ORIGINAL ARTICLE

IMMUNOSTAINING PATTERNS OF CD31 AND PODOPLANIN IN PREVIOUSLY UNTREATED ADVANCED ORAL/ OROPHARYNGEAL CANCER: PROGNOSTIC IMPLICATIONS

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Abstract: *Background.* The objective of this study was to assess angiogenesis and lymphangiogenesis patterns in advanced oral/oropharyngeal cancer by immunohistochemical techniques.

Methods. Forty-five patients with advanced oral/oropharyngeal cancer, treated by primary surgery between January 1996 and December 2005, were selected. All cases were followed for at least 24 months. Angiogenesis and lymphangiogenesis were evaluated with antibodies against CD31 and podoplanin, respectively. Survival outcomes were calculated by the Kaplan–Meier method, whereas univariate comparisons were obtained by log-rank, chi-square, and Mann–Whitney tests.

Results. Survival correlated with the area of peritumoral blood vessels ($p = .02$), whereas the number of intratumoral lymphatics ($p = .02$) correlated with the occurrence of nodal metastasis. The risk for distant metastasis correlated with the perimeter of intratumoral lymphatics ($p = .02$).

Conclusions. Peritumoral angiogenesis presented different expression patterns between survivors and patients who died of disease. Intratumoral lymphangiogenesis was correlated with a higher risk of developing lymph node (LN) and distant metastasis. *Head Neck* **32:** 786–792, 2010

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Although in the recent decades early diagnosis of neoplastic diseases and their profound social awareness has dramatically improved disease-specific survival (DSS), lymph node and distant spread of the disease represent the main prognostic indicators.

Nodal status is generally considered 1 of the most important prognostic factors in patients with head and neck squamous cell carcinoma (HNSCC).[1] Tumor cells may invade both blood and lymphatic vessels, causing regional and distant spread of disease. The mechanisms underlying metastatic dissemination of disease have recently become a challenging area of research. Many investigations have studied the molecular basis of blood and neoformation of lymphatic vessels,[2–4] and have outlined a complex scenario in terms of physiologic mechanisms and a potential role in neoplastic progression. Specifically,

in patients with head and neck cancer, special attention was given to the potential prognostic significance of intra- and peritumoral angiogenesis and lymphangiogenesis by examining vascular density through the application of specific markers such as podoplanin, LYVE-1, prox-1, and VEGFR3 for lymphatic endothelial cells[2,3] and CD31 and CD34 for hematic cells.[5,6]

The present study used immunohistochemistry (IHC) for CD31 and podoplanin to evaluate angiogenesis and lymphangiogenesis and their impact on nodal and distant spread in a cohort of patients affected by previously untreated advanced oral/oropharyngeal cancer.

MATERIALS AND METHODS

Patients. Between January 1996 and December 2005, 45 patients (male/female, 5/1; mean age, 59 years; range, 37–77 years) affected by previously untreated advanced (stage III and stage IV) oral (62%) or oropharyngeal (38%) SCC, underwent surgical treatment at the Department of Otorhinolaryngology, University of Brescia (Italy). Unilateral (64%) or bilateral (36%) neck dissections were also performed. In general, patients received bilateral neck dissection when the lesion was crossing the midline, or preoperative ultrasound examination detected contralateral lymph nodes that were suspicious for malignancy. The extent of neck dissection was modulated according to the site of the primary tumor and clinical neck status. Clinical classification of disease was accomplished by physical examination, combined with ultrasound and CT or MRI. Neck dissections were classified according to the latest neck dissection classification update.[7]

Other inclusion criteria were (1) no previous surgery on the lateral compartment of the neck for either benign or malignant lesions and (2) no previous treatment for other head and neck primaries. Patients were followed until death or for at least 24 months after the end of treatment.

