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Association of eIF4E and SPARC Expression with Lymphangiogenesis and Lymph Node Metastasis in Hypopharyngeal Cancer

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Abstract. *Background/Aim: Head and neck squamous cell carcinomas (HNSCC) are characterized by aggressiveness, early recurrence and lymph node metastasis. Therefore, there is an urgent need to identify new biomarkers and drug targets. Materials and Methods: Neck dissection specimens from 11 patients diagnosed with hypopharyngeal cancer were analyzed for their lymphatic vessel density (LVD) by lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) immunostaining, expression of eukaryotic initiation factor 4E (eIF4E) and levels of secreted protein acidic and rich in cysteine (SPARC) using immunoblot analysis. Results: Compared to lymph node biopsies of healthy controls, LVD was significantly increased in metastatic lymph nodes as well as in advanced primary tumors. Overexpression of eIF4E and SPARC was demonstrated in all hypopharyngeal cancer specimens. Notably, we observed that increased LVD significantly correlated with the expression of eIF4E as well as SPARC levels. Conclusion: eIF4E- and SPARC-associated signaling pathways may be associated with lymphangiogenesis and could be exploited to counteract the spread of hypopharyngeal cancer cells.*

In spite of improvements in diagnostics, surgical techniques and expertise in chemoradiotherapy, locally advanced squamous cell carcinomas of the head neck (HNSCC) remain a therapeutic challenge, with no significant improvement in 5-year survival rates for several decades. In particular, the metastatic spread to cervical lymph nodes is associated with a dramatic prognostic decline to 5-year survival rates of less than 50%, especially in hypopharyngeal cancer (1, 2). These metastases often occur metachronically after complete resection and adjuvant chemoradiotherapy. This might indicate occult tumor dissemination already at early stages or minimal residual disease following primary therapy (3).

Recently, improvements in response to chemotherapeutic agents have been seen in palliative chemotherapy of patients with irresectable or metastatic hypopharyngeal cancer using targeted therapies aiming at the endothelium derived growth factor receptor (EGFR) pathway since EGFR is found to be overexpressed in more than 90% of these tumors (4, 5). Due to the limited number of patients experiencing partial or complete remissions and long-term responses to anti-EGFR agents, there is a great need to identify alternate targets as well as innovative combination therapies in order to overcome resistance to single agents (6). Besides their use in palliative settings, new agents may also be applied as an adjuvant, maintenance or low dose metronomic therapy for high-risk patients (7-10).

Mechanisms that favor tumor dissemination to regional lymph nodes are not yet fully understood, but may essentially be governed by lymphangiogenesis, not only in the primary tumor microenvironment, but also in the more distant lymphatic system draining to cervical nodes. There is evidence that lymph node sites have to meet certain requirements for successful metastatic spread and that prior lymphangiogenesis might be a crucial factor rather than a coincidental process (11, 12). Due to the dramatic decrease in overall survival in the patients with cervical lymph node metastases, new targeted therapies to block the development of a 'metastatic niche' in the neck would have high therapeutic potential.

Among possible targets identified in primary HNSCC including hypopharyngeal cancer is the mammalian target of rapamycin (mTOR) pathway (13). Due to their anti-angiogenic and immunosuppressive properties, mTOR

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inhibitors are already established in immunosuppression after organ transplantation and in chemotherapy of breast cancer, renal cell and neuroendocrine pancreatic carcinomas (14-16). Furthermore, there is evidence that the mTOR pathway plays a critical role in primary tumor-driven lymphangiogenesis in the majority of HNSCC (17). Animal studies showed that mTOR inhibition using tacrolimus resulted in reduced intra- and peritumoral lymphatic vessel density (LVD). In addition, reduced invasion of lymphatic vessels and consequently a reduced rate of lymph node metastases was observed (18, 19). On the other hand, the directly mTOR-dependent proto-oncogene eukaryotic initiation factor 4E (eIF4E) is known to be overexpressed in several malignant diseases, including hypopharyngeal cancer (13, 20). It may also be found in premalignant lesions of the upper airway tract, but is not expressed in intact, healthy mucosa (21, 22). eIF4E has been shown to be an independent prognostic marker for recurrent disease and overall survival in patients with HNSCC with regard to its expression in surgical margins after complete tumor resection (20). A direct association of eIF4E expression with neo-angiogenesis and lymphangiogenesis has not yet been shown in hypopharyngeal cancer.

