Wydawnictwo UR 2024
ISSN 2544-1361 (online)
doi: 10.15584/ejcem.2024.1.15

http://www.ejcem.ur.edu.pl
European Journal of Clinical and Experimental Medicine
Eur J Clin Exp Med 2024; 22 (1): 82–87

ORIGINAL PAPER

Expression of vascular endothelial growth factor and microvessel density in oral squamous cell carcinoma and its correlation with various clinico-pathological parameters

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ABSTRACT

Introduction and aim. Angiogenesis, which is accomplished by capillary sprouting, is the process by which new vessels are created from pre-existing ones. In tumor, once their initial blood supply is depleted, a tumour is unable to grow without additional blood flow. Additionally, a tumor's microvasculature, or microvessel density (MVD), increases along with its capacity to produce angiogenesis. We aimed to observe the relationship between the expression of vascular endothelial growth factor (VEGF) and MVD (using CD34) in oral squamous cell carcinoma (OSCC).

Material and methods. The expression of VEGF and CD34 antibodies was analysed using immunohistochemistry method on 50 cases of histopathologically proved OSCC. The expression was correlated with clinicopathological parameters.

Results. A significant correlation was observed between VEGF expression and gender, LVSI. No correlation between any other factors and the difference in VEGF expression was statistically significant. Similarly, the MVD expression was not found to be statistically significant in any of the pathological parameters.

Conclusion. VEGF positivity as well as MVD were found to be independent of the tumor pathology. Tumor MVD was found to be independent of the expression of VEGF. Further studies in a larger study group may establish a significant association so that antiangiogenic targeted therapy may be initiated.

Keywords. microvessel density, oral squamous cell carcinoma, vascular endothelial growth factor

Introduction

Cancer of the lips and oral cavity (CLOC) is one of the most common types of cancer in the world. In 2020, over 177,000 people died from CLOC, with Southeast Asia having the highest number of deaths. India also has a high number of CLOC cases, accounting for over 10% of all cancer cases in the country.[1]

Oral squamous cell carcinoma (OSCC) is the most common type of CLOC.[2] It is more common in older adults and men.[3,4] Although there have been advances in diagnosis and treatment, the 5-year survival rate for OSCC remains low, at about 50-60%.[5] This is largely because OSCC often spreads to the lymph nodes. Recent research has identified a number of cellular events that play a role in tumor progression, which may lead to new treatment options in the future.[6]

Tumors need blood vessels to grow and spread. Angiogenesis is the process by which new blood vessels form from existing ones. This process is called capillary sprouting. The number of blood vessels in a tumor is linked to how aggressive the tumor is, and the number of blood vessels in a tumor has been shown to be an

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Received: 28.07.2023 / Revised: 16.11.2023 / Accepted: 21.11.2023 / Published: 30.03.2024

Panigrahi R, Jha NK, Hota SK. Expression of vascular endothelial growth factor and microvessel density in oral squamous cell carcinoma and its correlation with various clinico-pathological parameters. *Eur J Clin Exp Med.* 2024;22(1):82–87. doi: 10.15584/ejcem.2024.1.15.

independent prognostic factor. Once a tumor's original blood supply is used up, it cannot grow beyond 1-2 mm without a new blood supply. The initial 1-2 mm zone is the farthest distance that oxygen and nutrients can diffuse from blood vessels.[7]

Therefore, angiogenesis is essential for tumor progression and metastasis. Although angiogenesis is difficult to measure directly in human tumors, there is increasing evidence that MVD may be considered as an indirect marker of neoangiogenesis. Although the most common antibodies used for microvessel staining so far are those against Von Willebrand factor VIII, CD31, and CD34, but CD34, a glycoprotein found in the membrane of endothelial cells (ECs), is considered to be highly sensitive for endothelial cells and produces minimum background staining.[8]

Vascular endothelial growth factor (VEGF) is the most important factor for the formation of new blood vessels in tumors. However, tumors do not start forming new blood vessels right away. This is called vascular quiescence. Vascular quiescence is ended by the "angiogenic switch", which is when the tumor starts to produce angiogenic factors. The angiogenic switch is "on" when there are more angiogenic factors than antiangiogenic factors, and it is "off" when the two types of factors are balanced.[8,9,10]

Aim

Thus this study was undertaken to evaluate the immunohistochemical expression of VEGF and CD 34 in OSCC and correlate the expression with histologic features of the tumor. The rationale behind this was that if the expression could be demonstrated in OSCC, this could offer an additional therapeutic strategy in the form of newer antiangiogenic drugs to prevent and treat cancer that are used as an adjunct to the currently available modalities.