Reconstruction at the primary site was always performed with pedicled (40%) or free flaps (60%). Forty (90%) patients underwent adjuvant treatment: radiotherapy (RT) alone in 37 (93%) cases and concomitant chemo-RT in 3 cases (7%), with extracapsular spread (ECS) and/or multiple lymph node metastases involving the lower neck levels (levels IV and VB). All primary tumors and neck metastases were staged in accord with the sixth edition of the American Joint Committee on Cancer (AJCC) TNM staging system.[8]

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

Immunohistochemistry. Angiogenesis and lymphangiogenesis were evaluated using IHC on 3-μm sections from formalin-fixed, paraffin-embedded tissue blocks using monoclonal antibodies against CD31 (NCL-CD31-1A10; Novocastra, Newcastle upon Tyne, UK) and podoplanin (D2-40, MCA2543; AbD Serotec Morphosis, Oxford, UK), respectively. Sections were dewaxed in xylene and rehydratated using a series of graded ethanol solutions. Endogenous peroxidase activity was blocked with 0.3% H_2O_2 solution in methanol for 20 minutes. The slides were heated in a microwave oven for 3 cycles of 5 minutes each at 750 W in ethylenediaminetetraacetic acid (EDTA, pH 8.0) for antigen retrieval. After washing with Tris-HCl buffer solution, the primary antibodies against CD31 (1:50 dilution) or podoplanin (1:40 dilution) were applied for 60 minutes. The Super Sensitive IHC Detection System (BioGenex, San Ramon, CA) and the EnVision System (EnVision+ System HRP Labeled Polymer K4000; DakoCytomation, Carpinteria, CA) were adopted for CD31 and podoplanin, respectively, to visualize primary antibodies. Diaminobenzidinetetrahydrochloride was used as the chromogen; sections were subsequently counterstained with hematoxylin (see Figure 1).

Evaluation of Staining. Four ×10 magnification fields, representative of peritumoral (2) and intratumoral (2) areas showing the highest vascular density, were digitally acquired using an Olympus BX-60 microscope equipped with a DP-70 camera (Olympus Optical Corp., Tokyo, Japan). No correspondence between the spots with highest density of CD31 and podoplanin was observed, and consequently different areas were selected for the analysis of these molecules. Moreover, CD31 hotspots showed a negligible number of lymphatics, allowing us to use CD31

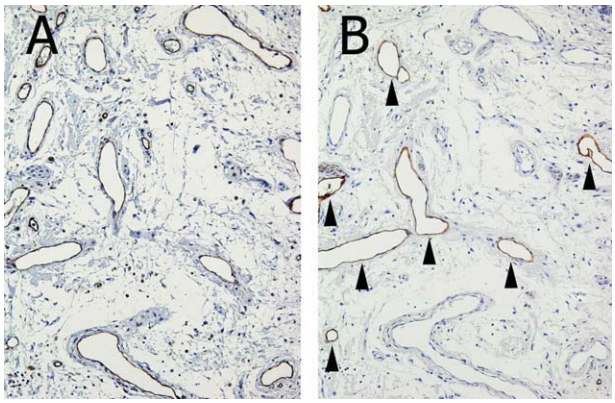

FIGURE 1. Immunohistochemical staining of vessels with CD31 and podoplanin in 2 sequential sections of normal submucosal tisuue. (**A**) Both blood and lymphatic vessels express CD31. (**B**) In contrast, lymphatic vessels (black arrowheads) were selectively stained by antibody D2-40 against podoplanin (counterstained with hematoxylin; original magnification, × 10). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

as a specific marker for blood vessels. For each field, the number, the mean perimeter, and area of podoplanin-positive lymphatic and CD31-positive blood vessels were measured by using CELL^F 2.5 software (Olympus Soft Imaging Solutions, Munich, Germany). Lymphatic vessel invasion by tumor nests was also assessed on slides immunostained with D2-40 antibody.

Statistical Analysis. Statistical analysis was performed using 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 with use of the log-rank test. The influences on DSS of different clinicopathologic factors such as patient sex, primary site, grading, pathologic T (pT) status, pathologic N (pN) status, stage, ECS, and endolymphatic embolization were evaluated. 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 if $<.05$.

