy) revealed that a higher mean vessel area and a higher vascular surface per mm^2 were both related with a higher risk of dead for the disease ($p = .043$ and .034, respectively) (Tables 2 and 3). Moreover, a higher number of vessels was related to a higher risk of local relapse ($p = .011$) (Table 4).

TABLE 3. Correlation between mean area of blood vessels expressed in μm^2 and outcome.

	Mean area of blood vessels, μm^2		
	Patient with poor prognosis	Patient with favorable prognosis	*p* value
Dead of disease	1655	1101	.043
Local relapse	1217	1068	.469
Regional relapse	843	1167	.169
Need for total laryngectomy	1122	1138	.451

TABLE 4. Correlation between mean blood vessel number/mm^2 and outcome.

	Mean blood vessel number/mm^2		
	Patient with bad prognosis	Patient with good prognosis	*p* value
Dead of disease	110	84	.578
Local relapse	89	70	.011
Regional relapse	78	78	.295
Need for total laryngectomy	93	74	.179

With regard to lymphatic vessels, we found peritumoral podoplanin–positive lymphatic vessels in 11/28 (39%) patients with good prognosis and in 19/27 (70%) patients with poor prognosis. The presence of lymphatic vessels significantly correlated with local relapse ($p = .004$) (Table 5).

In 9/55 (16%) patients (2 with poor prognosis and 7 with favorable prognosis), we could not measure peritumoral lymphatic vessels because of diffuse stromal peritumoral cells podoplanin staining. The presence of podoplanin-positive peritumoral stromal cells was not related to any of the previously mentioned outcomes.

Characterization of stromal peritumoral podoplanin–positive cells

Because we found stromal peritumoral podoplanin–positive cells in 22 cases (13/28 patients with favorable prognosis and 9/27 patients with poor prognosis), we studied these cells with serial immunohistochemical staining for CD31, podoplanin, cytokeratin B903, and calponin, together with double podoplanin–smooth muscle actin immunofluorescence staining. These cells showed expression of podoplanin and calponin (Figures 2 and 3) and coexpression of podoplanin and smooth muscle actin in stromal peritumoral cells (Figure 4). As a consequence of this profile, these cells were finally defined as peritumoral-activated myofibroblasts.

TABLE 5. Correlation between the presence of peritumoral lymphatic vessels and outcome.

	Presence of peritumoral lymphatic vessels
Dead of disease	$p = 1$
Local relapse	$p = .004$
Regional relapse	$p = 1$
Need for total laryngectomy	$p = 0.493$

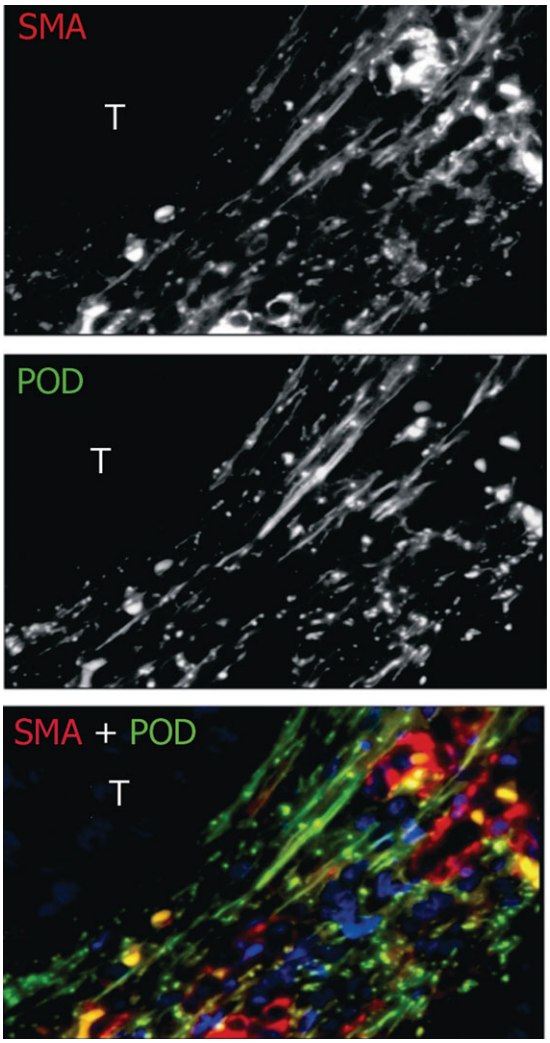

FIGURE 2. Serial immunohistochemical staining for podolanin, CD31, cytokeratin B903 and calponin (original magnification × 10). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

