Circulating Vascular Endothelial Growth Factor and Vascular Cell Adhesion Molecule in Laryngeal Squamous Cell Carcinomas

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Abstract: *Objective*: To assess the relationship between the immunohistochemical expression of vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule (VCAM) with the clinical feature of laryngeal squamous cell carcinoma (LSCC).

Study Design and Methods: Twenty-nine patients with LSCC were examined as the LSCC group. Twenty-one healthy people who showed no signs of regional or systemic disorders were used as the control group. The staging system for LSCC was determined using American Joint Committee on Cancer (AJCC) 2010 criteria. A quantitative enzyme-linked immunosorbent assay (ELISA) was performed to measure the concentration of soluble serum VEGF and VCAM-1.

Results: VEGF levels were higher in the LSCC group compared to the control group ($p = 0.001$). VCAM levels were not significantly different ($p = 0.617$). VEGF levels were not correlated to the T and N stage in the LSCC group ($p = 0.402$). VCAM levels were significantly correlated with the T and N stage ($p < 0.0001$).

Conclusions: VCAM levels could not be used as a tumor marker for diagnosis but were correlated with the stage and, therefore, the aggressivity of the cancer disease.

Keywords: Larynx, squamous cell carcinomas, vascular endothelial growth factor, vascular cell adhesion molecule, tumor marker, stages, lymph node metastasis, aggressivity.

INTRODUCTION

The latest treatment modalities have facilitated the early detection of laryngeal carcinomas, changing the treatment options that provided an important contribution for the improvement of the patient's quality of life. Cancer cells secrete many biochemically detectable molecules. There are many molecules that have been studied for the use of early detection of laryngeal carcinomas. However, no tumor marker for laryngeal carcinomas, highly specific as well as sensitive, has been found until now.

Two important mechanisms controlling the tumor progression and metastasis in tumor biology are angiogenesis and adhesion processes. Angiogenesis is needed for the growth of both primary and metastatic tumors demanding blood supply to grow over the size of 1 or 2 mm [1-3]. The major positively acting angiogenesis molecule is vascular endothelial growth factor (VEGF) [3-6]. There are four different VEGF forms, but the most commonly used forms are 121 and 165 aminoacids. These forms differ by the heparin binding's capability. VEGF is also found in normal and embryogenic tissues such as lungs, kidneys, heart, and adrenal glands [4]. Some other factors, such as interleukin 1, interleukin 6 and interleukin 8, are secreted from the vessels with VEGF and inducing angiogenesis. In previous studies, VEGF and platelet derived growth factor (PDGF) have the major roles in angiogenesis [7]. In recent years, angiogenesis has been thought to occur from the disbalance between the activating and inhibating factors of angiogenesis [3].

Overexpression of VEGF has been found in many cancer types. In some types, like head and neck carcinomas, it is regarded as a bad prognostic factor [1, 8, 9]. Recently, new treatment options against angiogenesis have been introduced for cancer therapy; therefore; the determination of angiogenesis profiles in laryngeal carcinomas has become increasingly more important. Angiogenesis factor profiles and the relationships of these profiles with the clinicopathological parameters have not been clear until now. Microvessel densities and angiogenesis profiles must be clearly established for anti-angiogenesis therapy for carcinomas [3].

Another important mechanism in cancer biology is the adhesive relationship between extracellular matrix and the cells. There are three different adhesive molecule groups for this mechanism. The first group is called the immunglobulin upper family, which consists of the vascular cell adhesion molecule (VCAM), the

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intercellular adhesion molecule (ICAM), and the neural cell adhesion molecule (N-CAM). These molecules are found on the surfaces of different cell types and act for lymphositic and leucocytic adhesion. The second group is the integrin molecules and the third group is the selectin family. All of these molecules are thought to take part in tumor invasiveness and tumor aggressivity, but the mechanism of this function is still unclear [10-12].

Some studies show that adhesion molecule levels, especially the VCAM levels in patients with cancer, is higher in circulation than in the normal population, but the cellular source of these molecules cannot be found. All of these molecules are thought to take part in the process of metastasis, but the mechanism is unknown. The possible roles of soluble adhesion molecules in the prognosis of head and neck carcinoma deserve further elucidation and evaluation, but some studies show that the circulating adhesion molecules such as VCAM is higher in patients with cancer than in the normal population [10-12].

