ORIGINAL ARTICLE UDC: 616.311.4+616.313]:616-006.6-036

Clinical significance of vascular endothelial growth factor expression in patients with carcinoma of the mouth floor and tongue

Klinički značaj ekspresije vaskularnog endotelnog faktora rasta kod bolesnika sa karcinomom poda usne duplje i jezika

Miroslav Brećić*, Ružica Kozomara*, Snežana Cerović†, Nebojša Jović*, Slobodanka Vukelić-Marković*, Srboljub Stošić*

Military Medical Academy, *Clinic for Maxillofacial, Oral Surgery and Implantology, †Institute of Pathology, **Belgrade, Serbia**

Abstract

Background/Aim. Although there are several types of malignant oral cancers, more than 90% of all diagnosed oral cancers are squamous cell carcinoma (OSCC). Angiogenesis is a cascade-like mechanism which is essential for tumor growth and metastasis. Therefore, vascular endothelial growth factor (VEGF) expression in OSCC and its effect on clinicopathological characteristics and prognosis is of major interest. So far researches have shown that increased expression of this gene, in other words enhanced sinthesis of this protein (VEGF), independently on other factors, increases a chance for local relapse, and distant metastasis. Consequently, patients with OSCC have poor disease-free survival, as well as poor overall survival. The aim of the study was to determine clinical significance of VEGF expression in patients with stage II and III OSCC. **Methods.** This retrospective study analysed 40 patients who had been operated for OSCC of their tongue and the mouth floor. Of these patients, some had stage II and III OSCC with histological grade, G1-G3 and nuclear grade Ng1-Ng3. Two high quality tissue samples were obtained and immunohistochemical expression of VEGF was quantitatively determined by using high microscope amplification. The value of VEGF expression of 20% was rated as significant expression, whereas tumor cells reactivation less than 20% was considered very low or no expression at all. The patients were followed up for a 3-year period. **Results.** The obtained results showed that 11 (17.5%) patients had VEGF expression less than 20% and 29 (82.5%) above 20%. A statistical significance was immanent with positive nodal status ($p < 0.05$) and disease stage ($p < 0.05$). No statistical correlation was found between the level of VEGF expression and histological and nuclear grade, tumor size, disease relapse or patients overall survival. **Conclusion.** Inspite the controversy about the prognostic relevance of VEGF our results as well as the results of previous studies, suggest that the expression of VEGF is not reliable as a clinical parameter for the prognosis and disease outcome but it is one of the important factors for the disease progression.

Key words:
vascular endothelial growth factors; carcinoma, squamous cell; disease progression; prognosis.

Apstrakt

Uvod/Cilj. Iako postoji nekoliko vrsta malignih oralnih tumora, više od 90% svih dijagnostikovanih oralnih karcinoma čini karcinom skvamoznih ćelija (*oral squamous cell carcinoma* – OSCC). Angiogenezu predstavlja kaskadni mehanizam koji je važan za razvoj tumora i metastaza. Stoga, ekspresija vaskularnog endotelnog faktora rasta (*vascular endothelial growth factor* – VEGF) kod OSCC i njegov uticaj na kliničkopatološke karakteristike i prognozu imaju poseban značaj. Dosadašnja istraživanja pokazala su da povećana ekspresija ovog gena, tj. povećana sinteza ovog proteina (VEGF) uvećava šanse za lokalnim relapsom i udaljenim metastazama nezavisno od ostalih faktora. Sledstveno tome, bolesnici sa OSCC imaju lošu prognozu preživljavanja. Cilj studije bio je da utvrdi klinički značaj ekspresije VEGF kod bolesnika sa OSCC stadijuma bolesti II i III. **Metode.** Ovom retrospektivnom studijom analizirano je 40 bolesnika operisanih zbog OSCC jezika i poda usne duplje. Neki od njih bili su stadijuma bolesti II i III OSCC histološkog stepena G1-G3 i nuklearnog Ng1-Ng3. Dobijena su dva visokokvalitetna uzorka tkiva, te je kvantitativno utvrđena imunohistohemijska ekspresija VEGF uz primenu uvećanja mikroskopom. Vrednost ekspresije VEGF od 20% rangirana je kao značajna, dok je reaktiviranje ćelija tumora ispod 20% smatrano veoma niskim ili se uopšte nije smatralo ekspresijom. Bolesnici su praćeni tri godine. **Rezultati.** Analizom parametara utvrđeno je da je 11 (17,5%) bolesnika imalo ekspresiju VEGF ispod 20%, dok je 29 (82,5%) imalo iznad 20%. Statistička značajnost postojala je kod pozitivnog nodusnog statusa ($p < 0,05$) i stadijuma bolesti bolesti ($p < 0,05$). Nije nađena statistički značajna korelacija između stepena ekspresije VEGF i histološkog i nuklearnog gradusa, veličine tumora, relapsa bolesti, niti ukupnog preživljavanja bolesnika. **Zaključak.** Iako je prognostički značaj VEGF kontroverzan, rezultati ove studije, kao i prethodnih ukazuju da ekspresija VEGF nije klinički pouzdan parametar prognoze i ishoda bolesti, ali jeste važan faktor progresije bolesti.

Ključne reči:
vaskularni endotelni faktori rasta; karcinom, skvamocelularni; usta, neoplazme; bolest, progresija; prognoza.

Correspondence to: Miroslav Brećić, Military Medical Academy, Clinic for Maxillofacial, Oral Surgery and Implantology, Crnotravska 17, 11 000 Belgrade, Serbia. Phone: +381 63 817 3785. E-mail: broci@bitsyu.net

Introduction

The etiology of oral squamous cell carcinoma (OSCC) is not well known. Major risk factors for OSCC are smoking, alcohol use, poor oral hygiene, oral human papillomavirus (HPV), AIDS and any other chronic irritation, such as chronic infections, dental caries, etc [1–4]. The hallmark of squamous cell carcinoma is its fast and infiltrative growth, invasion and destruction of adjacent structure, and very high potential for metastatic lymphatic and haematogenous spread.

There is a widespread opinion that malignancy development is multifactorial in which environmental and genetic cofactors influence on molecular and genetic alterations of the cell [5–7].

