Characterization and prognostic value of lymphatic vessels in an oral tongue squamous cell carcinoma cohort

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ABSTRACT

Background

Tumor lymphangiogenesis increases the area of interaction between lymphatic vessels and tumor cells, and may thereby facilitate metastases. In oral cancer high lymphatic vessel density (LVD) and large lymphatic vessel area (LVA) have been associated with more aggressive tumors, but results from different studies are not conclusive. The aim of this study was to assess the association of tumor-associated LVD and LVA with clinical-pathological data, including patient survival, in a homogenous cohort of oral tongue squamous cell carcinoma (OTSCC) patients.

Methods

Formalin-fixed, paraffin-embedded tumour samples from 125 OTSCC patients were immunohistochemically stained with the D2-40 antibody to recognize lymphatic vessels. The mean LVD and the mean LVA were analysed in five hotspots per tumour sample and compared to clinical-pathological data as well as 5-year disease-specific survival (DSS).

Results

Neither LVD nor LVA were significantly associated with the clinical-pathological variables or 5-year DSS in our patient cohort. In univariate analyses, the N status, tumour stage, tumour differentiation as well as lymphocyte infiltration were the only significant predictors for patient outcome ($p<0.001$, $p<0.001$, $p<0.001$ and $p=0.043$ respectively). In multivariate analyses, only the N status and tumour differentiation were independent prognostic factors.

Conclusions

LVD and LVA are not indicative of 5-year DSS in our OTSCC cohort. More studies on the immunoregulatory, location-dependent role of lymphatic vessels in large, homogeneous OTSCC patient cohorts are needed.

KEYWORDS: Prognostic marker, oral tongue squamous cell carcinoma, D2-40, lymphatic vessel

BACKGROUND/INTRODUCTION

Worldwide about 355 000 persons are diagnosed with oral cancer each year (1), and more than 90% of these cancers are squamous cell carcinomas (SCCs). The mobile tongue is the most common intraoral site (2-5), and the incidence of oral tongue (OT)SCCs is rising in several countries (6), also among younger persons (7, 8). OTSCC is an aggressive disease, characterized by high treatment morbidity and poor prognosis. The 5-year survival rate of OTSCCs in Norway is about 50% (9), and the prognosis is influenced by factors such as primary tumour size, differentiation and presence and extent of regional and distant metastases. The tumour cells typically metastasize in early stages of the disease, mainly through lymphatic vessels to draining lymph nodes on the neck (10).

In human cancers, lymphatic vessels can undergo changes that facilitate metastasis, such as formation of new lymphatic vessels from pre-existing lymphatics, so-called lymphangiogenesis, as well as structural and morphological alterations including lymphatic enlargement (11). Numerous molecules may modulate lymphangiogenesis, but the best-established once are Vascular Endothelial Growth Factor (VEGF)C and VEGFD. These growth factors may be produced by tumour cells, immune cells, and fibroblasts in the tumour microenvironment, and after proteolytic activation, they both signal through VEGF receptor (VEGFR)3 and induce proliferation of lymphatic endothelial cells. This promotes both the enlargement of existing lymphatic vessels as well as the sprouting of new ones, and increases the area where tumour cells can enter the lymphatic vessels. Furthermore, lymphatic endothelial cells may express chemokines such as CXCL12 and CCL21 which can attract tumour cells that express the matching chemokine receptors CXCR4 and CCR7, thereby facilitating metastases. Production of lymphangiogenic factors, lymphatic remodelling and lymphangiogenesis have been associated with lymph node metastasis and poorer survival rate in mouse models as well as in various human cancers (11). Blocking lymphangiogenic signalling pathways might be a

therapeutic strategy to restrict metastatic spread (12). However, the complex mechanisms of lymphatic tumour metastasis and the interplay between tumour cells, lymphatic vessels and the surrounding immune microenvironment are still poorly understood (13).

