

Presence of tumour high-endothelial venules is an independent positive prognostic factor and stratifies patients with advanced-stage oral squamous cell carcinoma

Running title:

High-endothelial venules in oral cancer

Anna M. Wirsing¹, Oddveig G. Rikardsen^{1,2}, Sonja E. Steigen^{1,3}, Lars Uhlin-Hansen^{1,3} and Elin Hadler-Olsen^{1,3}

¹Department of Medical Biology, Faculty of Health Sciences, University of Tromsø – The Arctic University of Norway, 9037 Tromsø, Norway

²Department of Otorhinolaryngology, University Hospital of North Norway, 9038 Tromsø, Norway

³Department of Clinical Pathology, University Hospital of North Norway, 9038 Tromsø, Norway

Corresponding author:

Anna M. Wirsing

Department of Medical Biology, Faculty of Health Sciences,

University of Tromsø – The Arctic University of Norway, 9037 Tromsø, Norway

Telephone: +47 77644644

Fax: +47 77644680

E-mail: anna.wirsing@uit.no

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Ethics, Northern Norway (REK-number 22/2007) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. This article does not present any animal studies.

Informed consent: The Regional Committee for Medical and Health Research Ethics, Northern Norway approved the study without requiring informed consent from the patients, as many of them were dead when the study was initiated.

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Abstract

Background: Staging of oral squamous cell carcinoma is based on the TNM system, which has been deemed insufficient for prognostic purposes. Hence, better prognostic tools are needed to reflect the biological diversity of these cancers. Previously, high numbers of specialized blood vessels called high-endothelial venules have been reported to be associated with prolonged survival in patients with breast cancer. In this study, we analysed the prognostic value and morphological characteristics of tumour-associated high-endothelial venules in oral cancer.

Methods: The presence of tumour-associated high-endothelial venules was evaluated by immunohistochemistry in 75 patients with oral squamous cell carcinoma and analysed with correlation to clinicopathological parameters, patients' survival, and vessel morphology. Ten of the samples were analysed at multiple levels to evaluate intratumoural heterogeneity.

Results: The presence of tumour-associated high-endothelial venules was found to be associated with lower disease-specific death in multivariate regression analyses ($P=0.002$). High-endothelial venules were present in all ($n=53$) T1-T2 tumours, but only in two-thirds ($n=14$) of the T3-T4 tumours. The morphology of high-endothelial venules was heterogeneous and correlated with lymphocyte density. High-endothelial venules were found to be distributed homogeneously within the tumours.

Conclusion: We found the presence of tumour-associated high-endothelial venules to be an easy-to-use, robust, and independent positive prognostic factor for patients with oral cancer. Absence of these vessels in advanced-stage tumours might identify patients with more aggressive disease. Evaluating the presence of tumour-associated high-endothelial venules might help to tailor the treatment of oral cancer patients to their individual needs.

Keywords

Oral squamous cell carcinoma, prognostic factor, high-endothelial venules, peripheral node addressin, inflammation, lymphocyte trafficking

Introduction

The far majority (>90%) of malignancies in the oral cavity and the oropharynx are squamous cell carcinomas (SCCs), which are generally aggressive and frequently metastasize to lymph nodes at an early phase (1). The TNM staging system, which describes tumours based on the size and invasion features of the primary tumour (T), the presence of lymph node metastases (N), and distant metastases (M) (2) is currently the most reliable way to predict the outcome for patients with oral SCCs (OSCCs) (3, 4). However, the TNM grading does not reflect the considerable biological diversity of these tumours (5). Numerous studies on potential prognostic markers to foresee the outcome and therapeutic needs of individual OSCC patients have been performed (6-8). Despite concerted efforts, none of these biomarkers are routinely used in clinical practice. Thus, there is a great need to identify new and better prognostic tools that are both reliable and easy to implement in clinical routines. Furthermore, a better understanding of the biology of OSCCs could guide the development of new, targeted therapies for OSCC patients.

Angiogenesis is one of the hallmarks of cancer and, as such, generally associated with tumour progression and poor clinical outcome (9). However, formation of specialized blood vessels called high-endothelial venules (HEVs) has recently been found to be associated with a favourable prognosis in breast cancer patients, probably by facilitating anti-tumour responses through recruitment of cytotoxic lymphocytes to the tumour site (10, 11). HEVs appear normally in lymph nodes where they support high levels of lymphocyte extravasation from the blood (10, 12). As their name implies, HEVs are characterized by cuboidal endothelial cells, which express specialized ligands for lymphocytes such as the chemokine peripheral node addressin (PNAd) on their luminal surface (13). By binding to L-Selectin, PNAd anchors circulating, naive lymphocytes to the HEV wall (14). Lymphocyte extravasation is mediated through discontinuous, 'spot-welded' junctions, which are characteristic for HEV endothelial cells (12, 15, 16), and which differ from the tight-junctions that characterize capillary and arterial endothelium (17). High density of tumour-infiltrating lymphocytes (TILs) has earlier been shown to have beneficial effects on patient survival in several human solid tumours (18, 19). TILs are sometimes organized in so-called tertiary lymphoid structures (TLSs), which resemble lymphoid follicles in lymph nodes, but typically appear in non-lymphoid tissue under terms of chronic inflammation (20). HEVs are thought to be key players in the recruitment of lymphocytes to the TLSs, and TLS formation has been associated with improved survival rates in lung-, breast-, colorectal- and oral cancer (11, 21-23). In human cancers, presence of HEVs, both in TLSs and independent of these structures, is a recent discovery (10). Tumour HEV density and phenotype have been found to be highly heterogeneous and dependent on the surrounding tissue, suggesting that HEVs shape their tissue microenvironment and vice versa (24). HEVs were seen to form independently from T- and B-lymphocytes but