Despite advancement in diagnostics, surgical technique and postsurgical oncologic protocols, it is shown in the past thirthy years that a 5-year survival rate in patients with OSCC is approximately 50% [2]. Therefore, ongoing efforts to answer the questions regarding biological effect of this malignant tumor still persist.

The main goals of up-to-date research are to find the algorithm that will help in understanding process of cancerogenesis and to find specific biomarkers which will facilitate accurate identification of disease progression and outcome, as well as specific agents that will help in treatment of OSCC. A number of genetic and genetically regulatory factors have been recognized showing important roles in cancerogenesis and regulated by a balance between angiogenic stimulators and inhibitors. Among them, there are multiple growth factors and their receptors [8]. It is well known that neovascularization is the main contributory factor for tumor growth.

One of the most potent proangiogenic factors is vascular endothelial growth factor (VEGF). VEGF and its receptors play important role in both pathological and normal angiogenesis.

Activation of VEGF and its receptors promote a cascade in intracellular signaling to stimulate endothelial cell mitogenesis, cell migration and differentiation, vascular permeability and enhancing mobilisation of endothelial progenitor cells from the bone marrow to peripheral circulation [9–11].

VEGF family consists of seven different types: VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E, VEGF-F placentar growth factor – 1 and – 2 (PlGF).

VEGF mediates their angiogenic effects by binding to specific VEGF receptors. VEGF receptors vary among the three primary receptors; VEGFR-1, -2, -3. All of them are type III receptor tyrosine kinases [12]. VEGF has an important role in neovascularization of oral carcinoma, and its metastasis being a potent predictor in disease progression [13–29].

Based on previous acknowledgement regarding VEGF role in molecular cancerogenesis in OSCC, we hypothesized that patients with carcinoma of the mouth floor and tongue stage II and III an increased VEGF expression could have frequent local and/or regional disease relapse and shorter overall survival as compared to patients with no evidence of VEGF expression.

The aim of this study was to assess the level of VEGF expression in tumor tissues in all patients with OSCC of the mouth floor and tongue stage II and III, to evaluate the rate of VEGF expression in correlation with local and regional disease relapse, and incidence of distant metastasis, as well as a 3-year survival rate, and to investigate a relationship between the level of VEGF expression with histological type and tumor grading.

Methods

In the retrospective study we analysed 40 patients operated on in a period between July 2001 and May 2003 at the Maxillofacial Clinic in the Clinic for Oral and Maxillofacial Surgery, Military Medical Academy, Belgrade. All of those patients were operate for OSCC of their tongue and the mouth floor (stage II and III OPCC with histological grade G1-G3 and nuclear grade Ng1-Ng3).

Each of them underwent postsurgical radiation therapy according to the protocol for this type of cancer and had a regular 3-year follow-up. The patients were analysed based on their age, sex, their general well-being (Karnofsky scale), cigarette smoking, alcohol consuming, and oral hygiene status.

Clinical parameters relevant for the disease course follow-up included identification of cancer staging (TNM), tumor characteristics (size, location, histological and nuclear grading), type of surgical procedure and postoperative period. Regarding the therapy effectiveness in terms of the apperance of local recidives, regional and/or distant metastasis, as for length of disease remission and patients overall survival, we compared two categories: patients with VEGF expression of $\leq$ 20%, and another with VEGF expression > 20% in their tumor tissue.

Pathohistological parameters

All tissue samples were processed by using the macroscopic topographic method for diagnosing a type, grade and postoperative-pathologic staging based on TNM classification [30]. The tissue specimens obtained from tumor resection and from lymphatic nodes were fixed by 5% puffer formalin, inserted into paraffin blocks and sliced to 4 μm. Tissue specimens were processed in a Sakura V.I.P. machine apparatus designed for automatic fixation, dehydration, clearing and impregnation. For tissue preparation the standard hematoxylin-eosin color (H&E) was used.

Accorging to the WHO classification tumor was staged from 1–3.

Keratin type of squamous cell carcinoma was marked as G1 (Figure 1), squamous carcinoma composed of cohesive mildly polymorphic tumor cells as G2 (Figure 2) and as G3 tumor in trabecular or solid layers characterized with polymorphic nucleus and multiple mitotic figures (Figures 3 and 4).

Immunohistochemical analysis of VEGF

Epitop demasking was performed by using microwave pretreatment and immersing slides in the Target Retrieval Solution – Solution pH 9.0 (Dako S 2367). As primary anti-

gen, we used commercial mouse monoclonal antihuman antibody – VEGF (DAKO M7273), concentration VG1 with dilution of 1:25. For visualization, LSAB+(DAKO K0690) system and chromogen DAB Liquid (K3466) were used.

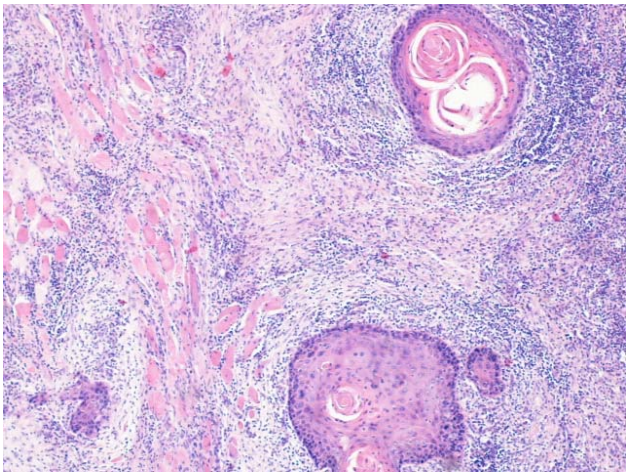

Fig. 1 – Well differented squamous cell carcinoma (H&E ×40)

