Lymphatic Vessel Density in Correlation to Lymph Node Metastasis in Head and Neck Squamous Cell Carcinoma

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Abstract. *Background: In head and neck squamous cell carcinoma (HNSCC), metastatic dissemination to regional lymph nodes serves as a major prognostic indicator for incipient disease progression and constitutes the guideline for subsequent therapeutic strategies. In this study, whether intratumoral (IT) and peritumoral (PT) lymphatic vessel density (LVD) might be a predictive indicator to the risk of lymph node metastasis was investigated. Patients and Methods: Tumour lymph vessels in fresh frozen sections of 105 head and neck cancer were quantified by immunostaining for the lymphatic endothelial marker LYVE-1. These results underwent correlation with the nodal status of the patient. Results: There was a significant relationship between a high IT LVD and nodal metastasis (N+) (p=0.049, Mann-Whitney test). Analysed separately by anatomic regions, a significant correlation was only shown in oral carcinoma (p=0.032, Mann-Whitney test). Intratumoral LVD was lower compared to peritumoral LVD. Logistic regression, however, showed that the only predictive parameter for the nodal status was the localisation of the primary tumour but not LVD. Conclusion: This study confirmed that IT LVD is low in HNSCC. In this group of tumours there was a significant correlation between IT LVD and nodal involvement.*

The estimated incidence worldwide of head and neck squamous cell carcinoma (HNSCC) is approximately 900,000 and HNSCC is the sixth most prevalent neoplasm in the world (1, 2). Tobacco and alcohol are crucial in the development of these tumours (3, 4). Despite aggressive and often mutilating therapeutic regimens, overall long term survival is still less than 50% (5, 6). During the last decade, intensive research revealed several molecular, cytogenetic and immunohistochemical markers to better predict the clinical course of this malignancy and to better evaluate the individual aggressiveness (7). The aim of these studies, from a clinical point of view, is to improve the selection of the appropriate individual therapeutic strategy, as well as to prevent patients from possible overtreatment (8-11).

In many tumour types including HNSCC, lymphatic vessels serve as a major route for tumour metastasis. The dissemination of malignant cells to the regional lymph nodes is an early step in the progression of many solid tumours and an important determinant of prognosis (12). Lymphangiogenesis (formation of new lymphatic vessels) is thought to be crucial for cancer cells to metastasise to the regional lymph nodes (13, 14). However, research in this important process has been neglected largely due to the lack of molecular markers specific to the lymphatic endothelium. Therefore, with the identification of LYVE-1, a specific marker for lymphatic endothelium, this problem has been overcome (15, 16). LYVE-1 is the lymphatic receptor for the extracellular matrix mucopolysaccharide hyaluronan. It is member of the Link protein family whose only other major hyaluronan receptor is directly involved in leukocyte migration and tumour metastasis (18).

Two previous studies have investigated the correlation of LYVE-1 expression with lymph node metastasis and prognosis in HNSCC. Beasley *et al.* (8) identified discrete "hotspots" of intratumoral (IT) small proliferating lymphatics in all HNSCC and a high IT lymphatic vessel density was associated with neck node metastases. Maula *et al.* (9) found that intratumoral LYVE-1-positive lymphatic vessels were clearly associated with a higher risk for local relapse as well as with poor disease-specific prognosis. In contrast, a high density of peritumoral (PT) LYVE-1-positive vessels was a sign of favourable survival.

As immunostaining with this highly specific and now commercially available marker LYVE-1 is a fast procedure, theoretically this marker would be an ideal candidate for clinical routine to assess the individual risk of lymph node metastasis and prognosis out of biopsies from the primary

Abbreviations: HNSCC, head and neck squamous cell carcinoma; LYVE-1, lymphatic vessel endothelial hyaluronan receptor 1.

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tumour of HNSCC. The aim of this study was to assess the relationship between intra- and peritumoral LYVE-1 expression in primary HNSCC and the presence of lymph node metastasis in a large group of tumours (n=105) at different sites of the upper aerodigestive tract to verify or disprove previous findings.