Findings from earlier studies point towards an association between lymphangiogenesis and remodeling of tumor-associated lymphatic vessels with more aggressive OSCC (14-19), but results are not conclusive (20, 21). The aim of the current study was to determine the associations of the tumor-associated lymphatic vessels density (LVD) and area (LVA) to clinical-pathological data including lymph node metastases, lymphocyte infiltration and 5-year disease-specific survival (DSS) in a large, homogenous cohort of OTSCC patients.

METHODS

Patients and material

In the current study we used a cohort of 125 OTSCC patients derived from the retrospective Norwegian Oral Cancer (NOROC) study (9). It included patients with primary, treatment naïve SCC confined to the anterior two-thirds of the oral tongue diagnosed between January 1st 2005 and December 31st 2009 at the four head and neck cancer centers in Norway (the university hospitals of Oslo, Bergen, Trondheim, and Tromsø). The last day of follow-up was 1 June 2015, when all patients were followed up for a minimum of five years or until death. We retrieved the cause of death from the Cause of Death Registry if it was not reported in the patients' files. Experienced head and neck surgeons collected relevant clinical data and TNM classification from the patients' hospital files. All tumors were reclassified by experienced pathologists in accordance with the eighth edition of the TNM classification (22), with the T status based on histopathological examination including tumor depth. The N status was based on histopathological analysis (pN) for patients who underwent neck surgery, otherwise it was based on clinical/radiological examination (cN).

All patient information was deidentified prior to analysis. The study was approved by the Regional Ethics Committee of Northern Norway (Protocol number 2013/1786 and 2015/1381), which waived the need to obtain written or oral consent from the patients still alive, though they had the opportunity to opt-out.

Immunohistochemistry

We used 4μm-thick formalin-fixed, paraffin-embedded tumour specimens from our 125 OTSCC patients for immunohistochemical staining. The mouse anti-Podoplanin, clone D2-40 antibody (Dako, Glostrup, Denmark) was used for visualization of lymphatic vessels. Immunohistochemistry was performed on full tumour sections on a Ventana Benchmark Ultra

automated immunostainer (Ventana Medical Systems, VMS, Tucson AZ, USA) at the Diagnostic Clinic – Clinical Pathology, University Hospital of North-Norway (UNN), which is accredited according to the ISO/IEC 15189 standard for the D2-40 staining, using the same protocols, positive and negative controls as in the clinical routines. Prior to staining, all sample sections were incubated overnight at 60°C before deparaffinization in xylene and rehydration in graded alcohol baths. Inherent peroxidase activity in the tissue was blocked with 3% H_2O_2 (Ventana Medical Systems, France or Dako Glostrup, Denmark). Subsequently, the slides were incubated with the primary D2-40 antibody at 1:25 dilution for 32 min at room temperature. A cocktail of HRP labelled goat anti-mouse IgG/IgM together with diaminobenzidine from the Ventana UltraView Universal DAB Detection Kit (#760-500, Ventana) were applied for detection. The slides were then rinsed in distilled water and counterstained with hematoxylin.

Scoring

Assessment of lymphatic vessel density

The number of D2-40 positive vessels was evaluated by the hotspot method. Slides were scanned using an Olympus VS120 slide scanner (Olympus, Germany), and visualized by the Olympus OlyVIA software version 1.06 (Olympus, Germany). All sections were meticulously scanned at low power magnification to recognize areas with high density of lymphatic vessels, so-called hotspots. For each tumour slide five hotspots were photographed at high-power magnification (400x). We assessed lymphatic vessels in intra-tumoural stroma or in the tumour periphery. Vessels embedded within tumour islands were rare and were not assessed. We only assessed vessels that were within one high power field distance from tumor cells, meaning that there had to be some tumor cells present in the visual field. We defined a lymphatic vessel as an immunohistochemically stained ring-structure with thin walls to exclude staining artefacts and uncertain vessels. The wall could not be disrupted and there should be a clear lumen. If two

adjacent lymphatic vessels were connected, they were counted as one. Hotspots from areas with a high background staining or destruction of the tissue were excluded.

