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HEAD AND NECK

High relative density of lymphatic vessels predicts poor survival in tongue squamous cell carcinoma

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Abstract Tongue cancer has a poor prognosis due to its early metastasis via lymphatic vessels. The present study aimed at evaluating lymphatic vessel density, relative density of lymphatic vessel, and diameter of lymphatic vessels and its predictive role in tongue cancer. Paraffin-embedded tongue and lymph node specimens ($n = 113$) were stained immunohistochemically with a polyclonal antibody von Willebrand factor, recognizing blood and lymphatic endothelium and with a monoclonal antibody podoplanin, recognizing lymphatic endothelium. The relative density of lymphatic vessels was counted by dividing the mean number of lymphatic vessels per microscopic field (podoplanin) by the mean number of all vessels (vWf) per microscopic field. The high relative density of lymphatic vessels (≥80 %) was associated with poor prognosis in tongue cancer. The relative density of lymphatic vessels predicted poor prognosis in the group of primary tumor size T1–T2 and in the group of non-metastatic cancer. The lymphatic vessel density and diameter of lymphatic vessels were not associated with tongue cancer survival. The relative density of lymphatic vessels might

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have clinically relevant prognostic impact. Further studies with increased number of patients are needed.

Keywords Tongue cancer · Head and neck cancer · Lymphatic vessels · Survival · Immunohistochemistry

Introduction

Tongue squamous cell carcinoma (tongue cancer) accounts for more than half of the total oral cancer cases diagnosed each year worldwide [1]. Tongue cancer is one of the most aggressive cancers in the oral cavity [1]. Its 5-year survival rate is only about 50 % [2]. Lymph node metastasis strongly correlates with the survival in patients with oral cancer [3]. The incidence of neck lymph node metastasis in oral cancer varies from 25 to 65 % [4, 5]. Tongue cancer typically metastasizes to cervical lymph nodes [6] due to the presence of a rich lymphatic network and the high number of lymph nodes in the neck region [3, 7, 8]. Tumor cells are able to migrate to lymphatic vessels and, therefore, migrate to the lymph nodes [9]. Blocking the lymphangiogenesis of the tumor might be important in preventing the metastatic cascade of the cancer. Thus, understanding the pathophysiology of lymph node metastasis in tongue cancer is essential for early diagnosis and treatment.

Quantification of blood and lymphatic vessel density has been used to understand the cancer biology and as a prognostic factor, for example, in melanoma and head and neck cancer [10, 11]. There are several antibodies which recognize blood and/or lymphatic vessel antigens. Von Willebrand factor (vWf) is a large glycoprotein with a multimeric structure which mass ranging from 500 kDa up to over 10 000 kDa [12]. The encoding gene of vWf is located in chromosome 12p13.2 [13]. vWf is produced through a multiphase process by endothelial cells and megakaryocytes [13]. The primary product of the gene is a 2813 amino acids protein which is composed of a signal peptide of 22 amino acids, a large propeptide of 741 amino acids, and a mature vWF molecule containing 2050 amino acids [12]. vWf is expressed by blood endothelium and also weakly expressed on lymphatic endothelium [14–19]. Results about vWf expression in cancers are ambivalent [20]. Studies have shown that colorectal and gastric cancers have a higher density of vWf stained vessels than healthy tissue. The high density of vessels correlates with poor survival [13, 21]. In contradiction, previous experimental studies have demonstrated that vWf could reduce metastases by inducing tumor cell death [22]. In addition, vWf might have pro-apoptotic properties, thus its deficiency enhances metastatic potential in mice [23].

Monoclonal antibody podoplanin (clone D2-40) recognizes podoplanin in lymphatic endothelium. D2-40 was originally recognized as an oncofetal antigen, M2A antigen, which is an *O*-linked sialoglycoprotein with a simple mucin-type carbohydrate epitope associated with germ-cell neoplasms [24, 25]. Currently, it is known that podoplanin and M2A antigen are identical proteins [25]. Podoplanin is expressed in lymphatic endothelial cells, but is not expressed in blood endothelial cells [26]. Therefore, it is a specific marker for lymphatic endothelium [26]. The overexpression of podoplanin has been reported in oral premalignacies, such as oral leukoplakia, oral erythroleukoplakia, carcinoma in situ, and lichen planus [27–31].

Tongue cancer is often aggressive, invasive, and has high tumor growth capacity which generates the considerable risk of lymphatic invasion by tumor cells [32, 33]. Lymphangiogenesis, richness of lymphatic vessels, and muscularized structures have shown to be crucial in progression of tongue cancer [34]. To understand more of these pathways, this study aimed at evaluating lymphatic vessel density, lymphatic vessel diameter, the relative density of lymphatic vessels, and its predictive role in tongue cancer. We hypothesized that lymphatic vessel density associates with tongue cancer survival.

Materials and methods

Clinical samples

This study was performed at the Departments of Otorhinolaryngology and Pathology, Tampere University Hospital and Haartman Institute, University of Helsinki, Finland. The study protocol was approved by the Hospital's Ethical Committee. The formalin-fixed paraffin-embedded samples were collected retrospectively from the archives of the hospital. The samples were taken from 61 patients who had been treated for tongue squamous cell carcinoma during the years 1999–2007. The control group consisted of 29 patients who had been treated for tongue squamous cell hyperplasia during the same time interval. All samples were reviewed by a pathologist. The demographic data, collected from patient records, are shown in Table 1. The power analysis was performed by approximated 75 % increase in median lymphatic vessel density in the non-surviving group. With approximated standard deviation of 1.0, the estimated sample size was 56 ($\alpha = 0.05$, $\beta = 0.2$).