Patients and Methods

Patients. Tumour specimens from 105 previously untreated patients with HNSCC were snap frozen at the time of surgery. All tumours were resected at the Department of Otorhinolaryngology, Head and Neck Surgery, University Hospital Mannheim, Germany, between 1997 and 2001. These 105 specimens consisted of 15 (14.3%) tumours of the oral cavity, 18 (17.1%) tumours of the oropharynx, 34 (32.4%) tumours of the hypopharynx and 38 (36,2%) tumours of the larynx. The median age was 61 years (range 40-86 years) at the time of diagnosis; the group included 87 (83%) men and 18 (17%) women. Seventy one (67.7%) were stage IV tumours, 2 (1.9%) were stage III tumours and 32 (30.5%) were stage I or II tumours. Thirty five were N0 at presentation and 70 had metastatic spread to the neck nodes (N+). All patients had surgery as their first line of management, with some receiving postoperative radiotherapy. Clinical data selection included the patient's age, gender, nodal status and recurrence at time of diagnosis.

Immunostaining. Seven μm sections of frozen tissue were cut onto adhesive glass slides and were left to dry for half an hour. They were then fixed in acetone for 15 min and washed in PBS buffer. One set of sections was routinely stained with haematoxylin and eosin to confirm the pathological diagnosis as well as to estimate the ratio of tumour cells in the specimen. Non-neoplastic biopsies from human mucosa were stained for the control of specific staining. A second set of sections was immunostained with the polyclonal LYVE-1 antibody. For the staining of blood vessels, a monoclonal mouse CD34 antibody was used in a third set of sections. After inactivation of endogenous peroxidase with 100 μL peroxidase blocking reagent for 30 min, sections were treated with normal bovine serum (Dako, Hamburg, Germany) for 30 min.

Staining for LYVE-1: For the detection of lymphatic endothelium, slides were incubated with 1:50 dilution of rabbit polyclonal LYVE-1 antibody (Acris, Hiddenhausen, Germany) overnight at 4˚C. A biotinylated anti-rabbit-immunoglobulin (Amersham, Braunschweig, Germany) as a second antibody was added for 45 min and a streptavidin peroxidase protocol was used for 45 min (Amersham, Braunschweig, Germany). After that the sections were stained with AEC-substrate chromogen.

Staining for CD34: For the detection of blood vessels, slides were incubated with 1:50 dilution of mouse monoclonal CD34 antibody (Calbiochem, Schwalbach, Germany) overnight at 4˚C. The remaining steps were as mentioned for LYVE-1 stainig. Slides were counterstained with haematoxylin (Harris, Burgdorf, Germany) and mounted with Crystal/Mount™ (Biomeda, Fostercity, USA). Negative controls were integrated in all staining procedures. Intratumoral and peritumoral vessel density were analysed separately. The number of lymphatic vessels was counted within 3 microscopic fields at a magnification of ×200 and the median was used for statistical analysis. All specimens were analysed by the same investigator (S.F.). Examples of LYVE-1 expression are given in Figure 1A.

Statistical analysis. The Mann-Whitney test was employed to examine the association between lymphatic vessel density and lymph node status. Logistic regression analysis was employed to determine which clinicopathologic factors were predictive of lymph node metastasis. The χ^2-test was used to examine the association between biological variables and pathological parameters. A p-value <0.05 was considered significant. All statistical tests were performed using SAS release 8.02 (SAS Institute Incorporated, Cary, USA).