If there were no lymphatic vessel hotspots in the tissue sample, the patient was still included but LVD was counted as zero. If there were fewer than five hot spots, the available hotspots were included and LVD was calculated as total number of vessels divided by the number of hotspots available for the patient. For some patients there were two available tissue samples for scoring. For these, both were scored, and the sample with the highest score was chosen.

The number of lymphatic vessels in each hotspot picture was counted by two independent observers who were trained and calibrated by an experienced pathologist prior to the scoring. The scores of the two observers were compared. In cases where the number of lymphatic vessels differed by ≤ 2, the mean of the two observers' counts was used. If the count differed with ≥3 lymphatic vessels the hotspot was revaluated in unity.

Measurement of lymphatic vessel lumen area.

We used the open-source software QuPath version 0.1.2 (23) for measurement of LVA. We downloaded the digital image files of the lymphatic vessel hotspots into QuPath, and manually annotated the lumen area of D2-40-positive tumour-associated vessels using QuPath's wand tool. The annotated areas were automatically calculated by QuPath. Then, the mean LVA for each patient was calculated manually by dividing the total LVA per patient by the number of hotspots available.

For statistical analyses, we dichotomized the results for lymphatic vessel density and lumen area in low vs. high, and small vs. large, respectively. For each variable we tested the cut-off between low/high, and small/large at each quartile: 25% lowest vs. rest; 50% (median); and 75% highest vs. rest. For survival analyses, we reported the results for the quartile that gave the

best separation of survival between the groups. For correlation analyses, we reported the results for LVD and LVA as continuous variables.

Statistical analyses

We used SPSS software version 28.0 for Windows (IBM, Armonk, NY, USA) for all calculations. Inter-observer variability for lymphatic vessel count was analysed using the Spearman correlation test. Correlation between two continuous variables was assessed by the Pearson's Chi-square test, and between continuous and discrete variables by Spearman bivariate correlation analyses. We used Kaplan-Meier analyses to calculate 5-year disease-specific survival (DSS) rates and to plot survival curves. The log rank test was used to evaluate the statistical significance. Multivariate analyses were performed using multiple Cox regression model and the enter method. The significance level was set to $p < 0.05$.

We followed the reporting recommendations for tumour marker prognostic studies (REMARK) to assure reproducibility and transparency of our study (24, 25).

RESULTS

Immunohistochemical staining and scoring of lymphatic vessels

We used the D2-40 antibody for visualization of lymphatic vessels. Representative pictures of immunohistochemical staining and scoring for D2-40 are shown in Figure 1. D2-40-positive lymphatic vessels showed a distinct membranous brown staining and were mostly located in the tumour stroma (Figure 1A). In some cases, there were both background staining and staining of cancer tissue present from the D2-40 antibody. The mean LVD per hotspot ranged from 0.20 to 25.40, with the median being 6.80. The reproducibility for hotspot analyses between the two observers (CEW and IAC) was very good with the Spearman's rho correlation coefficient for mean lymphatic vessel count per tumour section being 0.933. The mean LVA per hotspot ranged from 52 to 8021 μm^2 with the median being 1211 μm^2. Increased LVD significantly correlated with increased LVA (Pearson correlation coefficient $r=0.645$; $p<0.001$; data not shown).

Prognostic value of clinical-pathological variables including lymphatic vessels

As cut-off between high and low mean LVD and LVA we chose the 25 percentile as that yielded the best separation of 5-year DSS. Neither LVD nor LVA were significantly associated with 5-year DSS in univariate Kaplan-Meier survival analyses (Table 1). The absence of lymph node metastases, a low tumor stage and low-grade tumor differentiation showed a highly significant association with longer patient survival ($p<0.001$ for the respective variables). Patients with a low T status as well as patients with abundant tumor-associated lymphocyte infiltration also had significantly improved 5-year DSS survival ($p=0.026$ and $p=0.043$, respectively).