RESULTS

Oncologic Outcomes. Data concerning postoperative T and N status are summarized in Table 1. Staging of lesions was as follows: stage III in 6 (13%) cases, IVa in 38 (85%), and IVb in 1 (2%). Fifteen (33%) patients had no lymph node metastasis (pN0), and 30 (67%) were positive for nodal disease (pN+); ECS was detected in 17 (38%) cases. The mean follow-up was 54 months (range, 4–136 months). Twenty-three (51%) patients were free of disease, and 22 (49%) died of disease: 12 (27%) for local recurrence, 6 (13%) for distant metastases (lung in 5 cases, bones and liver in 1 case), 2 (4%) for locoregional recurrence, and 1 (2%) each for regional recurrence and a second tumor. Five-year DSS was 59.5%. Among all factors considered (Table 2) only stage IV ($p = .02$), presence of neck metastasis ($p = .04$), and ECS ($p = .001$) correlated with poor outcome.

Immunohistochemistry Findings. The mean number, perimeter, and area of intratumoral and peritumoral podoplanin-positive lymphatic and CD31-positive blood vessels are reported in Tables 3 and 4. The number of peritumoral blood and lymphatic vessels was significantly increased compared with intratumoral values ($p = .001$). Moreover, the perimeter of intratumoral blood vessels was significantly increased compared with the peritumoral value ($p = .01$).

The number, perimeter, and area of both blood and lymphatic vessels were statistically compared in relation to different clinical parameters, such as patient status (alive vs died of disease), postoperative nodal status, and detection

Table 1. pT and pN staging of the patient population ($n = 45$).

	No. (%) by pathologic N classification					
T classification	pN0	pN1	pN2a	pN2b	pN2c	Total
pT3	4 (31)	2 (15)	—	5 (39)	2 (15)	13 (29)
pT4a	11 (35)	5 (17)	1 (3)	12 (39)	2 (6)	31 (69)
pT4b	—	—	—	1 (100)	—	1 (2)
Total	15 (33)	7 (16)	1 (2)	18 (40)	4 (9)	45

Abbreviations: pT, pathologic T (primary classification); pN, pathologic N (classification correlates with the number of nodes examined).
Note: All rates represent fractions of totals in each row, unless indicated otherwise.

Table 2. Oncological outcomes: influence of different clinicopathologic factors on DSS.

Factor	No. of patients (%)	5-year DSS	*p* value
Sex			.27
Male	38 (84)	57.5%	
Female	7 (16)	71.43%	
Primary site			.12
Oral cavity	28 (62)	53.1%	
Oropharynx	17 (38)	70.1%	
Grading			.63
G2	21 (47)	61.5%	
G3	24 (53)	58.3%	
pT status			.17
T3	13 (28.9)	69.2%	
T4	32 (71.1)	55.1%	
pN status			.007
N0	15 (33)	79.4%	
N1	7 (15)	85.7%	
N2	23 (52)	38.4%	
pN status			.04
N0	15 (33)	79.4%	
N1–N2	30 (67)	49.2%	
Stage			.02
III	6 (13)	100.0%	
IV	39 (87)	53.1%	
Extracapsular spread			.001
Negative	13 (43)	76.9%	
Positive	17 (57)	26.5%	
Lymphatic embolization			.16
Negative	19 (42)	63.2%	
Positive	26 (58)	57.7%	

Abbreviations: DSS, disease-specific survival; pT, pathologic T (primary classification); pN, pathologic N (classification correlates with the number of nodes examined).

of distant metastasis during follow-up (Tables 5 and 6). Survival significantly correlated with the area of peritumoral CD31-positive blood vessels, with higher values in patients who died of disease ($p = .02$). A positive trend was also observed in relation to postoperative nodal status ($p = .06$) and detection of distant metastasis during the follow-up ($p = .08$). An increased number of intratumoral lymphatics was associated with a higher risk of developing nodal disease ($p = .02$). Moreover, the risk for distant metastasis was increased with lower perimeter values of intratumoral lymphatics ($p = .02$); a positive trend was also observed for area of intratumoral lymphatics ($p = .09$).

Detection of neoplastic embolization (see Figure 2) in lymphatic vessels correlated with the presence of nodal disease ($p = .003$).

