How to Cite:
Alqalshy, E., El-Rahman, K. A., Ibrahim, A., Alazzazi, M. A., Abdel-Wahab, A. S., Mahmoud, H. M., Abdelhameed, W. B., & Esmail, A. E. A. E. M. (2021). Immunohistochemical evaluation of CD31 and D2-40, expression in oral squamous cell carci-noma. *International Journal of Health Sciences*, *5*(S2), 514–525. https://doi.org/10.53730/ijhs.v5nS2.12762

Immunohistochemical evaluation of CD31 and D2-40, expression in oral squamous cell carcinoma

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International Journal of Health Sciences ISSN 2550-6978 E-ISSN 2550-696X © 2021.
Manuscript submitted: 18 Sept 2021, Manuscript revised: 9 Nov 2021, Accepted for publication: 12 Dec 2021

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Abstract---Background: The present study was carried out to evaluate the relation between angiogenesis, and tumor stage of oral squamous cell carcinoma (OSCC). Materials and methods: Thirty formalin-fixed paraffin embedded blocks were used, ten of them were previously diagnosed as well differentiated OSCC, ten moderately differentiated OSCC and ten poorly differentiated OSCC. To determine the expression of CD31 and D2-40 proteins, streptavidin-biotin immunoperoxidase staining technique was used. The areas with the most vascular density (hot spots) were determined. The stained vessels were counted independently in intratumoral and peritumoral stroma. Results: CD 31 protein showed positive expression in the peritumoral and intratumoral blood vessels subjacent to the malignant invading nests. D2-40 expression was positive in lymphatic vessel in the peritumoral and intratumoral stroma subjacent to the invading nests. The highest mean values of both CD31 and D2-40 proteins expression were recorded in poor differentiated OSSC followed by moderate differentiated OSSC then well differentiated OSSC. There was statistically significant difference found between the three studied groups regarding CD31 and D2-40 levels. Also there was statistically significant positive correlation found between CD31 level and D2-40 level.Conclusion: CD31 and D2-40 are related to stage of OSCC and are consistent with angiogenesis in tumor progression.

Keywords---immunohistochemistry, CD31, D2-40, tumor angiogenesis, OSSC.

Introduction

Oral carcinoma is the sixth most common malignant tumor worldwide and the third most prevalent cancer in developing countries, with a particularly high incidence in South-East Asian countries and India [1]. Oral cancer arises as a result of an increase in genetic instability, which involves the activation of oncogenes and the suppression of tumor suppressor genes. Most human malignancies are defined by the loss of biological mechanisms that regulate cell cycle progression, cell death vs growth balance, and apoptosis in their early stages [2]. OSCC is the most common head and neck cancer, with the greatest mortality rate of all carcinomas [3].

OSCC is most common in people between the ages of 45 and 75, and is gradually increasing [4]. The most common etiological and predisposing factors for OSCC are smoking and drinking habits, as well as ultraviolet radiation (particularly for lip cancer), but other factors such as human papillomavirus (HPV) and Candida infections, nutritional deficiencies, and genetic predisposition have been also

associated [5, 6]. OSCC is a disease that affects adults and the elderly, with the most common clinical manifestation being an ulcerated lesion with a necrotic central area and raised rolling edges [7].

The five-year survival rate showed a little improvement over the years [8]. Identifying reliable prognostic factors remains challenging. The multistep accumulation of diverse genetic alterations in squamous cells is thought to be the source of OSCC development. Transformed cells can proliferate and invade as a result of these progressive changes [9]. The biological and clinical behaviours of OSCC and head and neck squamous cell carcinoma have been studied utilizing a variety of biological markers. Clinical, radiological, and histopathological investigations are used to determine a patient's prognosis and treatment options. The primary tumor's location, as well as the presence or absence of local metastasis in cervical lymph nodes and distant metastases, are all significant characteristics to consider. [10].