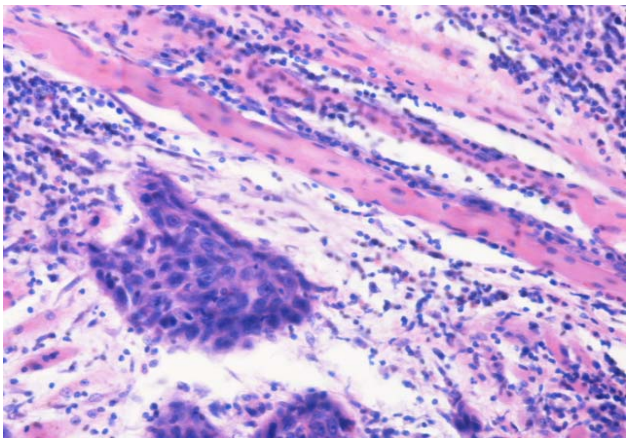

Fig. 2 – Middle differented squamous cell carcinoma (H&E ×75)

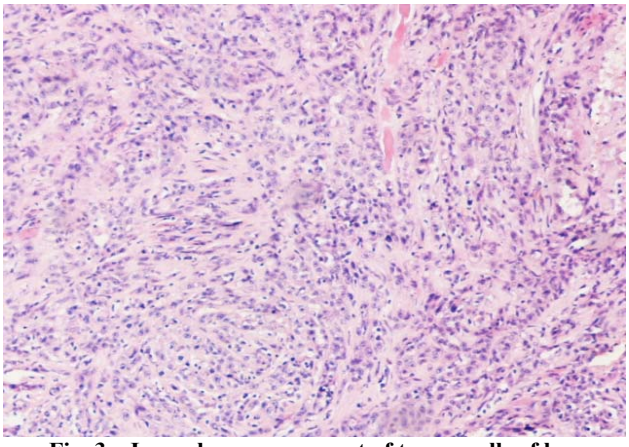

Fig. 3 – Irregular arrangement of tumor cells of low differented squamous cell carcinoma (H&E ×75)

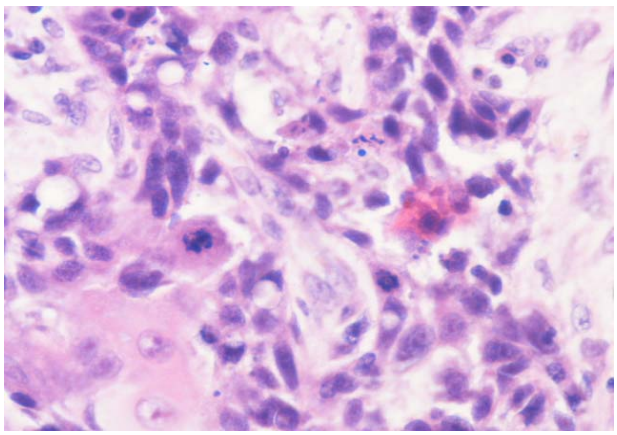

Fig. 4 – Large number of mitotic figures of low differented squamous cell carcinoma (H&E ×100)

Immunohistochemical (IMH) reaction of VEGF was performed on two tumor tissue specimens, one of them obtained from the central area without necrosis and the other one from the periphery, close to the nontumor edge.

Under a high resolution microscope (×100), marginal value of 20% immunoreactive cells (total of 1 000 analized cancerous cells) was identified. This model was charaterized as a significant level of exspression, while a value of 20% immunoreactive cells showed very low or no expression at all [31]. In our study we specified significant level of VEGF exspression above > 20% (VEGF > 20%) as VEGF 2, and level below 20% for VEGF < 20% as (VEGF 1).

Statistical analysis was done by using nonparametric methods. Level of VEGF exspression in relation to the known risk factors for developing oral planocellular cell carcinoma of tongue and the mouth floor and in relation to tumor parameters was analysed with χ^2 (chi-square) test with Yates' Correction Factor and Fisher's exact test. A period without disease relapse and overall survival rate was calculated by the Kaplan-Meier method. A period without disease relapse and overall survival rate between two groups was compared by the Log-Rank test.

Results

Our study included 40 patients of which 36 were males, aged between 43 and 77, mean age 60.6 years, median 55.4 years. According to sex, median age for the males was 59.6 years and for the women 54 years. The majority of those patients (80%) were long-term smokers – consumed more than 20 cigarettes per day, and 60% of them had long-term alcohol intake.

According to the TNM system we identified that 7/40 (17.5%) patients were with stage II disease and 33/40 (82.5%) with stage III. Clinicopathological parameters of the patients are shown in Table 1, size of regional metastasis in Table 2, and primary tumor location in Table 3. Dispersion of parameters specific for primary tumor with different disease stages are illustrated in Figure 5. This indicates that 18 (45%) patients had poor oral hygiene, but 22 (55%) had adequate one at the time of their first physical exam. Of all

Table 1

Primary tumor characteristics in patients with oral squamous cell carcinoma

Tumor caracteristics	Number of patients
Tumor size (T)	2
T1	28
T2	10
T3	
Tumor growth	
Infiltrative	32
Exophytic	8
Histological grade (G)	
G1	14
G2	24
G3	2
Nuclear grade (Ng)	
Ng1	18
Ng2	8
Ng3	14

Table 2

Frequency of regional/neck metastatic lymph node

Regional neck metastatic lymph node	Number of patients
N0	10
N+	30
Total	40

Table 3

Pimary tumor localisation

Primary tumor of tongue and mouth floor localisation	Number of patients
Lateral edge of tongue – middle third	25
Lateral edge of tongue – back third	9
Lateral edge of tongue – front third and mouth floor	4
Mouth floor	2
Total	40

above, 50% patients (20/40) had disease relapse, local recurrence in 55% (11/20), whereas local and regional relapse observed in 45% patients (9/20). Twenty-three tumors (41%) were positive for VEGF expression.

