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Original Article / Çalışma - Araştırma

Quantifying the expression of VEGF using qRT-PCR, evaluation of MVD and their correlation with clinicopathological factors in laryngeal squamous cell carcinoma

Larengeal skuamöz hücreli karsinomalarda qRT-PCR kullanılarak VEBF ekspresyonu ölçümü, MDY değerlendirilmesi ve bunların klinikopatolojik faktörlerle ilişkisi

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Objectives: In this study we quantified the expression of vascular endothelial growth factor (VEGF) using quantitative real-time polymerase chain reaction in laryngeal squamous cell carcinoma (LSCC) tissues and evaluated the correlation between the level of VEGF and microvessel density (MVD), and clinicopathological factors.

Patients and Methods: Twenty-seven patients with LSCC undergoing total or partial laryngectomy at the Ear, Nose, and Throat and Head and Neck Surgery Department of the Izmir Tepecik Training and Research Hospital between September 2006 and July 2008. There was no VEGF expression in two patients that were excluded from the study. Twenty-five patients (24 males, 1 female; mean age 61 years; range 43 to 82 years) were included in this study, but MVD levels of 10 patients could not be determined.

Results: As defined by the 2003 American Joint Committee on Cancer (AJCC) TNM classification, seven patients (28%) were stage 1, six patients (24%) were stage 2, four patients (16%) were stage 3, and eight patients (32%) were stage 4. Thirteen patients (52%) had well-differentiated (G1) tumors, and twelve had moderately differentiated tumors. Among the 15 patients for whom the MVD was determined, the median value was 48, with a (range 13-78; vessels / 3.76 mm^2). Among the 25 patients for whom the VEGF level was determined, the median value was 0.035 vessels / 3.76 mm^2 (range 0.010-0.127).

Conclusion: We could not find a statistical correlation between clinicopathological factors and either VEGF or MVD. Our study demonstrates that VEGF is expressed by LSCC.

Key Words: Laryngeal squamous cell carcinoma; quantitative real-time polymerase chain reaction; vascular endothelial growth factor.

Amaç: Bu çalışmada, larenks skuamöz hücreli kanser (LSHK) dokularında kantitatif gerçek zamanlı polimeraz zincir reaksiyonu kullanılarak vasküler endotelyal büyüme faktörü (VEBF) düzeyleri ölçüldü, VEBF, mikrodamar yoğunluğu (MDY) ve klinikopatolojik faktörler arasındaki ilişki değerlendirildi.

Hastalar ve Yöntemler: Eylül 2006 - Temmuz 2008 tarihleri arasında İzmir Tepecik Eğitim ve Araştırma Hastanesi Kulak Burun Boğaz Baş-Boyun Cerrahisi Kliniği'ne başvuran, 27 LSHK hastasına total veya parsiyel cerrahi uygulandı. Yirmi yedi hastanın ikisi, VEBF ekspresyonu olmadığı için çalışmaya dahil edilmedi. Çalışmaya 25 hasta (24 erkek, 1 kadın; ort. yaş 61 yıl; dağılım 43-82 yıl) alındı, fakat bu hastaların 10'unda MDY seviyesi belirlenemedi.

Bulgular: Amerikan Kanser Komitesi (AJCC)'nin 2003 yılında tanımladığı TNM sınıflandırmasına göre; yedi hasta (%28) evre 1, altı hasta (%24) evre 2, dört hasta (%16) evre 3, sekiz hasta (%32) evre 4 idi. On üç hasta iyi derece diferansiye (G1), 12 hasta orta derece diferansiye (G2) tümöre sahipti. Mikrodamar yoğunluk değerleri belirlenebilen 15 hasta içinde, median değer 48 idi (dağılım 13-78; damar / 3.76 mm^2). Vasküler endotelyal büyüme faktörü seviyeleri tespit edilebilen 25 hasta arasında, median değer 0.035 damar / 3.76 mm^2 (dağılım 0.010-0.127) idi.

Sonuç: Klinikopatolojik faktörler ve ayrıca VEBF ve MDY arasında istatistiksel olarak anlamlı bir ilişki bulunamadı. Bizim çalışmamız LSKH'den VEBF salındığını gösterdi.