Tissue handling and immunohistochemistry

Von Willebrand factor and podoplanin staining were performed on 113 samples. Before performing immunohistochemistry, the pathologist (TP) reviewed all samples by

Table 1 Characteristics of the patients

	Tongue hyperplasia, $n = 29$		Tongue cancer, $n = 61$		p value
	n	%	n	%	
Gender					
Male	17	58.6	33	54.1	0.821
Female	12	41.4	28	45.9	
Age					
<60 years	19	65.6	27	44.3	0.073
≥60 years	10	34.5	34	55.7	
Smokers					
No	22	75.9	29	47.5	0.013
Yes	7	24.1	32	52.5	
Heavy alcohol users					
No	24	85.7	44	72.1	0.189
Yes	4	14.3	17	27.9	
Previous lichen ruber planus	2	6.9	11	18.0	0.033

p values by Fisher's exact test

light microscope and selected the samples with the most representative pathological signs of cancer or hyperplasia with no or very little necrosis. The samples were cut into 4–5 μm-thick paraffin sections and were placed on Superfrost Plus microscope slides (Menzel-Gläser, Braunschweig, Germany). Fully automated immunostaining was performed by Ventana BenchMark LT Automated IHC Stainer (Ventana Medical System, Tucson, Arizona, USA). Ultraview Universal DAB detection kit (catalog no. 760-500, Ventana Medical System) was used. Ventana EZ Prep solution (catalog no. 950-100, Ventana Medical System) was used for deparaffinisation. For epitope retrieval CC1: Tris-EDTA buffer pH 8.0 (catalog no. 950-124, Ventana Medical System) was used at 95 to 100 °C for 30 min with paraffin-embedded tissue sections. Endogenous peroxidase was blocked with UV-inhibitor 3 % H202 (Ventana Medical System) for 4 min at 37 °C. Tissue slides were rinsed between steps with Ventana Tris-based Reaction buffer (catalog no. 950-300, Ventana Medical System). Slides were incubated at 37 °C for 24 min with pAb anti-Von Willebrand factor (1:2000, A0082, DAKO Corporation, Carpinteria, CA, USA), and for 32 min with mAb anti-podoplanin (1:50, Clone D2-40, M3619, DAKO Corporation, Carpinteria, CA, USA) followed by the application of Ventana Ultraview HRP Universal Multimer (8 min at 37 °C). Diaminobenzidine (DAB) was used as a chromogen and hematoxylin as a nuclear stain. The known positive tissue samples from sinonasal mucosa were also used to confirm the staining reliability of all separate staining patches. Specificity of immunohistochemistry was controlled by omitting the primary antibodies or replacing them with irrelevant antisera.

Light microscopic evaluation

Cytoplasmic staining of lymphatic and blood endothelial cells were examined with a Leica DM 2000 light microscope (Leica Microsystems GmbH, Wetzlar, Germany) by two observers without knowledge of clinical status and outcome data.

In the tongue cancer specimens, the vessel density was calculated in intratumoral area and whole tumor (including intra- and peritumoral) from five microscopic fields (0.785 mm^2/field). The relative density of lymphatic vessels (RDLV) was counted by dividing the mean number of lymphatic vessels per microscopic field (podoplanin) by the mean number of all vessels (vWf) per microscopic field. vWF is weakly expressed on lymphatic endothelium. Therefore, the positive lymphatic vessel staining of vWf was assured by the lack of erythrocyte or smooth muscle within the lymphatic vessel walls. Analysis was performed at 200× (20× objective lens and 10× ocular lens) magnification.

The mean diameters of lymphatic vessels were determined in five fields with 400 (40× objective lens and 10× ocular lens) magnification in intratumoral area and whole tumor. The mean diameter of the samples was graded as $S \leq 50$ μm, $M = 51$–130 μm, $L < 130$ μm.

Data analysis

Descriptive statistics for patients and tumor characteristics were presented in tongue cancer and in tongue hyperplasia. Fisher's exact test (2-tailed) was used to compare patient and tumor characteristics. The correlations were analyzed by the Spearman rank correlation test. The Kruskal–Wallis test (more than two groups) and Mann–Whitney U test

(two groups) were used to study the comparison of the vessels densities, the relative density of lymphatic vessels, and diameters of lymphatic vessels in the different groups. Survival curves were drawn by the Kaplan–Meier method, and analyzed by the log-rank test. Overall, survival was calculated from the date of diagnosis to death, while disease-specific survival was calculated from date of diagnosis to death from tongue cancer or the end of June 2014 whichever came first. Two-tailed p-values of <0.05 were considered statistically significant. Statistical analysis was carried out by the SPSS Base 15.0 Statistical Software Package (SPSS Inc., Chicago, IL, USA).

Results

Patient and tumor characteristics

The average age of the patients in the tongue hyperplasia group was 56 years (min–max 31–82 years) and in the tongue carcinoma group, it was 61 years (min–max 17–91 years). The difference was not statistically significant (Table 1). The number of smokers and patients with previously diagnosed lichen ruber planus in the oral mucosa was higher in the tongue cancer group than the tongue hyperplasia group (Table 1). No significant differences in other demographic factors were found.

