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Original Contribution

CD105- and CD31-assessed microvessel density in laryngeal carcinoma biopsies as a predictor of recurrence after exclusive primary surgery

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ABSTRACT

Purpose: Surgery is currently indicated as a unimodal therapeutic approach with curative intent in selected laryngeal squamous cell carcinomas (LSCCs) ranging from stage I to III. The main aim of this study was to evaluate the prognostic role of CD105- and CD31-assessed microvessel density (MVD) in biopsy and in surgical specimens from a cohort of consecutive stage I-III LSCCs who had undergone exclusive primary surgery, according to current guidelines.

Materials and methods: CD105- and CD31-assessed MVD were analyzed in paired biopsies and surgical specimens of 24 consecutive cases of LSCC who underwent exclusive surgery.

Results: On biopsy specimens, CD105- and CD31-assessed MVD were positively associated with recurrence risk (hazard ratio [HR] 1.266, $p = 0.0034$ and HR 1.265, $p = 0.0081$, respectively). In surgical specimens, CD105- and CD31-assessed MVD were significantly associated with disease-free survival (DFS) (HR 1.213, $p = 0.0016$ and HR 1.237, $p = 0.0023$ respectively). Considering a stratification based on median value, recurrence risk was higher in patients with a CD105-assessed MVD > 0 in both biopsies and surgical specimens (HR 11.005, $p = 0.0326$ and HR 34.483, $p = 0.0311$). No significant differences in terms of recurrence risk were found for CD31-assessed on biopsies or on surgical specimens.

Conclusions: This study supports the role of biopsy CD105-MVD as a predictor of recurrence after exclusive surgery for LSCCs. Further prospective studies are mandatory to better characterize the prognostic role of CD105-MVD evaluated on biopsies to develop novel criteria to identify patients at higher risk of recurrence for more aggressive approaches or adjuvant treatment.

1. Introduction

Surgery is currently indicated as a unimodal therapeutic approach with curative intent in selected laryngeal squamous cell carcinomas (LSCCs) ranging from stage I to III [1]. Nowadays, surgical approaches include endoscopic [2] or open partial and reconstructive laryngectomies [3,4], or total laryngectomy [5], with or without neck dissection and/or thyroidectomy. However, despite diagnostics and surgical techniques having undergone constant evolution, long-term survival rates have not significantly improved in the recent years [6,7].

In order to identify patients with higher recurrence risk after primary surgery who could benefit from adjuvant postoperative treatments, interest in predictive markers is rising, especially molecular ones [7-9]. Neoplasm angiogenesis represents a promising research field for both prognostic and therapeutic purposes. Neo-angiogenesis is essential to tumor growth and progression [6,10]. The assessment of microvessel density (MVD) is a well-established approach to obtain quantitative data about neoplastic angiogenesis processes, and it is currently often based on the immune-staining of vascular endothelial cell markers [6,11]. CD31, a widely-used marker for MVD evaluation, is a member of the immunoglobulin-superfamily platelet endothelial cell adhesion molecule-1 [12], which is highly expressed on the surface of endothelial cells, and is involved in angiogenesis processes of several malignancies, including early breast cancer [13], nasopharyngeal cancer [14], and head and neck carcinomas with deep invasion [15]. CD105 is an auxiliary receptor of transforming growth factor-β (TGF-β), which binds to TGF-β1 and TGF-β3, modulating their signaling by interacting with type I and type II TGF-β receptors. It is involved in activating a complex signaling pathway involving the proliferation, migration and adhesion of endothelial cells [16-18], resulting in increased angiogenesis. Since it

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appears to be specifically expressed on the surface of active endothelial cells, it has been proposed as a reliable marker for neoplasms MVD assessment [6,11]. The assessment of MVD, by means of CD105 and CD31 immunostaining, has been found to be relevant for LSCC prognosis, high MVD values being associated with increased recurrence risk [6,9]. However, in the literature, assessment of the prognostic role of MVD has been based mainly on the analysis of surgical specimens, while data based on preoperative LSCC biopsies are lacking [19]. The question of whether information obtainable from studies on surgical specimens could be gleaned in advance by analyzing pretreatment biopsies is still unanswered [20]. With this possibility in mind, the larynx as a site of disease could be particularly challenging because biopsies obtained by micro-laryngoscopy are extremely small [21].