Anahtar Sözcükler: Larenks skuamöz hücreli kanser; kantitatif gerçek zamanlı polimeraz zincir reaksiyonu; vasküler endotelyal büyüme faktörü.

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Head and neck cancers are the 6th most prevalent type of cancer in the world, with a global yearly incidence of 500.000.[1] Head and neck squamous cell carcinoma (HNSCC) is the most common malignancy of the upper aerodigestive tract, accounting for more than 90% of malignancies at this site,[2] and laryngeal squamous cell carcinomas (LSCC) comprise the vast majority (approximately 96%) of laryngeal malignancies.[3]

The five-year survival rates for head and neck cancers have not improved significantly, remaining at 52 percent. Due to the high rate of local-regional recurrence and mortality in HNSCC, interest has developed in identifying molecular markers that predict patients at risk for disease progression after standard therapy. Molecular markers that predict local recurrence in surgically treated patients would be helpful in identifying those who may benefit from postoperative radiotherapy.[1]

Despite the evolution and refinement of multimodal treatments for head and neck cancer (surgery, radiation therapy, and chemotherapy), the overall survival rate in stages III and IV is less than 40 percent. The most important factor in the prognostic evaluation of HNSCC is tumor node metastasis (TNM) staging, the nodal stage being the most relevant. However, it is well known that having the same TNM stage does not always guarantee similar outcomes in two different patients; there is significant prognostic variation within each TNM stage. This has led to a concerted effort to further define categories with consistent outcomes within each stage or to discover categories of prognostic outcome that are independent of the TNM stage.[4]

To date, a number of protein and deoxyribonucleic acid (DNA) markers in HNSCC have been explored using immunohistochemistry, polymerase chain reaction (PCR)-based techniques, and flow cytometry. The four categories of molecular markers that have received the most attention are: *(i)* p53; *(ii)* angiogenesis- related markers; *(iii)* cyclin D1; and *(iv)* epidermal growth factor receptor (EGFR). Although the data remain inconclusive, these markers have potential clinical significance in determining patient prognoses and selecting appropriate therapeutic modalities.[5]

Angiogenesis is considered a very important biological process in many neoplasms: it is crucial both for the growth of a primary tumor and for the development of distant metastasis. Among the factors causing tumor angiogenesis, vascular endothelial growth factor (VEGF) is a leading candidate. Vascular endothelial growth factor induces the proliferation, differentiation, and migration of vascular endothelial cells, increases capillary permeability, and enhances endothelial cell survival by preventing apoptosis.[6] The staining of microvessels has also been employed to assess the extent of angiogenic activity in a tumor specimen. Antibodies directed to markers such as factor 8, CD-31, and CD-34, found specifically on endothelial cells, can be used to count and average out the density of microvessels in the areas of most intense neovascularization.[1]

Several methods for measuring VEGF expression in tissues or cells have been reported. At the protein level, immunohistochemical staining (IHS) has been used for semiquantitative analysis of VEGF expression and ELISA has been used to quantify cytosolic VEGF protein expression. At the transcriptional level, Northern blots and PCR-based quantitative methods have been used to assess the expression of VEGF messenger ribonucleic acid (m-RNA) in tissues. Polymerase chain reaction-based quantification methods have proved to be powerful tools for the quantitative analysis of the copy number of a given target gene or mRNA, especially when only a limited amount of tissue is available, as is the case in a small early-stage tumor or an aspiration biopsy specimen. Quantitative reverse transcription-PCR is conventionally performed by evaluating the amount of product after a given number of cycles (end-point quantitative PCR). A novel quantitative PCR approach using real-time detection has been recently developed: quantitative real-time polymerase chain reaction (qRT-PCR).

Quantitative reverse transcription-PCR is a combination of three steps: *(i)* the reverse transcriptase (RT)-dependent conversion of RNA into complementary DNA (cDNA); *(ii)* the amplification of the cDNA using PCR; and *(iii)* the detection and quantification of amplification products in real time using fluorescent reporter dyes to combine the amplification and detection steps of the PCR reaction in a single tube.[7]

Our study focused on quantifying the expression of VEGF using qRT-PCR in LSCC tissues and finding a correlation between the level of VEGF and microvessel density (MVD), and

clinicopathological factors. We hypothesized that high levels of VEGF and MVD could correlate with high TNM staging. Vascular endothelial growth factor and MVD measurements can contribute to TNM staging system.

