Expression of Vascular Endothelial Growth Factor and Microvessel Density in Head and Neck Tumorigenesis

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ABSTRACT

Angiogenesis is a fundamental process in tumor growth and metastasis, and its significance and that of vascular endothelial growth factor (VEGF) expression as prognostic indicators have been documented for various types of human tumors. However, the mechanisms responsible for angiogenesis in head and neck squamous cell carcinoma are not well defined. To examine the relationship between angiogenesis and the phenotypic progressions of head and neck tumorigenesis, we used immunohistochemistry to analyze VEGF expression and microvessel density in 70 paraffin-embedded specimens that contained adjacent normal epithelium, premalignant lesions, or both from 57 patients with head and neck squamous cell carcinoma. Ten samples of normal oral mucosa were obtained from people who did not smoke or drink alcohol and included in the analysis as normal controls. Microvessel density was evaluated by averaging 10 microscopic fields (×400) in a defined area of each specimen. The degree of VEGF expression was assessed on a cell-by-cell basis in 10 microscopic fields (×200) in a defined area on a scale ranging from 0 (no expression) to 3+ (highest level of expression). In addition, the weighted mean index of VEGF expression was calculated. The mean ± SD weighted mean index of VEGF expression in normal control epithelium (1.10 ± 0.38, n = 10) was higher than it was in adjacent normal epithelium (0.82 ± 0.27, n = 13; P = 0.04). VEGF expression decreased as samples ranged from normal adjacent epithelium to hyperplasia (0.78 ± 0.28, n = 21), mild dysplasia (0.70 ± 0.29, n = 28), moderate dysplasia (0.67 ± 0.29, n = 11), severe dysplasia (0.51 ± 0.39, n = 6), and squamous cell carcinoma (0.20 ± 0.27, n = 70; overall P = 0.0001). VEGF expression was two times lower in cases with nodal disease (0.17 ± 0.26, n = 29) than it was in nonnodal disease (0.32 ± 0.29, n = 16; P = 0.02). Microvessel density showed no significant difference from adjacent normal epithelium premalignant lesions to cancer. In tumor, no correlation was seen between VEGF expression or microvessel density and differentiation, primary tumor site, T stage, or smoking status. These findings indicate that VEGF expression is down-regulated during head and neck tumorigenesis. However, further studies are required to better understand the mechanism of VEGF down-regulation in head and neck tumorigenesis.

INTRODUCTION

The development of head and neck cancer exemplifies the theories of field cancerization and of the multistep process of tumorigenesis (1, 2). The concept of field cancerization is that the entire expanse of the aerodigestive tract that is exposed to carcinogenic insult has an increased risk of multiple cancer development (1). In addition, the sequential presence of a variety of premalignant lesions and malignant lesions in the same patient reflects progressive phenotypic and genotypic alterations associated with tumorigenesis. In multistep tumorigenesis, the accumulation of genetic alterations involves activation of proto-oncogenes and inactivation of tumor suppressor genes and results in phenotypic changes. Although many steps are associated with tumor progression, neo-angiogenesis is an essential factor for tumor growth and metastasis.

Angiogenesis, the formation of new microvasculature, is an important component in many biological processes, both in physiological conditions, such as proliferating endometrium, corpus luteum formation, and embryogenesis, and in pathological conditions, such as rheumatoid arthritis, diabetic retinopathy, and neoplastic disease (3). It has been proposed that angiogenesis is needed for the growth of both primary and metastatic tumors of the demand for blood supply of lesion size beyond 1 or 2 mm³ (4, 5). Based on data from experimental animal studies, angiogenesis precedes overt tumor formation in animal models involving chemically induced carcinogenesis and in transgenic mice, suggesting that tumor progression depends on a switch from a prevascular to an angiogenic phase (6). Folkman et al. (7) found that a phenotype of tumor angiogenesis was switched on in the early stage of tumor progression, reporting that angiogenic activity first appeared in a subset of hyperplastic islets before the onset of tumor formation in a study using transgenic mice expressing an oncogene in the β-cells of the pancreatic islets.