The characteristics of the tumors are shown in Table 2. 32.8 % of tongue cancer patients had tumor size over 4 cm (T3–T4), 50.8 % had cervical lymph node metastasis, 63.9 % had tumor depth of at least 4 mm, 3.3 % had perineural invasion, and 19.7 % had poorly differentiated cancer (grade III). 88.5 % of tongue cancer patients underwent operation. 54.1 % received chemoradiotherapy. 85.2 % of the operated patients underwent additional cervical lymph node management. Growth into one resection margin was detected in 5.6 % of all resected tumors. Cancer recurrence occurred in 47.5 % of cases. 36.1 % of tongue cancer patients were alive after 5 years of follow-up.

The lymphatic and blood vessel density in tongue hyperplasia and tongue cancer

Figure 1 shows von Willebrand factor and podoplanin expression in tongue specimens. In the whole tumor, the mean density of all vessels (vWf) was 35.7/field in the tongue hyperplasia group and 30.6/field in the tongue cancer group. The difference was not statistically significant (Table 3). The mean density of lymphatic vessels was 15.3/field in the tongue hyperplasia group and 16.6/field in the tongue cancer group. The difference was not statistically

Table 2 Characteristics of the tumors

	Tongue cancer, $n = 61$	
	n	%
Primary tumor size		
T1	14	23.0
T2	27	44.3
T3	17	27.9
T4	3	4.9
cN classification		
cN0	44	72.1
cN1	12	19.7
cN2	5	8.2
pN classification		
pN0	19	31.1
pN1	7	11.5
pN2	20	32.8
pNx	15	24.6
Cervical LN metastasis[a]		
LN−	30	49.2
LN+	31	50.8
Tumor operation		
Resection	20	32.8
Resection and flap reconstruction[b]	34	55.7
Inoperable	7	11.5
Cervical lymph node management		
No	15	24.6
Functional[c]	44	72.1
Radical	2	3.3
Resection margin		
Clear (≥3 mm)	33	54.1
Close (<3 mm)	18	29.5
Involved	3	4.9
Unknown	7	11.5
Tumor grade		
I	24	39.3
II	25	41.0
III	12	19.7
Perineural invasion		
No	57	93.4
Yes	2	3.3
Unknown	2	3.3
Lymphatic vessel invasion		
No	58	93.4
Yes	1	1.6
Unknown	2	3.3
Tumor depth		
Low (<4 mm)	7	11.5
High (≥4 mm)	39	63.9
Unknown	15	24.6

Table 2 continued

	Tongue cancer, $n = 61$	
	n	%
Chemotherapy		
No	28	45.9
Yes	33	54.1
Recurrence		
No	32	52.5
Yes	29	47.5
Status (5 years of follow-up)		
Alive	22	36.1
Dead of tongue cancer	27	44.3
Dead of other causes	12	19.7

[a] Positive cervical lymph node metastasis (LN+) was determined by pN+; or cN+ in case of pNx

[b] Microvascular reconstruction by radial forearm flap

[c] Includes removal of sentinel lymph nodes

significant (Table 3). When observing density of all vessels, and lymphatic vessels in the subgroups of primary tumor diameter, tumor grades, cervical lymph node metastasis, perineural invasion, tumor depth, or 5-year mortality, a significant difference was not found (Table 3).

In intratumoral area, the mean density of all vessels was 33.2/field and the mean density of lymphatic vessels was 22.9/field. When observing the mean density of all vessels and lymphatic vessels in the subgroups of primary tumor sizes, tumor grades, and cervical lymph node metastasis, a significant difference was not found. The mean density of all vessel and lymphatic vessel in intratumoral area was significantly higher than in whole tumor ($p = 0.037$, $p = 0.001$, retrospectively, data not shown).

The lymphatic vessels diameter in tongue cancer and tongue hyperplasia

The mean diameter of lymphatic vessels was 67 µm (min–max 13–182 µm) in tongue cancer and 60 (min–max 13–150) in tongue hyperplasia. The diameter of lymphatic vessels did not differ between the tongue hyperplasia group and the tongue cancer group. The diameter of lymphatic vessels in whole tumor and intratumoral area did not differ in the subgroups of primary tumor size, tumor grade, and cervical lymph node metastasis. When comparing the cancer subgroups with RDLV ≥80 % and <80 %, the diameter of lymphatic vessel in the whole tumor area, or in the intratumoral area, did not differ significantly.