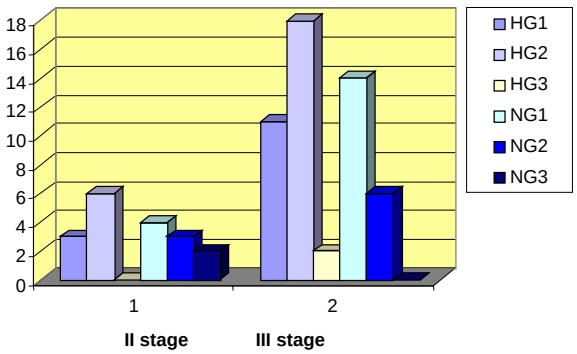

Fig. 5 – Frequency of histological (H) and nuclear (N) grade of II and III disease (oral squamous cell carcinoma) stage

Regarding location for primary tumor relapse, 45% of the patients had relapse at the edge of the anterior two-thirds of tongue, 36% on posterior one third, and 18% had primary cancer at the floor of the mouth. The appearance of local and regional relapse was noticed in 45% surgically treated patients, mainly where primary cancer involved edge of the posterior third tongue (55%). We did not find a significant statistical correlation between location of disease relapse and site of primary resected tumor (χ^2 test, $p > 0.05$), nor a connection between frequency of relapse and onset of occurrence time interval (χ^2 test, $p > 0.05$). In our specific group of patients a 3-year-disease-free survival were identified in 42% (17/40) patients, while 82% (14/17) stage III and 18% (3/17) patients at stage II died in a 3-year follow-up period.

By analyzing tumor specimens we obtained that 11 of 40 tumors (28%) were positive for VEGF 1 expression (Figures 6 and 7) and 29/40 (72%) positive for VEGF 2 (Figures 8 and 9).

Statistical data analysis did not releave any significant correlation between patients age and VEGF expression (χ^2 test, $p > 0.05$). Although VEGF 2 expression was found in 22 nonsmokers and 7 smokers no statitical connection was estimated in terms of this risk factor and VEGF expression (Fisher test, $p > 0.05$).

In addition, alcohol consumption, smoking or poor oral hygiene did not demonstrate any close correlation with VEGF expression (Fisher test, $p > 0.05$).

Further statistical analysis did not show VEGF expression of any significance in relation to tumor size (χ^2 test, $p > 0.05$) or histological parameters, or nuclear gradus and VEGF expression (Fisher test, $p > 0.05$).

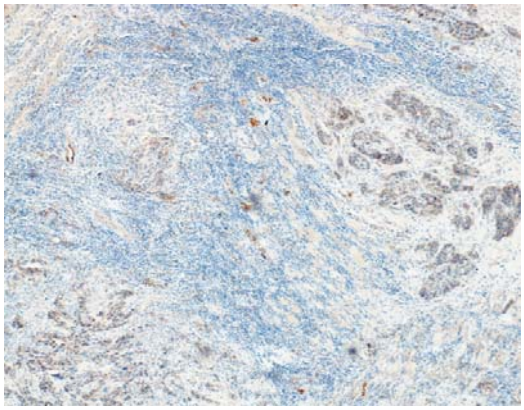

Fig. 6 – Low immunohistochemical expression of vascular endothelial growth factor in squamous cell carcinoma (LCA ×40)

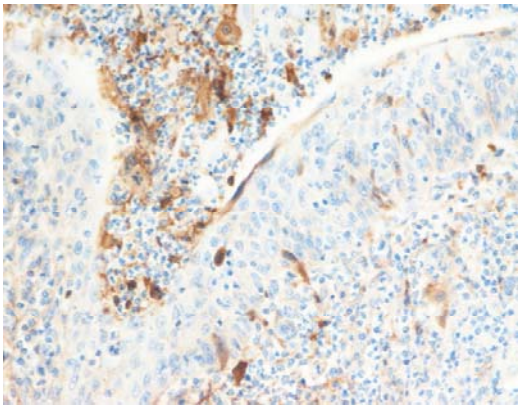

Fig. 7 – Low immunohistochemical expression of vascular endothelial growth factor (VEGF) in squamous cell carcinoma (LCA ×70)

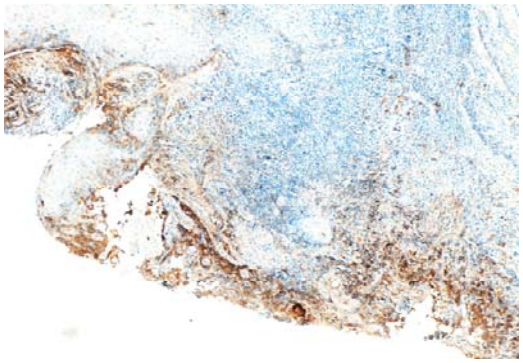

Fig. 8 – Significant immunohistochemical expression of vascular endothelial growth factor (VEGF) in squamous cell carcinoma (LCA ×40)

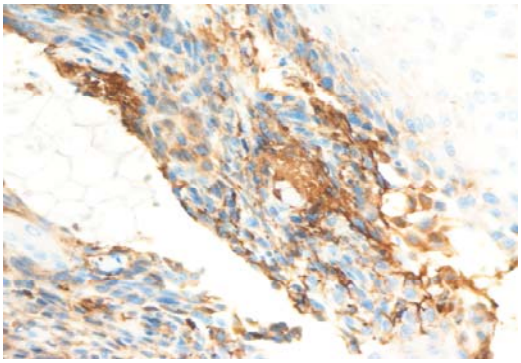

Fig. 9 – Significance immunohistochemical expression of vascular endothelial growth factor (VEGF) in squamous cell carcinoma (LCA ×100)

This study showed that the percentage of patients with lymphadenopathy on initial physical exam was 75%. Analysis concluded that there was a significantly high difference between VEGF 2 expression in the patients with metastatic neck lymph node involment (in 26 of 40 patients or 65%), as compared to the patients without neck involvement (26 *vs* 3, Fisher test, $p < 0.05$) (Table 4).

Our results also demonstrate important statistical difference in VEGF 2 expression between the disease stage II and III. Of 40 patients, 8 (20%) were at the stage II, while 32 (80%) were at the stage III. Our study revealed that 27 patients with stage III had VEGF expression > 20% (VEGF 2and only two with stage II (27 *vs* 2, Fisher test, $p < 0.05$) (Table 5).

In the study and during the regular follow-up we noticed that 50% of the patients with OSCC had disease relapse. Comparing two groups of patients, one with relapse and other without, there was no statistical significance in frequency of VEGF expression (Fisher test, $p > 0.05$).

Following patient's disease-free interval (DFI) we have not observed dissimilarity among the subjects with type VEGF 1 expression and the group of patients with VEGF expression above 20% (VEGF 2) (Log Rank test, $p > 0.05$). This analysis was complemented by Hazard Ratio (HR) test for DFI with 95% CI.

