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MAIN ARTICLE

Prognostic value of lymphangiogenesis in supraglottic laryngeal carcinoma

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Abstract

Background: Metastasis to regional lymph nodes via lymphatic microvessels plays a key role in cancer progression, and is an important prognostic factor in many cancers. Recent evidence suggests that tumour lymphangiogenesis promotes lymphatic metastasis.

Aims: To investigate whether tumour lymphatic microvessel density correlates with clinicopathological factors and serves as a prognostic indicator of supraglottic laryngeal carcinoma progression.

Methods: The lymphatics of 84 supraglottic laryngeal carcinoma cases were investigated by immunohistochemical staining for podoplanin (also termed D2-40). The relationships between (intra- and peritumoural) lymphatic microvessel density, clinicopathological parameters and clinical prognosis were analysed.

Results: There was a significant relationship between high intratumoural lymphatic microvessel density and aggressive tumour node stage ($p < 0.0001$), distant metastasis ($p = 0.037$) and poor prognosis ($p = 0.011$), and between high peritumoural lymphatic microvessel density and node stage ($p = 0.004$) and poor prognosis ($p = 0.029$). Patients with high lymphatic microvessel density also had significantly worse disease-free survival ($p = 0.003$) and overall survival ($p = 0.005$). Intratumoural lymphatic microvessel density was found to be an independent prognostic factor for overall survival ($p = 0.008$) and disease-free survival ($p = 0.005$) (multivariate analysis).

Conclusion: Lymphatic microvessel density (detected by podoplanin immunohistochemistry), especially intratumoural density, may be an independent predictor of lymphatic tumour spread and survival in supraglottic laryngeal carcinoma patients, and may be useful to guide decisions regarding additional surgery.

Key words: Lymphangiogenesis; Carcinoma, Squamous Cell; Otolaryngology; Podoplanin Protein, Human; Prognosis

Introduction

Head and neck squamous cell carcinoma (SCC) is one of the most common types of cancer, with more than 500 000 new cases predicted annually worldwide.[1] Patients with head and neck SCC experience severe disease- and treatment-related morbidity, and have only a 50 per cent five-year survival rate. The latter statistic has not improved in more than two decades.[2]

Head and neck SCC frequently metastasises to the regional lymph nodes, and this is the strongest predictor of disease prognosis. For most human malignancies, precise lymph node staging is necessary to establish an accurate clinical prognosis, and such staging often guides therapeutic decisions. However, currently available pre-operative clinical staging methods (including newer radiographic techniques) are suboptimal, and often misdiagnose the presence or absence of cervical nodal metastasis.[2,3] It is essential to improve our knowledge of the underlying molecular mechanisms by which tumours spread via the lymphatic vessels to the distal lymph nodes and beyond, in order to develop effective therapeutic modalities for head and neck SCC.

One of the shortcomings of previous studies of immunohistochemically assessed lymphangiogenesis has been an inability to distinguish between blood and lymphatic vessels; this is significant, as the two types of vessel play different roles in cancer progression.[4,5]

In contrast, the development of a polyclonal antibody recognising podoplanin[6] has enabled the current authors to stain lymphatic vessels selectively. Podoplanin (also termed D2-40) is a mucin-like transmembrane glycoprotein consisting of an extracellular

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domain, a single transmembrane portion, and a short cytoplasmic tail for protein kinase C and cyclic adenosine monophosphate phosphorylation.[7,8] Podoplanin is highly and specifically expressed in lymphatic endothelial cells and during tumour-associated lymphangiogenesis, but is not expressed in blood endothelial cells.[9] Podoplanin is widely used in histopathology as a specific marker for lymphatic endothelium.[8] Its expression has been reported in carcinomas of the skin, lung, uterus and thyroid. A high level of podoplanin expression is significantly associated with lymph node metastasis and a poor survival rate.[10–13] This raises the possibility that podoplanin may have biological functions in tumour cells, and may play a role in tumour-associated lymphangiogenesis and malignant transformation.

The current study used monoclonal antibody D2-40, which specifically recognises podoplanin, to determine the expression of podoplanin in patients with supraglottic laryngeal carcinoma. The study then analysed potential associations between podoplanin expression and clinicopathological factors. The potential value of lymphangiogenesis assessment in predicting disease outcome was also examined.

Materials and methods

Patients and samples

The study enrolled an initial cohort of 108 patients with supraglottic laryngeal carcinoma who were treated at the Kurume University Hospital between October 1995 and October 2004.