Material and methods

This hospital-based retrospective study was carried on cases of OSCC from October 2018 to September 2022 that fit the selection criteria were included in the study. The Institutional Ethics Committee approved the study protocol (meeting date; 18/06/2021, decision number; 2021/3309). Written informed consent could not be obtained due to the retrospective nature of the study.

Inclusion

Patient with both sex, age range from 18 to 80 years and histologically confirmed radical excised cases of oral squamous cell carcinoma were included.

Exclusion

Recurrent OSCC, punch and incisional biopsy cases, unknown T-stage and N-stage cases, cases with neoadjuvant treatment, and poorly preserved cases were excluded from this study.

Histopathological diagnosis

Archival blocks from the pathology department were retrieved between October 2018 and September 2022. Relevant patient data were obtained from the hospital database. Histological evaluation with tumor grading was performed according to the World Health Organization (WHO) criteria. Tumor depths of invasion, lymphovascular invasion, and perineural invasion were recorded. Pathological staging was then performed according to the AJCC 8th edition. All included cases were grouped into two categories based on depth of invasion: ≤1 cm and >1 cm.

Immunohistochemistry procedure

Immunohistochemical evaluation of VEGF and CD34 was performed on 4–5 µm thick formalin-fixed paraffin-embedded tissue sections on poly-L-lysine-coated slides.

After deparaffinizing in three changes of xylene for 5 minutes each and rehydrating in a graded series of alcohol, the microwave antigen retrieval was performed using Tris EDTA (target retrieval buffer, pH 9) at 700 watts for 5 minutes, 600 watts for 5 minutes, and 600 watts for 5 minutes. Then, the slides were washed with Tris-buffered saline (TBS, pH 7.4). An endogenous peroxidase block was performed by adding 100 µL of peroxidase block to each tissue section and incubating at room temperature for 8–10 minutes. The slides were then washed in TBS for 5 minutes. Primary antibodies against CD34 (monoclonal mouse antibodies; ProTaqs Cat. No. 401602092; Quartett GmbH, Germany) and anti-VEGF (monoclonal mouse antibodies; clone VG-1; Diagnostic Biosystems, The Netherlands) were added to each tissue section (100 µL) and incubated in a humid chamber for 45 minutes. After washing in TBS for 5 minutes, HRP polymer (100 µL) was added to each tissue section, and the slides were kept in a humidity chamber for 30 minutes. After washing, a freshly prepared DAB solution was added and incubated for 15 minutes. The slides were then washed with wash buffer and distilled water. The counterstain was done with Harris hematoxylin for 1 minute, followed by washing with running tap water. Dehydration was done with a graded series of isopropyl alcohol (70%, 85%, and 100%) for 5 minutes each, followed by a xylene wash. Finally, the slides were mounted in DPX. Positive and negative controls were included in each batch.

Expression or scoring

VEGF

VEGF was expressed as a cytoplasmic stain in the tumor cells. The stained slides were interpreted as described by

Soini et al.[11] Scoring was based on the intensity of immunostaining in the lining endothelial cells (ECs; I) and the percentage of positive cells (P). The final immunostaining score was determined by the sum of the intensity of immunostaining (I) and the P. Final scores ranged from 0 to 7.

MVD

Vascular hotspots were evaluated under low magnification, and microvessel counting was performed manually under high power. The average was calculated for statistical evaluation. The mean of all microvessel counts was calculated as 21. All specimens were classified as "LOW MVD" for values ≤21 and "HIGH MVD" for values >21.

Statistical analysis

Measurement data were expressed as the mean ± standard deviation. Count data were expressed as percentages. Associations between VEGF and MVD expression and clinicopathologic factors were tested using the chi-square test. To assess the correlation between VEGF and MVD, the Karl-Pearson correlation coefficient was calculated. A p value of <0.05 was defined as statistically significant. Stata Version 15.1 software was used (StataCorp LLC, Texas, USA).

