EXPRESSION OF VASCULAR ENDOTHELIAL GROWTH FACTOR IN ORAL SQUAMOUS CELL CARCINOMA: A CLINICOPATHOLOGICAL STUDY

Ahmed M. Gouda BDS, Fatma H. El-Didi PhD, Zeinab E. Darwish PhD, Ibrahim M. Zeitoun PhD

ABSTRACT

INTRODUCTION: Oral squamous cell carcinoma (OSCC) is one of the most widely occurring cancer worldwide. It represents the tenth most common cancer affecting the world population. Like all other tumors, malignant epithelial cells of the OSCC need adequate blood supply and thus tend to recruit new blood vessels by Angiogenesis (the formation of new vessels by sprouting of the pre-existing endothelium). The major regulators of blood and lymph vessel development are the members of the Vascular Endothelial Growth Factor (VEGF) family. These are multifunctional proteins mainly involved in normal and pathologic angiogenesis. Accordingly, there is an increasing interest in evaluating the diagnostic and prognostic value of VEGF family.

OBJECTIVES: To evaluate the expression of VEGF-A in OSCC and to correlate it with both histopathological grading and clinical data.

MATERIALS AND METHODS: This study includes 20 patients with OSCC. The lesions of concern were clinically examined and biopsied. The tissue biopsies, as well as five negative control specimens, were processed and paraffin sections were prepared. Hematoxylin and eosin-stained sections were examined for grading of the carcinoma. The immunohistochemical expression of VEGF was evaluated by the use of anti-VEGF-A antibody using the Strept-Avidin-Biotin method on paraffin sections. Immunohistochemical results were evaluated using an image analyzer. Results were recorded and statistically analyzed and correlated with both clinical and histological grading of the tumors.

RESULTS: The expression of VEGF was found to be significantly related to the grade of differentiation of the tumor, where the poorer the differentiation, the more the expression the antibody. On the contrary, no significant relation between VEGF expression and clinical data was found.

CONCLUSIONS: The expression of VEGF is of a great value as a means of diagnosis concerning histological grading of the tumor, but cannot be used as a sole method for evaluating the case prognosis.

KEYWORDS: OSCC, VEGF, Immunohistochemistry, angiogenesis

INTRODUCTION

Squamous cell carcinomas (SCCs) are neoplasms which originate from epithelial cells of various organs and their biologic behavior depends on different factors either external or internal. (1)

Worldwide, over 500,000 new cases of head and neck squamous cell carcinoma (HNSCC), including oral and oropharyngeal squamous cell carcinomas, are reported annually. It accounts for about 5% of all neoplasms of the body (2).

Many factors are involved in the etiopathogenesis of OSCC such as tobacco (smoked or chewed), drinking alcohol, spicy food, unhealthy diet, presence of local trauma factors, viruses such as human papilloma virus (HPV) and Epstein–Barr virus (EBV), development of premalignant lesions, inherited genetic mutations, hormonal disturbances, disturbance in immune system and chronic exposure to sunlight which has been suggested as an important element in SCC of the lower lip (3).

The tumor microenvironment has gained increasing interest in cancer research over the last decades, and it is now generally accepted that the microenvironment plays an important role in the development and progression of cancer (4).

In normal healthy condition, mammalian cells require oxygen and nutrients for their survival and are therefore located within 100 to 200 μm of blood vessels which is the diffusion limit for oxygen (5), accordingly, without blood vessels, tumors cannot grow beyond a critical size or metastasize to other organs. Similarly, without an efficient blood supply we may not be able to deliver anti-cancer drugs to all regions of a tumor in effective quantities (6).

Angiogenesis - defined as the formation of new vessels by sprouting of the pre-existing endothelium - is fundamental not only in tumor growth, but also in inflammatory reactions, immune reactions, embryonic development, reproductive cycle and wound healing. It is, also, vital in pathologic conditions such as rheumatoid arthritis, inflammatory disorders, retinopathies, obesity, asthma, diabetes, cirrhosis, multiple sclerosis, psoriasis and other autoimmune diseases (6, 7).

Physiological angiogenesis rarely occurs in adults, except during episodes of wound healing and in ovaries and endometrium during the reproductive life of females (8).