We performed multivariate Cox regression analyses using the enter method for variables that were significant in univariate analyses (T status, N status, tumour differentiation, and lymphocyte infiltration), and ran separate analyses for LVD and LVA. All variables included into the models fulfilled the proportional hazards assumption (Figure S1). The only significant,

independent prognostic factors for 5-year DSS were N status and tumour differentiation (Table 2).

Correlation of lymphatic vessels with clinical-pathological variables

No significant correlations were observed between LVD or LVA as continuous variables and the clinical-pathological variables included in our study. An overview of the results are presented in Table 3.

DISCUSSION

Metastasis to cervical lymph nodes through lymphatic vessels is a common and early event in OTSCC, and is one of the most reliable prognostic factors (26-28). Thus, understanding the role of lymphatic vessels in OTSCC may help to improve patient outcome. In the present study, we show that tumour-associated lymphatic vessels have no prognostic value in our patient cohort, which is in line with several earlier published studies on OTSCC (20, 21, 29). However, other studies have pointed towards an association between increased expression of lymphatic markers and tumour progression and/or poor patient prognosis in OTSCC (30-33). In a recent systematic review by Almahmoudi et al. (34), high expression of lymphatic markers was commonly but not always found to predict poor survival in OTSCC. The authors did not recommend to implement lymphatic markers as prognosticator in clinical practice due to differences in the use of methods and assessment criteria of many studies, small patient cohorts or cohorts of patients with cancers from numerous anatomical subsites, as well as poor reporting in the studies. Our results on the prognostic value of lymphatic vessels in OTSCC derive from a large patient cohort of tumours confined to the anterior two-thirds of the tongue, with clinical-pathological data being carefully verified by clinical specialists (9). We followed the REMARK guidelines (24, 25) to allow transparency and reproducibility of our results, which need to be verified in large, homogeneous OTSCC cohorts.

There is no consensus on how to assess LVD in tumors. Lymphatic vessels within tumour islands are often poorly functional due to intratumoural pressure, and are not considered to be crucial for lymph node metastasis (11). Nevertheless, both Chen et al. and Maula et al. have demonstrated that intra- but not peritumoural lymphatic vessels were associated with a poor prognosis in head and neck squamous cell carcinoma (35, 36). In contrast, peritumoural lymphatic vessels are main routes for tumour metastatic spread (37), and most published studies seem to calculate peritumoural vessel density (34). In addition to differences in where to count

lymphatic vessels, the strategy of which vessels to include in the count may differ between studies, for instance how to handle collapsed vessels, vessels with incomplete walls or neighboring vessels where it is difficult to decide whether they are the same or separate vessels. These issues may render the counting subjective. However, the two independent observers in our study had a very high inter-observer agreement on the counts, suggesting that by training and calibration it is possible to define criteria that allow reproducibility in counting. Nevertheless, measuring LVA may be an easier method to standardize, and probably less prone to different approaches between studies. We tested both approaches and LVD and LVA were significantly correlated. However, neither LVD nor LVA significantly correlated with 5-year DSS or any of the clinical-pathological variables included in our study.

D2-40 (podoplanin), as used in our study, and lymphatic vessel endothelial hyaluronan receptor (LYVE)-1 are widely used markers for the detection of lymphatic endothelium, but their specificity has been challenged through staining of non-lymphatic tissue, suggesting a combination of at least two lymphatic endothelium markers for accurate detection (38). Of note, we only reported D2-40-positive lymphatic vessels in the tumour stroma and not within tumour isles, thus the occasional D2-40 staining of the cancer tissue probably did not impair the sensitivity of our results.