In this study, the relationship between VEGF and VCAM with the clinical feature of laryngeal squamous carcinoma was evaluated with immunohistochemical examination to conclude whether VCAM and VEGF molecules can be used as a tumor marker.

MATERIAL AND METHODS

Patient Population

Twenty-nine patients with laryngeal squamous cell carcinomas were examined in the laryngeal squamous cell carcinoma (LSCC) group (27 males and 2 females). The median age was 58 years old, the youngest patient being 30 years old and the oldest patient being 75 years old. Twenty-one healthy people (18 males and 3 females) showing no signs of regional or systemic disorders were used as the control group. Primary localization of the tumor in all patients was the larynx. The stage of the disease was determined using AJCC 2010 criteria. The staging system for laryngeal cancer is clinical and based on the best possible estimate of the extent of the disease before treatment. The assessment of the primary tumor was based on inspection and palpation, when possible, and by both indirect mirror examination and indirect endoscobic examination, when necessary. Then, if a tumor mass or an irregular mucosal appearance was exposed, the biopsy was carried out with direct laryngoscopy using the microlaryngeal surgery procedure. It was confirmed that the tumor histologically and any other pathological data obtained on the biopsy were included. In all patients, the pathological diagnosis was squamous cell carcinoma. N stage was noted after a histopathologic examination of neck dissection. Blood samples were obtained *via* intravenous cathetors and put into an EDTA tube and transferred to Immunology Laboratory.

Serum VEGF-VCAM Immunoassay

A quantitative enzyme-linked immunosorbent assay (ELISA) was performed to duplicate each sample to measure the concentration of soluble serum VEGF and soluble serum VCAM-1. A commercially available ELISA kit was used (Cytelisa Human VEGF Immunoassay). Serum samples from all patients were incubated for 30 minutes at room temperature and centrifuged for 10 minutes at 5000 rpm speed. The Cytelisa kit catches the free cytokine in the serum samples using the sandwich enzyme immunoassay technique. The kit includes a VEGF 165 variant. Serums were duplicated on micro-titer plates coated with a monoclonal antibody specific for VEGF. Next, any unbound substances were washed away and an enzyme-linked polyclonal antibody specific for VEGF was introduced. This incubated for 2 hours at room temperature and the plates were washed to remove unbound antibody. A substrate solution was added and color development was stopped after 25 minutes at room temperature. A microplate reader was then used to determine calorimetric densities at 490 nm for each sample. Results were calculated from a standard curve generated by a form parametric logistic curve fit and expressed in pg/mL of serum. A Biosource kit was used to determine the VCAM levels, according to the manufacturer's instructions.

Statistical Findings

The SPSS 20 software programs were used for the statistical analysis. In addition to statistical methods (mean, standard deviation, median, and correlation coefficient), the following independent sample tests were used to evaluate the study data: the Mann–Whitney U test, the Kolmogorov-Smirnov test, the Shapiro-Wilkinson test, and the Jonckheere Tersptra test. Statistical significance levels were set as $p < 0.05$ and $p < 0.01$.

RESULTS

The statistical analysis of the study included 45 males (88.9%) and 5 females (11.1%). Twenty-nine of

these patients were in the LSCC group. The mean age was 57±5,1 years. The stages of the laryngeal squamous cell carcinomas were T0N0 in 21 patients, T1N0 in 5 patients, T2N0 in 19 patients, T3N0 in 2 patients, T3N1 in 2 patients, and T4N2 in 1 patient.

The mean serum concentration of VEGF for the control group was 19 pg/mL (11-160 pg/mL) and the interquartil range (IQR) was 10 pg/mL. The mean serum VEGF level for the LSCC group was 39 pg/mL (26-470 pg/mL) and the IQR was 73 pg/mL. The mean serum concentration of VCAM for the healthy group was 415.90 ± 101.92 ng/mL (260-550 ng/mL). The mean serum VCAM level for the patient group was 442.50 ng/mL (260-1125 ng/mL) and the IQR was 157.50. VCAM levels were not uniformly distributed in the LSCC group; VCAM levels were uniformly distributed in the control group.

When the mean VEGF levels were compared with the Mann-Whitney U test between the patient and the control group, there was a significant increase in the LSCC group ($p = 0.001$). However, when the mean VCAM levels were compared, no significant difference was observed between the groups ($p = 0.617$).