Results

To characterize lymphatic vessels in HNSCC, immunostaining of squamous cell carcinoma of the oral cavity, oropharynx, hypopharynx and larynx was performed with antibodies to LYVE-1 HA receptor, previously identified as a marker for lymphatic vessel endothelium in human and murine tissue (16, 19). Consistent to previous reports (8, 9), lymphatic vessels were observed both within the tumour mass and in the peritumoral area, and they were congruently thin-walled. These were clearly distinguished from adjacent blood vessels, which did not stain significantly for LYVE-1 and which were regular in shape and contained RBCs (Figure 1B). The intratumoral vessels often had a distinctive reticular architecture with numerous tiny or ill-defined lumina (Figure 1C) that distinctly differed from the larger and more dilatated peritumoral vessels (Figure 1D) or vessels in the normal tissue area (Figure 1E). The peritumoral (PT) vessels were mostly identified in the inflammatory area surrounding the tumour. Intratumoral LVD was lower compared to peritumoral LVD (mean in N+ tumors: 1.34 vessels/intratumoral spot, 3.86 vessels/peritumoral spot). No immunostaining was identified in the tumour cells. The patients were divided into two groups. One group contained the patients that presented no lymph node metastasis at time of diagnosis (N0); the second group showed lymph node metastasis at time of diagnosis (N+) and N1-, N2- and N3-tumors were subsumed in N+. A significant relationship was identified between the presence of IT lymphatic vessel density and nodal metastases (p=0.049, Mann-Whitney test). Analysed separately by anatomic regions, a significant correlation was only shown for patients with carcinoma of the oral cavity (p=0,032; Mann-Whitney test). A similar relationship for patients with oropharyngeal, hypopharyngeal or laryngeal carcinoma was not found ($p>0.05$). There was no significant association between the peritumoral LVD and lymph node metastasis. A significant correlation between LVD and lymph node metastasis was only found in the subgroup of the hypopharyngeal carcinomas (p=0.015). There was no significant association between the IT or PT LVD and the patient's age, gender, tumour size and recurrence. To determine which of the observed parameters best predicted the presence of lymph node metastasis in the current series of HNSCC cases, a logistic regression analysis was performed. The results demonstrated that only the tumour

localisation implied a significantly higher risk of lymph node metastasis (p=0.014). The LVD was not found to be associated with an increased risk of lymph node metastasis as a predictive marker in this study.

Discussion

HNSCC is one of the most common cancers. Although these tumours are potentially curable by local radiotherapy and surgical resection, the overall 5-year survival rate reaches only 50% (5, 9). The existence and biological function of lymphatic vessels in human tumours is discussed controversially, mainly because of the lack of specific markers for lymphatic endothelium. Lymph node involvement is one of the strongest predictors of poor prognosis. Therefore, there is an exigency to identify characteristics of the primary tumour that might predict nodal metastasis. Recently, antibodies specific for lymphatic endothelium were discovered. This provides important new insights into the process of tumour associated lymphatic formation and its possible clinical relevance. The most commonly used lymphatic marker is the LYVE-1 antibody, which recognizes a hyaluronan receptor that is present on normal and tumour associated lymphatic endothelial cells. Hence, LYVE-1 is the first lymph-specific HA receptor to be characterized and is a uniquely powerful marker for lymph vessels themselves (16). Indeed, LYVE-1 is also present at a very low degree in normal hepatic blood sinusoidal endothelial cells in mice and humans (16, 17). LYVE-1 is a homologue of the CD44 HA receptor and a new member of the Link protein superfamily. In the current study, this polyclonal antibody LYVE-1 was used to examine whether there is a relationship between the LVD in and around HNSCC and metastasis. It was asserted that intratumoral and peritumoral lymphatics are present in specimens of HNSCC and that intratumoral LVD is lower compared to peritumoral LVD.