The first description of a link between human tumors and their blood supply occurred more than 100 years ago (9), but it was only in 1939 that the tumor cells themselves were hypothesized to release a blood vessel growth stimulating factor, that was later associated with rapid growth of tumors (10).

Vascular endothelial growth factors (VEGFs) comprise a family of proteins mainly involved in normal and pathologic angiogenesis. The VEGF family includes VEGF-A (known as vascular permeability factor (VPF) or VEGF), VEGF-B, VEGF-C, VEGF-D, VEGF-E, and


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placental growth factors (PGF). From those six members, VEGF-A plays essential roles in angiogenesis (11).

Many studies have been done on the expression of VEGF in OSCC cases, but few were reported on its expression concerning area percent, optical density and micro vessels count.

The aim of the present work was to evaluate VEGF-A expression in oral squamous cell carcinoma, to correlate it with the histological grading and clinical findings of oral squamous cell carcinoma.

MATERIALS AND METHODS
The study was performed in the Faculty of Dentistry, Alexandria University after gaining the approval of the Research Ethics Committee. Twenty OSCC patients collected from the Cranio-Maxillofacial and Plastic Surgery Department were included. Surgical biopsies were taken for histological and immunohistochemical examination as well as five surgical specimens were taken from free safety margins serving as negative control. All patients signed informed consents for the agreement to participate in the study. The patients who presented with signs of autoimmune diseases (e.g. Rheumatoid Arthritis, Systemic Lupus Erythematosus and Multiple Sclerosis), having a history of radiotherapy, chemotherapy or other cancers were excluded from the study.

Biopsies were taken from the tumor tissues in cancer patients and from free safety margins. The specimens were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax using the conventional procedures. Serial sections of 3-4 μm thickness were placed on glass slides and stained using Hematoxylin and Eosin (H&E). Immunohistochemical (IHC) staining using Anti-VEGFA antibody was also performed using the Labeled Strept- Avidin Biotin complex method (LSAB). Then, the sections were examined by the image analyzer computer system using the software Leica Qwin 500.

STATISTICAL ANALYSIS
The difference in mean area percent and mean optical density of VEGF in OSCC samples of different grades as well as normal control samples was estimated using ANOVA test.

The difference in mean micro vessels density in OSCC samples of different grades as well as normal control samples was estimated using ANOVA test.

A (P) value less than 0.05 was considered significant. The values were given as a mean value ± SD (standard deviation).

RESULTS
1. Clinical results
The age range of patients was from 32 to 68 years. The Mean ± SD age was found to be 58.5 ± 9.91 years. Ten patients (50%) were males and the remaining ten (50%) were females.

The most common site of OSCC was found to be the cheek mucosa, representing 45% (9 cases) followed by the lateral side of tongue representing 35% (7 cases), then the retromolar area representing 10% (2cases). Finally, both the maxillary tuberosity and the mandibular alveolar ridge were represented by only (5%) one case each.

Regarding the clinical staging of the patients, stage IV was found to be the most predominant (55%), followed by stage II and stage III (20% each) while stage I represented only 5% of the studied cases.

2. Histopathological Results
Histological examination revealed that moderately differentiated OSCC was the most predominant entity (50%), followed by well differentiated OSCC (40%) (Fig1) and only 10% of the studied cases were diagnosed as poorly differentiated OSCC (Fig 2).

Figure (1): Photomicrograph of well differentiated squamous cell carcinoma, where epithelial pearls could be seen (H&Ex100).

Figure (2): Photomicrograph of poorly differentiated squamous cell carcinoma showing normal and abnormal mitosis (H&Ex400).

3. Immunohistochemical Results
Normal control tissues (n=5) showed negative to very weak immunoreactivity for VEGF antibody in both epithelial and connective tissue cells (Fig.3), while OSCC showed immunopositivity reaction in both cytoplasm and cell membrane of malignant epithelial cells as well as connective tissue cells which presented as weak to moderate reaction in well differentiated type (Fig.4), moderate reaction in moderately differentiated type (Fig.5) and intense reaction in poorly differentiated type (Fig.6).