Table 4

Frequency of vascular endothelial growth factor (VEGF) expression level of meatstatic lymph node

Nodal status	VEGF1	VEGF2	Number of patients
N+	7	3	10
N-	4	26*	30
Total	11	29	40

*$p < 0.05$

Table 5

Correlation of disease stage and frequency of vascular endothelial growth factor (VEGF) expression level

Disease stage	VEGF1	VEGF2	Number of patients
II	6	2	8
III	5	27*	32
Total	11	29	40

*$p < 0.05$

The results obtained in this study showed that patients with VEGF 1 expression had 30% less risk of having disease reactivation (Figure 10).

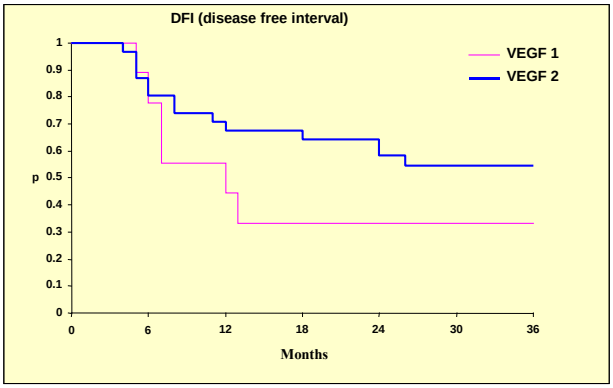

Fig. 10 – Disease free interval in patients with vascular endothelial growth factor 1 (VEGF 1) (n=11) and vascular endothelial growth factor 2 (VEGF 2) (n=29) Log Rank test ($p > 0.05$)

In this study, no statistical correlation between VEGF expression and the length of overall survival was observed in patients with OSCC (Log Rank test, $p > 0.05$). This test was supplemented, as well, by HR analysis which concluded that patients with VEGF 1 expression had more than double lenght of overall survival (Figure 11).

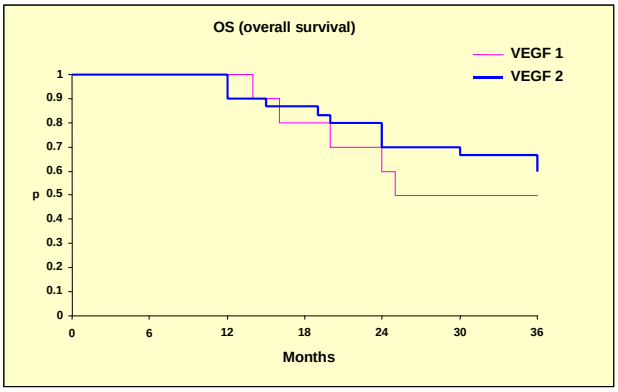

Fig. 11 – Overall survival in patients with vascular endothelial growth factor 1 (VEGF 1= (n = 11) and vascular endothelial factor 2 (VEGF 2) (n = 29), Log Rank test, ($p > 0.05$)

Discussion

Oral squamous cell carcinoma is the most common malignancy of the upper aerodigestive tract. The most commonly affected organs are the mouth floor and the tongue rendering about 90% of all malignant tumors in this regional distribution. It is estimated that around 350 000 people worldwide are newly diagnosed with OSCC each year.

Oral squamous cell cancer consistently ranks as one of the top eleven cancers worldwide [31–33].

So far, TNM clinical classification and pathohistological findings were essential parameters of the disease prognosis and outcome; however, there is a limit in understanding the evolution of this malignancy.

Application of molecular biology and genetics has significantly advanced the field of human cancer research in OSCC.

This explosion in technology has fueled the current expansion of knowledge into the working of the human cell. Clinical data so far have demonstrated that genetic and molecular cells alteration has been caused by multiple factors [2].

A combination of surgery radiation therapy and/or chemotherapy are the mainstay of OSCC therapy.

Despite advancement in diagnostics, surgical technique and postsurgical oncologic protocols a 5-year survival rate in patients with OSCC is approximately 50%, in the past thirthy years [2]. Thus, ongoing efforts to answer the questions regarding biological effect of this malignant tumor have been subject to numerous studies.

The main goals of many studies last years have been to understand the process of malignant transformation and to establish an algorithm that explains the transformation of normal cell to cancer one under specific conditions. A number of genetic and genetically regulated factors have been recognized showing important role in cancerogenesis and regulated by a balance between angiogenic stimulators and inhibitors, such are multiple growth factors and their receptors.

One of the most potent proangiogenic factor is vascular VEGF. Among the factors causing tumor angiogenesis, VEGF is considered crucial for both the growth of the primary tumor and the development of metastases.

For the past three decades VEGF has been investigated and research has shown its crucial contribution to carcinogenesis. Vasculogenesis is the key factor for almost all malignancy development, so as for OSCC. Multiple studies have investigated a correlation between VEGF and its receptors with OSCC development. The aim of numerous studies was to point out the association of VEGF and OSCC, including tumor incidence, growth, progression, metastatic potential, invasion, recurrence and overall patient's survival.

VEGF has been tested as an isolated factor or in combination with others such as growth factors, multiple gene alterations, as well as molecular changes in patients who were diagnosed with oropharyngeal squamous cell carcinoma. There has been tendency to find highly specific biomolecular markers as valuable prognostic relevance of this malignancy.

The aim of that study was also to review the current knowledge on the prognostic value of tumor marker in the treatment of OSCC. The literature of the past 5 years (1997–August 2002) was screened. One hundred and sixty-nine articles were included in this review, that analyzed markers of relevance for OSCC.

Twenty-nine molecular markers of relevance were identified. Tumor markers were allocated to four groups according to their function: the first group included markers responsible for enhancement of tumor growth, in other words cell cycle acceleration and proliferation; the second group – tumor suppression and anti-tumor defence with the role in immune response and apoptosis; the third one were factors that influence angiogenesis, and the forth group constituted

markers for tumor invasion and metastatic potential such as adhesion molecules and matrix degradation.