The process of angiogenesis involves several sequential steps, including degradation of the basement membrane of the parent vessel and extracellular matrix, locomotion of endothelial cells toward a tumor implant, mitosis, lumen formation, development of sprout loops and of a new basement membrane, and finally, recruitment of pericytes (8, 9). Each of these distinct
Proliferation steps is likely to be regulated differentially (10). The induction of angiogenesis is mediated by positive and negative regulatory molecules released by both tumor and host cells and depends on a net balance between positive and negative angiogenic factors (6, 11). At present, many angiogenic molecules have been identified. The major positively acting angiogenic molecules include basic fibroblast growth factor, VEGF, interleukin-8 (II-8), platelet-derived endothelial cell growth factor, and hepatocyte growth factor (9, 12). Major angiogenic inhibitors include thrombospondin-1, platelet factor-4, angiostatin, endostatin, IFN-α, and tissue inhibitors of metalloproteinases (9).

The human VEGF gene has been mapped to chromosome 6p21.3 (13). Biochemically, VEGF is a heparin-binding glycoprotein that occurs in least four molecular forms; these consist of 121, 165, 189, or 206 amino acids from the same gene by alternative mRNA splicing (14, 15). VEGF is also known as vascular permeability factor and was originally discovered as one of the most powerful agents causing vascular permeability. It is a potent, multifunctional cytokine that exerts several important and possibly independent actions on vascular endothelium (16). VEGF regulates multiple endothelial cell functions, including mitogenesis, permeability, vascular tone, the production of vasoactive molecules, and the stimulation of monocyte chemotaxis (14, 17).

The significance of tumor angiogenesis as a prognostic indicator has been documented in various types of human tumors. The first report that the level of angiogenesis in a human tumor could predict the likelihood of metastasis was found in cutaneous melanoma (18). Evidence has been presented to indicate that angiogenesis levels of primary invasive carcinomas and overexpression of VEGF correlated with the simultaneous presence of metastatic disease and recurrence as well as with poor survival rates, in patients with several human tumors, including breast cancer, lung cancer, prostate cancer, ovarian cancer, cervical cancer, esophageal cancer, and colon cancer (19–28). In several studies, increased microvessel density and overexpression of VEGF in head and neck squamous cell carcinoma was also associated with metastasis, recurrence, and poor prognosis (29–35). However, the association between angiogenesis and tumorigenesis of head and neck squamous cell carcinoma is not well defined. To examine the relationship between angiogenesis and the histological progression during head and neck tumorigenesis, we analyzed VEGF expression and microvessel density in head and neck squamous cell carcinoma specimens that contained adjacent normal epithelium and premalignant lesions and correlated them with clinicopathological parameters.

### PATIENTS AND METHODS

#### Patients and Tumor Samples.

The patients whose tumor specimens were selected for this study underwent surgery at some time between 1990 and 1998 at the University of Texas M. D. Anderson Cancer Center. The selection criteria included available tissue blocks and the tissues containing squamous cell carcinoma of head and neck and either adjacent normal epithelium or nearby premalignant lesions (hyperplasia and/or dysplasia). Seventy formalin-fixed, paraffin-embedded tissue specimens of head and neck squamous cell carcinomas were obtained from the Department of Pathology at the University of Texas M. D. Anderson Cancer Center. The areas of normal squamous epithelium, hyperplasia, and/or dysplasia adjacent to the cancerous area were identified and marked by the pathologist (A. K. E. N.) according to the criteria described previously (36–38). As controls, 10 normal oral mucosa tissue specimens were obtained from people who had had a tonsillectomy or uvulopalatopharyngoplasty for a benign condition; these individuals (mean age, 18; range, 5–42) neither smoked nor drank alcohol. Biopsy specimens were placed in 10% buffered formalin for at least 4 h and processed for paraffin embedding. In these samples of normal epithelium and premalignant lesions, the three epithelial layers (basal, parabasal, and superficial) were identified according to their histological characteristics and assessed separately (36). Tissue sections 4 μm thick were mounted on aminolysis-lsilane-coated slides (Histology Control System, Glen Head, NY). Formalin-fixed, paraffin-embedded human placental tissue known to express VEGF was attached on the same slide beside each tumor section to serve as an internal control for measurements of VEGF expression. To obtain clinical information, we reviewed medical records, including age, sex, history of tobacco consumption, primary tumor sites (oral cavity, oropharynx, larynx, and hypopharynx), T stage (primary tumor size), and N stage (lymph node status) according to American Joint Committee on Cancer and histological grade of tumor differentiation (well, moderately, or poorly differentiated).