In the LSCC group, when VEGF levels were compared on the basis of the T stage of the disease, a statistically significant difference between the groups ($p=0.402$) was found, as shown in Table **1**. No difference was observed between the groups when the VEGF levels were compared on the basis of the N stage of the disease in the LSCC group ($p = 0.167$), as shown in Table **2**.

Table 1: Comparison of VEGF Levels and T Stage Cancer

T Stage	N	Mean VEGF Level (pg/ml)	p
T1	5	38.5	0.402
T2	19	70	
T3	4	39	
T4	1	37	

Table 2: Comparison of VEGF Levels and N Stage

N Stage	N	Mean VEGF level (pg/ml)	P
N0	26	40	0,167
N1	2	39	
N2	1	37	

The difference of VCAM levels between the groups based on the T stage of the disease was statistically significant in the LSCC group ($p < 0.0001$), as shown in Table **3**. There was also a statistically significant difference when VCAM levels were compared on the basis of the N stage of the disease in the LSCC group ($p < 0.0001$), as shown in Table **4**.

Table 3: Comparison of VCAM Levels and T Stage

T Stage	N	Mean VEGF level (pg/ml)	P
T1	5	345	<0.0001
T2	19	420	
T3	4	520	
T4	1	1125	

Table 4: Comparison of VCAM Levels and N Stage

T Stage	N	Mean VEGF level (pg/ml)	P
N0	26	400	<0.0001
N1	2	522,5	
N2	1	1125	

DISCUSSION

Prognosis in laryngeal carcinomas are related to the tumor size, lymph node metastasis, and the presence of capsular invasion; however, no reliable microscopic appearance or biochemical parameters is found for the purpose of evaluating the prognosis of laryngeal carcinomas. It is speculated that the treatment protocols can be more decisive and certain *via* the determination of more reliable and specific parameters for prognosis and lymph node metastasis. Newly discovered and clinically important prognostic parameters are very important for the use of different treatment modalities [13]. Recently, some improvements have been made in the level of regulatory molecules. However, there is still a debate on these molecules for the use of clinical purposes [7].

Angiogenesis, which causes the regeneration of new vessels, must begin for metastasis to occur. VEGF is needed for vasculogenesis endothelial cell proliferation and migration. VEGF is the main regulator of tumor angiogenesis. VEGF does not directly affect tumor development and transformation. Indirect proliferative effect of angiogenesis stimulates development and the spread of tumors [14]. Ninck *et al*. studied angiogenesis profiles in head and neck

carcinomas and found that therapies targeting only one factor can fail because there are many other factors that affect the angiogenesis [3]. Angiogenesis is characterised by basement membrane damage with new basement membrane formation, endothelial cell migration, and new blood vessel formation [15]. Borgstörm *et al*. studied the relationship between tumor angiogenesis and metastases in patients with invasive prostate carsinoma. They found that microvessel formation is much less in the patients without metastasis [16]. Akdeniz *et al*. found in their research about cervical metastasis and its relationship with VEGF that two of five patients had a high value of VEGF positive straining pattern [17].

Mineta *et al*. found that an elevated expression of VEGF had a negative effect on the prognosis of tongue squamous cell carcinomas [7]. Bowden *et al*. also found that an elevated expression of VEGF mRNA increases the tumor agressivity and risk of metastasis in skin squamous cell carcinomas [13]. Takeichi *et al*. found a 56% increase in VEGF expression at laryngeal carcinoma with 60 patients having squamous cell carcinoma of the head and neck region [18]. In other research on VEGF's role on metastasis, a correlation between lymph node metastasis and VEGF at primer laryngeal carsinoma was found [19].

There are some studies concerning the levels of VEGF in the circulation of different cancer types. Teknos *et al*. studied serum VEGF levels in patients with high-stage laryngeal carcinoma and showed that serum VEGF levels can be associated with a prognostic value because high levels of VEGF correlate with the low-survival rate. However, they postulated that the use of chemotherapy decreases tumoral microvessel density and VEGF levels also decrease in the circulation [2]. They stated that VEGF levels can be a predictive factor.