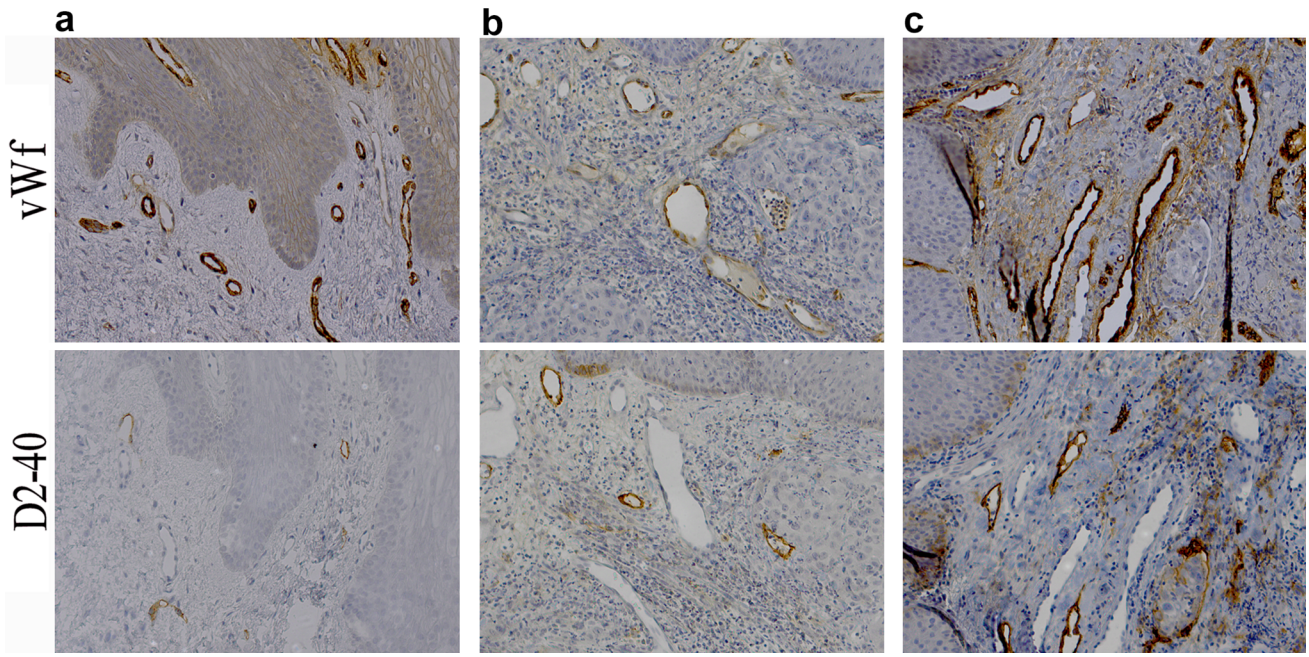

Fig. 1 Von Willebrand factor and podoplanin (clone D2-40) expression in tongue specimens by immunohistochemical methods. The *upper row* detects all vessels stained by von Willebrand factor and the *lower row* detects lymphatic vessels stained by podoplanin. **a** <80 % relative density of lymphatic vessel in tongue hyperplasia. **b** <80 % relative density of lymphatic vessel in tongue cancer. **c** ≥80 % relative density in tongue cancer. Original magnification was 200×

Table 3 Median values of vessels densities and relative densities of lymphatic vessel by characteristics of the patients

	All vessels (N)	*p* value	Lymphatic vessels (*N*)	*p* value	RDLV (%)	*p* value
Diagnosis		0.207		0.959		0.300
Hyperplasia	35.7		15.3		50.0	
Cancer	30.6		16.6		62.5	
Primary tumor size		0.991		0.856		0.775
T1	29.9		15.3		52.5	
T2	35.7		16.6		70.5	
T3	30.6		19.1		58.8	
T4	25.5		33.1		74.3	
Grade		0.565		0.257		0.443
I	30.0		14.0		48.9	
II	33.1		19.1		68.6	
III	28.0		17.3		73.2	
Cervical LN metastasis[a]		0.540		0.633		0.306
LN−	33.1		19.1		56.1	
LN+	29.3		15.3		72.0	
Perineural invasion		0.307		0.202		0.379
No	31.8		17.8		64.0	
Yes	21.0		8.9		39.0	
Tumor depth		0.217		0.828		0.006
<4 mm	35.7		14.0		39.3	
≥4 mm	29.3		16.6		72.0	
Mortality related to tongue cancer (5 years)		0.964		0.319		0.065
No	31.8		14.0		48.9	
Yes	31.8		19.1		71.4	
Mortality related to other cause (5 years)		0.527		0.148		0.080
No	31.9		14.0		48.9	
Yes	29.9		18.5		70.8	

vWf indicates blood and lymphatic vessel density in microscopic field and podoplanin indicates lymphatic vessel density in microscopic field (0.785 mm^2/field; original magnification 200×). The relative density of lymphatic vessel (RDLV) indicates proportion of lymphatic vessels (podoplanin) to all vessels (vWf)

RDVL the relative density of lymphatic vessels, *y* years

* *p* values by Mann–Whitney *U* test (dichotomous variables) and Kruskall–Wallis *H*'s test (continuous variables)

[a] Positive cervical lymph node metastasis (LN+) was determined by pN+; or cN+ in the case of pNx

The relative density of lymphatic vessels in tongue hyperplasia and tongue cancer

The mean RDLV was 50 % in the tongue hyperplasia group and 62.5 % in the tongue cancer group. The difference was not statistically significant (Table 3). The median RDLV was statistically significantly higher in the subgroup of tumor depth of ≥4 mm, compared to the group having tumor depth of <4 mm (Table 3). When comparing RDLV in the subgroups based on primary tumor size, tumor grades, cervical lymph node metastasis, perineural invasion, or 5-year mortality, a significant difference was not found (Table 3). Similarly, when comparing intratumoral RDLV in the subgroup based on primary tumor size, tumor grade, and cervical lymph node metastasis, no significant difference was detected.