strongly required signalling from dendritic cells (13, 25, 26). Remodelling of tumour HEVs from lymphocyte-carrying vessels into dilated, blood-carrying vessels with thin walls has been proposed as an early prognostic marker of sentinel lymph node metastasis in breast and oral cancer patients (27, 28). Different from their counterparts within peripheral lymph nodes, tumour-associated HEVs and HEVs within TLSs are still poorly understood (29). However, a better understanding of the mechanisms regulating tumour HEVs might have a promising potential in modulating tumour growth and developing new therapeutic strategies for cancer patients (24).

The present study was performed to assess the morphology and prognostic value of tumour-associated HEVs in OSCCs as well as their correlation to clinicopathological characteristics. Immunohistochemical staining for HEVs was conducted on tumour samples from 75 OSCC patients. We found that the presence of HEVs is an independent prognostic marker for lower disease-specific death (DSD), and that lack of these vessels in advanced-stage tumours identifies patients with more aggressive disease. Thus, the presence of HEVs might be a useful hallmark to stratify OSCC patients and to help select patient subsets for individual therapeutic approaches.

Materials and Methods

Patients

The study broadly adheres to the REMARK recommendations for tumour marker prognostic studies (30). Formalin-fixed, paraffin-embedded tumour samples from 75 patients with histologically verified primary SCC of the oral cavity were collected from the archives of the Department of Clinical Pathology, University Hospital of North Norway (UNN). Inclusion criteria were availability of both tissue from the primary tumour and clinical information. Patients with prior radiotherapy to the head and neck area and patients with previous oral and pharyngeal cancer were excluded from the study. Large biopsies covering the subepithelial areas were evaluated from the patients who were not resected. Data on clinicopathological features, including treatment procedures and the HPV status were acquired from the patients' hospital files, pathology reports and the Statistics of Norway, Cause of Death Registry, and are presented in Table 1. The tumours were staged according to the newest TNM classification at the time of diagnosis (31-34). The classification of cancers of the lip and oral cavity remained unchanged between the different editions during the registration periods thus giving no consequence for the study. The patients were diagnosed in the period 1986–2002 and the last day of follow-up was January 1st, 2012. The Regional Committee for Medical and Health Research Ethics, Northern Norway, approved the use of the patients' tissue and the collection of the clinical information (REK-number 22/2007).

Immunohistochemistry

Immunohistochemical studies of HEVs were performed on formalin-fixed, paraffin-embedded, four-micrometer-thick tumour tissue sections. Manual staining for HEVs, including evaluation of antibody specificity, were carried out as previously described (23, 35). In brief, after rehydration, heat-induced antigen retrieval, and blocking steps, sections were incubated with the PNAd primary antibody (#120801, Rat anti-PNAd, clone MECA-79, Biologend, San Diego, diluted 1:25) for 30 minutes. Afterwards, HRP-labelled goat anti-rat light chain secondary antibody (#AP202P, Millipore, Temecula, CA, diluted 1:250, incubated 30 minutes) and diaminobenzidine (Dako EnVision + System-Horseradish Peroxidase, Dako,) were used for detection. Counterstaining was done with Harris hematoxylin (Sigma-Aldrich, St. Louis, MO). As previously described(23), formalin-fixed, paraffin-embedded human lymph nodes served as positive controls. Antibody specificity was evaluated by immunohistochemical staining of consecutive sections of OSCC cancer tissue from six different patients as well as three normal oral mucosa samples with the PNAd antibody, the blood vessel marker CD34, and the lymphatic endothelial cell marker D2-40. A few CD34+ vessels, but no D2-40+ lymphatic vessels displayed sporadic PNAd-staining in consecutive

OSCC tissue sections, whereas the three normal oral mucosa tissue sections were entirely negative for the PNAd antibody. No other HEV markers were used for verification of PNAd-positive vessels, as the PNAd antibody displayed only little unspecific staining. Sections where the primary antibody was omitted were used as negative controls.

High-endothelial venule count and morphological analysis

Seventy-five patients were included in the study. In 65 of them, the presence of HEVs was assessed based on evaluation of the PNAd staining at a single level in the tumour tissue block. In the remaining 10 patients, the blocks were cut down completely and presence of HEVs was assessed at 100µm distance throughout the tumour sample. All sections were stained with the endothelial cell marker PNAd as described above. The cutoff-value for PNAd-positivity was obtained from hotspot analyses, as a modification of the method published by Weidner et al (36). The sections were assessed by light microscopy at low power magnification (100×) to recognize the areas with highest HEV density (hotspots). Only distinct brown PNAd-staining in clusters of more than one cell were considered as HEVs. Micrographs of the five areas with highest HEV density were taken with a Leica DFC 420 camera on a Leica DM2000 microscope (Leica, Wetzlar, Germany) at high power magnification (400×), and the number of HEVs in the photographs was counted manually. For each tumour, the number of HEVs in each hotspot was added and the total number was then divided by five, giving a mean number of tumour-associated HEVs per section. Some tumours had less than five HEV positive areas. In these cases, the total sum of HEVs was also divided by five. The median number of HEVs per hotspot for the whole group of patients was used as cutoff-value for positive and negative HEV count, respectively. To verify PNAd staining, whole-slide digital images of the same tumour sections were manually reinvestigated for presence of HEVs at very high magnification under the virtual objective of the virtual microscope using the same cutoff-value for HEV-positivity as derived by hotspot analyses. This approach mainly aimed to avoid false-negative results due to weak PNAd staining in sporadic sections, which might be hard to detect using conventional light microscopy. Moreover, there is a growing interest in using whole-slide imaging for different applications in pathology practice (37), and we wanted to test practical issues including efficiency of PNAd detection. For these reasons, and for being able to study HEV morphology, all slides were scanned with a Zeiss Mirax Scanner at 400× magnification, and the whole-slide digital images were investigated at high-magnification (up to 63.76×) using the image analysis Mirax Viewer Software (3d Histech, Budapest, Hungary). Digital line measurement-tools, developed in the Mirax Viewer Software, were used to analyse (minimal) distances between outer vessel wall of HEVs and nearest tumour cells, vessel wall thickness, and inner vessel diameter. For quantitative estimation of the association between morphologic alteration of HEVs