A total of 24 patients who did not undergo surgery, or who received radiotherapy before surgery, were excluded.

Therefore, the actual analysis was performed on 84 patients who underwent total laryngectomy either with or without radical or modified radical neck dissection. Of these 84 patients, 77 were men and seven women. Patients' ages ranged from 43 to 95 years, with a mean age of 67 years.

Histopathologically, all patients had supraglottic laryngeal carcinoma, which was well differentiated in 15 patients, moderately differentiated in 48 and poorly differentiated in 21. Patients were classified according to the 2002 tumour–node–metastasis (TNM) classification, as follows: for tumour stage, T_1 = four patients, T_2 = 22, T_3 = 17 and T_4 = 41; for node stage, N_0 = 50 and N_+ = 34 (N_1 = 6, N_2 = 27 and N_3 = 1); and for metastasis stage, M_0 = 71 and M_1 = 13.

During follow-up examination, recurrence was detected in 11 patients, while the remaining 73 patients were free of recurrence. Overall, 84 patients had a three-year survival rate of 70.72 per cent and a five-year survival rate of 65.74 per cent.

The 84 patients' histopathological findings were initially screened at a low magnification. Cancerous and noncancerous tissues were then extracted from the original paraffin blocks for further study.

To provide a control for this study, normal supraglottic laryngeal mucosa tissue was obtained from five patients who had undergone total laryngectomy for advanced tongue cancer.

Immunohistochemical analysis

Consecutive, 3 μm thick tissue sections were placed on microscope slides, deparaffinated and dehydrated in xylol with graded alcohol. After rinsing with phosphate-buffered saline, the sections were placed in hot 10 mmol/l citrate buffer (pH 6.0) and heated in a microwave oven (twice, for 5 minutes each time) to facilitate antigen retrieval.

The Dako EnVision™ immunohistochemistry method was then utilised to stain the lymphatic microvessels with the D2-40 monoclonal antibody (antipodoplanin) (1:100, M0876; Dako, Tokyo, Japan). Using the Dako TechMate™ Horizon automated immunostainer, the slides were incubated with primary antibodies for 60 minutes. EnVision system peroxidase was used during the immunohistochemical staining process. Diaminobenzidine substrate chromogen solution was applied, followed by counterstaining with haematoxylin.

Determination of podoplanin expression

Podoplanin expression was evaluated by an independent investigator unfamiliar with the patient's history, using a digital optical microscope (VHX-200; Keyence, Osaka, Japan) at ×400 magnification. Lymphatic microvessels were counted in (1) cancer nests and stroma (i.e. intratumoural lymphatic microvessels), and (2) along the tumour–myometrial junction (i.e. peritumoural lymphatic microvessels). To determine the lymphatic microvessel count, each section was scanned at low magnifications (i.e. ×40 and ×100), and three representative areas were identified. Lymphatic microvessels in these three areas were then counted at ×200 magnification, and the mean count for the three areas was used for statistical analysis. Only lymphatic vessels containing podoplanin-expressing cells displaying cell membrane immunoreactivity were counted.

Sections of tongue SCC served as positive controls for immunohistochemical analysis. Sections incubated without the primary antibody served as negative controls.

Statistical analysis

For immunohistochemical markers, the cut-off value for the definition of subgroups was the median value.[14] Any correlation between immunohistochemical results and clinicopathological features was assessed using the chi-square test. Cumulative survival time was calculated using the Kaplan–Meier method, and compared using the log-rank test.

Overall survival was defined as the period from the primary surgery until the death of the patient. Death from a cause other than supraglottic laryngeal

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carcinoma, and survival until the end of the observation period, were considered as censoring events. Disease-free survival was defined as the period from the end of the primary therapy until the first evidence of progression of disease. Univariate analysis of overall survival and disease-free survival was performed as outlined by Kaplan and Meier.[15] Multivariate analysis was based on the Cox proportional hazards regression model. A two-tailed p value of less than 0.05 was considered statistically significant.