4. Correlation between histopathological results and immunohistochemical results
The difference in mean VEGF Area Percent (A%) and Mean micro vessel Density (MVD) between the well, moderately and poorly differentiated groups and each other using the F test (ANOVA) revealed high statistically significant difference, (p<0.01), with the highest mean of A% and the highest mean of MVD equal to 68.82 and 47.50 respectively, for poorly differentiated SCC, Tables (1, 3).
Figure (3): Photomicrograph of normal epithelium showing very weak staining reaction of VEGF antibody (x400).

Figure (4): Photomicrograph of a well differentiated squamous cell carcinoma case showing weak immunopositivity for VEGF antibody in both cytoplasm and cell membrane of malignant epithelial cells forming epithelial pearls (x100).

Figure (5): Photomicrograph of a moderately differentiated squamous cell carcinoma case showing moderate immunopositivity for VEGF antibody in both cytoplasm and cell membrane of malignant epithelial cell. (x100).

Figure (6): Photomicrograph of a poorly differentiated squamous cell carcinoma case showing intense immunopositivity for VEGF antibody in cytoplasm, cell membrane and perinuclear of malignant epithelial cells (x400).

Regarding the difference in mean VEGF Optical Density (OPD) between the different groups, results showed high statistical significance at (p ≤ 0.001) with the highest mean of OPD (068.52) for poorly differentiated SCC. This was with the exception of the comparison between poorly differentiated and moderately differentiated groups, where the results showed no statistical significance (p=0.579). Table (2)

Table (1): Relation between grades of differentiation and Area%.

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Poorly D (n = 2)</th>
<th>Moderately D (n = 10)</th>
<th>Well D (n = 8)</th>
<th>Control (n = 5)</th>
<th>F</th>
<th>P</th>
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<tbody>
<tr>
<td>Area %</td>
<td></td>
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<tr>
<td>Min. –</td>
<td>66.0 –</td>
<td>46.0 –</td>
<td>28.36 –</td>
<td>13.68 –</td>
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<tr>
<td>Max.</td>
<td>71.64</td>
<td>58.61</td>
<td>39.82 –</td>
<td>17.96 –</td>
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<tr>
<td>Mean ±SD</td>
<td>68.82 ±5.364 ±4.60</td>
<td>34.49 ±4.07</td>
<td>16.07 ±1.72</td>
<td>16.82</td>
<td>138.312</td>
<td>&lt;0.001 *</td>
</tr>
<tr>
<td>Median</td>
<td>68.82</td>
<td>55.51</td>
<td>35.72</td>
<td>13.68</td>
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<tr>
<td>Sig. bet. Grps</td>
<td>p1&lt;0.001*, p2&lt;0.001*</td>
<td>p3&lt;0.001*</td>
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</table>

F: F and p values for ANOVA test, Sig. bet. grps was done using Post Hoc Test (Tukey)
P: p value for comparing between control and each other group
p1: p value for comparing between poor and moderate
p2: p value for comparing between poor and well
p3: p value for comparing between moderate and well
* Statistically significant at p ≤ 0.05

Table (3): Relation between grade of differentiation and MVD.

<table>
<thead>
<tr>
<th>Differentiation</th>
<th>Poorly (n = 2)</th>
<th>Moderately (n = 10)</th>
<th>Well (n = 8)</th>
<th>Control (n = 5)</th>
<th>F</th>
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<td>MVD</td>
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<tr>
<td>Min. –</td>
<td>45.0 –</td>
<td>20.0 – – 23.0</td>
<td>12.0 –</td>
<td>3.0 –</td>
<td>463.794</td>
<td>&lt;0.001 *</td>
</tr>
<tr>
<td>Max.</td>
<td>50.0</td>
<td>21.60 ±14.0</td>
<td>14.0 ± 4.0</td>
<td>6.0 ± 4.0</td>
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<tr>
<td>Mean ±SD</td>
<td>47.50 ±3.54</td>
<td>1.10 ±1.51</td>
<td>1.51 ±1.14</td>
<td>4.0 ± 1.14</td>
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<tr>
<td>Median</td>
<td>47.50</td>
<td>22.0 ±13.50</td>
<td></td>
<td>4.0 ± 1.14</td>
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<tr>
<td>Sig. bet. Grps</td>
<td>p1&lt;0.001*, p2&lt;0.001*, p3&lt;0.001*</td>
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</table>

F: F and p values for ANOVA test, Sig. bet. grps was done using Post Hoc Test (Tukey)
P: p value for comparing between control and each other group
p1: p value for comparing between poor and moderate
p2: p value for comparing between poor and well
p3: p value for comparing between moderate and well
* Statistically significant at p ≤ 0.05
5. Correlation between immunohistochemical results and clinical results:

Upon comparing the mean, A%, mean OPD and mean MVD with different clinical parameters (sex, site, size, lymph node status and staging of cases) using Student test for statistical analysis, all results were found to be statistically insignificant.