and level of inflammation, vessel wall thickness and lumen diameter of 100 HEVs were analysed in areas with low and high levels of inflammation, respectively. Level of inflammation was evaluated semi quantitatively in the digitized PNAd stained sections with a cutoff of 50 lymphocytes/field of vision at high power magnification. Branched vessels and vessels without evident lumina were not taken into account when evaluating vessel wall thickness and inner lumen diameter. In general, no PNAd-positive single cells were counted as HEVs. HEVs have earlier been found in the same tissue sections in relation to TLSs (23), and these lymph node-like structures might also arise in the salivary glands, unrelated to the tumour. Thus, to exclude HEVs that were not tumour-associated, only HEVs within 700µm distance from the tumour front were taken into account. A trained pathologist histologically evaluated this. All studies were carried out manually.

Statistical Analysis

Statistical analyses were performed using the SPSS software version 22.0 for Windows (IBM, Armonk, NY) and Microsoft Excel 2013 (Microsoft, Redmond, WA). Inter-observer variability for HEV count was quantified by the Spearman correlation test. Vessel wall and lumen diameter data produced by image analysis deviated from a normal distribution. Therefore, a Mann-Whitney U test was used to determine if there was a significant difference in vessel wall thickness and lumen diameter between HEVs found in areas with high and low levels of inflammation, respectively. Correlation analyses for possible associations between different variables were performed using the Pearson's Chi-square test. Univariate Kaplan Meier analyses were used to evaluate disease-specific death (DSD) rates and disease-specific survival (DSS) curves. Significant differences between the groups of patients were estimated by the log-rank test. Statistically significant determinants in the univariate analysis were entered into multivariate Cox regression analyses. A stepwise forward multiple Cox regression analysis was carried out to determine independent prognostic factors. Validity of the proportional hazards assumption was tested by plotting log-minus-log plots. P-values <0.05 were considered statistically significant.

Results

High-endothelial venules were present in the majority of oral squamous cell carcinomas and homogeneously distributed within each tumour

Seventy-five patients with SCC of the oral cavity were evaluated for presence of HEVs by immunohistochemical staining for the MECA-79-reactive ligand PNAd. We first defined a cutoff value for PNAd-positivity by analogue hotspot analysis. The total number of tumour-associated HEVs was counted in five hotspots from each section. According to a median count of 0.5 HEVs per hotspot, we defined tumours with a total number of < 3 tumour-associated HEVs per section as HEV-negative and those with a total number of ≥ 3 HEVs per section as HEV-positive. The reproducibility for hotspot analysis between the two investigators (EHO and AMW) was very good with the Spearman's Rho correlation coefficient for mean HEV count per tumour section being 0.970. The cutoff value was subsequently applied to whole-slide digital images of the same tissue sections. Digital image analyses at high-power magnification using the designed cutoff-value for PNAd-positivity revealed that 68 (90.7%) of the OSCC samples were positive and the remaining 7 (9.3%) were negative. The final evaluation of the sections with high magnification revealed more vessels than the first hotspot analyses. We therefore recommend using high-power magnification to detect vessels with less intensive staining and avoid false-negative results. HEVs were preferentially located peripherally in the tumour stroma within marked accumulations of inflammatory cells. The tissue samples that were investigated for HEVs on multiple levels showed a full correlation in HEV score (positive/negative) between the levels. Nine of the 10 tissue blocks were positive for HEVs on multiple levels throughout the tumour sample, whereas one tissue block was entirely negative for HEVs on multiple levels.

High-endothelial venules in oral squamous cell carcinoma displayed heterogeneous morphology related to the inflammatory infiltrate in oral squamous cell carcinoma

The HEVs in our OSCC patient cohort displayed a heterogeneous morphology, and the three main phenotypes were: vessels with a thick wall and a small lumen (Figure 1A), vessels with a thin wall and a larger lumen (Figure 1B), and branched vessels (Figure 1C). Dilated and especially branched vessels were characterized by a gradual loss of their specific marker PNAd (Figure 1B and C, arrows). Morphologic alterations of HEVs were also evaluated in association to their surrounding tissue microenvironment (Figure 2). In highly inflammatory regions, mainly HEVs with a thick wall and a small or no lumen were seen (Figure 2A), whereas HEVs in regions with less infiltrating immune cells usually presented with thinner walls and a larger lumen (Figure 2B). Quantitative analysis of vessel wall thickness and lumen diameter of 100 HEVs in areas with low and high level of

inflammation, respectively, showed that vessel walls of HEVs were significantly thicker in areas with high compared to areas with low grade of inflammation ($U=1724.5$; $P<0.001$; Figure 2D). Lumen diameter, however, was significantly larger in HEVs located within low-grade inflammatory regions ($U=3219.0$; $P<0.001$; Figure 2E). Branched vessels were found in locations with various levels of inflammation (Figure 2, asterisks). Only few HEVs were seen in areas with very low inflammation (Figure 2C). Each of the tumour samples usually contained a heterogeneous mixture of the 3 HEV morphologic phenotypes (Figure 2, overview picture) and no single phenotype was exclusively found in any of the tumours. In association with the TLSs, however, only thick-walled HEVs with a very small or no lumen were found (data previously presented in (23)).