The data showed that the prognostic relevance of most tumor markers still remains not quite clear. Only 12 of 23 reports on the prognostic relevance of markers from the first group indicated a significant association with prognosis while 20 of 29 studies on markers from the second group showed a prognostic relevance. Based on the majority of the previous studies, markers of angiogenesis exhibited only minor importance for the prognosis and treatment of OSCC. The results on markers of tumor invasion and metastatic potential appeared not to be enough valuable indices for a statement regarding their prognostic value. This analysis stipulated that the role of isolated molecular markers within the tumor was not significant for prognosis of OSCC. On the other hand, molecular markers together with histopatological results are of great importance for prognosis and follow-up of patients outcome.

So far, no specific marker has been discovered any as an important prognostic indicator of squamous cell carcinoma or for follow disease outcome in patients with OSCC.

Therefore, our study as many previous ones used standardized, well known parameters and criteria that are important in application for diagnosis, prognosis and disease progression in the head and neck planocellular carcinoma. We observe the same with the level of expression by using only one biomolecular marker that is VEGF responsible for angiogenesis.

There was no statistical significance between VEGF expression and patient's age (χ^2 test, $p > 0.05$), cigarette smoking, alchohol consuming, and poor oral hygiene (Fisher test, $p > 0.05$).

Tumor size is one of the crucial parameters defying disease progression, outcome and treatment modalities in patients with OSCC, but tumor dimension is not always a valid index of behaviour of planocellular carcinoma. Tumor biology is very complex process and tumor size is compared with other factors that have significant role in OSCC pathogenesis. In our study we compared tumor size with VEGF expression. The results obtained from this analysis did not showed significant correlation between of VEGF expression level and tumor size (χ^2 test, $p > 0.05$). The same results were releaved by Shintani et al. [13] in their study from 2004. On the contrary, Shang et al. [34], in their study from 2006, describing negative correlation between tumor size (T stage) and VEGF expression showed a significant relationship between VEGF expression and size of the tumor.

A degree of differentiation of planocellular carcinoma is the most important parameter for clinical course of disease, and treatment modalities of OSCC depend on histological and nuclear grading.

Significance, VEGF expression in relation to the stated histological parameters is controversial. A study done by Kyzasa et al. in 2005 did not show a significant statistical relevance between VEGF expression and histologic grade. Opposed to that, Johnstone and Logan in 2007 signified a correlation between the degree of tumor differentiation and VEGF expression.

In our study, 20 patients with histological grade G2 showed high level of VEGF expression (VEGF 2), 7 patients with histological grade G1 had level VEGF 1 and VEGF2 expression. Also, the group with histological grade G3 releaved VEGF 2 expression or rather > 20%.

Nuclear grade is the parameter which we have also analyzed in comparison with VEGF expression. In our study, there were 16 patients with Ng1, level VEGF 2 expression; 4 patients with Ng2 and 9 with Ng3 also showed VEGF2. Statistical analysis observing histological and nuclear grading did not provide any connection with VEGF expression (Fisher test, $p > 0.05$).

The presence of enlarged regional lymph nodes (LN), observed on the first physical exam confirmed and metastasis in the same region were reliable signs of disease advancement, and very important factor that treatment success depends on. A previous opinion was that metastatic lymphatic spread was a result of tumor cells penetration into nearest lymphatic nodes. Recent studies show that this process is in relationship with lymphangiogenesis, which promotes metastatic spread. It has been confirmed that lymphangiogenesis persist in the nearest tumor location or in tumor itself and influence metastatic lymphatic node spread. Therefore, lymphangiogenesis has been the subject of numerous studies in the past several years and VEGF is characterized as a key factor in that process.

VEGF-C is the cardinal member of the VEGF family and plays an important role in the complexity of lymphangiogenesis [17, 18]. The studies done by Shintani et al. [13], Sauter et al. [16], Siriwardena et al. [17], O-charoenrat [18] have shown significant association between lymph nodes metastasis and VEGF expression from a certain members of VEGF family.

As distinct from above studies, the results obtained by Kyzas et al. [29], Tsea et al. [35] did not releave statistically significant connection between lymph nodes metastasis and VEGF expression. Our study showed that 75% patients had enlarged LN on initial physical examination.

Our analysis shows a significant statistical difference between two groups of patients in terms of the level of VEGF 2 expression. The first group with the lymph nodes metastasis, 26 of 40 (65%), showed VEGF 2 expression as compared with group of patients without neck LN metastasis (26 *vs* 3, Fisher test, $p < 0.05$).

Disease stage is an important parameter for disease follow-up and prognosis and TNM classification is used as a basic tool [13]. The TNM staging, as well as other factors that represent disease progression in patients with OSCC were compared in regards to the level of VEGF expression. Numerous studies indicate a significant connection between disease stage and excessive expression of VEGF [28, 36].

Our results also demonstrate an important statistical difference in VEGF 2 expression between disease stage II and III. Of 40 patients, 8 (20%) were at stage II, while 32/40 (80%) at stage III. Our study revealed that 27 patients with stage III had VEGF expression > 20% (VEGF 2), whereas only two patients with stage II (27 *vs* 2, Fisher test, $p < 0.05$) (Table 5).

During the regular patients follow-ups we noticed that 50% of the patients with OSCC had disease relapse. Comparing two groups, one with relapse and the other without there was no statistical significance in frequency of VEGF expression (Fisher test, $p > 0.05$).

Some studies indicate that excessive VEGF expression is in correlation with the lenght of disease-free-interval [37–41]. In our study, we did not observe dissimilarity among the subjects with type VEGF 1 expression and the group of patients with VEGF expression above 20% (VEGF 2) (Log Rank test, $p > 0.05$). This analysis was complemented by Hazard Ratio test for DFI with 95% CI.

The results obtained in this study show that the patients with VEGF 1 expression have 30% less risk of having the disease reactivation (Figure 9).

Overall survival (OS) in OSCC patients lies between 50–55% [5]. There are multiple factors that affect overall survival in patients with OSCC and one of them is excessive VEGF expression, according to numerous studies [29, 35, 37, 38, 42]. Our study showed no statistical correlation between VEGF expression and length of overall survival in the patients with OSCC (Log Rank test, $p > 0.05$). This test was supplemented by HR analysis which concluded that the patients with VEGF 1 expression have more than a double lenght of overall survival.