Finally, the extracellular matrix protein secreted protein acidic and rich in cysteine (SPARC) has been described as a possible target as well as an independent indicator of poor clinical outcome in patients with HNSCC (23-25). Besides its function as a mediator of cell–matrix interactions the degree of SPARC expression has been shown to modulate crucial signaling pathways, including mTOR-related pathways (26). SPARC overexpression and its independent prognostic value regarding recurrence and overall survival have already been shown in primary HNSCC (23, 27). In contrast, no up-regulation of basal SPARC expression is seen in normal tissue of the upper airway (23, 24). Intriguingly, a targeted therapy for SPARC-expressing cancers is available using nano-albumin-bound paclitaxel (28, 29). The association of SPARC as a possible target in the process of lymphatic spread of HNSCCs has not been shown.

Representing a consistent group with very poor prognosis, a high rate of metastases and a lack of correlation with HPV status, we focused specifically on hypopharyngeal cancer instead of on the quite heterogenous group of HNSCCs. The aim of this study was to investigate the correlation of LVD with expression rates of the mTOR signaling product eIF4E, as well as of SPARC in metastatic lymph nodes of hypopharyngeal cancer in order to evaluate their potential as new therapeutic targets.

Materials and Methods

Patients and samples. Biobank specimens derived from surgery for lymphatically metastasized hypopharyngeal cancer between 1995 and 2001 in the Department of Otolaryngology of Goethe-University at Frankfurt/Main, Germany, were used (30). Inclusion criteria contained patients with lymphatically metastasized squamous cell carcinoma of the hypopharynx of whom ipsilateral neck dissection specimen were available in our tumor tissue database. Exclusion criteria were systemic spread of the disease (*i.e.* metastases to the lung, liver, *etc.*), multiple carcinomas, induction chemotherapy, or no valid histopathological evaluation confirming the disease. In addition, cervical parajugular lymph nodes from patients without malignant disease who underwent neck surgery for benign lesions (*i.e.* non-infected neck cysts, lipomas and laryngoceles) were obtained as an additional control group. The study protocol was approved by the Ethics Committee of the medical faculty of Goethe University Frankfurt/Main (ethics vote 217/13), in accordance with the Declaration of Helsinki dating from 1975 and revised in 1983.

The biobank samples consisted of ipsilateral neck dissection specimen from each patient containing metastatic and non-metastatic lymph nodes. For metastatic lymph nodes and control cervical lymph nodes, fresh frozen samples were stored at −80˚C after resection. Additionally, samples were fixed with 10% formaldehyde and embedded in paraffin. For non-metastatic lymph nodes within the neck dissection specimen, only formaldehyde fixed samples were available. Specimen from metastatic lymph nodes (n=11), non-metastatic cervical lymph nodes derived from the same neck levels in the same patient (n=9), respectively, as well as control cervical lymph nodes from patients without malignant disease (n=11) were analyzed for LVD using immunohistochemical staining for lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1). Metastatic lymph nodes and control cervical lymph nodes were also evaluated for their expression of eIF4E and SPARC using western blot analysis.

Staging parameters obtained from the database relied on the eighth edition of the Tumor-Node-Metastasis (TNM) classification (31) as well as the Union for International Cancer Control (UICC) (32) staging system. Histopathological parameters were obtained from the evaluation of an independent Board-certified pathologist referring to tumor and nodal status, the differentiation of the tumor and the resection status.