The contradictory findings on the prognostic value of LVD and LVA may be due to such vessels' ability to also generate anti-tumour immune responses through attracting antigen-presenting cells to the draining lymph nodes (37). The immunomodulatory effect of lymphatic vessels might however be temporarily regulated, as tumour-associated lymphatic endothelial cells may also suppress ongoing anti-tumour responses during tumour development (39). We found no significant correlation between LVD or LVA and lymphocytic infiltration in the present study. However, it would be interesting to study the location-dependent role of lymphatic vessels in OTSCC, especially in relation to various types of infiltrating immune cells

and lymphangiogenic factors in the tumour immune microenvironment including the TLS. Effective anti-tumour responses can also be generated at the tumour site through tertiary lymphoid structures (TLS) (40), which we have earlier shown to be associated with improved survival in OSCC patients (41). Formation of lymphatic vessels eventually occurs in TLS, but much remains unknown (42).

CONCLUSION

In conclusion, LVD and LVA were not associated with patient survival or any clinical-pathological variables in our OTSCC cohort. Evidence suggests that tumour-associated lymphatic vessels have immunomodulatory functions (39), and it would be interesting to study the location-dependent role of lymphatic vessels in OTSCC, especially in relation to various types of infiltrating immune cells and lymphangiogenic factors in the tumour immune microenvironment including the TLS. The immunomodulatory functions may however be temporarily regulated (39), which requires studies on the role of lymphatic vessels at different stages during tumour development.

ADDITIONAL INFORMATION

Conflict of interest

The authors declare no conflicts of interest, financial or non-financial, for this article.

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Authorship

Conceptualization, E.H.-O. and A.M.W.; methodology, C.E.W., I.A.C.; validation A.M.W. and E.H.-O.; statistical analysis, A.M.W.; data curation, I.-H.B., S.E.S. and L.U.-H.; writing – original draft preparation, E.H.-O., C.E.W., I.A.C. and A.M.W.; writing – review and editing, S.N.M., L.U.-H., S.E.S., I.A.C., C.E.W. E.H.-O. and A.M.W; project administration S.N.M., E.H.-O. and A.M.W.; funding acquisition, L.U.-H.