The prognostic relevance of relative density of lymphatic vessels in tongue cancer

In the present study, RDLV was associated with poor survival in tongue cancer (Fig. 2). When observing all cancer patients, RDLV ≥80 % predicted poor overall and disease-specific survival (Fig. 2a, b). When observing the subgroup of patients with primary tumor size T1–T2, RDLV ≥80 % predicted poor overall and

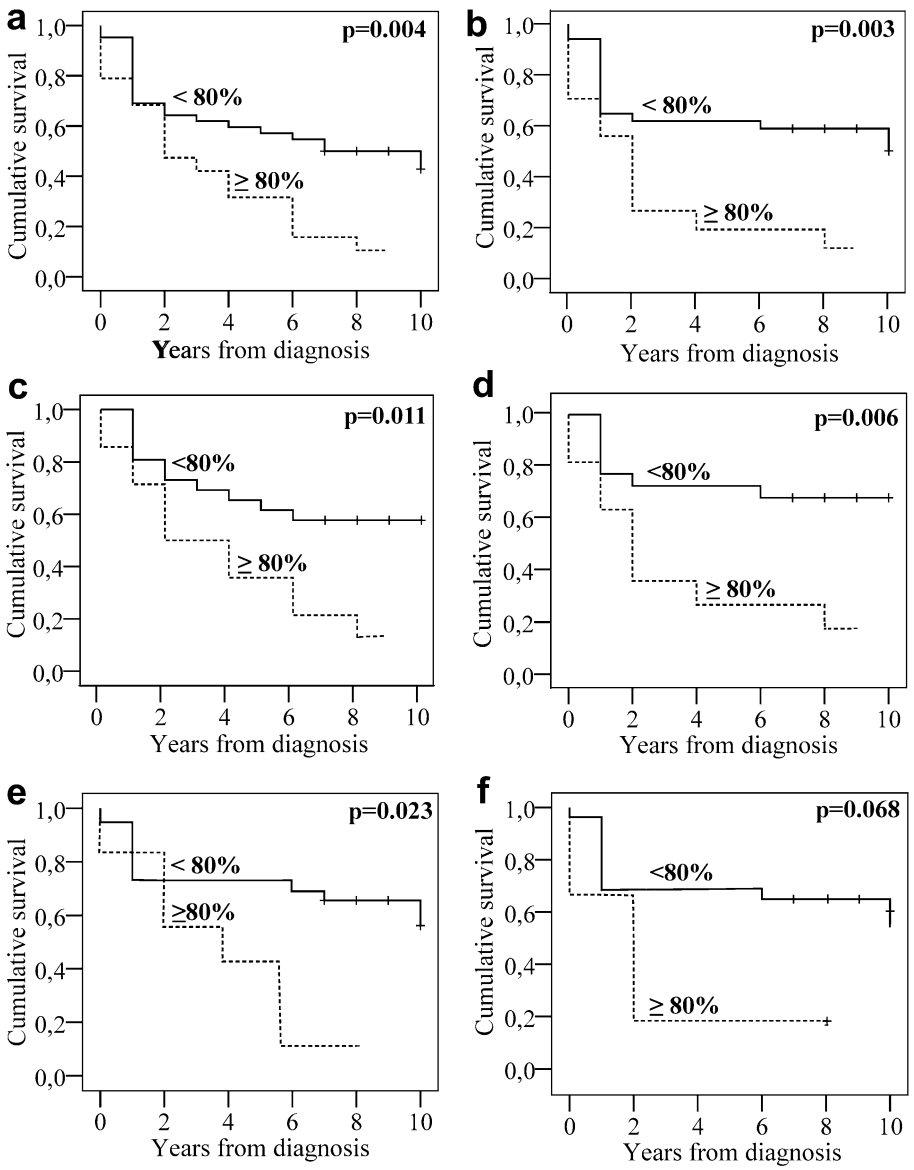

Fig. 2 Relative density of lymphatic vessel to all vessels and survival curves of patients with tongue cancer according to the Kaplan–Meier method. **a** Overall survival for the tongue cancer patients ($n = 61$). **b** Disease-specific survival for the tongue cancer patients ($n = 49$). **c** Overall survival for the patients with primary tumor size T1–T2 ($n = 40$). **d** Disease-specific survival for the patients with primary tumor size T1–T2 ($n = 33$). **e** Overall survival for the patients with negative cervical lymph node metastasis (LN−) ($n = 38$). **f** Disease-specific survival for the patients with LN− ($n = 33$). LN− was determined by pN−; or by cN− in the cases of pNx. *p*-values by the log-rank test

disease-specific survival (Fig. 2c, d) When observing the subgroup without cervical lymph node metastasis, RDLV ≥80 % associated significantly with poor overall survival, and insignificantly with poor disease-specific survival (Fig. 2e, f). In contrast, when observing the subgroup of patients with cervical lymph node metastasis, RDLV did not affect patients' prognosis. In addition, the same analysis was performed with the cut-off value of ≥90 %, and even poorer survival rates were detected ($p \leq 0.001$, data not shown). We also performed all analyses with RDLV using cut-off values lower than 80 %. However, cut-off values not bring significant difference to the survival rate. When we observed only the intratumoral area, RDVL had no effect on patients' survival. The mean density and diameter of lymphatic vessels did not have an effect on patient survival, when it was assessed in the whole tumor or in the intratumoral area.