Immunohistochemical staining. Paraffin-embedded slices of 2 μm were prepared using a Paraclear® and re-diluted using ethanol baths. For further removal of formaldehyde and unmasking of antigens tissue samples were boiled for 20 minutes in a 0.01 M citrate buffer solution (pH 6.0). Subsequently, endogenous enzymes were blocked by incubating samples in 3% H_2O_2 for 5 min and washing twice with 1x Tris-buffered saline and Tween 20 (TBST) washing buffer. The primary antibody for LYVE-1 (rabbit polyclonal antibody clone; Cell Signaling Technology, Boston, MA, USA) was diluted (1:350) using Antibody Diluent (Dako, Carpinteria, CA, USA) and incubated with the sections for 45 min at room temperature. Sections were rinsed using washing buffer for 5 min, incubated with a polylink secondary antibody (DCS, Hamburg, Germany) for 30 min at room temperature before incubation with alkaline phospatase. In order to induce the fluorescent reaction, New Fuchsin Substrate System (Dako) was applied and sections incubated for 1 min. Sections were then counterstained with Mayer's hematoxylin (Applichem, Darmstadt, Germany).

Evaluation of LVD was performed using the microscopic hot-spot method which describes a manual count of the target structure (in this case lymphatic vessels) in three representative regions of interest (ROI) (33). Counting was performed at 20-fold

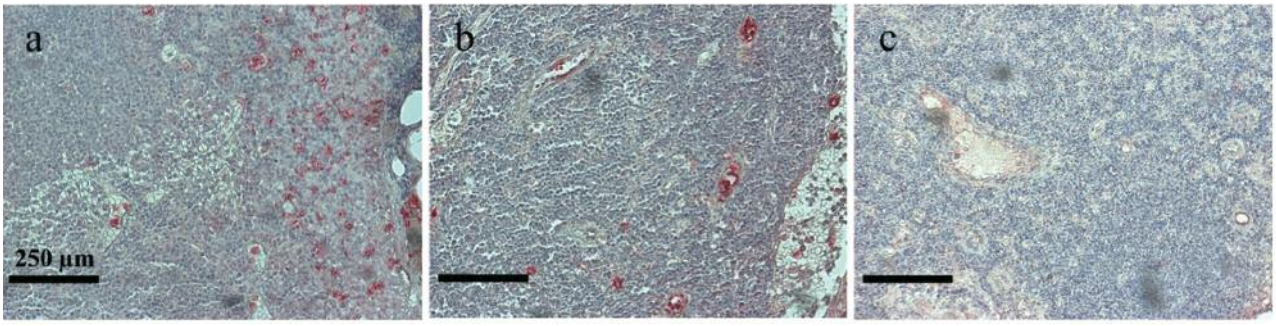

Figure 1. *Evaluation of the lymphatic vessel density (LVD) of metastatic and non-metastatic lymph nodes of the same level of the same patient's neck using anti-lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) stain. Immunohistochemical staining of lymphatic vessels in metastatic (a), non-metastatic (b) and control lymph nodes (c).*

magnification twice for each slice independently by two medical doctors in a blinded manner. LVD data are shown as mean±SD of lymphatic vessels per ROI.

Protein extraction and western blot analysis. Fresh frozen tissue samples from neck dissection specimen were lysed in order to extract protein using ready-to-use Cell Lysis Buffer (Cell Signaling Technology) and phenylmethylsulfonylfluoride in a Tissuelyser LT (Qiagen, Venlo, the Netherlands) at 50 Hz for 4 min. Tissue samples were then homogenized by vortex (IKA-Werke, Staufen im Breisgau, Germany), sonicated for 5 min and then centrifuged at 20,000 × *g* for 10 min at 3˚C. Total protein in the supernatant was determined using the Bradford method (34).