PATIENTS AND METHODS

Patients and fresh tissue samples

The ethical committee of the İzmir Tepecik Training and Research Hospital approved the protocol for the study. Fresh tissue samples were obtained from 27 patients with LSCC undergoing total or partial laryngectomy at the Ear, Nose, and Throat (ENT) and Head and Neck Surgery departments of the İzmir Tepecik Training and Research Hospital between September 2006 and July 2008. Patients with carcinoma in situ, verrucous carcinoma, or other types of carcinoma were excluded. None of the patients had received radiotherapy or chemotherapy before surgical intervention, and none of them had a prior history of HNSCC. Laryngeal squamous cell carcinoma cases were classified according to the American Joint Committee on Cancer TNM classification of 2003. In each case, a portion of the tumor was resected near the advancing edge, avoiding its necrotic center. After excision, the tissues were immediately immersed in RNA-later solutions and stored at +4 ºC for 24 hours and then at –70 ºC until use. The adjacent tissues were submitted for histopathological study.

The resection specimens were fixed in formalin and processed routinely; slides with 5 μm sections were prepared from each selected tumor block. All of the slides were stained with hematoxylin and eosin before viewing. Two observers using a multi-head microscope confirmed diagnosis. The slides were reviewed in double-blinded fashion, and the observers were not aware of the diagnosis. The tumor was graded as well (G-1), moderately (G-2), or poorly differentiated (G-3). Well-differentiated tumors had mild to focally moderate nuclear pleomorphism, with low mitotic counts and frequent keratin formation; moderately differentiated tumors showed moderate nuclear pleomorphism; and poorly differentiated tumors showed no evidence of keratinization, with large and pleomorphic nuclei and sometimes bizarre giant tumor nuclei and increased mitotic activity.

In order to assess microvascular density, one representative slide from each tumor was selected and stained for CD34 (Thermospecific, Clone QBEnd/10, prediluted in 1/200 Lab Vision Ab Diluent, catalog# TA-125-UD), an endothelial cell marker, by using standard avidin biotin methods with microwave antigen retrieval. Each CD-34-positive structure on the stained slide irrespective of any identifiable lumen was counted as a microvessel. Ten random fields of 3.76 mm^2 were examined at low magnification power (H-E x 100; Figure 1).

Although the human β-actin levels of all patients were obtained, we were unable to determine VEGF levels of two of the 27 patients meaning that there was no VEGF expression in these patients. We therefore excluded these two patients from the analysis.

The MVD levels of 15 of the remaining 25 patients were determined, but unfortunately, the MVD levels of the 10 patients could not be determined because we were unable to stain the corresponding slides.

qRT-PCR Homogenization and isolation

Between 50 mg - 100 mg of frozen tissue was placed in polypropylene centrifuge tubes with MagNa Lyser Green Beads and 1 ml of tripune isolation reagent. These tubes were homogenized at 6.500 rpm (speed) for 45 seconds with a MagNA Lyser Instrument (Roche Diagnostics GmbH, Mannheim Germany). Samples were stored at +4 ºC on cold blocks for two minutes and then incubated at +15 ºC to +25 ºC for five minutes. Then, 0.2 ml of chloroform was added to each sample, and the samples were shaken vigorously for 15 seconds. To

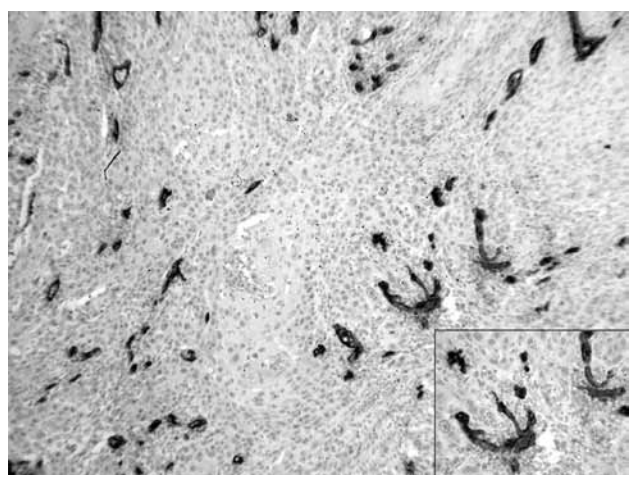


Figure 1. *Expression of CD34 marker in the endothelium of the blood vessels along with the negativity in the LSCC areas (H-E x 100).*