#### Determination of VEGF Expression.

VEGF expression was determined using a mouse monoclonal anti-VEGF antibody corresponding to amino acids 1–140 on human VEGF (Santa Cruz Biotechnology, Santa Cruz, CA) by immunohistochemistry. In brief, after deparaffinization with xylene and rehydration with graded alcohols, endogenous peroxidase activity was blocked by incubating the slides in 3% hydrogen peroxide (H2O2) with methanol for 15 min. To retrieve the antigens, we heated the slides in a microwave oven in 10 mM of sodium citrate buffer at pH 6.2 for 10 min and then allowed them to remain at room temperature for 30 min (39). After being washed in PBS, the slides were incubated with universal blocking solution (BioGenex, San Ramon, CA) to decrease the background signal, rinsed in PBS, and incubated a 1:60 dilution of anti-VEGF mouse monoclonal antibody overnight at 4°C. Slides were left at room temperature for 20 min and washed with PBS. Then the slides were incubated with a biotinylated secondary antibody for 30 min at 37°C and with biotin-avidin peroxidase conjugate (ABC kit, Vector Laboratories, Burlingame, CA) for 30 min at room temperature. After being washed in PBS, the VEGF antigen was visualized with a 0.1% 3,3’-diaminobenzidine solution (Sigma Chemical Co., St. Louis, MO) in a 1X solution of PBS and 0.01% hydrogen peroxide. Finally, the sections were counterstained with Mayer’s hematoxylin for 5 min and mounted in Eukitt (Calibrated Instruments, Inc., Hawthorne, NY).

We defined the degree of VEGF expression on a semiquantitative scale ranging from 0 (no expression) to 3+ (highest level of expression) on a cell-by-cell basis in 10 microscopic fields of view using a light microscope. The degree of expression was determined in a 100-μm² area around each invasive front.
fields (×200) in a defined area. The labeling index of VEGF was calculated as the total number of positively stained cells divided by total number of cells counted (mean number of cells counted, 4,500; range, 115–10,400). Also, the WMI of VEGF expression was calculated as the sum of the number of counted cells multiplied by each the degree of intensity of each cell (0–3+) and divided by the total number of cells counted, as follows: WMI of VEGF expression = (number of cells counted × degree of intensity)/total number of cells counted.

**Determination of Microvessel Density.** Microvessels were highlighted by staining endothelial cells with anti-CD34 antibody (BioGenex) by immunohistochemistry. Briefly, after deparaffinization, the slides were incubated with 3% hydrogen peroxide with methanol and nonimmune horse serum. Then, the slides were incubated with a 1:2 dilution of prediluted anti-CD34 antibody overnight at 4°C. Next, the slides were incubated sequentially with a biotinylated second antibody, biotin-avidin peroxidase conjugate (ABC kit, Vector Laboratories), and a 0.1% 3,3'-diaminobenzidine solution. Microvessel density was assessed without knowledge of any patient’s medical information. The endothelial cell or endothelial cell cluster that was clearly separated from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. In normal oral epithelium and premalignant lesions, microvessels were mainly located just underneath the epithelium. We counted two or three microscopic fields at ×400 magnification with a one-field depth from the basement membrane of the epithelium in the control tissues and in premalignant lesions and calculated the mean value of those two or three fields in each specimen. After the area of optimum staining within the tissues was marked, individual microvessels that were located in the stromal tissues surrounding the tumor nests were counted at ×400 microscopic magnification. In defined tumor areas, 10 microscopic fields (×400) were counted for evaluation of microvessel density, and the mean number of microvessels in 10 of the microscopic fields was calculated for the analyses.