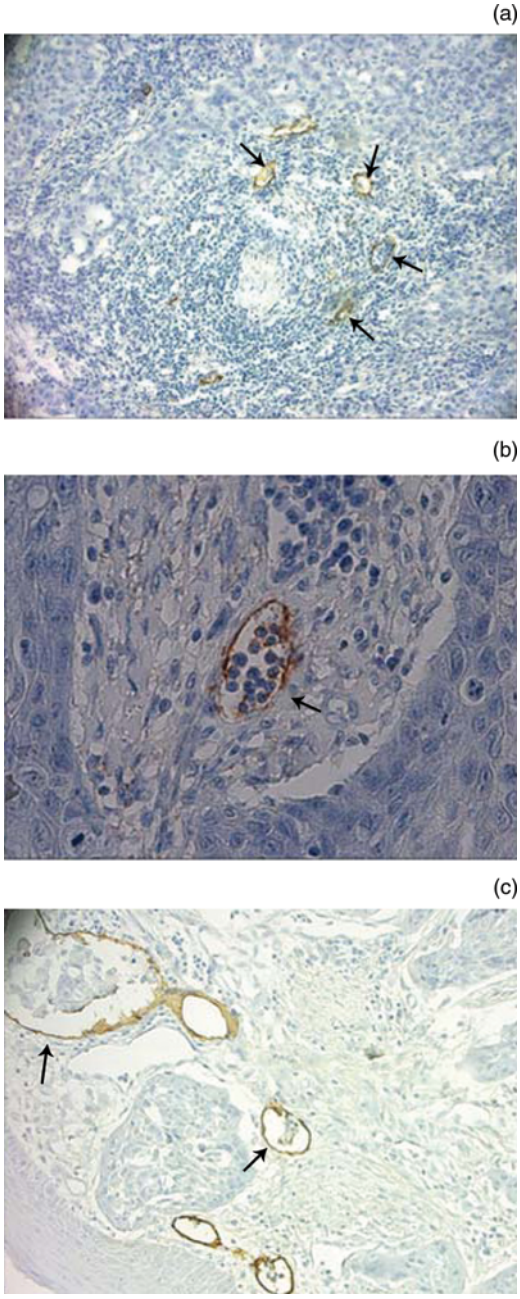

FIG. 1

Photomicrographs of immunohistochemical analysis of podoplanin expression in supraglottic laryngeal carcinoma, showing (a) podoplanin-positive lymphatic vessels (arrows) (×100), (b) lymphocytes within podoplanin-positive lymphatic vessels (arrows) (×400), and (c) podoplanin-positive lymphatic vessels containing tumour cells (arrows), indicative of lymphatic invasion (×100). Note the presence of unstained blood vessels.

All statistical analyses were performed using the Statistical Package for the Social Sciences version 12.0 for Windows software program (SPSS Inc, Chicago, Illinois, USA).

Results

Podoplanin expression in supraglottic laryngeal carcinoma

Immunohistochemical analysis for podoplanin was conducted in order to calculate the lymphatic microvessel density within the supraglottic laryngeal carcinoma samples (Figure 1). Podoplanin immunoreactivity was detected in the cytoplasm of lymphatic endothelial cells but not blood endothelial cells.

The majority of intratumoural lymph vessels were small and collapsed. In contrast, the peritumoural lymph vessels were often enlarged, with wide, open lumina. Lymph vessels were unevenly distributed throughout the tumour.

Relationship between lymphatic microvessel density and clinicopathological factors

For the 84 study patients, the median intratumoural lymphatic microvessel density was 0 microvessels per field (range, 0–26 vessels). As regards intratumoural vessels, 48 (57 per cent) patients were considered to have low lymphatic microvessel density, and 36 (43 per cent) to have high lymphatic microvessel density. The median peritumoural lymphatic microvessel density was four microvessels per field (range, 0–32 vessels). As regards peritumoural vessels, 46 (55 per cent) patients were considered to have low lymphatic microvessel density, and 38 (45 per cent) to have high lymphatic microvessel density.

The association between lymphatic microvessel density and clinicopathological features is summarised in Table I. A high intratumoural lymphatic microvessel density was significantly associated with a more aggressive tumour N stage ($p < 0.01$) and M stage ($p = 0.037$), and a poorer prognosis ($p = 0.011$) at the time of diagnosis. A high peritumoural lymphatic microvessel density was significantly associated with a more aggressive tumour N stage ($p = 0.004$) and a poorer prognosis ($p = 0.029$) at the time of diagnosis.

There was no statistically significant association between intratumoural lymphatic microvessel density and patient sex, histological differentiation, tumour T stage or recurrence. Peritumoural lymphatic microvessel density showed no significant association with sex, histological differentiation, tumour T stage, tumour M stage or recurrence.