Twenty micrograms of protein were then separated using a 10% polyacrylamide gel at 120/100 V for 90 min followed by western blotting on a nitrocellulose membrane at 80 mA and 16-20 V for 60 min (35, 36). Incubation with validated primary antibodies to eIF4E, SPARC- or β-actin (rabbit polyclonal antibody clone, dilution 1:1,000; Cell Signaling) following protein saturation of the binding sites of the matrix was carried out overnight at 4˚C. After thoroughly rinsing the samples with TBST, they were incubated with secondary antibody (rabbit polyclonal antibody, dilution 1:2,000; Cell Signaling) for 60 min at room temperature. Chemiluminescence reaction was induced by adding a solution containing Western Lightning Oxidizing Reagent (PerkinElmer, Waltham, MA, USA) and Enhanced Luminol (Sigma-Aldrich, St. Louis, MO, USA) (dilution 1:1) which was catalyzed by the horseradish peroxidase linked to the secondary antibody.

The band intensity of the chemiluminescence reaction for eIF4E (25.0 kDa), SPARC (42.0 kDa) and β-actin (42.0 kDa) was quantified using a Kodak Image Station 440 and Kodak 1D Image Analysis Software (Eastman Kodak, New Haven, CT, USA). The housekeeping protein β-actin served as loading control. Results were normalized to the intensity of β-actin.

Statistical analysis. Data are reported as the mean±SD. Student's *t*-test and Mann–Whitney rank-sum test were used to compare eIF4E and SPARC expression as well as LVD values. Linear regression analysis was applied to determine correlations. A *p*-value of less than 0.05 was considered to be statistically significant. All statistical analyses were performed using SigmaPlot 12 (Systat Software, Inc., San Jose, CA, USA).

Results

The analyzed patient population consisted of 10 males and one female, aged from 46 to 58 years (median 53 years). All patients suffered from lymphatically metastasized HNSCCs UICC stage III-IVB. The primary tumor site was mainly the piriform sinus (n=9) as well as the lateral hypopharyngeal wall (n=2). No distant metastases were present as determined by computed tomography of the neck, thorax and abdomen. None of the patients had undergone prior tumor-specific therapy. The obtained specimens included ipsilateral metastatic as well as non-metastatic cervical lymph nodes from the same neck level in patients as well as control cervical parajugular lymph nodes from patients without malignant disease. The results of the initial staging as well as clinical and pathological characteristics are shown in Table I.

Lymphangiogenesis. All available formalin-fixed tissue samples were immunohistochemically stained for lymphatic vessels using LYVE-1 as lymphatic endothelial cell marker (37). Staining of LYVE-1 resulted in a brown-red labeling of thin-walled non-erythrocyte filled vessels. Stained vessels were mainly located in the periphery of the lymph node or at the border of the metastasis with regular tissue (Figure 1).

The extent of lymphangiogenesis was measured by determining the mean LVD within the samples. Lymphatic vessels were present in all 31 samples of lymph node metastases, non-metastatic lymph nodes as well as control cervical lymph nodes. In samples of lymph node metastases, the mean LVD ranged from 4.3 to 19 per ROI. Mean LVD in non-metastatic lymph nodes ranged from 0 to 14 per ROI and in control cervical lymph nodes of healthy individuals from 0 to 4 per ROI. The difference between the LVD in lymph node metastases compared to non-metastatic lymph nodes as well as healthy cervical lymph nodes was highly statistically significant ($p<0.001$, Table I). Furthermore, non-metastatic lymph nodes from patients with cancer had a significant