Studies that were conducted to show the relationship between the VEGF levels, tumor agressivity, and metastatic capability in premalign lesions and displasias all concluded that VEGF levels cannot predict the tumoral agressivity for premalign lesions [1]. Akdeniz *et al*. found a VEGF expression of 52.63% in well-differantiated tumors and 60% in poor-differantiated tumors. In this research, they evaluated the relationship between VEGF expression and tumoral stages. In late-stage tumors (stages III and IV), the ratio of positive VEGF expression was 66.67%; but in early-stage tumors (stages I and II), it was 43.75%. The VEGF value was found to be positively correlated with the stage of tumor, but it was not statistically significant [17].

In this study, it was found that VEGF levels in the LSCC group was significantly higher than in the control group ($p = 0.001$); however, when comparing the VEGF levels, a statistically significant difference between the stages was not found. Based on these findings, determination of serum VEGF levels can provide sufficient information for the early diagnosis of the disease, but the assignment of prognosis and tumor aggressivity with this molecule, unlike other all studies, does not seem to be possible. With these findings, it can be concluded that VEGF cannot be used as a prognostic factor, but further studies are needed to confirm the results.

In tumor biology, the importance of the adhesion process is understood as a result of studies recently conducted [12]. The existence of adhesion molecules in circulation has been introduced in several studies conducted on head and neck carcinomas [11-13]. One of the most important adhesion molecules is VCAM. VCAM in serum causes tumor cell adhesion to the vascular endothelium. Although a positive correlation of the elevated VCAM concentration with the late stages and metastatic cases has been found in the research, its effect on patient survey is unclear [20].

Liu *et al*. published one of the earliest studies on adhesion molecules in circulation and stated that transformation of the tumor from the benign form to the malign form, tumoral cell invasion, extracellular matrix degradation, and tumoral cell adhesion processes should be functional. They also showed that VCAM levels of patients with laryngeal carcinoma in circulation is higher than in the control group, but the difference of VCAM levels between disease stages is statistically insignificant [12]. In another study on adhesion molecules, Banks *et al*. postulated that adhesion molecules in circulation facilitates the metastatic process of the neoplasms [11]. Kuzu *et al*. showed that VCAM only appears in tumoral tissues, not in normal tissues [10].

Kawano *et al*. found in their research that the cancers of the head and neck region showed that pretreatment serum VCAM and ICAM levels are high according to the healthy volunteers in the control group. They could not find any significant correlation between serum VCAM and ICAM values and TNM staging, distant metastasis, and lymph node status. They found that the average serum VCAM and ICAM

values after treatment were less than the pretreatment values, but were not statistically significant ($p > .05$) [20].

In this study, the results showed that the difference of VCAM levels between the LSCC group and the control groups were statistically insignificant ($p = 0.617$). With this result, it is controversial to use the VCAM molecule as a tumor marker, but to make distinction with other positive studies, it is obligatory to wait for future studies. In this study, it was interesting to find that VCAM levels between disease stages were statistically significant ($p < 0.0001$). VCAM levels were also significant between the stages on the basis of lymph node metastasis.

CONCLUSIONS

In this study, in accordance with other studies reviewed; it was found that the VEGF's value in patients with laryngeal cancer was statistically higher than the values in control group, but statistically significant differences with the stage of the tumor and lymph node metastases were not found. The studies that will be made with a larger series of samples in the future will be able to show that the relationship between TNM staging and metastasis has statistically significant differences with the level of VCAM. In previous studies; the serum concentration of VCAM in the head and neck region cancers was thought to be useful as a screening test instead of a prognostic factor. Although no statistically significant differences between the head and neck cancer and the control group could be found, a proportional increase in VCAM levels in patients with cancer at higher stages and lymph node metastasis, showed that VCAM levels were not specific and sensitive as a tumor marker, but it is a sufficient factor to determine the prognosis and tumor aggressivity in patients with cancer.

SUMMARY

The relationship between vascular endothelial growth factor (VEGF) and vascular cell adhesion molecule (VCAM) with the clinical feature of larengeal squamous cell carcinoma (LSCC) was evaluated with immunohistochemical examination to conclude whether these molecules can be used as a tumor marker. Although no statistically significant differences between the LSCC group and the control group were found, a proportional increase in VCAM levels in patients with cancer at higher stages and lymph node metastasis showed that VCAM levels were not specific and sensitive as a tumor marker However, it is a sufficient factor to determine the stages of cancer, the lymph node metastasis, and the tumor aggressivity in patients with cancer.

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