DISCUSSION

Early-stage laryngeal SCC has good oncologic outcomes after surgical or radiation therapy in terms of local and locoregional relapse other than distance metastasis. Nevertheless, few patients with unfavorable prognosis are commonly observed, suggesting a different tumor biologic behavior, which may be related to angiogenic and lymphangiogenic mechanisms.

Angiogenesis has been reported to be a prognostic indicator of outcome in a variety of head and neck SCCs,[6,9,10] but many controversies are reported in the literature. In 2000, Homer et al[1] analyzed 16 studies in a review on angiogenesis in head and neck SCC, and found in 8 a positive correlation between tumor microvascular density (MVD) and T/N classification, T/N relapse, and radiosensitivity of the tumor. Although several studies supported the crucial role of angiogenesis in laryngeal SCC and its impact on prognosis,[3,5,11] other authors did not found any correlation.[12,13] As suggested by Martone et al,[5] this wide range of results could be explained by heterogeneity of tumor stage and size and differences in the method adopted. In this setting, our belief is that some early-stage tumors with particular biologic features may be associated with a more aggressive behavior, probably for the fact that the growth and development of new blood vessels could be a crucial event even in early-stage lesions. Our hypothesis is confirmed by the observations of Sion-Vardy et al,[14] who found no difference in MVD in relation to the stage of disease in 59 patients with stage I–IV laryngeal cancer. Furthermore, Marioni et al[11] in their study on 43 patients observed the absence of correlation between staging and angiogenesis.

All reports in the literature studied angiogenesis in terms of vascular density at the periphery or in the context of the tumor without objective morphometric measurements of the vessels. By contrast, as previously reported in a study of our group on angiogenesis and lymphangiogenesis of advanced oral/oropharyngeal cancer,[4] a morphometric evaluation of the tumor vessels was performed, and the area and perimeter other than the vascular surface/mm^2 were measured. With regard to MVD (number/mm^2), many studies on laryngeal SCC (stage I–IV) revealed a correlation between this parameter and locoregional recurrence,[11] disease-free survival,[15] and regional recurrence in node-negative patients.[3] Nevertheless other studies denied any correlation between MVD and prognosis, tumor recurrence rate, and tumor-related death.[12,13] In our study we demonstrated a significant correlation between vessel number/mm^2 and local ($p = .011$) or locoregional ($p = .011$) recurrence, showing a possible crucial role of angiogenesis also in early-stage lesion of the larynx. We attempted to define other angiogenic parameters that could predict the aggressive behavior in early phases of tumor progression to identify patients requiring a more aggressive treatment. A higher mean area of the vessels and a higher vascular surface/mm^2 correlated with death of disease ($p = .043$ and $p = .034$, respectively). The presence of vessels with larger caliber, and consequently a higher vascular surface/mm^2, could promote tumor growth with a higher local and locoregional aggressiveness.

The presence of regional lymph node metastases is an event with a strong impact on prognosis in head and neck SCC,[16] and laryngeal SCC as well.[17] Consequently, the knowledge and the role of tumor lymphangiogenesis in tumor cells spreading and locoregional relapse could be a good indicator to identify tumors with potentially more aggressive behavior. Lymph node involvement in early-stage glottic laryngeal SCC is a rare finding because of the low density of lymphatic vessels, particularly at the level of the mucosa.[18–20] The study of lymphangiogenesis has only recently become a relevant issue in cancer research to identify markers predictive of locoregional spread. The identification of antigens expressed selectively by lymphatic cells has allowed the study of these mechanisms. Several authors evaluated tumor lymphangiogenesis in terms of peritumoral or intratumoral lymphatic density.[21,22] The correlation between the presence of a high density of peritumoral lymphatic vessels and locoregional recurrence was frequently reported in head and neck SCC.[22,23] However, intratumoral lymphatic