Cox regression analysis

Disease-specific survival was also tested for the following 12 factors by Cox regression: age, gender, smoking, heavy alcohol abuse, previous lichen ruber planus, tumor size, nodal metastasis, perineural invasion, lymphatic vessel invasion, tumor depth, resection marginal, and RDLV. In the unadjusted model, only the three following factors associated significantly with tongue cancer death: tumor size ≥20 mm ($p = 0.044$, OR 2.90, CI 1.03–8.18), the presence of cervical metastasis ($p = 0.021$, OR 2.54, CI 1.15–5.62), and RDLV ≥80 % ($p = 0.049$, OR 2.27, CI 1.00–5.11). When analyzing these three factors in the same

adjusted model, only RDLV ≥80 % associated significantly with tongue cancer death ($p = 0.050$, OR 2.24, CI 1.00–5.02). Thus, the association of RDLV with tongue cancer survival remained significant when adjusted by cervical metastasis and tumor size.

Discussion

This study was implemented to evaluate lymphatic vessel density and its predictive role in tongue cancer. The most significant finding was that high RDLV (≥80 %) associates with poor survival in tongue cancer, as well as in the subgroups with primary tumor size T1–T2 and non-metastasized tongue cancer. High proportion of lymphatic vessels might enable invasion of tongue cancer and thus lead to patients' poor outcome. Interestingly, we detected an association between high depth of tumor invasion and high proportion of lymphatic vessels. This is in line with observations, that crosstalk between invasive tumor and microenvironment might result in lymphangiogenesis [35]. We found that high RDLV did not affect patients' survival in the subgroup with metastatic tongue cancer. This might be due to the fact that metastatic tongue cancer has overall poorer prognosis than non-metastatic tongue cancer. To the knowledge of the authors, RDLV in tongue cancer has not previously been studied, and further studies in enlarged sample size and other populations are required to prove the results.

Despite the fact that the knowledge of the role of RDLV in tongue cancer is minor, the absolute lymphatic vessels density has previously been studied. We did not detect an association between high lymphatic vessel density and tongue cancer survival. The results of the predictive role of lymphatic vessel density have been demonstrated to be controversial also in other studies. A Japanese study group performed VEGF-C immunostainings in oral cancer samples, and they demonstrated that high lymphatic vessel density in whole tumor area predicts poor survival [36]. Another study group performed podoplanin immunostaining in head and neck tumors, including samples from oral cavity, oropharynx, and larynx, in population of Italy, and showed that high peritumoral lymphatic vessel density predicts poor survival [37]. On the other hand, another study group performed podoplanin immunostaining in head and neck tumors, including samples from oral cavity, lower lip, and larynx, in population of Greece, and they did not find a predictive role of peritumoral lymphatic vessel density [38]. Furthermore, Muñoz-Guerra et al. stained oral cancer specimens' immunohistochemically by PA2.26, and they suggested that high density of intratumoral lymphatic vessels associates with poor prognosis [39]. In addition, O'Donnell et al. studied population of the US and stained oral cancer specimens' immunohistochemically by podoplanin. They detected that the number of intratumoral and peritumoral lymphatic vessels is similar in oral cancer [38, 40]. Our finding was that lymphatic vessels density in intratumoral area is significantly higher than in whole tumor. However, in our study, intratumoral lymphatic vessel density did not affect patients' survival. These controversial results might be due to differences in the populations, tumor locations, patient treatments, and immunohistochemical or calculation techniques, such as calculating vessels in intra- or peritumoral area. Thus, the question of the role of the lymphatic vessels in tongue cancer arises and requires additional studies.

Previous studies have indicated that the cells of the immune system might prevent the cancer from spreading through the blood vessels [20]. In addition, vWf might have pro-apoptotic properties, and it might reduce metastases by inducing tumor cell death [20, 22]. This could be indirectly detected from our results, thus high proportion of lymphatic vessels to all vessels predicts poor survival. However, in our study, the vessels stained by vWf did not predict better prognosis. Some other studies have suggested that the interaction between tumor cells and coagulation cascade factors (such as vWf) and/or platelets might promote invasiveness and metastasis capacity of cancer cells [20, 41–44]. Hence, the role of blood and lymphatic vessels in cancers remains unsolved, and additional studies are needed.

Monoclonal anti-podoplanin, clone D2-40, is a reliable lymphatic endothelial cell marker which identifies podoplanin in lymphatic endothelium [24, 25, 45]. In many cancers, the role of podoplanin has associated with tumor progression and metastasis [46]. Several studies have revealed altered podoplanin expression in various cancers, including oral squamous cell carcinoma [46]. In addition, previous studies have indicated the potential role of this overexpressed protein in carcinogenesis, in hyperplastic and dysplastic areas in oral mucosa [26, 32, 47]. Instead, podoplanin expression has not been detected or was extremely low in normal epithelium indicating its role in tumorgenesis [26]. In this particular study, lymphatic vessel density did not differ between tongue hyperplasia and tongue cancer. Keeping this in mind, healthy tongue samples are also provided in future studies in cancer pathogenesis and early diagnostics.

We acknowledge that the number of patients was small, and that no healthy tongue tissue samples were available in this study. These results should be confirmed by a greater patient data and cross checked with healthy control group. The proportion of lymphatic vessels to all vessels might consume more time from the pathologist, which might limit the clinical feasibility of RDLV as a prognostic marker. On the other hand, microscopy might

be easier based on our findings that RDLV can be assessed from the whole sample instead of intratumoral or peritumoral locations, which in some cases might be difficult to differentiate. Due to the low number of patients, we were not able to completely analyze the co-effect of several factors.