Table (2): Relation between grades of differentiation and optical density.

<table>
<thead>
<tr>
<th>Grps</th>
<th>Poorly D (n = 2)</th>
<th>Moderately D (n = 10)</th>
<th>Well D (n = 8)</th>
<th>Control (n = 5)</th>
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<td>(OPD)</td>
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<tr>
<td>Min. –</td>
<td>66.82 –</td>
<td>70.22 –</td>
<td>50.82 –</td>
<td>29.20 –</td>
<td></td>
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<tr>
<td>Max. –</td>
<td>2.40</td>
<td>3.77</td>
<td>3.31</td>
<td>5.32</td>
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</tr>
<tr>
<td>Mean ±SD.</td>
<td>68.52 ± 2.40</td>
<td>65.43 ± 0.77</td>
<td>57.87 ± 3.31</td>
<td>53.73 ± 5.32</td>
<td></td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Media n</td>
<td>68.52</td>
<td>65.55</td>
<td>58.73</td>
<td>30.85</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dcontrol</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>128.431</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Sig. bet. Grps</td>
<td>p=0.579, p2=0.001*, p3&lt;0.001*</td>
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</table>

F,p: F and p values for ANOVA test, Sig. bet. Grps was done using Post Hoc Test (Tukey)
p1: p value for comparing between poor and moderate
p2: p value for comparing between moderate and well
p3: p value for comparing between poor and well
* : Statistically significant at p ≤ 0.05

DISCUSSION

Oral Squamous Cell Carcinoma(OSCC) is the sixth most common cancer worldwide and it encompasses at least 90% of all oral cavity malignancies (12). Increasing mortality rates due to OSCC has been observed for at least two decades and represents a real public health issue. This fact motivates the search for factors with prognostic relevance in order to get better individual management for every OSCC patients (13).

Although it has been accepted for a long time that carcinomas are associated with old age (14), in the present study the age range of the OSCC patients was between 32 and 68 with a mean age of 58.5 years. However, other studies recorded high prevalence of OSCC in younger individuals (less than 40 years). This was linked to heavy smoking, alcohol consumption, genetic risk factors or may be associated with increased exposure to carcinogenic agents (15).

Although most OSCC cases are located in the lateral margins of the tongue according to the literature (16, 17), in our study the most common site was the cheek mucosa followed by the lateral margin of the tongue. This finding is supported by Agarwal et al (18).

Concerning the gender in this study, male to female ratio was 1:1. This was in contradicton to Warnakulasuriya S. and Acharya S. et al., who supported the fact that oral cancer is more common in males than in females (14, 19). However, Patel et al., found that there is an increasing incidence of OSCC in females especially young ones (20). This is attributed to increasing in the use of tobacco and drinking alcohol in females nowadays, in addition to the fact that female patients tend to seek medical care more than males, thus more OSCC cases are now discovered in females (21).

Regarding the size of the primary tumors in the present study, T2 was the most prevalent followed by T4. This was in accordance with Kimura et al (22). On the other hand, Lwin et al., found that T4 was the most common size followed by T1 then T2 sizes (23).

Concerning lymph node status of our study cases, N2 was the most prevalent followed by N0 then N1. This was in controversy with Gervasio et al., who found that higher frequency of N3 lesions was seen with the progression of the disease, thus leading to a poorer prognosis and a more difficult treatment (24).

In the present study, stage IV was the most common clinical staging followed by stage II and stage III. This finding is in accordance with Gadbail AR et al. (25), but is contradicted with Rodrigues et al. who found that patients were classified as early stage (stages I and II) were of higher percentage (26).