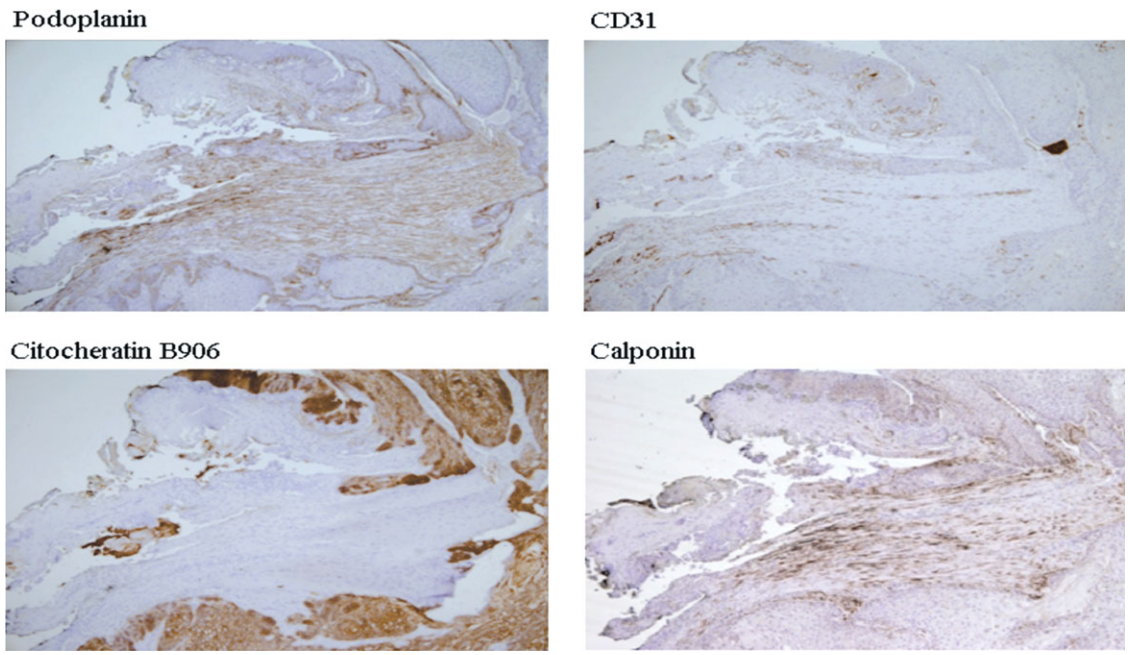

FIGURE 3. The same peritumoral stromal cell in serial sections: immunohistochemical coexpression of podoplanin and calponin (original magnification × 30). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

density is negligible when compared with the peritumoral density,[24,25] and in laryngeal SCC intratumoral lymphatics can be rarely demonstrated.[19] Even in our specimens, the intratumoral vessel positivity for podoplanin was an unusual finding. This prompted us to evaluate only the presence or absence of peritumoral lymphatic vessels as marker of lymphangioenesis. Moreover, a morphometric evaluation as we did for angiogenesis was not feasible in view of the low number of lymphatic vessels.

Our results showed a significant correlation between the presence of peritumoral lymphatic vessels and locoregional relapse ($p = .013$) and relapse at the primary site ($p = .004$). It is not clear why the tumor lymphangiogenic activity did not correlate with the occurrence of lymph node metastasis, but this finding may depend on the low prevalence of this event in our series. Similar results have been observed for angiogenesis, thus leading to the hypothesis suggesting that tumor-induced proliferation of lymphatic and blood vessels may be mediated by similar molecules, as suspected by Homer et al[26] for head and neck SCC. It is indeed well known that the tumor produces growth factors as vascular endothelial growth factor (VEGF) and VEGF-C (a closely related protein belonging to the VEGF family) that can promote angiogenesis and lymphangiogenesis simultaneously.[1] The presence of both lymphatic and blood vessels may facilitate local spread and consequently a more aggressive local behavior of early-stage laryngeal SCC.