Results

A total of 50 cases were included, with males predominating over females. The male-to-female ratio was 4.5:1. The mean age of the patients was 55 years, with an age range of 28 to 80 years. Buccal mucosa (54%, 27/50) was the most common tumor site, followed by tongue (34%, 17/50), gingivobuccal sulcus (10%, 5/50), and lip (2%). Most of the cases (84%, 42/50) were grade I. Most of the cases (64%, 32/50) had a depth of invasion (DOI) of 1 cm. Only 8% (4/50) of the cases showed evidence of lymphovascular invasion. Almost equal proportions of cases had and did not have evidence of perineural invasion (PNI). The majority of cases (34.0%, 17/50) were T2, followed by 30% (15/50) cases of T4. An equal number of cases (50%, 25/50) had and did not have nodal metastasis (Table 1).

VEGF expression in OSCC

VEGF expression was found in 47 (94%) cases, of which 28 (56%) had strong expression and 19 (38%) had weak expression. A significant correlation was observed between VEGF expression and sex and LVSI. No other factors were significantly correlated with the difference in VEGF expression (p>0.05).

MVD expression in OSCC

Overall, equal percentages of low and high MVD were observed. There was no significant correlation between MVD and clinicopathologic parameters (Table 2).

Table 1. Correlation between VEGF expression and clinicopathological parameters

Clinicopathological parameters		Numbers (%)	VEGF		p
			Weak	Strong	
Age	<60	36 (72)	14	22	0.219
	>61	14 (28)	8	6	
Sex	Male	41 (82)	15	26	0.021
	Female	9 (18)	7	2	
Site	BM	27 (54)	8	19	0.091
	Gingivobuccal sulcus	5 (10)	3	2	
	Lip	1 (2)	1	0	
	Tongue	17 (34)	10	7	
Grade	Well differentiated	42 (84)	19	23	0.132
	Moderately differentiated	7 (14)	2	5	
	Poorly differentiated	1 (2)	1	0	
Depth of invasion	DOI< I cm	32 (64)	14	18	>0.999
	DOI> I cm	18 (36)	8	10	
Lymphovascular invasion	Present	4 (8)	4	0	0.044
	Absent	46 (92)	18	28	
Perineural invasion	Present	24 (48)	11	13	0.257
	Absent	26 (52)	11	15	
T stage	T1	11 (22)	5	6	>0.999
	T2	17 (34)	8	9	
	T3	7 (14)	4	3	
	T4	15 (30)	5	10	
N stage	0	25 (50)	11	14	0.973
	1	6 (12)	2	4	
	2a	4 (8)	2	2	
	2b	9 (18)	4	5	
	3b	6 (12)	3	3	

Table 2. Correlation between MVD expression and clinicopathological parameters

Clinicopathological parameters		Numbers (%)	MVD		p
			Low	High	
Age	<60	36 (72)	16	20	0.533
	>61	14 (28)	8	6	
Sex	Male	41 (82)	19	22	0.721
	Female	9 (18)	5	4	
Site	BM	27 (54)	11	16	0.277
	Gingivobuccal sulcus	5 (10)	2	3	
	Lip	1 (2)	0	1	
	Tongue	17 (34)	11	6	
Grade	Well differentiated	42 (84)	20	22	0.845
	Moderately differentiated	7 (14)	3	4	
	Poorly differentiated	1 (2)	1	0	
Depth of invasion	DOI< I cm	32 (64)	16	16	0.774
	DOI> I cm	18 (36)	8	10	
Lymphovascular invasion	Present	4 (8)	3	1	0.340
	Absent	46 (92)	21	25	
Perineural invasion	Present	24 (48)	12	12	>0.999
	Absent	26 (52)	12	14	
T stage	T1	11 (22)	4	7	0.127
	T2	17 (34)	11	6	
	T3	7 (14)	1	6	
	T4	15 (30)	8	7	
N stage	0	25 (50)	10	15	0.795
	1	6 (12)	4	2	
	2a	4 (8)	2	2	
	2b	9 (18)	5	4	
	3b	6 (12)	3	3	