**Statistical Analysis.** Data are presented as mean ± SD. The variable containing the histological information was fitted into a repeated measurement model, and the differences among adjacent normal, hyperplastic, dysplastic, and cancerous tissue groups were detected. The relationship between VEGF expression and microvessel density in cancer or premalignant lesions was determined with Pearson’s coefficient of correlation and a linear regression analysis. The comparisons between the various histological groups and between the nodal disease group and the nonnodal disease group were done using the Mann-Whitney U test. The Kruskal-Wallis test was used when comparing VEGF expression in the T stages of cancerous tissue group. All tests were two-tailed, and P ≤ 0.05 was considered to indicate statistical significance.

**RESULTS**

**Patients and Tissue Characteristics.** Tumor specimens were obtained from either biopsy material or surgically resected specimens from 57 patients with head and neck squamous cell carcinoma. Of the 70 tumor specimens, 57 specimens were derived from primary tumor site, and 13 specimens were derived from metastatic lymph nodes. Of the 57 specimens of primary tumor site, 13 exhibited histologically adjacent normal epithelium, 21 exhibited hyperplasia, and 45 showed dysplasia (28, mild dysplasia; 11, moderate dysplasia; 6, severe dysplasia). As shown in Table 1, there were 41 men and 16 women, and the average age of these patients was 55 years, with a range of 26–75 years. Primary tumor sites were as follows: 19 in the oral cavity, 20 in the oropharynx, 14 in the larynx, 3 in the hypopharynx, and 1 in tumor of unknown primary origin. Five tumors were histologically well differentiated, 36 were moderately differentiated, and 16 were poorly differentiated. Samples were grouped by T and N stages as follows: 12 in T1, 19 in T2, 15 in T3, and 9 in T4 and 16 in N0, 10 in N1, 25 in N2, and 5 in N3.

### Table 1  Characteristics of patients  |  No. (%)  
--- | ---  
No. of patients | 57 (100)  
Median age (range) | 55 (26–75)  
Sex |  
Male | 41 (72)  
Female | 16 (28)  
Primary site |  
Oral cavity | 19 (33)  
Oropharynx | 20 (35)  
Larynx | 14 (25)  
Hyoppharynx | 3 (5)  
Unknown | 1 (2)  
T stage |  
T0 | 1 (2)  
T1 | 12 (21)  
T2 | 19 (33)  
T3 | 15 (26)  
T4 | 9 (16)  
Unknown | 1 (2)  
N stage |  
N0 | 16 (28)  
N1 | 10 (18)  
N2 | 25 (44)  
N3 | 5 (9)  
Unknown | 1 (2)  
M stage |  
M0 | 55 (96)  
M1 | 1 (2)  
Histological grade |  
Well differentiated | 5 (9)  
Moderately differentiated | 36 (63)  
Poorly differentiated | 16 (28)  
Smoking status |  
Current smoker | 31 (54)  
Former smoker | 9 (16)  
Nonsmoker | 8 (14)  
Unknown | 9 (16)  