Immunohistochemical evaluation revealed 22 specimens with peritumoral podoplanin–positive stromal cells. Because we assumed that they could be activated myofibroblasts, we decided to characterize these cells with further investigations in 5 cases. Serial immunohistochemical staining with anti-CD31, anti-podolanin, anti-cytocheratin B903, and anti-calponin suggested that these cells were stromal peritumoral cells. The double podoplanin–smooth muscle actin immunofluorescence staining showed coexpression in stromal peritumoral cells. This finding confirmed that these cells were activated myofibroblasts. The

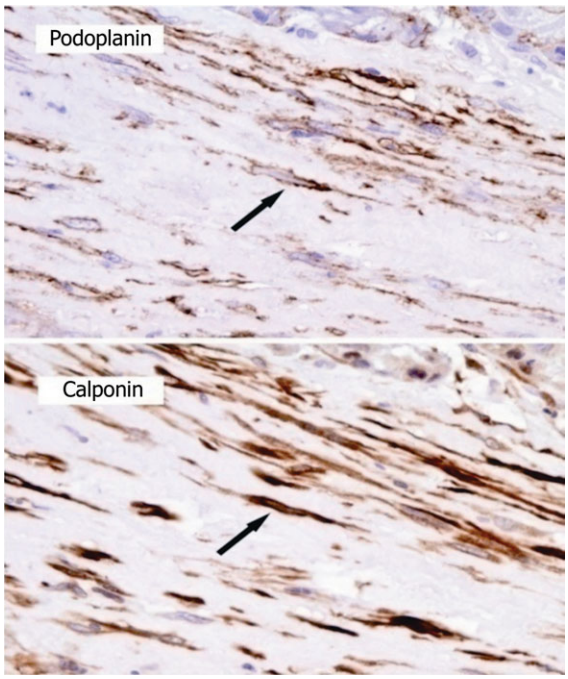

FIGURE 4. Double podoplanin–smooth muscle actin immunofluorescence staining of the peritumoral field. POD, podoplanin; SMA, smooth muscle actin; T, tumor. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

literature offers a contrasting view on the role of these cells. In 1 study on colon cancer, the detection of this cell population was interpreted as having a protective role, producing an abundant amount of extracellular matrix, able to circumscribe cancer growth.[27] In other studies on undifferentiated colon cancer or adenocarcinoma of the lung, these cells were associated with a bad prognosis, producing hematic and lymphatic vessel growth factors.[27,28] In a study on laryngeal cancer, Zidar et al[29] detected myofibroblasts only around the cancer island but not around dysplastic areas, indicating that invasion beyond the basement membrane is necessary to evoke a myofibroblastic stromal reaction. They observed at least 2 patterns of stromal reaction: one, in moderately differentiated cancer, was characterized by a marked proliferation of myofibroblasts and desmoplasia, with scarce lymphocytic infiltration; one, in poorly differentiated cancer, was characterized by few fibroblast, weak desmoplasia, and dense lymphocytic infiltration.

In our material, no significant correlation between the presence of these cells and prognostic outcome was found. Nine of 22 patients with activated myofibroblast had development of regional or locoregional relapse, and other 13 were free of disease without any recurrence.

CONCLUSION

In conclusion, having clear in mind the limitations of this study, that is, the small group of patients and possible bias caused by selection of the 2 groups, a simple and quick IHC evaluation of the specimen for CD31 and podoplanin could offer adjunctive information on early stage laryngeal SCC to more clearly depict the features of the lesion to properly modulate the treatment and to modify follow-up strategy on a case-specific basis. Further studies are needed to validate these findings that, if confirmed, could delineate this easy IHC procedure as a promising tool in the management of early stage laryngeal SCC.

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