Presence of tumour-associated HEVs is an independent positive prognostic factor in patients with oral squamous cell carcinoma

Presence of HEVs was analysed with correlation to the patients' clinicopathological features and 5-year DSD (Tables 1 and 2). As presented in Table 1, presence of HEVs was significantly associated with tumour differentiation, T stage, and HPV-status. In univariate analysis, positive HEV status was significantly associated with longer 5-year survival rate (73.5% compared to 14.3% in HEV-negative patients, $P<0.001$; Figure 3B). Significant adverse prognostic factors in univariate analysis included tumour site different from the mobile tongue as well as increasing T stage and positive N- and M stages, as indicated in Table 2. The T- and the N stages as well as the tumour site and the HEV-status were entered into multivariate Cox regression analyses. The cases with the unknown T- and N stages as well as the M status were taken out from the calculations because of unequal group size. All variables satisfied the proportional hazards assumption (Figure S1). Both the T stage ($P<0.001$) and the HEV-status ($P=0.002$) were independently predictive for DSD (Table 3). HEVs were found in all ($n=53$) of the T1-T2 tumours, but only in two-thirds ($n=14$) of the T3-T4 tumours (Table 1). For this reason, separate univariate analyses were conducted on the 21 patients with T3-T4 OSCCs. In these tumours, the absence of HEVs was a statistically significant predictor for lower 5-year survival (14.3% compared to 28.6% in HEV-positive patients, $P=0.004$; Figure 3A). No multivariate analyses were carried out on this patient subset due to small sample size.

Discussion

In this study, we have presented tumour-associated HEVs as a robust and easy-to-use, independent positive prognostic marker for patients with oral cancer. Sixty-eight (90.7%) of the 75 OSCC samples were defined as HEV-positive and the remaining 7 (9.3%) were negative. Univariate analysis showed that patients with HEVs had a significantly lower 5-year DSD rate compared to patients without HEVs ($P < 0.001$). In multivariate analyses both the HEV-status and the T stage were significant independent predictors of DSD ($P = 0.002$ and $P < 0.001$, respectively). HEVs were found in all T1-T2 tumours, but only in two-thirds of the T3-T4 tumours, suggesting tumour-associated HEV-status as a promising prognostic factor in patients with advanced cancer. Previously, tumour HEVs were found to be independently associated with favourable clinical outcome in breast cancer (10). To the best of our knowledge, this is the first study to report HEVs as an independent positive prognostic factor for primary OSCC cancer patients.

Tumour HEVs have earlier been shown to display a broad heterogeneity (10, 38). Previously, tumour HEVs were observed in human solid cancers, such as melanomas, breast cancers, colon, lung, and ovarian carcinomas in approximately 70% of the patients (10, 11, 24, 39). In oral cancer, however, we found approximately 90% of the patients to be HEV-positive. This might be due to intertumoural heterogeneity as well as different methodical approaches including individual cut-off points for HEV-positivity. The majority of the HEV negative tumours were found in oral locations other than the tongue, suggesting that HEVs may form more readily in some environments than others. In non-small-cell lung cancer, HEVs have been exclusively associated with ectopic lymphoid accumulations of immune cells called TLSs (40). We have previously reported the presence of HEVs in association with TLSs in the same OSCC patient cohort as in the present study (23). In contrast to the report from non-small-cell lung cancer, we here demonstrate that the majority of HEVs were unrelated to TLSs, as 68 of the 75 patients were HEV-positive while TLSs were only found in 17 of the tumours (23). Presence of HEVs was significantly associated with HPV-status, assessed by p16 immunohistochemical staining, where a higher proportion of the HPV-negative tumours were HEV-positive. Due to the low number of HPV-positive tumours in our study, this correlation needs to be confirmed in larger studies. Intratumour heterogeneity displays great challenges for the identification of suitable prognostic markers (41). In our study, we therefore performed multiple level assessments of 10 tissue blocks showing that analysis for HEV-positivity on a single level can be considered representative for the whole tumour. These results underpin the value of HEVs as a simple and robust, positive prognostic marker that might be easily implementable in clinical routines for patients with oral cancer.