**VEGF Expression.** VEGF expression was observed mainly in the cytoplasm of cells. The placental tissues that served as internal positive control tissues consistently showed 1+ to 2+ VEGF expression. The immunostaining pattern of VEGF in the cancer cells was heterogeneous. Some portions of normal salivary gland tissues and muscle tissues were stained positively. Some of the tumor-infiltrating inflammatory cells showed strong VEGF expression. Occasional weak staining for VEGF was also observed in the capillary endothelial cells of the tumors. Table 2 shows VEGF expression in each layer of
epithelium of various histology. VEGF expression in normal control epithelium was predominantly detected in the basal layer and seemed to decrease gradually from the basal layer to the superficial layer. In hyperplastic and dysplastic lesions, VEGF expression was predominantly detected in the parabasal layer. The WMI of VEGF expression in normal control epithelium (1.10 ± 0.38) was significantly higher than it was in adjacent normal epithelium (0.82 ± 0.27; P = 0.04). VEGF expression was remained at low levels in adjacent normal epithelium, hyperplasia (0.78 ± 0.28), mild dysplasia (0.70 ± 0.29), and moderate dysplasia (0.67 ± 0.29). VEGF expression levels were further decreased in severe dysplasia (0.51 ± 0.29) and in squamous cell carcinoma (0.20 ± 0.27; Fig. 1), and the overall decreasing VEGF expression from adjacent normal epithelium to squamous cell carcinoma was statistically significant (P = 0.0001). The association between VEGF expression in tumor cells and primary tumor site, T stage, and differentiation was analyzed, but no significant association was found in these.

### Table 2  Relationship of VEGF expression to histological type

<table>
<thead>
<tr>
<th>Histological type</th>
<th>Basal</th>
<th>Parabasal</th>
<th>Superficial</th>
<th>Total</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>NC (n = 10)</td>
<td>1.52 ± 0.53</td>
<td>1.20 ± 0.36</td>
<td>0.41 ± 0.23</td>
<td>1.10 ± 0.38</td>
<td>0.04</td>
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<tr>
<td>ANL (n = 13)</td>
<td>0.96 ± 0.26</td>
<td>0.90 ± 0.28</td>
<td>0.46 ± 0.33</td>
<td>0.82 ± 0.27</td>
<td>0.92</td>
</tr>
<tr>
<td>HYP (n = 21)</td>
<td>0.79 ± 0.27</td>
<td>0.85 ± 0.31</td>
<td>0.45 ± 0.29</td>
<td>0.78 ± 0.28</td>
<td>0.18</td>
</tr>
<tr>
<td>DYP-1 (n = 28)</td>
<td>0.72 ± 0.31</td>
<td>0.81 ± 0.31</td>
<td>0.30 ± 0.32</td>
<td>0.70 ± 0.29</td>
<td>0.36</td>
</tr>
<tr>
<td>DYP-2 (n = 11)</td>
<td>0.69 ± 0.27</td>
<td>0.73 ± 0.31</td>
<td>0.35 ± 0.32</td>
<td>0.67 ± 0.29</td>
<td>0.11</td>
</tr>
<tr>
<td>DYP-3 (n = 6)</td>
<td>0.53 ± 0.37</td>
<td>0.57 ± 0.47</td>
<td>0.56 ± 0.49</td>
<td>0.51 ± 0.39</td>
<td>0.06</td>
</tr>
<tr>
<td>Cancer (n = 70)</td>
<td></td>
<td></td>
<td></td>
<td>0.20 ± 0.27</td>
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</table>

* NC, normal control; ANL, adjacent normal epithelium; HYP, hyperplasia; DYP-1, mild dysplasia; DYP-2, moderate dysplasia; DYP-3, severe dysplasia.