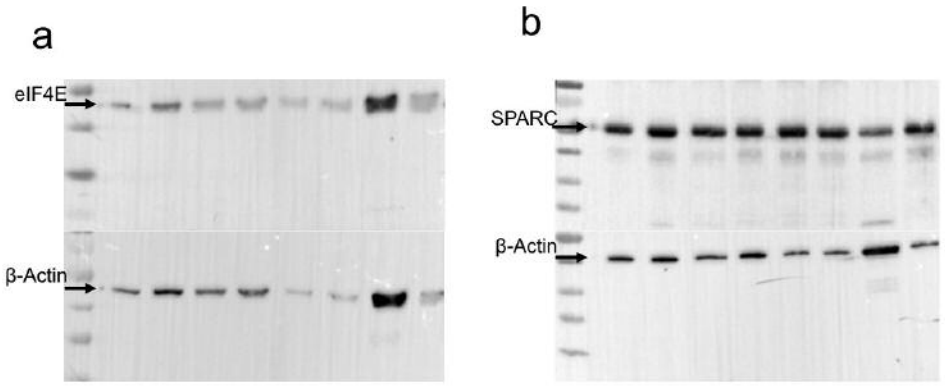

Figure 2. *Representative immunoblot analysis of metastatic lymph nodes (n=8). Expression of eukaryotic initiation factor 4E (eIF4E) (a) was detected in all metastatic lymph specimens at 25 kDa. Expression of secreted protein acidic and rich in cysteine (SPARC) (b) was detected in all specimens at 42 kDa. β-Actin was used as loading control.*

Table I. *Lymphatic vessel density (LVD), and expression of eukaryotic initiation factor 4E (eIF4E) and secreted protein acidic and rich in cysteine (SPARC) in metastatic, non-metastatic and control lymph nodes in relation to staging parameters in patients with lymphatic metastasized hypopharyngeal carcinomas.*

Parameter	Subclass	Tissue	n	LVD (n/ROI)	eIF4E (AU)	SPARC
Lymph nodes		Metastatic	11	9.71±5.67*	4.82±3.07#	3.7±1.88#
		Non-metastatic	9	3.89±3.71#	n.a.	n.a.
		Control	11	1.375±1.41	0.32±0.17	0.12±0.07
Histopathological differentiation	G2	Metastatic	8	10.79±6.27	5.31±64	3.93±2.17
		Non-metastatic	6	2.78±1.96	n.a.	n.a.
	G3	Metastatic	3	7.17±3.37	3.83±0.48	3.17±0.57
		Non-metastatic	3	6.11±3.37	n.a.	n.a.
T-Status	T2	Metastatic	5	7.5±3.61	4.54±1.93	3.44±1.98
		Non-metastatic	5	4.6±4.34	n.a.	n.a.
	T3-4	Metastatic	6	11.72±6.63+	5.18±4.03	3.96±1.74
		Non-metastatic	4	3±2.45	n.a.	n.a.
N-Status	N1	Metastatic	2	13.08±6.74	7.75±4.35	5.55±1.65
		Non-metastatic	1	11.05±0	n.a.	n.a.
	N2	Metastatic	6	9.22±6.01	3.25±0.95	2.9±0.78
		Non-metastatic	5	3.17±2.16	n.a.	n.a.
	N3a	Metastatic	3	8.78±3.81	5±2.29	3.8±2.36
		Non-metastatic	3	2.55±1.3	n.a.	n.a.

AU: Arbitrary unit; ROI: region of interest; n.a.: not available. *Significantly different to non-metastatic and control lymph nodes at $p<0.05$. #Significantly different to control lymph nodes at $p<0.05$. +Significantly different to metastatic lymph nodes of T2 hypopharyngeal carcinomas at $p<0.05$.

higher LVD than lymph nodes from patients without malignant disease ($p<0.001$, Table I). Interestingly, patients with T3-T4 staged primary tumors exhibited a significantly higher LVD of their lymph node metastases compared to those withT2 primary tumors ($p=0.009$, Table I). In addition, we analyzed the LVD in comparison to staging parameters (see Table I). In contrast to metastatic lymph nodes no stage-dependent increase in LVD was observed in non-metastatic lymph nodes of patients with hypopharyngeal cancer.

eIF4E. In order to evaluate the putative association of eIF4E expression with lymphangiogenesis in hypopharyngeal tumors, we performed western blot analysis for all metastatic lymph nodes of the cohort. We detected eIF4E protein in all metastatic lymph nodes, with expression levels ranging from 1.9 to 12.1 arbitrary units (AU) (mean 4.82±3.07 AU, Table I, Figure 2a). Importantly, eIF4E expression in metastatic lymph nodes was significantly higher than in control lymph nodes of healthy individuals ($p<0.001$; Table I).