The main aim of this study was to evaluate the prognostic role of CD105- and CD31-assessed MVD in biopsy and surgical specimens from a cohort of consecutive stage I-III LSCCs who had undergone exclusive primary surgery, according to current guidelines.

2. Materials and methods

2.1. Patients

The study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in agreement with the Italian privacy and sensitive data laws and the University of Padova Otolaryngology Section's internal rules. Before undergoing surgery, all patients signed a detailed informed consent form.

The investigation involved 24 consecutive cases (22 males, 2 females; mean age 64.3 ± 7.7 years) of LSCC (stages I to III), treated with primary partial or total laryngectomy - combined with unilateral or bilateral cervical lymph node dissection in 22 cases - at the Otolaryngology Section of Padova University. None of the patients showed pathological findings that indicated post-operative adjuvant treatment.

Following our institutional diagnostic recommendations [11], all patients had undergone microlaryngoscopy with laryngeal biopsy, upper aero-digestive tract endoscopy, neck ultrasonography (with or without fine needle aspiration biopsy), head and neck contrast-enhanced CT and/or MRI, chest X-ray and liver ultrasonography. No distant metastases (M) were detected. LSCCs were staged according to the 8th edition of the TNM Classification of Malignant Tumors [22].

As previously described [23,24], clinical follow-up controls after treatment at our institution (adjustable to patients' individual characteristics) were scheduled: once a month for the 1st year; every 2 months in the 2nd year; every 3 months in the 3rd year; every 4 months in the 4th year; every 6 months in the 5th year; every 12 months thereafter. Contrast-enhanced CT of the neck, total body positron emission tomography, chest CT, and neck and liver ultrasonography were repeated if clinically indicated. The median follow-up was 68.5 months (IQR 59.5 months).

2.2. Immunohistochemistry

2.5-μm sections were cut from formalin-fixed paraffin embedded (FFPE) tissue of each LSCC surgical sample and paired pre-operative biopsy; immunohistochemical analysis was performed in an automated system (Benchmark-Ultra, Ventana, Tucson, AZ, US). The following primary antibodies were used: CD105 (Mouse Monoclonal, clone SN6, dilution 1:100, Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) and CD31 (Mouse Monoclonal, clone JC70A, dilution 1:50, Dako, Glostrup, Denmark).

Slides stained for CD105 and CD31 were examined to determine MVD in tissue specimens (biopsies and paired surgical samples), considering intra-tumor and peri-tumor areas (< 500 μm within the tumor border). Each slide was initially examined by light microscope at 100x magnification identifying three areas with the highest number of stained vessels, defined "hot spots" [25]. Sections in which at least three "hot spots" could not be identified (especially small biopsy samples) were not included in the study. Vessels in each of these "hot spots" were counted at 400x magnification (High Power Field [HPF]) (0.237 mm^2/field under the light microscope). MVD was calculated as the arithmetic mean of the number of micro-vessels counted.

Countable micro-vessels were defined as each immune-positive structure (round, oval and irregular) separated from other profiles or connective tissue elements and from each other. Vessels with a muscular layer were excluded from the count, as well as areas of necrosis.

The pathologist interpreting the sections was unaware of the patients' clinical outcomes.

2.3. Statistical analysis

Data have been presented as mean and standard deviation, median and interquartile range for quantitative variables, with count and percentages for those categorical. Association of CD105 and CD31 MVD in both biopsies and complete resection specimens with the dichotomized clinical-pathological features was assessed with Wilcoxon rank sum test.

We applied univariate Cox regression to identify the potential predictors of LSCC recurrence and the results were expressed as p-value, hazard-ratio (HR) and 95% confidence interval.

The time to LSCC recurrence was calculated as the time from completing treatment for the primary tumor to LSCC recurrence, or to the last follow-up for patients experiencing no recurrence. Kaplan-Meier method was used to graphically represent the time to LSCC recurrence according to CD105- and CD31-assessed MVD for both kinds of specimens considering the categorization of MVD at the median value.

A p-value < 0.05 was considered significant, while values in the range between 0.05 and 0.09 were assumed to indicate a statistical trend.

The statistical analyses were performed with SAS 9.4 (SAS Institute Inc., Cary, NC, USA).

3. Results

3.1. Overall clinical outcome and conventional clinical-pathological variables

In this series, 6 patients underwent total laryngectomy, 15 open partial horizontal laryngectomy (of which 3 OPHL I, 11 OPHL II and 1 OPHL III), and 2 endoscopic CO_2 laser-assisted cordectomy. Data on the laryngectomy type were not available for one patient.