Fig. 1  Immunohistochemical demonstration of VEGF expression in head and neck tumorigenesis. Immunohistochemistry was performed on paraffin sections with monoclonal anti-VEGF antibody. VEGF expression was highest in normal control oral epithelium (A) and decreased as lesions progressed from adjacent normal epithelium (B) to hyperplasia (C), dysplasia (D), and cancer (E). It is noteworthy that VEGF expression focally increased in dysplasia and carcinoma.
variables and degree of VEGF expression in cancerous tissue. By N stage, VEGF expression in tumors was as follows: $N_0$ ($n = 16$), $0.32 \pm 0.29$; $N_1$ ($n = 10$), $0.25 \pm 0.36$; $N_2$ ($n = 25$), $0.14 \pm 0.22$; and $N_3$ ($n = 5$), $0.07 \pm 0.07$. Grouping the samples as either node negative (N–) or node positive (N+) VEGF expression was $0.17 \pm 0.26$ in the N+ samples and $0.32 \pm 0.29$ in the N– group. There was statistical significance between the N– and N+ groups ($P = 0.02$; see Table 4).

**Microvessel Density.** In normal oral mucosa and premalignant lesions, microvessels were located mainly just underneath the epithelium. After counting these microvessels, we determined the mean $\pm$ SD of microvessel density to be $26 \pm 6.7$ in control normal tissue, $18 \pm 4.3$ in adjacent normal tissue, $19.7 \pm 6.9$ in hyperplasia, $18.6 \pm 7.4$ in mild dysplasia, $15.1 \pm 5.3$ in moderate dysplasia, $19.2 \pm 11.2$ in severe dysplasia, and $19.6 \pm 6.8$ in cancer (Table 3). Microvessel density in normal control tissues was significantly higher than that in premalignant lesions and cancer ($P = 0.003$; Table 3). To compare microvessel density in different histological types, we looked at patients in two groups: those with cancer and those with premalignant lesions. Microvessel density in case of cancer was not correlated with smoking status, primary tumor site, differentiation, T stage, or N stage (Table 4). There was also no correlation between the histological type and microvessel density in premalignant lesions (Fig. 2).

**Correlation between VEGF Expression and Microvessel Density.** We analyzed the relationship between VEGF expression and microvessel density in cancer and in premalignant lesions. Microvessel density did not correlate with VEGF expression in either group.

**DISCUSSION**

The process of angiogenesis is the outcome of an imbalance between positive and negative angiogenic factors produced by both tumors and normal cells. Although numerous angiogenic factors have been described, the ones responsible for angiogenesis in head and neck cancer are still unknown.

We analyzed VEGF expression in samples of head and neck squamous cell carcinoma, premalignant lesions, and, for control purpose, normal oral epithelium. Levels of VEGF expression were highest in normal control oral epithelium and decreased as lesions progressed from adjacent normal epithelium to hyperplasia, to dysplasia, and to invasive cancer. VEGF expression in cancer showed no correlation with differentiation, smoking status, or T stage. However, the N– group had a level of VEGF expression twice as high as that of the N+ group. Many human cancers showed increased VEGF expression compared with VEGF expression in corresponding normal tissues. But some authors reported opposite results, demonstrating that VEGF expression levels were higher in corresponding normal tissues (40). One study of VEGF expression in prostate lesions revealed that VEGF expression was higher in samples of normal epithelium and premalignant lesions than it was in cancer (40). In skin lesions, vulva lesions, and salivary gland lesions, VEGF was also expressed as highly in normal tissue and benign lesions as it was in cancer (41–43). In case of head and neck cancer, some authors reported that VEGF expression in cancerous tissue was elevated over that in normal tissue (33, 44). But in one study, positive staining for VEGF was shown in normal and hyperplastic squamous epithelium, as well as in cancerous lesions (45).