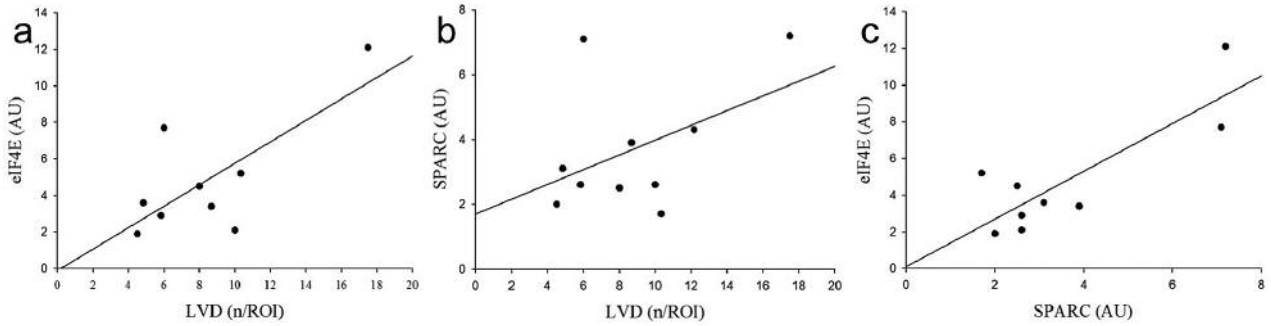

Figure 3. *Correlation of lymphatic vessel density (LVD) with expression of eukaryotic initiation factor 4E (eIF4E) (a; p=0.027, R^2=0.528) and secreted protein acidic and rich in cysteine (SPARC) (b; p=0.003, R^2=0.794) in cervical lymph node metastases. Correlation between the expression of SPARC and the expression of eIF4E in cervical lymph node metastases (c; p=0.004, R^2=0.782). AU: Arbitrary unit; ROI: region of interest.*

SPARC. Next, we wanted to investigate the potential role of SPARC in the process of lymphatic spread. Similarly, western blot analysis was performed for all metastatic lymph nodes of the 11 cases. All specimens showed SPARC expression ranging from 1.7 to 7.2 AU (Table I, Figure 2b). Again, we found that SPARC expression in metastatic lymph nodes was significantly higher than in control lymph nodes of healthy individuals ($p<0.001$, Table I).

Correlation between LVD, eIF4E and SPARC. In order to finally evaluate the significance of our expression analysis, we performed correlation analyses using the determined LVD, eIF4E and SPARC expression as well as independent staging parameters of the patient cohort. Our data showed a significant positive correlation between the LVD and the expression of eIF4E in metastatic lymph nodes ($p=0.027$, $R^2=0.528$, Figure 3a): lymph nodes with high lymphatic vessel density also exhibited increased eIF4E expression. Moreover, we found a significant positive correlation between the LVD and the expression of SPARC in metastatic lymph nodes ($p=0.003$, $R^2=0.794$, Figure 3b). Additionally, we found a significant positive correlation between the expression of eIF4E and the expression of SPARC ($p=0.004$, $R^2=0.782$, Figure 3c).

Discussion

There is evidence that lymphangiogenesis within the cervical tumor microenvironment is a major prerequisite for tumor spread of hypopharyngeal cancer to lymph nodes (13, 38). The presence of regional lymph node metastases is associated with a poor prognosis and a significantly higher rate of recurrence (1, 2). Much data is available showing the involvement of signaling pathways regulating angiogenesis and lymphangiogenesis within the primary tumor site (13, 38, 39). In contrast, the mechanisms promoting metastatic spread into lymph nodes and their surrounding microenvironment have not been studied in detail and remain poorly understood. Despite its high prognostic and possibly therapeutic relevance there is a lack of data concerning microenvironmental changes and predispositions in cervical lymph nodes that favor the rise of metastases. Stacker *et al*. pointed out that tumor-mediated lymphangiogenesis mainly consists of three mechanisms (38): i) The *de novo* generation of lymphatic vessels (*i.e*. lymphangiogenesis), ii) the enlargement of pre-existing collecting lymphatic vessels, and iii) the generation of new lymphatic vessels and enlargement of pre-existing lymphatic vessels within the draining lymph nodes. These mechanisms can be verified prior to the actual dissemination of metastatic cells and may be understood as essential steps in facilitating the lymphatic spread.