separate the solution into three phases, tubes were centrifuged at 12.000 g for 15 minutes at +4 ºC. After centrifugation, the upper aqueous phase was transferred to a new polypropylene tube; 0.5 ml of isopropanol was added to each tube and the tubes were inverted several times. Afterwards, samples were incubated for five minutes at +15 ºC to +25 ºC to allow the RNA to precipitate. The samples were centrifuged at 12.000 g for 15 minutes at +4 ºC. The supernatant was discarded, and 1 ml of 75% ethanol was added. The RNA pellet was then washed in ethanol by vortexing. Samples were centrifuged at 7.500 g for five minutes at +4 ºC, and the supernatant was discarded again. Excess ethanol was removed from the RNA pellet by using a thermoshaker for 1 minute at +57 ºC. Afterwards, RNase-free water was added and the samples were incubated for five minutes at +57 ºC to allow the pellets to dissolve. The RNA samples were cooled at +4 ºC on cold blocks and stored at –80 ºC.

Synthesis of cDNA

Transcriptor first strand cDNA synthesis kits (Roche, Mannheim, Germany) and thermal cycler were used. Ribonucleic acid obtained from every sample was equalized to 1 µl and completed to 11 µl with water. A random hexamer primer (2 µl) was added, resulting in a 13 µl reaction mix. The samples were subjected to denaturation at +65 ºC for 10 minutes in a thermal block cycler and then immediately cooled at 0 ºC for one minute. Each reaction mix was completed with 4 µl of transcriptor reverse transcriptase reaction buffer, 0.5 µl of protector RNase inhibitor, 2 µl of deoxynucleotide mix, and 0.5 µl of transcriptor reverse transcriptase, resulting in a final reaction volume of 20 µl. The samples were then incubated for 10 minutes at +25 ºC and for 60 minutes at +50 ºC, after which transcriptor reverse transcriptase was inactivated at +85 ºC for five minutes. The samples were then cooled to +4 ºC, and the cDNAs were stored at –20 ºC. The samples were diluted 1:10 with water for real-time PCR.

qRT-PCR Amplification of cDNA (VEGF and β-actin)

Quantitative RT-PCR was performed on a LightCycler 1.5 instrument (Roche, Mannheim, Germany) using LightCycler Fast-Start DNA Master SYBR Green I (Roche, Mannheim, Germany). Parameter specific primer sets, human VEGF as the target gene and human β-actin as the house-keeping gene, optimized for the LightCycler instrument were developed and obtained from Search-LC (Heidelberg, Germany). Preparing for the VEGF and β-actin PCR mixtures, following reagents were added in a light protected tube on ice: 6 µl of water, 2 µl of parameter specific primer set, and 2 µl of Light-Cycler Fast-Start DNA Master SYBR Green I. The resulting 10 µl reaction mixtures were injected into pre-cooled Light-Cycler capillaries and, 10 µl diluted cDNA was added. The reaction mixtures were then centrifuged at 2.000 rpm for 30 seconds. The samples were cycled to amplify the VEGF and β-actin genes according to protocol provided with the parameter specific primer sets (Figure 2).

The copy number of the specific transcripts were calculated from a standard curve obtained by plotting known input concentrations of four plasmids at log dilutions against the PCR cycle number (crossing point; CP). These plasmids were provided with the parameter specific primer sets. The copy number of the VEGF transcripts was normalized by the house keeping gene β-actin.

Melting curve analysis

The specificity of qRT-PCR products were determined by the melting curve profiles. The melting point of every amplicon depends on its sequence, length, guanine and cytosine content, allowing us to use melting curve profiles to identify DNA products. As the temperature was slowly increased, the fluorescence of the sample was monitored to determine the melting temperature profiles. When the PCR reaction was finished, the temperature was first reduced from 95 ºC to 58 ºC at –0.1 ºC per second and then increased again to 95 ºC. The specific melting points for VEGF and β-actin were 88 ºC and 90 ºC, respectively (Figure 3).