DISCUSSION

Studies on patients with head and neck cancer suggest that histopathologic detection and quantification of blood and lymphatic angiogenesis on formalin-fixed specimens by simple IHC techniques could lead to the identification of specific staining profiles correlated with a significantly higher risk of developing nodal and distant metastasis.[4–6,9–15]

Our results confirmed the well-known prognostic impact of pT classification, lymph node involvement, and ECS on DSS.[9–11] Even though it is still a matter of debate whether nodal spread evolves through preexisting lymphatic vessels or by a lymphangiogenetic process elicited by the tumor, these data nonetheless emphasize the concept that lymphatic spread plays a crucial role in tumor dissemination.

In examinations of blood and lymphatic vascular density, different patterns of expression were observed in intratumoral and peritumoral areas. A higher density of blood and lymphatic vessels was detected in peritumoral spots ($p = .001$) (Tables 3 and 4), whereas in 28 (62%) patients we found no intratumoral lymphatic vessels. Nevertheless, in 5 (11%) patients, no peritumoral lymphatic vessels were observed. In a study of 52 patients affected by HNSCC, Franchi et al[12] observed a significant decrease of lymphatic vessels in intratumoral spots. Ohno et al[13] reported comparable results, with a higher peritumoral density of lymphatics, especially in the superficial component of neoplastic tissue.

In contrast, a uniformly elevated blood vascular density was found in both intratumoral and peritumoral fields. These observations were confirmed by other studies demonstrating no

Table 3. Number, mean perimeter, and area of intratumoral and peritumoral + blood vessels.

	Intratumoral	Peritumoral	*p* value
Mean number	37.7 (Range, 16–66)	55.9 (Range, 23–103)	.001 (37.7 vs 55.9)
Mean area, μm^2	938.1 (Range, 248.5–3387.8)	907.4 (Range, 273–2476.5)	.75 (938.1 vs 907.4)
Mean perimeter, μm	137.1 (Range, 73.1–252.6)	118.8 (Range, 61.5–162.2)	.01 (137.1 vs 118.8)

Note: Statistical comparisons between intratumoral and peritumoral values were performed with the Mann–Whitney test.

Table 4. Number, mean perimeter, and area of intratumoral and peritumoral podoplanin + lymphatic vessels.

	Intratumoral	Peritumoral	*p* value
Mean number	5.3 (Range, 0–29)	15.5 (Range, 0–40)	.001 (5.3 vs 15.5)
Mean area, μm^2	562.7 (Range, 218.4–1160.6)	748.1 (Range, 86.5–2815.9)	.22 (562.7 vs 748.1)
Mean perimeter, μm	122.2 (Range, 79.6–186.8)	141.3 (Range, 36–276.4)	.06 (122.2 vs 141.3)

Note: Statistical comparisons between intratumoral and peritumoral values were performed with the Mann–Whitney test.

significant difference for blood and lymphatic vascular density in the comparison of intratumoral and peritumoral spots.[11]

In our study, the area of peritumoral blood vessels was significantly correlated with patient status (alive vs died of disease), suggesting that the presence of enlarged blood vessels at this site might enhance tumor spread and progression of disease. The observation of a positive trend between the area and nodal status as well as the development of distant metastasis supports this hypothesis (Table 5). No data are available concerning this issue in the literature, in which vascular density is considered the main parameter for assessment of neoplastic angiogenetic activity. Even though the prognostic value of the vascular area needs to be confirmed by further investigations, a multiparametric approach in the angiogenetic process—not limited to the standard vascular density—could be more sensitive to define further correlations with clinicopathologic and prognostic parameters.