Head and neck tumorigenesis is characterized by cumulative genetic changes from normal epithelium to cancer, including activation of oncogenes and inactivation of tumor suppressor genes. Our previous studies showed that levels of epidermal growth factor receptor and p53 protein expression increased as tissues progressed from adjacent normal epithelium to hyperplasia, to dysplasia, and then to squamous cell carcinoma (37, 38). In this study, however, VEGF expression patterns were the opposite of those of epidermal growth factor receptor and p53 expression. According to our results, VEGF expression by itself apparently does not account for an invasive or aggressive tumor phenotype, because VEGF protein was expressed in normal oral epithelium, hyperplasia, and dysplasia and was not associated with clinicopathological parameters. With respect to the function of VEGF derived from normal oral epithelium, we suggest that it might regulate mucosa function under normal physiological conditions. It is evident that VEGF has a role in the embryonic angiogenesis and in physiological angiogenesis processes, such as corpus luteum development and wound healing (3, 46, 47). VEGF is expressed in embryo and many human and mouse adult organs, including lung, kidney, adrenal gland, and heart (48). VEGF is also detected in the cultured human primary keratinocytes and the saliva of healthy individuals (43, 49). In our study, VEGF expression was higher in the N– group than it was in the N+ group. We do not know whether this result is associated with actual VEGF function or whether it is just a coincidental phenomenon.

We analyzed microvessel density in premalignant lesions and cancer. Microvessel density in cancer has no correlation with smoking status, differentiation, or TNM staging. Microvessel density in premalignant lesions and cancer. Microvessel density in case of cancer was not correlated with smoking status, primary tumor site, differentiation, T stage, or N stage (Table 4). There was also no correlation between the histological type and microvessel density in premalignant lesions (Fig. 2).

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significantly associated with metastasis and poor prognosis in various tumor types. Other reports, however, showed no association between high vascularity of the primary tumor and clinicopathological parameters (50, 51). A number of studies have compared normal tissues with either premalignant lesions or malignant lesions and have shown significantly higher vascularity in dysplasia and cancer (24, 32, 52). In head and neck cancer, there are also conflicting data about the correlation between tumor

**Table 4**  Relationship of various clinicopathological factors to VEGF expression and microvessel density

<table>
<thead>
<tr>
<th>Clinicopathological factor</th>
<th>WMI of VEGF</th>
<th>P</th>
<th>Microvessel density*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumor site</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Oral cavity (n = 19)</td>
<td>0.72</td>
<td></td>
<td>21.4 ± 8.5</td>
<td>0.48</td>
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<tr>
<td>Oropharynx (n = 20)</td>
<td>0.16 ± 0.26</td>
<td></td>
<td>17.8 ± 5.3</td>
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<tr>
<td>Larynx (n = 14)</td>
<td>0.20 ± 0.25</td>
<td></td>
<td>19.9 ± 8.1</td>
<td></td>
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<tr>
<td>Hypopharynx (n = 3)</td>
<td>0.10 ± 0.13</td>
<td></td>
<td>21.0 ± 5.1</td>
<td></td>
</tr>
<tr>
<td>Unknown (n = 1)</td>
<td>0.64</td>
<td></td>
<td></td>
<td>0.13</td>
</tr>
<tr>
<td>Smoking status</td>
<td></td>
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<tr>
<td>Current smoker (n = 31)</td>
<td>0.13 ± 0.19</td>
<td></td>
<td>18.1 ± 6.6</td>
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<tr>
<td>Former smoker (n = 9)</td>
<td>0.15 ± 0.25</td>
<td></td>
<td>18.7 ± 4.3</td>
<td></td>
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<tr>
<td>Nonsmoker (n = 8)</td>
<td>0.03 ± 0.02</td>
<td></td>
<td>24.5 ± 4.8</td>
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<tr>
<td>Unknown (n = 9)</td>
<td>0.18 ± 0.29</td>
<td></td>
<td>21.3 ± 10.9</td>
<td></td>
</tr>
<tr>
<td>Histological grade</td>
<td>0.97</td>
<td></td>
<td></td>
<td>0.92</td>
</tr>
<tr>
<td>Well differentiated (n = 5)</td>
<td>0.20 ± 0.36</td>
<td></td>
<td>20 ± 4.2</td>
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<tr>
<td>Moderately differentiated (n = 36)</td>
<td>0.13 ± 0.20</td>
<td>19.7 ± 7.7</td>
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<tr>
<td>Poorly differentiated (n = 16)</td>
<td>0.13 ± 0.23</td>
<td>19.4 ± 5.8</td>
<td></td>
<td></td>
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<tr>
<td>T stage</td>
<td>0.08</td>
<td></td>
<td></td>
<td>0.71</td>
</tr>
<tr>
<td>T&lt;sub&gt;1&lt;/sub&gt;, T&lt;sub&gt;2&lt;/sub&gt; (n = 31)</td>
<td>0.14 ± 0.23</td>
<td>18.9 ± 7.0</td>
<td></td>
<td></td>
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<tr>
<td>T&lt;sub&gt;3&lt;/sub&gt;, T&lt;sub&gt;4&lt;/sub&gt; (n = 24)</td>
<td>0.27 ± 0.29</td>
<td>19.4 ± 5.3</td>
<td></td>
<td></td>
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<tr>
<td>N stage*</td>
<td>0.02</td>
<td></td>
<td></td>
<td>0.67</td>
</tr>
<tr>
<td>N&lt;sub&gt;0&lt;/sub&gt; (n = 16)</td>
<td>0.32 ± 0.29</td>
<td></td>
<td>18.5 ± 7.1</td>
<td></td>
</tr>
<tr>
<td>N&lt;sub&gt;1&lt;/sub&gt;,&lt;sub&gt;3&lt;/sub&gt; (n = 29)</td>
<td>0.17 ± 0.26</td>
<td>19.6 ± 6.1</td>
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</table>