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Figures

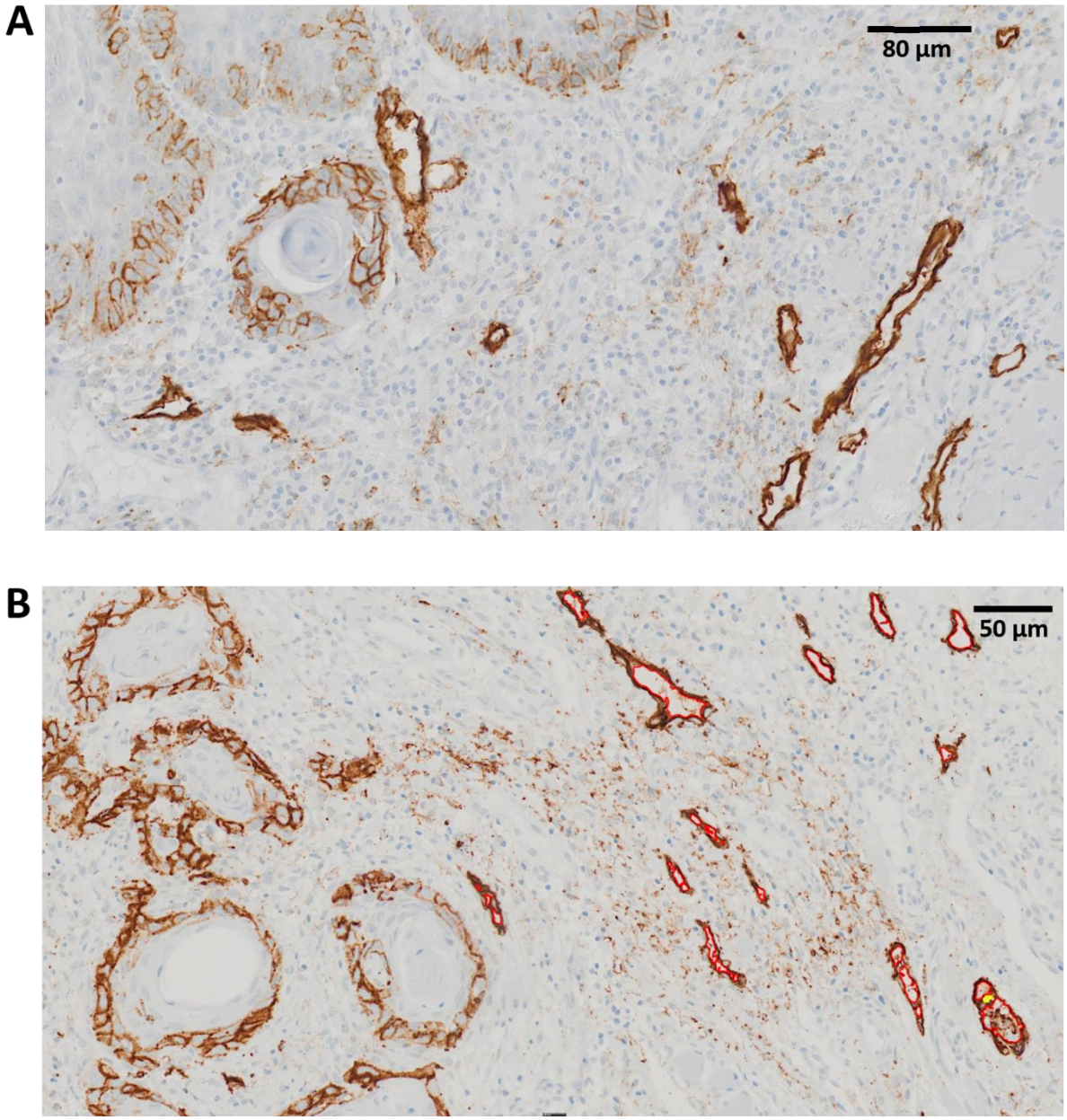

Figure 1. Representative immunohistochemical staining and scoring for D2-40. A) shows a representative picture of a hotspot containing D2-40 positive vessels. B) shows a representative picture of measurement of D2-40 positive vessel lumen area. Red color indicates lumen area detected by the QuPath software.

Tables

Table 1. Clinical-pathological characteristics of oral tongue squamous cell carcinoma patients (n=125) including lymphatic vessel density (LVD) and area (LVA), and their association with 5-year disease-specific survival (DSS) in Kaplan-Meier analysis. The p-value was calculated using the log-rank test, with the missing/unknown cases for the respective variables omitted, and the significance level set to 0.05.

	N	5-year DSS %	p-value
Gender			
Male	76	68.4	0.783
Female	49	71.4	
Age at diagnosis, years			
< 60	48	70.8	0.444
≥ 60	77	68.8	
Smoking			
Never	34	79.4	
Current	49	69.4	0.110
Former	30	53.3	
Missing	12	83.3	
T status			
T1	42	83.3	
T2/T3	80	65.0	*0.026*
Unknown	3	0.0	
N status			
N0	91	79.1	
N+	32	40.6	*<0.001*
Nx	2	100.0	
Stage			
Low stage (stage I or II)	73	80.8	
High stage (stage III or IV)	50	54.0	*<0.001*
Nx/Unknown	2	50.0	
Differentiation, whole tumour			
Low-grade (well or moderate)	107	73.8	
High-grade (poor)	11	27.3	*<0.001*
Missing	7	71.4	
Lymphocyte infiltration			
Little	41	58.5	
Abundant	75	74.7	*0.043*
Missing	9	77.8	
LVD			

Low	32	62.5	0.158
High	93	72.0	
LVA (µm²)			
Small	30	60.0	0.186
Large	92	71.7	
Missing	3	100.0	

[1] Combination of cN and pN. In case of neck dissection, the result on pN was superior to cN.

[2] The best separation cutoff was 25% for lymphatic vessel density and lymphatic vessel lumen area.