Nineteen patients (79.2%) experienced no disease recurrence after surgery, while 5 relapsed after a median period of 12 months (IQR 10.0-18.0 months).

Cox proportional hazards model ruled out any significant associations between DFS and conventional clinical-pathological variables, in terms of pT category, pathological grading, N status and stage (p = 0.4720, p = 0.3426, p = 0.6974, p = 0.6398, respectively).

3.2. Association between LSCC biopsies MVD and clinical outcome

On biopsy specimens, CD105- and CD31-assessed MVD were significantly associated with recurrence risk in the Cox regression model (p = 0.0034 and p = 0.0081, respectively), with a hazard ratio of 1.266 (95% C.I. 1.081-1.481) for CD105-assessed MVD and 1.265 (95% C.I. 1.063-1.505) for CD31-assessed MVD.

Considering the categorization at median value, recurrence risk was higher in patients with a CD105-assessed MVD in biopsies > 0 vs. those with MVD = 0 (p = 0.0326), with a hazard ratio of 11.005 (95% C.I. 1.219-99.334). Instead, categorization at the median CD31-assessed MVD value (MVD ≤ 4.84 vs. MVD > 4.84) did not show any significant

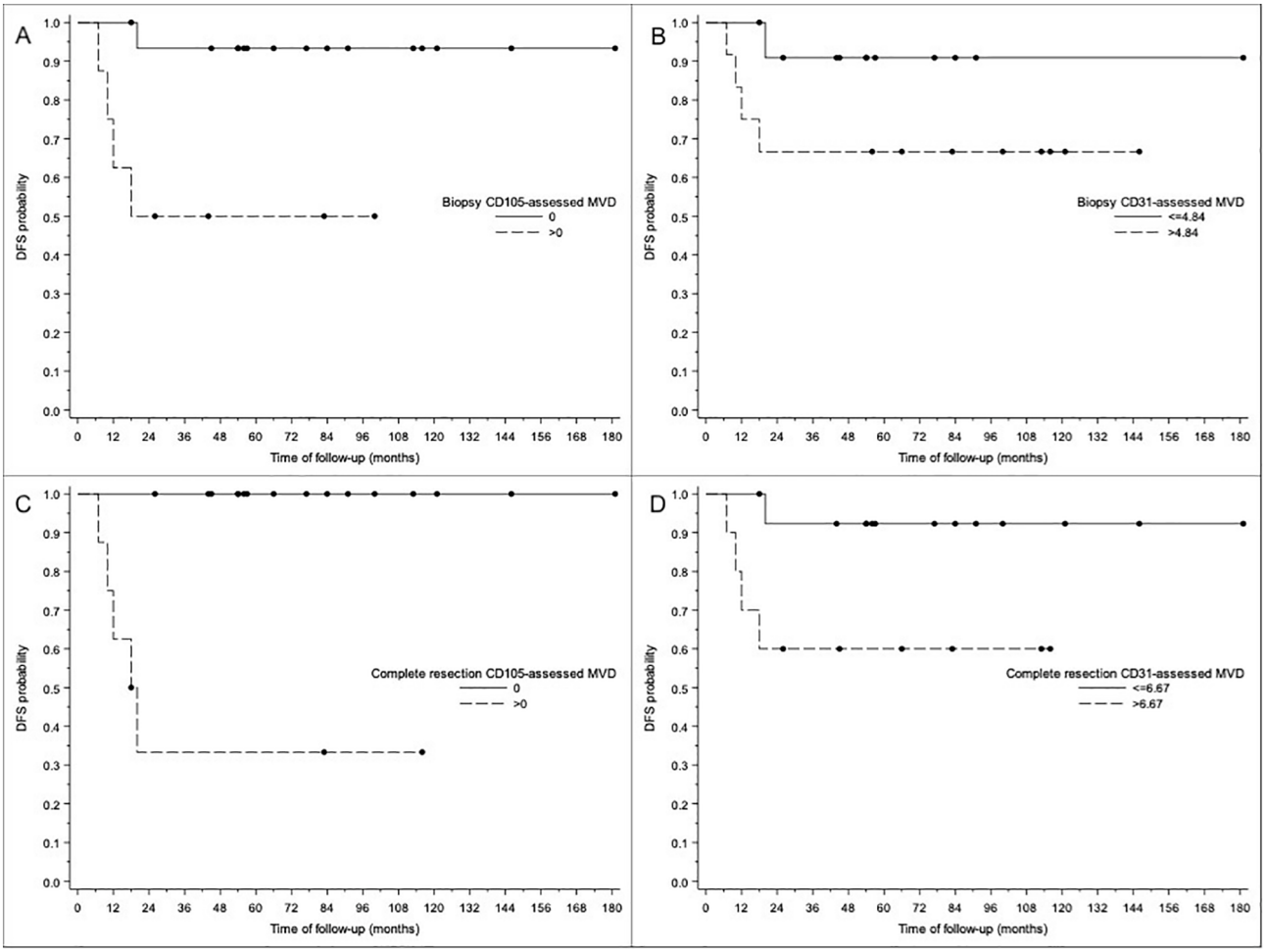

Fig. 1. Kaplan-Meier graphs showing differences in disease-free survival: A. between cases with CD105-assessed MVD > 0 vs. MVD = 0 in LSCC biopsy; B. between cases with CD31-assessed MVD MVD ≤ 4.84 vs. MVD > 4.84 in LSCC biopsy; C. between cases with CD105-assessed MVD > 0 vs. MVD = 0 in complete resection; D. between cases with CD31-assessed MVD > 6.67 vs. MVD ≤ 6.67 in complete resection.