Our data provide evidence, that lymphangiogenesis is indeed a phenomenon that occurs during the spread of hypopharyngeal cancer. Moreover, it might be a key mediator in the development and progression of cervical lymph node metastases (40, 41). In our study, the overall degree of lymphangiogenesis represented by the LVD was significantly higher in lymph node metastases when compared to non-metastatic lymph nodes. Importantly, metastatic as well as non-metastatic lymph nodes were derived from corresponding cervical levels of the same patients. In addition, our data strongly support the hypothesis that activation of lymphangiogenesis begins before the actual metastatic dissemination since LVD in non-metastatic cervical lymph nodes of patients with hypopharyngeal cancer was significantly higher than in control lymph nodes of patients without malignant disease. Moreover, patients suffering from larger primary hypopharyngeal carcinomas (T3 to T4) had a significantly higher LVD within their cervical lymph nodes than did patients with smaller primary tumors. In contrast, no increase in LVD was observed comparing non-metastatic lymph nodes of early and advanced primary tumors.

Nathan *et al*. demonstrated a significant decrease in intratumoral LVD, and a reduced lymphatic vessel invasion rate, as well as an overall decrease in the number of metastasis-positive lymph nodes following mTOR inhibition using rapamycin in an orthotopic mouse model (13). In addition, a significant attenuation of metastatic lymph node spreading was observed. On the contrary, our data showed a clear and significant correlation between expression of the mTOR-dependent proto-oncogene eIF4E and the LVD within metastatic lymph nodes (Figure 3a).

The role of SPARC in carcinogenesis and tumor progression is not yet well understood. It appears to play an important role during transformation as SPARC was shown to be an independent prognostic parameter for overall and progression-free survival in HNSCC (23). There are some data indicating the involvement of SPARC in regulating central cellular pathways as well as crosstalk in hematological neoplasia (42-44). In this study, we were able to show for the first time that the expression of SPARC and the overall LVD in metastatic lymph nodes are significantly correlated in hypopharyngeal cancer. To date, SPARC expression has not been associated with lymphangiogenesis. A possible regulation of the mTOR pathway by SPARC has only been investigated by few experimental studies (45, 46).

Paget's seed and soil hypothesis states that the target tissue of the metastatic spread needs to fulfill certain requirements regarding the tumor microenvironment. Thus, metastatic spread is not only a tumor cell-dependent process in which a malignant clone metastasizes to blood or lymphatic vessel networks within the drainage pathway (11, 12). It may rather be the net result of coinciding appropriate circumstances within both the microenvironment and in drainage pathways. The fact that metastatic lymph nodes showed an increase of lymphangiogenesis compared to adjacent non-metastatic lymph nodes and lymph nodes from patients without malignant disease may support this hypothesis (11, 12). It has to be determined which mediators are involved in the development of a tumor microenvironment becoming an activated metastatic niche. A further evaluation of possible mediators that contribute to increased lymphangiogenesis prior to metastatic dissemination is of great clinical relevance. This would provide a therapeutic rationale in order to prevent cervical lymph node metastasis or cervical recurrence. Thus, targeted therapies aiming at lymphatic vessels might imply salvage measures for patients that are not eligible for surgery or (re-)irradiation.

In conclusion, we showed, for the first time, that lymphangiogenesis within cervical lymph nodes seems to play a crucial role in the development and progression of cervical lymph node metastases of hypopharyngeal cancer. Possible targets in this process are mTOR-dependent eIF4E and the extracellular matrix protein SPARC.

Compliance with Ethical Standards

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. The study protocol was approved by the ethics committee of the medical faculty of the Goethe-University Frankfurt/Main (ethics vote 217/13). For this type of study formal consent is not required.

Conflicts of Interest

The Authors declare that they have no conflict of interest in regard to this study.

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