Table 2. Multivariate analysis of 5-year disease-specific survival in oral tongue squamous cell carcinoma (OTSCC) according to Cox's proportional hazards model. T status, N status, tumour differentiation and lymphocyte infiltration were adjusted for LVD and LVA separately. The missing/unknown cases (n=14) were excluded from the OTSCC cohort (n=125).

	Adjusted for LVD			**Adjusted for LVA**		
Variable n	**Hazard ratio**	**95% CI**	**P-value**	**Hazard ratio**	**95% CI**	**P-value**
T status T1, n=35 vs T2/T3, n=76	1.848	0.718-4.755	0.203	1.893	0.745-4.808	0.180
N status N+, n=29 vs N0, n=82	3.003	1.429-6.308	*0.004*	3.474	1.632-7.396	*0.001*
Differentiation, whole tumour High-grade (poor), n=10 vs low-grade (well or moderate), n=101	3.277	1.369-7.840	*0.008*	3.433	1.480-7.966	*0.004*
Lymphocyte infiltration Abundant, n=70 vs little, n=41	0.597	0.291-1.227	0.160	0.648	0.317-1.322	0.233
LVD High, n=84 vs low, n=27	0.695	0.318-1.519	0.361			
LVA Large, n=75 vs small, n=25				0.499	0.227-1.095	0.083

Table 3. Correlation between clinical-pathological variables of oral tongue squamous cell carcinoma patients and lymphatic vessel density (LVD) as well as lymphatic vessel area (LVA) using Spearman bivariate correlation analyses. The p-value for the Spearman rank correlation coefficient was calculated with the missing/unknown cases for the respective variables omitted, and the significance level set to 0.05.

	LVD, n=125		**LVA (µm²), n=122**	
	Mean (±SD)	*P*	*Mean (±SD)*	*P*
Gender				
Male	7.19 (4.05)	0.549	1447.41 (1502.47)	0.105
Female	7.19 (3.82)		1552.28 (1104.93)	
Age at diagnosis, years				
≤ 60	7.32 (3.83)	0.716	1513.35 (1423.70)	0.496
> 60	7.11 (4.03)		1469.82 (1330.57)	
Smoking				
Never	6.65 (2.43)	0.388	1313.84 (735.18)	0.968
Current	6.75 (4.41)		1407.90 (1485.01)	
Former	7.83 (3.56)		1695.47 /1696.13)	
Missing	8.90 (5.78)		1743.50 (1256.84)	
T status				
T1	6.78 (2.30)	0.545	1154.95 (1095.60)	0.261
T2	7.86 (4.89)		1719.59 (1281.66)	
T3	6.67 (3.71)		1615.29 (1780.43)	
Unknown	8.07 (6.22)		761.00 (638.160)	
N status[1]				
N0	7.03 (3.53)	0.746	1435.77 (1411.78)	0.170
N+	7.68 (5.02)		1634.22 (1240.59)	
Nx	6.60 (3.39)		1382.50 (1581.80)	
Stage				
Low stage (stage I or II)	7.07 (3.54)	0.935	1413.10 (1263.62)	0.630
High stage (stage III or IV)	7.30 (4.47)		1615.80 (1509.66)	
Nx/Unknown	8.70 (6.36)		850.50 (829.44)	
Differentiation, whole tumour				
Low-grade (well or moderate)	7.40 (4.12)	0.101	1545.44 (1431.73)	0.456
High-grade (poor)	5.55 (2.65)		1061.82 (535.62)	
Missing	6.57 (2.03)		1091.50 (689.71)	
Lymphocyte infiltration				
Little	6.68 (2.84)	0.677	1270.93 (841.67)	0.585
Abundant	7.62 (4.53)		1642.00 (1601.67)	
Missing	5.89 (2.42)		1025.00 (527.65)	

[1] Combination of cN and pN. In case of neck dissection, the result on pN was superior to cN.