Intratumoral lymphatic vessels. The presented findings in squamous cell carcinoma of the oral cavity demonstrate that a high intratumoral lymphatic vessel density correlates with neck node metastasis. This adds further support to previous studies (8, 11, 20) reporting that intratumoral lymphatics are important in the spread of head and neck squamous cell carcinoma to regional lymph nodes. Two previous studies have examined tumour lymphatics in HNSCC employing the LYVE-1 antibody (8, 9). Both reported the presence of intratumoral and peritumoral lymphatics. In one report, a positive correlation was found between the intratumoral LVD and lymph node metastasis in the subgroup of oropharyngeal squamous cell carcinoma (8), in the second report this correlation was observed in laryngeal carcinoma (10). Other groups found these results in the entire series of all carcinomas (9, 14). Beasley *et al*. also showed that the presence of LYVE-1 positive intratumoral lymphatics was associated with poor 5-year survival (8). Nevertheless, in a study by Padera *et al*. (21), metastases were detected despite no observable intratumoral LYVE-1+ vessels. They proposed that functional lymphatics available at the tumour margin are sufficient for promoting metastasis by offering a larger area for tumour cell escape. The negative results in the oropharyngeal, hypopharyngeal and laryngeal subgroups in the present study underline the problem of predicting tumour behaviour in the clinical setting. Additionally, problems occurred by examining single sections of a tumour taken at a single point in time without considering the interaction of other biological systems on the process of tumour metastases. Maybe the HNSCC subgroups might vary in their capacity to invade lymphatics. All these different results show that the link between lymphangiogenesis and dissemination to the lymph nodes is complex.

Peritumoral lymphatic vessels. Concerning the importance of PT lymphatic vessels in the metastatic spread of HNSCC, the results of current and previous studies are controversial. In this study a correlation between the PT LVD and nodal metastasis could not be demonstrated. Only in the hypopharyngeal subgroup significant results could be found. Kyzas *et al*. also demonstrated a significant correlation between higher PT LVD and lymph node involvement. Some investigators (11) suggest a significant relationship between high PT LVD with shorter disease-free survival, whereas a relationship between higher PT LVD and more favourable outcome has also been reported (9, 22). These results could be explained by an enhanced immune response to the tumour cells. In the present study an augmented inflammatory infiltrate was found in the area of the PT lymphatics. Cases of high PT LVD have been reported to be frequently related with an increased lymphocytic infiltrate (23). Hinojar-Gutiérrez *et al*. supposed that this infiltrate may act as a supplementary source of VEGF-C, leading to an increase in vascular permeability and tumour interstitial pressure that would randomly induce some tumour cells to enter the initial lymphatic vessels passively (23).

An association between the LVD or lymph node involvement and patient's age, gender, tumour size and the presence of relapse was not found. This adds further support to results of previous studies (8, 10, 24).

Prognosis of HNSCC. A logistic regression analysis was conducted with LVD, tumour location and presence of relapse. The results showed that the only predictive parameter for the nodal status was the localisation of the primary tumour but not LVD.

Evaluation of the impact of an association between LVD and lymph node metastasis has led to controversial results. This study showed that intratumoral LVD is low in HNSCC. In the current series of HNSCC, there was a significant correlation between LVD and nodal involvement in the subgroup of carcinoma of the oral cavity. The results of this