Table 1
Conventional clinical-pathological variables vis-à-vis CD105- and CD31-assessed MVD in biopsies and surgical specimens.

	pT			Grading			Staging		
	T1-T2	T3		G1-G2	G3		I-II	III	
	No. = 18	No. = 6	P-value	No. = 18	No. = 6	P-value	No. = 17	No. = 7	P-value
CD105 MVD (biopsy)									
Mean	2.98	1.83	0.4503	2.32	3.83	0.3608	2.51	3.14	1.000
(SD)	(4.77)	(4.49)		(4.67)	(4.75)		(4.47)	(5.37)	
Median	0.00	0.00		0.00	2.00		0.00	0.00	
(IQR)	(0.00–4.00)	(0.00–0.00)		(0.00–2.34)	(0.00–8.00)		(0.00–3.00)	(0.00–11.00)	
CD31 MVD (biopsy)									
Mean	5.85	4.67	0.4032	5.45	5.89	0.8936	5.43	5.86	0.8986
(SD)	(4.30)	(4.52)		(4.43)	(4.18)		(4.03)	(5.19)	
Median	4.84	3.67		4.84	5.00		4.34	5.34	
(IQR)	(3.00–7.00)	(2.00–6.00)		(3.00–6.34)	(2.00–8.67)		(3.00–6.34)	(2.00–12.67)	
CD105 MVD (surgical specimen)									
Mean	4.19	1.95	0.7810	3.48	4.06	0.9683	3.57	3.76	0.7621
(SD)	(7.14)	(3.51)		(6.54)	(6.66)		(6.85)	(5.78)	
Median	0.00	0.00		0.00	0.00		0.00	0.00	
(IQR)	(0.00–4.34)	(0.00–3.00)		(0.00–3.67)	(0.00–8.67)		(0.00–3.67)	(0.00–8.67)	
CD31 MVD (surgical specimen)									
Mean	8.04	6.84	1.000	7.17	9.45	0.3669	7.51	8.29	0.6330
(SD)	(7.07)	(3.76)		(6.19)	(7.11)		(6.92)	(5.15)	
Median	6.01	6.67		5.84	7.17		5.34	6.67	
(IQR)	(3.67–7.67)	(3.34–9.67)		(3.34–7.00)	(5.34–12.34)		(3.67–7.00)	(3.34–12.34)	

MVD: microvessel density; SD: standard deviation; IQR: interquartile range.