Recent findings indicate that HEVs have an important role in cancer progression and that their plasticity might be a critical feature (24, 27, 28). Martinet et al. conducted a study on breast carcinoma sections, where the highest density of tumour HEVs was found within the in situ and not the invasive component (42). In our study, HEVs were found in all of the T1-T2 tumours, but were not observed in one-third of the T3-T4 tumours, suggesting that these vessels might play an important role for the progression of oral cancer, perhaps by suppressing tumour growth. HEVs have however been shown to be plastic and able to differentiate and dedifferentiate upon various stimuli, such as from the tumour microenvironment (13). Thus, in larger tumours, HEVs might transdifferentiate to normal blood vessels and gradually lose their specific marker PNAd. Several studies have reported that such a remodelling is associated with poor prognosis (27, 28). Dendritic cells are thought to be important regulators of HEV-mediated lymphocyte trafficking to lymph nodes (24). In the absence of dendritic cells, mature HEVs were seen to revert to an immature phenotype, and HEV-mediated lymphocyte recruitment to lymph nodes was inhibited (26). Thus, it might be that more advanced tumours have found ways to disarm the dendritic cells causing HEV regression. Generation and maturation of dendritic cells, however, might vary with tumour grade and also within the tumours, which might then result in heterogeneous HEV phenotypes. We found dilated and especially branched HEVs to be characterized by a gradual loss of their PNAd staining. Moreover, these morphologically altered HEVs were generally associated with low grade of inflammation. Accordingly, HEVs with a thick wall and a small lumen – resembling the lymphocyte trafficking functional phenotype – were mostly located in areas with more prominent lymphocytic infiltration. These findings are consistent with previous results, where HEVs lined by a cuboidal endothelium were surrounded by more lymphocytes than those lined by a flat endothelium (39). In our patient cohort, HEVs with different morphologies were found in the same tumours. Tumour growth from a single cell is a stepwise progression, and the heterogeneity of HEV morphology within a single tumour might be due to transition processes in tumour immunogeneity.

It has earlier been shown that remodelling of HEVs in lymph nodes of human breast cancer and OSCC patients is a poor prognostic factor (27, 28). In the present study, we have not investigated the presence and morphology of HEVs in metastatic lymph nodes of these patients. However, in future studies, it would be of great interest to investigate the HEV status in the respective lymphatic basin. Further, we did not evaluate the association between specific morphological phenotypes and clinicopathological characteristics, but rather the presence of HEVs irrespective of their morphologic features, as many tumours displayed several morphological HEV phenotypes. Hence, we can only hypothesize that morphologically altered vessels might have adverse effects on tumour progression. Additionally, it would be interesting to see if HEVs with weak PNAd-staining start expressing blood

vessel markers, and if HEV phenotype is associated with presence or absence of dendritic cells. Another weakness of our study is that we conducted semi-quantitative analyses of HEV surrounding lymphocytes, but did not distinguish between different subsets of lymphocytic cells. In future studies, it would be of great interest to better characterize the inflammatory infiltrate in which HEVs arise, as cell-type-specific transcription factors are probably involved in the development and maintenance of HEVs (13). Studying different subsets of immune cells and specific chemokines might be an interesting supplement to better understand possible interactions between HEVs and the tumour microenvironment. Finally yet importantly, our results have to be confirmed in larger studies.

In conclusion, we report that the presence of tumour-associated HEVs in OSCCs is significantly correlated with lower DSD. Tumour HEVs were commonly found in early-stage OSCCs, but only in two-thirds of the T3-T4 tumours. Thus, evaluation of this biomarker might be important to identify patients with more aggressive T3-T4 OSCCs. Our results on HEV morphology indicate an intricate relationship between HEVs and the surrounding microenvironment. HEVs are highly plastic and shifting from a lymphocyte- to a blood-carrying vessel might be associated with tumour progression. Thus, identification of markers and genes for HEV development and regulation in cancer-associated inflammation may open new opportunities for targeted therapies and genetic manipulation. As immunohistochemical detection of HEVs is simple, and the distribution of these vessels seems to be even throughout a tumour, this may be a valuable supplement to the TNM-staging for prognosis assessment and treatment stratification in OSCC patients.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

All procedures performed in this study involving human material were in accordance with the ethical standards of the Regional Committee for Medical and Health Research Ethics, Northern Norway (REK-number 22/2007) 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.

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Tables

Table 1: Comparison of clinicopathological variables between 75 OSCC patients with and without high-endothelial venules (HEVs) using Pearson's Chi-square test.

	HEV-negative (N = 7) (no. (%))	HEV-positive (N = 68) (no. (%))	P-value
Gender			
Male	6 (85.7)	37 (54.4)	0.227
Female	1 (14.3)	31 (45.6)	
Age at diagnosis, years			
Mean	68.29	62.26	0.795
0-59	2 (28.6)	26 (38.2)	0.706
≥ 60	5 (71.4)	42 (61.8)	
Smoking history			
Never smoker	1 (14.3)	16 (23.5)	0.821
Former smoker	1 (14.3)	9 (13.2)	
Current smoker	4 (57.1)	39 (57.4)	
Unknown	1 (14.3)	4 (5.9)	
Alcohol consumption			
Never	1 (14.3)	11 (16.2)	0.156
≤ 1 times weekly	0 (0.0)	27 (39.7)	
> 1 times weekly or daily	3 (42.9)	17 (25.0)	
Unknown	3 (42.9)	13 (19.1)	
Tumour site			
Mobile tongue	1 (14.3)	34 (50.0)	0.071
All others	6 (85.7)	34 (50.0)	
Tumour differentiation			
Well	3 (42.9)	25 (36.8)	0.036
Moderate	2 (28.6)	40 (58.8)	
Poor	2 (28.6)	3 (4.4)	
T stage			
T1/T2	0 (0.0)	53 (77.9)	<0.001
T3/T4	7 (100.0)	14 (20.6)	
Unknown	0 (0.0)	1 (1.5)	
N stage			
N0	3 (42.9)	48 (70.6)	0.326
N+	3 (42.9)	15 (22.1)	
Unknown	1 (14.3)	5 (7.4)	
M stage			
M0	6 (85.7)	63 (92.6)	0.668

M+	0 (0.0)	1 (1.5)	
Unknown	1 (14.3)	4 (5.9)	
HPV/p16			
Negative	4 (57.1)	61 (89.7)	
Positive	1 (14.3)	5 (7.4)	
Unknown	2 (28.6)	2 (2.9)	0.011

Table 2: Clinicopathologic variables as predictors for 5-year disease-specific death in univariate Kaplan-Meier analysis for 75 patients with OSCC.