The number of intratumoral lymphatic vessels correlated with lymph node metastasis, whereas no relationship was found between any lymphatic measurement and patient status (alive vs died of disease) (Table 6). Similar findings have been observed in previous studies,[12,16] whereas others have reported a significant correlation of high intratumoral lymphatic vascular density, not only with the prevalence of lymph node metastasis, but also with decreasing DSS. These discrepancies may depend on different criteria adopted to select patient populations, such as all head and neck primaries[9,10] or both early- and advanced-stage diseases.[9,10,14]

The role of peritumoral lymphatics in relation to nodal spread and survival is still an open issue, with contrasting results reported in the literature.[9,10] Moreover, endolymphatic embolization by tumor nests has been shown to be significantly related to nodal dissemination of the disease, as previously documented.[10,13]

The significant correlation between the perimeter value of intratumoral lymphatic vessels and the development of distant metastases reported herein is an original observation that is worthy of validation in additional studies on larger populations. Our results clearly showed a reduced prevalence of systemic spread when the mean perimeter value of intratumoral lymphatic vessels was increased. As a possible explanation, these vessels, characterized by a thin wall and a low luminal pressure when wide, could be more easily compressed and collapsed by proliferating cells as a result of high intratumoral pressure, with subsequent limited drainage activity. This hypothesis can also support the positive trend seen for distant metastases, considering an

Table 5. Counts (number) and measurements (perimeter and area) of intratumoral and peritumoral CD31 + blood vessels in relation to different clinical parameters (patient status, postoperative nodal status, and detection of distant metastasis during follow-up).

	Patient status (NED vs DOD)	Nodal status (pN0 vs pN+)	Distant metastasis (M0 vs M1)
Intratumoral			
No.	$p = .18$ (35 vs 41)	$p = .34$ (39 vs 37)	$p = .62$ (37 vs 41)
Perimeter, μm	$p = .33$ (146 vs 128)	$p = .96$ (137 vs 137)	$p = .55$ (137 vs 138)
Area, μm^2	$p = .25$ (1121 vs 746)	$p = .48$ (929 vs 943)	$p = .88$ (960 vs 820)
Peritumoral			
No.	$p = .22$ (60 vs 52)	$p = .43$ (59 vs 54)	$p = .15$ (58 vs 45)
Perimeter, μm	$p = .17$ (115 vs 123)	$p = .20$ (114 vs 121)	$p = .12$ (117 vs 130)
Area, μm^2	$p = .02$ (754 vs 1068)	$p = .06$ (725 vs 999)	$p = .08$ (859 vs 1172)

Abbreviations: pN, pathologic N (classification correlates with the number of nodes examined); NED, no evidence of disease; DOD, died of disease.
Note: The Mann–Whitney test was used for statistical comparisons.

Table 6. Counts (number) and measurements (perimeter and area) of intratumoral and peritumoral podoplanin + lymphatic cells in relation to different clinical parameters (patient status, pN status, and development of distant metastasis).

	Patient status (NED vs DOD)	Nodal status (pN0 vs pN+)	Distant metastasis (M0 vs M1)
Intratumoral			
No.	$p = .25$ (4.6 vs 6.0)	$p = .02$ (1.5 vs 7.2)	$p = .24$ (4.9 vs 7.5)
Perimeter, μm	$p = .69$ (115 vs 126)	$p = .88$ (118 vs 123)	$p = .02$ (130 vs 98)
Area, μm^2	$p = .48$ (512 vs 590)	$p = .30$ (673 vs 548)	$p = .09$ (616 vs 388)
Peritumoral			
No.	$p = .87$ (16.4 vs 14.6)	$p = .18$ (12.8 vs 16.9)	$p = .96$ (16.7 vs 14.5)
Perimeter, μm	$p = .60$ (133 vs 159)	$p = .98$ (139 vs 142)	$p = .97$ (142 vs 138)
Area, μm^2	$p = .42$ (663 vs 837)	$p = .31$ (646 vs 795)	$p = 1.00$ (765 vs 652)

Abbreviations: pN, pathologic N (classification correlates with the number of nodes examined); NED, no evidence of disease; DOD, died of disease.
Note: The Mann–Whitney test was used for statistical comparisons.

increased area of intratumoral lymphatics, suggesting that the smallest vessels, likely with improved drainage activity, could play a crucial role in systemic spread of disease (Table 6; Figure 3). The process leading or driving tumor cells into the systemic bloodstream is still unclear: the presence of shunts between the vascular and lymphatic tumor systems or a compromised filtering function of lymph nodes overloaded by high volume of neoplastic cells could both serve as possible explanations.[4]

A new computer-assisted multiparametric evaluation of tumor angiogenesis and lymphangiogenesis was adopted in our study, with the intent to assess not only the density of blood and lymphatic vessels, but also their area and perimeter. Such an approach might be helpful to identify possible adjunctive prognostic factors and, consequently, to properly plan adequate adjuvant treatment.