Statistical analysis

The statistical analyses were conducted using SPSS for Windows software version 15.0 (SPSS Inc., Chicago, Illinois, USA) Spearman's correlation test, the Kruskal-Wallis test, the Mann-Whitney U-test, and chi-squared tests were used.

RESULTS

Of the twenty-seven original patients, twenty-five patients (24 males, 1 female; range 43 to 82 years with a median of 61 years) were finally included in this study.

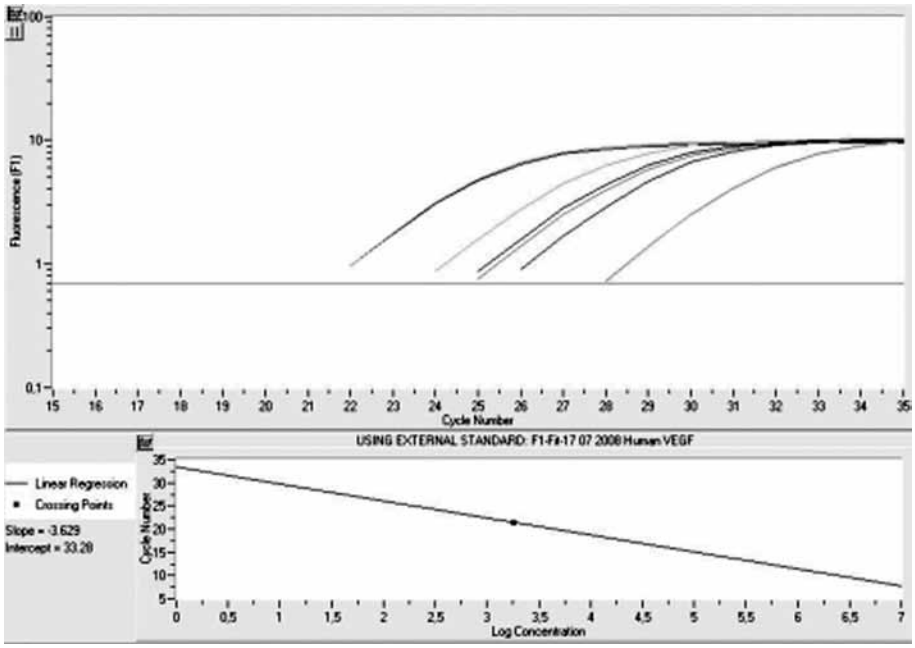


Figure 2. *Fluorescence history versus cycle number plot of vascular endothelial growth factor transcript by Light Cycler qRT-PCR assay based on SYBR Green I dye detection and standard curve for vascular endothelial growth factor cycle number values are plotted against vascular endothelial growth factor plasmid standards.*

All patients were smokers with a median of 50 packs per year of cigarette smoking (minimum 1 pack/year-maximum 135 packs/year). Eighteen patients (72%) had used alcohol.

All patients had LSCC and underwent surgical treatment. As defined by the 2003 AJCC TNM classification, seven tumors (28%) were stage I, six tumors (24%) were stage II, four tumors (16%) were stage III, and eight tumors (32%) were stage IV. Morover, seven patients (28%) were T_1, six (24%) were T_2, five (20%) were T_3, and seven (24%) were T_4. Twenty-two patients (88%) were N_0, and three (12%) were N_2 (Table 1).