	Patients (N= 75) (no. (%))	5-Year DSD (%)	P-value
Gender			
Male	43 (57.3)	37.2	0.206
Female	32 (42.7)	25.0	
Age at diagnosis, years			
0-59	28 (37.3)	28.6	0.578
≥ 60	47 (62.7)	34.0	
Smoking history			
Never smoker	17 (22.7)	23.5	0.654
Former smoker	10 (13.3)	20.0	
Current smoker	43 (57.3)	37.2	
Unknown	5 (6.7)	40.0	
Alcohol consumption			
Never	12 (16.0)	16.7	0.347
≤ 1 times weekly	27 (36.0)	29.6	
> 1 times weekly or daily	20 (26.7)	35.0	
Unknown	16 (21.3)	43.7	
Tumour site			
Mobile tongue	35 (46.7)	17.1	0.024
All others	40 (53.3)	45.0	
Tumour differentiation			
Well	28 (37.3)	28.6	0.679
Moderate	42 (56.0)	33.3	
Poor	5 (6.7)	40.0	
T stage			
T1/T2	53 (70.7)	15.1	<0.001
T3/T4	21 (28.0)	76.2	
Unknown	1 (1.3)	0.0	
N stage			
N0	51 (68.0)	21.6	<0.001
N+	18 (24.0)	66.7	
Unknown	6 (8.0)	16.7	
M stage			
M0	69 (92.0)	31.9	0.014
M+	1 (1.3)	100.0	
Unknown	5 (6.7)	20.0	

HPV/p16			
Negative	65 (86.7)	32.3	
Positive	6 (8.0)	16.7	0.570
Unknown	4 (5.3)	50.0	
HEV			
Negative	7 (9.3)	85.7	
Positive	68 (90.7)	26.5	<0.001

P-values were calculated using the log-rank test.

Table 3: Multivariate analysis of 5-year disease-specific death according to Cox's proportional hazards model*.

Variable	Hazard ratio	95% C.I.	P-value
T stage (T1/T2 [n= 49] v. T3/T4 [n=20])	8.296	3.120 – 22.062	<0.001
HEV (negative [n = 6] v. positive [n = 63])	0.147	0.044 – 0.490	0.002

*Only 69 patients were analysed because the cases with the unknown T- and N stages were taken out from the calculations.

Figures

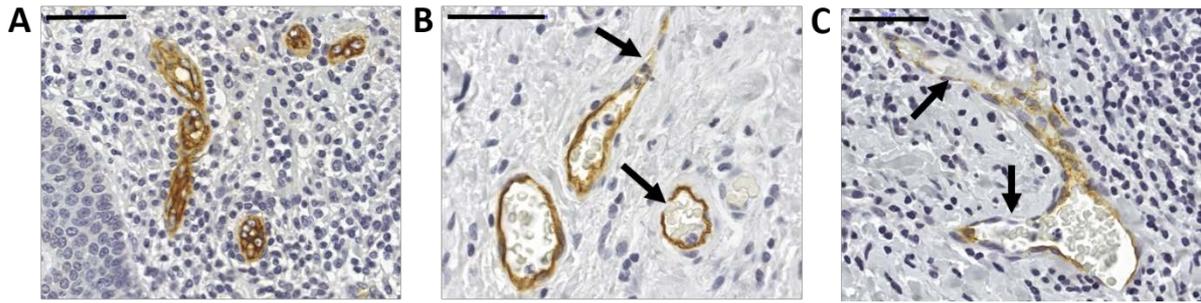


Figure 1: Different phenotypes of high-endothelial venules in oral squamous cell carcinoma. The pictures show representative immunohistochemical stainings for high-endothelial venules (HEVs). Both HEVs with a thick wall and a small or no lumen (A) as well as HEVs with a thinner wall and a larger lumen (B) were found. Some of the HEVs were also seen to branch out (C). Dilated and branched vessels gradually lost their specific marker PNAAd (arrows, B and C). PNAAd+ vessels are stained brown and cell nuclei are stained blue by hematoxylin. Scale bars indicate 50 μ m.