Regarding the histopathological grading in the present study moderately differentiated SCC was the most prevalent followed by well differentiated SCC then poorly differentiated SCC. These results were supported by Zargoun et al and Gadbail et al (25, 27). While Pandey et al., have found that well differentiated grade was the most common followed by moderately differentiated then poorly differentiated (28).

The progression process of a normal cell to malignancy involves numerous mechanisms; one of which is the capacity to stimulate angiogenesis through the increased secretion of vessel-inductors and suppression of vessel-inhibitors (29).

The behavior of tumors is consistent with the concept that tumor is angiogenesis dependent. Because of the obvious importance, we have chosen to look at the immunomodulatory molecules controlling this process.

Vascular endothelial growth factor (VEGF) is a cytokine that has been documented to control angiogenesis and considered as a prime mediator in it (30).

A study made by Eisma RJ. et al., revealed that high VEGF levels predicted a higher rate of disease recurrence and shorter disease-free interval in bivariate analysis (31).

Johnston S and Logan RM, indicated a significant upregulation of VEGF expression during the transition from normal oral epithelium through dysplasia to invasive OSCC, but no correlation was found between VEGF expression and the grade of dysplasia (32), the same as Barbosa NG et al. who found the same results (44), while Margaritescu et al., found a correlation between VEGF and the different degrees of dysplasia, to invasive carcinoma. (33)

In the present study immunohistochmical expression of VEGF was evaluated in the term of area percent (A %) and optical density (OPD) which were correlated with histopathological grading of invasive SCC. It was revealed that the highest intensity of VEGF was recorded in poorly differentiated SCC cases followed by moderately differentiated and the least intensity was found in well differentiated. These results were supported by Kyzas PA et al., who observed a correlation between the increase in VEGF positivity with poor histologic differentiation (34).

Another study done by Ascani G. et al., who had found that angiogenesis process was strictly related to the histological grade of differentiation and to the presence of
loco-regional metastases in oral carcinoma which also supports our results (35).

In contrary, Mărgăritescu C. et al., have demonstrated that VEGF expression was reduced in poor differentiated OSCC tumors when compared to moderate and well differentiated forms (33).

As Angiogenesis cannot be measured directly in human tumors, several studies have concluded that quantification of micro vessels in histological sections (vascularity) may be used as an index of angiogenesis in some tumors (36).

In the present study micro vessels were counted using image analyzer and correlated with histopathological grading of tumor tissues and the test revealed that the largest numbers of micro vessels were observed in poorly differentiated OSCC cases and the statistical results revealed high significant difference between the three grades of differentiation.

These results were supported by Wadhwan et al., who observed that Micro Vessel Density (MVD) in poorly differentiated OSCC was statistically significantly increased in comparison to moderately differentiated OSCC and well differentiated OSCC, while MVD was slightly increased in moderately differentiated OSCC and well differentiated OSCC, but the increase was not statistically significant (37).

Concerning the correlation between VEGF expression and clinical data of the studied cases, the statistical analysis revealed non-significant difference in comparing the expression with all parameters of clinical data.

These results were supported by the study of Naderi NJ. Et al., who showed that no correlation was seen between VEGF expression with lymph node involvement, tumor differentiation, gender, or age (38).

However, Kyzas PA et a., found correlations between the increase in VEGF positivity with higher clinical stage (TNM) and poor differentiation (34).

Regarding tumor microvasculature in the present study, MVD was correlated with the different parameters of clinical data and results revealed statistically non-significant difference with all parameters.

That was supported by the studies done by Ascani et al. and Shintani S. et al., which showed that there were no statistically significant association between MVD and clinical variables such as age, sex, tumor size and site(35, 39).

In contradiction Kyzas PA et a., found high MVD was correlated with the higher clinical stage (34).

CONCLUSIONS

Over expression of vascular endothelial growth factor (VEGF) can be detected in cases of oral squamous cell carcinoma (OSCC) in comparison to normal tissues. The Immunopositivity for VEGF was found to be inversely proportional to the degree of differentiation of the OSCC, where well differentiated OSCC showed weak to moderate immunopositivity while poorly differentiated OSCC showed intense reaction.

The correlations between immunohistochemical expression of VEGF and different parameters of clinical data revealed statistically non-significant difference.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

REFERENCES