Supplementary

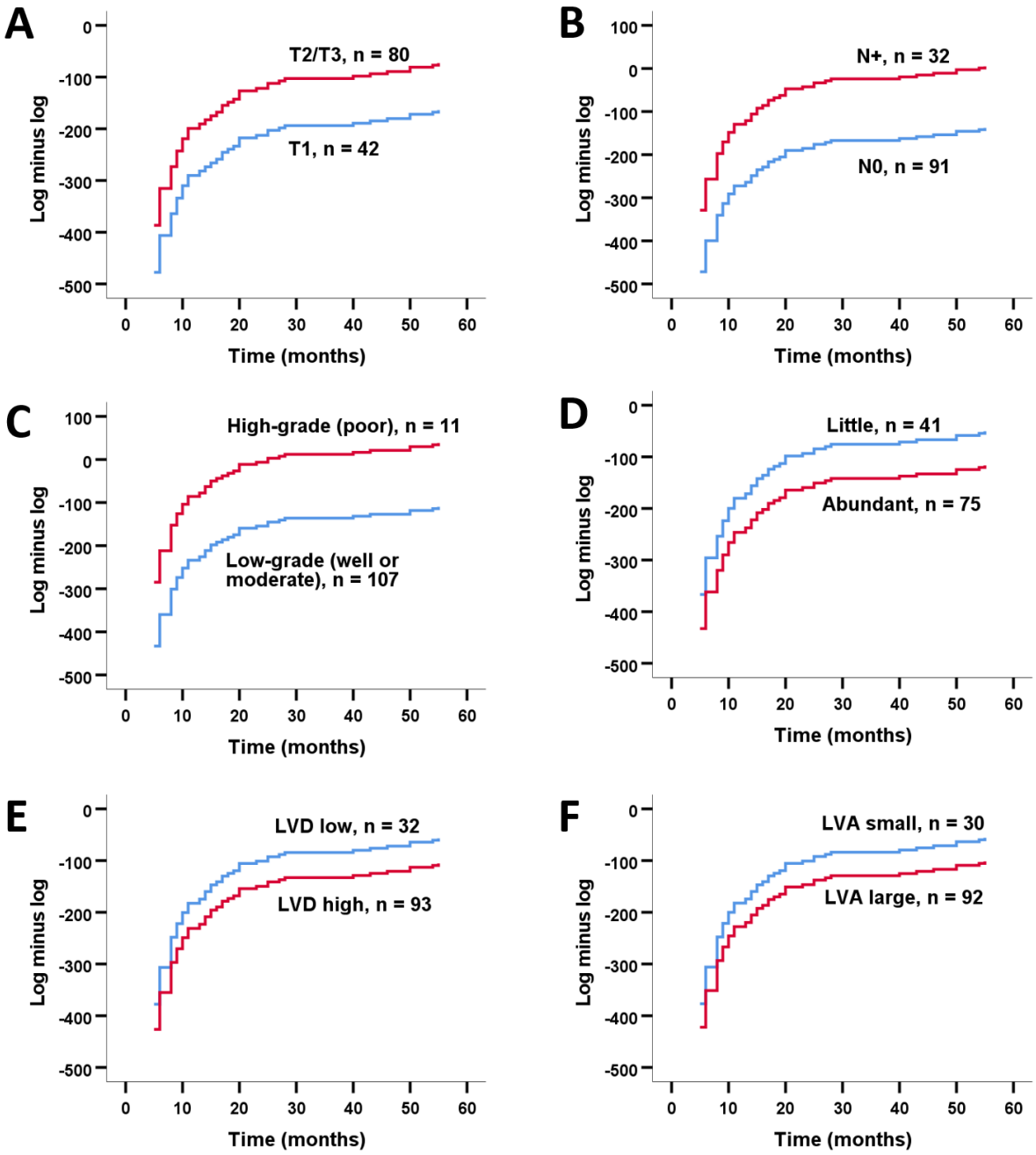

Figure S1. Log minus log plots for proportional hazards checking. A) pT status, B) N status, C) differentiation of whole tumour, D) lymphocyte infiltration, E) lymphatic vessel density (LVD), and F) lymphatic vessel lumen area (LVA).