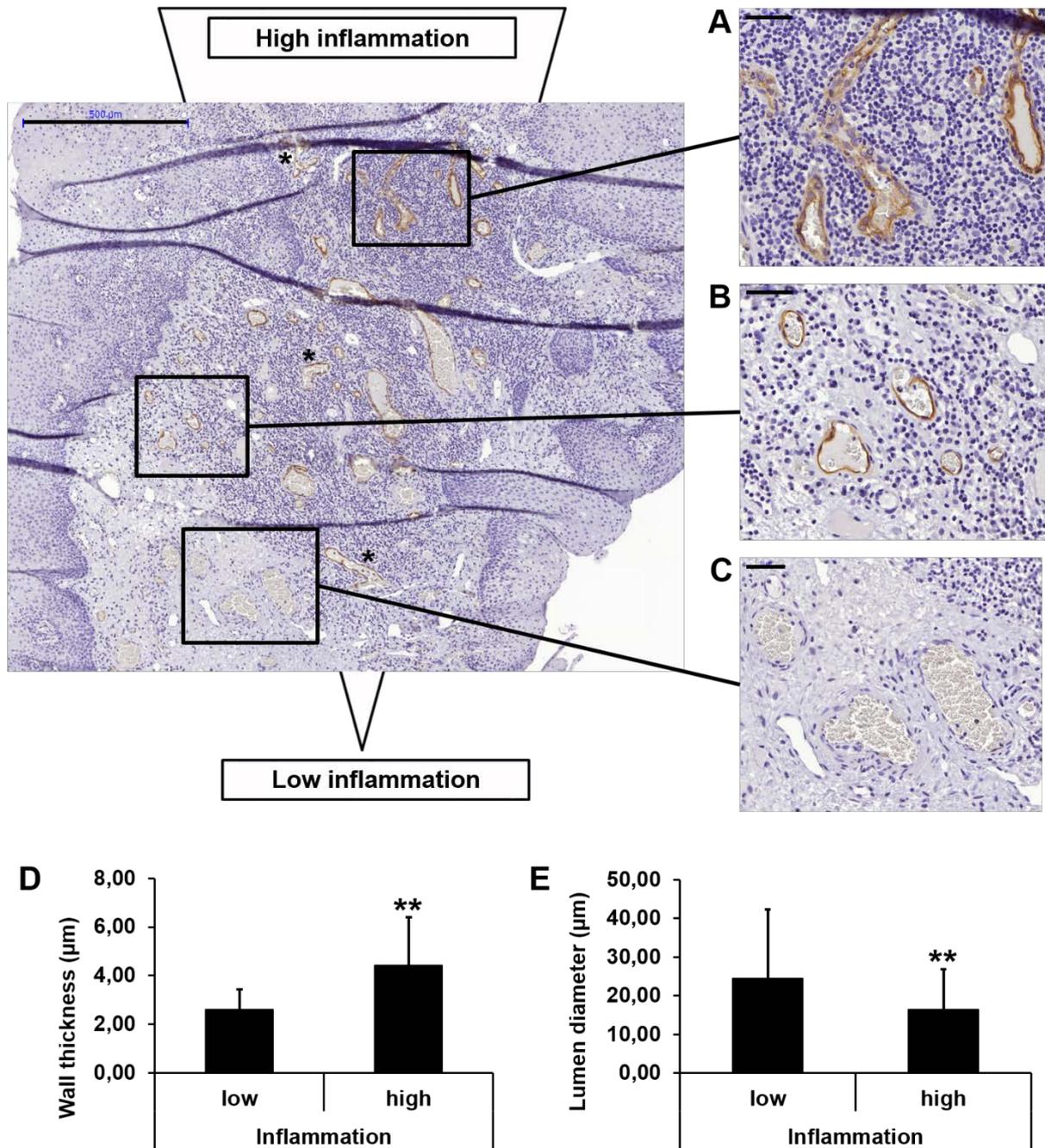


Figure 2: Qualitative and quantitative analysis of distribution of morphologic altered high-endothelial venules in the tumour microenvironment of oral squamous cell carcinoma. Pictures A-C show representative immunohistochemical stainings for high-endothelial venules (HEVs) in areas with various levels of lymphocyte infiltration. In sections with high level of inflammation (A), HEVs had a significantly thicker wall (D) and smaller lumen (E) compared to sections with lower level of inflammation (B). In regions with very low or no inflammation, very few or no PNA⁺ vessels were seen, respectively (C). Branched vessels were found in areas with various

levels of inflammation (asterisks in overview picture). PNA⁺ vessels are stained brown and cell nuclei are stained blue by hematoxylin. Scale bars indicate 500 μ m in the overview picture and 50 μ m in the enlarged sections (A-C). Columns indicate average vessel wall thickness (D) and lumen diameter (E). Error bars represent one standard deviation. **, $P < 0.001$ relative to HEVs found in areas with low level of inflammation.