In recent years, an increasing interest in the role of antiangiogenetic agents in cancer therapy has developed—with encouraging results.[17–19] Many angiogenetic factors, such as vascular endothelial growth factor, are believed to induce neoangiogenesis with formation of tortuous and leaky vessels. The possible role of antiangiogenetic drugs such as bevacizumab may be not only to reduce vessel formation, but also to stabilize tumor vessels, to improve the activity of chemotherapeutic agents within the tumor mass.[15,20] More recently, several studies on tumor lymphangiogenesis have emphasized a role other than angiogenesis in neoplastic progression, which may be a promising target for future cancer therapy.[4,20,21]

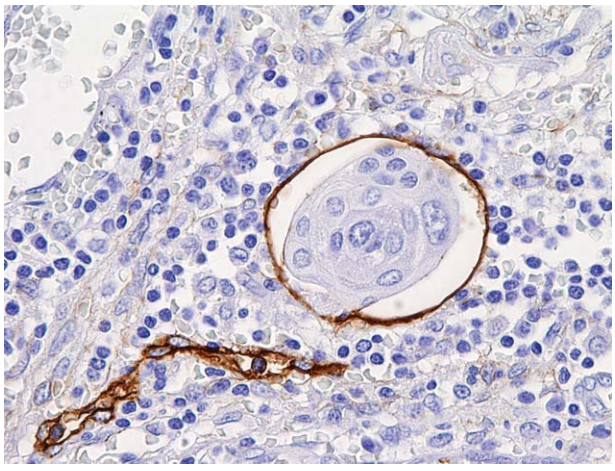

FIGURE 2. Detection of neoplastic embolization in a peritumoral lymphatic vessel immunostained for podoplanin (counterstained with hematoxylin; original magnification, ×40). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

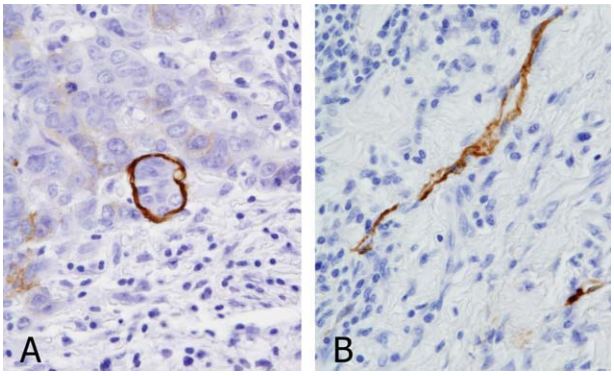

FIGURE 3. (**A**) Lymphatic vessels with lower perimeter values are more frequently characterized by a patent lumen often harboring neoplastic emboli. (**B**) In contrast, major lymphatic vessels are often collapsed with a virtual section area (immunostained for podoplanin; counterstained with hematoxylin; original magnification, ×40). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

The relatively small size of the patient sample, the retrospective nature of the study, and the reproducibility of the "hot spot" assignment—strictly dependent on the training and experience of the investigator—represent the limitations of the present study. Notwithstanding, 2 major issues of this study are worth emphasizing: (1) the stringent selection criteria adopted to define the patient population, and (2) the opportunity to capture not only vascular density but adjunctive morphologic information on blood and lymphatic vessels such as the area and perimeter, to identify possible correlations with clinicopathologic factors.

In conclusion, peritumoral blood angiogenesis correlated with patient status (alive vs died of disease) and intratumoral lymphangiogenesis, as well as the presence of lymphatic emboli correlated with lymph node metastasis, whereas the detection of enlarged intratumoral lymphatic vessels was associated with a lower risk of distant neoplastic spread. These data suggest that vascular proliferation is a significant event in the biology of head and neck cancer. As a consequence, its measurement might be useful to better assess prognosis and, consequently, to modulate treatment.

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