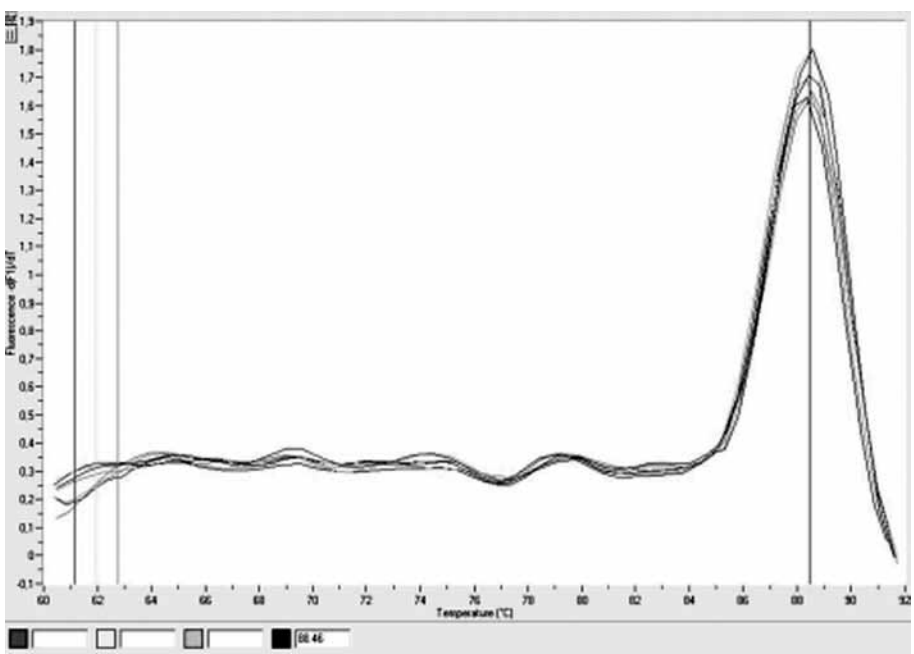


Figure 3. *Melting curve analysis of vascular endothelial growth factor amplification products.*

Table 1. Clinicopathological features of 25 patients with laryngeal squamous cell carcinoma

Characteristics	No. of patients	
	n	%
Tumor differentiation		
Well differentiated (G1)	13	52
Moderately differentiated (G2)	12	48
T classification		
T_1	7	28
T_2	6	24
T_3	5	20
T_4	7	28
Lymph node		
N_0	22	88
N_{2a}	1	4
N_{2c}	2	8
Clinical stage		
Stage I	7	28
Stage II	6	24
Stage III	4	16
Stage IV	8	32

Thirteen patients (52%) had well-differentiated (G1) tumors, and twelve had moderately differentiated tumors.

Among the 15 patients for whom the MVD was determined, the median value was 48, with a range of 13-78 (vessels/3.76 mm^2).

Among the 25 patients for whom the VEGF level was determined, the median value was 0.035 with a range of 0.010-0.127 (Table 2).

We analyzed the correlation between TNM classification, tumor size, lymph node, and differentiation on one hand and VEGF and MVD values on the other hand; we did not find any statistically significant relationships ($p>0.05$; Table 3-6).

DISCUSSION

Clinical TNM classification often underestimates the progression of the disease when compared to the real pathological TNM, which places a significantly higher proportion of tumors in the advanced stage (III-IV) group. A different approach to LSCC, based on genetics and molecular biology in addition to clinical and histological approaches, is required to overcome these obstacles and to reduce cancer-related mortality in LSCC patients.[3]

Table 2. Vascular endothelial growth factor and microvessel density levels

Variable	Minimum	Maximum	Median	SD
VEGF	0.010	0.127	0.035	0.313
MVD	13	78	48	18.97

SD: Standard deviation; VEGF: Vascular endothelial growth factor, levels of 25 patients; MVD: Microvessel density, levels of 15 patients.

Angiogenesis, the sprouting of new blood vessels from preexisting endothelium, is required to support the growth of tumors beyond microscopic size. Angiogenetic activity can be assessed in archival tumor tissue directly, by counting the density of microvessels perfusing the tumor, or indirectly, by staining with antibodies directed against angiogenesis-promoting factors. Many growth factors and cytokines, including VEGF, have been shown to promote angiogenesis.[1]

Although angiogenesis is difficult to measure directly in human tumors, the quantification of immunohistochemically-stained microvessels in histological sections has been suggested as an index. The most common antibodies used for microvessel staining so far are those against von Willebrand factor (factor 8), CD31, CD34, and more recently CD105.[6]

Vascular endothelial growth factor can be constitutively expressed by human tumors or it can be induced by other growth factors and cytokines, including TGF-α, EGF, and phorbol esters. Changes in environmental conditions, such as episodes of hypoxia, seem to be significant in the induction of VEGF. Thus, the expression of VEGF may depend on a variety of endogenous and exogenous factors that can vary greatly during tumor progression. Studies of non-SCC lesions have demonstrated a direct correlation between VEGF expression and MVD, which suggests that VEGF is the predominant growth factor in tumor vascularization. High MVD is associated with increasing tumor aggressiveness. However, this close relationship between MVD and metastatic potential has not been universally detected in human tumors, nor do all human tumors demonstrate increased expression of VEGF.[8]