* Microvessels were counted at ×400 magnification.

Values of VEGF expression in N stage are derived from primary tumor values.

**Fig. 2**  Immunohistochemical demonstration of microvessels with monoclonal anti-CD34 antibody in head and neck tumorigenesis. Microvessels were located mainly just underneath the epithelium in control normal oral mucosa (A), adjacent normal mucosa (B), hyperplasia (C), and dysplasia (D). There were no correlations between microvessel density and histology in control normal tissue, premalignant lesions, or cancerous tissue (E).
microvessel density and metastasis and prognosis. Some studies showed association between microvessel density and metastasis (29–32). In other studies, however, there was no such correlation with clinical parameters (53–56). A major difficulty in studying angiogenesis in humans is the lack of direct methods for measuring angiogenic activity. A commonly used indirect method consists of measuring the density of the microvasculature in histological sections of the tumor and considering this measurement to represent the angiogenic status of the tumor. This approach has led to conflicting results regarding the value of microvessel density as a prognostic indicator in solid tumors. Our results showed no correlation between angiogenesis and clincopathological parameters. Microvessel density showed no significant difference from adjacent normal epithelium, premalignant lesions to cancerous lesions. As a tumor grows, the total number of microvessels is increased in parallel with tumor volume. Therefore, microvessel density is maintained similarly during head and neck tumorigenesis. It is well known that normal vascular endothelial cells constitute a quiescent population in adult humans, and the turnover rate of resting blood vessels is extremely low. In this study, a pan-endothelial marker (CD34) was used to highlight the blood vessels. Such a marker cannot distinguish between resting and active angiogenic vessels. Thus, we needed new endothelial cell markers that would detect only active neangiogenic vessels. The search for specific markers of angiogenic vessels has identified certain molecules, such as the \( \alpha_v \beta_3 \) and \( \alpha_v \beta_3 \) integrins, as possible candidates (57, 58).

In conclusion, our results showed that VEGF expression is involved in normal mucosa epithelium and the early stages of premalignant lesions and is down-regulated during head and neck tumorigenesis. It can be postulated, first, that VEGF may have a physiological role in upper aerodigestive tract epithelium, and second, that VEGF may play an important role in the early stage of head and neck tumorigenesis, and other genetic factors might play a role in the later stages (37). Further studies are required to better understand the mechanism of VEGF down-regulation in head and neck tumorigenesis.

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