Survival and recurrence analysis

Intratumoural lymphatic microvessel density was the only significant prognostic factor for overall survival and disease-free survival in the supraglottic laryngeal carcinoma patients (log rank $p = 0.005$ and log rank $p = 0.003$, respectively) (Figures 2 and 3). The mean

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TABLE I
ASSOCIATION OF LYMPHATIC MICROVESSEL DENSITY WITH CLINICOPATHOLOGICAL PARAMETERS

Parameter	Pts (*n*)	Lymphatic microvessel density* (pts (*n*))					
		Intratumoural (mv/fld)			Peritumoural (mv/fld)		
		0	>0	*p*	≤4	>4	*p*
Pt sex							
– Male	77	44	33	1.000	42	35	1.000
– Female	7	4	3		4	3	
Ca diff							
– Well	15	9	6	0.811	6	9	0.447
– Mod	48	26	22		28	20	
– Poor	21	13	8		12	9	
T stage							
– T_{1-2}	30	21	9	0.076	15	15	1.000
– T_{3-4}	54	27	27		31	31	
N stage							
– N_0	50	39	11	0.000	34	16	0.004
– N_+	34	9	25		12	22	
M stage							
– M_0	71	44	27	0.037	41	30	0.236
– M_1	13	4	9		5	8	
Recurrence?							
– No	73	44	29	0.135	39	34	0.747
– Yes	11	4	7		7	4	
Prognosis							
– Lived	61	40	21	0.011	38	23	0.029
– Died	23	8	15		8	15	

*Assessed using podoplanin immunoreactivity. Pts = patients; mv/fld = microvessels per microscopy field; Ca dif = cancer differentiation; mod = moderate; T = tumour; N = node; M = metastasis

survival time was 34 months in patients with high intratumoural lymphatic microvessel density, and 42 months in those with low intratumoural lymphatic microvessel density.

In contrast, peritumoural lymphatic microvessel density showed no significant association with overall survival or disease-free survival.

Univariate and multivariate analyses

Univariate analysis showed that intratumoural lymphatic microvessel density was a significant predictor of overall survival and disease-free survival ($p = 0.005$ and $p = 0.003$, respectively) (Table II), whereas tumour TMN stage, tumour differentiation and peritumoural lymphatic microvessel density were not significant predictors of overall or disease-free survival ($p > 0.05$).

In the multivariate analysis, covariates for survival were tested using a Cox proportional hazards model (Table II). Patients with a low intratumoural lymphatic microvessel density had a significantly lower risk for disease-free survival (hazard ratio = 3.078, 95 per cent

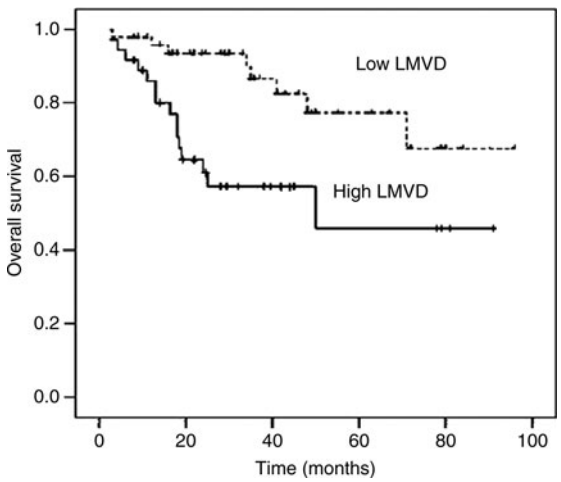

FIG. 2

Kaplan–Meier analysis of overall survival of patients with supraglottic laryngeal carcinoma who had high and low intratumoural lymphatic microvessel densities (LMVD). $p = 0.005$.