Comparing the results of our study with the results of other ones we observed certain differences of importance in VEGF expression related to tumor size, incidence of lymh nodes metastasis, disease stage, disease free interval and overall survival.

In our study we found a significant correlation with high level of VEGF expression (VEGF 2) disease stage and the presence of lymph nodes metastasis. Other studies [13, 17, 18, 28, 34] showed the same connection between the increased VEGF expression and metastatic LN in patients suffering from OSCC. Our study as well as other studies [28, 35, 36] confirmed that the disease stage is in correlation to VEGF expression. The rest of the clinical and pathohistologic parameters did not have statistical significance in linking it with level of VEGF expression. Although we contemplated a correlation of enhanced VEGF expression with DFI [19, 28, 29, 34, 35, 36] and OS, our results were negative, which was contradictory to some other studies. One of the reasons for negative results could be explained by a small number of patients analysed.

Angiogenesis is an essential requirement for tumor growth, invasion and metastasis [41]. Tumor angiogenesis is controled by angiogenic factors directly induced by tumor cell itself or indirectly induced from surrounding stromal tissue. There are several factors that have angiogenic activity, such as basic fibroblast growth factor (bFGF), platelet-derived endothelial growth factor (PDGF) and vascular endothelial growth factor (VEGF). They are highly specific for endothelial tissue and have a significant role as vascular permeability factor (VPF) [36]. It is well known for VEGF to be one of the most potent promoter of tumor angiogenesis [35]. The contribution of VEGF to the development of the head and neck squamous cell carcinomas and its role in angiogenesis was analyzed and confirmed on several occasions.

Due to controversy regarding VEGF effect, conflicting results within the literature still perzist [13].

The majority of authors emphasize VEGF role in OSCC angiogenesis, most importantly its effect in tumor agressivness, local and regional spreading, as well as overall survival. VEGF is believed to be a reliable marker for the outcome in many cancers, thus in OSCC.

Negative results obtained in certain sudies [20] confirm that there is no highly specific marker which would assure adequate prognosis and disease outcome for OSCC.

Conclussion

Inspite the controversy about the prognostic relevance of VEGF, our results as well as the results of previous studies suggest that the expression of VEGF is not reliable as a clinical parameter for the prognosis and disease outcome but it is one of the important factors for the disease progression.

Understanding the development and progression of oral cancer is critical in the quest for a successful therapeutic intervention. Therefore, further studies aimed at finding a highly specific and sensitive marker and its application in OSCC are necessary.

R E F E R E N C E S

1. *Llewellyn CD, Linklater K, Bell J, Johnson NW, Warnakulasuriya S.* An analysis of risk factors for oral cancer in young people: a case-control study. Oral Oncol 2004; 40(3): 304–13.
2. *Neville BW, Day TA.* Oral cancer and precancerous lesions. CA Cancer J Clin 2002; 52(4): 195–215.
3. *Kozomora R.* Importance of p53 antioncogene and human papilloma virus for radioresistance of oral squamous cell carcinoma of tongue and floor of the mouth [dissertation]. Belgrade: Military Medical Academy; 2003. p. 1-146. (Serbian)
4. *Ha PK, Pai SI, Westra WH, Gillison ML, Tong BC, Sidransky D,* et al. Real-time quantitative PCR demonstrates low prevalence of human papillomavirus type 16 in premalignant and malignant lesions of the oral cavity. Clin Cancer Res 2002; 8(5): 1203–9.
5. *Bishop JM.* The molecular genetics of cancer. Science 1987; 235(4786): 305–11.
6. *Cockerell GL, Cooper BJ.* Diorders of cll growth and cancer biology. In: *Slauson DO, Cooper BJ,* editors. Mechanisms of disease – a textbook of comparative general pathology. St. Lous: Mosby a Harcourt Health Sciences Company; 2002. p. 299–377.
7. *Cowell JK.* Basic principles in cancer genetics. In: *Cowell JK,* editor. Molecular Genetics of Cancer. 2nd ed. Oxford: Academic Press; 2001. p. 1–32.
8. *Schliephake H.* Prognostic relevance of molecular markers of oral cancer--a review. Int J Oral Maxillofac Surg 2003; 32(3): 233–45.
9. *Carmeliet P.* Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000; 6(4): 389–95.
10. *Carmeliet P, Jain RK.* Angiogenesis in cancer and other diseases. Nature 2000; 407(6801): 249–57.
11. *Hanahan D, Folkman J.* Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. Cell 1996; 86: 353–64.