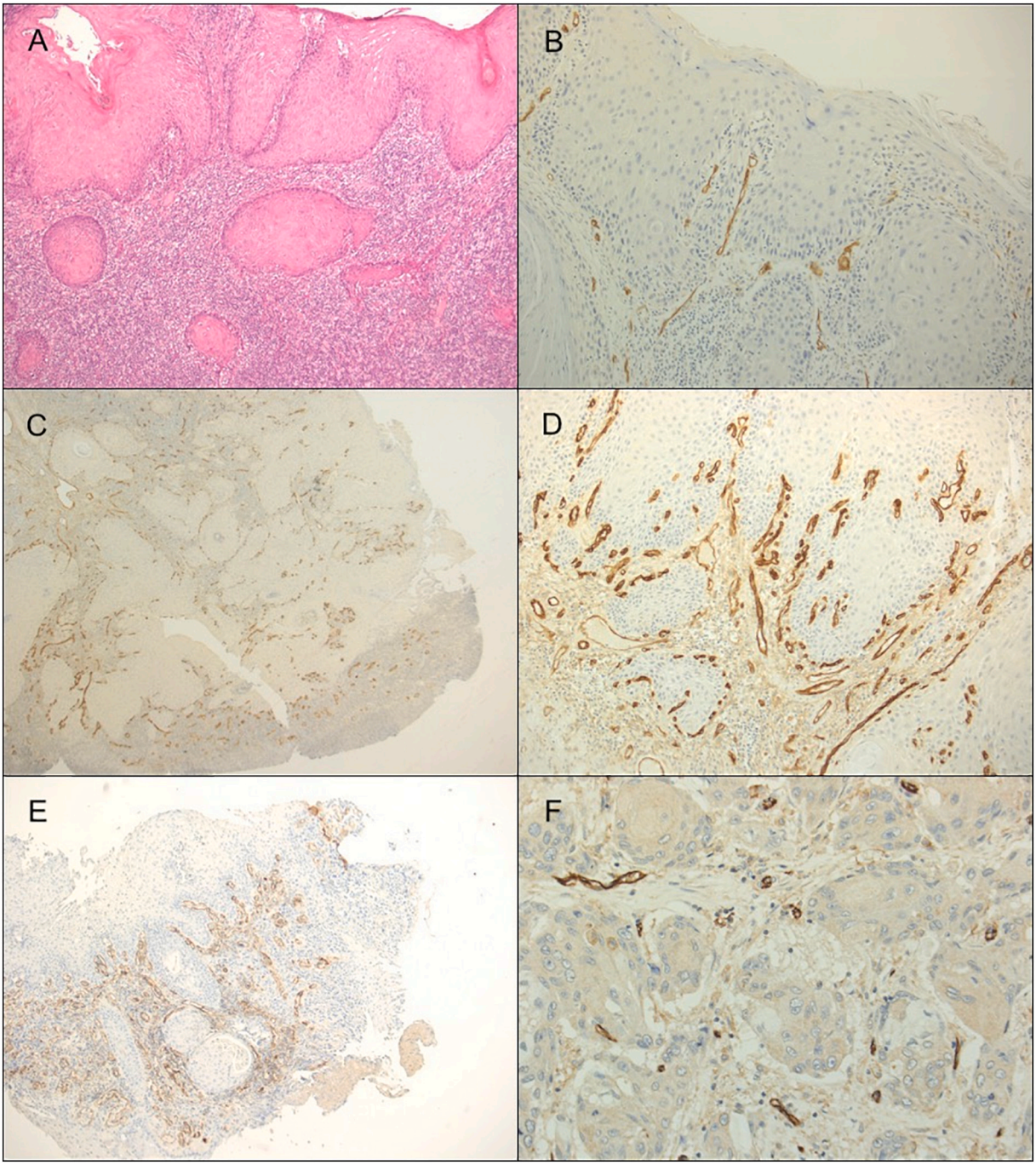

Fig. 2. A. Hematoxylin & Eosin (H&E) stained slide of well-differentiated LSCC, showing infiltration of the stroma (original magnification 50x); B. Same case as A, surgical specimen showing low MVD (CD 105 immunostaining, original magnification 100x); C. Pre-operative biopsy of LSCC showing high MVD (CD 105 immunostaining, original magnification 50x); D. Same case as C, surgical specimen showing high MVD (CD 105 immunostaining, original magnification 100x); E. Immunostaining for CD31 highlights a high MVD in an LSCC biopsy (original magnification 50x); F. Immunostaining for CD31 highlights a low MVD in an LSCC biopsy (original magnification 200x).

association with recurrence (Cox regression model, p = 0.1619). Disease-free survival of patients grouped by CD105- and CD31-assessed MVD on biopsy specimens is shown in Fig. 1A and B, respectively.