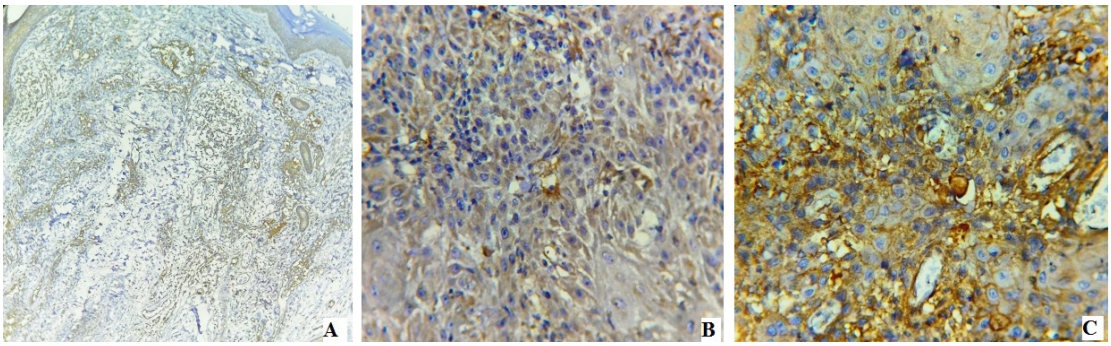

Fig. 1. A: Microsection shows strong cytoplasmic positivity of VEGF in tumour cells in angiosarcoma of skin which was taken as positive control (400x). B: Microsection shows weak cytoplasmic positive staining of VEGF in oral squamous cell carcinoma cells (400x). C: Microsection shows strong cytoplasmic positive staining of VEGF in oral squamous cell carcinoma cells (400x)

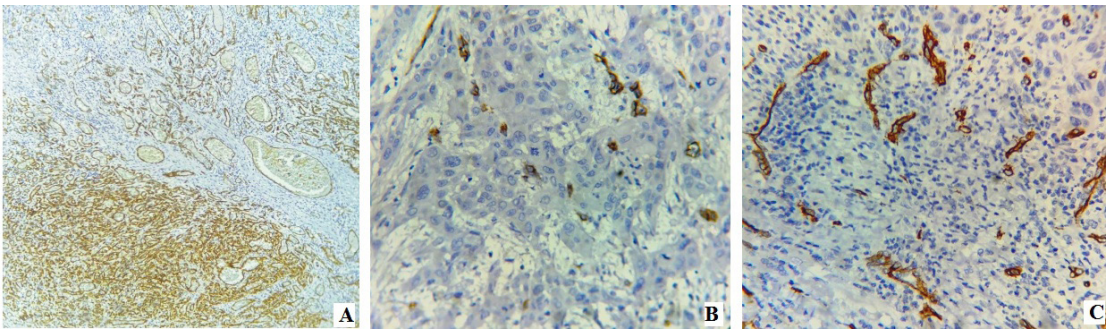

Fig. 2. A: Microsection shows strong membranous positivity of CD34 in endothelial cells of capillary hemangioma which was taken as positive control (400x). B: Microsection shows membranous staining of CD34 in endothelial cells of oral squamous cell carcinoma indicating low MVD (400x). C: Microsection shows membranous staining of CD34 in endothelial cells of oral squamous cell carcinoma indicating high MVD (400x)

The comparison of MVD scores with respect to VEGF expression

In our study, the majority of cases showed strong VEGF expression, among which 57.1% (16/28) showed strong MVD. Among tumor with absent VEGF expression, 66.7% (02/02) cases show low MVD. Low MVD was seen in 52.6% (10/19) of cases of tumor with weak VEGF expression. There was no significant correlation between MVD and VEGF expression (p=0.593) (Fig. 1 and 2).

Discussion

Tumors recruit new blood vessels from the existing circulation (angiogenesis), which contributes to tumor invasion and metastasis. Studies in the literature provide evidence that VEGF expression is necessary for neoangiogenesis, which is essential for tumor growth and metastasis.[12] Based on this evidence, we hypothesized that oral cavity tumors express VEGF for their growth and that MVD increases with increasing VEGF expression. To test our hypothesis, we evaluated VEGF expression and MVD using the CD34 marker in 50 OSCC cases. Both VEGF expression and MVD were correlated with known clinicopathological parameters.

Although we found strong VEGF expression in most OSCC cases with age ≤60 years, buccal mucosa, well-differentiated tumor, and DOI<1 cm, the difference was not statistically significant. Only sex and lymphovascular invasion (LVI) showed a significant correlation. All LVI-positive cases expressed VEGF, but 60.9% of LVI-negative cases also showed strong VEGF expression. We could not find similar studies that correlated VEGF expression with LVI.