12. *Ferrara N, Davis-Smyth T*. The biology of vascular endothelial growth factor. Endocr Rev 1997; 18(1): 4–25.
13. *Shintani S, Li C, Ishikawa T, Mihara M, Nakashiro K, Hamakawa H*. Expression of vascular endothelial growth factor A, B, C, and D in oral squamous cell carcinoma. Oral Oncol 2004; 40(1): 13–20.
14. *Bikfalvi A*. Angiogenesis: health and disease. Ann Oncol 2006; 10(17 Suppl): x65–70.
15. *Sasahira T, Kirita T, Bhawal UK, Ikeda M, Nagasawa A, Yamamoto K,* et al. The expression of receptor for advanced glycation end products is associated with angiogenesis in human oral squamous cell carcinoma. Virchows Arch 2007; 450(3): 287–95.
16. *Sauter ER, Nesbit M, Watson JC, Klein-Szanto A, Litwin S, Herlyn M*. Vascular endothelial growth factor is a marker of tumor invasion and metastasis in squamous cell carcinomas of the head and neck. Clin Cancer Res 1999; 5(4): 775–82.
17. *Siriwardena BS, Kudo Y, Ogawa I, Udagama MN, Tilakaratne WM, Takata T*. VEGF-C is associated with lymphatic status and invasion in oral cancer. J Clin Pathol 2008; 61(1): 103–8.
18. *O-charoenrat P, Rhys-Evans P, Eccles SA*. Expression of vascular endothelial growth factor family members in head and neck squamous cell carcinoma correlates with lymph node metastasis. Cancer 2001; 92(3): 556–68.
19. *Smith BD, Smith GL, Carter D, Sasaki CT, Haffty BG*. Prognostic significance of vascular endothelial growth factor protein levels in oral and oropharyngeal squamous cell carcinoma. J Clin Oncol 2000; 18(10): 2046–52.
20. *Schliephake H*. Prognostic relevance of molecular markers of oral cancer--a review. Int J Oral Maxillofac Surg 2003; 32(3): 233–45.
21. *Kishimoto K, Sasaki A, Yoshihama Y, Mese H, Tsukamoto G, Matsumura T*. Expression of vascular endothelial growth factor-C predicts regional lymph node metastasis in early oral squamous cell carcinoma. Oral Oncol 2003; 39(4): 391–6.
22. *Lalla RV, Boisoneau DS, Spiro JD, Kreutzer DL*. Expression of vascular endothelial growth factor receptors on tumor cells in head and neck squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2003; 129(8): 882–8.
23. *Mineta H, Miura K, Ogino T, Takebayashi S, Misawa K, Ueda Y*, et al. Prognostic value of vascular endothelial growth factor (VEGF) in head and neck squamous cell carcinomas. Br J Cancer 2000; 83(6): 775–81.
24. *Schimming R, Reusch P, Kuschnierz J, Schmelzeisen R*. Angiogenic factors in squamous cell carcinoma of the oral cavity: do they have prognostic relevance?. J Craniomaxillofac Surg 2004; 32(3): 176–81.
25. *Uehara M, Sano K, Ikeda H, Sekine J, Irie A, Yokota T*, et al. Expression of vascular endothelial growth factor and prognosis of oral squamous cell carcinoma. Oral Oncol 2004; 40(3): 321–5.
26. *López de Cicco R, Watson JC, Bassi DE, Litwin S, Klein-Szanto AJ*. Simultaneous expression of furin and vascular endothelial growth factor in human oral tongue squamous cell carcinoma progression. Clin Cancer Res 2004; 10(13): 4480–8.
27. *Arora S, Kaur J, Sharma C, Mathur M, Bahadur S, Shukla NK*, et al. Stromelysin 3, Ets-1, and vascular endothelial growth factor expression in oral precancerous and cancerous lesions: correlation with microvessel density, progression, and prognosis. Clin Cancer Res 2005; 11(6): 2272–84.
28. *Kyzas PA, Stefanou D, Batistatou A, Agnantis NJ*. Prognostic significance of VEGF immunohistochemical expression and tumor angiogenesis in head and neck squamous cell carcinoma. J Cancer Res Clin Oncol 2005; 131(9): 624–30.
29. *Kyzas PA, Cunha IW, Ioannidis JP*. Prognostic significance of vascular endothelial growth factor immunohistochemical expression in head and neck squamous cell carcinoma: a meta-analysis. Clin Cancer Res 2005; 11(4): 1434–40.
30. *Patel SG, Shah JP*. TNM staging of cancers of the head and neck: striving for uniformity among diversity. CA Cancer J Clin 2005; 55(4): 242–58.
31. *Kyzas PA, Stefanou D, Batistatou A, Agnantis NJ*. Potential autocrine function of vascular endothelial growth factor in head and neck cancer via vascular endothelial growth factor receptor-2. Mod Pathol 2005; 18(4): 485–94.
32. *Tang XH, Knudsen B, Bemis D, Tickoo S, Gudas LJ*. Oral cavity and esophageal carcinogenesis modeled in carcinogen-treated mice. Clin Cancer Res 2004; 10(1 Pt 1): 301–13.
33. *Ahrendt SA, Decker PA, Doffek K, Wang B, Xu L, Demeure MJ*, et al. Microsatellite instability at selected tetranucleotide repeats is associated with p53 mutations in non-small cell lung cancer. Cancer Res 2000; 60(9): 2488–91.
34. *Shang ZJ, Li ZB, Li JR*. VEGF is up-regulated by hypoxic stimulation and related to tumour angiogenesis and severity of disease in oral squamous cell carcinoma: in vitro and in vivo studies. Int J Oral Maxillofac Surg 2006; 35(6): 533–8.
35. *Tse GM, Chan AW, Yu KH, King AD, Wong KT, Chen GG,* et al. Strong immunohistochemical expression of vascular endothelial growth factor predicts overall survival in head and neck squamous cell carcinoma. Ann Surg Oncol 2007; 14(12): 3558–65.
36. *Li C, Shintani S, Terakado N, Klosek SK, Ishikawa T, Nakashiro K*, et al. Microvessel density and expression of vascular endothelial growth factor, basic fibroblast growth factor, and platelet-derived endothelial growth factor in oral squamous cell carcinomas. Int J Oral Maxillofac Surg 2005; 34(5): 559–65.
37. *Li YH, Hu CF, Shao Q, Huang MY, Hou JH, Xie D*, et al. Elevated expressions of survivin and VEGF protein are strong independent predictors of survival in advanced nasopharyngeal carcinoma. J Transl Med 2008; 6: 1.
38. *Smith BD, Smith GL, Carter D, DiGiovanna MP, Kasowitz KM, Sasaki CT,* et al. Molecular marker expression in oral and oropharyngeal squamous cell carcinoma. Arch Otolaryngol Head Neck Surg 2001; 127(7): 780–5.
39. *Matsuo K, Shintani S, Tsuji T, Nagata E, Lerman M, McBride J*, et al. p12(DOC-1), a growth suppressor, associates with DNA polymerase alpha/primase. FASEB J 2000; 14(10): 1318–24.
40. *Shintani S, Mihara M, Terakado N, Nakahara Y, Matsumura T, Kohno Y*, et al. Reduction of p12DOC-1 expression is a negative prognostic indicator in patients with surgically resected oral squamous cell carcinoma. Clin Cancer Res 2001; 7(9): 2776–82.
41. *Ng IO, Lam KY, Ng M, Regezi JA*. Expression of p21/waf1 in oral squamous cell carcinomas-correlation with p53 and mdm2 and cellular proliferation index. Oral Oncol 1999; 35(1): 63–9.
42. *Shang ZJ, Li JR*. Expression of endothelial nitric oxide synthase and vascular endothelial growth factor in oral squamous cell carcinoma: its correlation with angiogenesis and disease progression. J Oral Pathol Med 2005; 34(3): 134–9.

The paper received on October 21, 2008.