3.3. Association between MVD on surgical specimens and clinical outcome

In surgical specimens, CD105- and CD31-assessed MVD were significantly associated with DFS in the Cox regression model (p = 0.0016 and p = 0.0023, respectively), with a recurrence hazard ratio of 1.213 (95% C.I. 1.076-1.369) for CD105-MVD and 1.237 (95% C.I. 1.079-1.418) for CD31-MVD.

In surgical specimens, considering the categorization at median value, recurrence risk was higher in patients with a CD105-assessed MVD > 0 vs. those with MVD = 0 (p = 0.0311), with a recurrence hazard ratio of 34.483 (95% C.I. 1.379 - ∞). Instead, categorization at the median CD31-assessed MVD value (MVD ≤ 6.67 vs. MVD > 6.67) showed a trend towards lower recurrence risk in patients with MVD ≤ 6.67 vs. MVD > 6.67 (Cox regression model, p = 0.0810).

Disease-free survival of patients grouped by CD105- and CD31-assessed MVD on surgical specimens is shown in Fig. 1C and D,

Table 2
Hazard ratio of LSCC recurrence stratified by CD105- and CD31-assessed MVD (in biopsies and surgical specimens).

	Without LSCC recurrence	With LSCC recurrence	P-value	HR (95% CI)
	No. = 19	No. = 5		
CD105 MVD (biopsy)				
Mean	1.07	8.87	0.0033	1.266
(SD)	(2.68)	(5.56)		(1.081–1.481)
Median	0.00	10.34		
(IQR)	(0.00–0.00)	(8.00–11.00)		
CD31 MVD (biopsy)				
Mean	4.28	10.40	0.0081	1.265
(SD)	(2.92)	(5.49)		(1.063–1.505)
Median	4.00	12		
(IQR)	(2.34–6.00)	(8.67–12.67)		
CD105 MVD (surgical specimen)				
Mean	1.19	12.87	0.0016	1.213
(SD)	(3.50)	(6.91)		(1.079–1.418)
Median	0.00	15.67		
(IQR)	(0.00–0.00)	(8.67–17.00)		
CD31 MVD (surgical specimen)				
Mean	5.62	15.81	0.0023	1.237
(SD)	(3.77)	(8.08)		(1.079–1.418)
Median	5.34	18.67		
(IQR)	(3.00–7.00)	(12.34–22.34)		

MVD: microvessel density; SD: standard deviation; IQR: interquartile range; HR: hazard ratio; 95% CI: 95% confidence interval.

Table 3
Hazard ratio of LSCC recurrence stratified by dichotomized CD105- and CD31-assessed MVD values (on biopsies and surgical specimens), and by conventional clinical-pathological variables.

Variables	Without LSCC recurrence	With LSCC recurrence	P-value	HR (95% CI)
	No. = 19	No. = 5		
pT				
T1-T2	15 (78.9%)	3 (60%)	0.4720	1.929 (0.331–11.552)
T3	4 (21.1%)	2 (40%)		
Grading				
G1-G2	15 (78.9%)	3 (60%)	0.3426	2.381 (0.397–14.291)
G3	4 (21.1%)	2 (40%)		
Status N0\N+				
0	18 (94.7%)	5 (100%)	0.6974	1.876 (0.079–45.455)
1	1 (5.3%)	0 (0%)		
Staging				
I-II	14 (73.7%)	3 (60%)	0.6398	1.533 (0.256–9.183)
III	5 (26.3%)	2 (40%)		
CD105 MVD biopsy				
0	15 (78.9%)	1 (20%)	0.0326	11.005 (1.219–99.334)
> 0	4 (21.1%)	4 (80%)		
CD31 MVD biopsy				
≤4.84	11 (57.9%)	1 (20%)	0.1619	4.786 (0.534–42.924)
> 4.84	8 (42.1%)	4 (80%)		
CD105 MVD surgical specimen				
0	16 (84.2%)	0 (0%)	0.0311	34.305 (1.380-∞)
> 0	3 (15.8%)	5 (100%)		
CD31 MVD surgical specimen				
≤6.67	13 (68.1%)	1 (20%)	0.0810	7.062 (0.786–63.465)
> 6.67	6 (31.6%)	4 (80%)		

MVD: microvessel density; SD: standard deviation; IQR: interquartile range; HR: hazard ratio; 95% CI: 95% confidence interval.

respectively.

Table 1 summarizes the population's features in terms of conventional clinical-pathological variables, biopsy-assessed MVD, and histopathological surgical specimen-assessed MVD vis-à-vis recurrence risk.