We had the most cases in grade I, with 54.7% expressing strong VEGF expression. However, we could not establish a statistically significant correlation. Our study is consistent with previous reports.[7,13,14]

Astekar et al. found in their study that VEGF expression decreased from well-differentiated to poorly differentiated OSCC, but others have found a significant correlation between VEGF expression and tumor grade.[15] In their study, all poorly differentiated OSCC specimens and most moderately differentiated OSCC specimens expressed VEGF significantly with moderate to strong intensities, in contrast to our findings. They opined that the tumor cells in poorly and moderately differentiated OSCC exhibit angiogenic phenotypes, which could reflect a deregulated genotype.[7,16] Of our cases, 34% were classified as T2 and 14% as T3. We did not find a significant correlation between tumor stage and VEGF expression, but Sappayatosok et al. and Li et

al. did.[14,17] Larger sample sizes may be needed to establish a correlation in our study.

In our study, 50% of cases showed nodal metastasis, but the correlation between N stage and VEGF expression was not significant (p=0.973). Similar findings were observed by Nadir et al., but others have found a significant correlation between lymph node metastasis and VEGF expression.[14,17-19]

The current data showed no significant correlation between MVD and clinicopathological factors, which is in agreement with previous reports.[12,15,20]

When correlating MVD with age, we did not observe a statistically significant correlation (p=0.533). Of cases ≤60 years, 55.6% showed high MVD. Our findings are consistent with a previous report in which no statistically significant correlation was found.[21] However, Shahsaveri et al. found a significant correlation between MVD and age (p=0.029).[22] Two studies found a significant correlation between tumor grade and MVD (p<0.02, respectively).[23,24] We found no significant correlation between grade, DOI, LVI, or PNI with MVD. The possible reason for this lack of association in our study is the smaller sample size. We had the most cases in the T2 stage, but we did not observe a significant correlation between MVD and tumor stage (p=0.127). Our findings are consistent with those of a previous study.[15] However, Shieh et al. and Sappayatosok et al. found significant correlations (p<0.0001 and p<0.005, respectively).[17,26]

Artese et al. and Miyahara et al. found a significant correlation between MVD and lymph node metastasis.[27,28] In the current study, half of the cases showed nodal metastasis. We did not find a significant correlation between MVD count and nodal status (p=0.795). Our findings are consistent with those of Sappayatosok et al.[17] However, Elmorsy et al. found a significant correlation between MVD and nodal stage (p<0.001).[25]

Few studies have correlated MVD with VEGF. In our study, we did not find a significant correlation between VEGF and MVD (p=0.593). However, Astekar et al. found a significant correlation (p<0.001) in a similar study.[15] In our study, none of the clinicopathological parameters studied showed a significant correlation with MVD. However, some of the studies mentioned above found significant correlations with a few parameters. In future studies with larger sample sizes, MVD may help establish associations with a range of clinicopathological parameters. The density of tumor blood vessels measured in studies is primarily based on areas selected from the peripheral or central part of the tumor, or an even assortment of hotspot areas. This is another reason for the dissimilarity between reports. For achieving a precise result, more samples with more harmonized assessment methods are needed. Without strict standardization in methodology, conflicting results on the correlation between reports will continue.

In the present study, the most important limitation was the number of cases. By increasing the counts, more detailed results will be achieved.

Conclusion

Our study results demonstrate that VEGF expression in OSCC is independent of most clinicopathological variables, with the exception of patient sex and LVI. MVD (indirectly measured by CD34 expression) in OSCC cases is also uncorrelated with known clinicopathological factors and tumor VEGF expression. Further studies in a larger cohort may establish a significant association, which could be useful for applying antiangiogenic targeted therapy.

Declarations

Funding

This research received no external funding.

Author contributions

Conceptualization, S.K.H.; Methodology, N.K.J.; Formal Analysis, Investigation, S.K.H., R.P and N.K.J.; Data Curation, S.K.H. and N.K.J.; Writing – Original Draft Preparation, S.K.H., N.K.J., and R.P.; Supervision, S.H.K and R.P.

Conflicts of interest

The authors declare that there are no financial or other relations that could be construed as a potential conflict of interest.

Data availability

Datasets analyzed in this study are available from the corresponding author upon reasonable request.

Ethics approval

The approval of the Ethics Committee was obtained before initiation of the study (meeting date; 18/06/2021, decision number; 2021/3309). All procedures performed in this study involving human participants were in accordance with the ethical standards specified by the institutional and national research committee and with the Helsinki Declaration and its later amendments or comparable ethical standards.

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