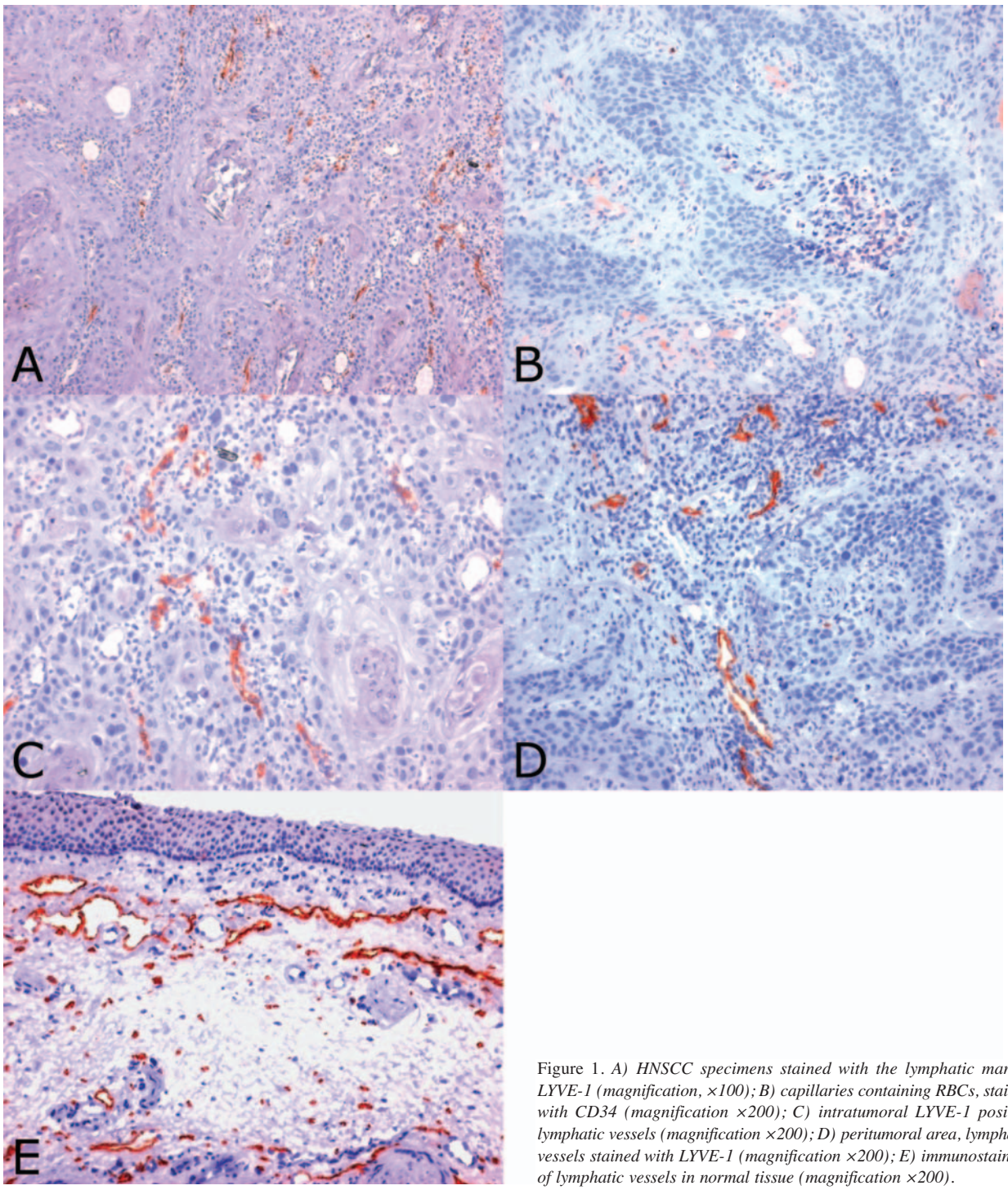

Figure 1. *A) HNSCC specimens stained with the lymphatic marker LYVE-1 (magnification, ×100); B) capillaries containing RBCs, stained with CD34 (magnification ×200); C) intratumoral LYVE-1 positive lymphatic vessels (magnification ×200); D) peritumoral area, lymphatic vessels stained with LYVE-1 (magnification ×200); E) immunostaining of lymphatic vessels in normal tissue (magnification ×200).*

study demonstrate that a definite statement about the correlation between intratumoral as well as peritumoral LVD and the occurrence of lymph node metastasis can not be made. The results for the subgroups larynx, hypopharynx, or oropharynx and oral cavity vary. It remains unclear whether the intratumoral lymphatics infiltrate from the tumour surrounding area or arise from lymphangiogenesis. More studies are required to determine whether lymphatic density will allow individual patient evaluation beyond lymph node status, clinical stage and tumour localisation on the basis of

immunohistochemical findings. The immunohistochemical marker LYVE-1 enables the investigation of the correlation of the PT and IT LVD and the involvement of lymph node metastasis in HNSCC. Furthermore, the identification of parameters that correlate with lymphatic metastasis in HNSCC could serve for the development of new anticancer strategies. One possibility would be to use inhibitors of lymphangiogenic factors and destroy intratumoral lymphatic vessels, which could inhibit lymphatic metastasis. These approaches are particularly attractive, as, in contrast to chemotherapy and radiation, they are of limited toxicity for non-tumoral tissue.

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