As a conclusion, the density and diameter of lymphatic vessels were not associated with tongue cancer survival. On the other hand, high relative density of lymphatic vessels associates with tumor invasiveness and poor survival. Further studies with increased population size are still required to assess clinical relevancy of the findings.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. For this type of study, formal consent is not required. This article does not contain any studies with animals performed by any of the authors.

Informed consent Informed consent was obtained from all individual participants included in the study.

References

1. Bello IO, Soini Y, Salo T (2010) Prognostic evaluation of oral tongue cancer: means, markers and perspectives (II). Oral Oncol 46(9):636–643
2. Scully C, Bagan J (2009) Oral squamous cell carcinoma: overview of current understanding of aetiopathogenesis and clinical implications. Oral Dis 15(6):388–399
3. Forastiere A, Koch W, Trotti A, Sidransky D (2001) Head and neck cancer. N Engl J Med 345(26):1890–1900
4. Siegel R, Naishadham D, Jemal A (2013) Cancer statistics, 2013. CA Cancer J Clin 63(1):11–30
5. Jemal A, Siegel R, Xu J, Ward E (2010) Cancer statistics, 2010. CA Cancer J Clin 60(5):277–300
6. Teichgraeber JF, Clairmont AA (1984) The incidence of occult metastases for cancer of the oral tongue and floor of the mouth: treatment rationale. Head Neck Surg 7(1):15–21
7. Kuriakose MA, Trivedi NP (2009) Sentinel node biopsy in head and neck squamous cell carcinoma. Curr Opin Otolaryngol Head Neck Surg 17(2):100–110
8. Clark JR, Naranjo N, Franklin JH, de Almeida J, Gullane PJ (2006) Established prognostic variables in N0 oral carcinoma. Otolaryngol Head Neck Surg 135(5):748–753
9. Storr SJ, Safuan S, Mitra A, Elliott F, Walker C, Vasko MJ et al (2012) Objective assessment of blood and lymphatic vessel invasion and association with macrophage infiltration in cutaneous melanoma. Mod Pathol 25(4):493–504
10. Tatti O, Gucciardo E, Pekkonen P, Holopainen T, Louhimo R, Repo P et al (2015) MMP16 mediates a proteolytic switch to promote cell-cell adhesion, collagen alignment, and lymphatic invasion in melanoma. Cancer Res 75(10):2083–2094
11. Audet N, Beasley NJ, MacMillan C, Jackson DG, Gullane PJ, Kamel-Reid S (2005) Lymphatic vessel density, nodal metastases, and prognosis in patients with head and neck cancer. Arch Otolaryngol Head Neck Surg 131(12):1065–1070
12. Denis CV (2002) Molecular and cellular biology of von Willebrand factor. Int J Hematol 75(1):3–8
13. Yang X, Sun H, Li Z, Zhang H, Yang W, Ni B et al (2015) Gastric cancer-associated enhancement of von Willebrand factor is regulated by vascular endothelial growth factor and related to disease severity. BMC Cancer 15(1):80
14. Sozio F, Rossi A, Weber E, Abraham DJ, Nicholson AG, Wells AU et al (2012) Morphometric analysis of intralobular, interlobular and pleural lymphatics in normal human lung. J Anat 220(4):396–404
15. Wada H, Shiozawa M, Sugano N, Morinaga S, Rino Y, Masuda M et al (2013) Lymphatic invasion identified with D2-40 immunostaining as a risk factor of nodal metastasis in T1 colorectal cancer. Int J Clin Oncol 18(6):1025–1031
16. Kahn HJ, Bailey D, Marks A (2002) Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. Mod Pathol 15(4):434–440
17. Fogt F, Zimmerman R, Ross H, Daly T, Gausas R (2004) Identification of lymphatic vessels in malignant, adenomatous and normal colonic mucosa using the novel immunostain D2-40. Oncol Rep 11(1):47–50
18. Miettinen M, Lindenmayer AE, Chaubal A (1994) Endothelial cell markers CD31, CD34, and BNH9 antibody to H- and Y-antigens—evaluation of their specificity and sensitivity in the diagnosis of vascular tumors and comparison with von Willebrand factor. Mod Pathol 7(1):82–90
19. Erhard H, Rietveld FJ, Bröcker EB, De Waal RM, Ruiter DJ (1996) Phenotype of normal cutaneous microvasculature. J Invest Dermatol 106(1):135–140
20. Franchini M, Frattini F, Crestani S, Bonfanti C, Lippi G (2013) von Willebrand factor and cancer: a renewed interest. Thromb Res 131(4):290–292
21. Lackner C, Jukic Z, Tsybrovskyy O, Jatzko G, Wette V, Hoefler G et al (2004) Prognostic relevance of tumour-associated macrophages and von Willebrand factor-positive microvessels in colorectal cancer. Virchows Arch 445(2):160–167
22. Terraube V, Pendu R, Baruch D, Gebbink M, Meyer D, Lenting P et al (2006) Increased metastatic potential of tumor cells in von Willebrand factor-deficient mice. J Thromb Haemost 4(3):519–526
23. Shavit J, Motto D (2006) Coagulation and metastasis–an unexpected role for von Willebrand factor. J Thromb Haemost 4(3):517–518
24. Michikawa C, Uzawa N, Kayamori K, Sonoda I, Ohyama Y, Okada N et al (2012) Clinical significance of lymphatic and blood vessel invasion in oral tongue squamous cell carcinomas. Oral Oncol 48(4):320–324
25. Marks A, Sutherland DR, Bailey D, Iglesias J, Law J, Lei M et al (1999) Characterization and distribution of an oncofetal antigen