4. Discussion

This investigation suggests a significant prognostic role of MVD evaluated on LSCC preoperative biopsies, showing an increased HR recurrence in patients with higher CD105- and CD31-stained MVD values (Fig. 2A–F). Moreover, the stratification of LSCC patients by CD105-MVD median value on biopsies led to the identification of a subgroup of cases (those with CD105 MVD > 0) with higher recurrence rate and shorter DFS. Similarly, a significant prognostic role of CD105- and CD31-assessed MVD also emerged from the analysis of surgical specimens, thus supporting the role of such markers as valuable tools for recurrence risk stratification in LSCC (Tables 2 and 3), in line with previous studies [6,9,11].

Biological markers analysis on biopsies may potentially provide relevant prognostic data to support clinical decision making [19]. However, possible concerns lie in the fact that a biopsy may not necessarily be representative of the entire neoplasm and could be affected by several artifacts due to its small size and the procedure itself. Despite this, for some types of malignancies, available evidence seems to depict biopsies as reliable for biomarker assessment purposes. In non–small cell lung carcinomas, programmed death ligand 1 (PD-L1) expression in biopsy samples appeared to be concordant with subsequent surgical specimens [26], and similar results were found by Szade et al. [27] for desmoglein 3, CK7, and p40 as well. Also in the head and neck carcinoma setting, even with the limitation of few available data, it seems possible to obtain reliable immunohistochemistry data from biopsies: in oropharyngeal SCC, Brcic et al. [28] found a high correlation of p16 and PD-L1 expression between small biopsies and surgical specimens. Instead, Takes et al. [19] found different degrees of concordance between biopsies and surgical specimens in LSCCs, depending on the tested markers.

The possibility to obtain prognostic information from biomarker evaluation on biopsies may be particularly relevant in LSCC surgical oncology, in which the criteria indicating postoperative radiation therapy are still somewhat debated. Current guidelines [1] consider adjuvant post-operative radiation therapy for laryngeal cancer in the presence of pathological risk factors, such as positive or close margins, perineural, lymphatic, or vascular invasion, pT4 and, only for supraglottic cancers, pT3 stages, and advanced nodal involvement (N stage ≥2) [22]. In the absence of such factors, also locally advanced tumors, even extending to the post-cricoid area, pre-epiglottic space, paraglottic space and/or inner cortex of the thyroid cartilage, may be considered radically treatable with an *en bloc* resection of all these structures by a total laryngectomy [5,29]. However, modern biological markers could be useful to pinpoint sub-groups of LSCC patients with higher risker of recurrence within the cohorts committed to exclusive upfront surgery according to conventional criteria. The preliminary evidence emerging from this study seems to support a promising prognostic role of CD105 evaluated on biopsies, suggesting novel applications of this biological marker in the preoperative work-up, as part of the biopsy histopathological evaluation.

The main weaknesses of the study concern the retrospective setting of the investigation, and the limited number of cases considered. Instead, the main strength of this investigation lies in the homogeneity of the series of patients considered because: (i) only squamous cell carcinomas located in a single head and neck structure (the larynx) were considered; (ii) all patients underwent primary laryngeal surgery; (iii) their surgical treatment was performed consecutively by the same team; (iv) none of the patients required postoperative radiotherapy or radio-chemotherapy; (v) the CD31- and CD105-assessed MVD was evaluated on both preoperative biopsies and surgical specimens; (vi)

patients' clinical-radiological follow-up criteria were defined. In addition, over the course of about 15 years [30,31], the pathologists in our research group have gained plenty of experience in assessing MVD with several modalities from the immunohistochemical expression of CD105 in head and neck malignancies.

In conclusion, data from this exploratory study suggest a role of biopsy MVD (in particular assessed by CD105 expression) as a predictor of recurrence after exclusive surgery for LSCCs. Further prospective studies are mandatory to better characterize the prognostic role of tumor angiogenesis markers (as CD105) evaluated on biopsies, with the aim of developing novel criteria - besides the traditional clinical-pathological risk factors – to identify in the preoperative staging phase patients at higher risk of LSCC recurrence after surgery, requiring more aggressive approaches or adjuvant treatment.

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Ethics approval

The study was conducted in accordance with the principles of the Helsinki Declaration. Data were examined in agreement with the Italian privacy and sensitive data laws and the University of Padova Otolaryngology Section's internal rules.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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