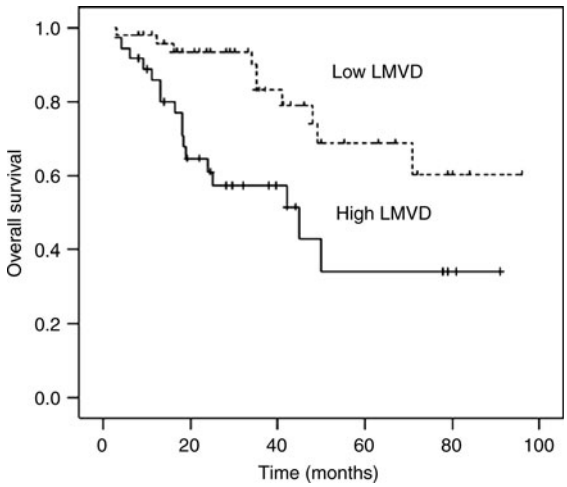

FIG. 3

Kaplan–Meier analysis of disease-free survival of patients with supraglottic laryngeal carcinoma who had high and low intratumoural lymphatic microvessel densities (LMVD). $p = 0.003$.

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TABLE II
PATIENTS' OVERALL AND DISEASE-FREE SURVIVAL*

Parameter	*p*		95% CI	RR
	Univariate†	Multivariate‡		
OS				
TNM stage				
– T stage	0.095	0.036	0.826–7.163	2.432
– N stage	0.384	0.385	0.632–3.264	0.385
– M stage	0.321	0.157	0.744–5.613	2.043
Diff	0.898	0.699	0.465–1.671	0.881
IT LMVD	0.005	0.008	1.359–7.634	3.221
PT LMVD	0.139	0.146	0.807–4.242	1.85
DFS				
TNM stage				
– T stage	0.081	0.194	0.735–4.534	1.826
– N stage	0.478	0.48	0.614–2.824	1.316
– M stage	0.106	0.0019	1.196–7.111	2.916
Diff	0.819	0.53	0.456–1.497	0.826
IT LMVD	0.003	0.005	1.401–6.761	3.078
PT LMVD	0.481	0.484	0.602–2.921	1.326

*84 patients with supraglottic laryngeal carcinoma. †Log rank test; ‡Cox regression. CI = confidence interval; RR = relative risk; univariate = univariate analysis; multivariate = multivariate analysis; OS = overall survival; TNM = tumour–node–metastasis; diff = tumour differentiation; IT = intratumoural; LMVD = lymphatic microvessel density; PT = peritumoural; DFS = disease-free survival

confidence interval (CI) = 1.401–6.761, $p = 0.005$) and for overall survival (hazard ratio = 3.221, 95 per cent CI = 1.359–7.634, $p = 0.008$), compared with patients with a high intratumoural lymphatic microvessel density. Moreover, multivariate analysis indicated that tumour M stage was an independent prognostic factor for disease-free survival ($p = 0.0019$), whereas tumour T stage was an independent predictor of overall survival ($p = 0.036$). Only intratumoural lymphatic microvessel density was found to be an independent predictor of both disease-free survival ($p = 0.003$) and overall survival ($p = 0.005$).

This suggests that intratumoural lymphatic microvessel density may be a stronger predictor of supraglottic laryngeal carcinoma prognosis than tumour histology.

Discussion

In patients diagnosed with supraglottic laryngeal carcinoma, the determination of lymph node status at the time of diagnosis is one of the major predictors of outcome, and is also used to guide therapeutic decisions. Despite recent advances in radiological imaging, the most reliable method of detecting lymph node metastasis remains the careful microscopic assessment of regional lymph nodes harvested from resection specimens.

However, the prognostic value of lymph node assessment is restricted by the variable yield of nodes harvested from the surgical specimens.[38] It may also be adversely affected by the current recommendation to examine microscopically only one slide from each node.[16]

Consequently, accurate assessment of the risk of lymphatic metastasis is not always possible. Therefore, a method which accurately predicted the risk of lymph node metastasis, based on characteristics of the primary tumour, would constitute a powerful prognostic tool for clinicians and pathologists.

In this study, intratumoural and peritumoural lymphatic microvessel densities were assessed within surgical specimens of supraglottic laryngeal carcinoma, using a specific lymphatic endothelial marker.[17,18] Previous findings have suggested that intratumoural lymphatics are nonfunctional, and that lymph node metastases occur in tumours lacking intratumoural functional lymphatics.[19] In contrast, the current study found that high densities of both intratumoural and peritumoural lymphatic microvessels were both significantly associated with the presence of lymph node metastasis. Furthermore, intratumoural lymphatic microvessel density (but not peritumoural lymphatic microvessel density) was shown to be a prognostic factor, and to be significantly correlated with overall survival and disease-free survival.

The hypothesis that intratumoural lymphatic microvessels are necessary for metastasis of tumour cells to lymph nodes is further supported by two important findings.