(M2A antigen) expressed on testicular germ cell tumours. Br J Cancer 80(3–4):569–578
26. Agarwal D, Pardhe N, Bajpai M, Gupta S, Mathur N, Vanaki S et al (2014) Characterization, localization and patterning of lymphatics and blood vessels in oral squamous cell carcinoma: a comparative study using D2-40 and CD-34 IHC marker. J Clin Diag Res JCDR 8(10):ZC86
27. Zhang G, Guo ZL, Gao Y (2009) Podoplanin expression in oral squamous cell carcinoma and leukoplakia and its correlation with lymph vessels density. Zhonghua Kou Qiang Yi Xue Za Zhi 44(8):488–491
28. de Vicente JC, Rodrigo JP, Rodriguez-Santamarta T, Lequerica-Fernández P, Allonca E, García-Pedrero JM (2013) Podoplanin expression in oral leukoplakia: tumorigenic role. Oral Oncol 49(6):598–603
29. Kreppel M, Kreppel B, Drebber U, Wedemayer I, Rothamel D, Zöller J et al (2012) Podoplanin expression in oral leukoplakia: prognostic value and clinicopathological implications. Oral Dis 18(7):692–699
30. Feng J, Mi J, Wu L, Ma L, Shi L, Yang X et al (2012) Expression of podoplanin and ABCG2 in oral erythroplakia correlate with oral cancer development. Oral Oncol 48(9):848–852
31. Funayama A, Cheng J, Maruyama S, Yamazaki M, Kobayashi T, Syafriadi M et al (2011) Enhanced expression of podoplanin in oral carcinomas in situ and squamous cell carcinomas. Pathobiology 78(3):171
32. Inoue M, Roan C, Abe T, Buery RR, Nagatsuka H, Katase N et al (2011) Localization and characterization of lymphatic vessels in oral and cervical squamous cell carcinoma. Exp Ther Med 2(5):793–797
33. Li H, Guo L, Chen S, Zhao X, Zhuang S, Wang L et al (2012) GOLPH3 overexpression correlates with tumor progression and poor prognosis in patients with clinically N0 oral tongue cancer. J Transl Med 10(1):168
34. Sano D, Myers JN (2007) Metastasis of squamous cell carcinoma of the oral tongue. Cancer Metastasis Rev 26(3–4):645–662
35. Schlereth SL, Refaian N, Iden S, Cursiefen C, Heindl LM (2014) Impact of the prolymphangiogenic crosstalk in the tumor microenvironment on lymphatic cancer metastasis. BioMed Res Int 2014:639058. doi:10.1155/2014/639058
36. Miyahara M, Tanuma J, Sugihara K, Semba I (2007) Tumor lymphangiogenesis correlates with lymph node metastasis and clinicopathologic parameters in oral squamous cell carcinoma. Cancer 110(6):1287–1294
37. Franchi A, Gallo O, Massi D, Baroni G, Santucci M (2004) Tumor lymphangiogenesis in head and neck squamous cell carcinoma. Cancer 101(5):973–978
38. Kyzas PA, Geleff S, Batistatou A, Agnantis NJ, Stefanou D (2005) Evidence for lymphangiogenesis and its prognostic implications in head and neck squamous cell carcinoma. J Pathol 206(2):170–177
39. Muñoz-Guerra MF, Marazuela EG, Martín-Villar E, Quintanilla M, Gamallo C (2004) Prognostic significance of intratumoral lymphangiogenesis in squamous cell carcinoma of the oral cavity. Cancer 100(3):553–560
40. O'Donnell RK, Feldman M, Mick R, Muschel RJ (2008) Immunohistochemical method identifies lymphovascular invasion in a majority of oral squamous cell carcinomas and discriminates between blood and lymphatic vessel invasion. J Histochem Cytochem 56(9):803–810
41. Franchini M, Lippi G, Montagnana M, Targher G, Zaffanello M, Salvagno GL et al (2009) Hemophilia and cancer: a new challenge for hemophilia centers. Cancer Treat Rev 35(4):374–377
42. Langer F, Amirkhosravi A, Ingersoll S, Walker J, Spath B, Eifrig B et al (2006) Experimental metastasis and primary tumor growth in mice with hemophilia A. J Thromb Haemost 4(5):1056–1062
43. Brüggemann LW, Versteeg HH, Niers TM, Reitsma PH, Spek CA (2008) Experimental melanoma metastasis in lungs of mice with congenital coagulation disorders. J Cell Mol Med 12(6b):2622–2627
44. Franchini M, Mannucci PM (2012) Thrombin and cancer: from molecular basis to therapeutic implications. Semin Thromb Hemost 38(1):95
45. Yonemura Y, Endou Y, Tabachi K, Kawamura T, Yun H, Kameya T et al (2006) Evaluation of lymphatic invasion in primary gastric cancer by a new monoclonal antibody, D2-40. Hum Pathol 37(9):1193–1199
46. Swain N, Kumar SV, Routray S, Pathak J, Patel S (2014) Podoplanin—a novel marker in oral carcinogenesis. Tumor Biol 35(9):8407–8413
47. Kawaguchi H, El-Naggar AK, Papadimitrakopoulou V, Ren H, Fan YH, Feng L et al (2008) Podoplanin: a novel marker for oral cancer risk in patients with oral premalignancy. J Clin Oncol 26(3):354–360