Although VEGF and MVD have documented importance in both the development and the progression of tumors in multiple models, their roles in HNSCC carcinogenesis are not clear.[8]

Table 3. Distribution of patients' median VEGF and MVD values according to TNM stage

TNM Stage	Number of patients	VEGF (median value)	MVD (median value)
Stage I	7	0.028	13 (n*=1)
Stage II	6	0.042	66 (n*=4)
Stage III	4	0.041	56 (n*=3)
Stage IV (4a+4b)	8	0.033	40 (n*=7)

VEGF: Vascular endothelial growth factor; MVD: Microvessel density; TNM: Tumor-node-metastasis; *: Number of patients whose MVD levels could be evaluated in this group; TNM stage and VEGF levels were compared using Kruskal-Wallis statistical analysis with p=0.8. TNM stage was compared to MVD with p=0.08.

Table 4. Distribution of patients' median VEGF and MVD values according to tumor size

Tumor size	Number of patients	VEGF (median value)	MVD (median value)
T_1	7	0.028	13(n*=1)
T_2	6	0.042	66(n*=4)
T_3	5	0.046	52(n*=4)
T_4	7	0.032	39(n*=6)

VEGF: Vascular endothelial growth factor; MVD: Microvessel density; *: Number of patients whose MVD levels could be evaluated in this group; Tumor size and VEGF levels were compared using Kruskal-Wallis statistical analysis with p=0.5. Tumor size was compared to MVD with p=0.1.

Studies examining the association of VEGF immunohistochemical expression and tumor angiogenesis with clinicopathological factors and outcomes in HNSCC have yielded inconclusive and conflicting results.

Kyzas et al.[9] could not find a statistically significant correlation between VEGF expression and histological grade, lymph node status, tumor status, or smoking habits in 50 patients with lip SCC. On the other, Teknos et al.[10] showed that tissue levels of VEGF and IL-8 were correlated with more aggressive disease, higher TNM stage, more recurrences, and shorter disease-free survival in head and neck cancer. In another study Lentsch et al.[11] found a strong correlation between MVD and tumor-stage in HNSCC.

In this prospective study, VEGF expression was detected in 25 of 27 cases (96.5%) using qRT-PCR. Tissue samples of 50-60 mg were sufficient for qRT-PCR, and this small amount of tissue did not affect TNM classification in the postoperative period.

In our study, we could not find a statistical correlation between clinicopathological factors (TNM stage, tumor size, lymph node metastasis, and differentiation) and either VEGF or MVD. There was also no correlation between VEGF and MVD.

Table 5. Distribution of patients' median VEGF and MVD values according to lymph node status

Lymph node status	Number of patients	VEGF (median value)	MVD (median value)
N_0	22	0.035	49 (n*=12)
N_2 (2a+2c)	3	0.032	40 (n*=3)

VEGF: Vascular endothelial growth factor; MVD: Microvessel density; *: Number of patients whose MVD levels could be evaluated in this group; Lymph node status and VEGF levels were compared using the Mann Whitney U-test with p=0.6. Lymph node status was compared to MVD with p=0.3.

Table 6. Distribution of patients' median VEGF and MVD values according to differentiation

Lymph node status	Number of patients	VEGF (median value)	MVD (median value)
G1	13	0.046	56 (n*=8)
G2	12	0.030	40 (n*=7)

VEGF: Vascular endothelial growth factor; MVD: Microvessel density; *: Number of patients whose MVD levels could be evaluated in this group; Differentiation and VEGF levels were compared using the Mann Whitney U-test with p=0.3. Differentiation was compared to MVD with p=0.08.

In conclusion, our study demonstrates that VEGF is expressed by laryngeal squamous cell carcinomas, but it cannot make a contribution to TNM staging, and neither can MVD. We reckoned these parameters would allow us to foresee different courses of tumors of the same stage. However, we are of the opinion that working with broader patient groups having the same stage will be required. We believe it will thereby be more convenient to compare the values that will be obtained with survey, secondary primer, distant metastasis and recurrance rates.

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