First, tumour cells have been observed to be present within intratumoural lymphatic microvessels. However, several studies have shown that intratumoural lymphatic vessels are often occluded by tumour cells at the time when lymph node metastases are detectable.[19,20] Hence, tumour lymphatic vessels may have normal drainage functions in the early stages of tumour development, but these functions may be blocked in the later stages of tumour progression.[21]

Second, the present study found that lymph node metastases occurred in 25 of the 36 (69.4 per cent) patients with a high density of intratumoural lymphatic microvessels, but in only nine of the 48 (18.8 per cent) patients with a low density of intratumoural lymphatic

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microvessels. Furthermore, 15 of the 36 (41.7 per cent) patients with a high density of intratumoural lymphatic microvessels died of their tumour, while only eight of the 48 (16.7 per cent) patients with a low density of intratumoural lymphatic microvessels died of their tumour. Intratumoural lymphangiogenesis was significantly associated with the presence of lymph node metastasis and with poor prognosis; this indicates that intratumoural lymphangiogenesis is an active element of human lymph node metastasis.

The detection of increased lymphatic microvessel density may provide a convenient and reliable means of determining the risk of lymphatic metastasis in solid tumours. Furthermore, lymphatic microvessel density may constitute a simple target for future anti-tumour therapies.

Previous studies have reported controversial results regarding the importance of peritumoural lymphatic microvessels for the metastatic potential of laryngeal SCC. Some investigators[22] have found a significant association between high peritumoural lymphatic microvessel density and shorter disease-free survival. In contrast, other studies have found a relationship between high peritumoural lymphatic microvessel density and more favourable outcomes.[23,24]

The authors of the current study did not find a significant association between peritumoural lymphatic microvessel density and overall survival or disease-free survival, although correlations were noted between higher peritumoural lymphatic microvessel density and lymph node involvement and worse prognosis.

Such contradictory results for the correlation between peritumoural lymphatic microvessels and prognosis may be due to differences in: patient selection; methods of counting lymphatic microvessels; the grade, stage and type of tumours analysed; and the local microenvironment present during the development of primary tumours.

In addition, the underlying mechanisms by which tumours induce lymphangiogenesis and lymphatic metastasis are complex, and vary significantly in tumours of different histological type and anatomical location.[25]

- **Head and neck squamous cell carcinoma frequently metastasises to the regional lymph nodes, and this is the strongest indicator of disease prognosis**
- **This study assessed the association of intra- or peritumoural lymphatic microvessels with clinicopathological factors and survival**
- **Intratumoural lymphatic microvessel density may be an independent predictor of lymphatic tumour spread**

However, a main contributor to the controversy over the prognostic significance of tumour lymphatic microvessels may be the lack of appropriate lymphatic vessel markers. Previous investigators have used nonspecific or nonsensitive markers for their studies, such as Lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1). Although this is considered a specific marker for lymphatic vessels, it has also been observed in the endothelium of blood vessels.[26,27] The current study used podoplanin, considered to be the most specific lymphatic endothelial marker,[28] and all podoplanin-positive vessels were observed to have morphological features consistent with lymphatics.

Some studies have analysed the presence of lymphangiogenesis in head and neck SCC. Maula *et al.*[29] studied patients with head and neck SCC, and found that intratumoural lymphatic vessels were associated with a poor disease-specific prognosis, but that a high density of peritumoural vessels was associated with favourable survival. Frech *et al.*[30] found that a high density of intratumoural lymphatic vessels correlated significantly with nodal metastasis in oral carcinoma patients, but showed no such association in patients with other types of head and neck SCC. The discrepancy between these two study findings may be due to differences in lymphatic vessel markers, and to the fact that these authors assessed a diverse range of tissue of varying histology arising from a variety of anatomical head and neck SCC sites.

Conclusion

These study findings establish podoplanin as a valid marker for lymphatic endothelium.

Although an increase in intratumoural lymphatic microvessel density was significantly associated with a worse prognosis in patients with supraglottic laryngeal carcinoma, the prognostic power of peritumoural lymphatic microvessel density was not strong enough to retain significance.

Intratumoural lymphatic microvessel density may have clinical utility in the evaluation of supraglottic laryngeal carcinoma, particularly for the estimation of lymph node metastatic risk. In patients with supraglottic laryngeal carcinoma, increased intratumoural lymphatic microvessel density may serve as an indicator of poor prognosis.

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