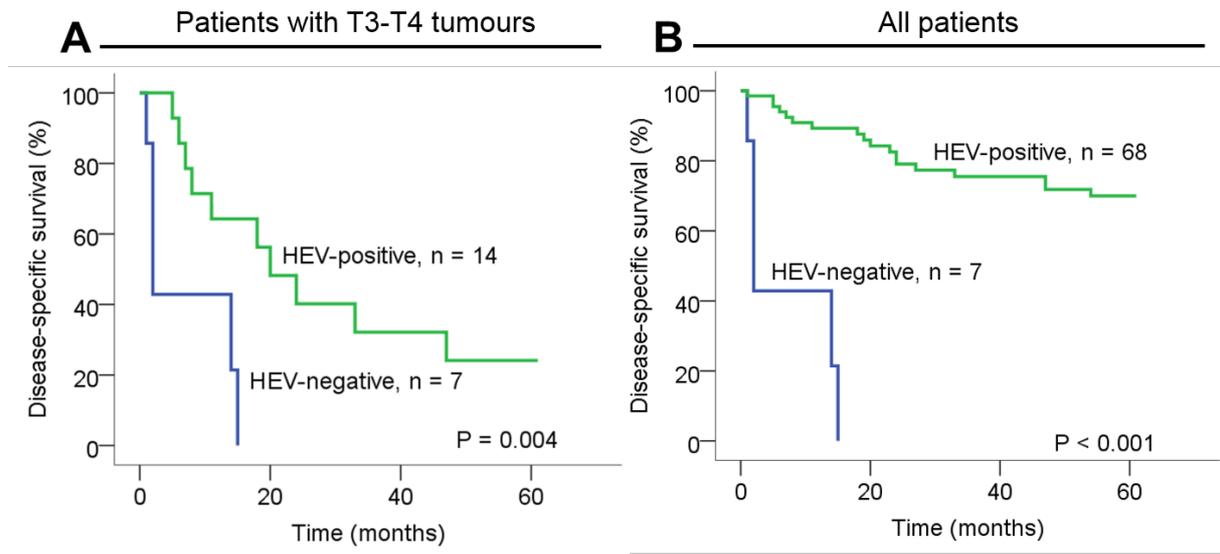


Figure 3: Kaplan-Meier analysis of 5-year disease-specific survival for patients with oral squamous cell carcinoma with and without high-endothelial venules. The presence of high-endothelial venules (HEVs) is associated with improved survival in A) 21 patients with T3-T4 oral squamous cell carcinoma (OSCC) ($P=0.004$) and in B) all 75 OSCC patients in our cohort ($P<0.001$). In the T3-T4 tumours (A), the Kaplan-Meier curve shows a 5-year disease-specific survival (DSS) rate of 28.6 % for HEV-positive patients and 14.3 % for HEV-negative patients. The 5-year DSS rate for all HEV-positive patients in our cohort (B) was 73.5 %. The P-value was calculated using the log-rank test.

Supplemental data

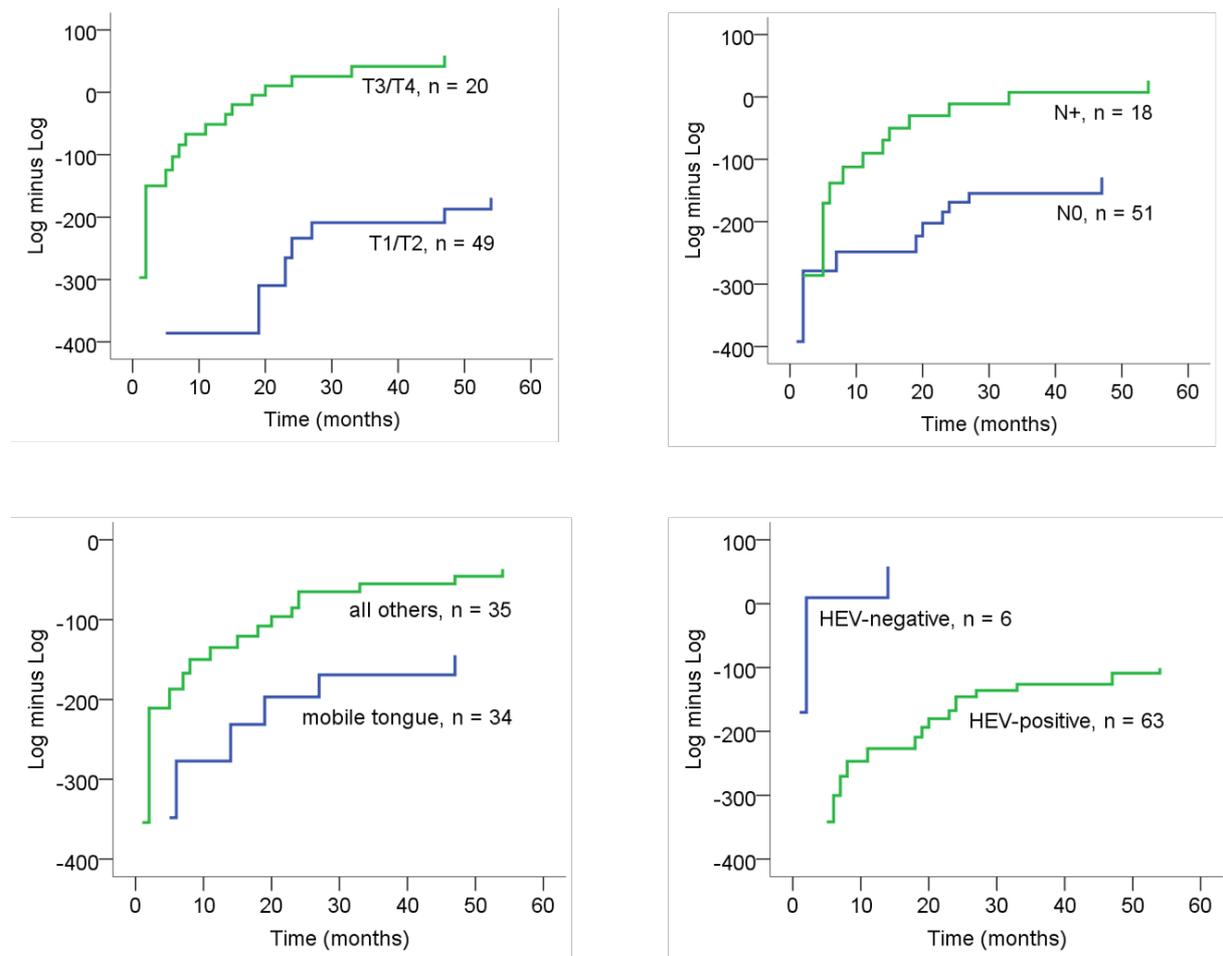


Figure S1: Log minus log plots for proportional hazards checking; (A) T stage; (B) N stage, (C) tumour site, (D) high-endothelial venules (HEVs).