Angiogenesis or is essential for development and progression of malignant tumors [11]. Angiogenesis can be utilized as a prognostic indicator, indicating the likelihood of tumor growth and metastasis. Rather than direct tumor cell inhibition, it could be exploited as a novel secondary target for anticancer therapy [12]. Some authors have reported the usefulness of microvessel density as prognostic tool for assessment of patients after surgical treatment [13, 14]. CD31 and vascular endothelial growth factor (VEGF) are well-defined angiogenesis indicators. CD31 is a protein that is highly expressed on the surface of endothelial cells and well established for the monitoring of vascular density in malignant tissue [15]. CD31 was found to be involved in angiogenesis of early breast carcinoma [16], and nasopharyngeal carcinoma [17]. Sion-Vaardy et al. found a significantly increased number of vessels in head and neck tumors with deeper invasion [18].

The lymph node status is one of the most important criteria in determining prognosis and treatment options. Although the importance of this component has been well established, the process by which tumor cells infiltrate the lymphatic system and cause lymph node metastases is still unknown[19]. Lymphocytic metastases were thought to be caused by a passive mechanism for decades, based on the basic architecture of lymphatic vessels. The identification of molecular markers that govern lymphatic metastasis is currently a major problem for using targeted therapies to improve therapeutic outcomes. The lymphatic endothelial cell marker podoplanin has been found to be expressed in a variety of malignancies, including oral tumors. Podoplanin plays a role in carcinogenesis and cancer progression in head and neck cancers, and its expression is not limited to the endothelium of lymphatic vessels [20]. D2-40 antibody detects human podoplanin uniquely and can thus be used to assess its expression in the development of malignant neoplasms and lymphatic invasion. [21]. Regardless of the exact role of podoplanin in cell migration and tumor growth, its expression in squamous cell carcinoma tumor cells was identified immunohistochemically [22].

Materials and Methods

Samples Selection

Thirty formalin-fixed paraffin embedded blocks were used, 10 of them were previously diagnosed as well differentiated OSCC, 10 moderate differentiated OSCC and 10 poorly differentiated OSCC. They were collected from the archives of Oral and Dental Pathology Department, Faculty of Dental Medicine, Boys, Al-Azhar University, Cairo. All sections were stained with H&E to confirm the diagnosis.

Immunohistochemistry

To determine the expression of CD31 and D2-40 proteins, streptavidin-biotin immunoperoxidase staining technique was used. The immunostaining procedures were carried out according to the manufacturer's instructions, with CD-31 immunostaining by the Avidin Biotin complex method (Biogenex life sciences ltd, California, USA) to demonstrate blood vessels and toluidine blue staining for mast cell identification [23]. The anti CD-31 antibody highlighted the microvessels by staining endothelial cell membrane. A vessel was defined as a cluster of endothelial cells that was clearly isolated from surrounding microvessels. Because lymphatic endothelial cells lack brown staining, it was easy to distinguish between blood and lymphatic vessels.

Microvessels staining and counting were carried out in accordance with previous investigations [24]. The stained sections were examined at a magnification of ×40 to identify the areas with the most vascular density (hot spots). The stained vessels were counted independently in intratumoral and peritumoral stroma in five areas of hot spot at ×400 magnification. Anti-D2-40 monoclonal antibodies (DakoCytomation, Carpinteria, CA, USA) were used to highlight lymphatic vessels and tumor cells expressing D2-40. Incubation with primary antibody, was followed by the use of labeled streptavidin biotin working system (LSAB+, DakoCytomation) and 3,3 diaminobenzidine as chromogen. A modified Lillie hematoxylin counterstain was used. The presence, morphology, and density of lymphatic vessels were all measured.

Staining Interpretations

Under 200 magnification, the lymphatic microvascular density (LMVD) was determined using the hot spot method [25]. Evaluation of immunostaining was done using image analysis computer system was used to assess positive of the immunostaining. This was done in the Oral and Dental Pathology Department - Faculty of Dental Medicine - Boys- Cairo - Al-Azhar University.

Statistical Analysis

Statistical presentation and analysis of the present study was conducted, using the mean, standard deviation, ANOVA, Tukey’s test and Spearman’s correlation coefficient was used to determine correlations between different measurements by

SPSS V204. Significant level: Non significant >0.05, significant <0.05* high significant <0.001*.

Results

CD 31 protein expression results

Immunohistochemical staining using CD 31 protein showed a high number of CD31-positive vessels in the peritumoral and intratumoral stroma subjacent to the malignant invading nests of poor differentiated OSSC which recorded the highest vessels count mean area (45.36 ± 8.57), when compared to moderate differentiated OSSC (34.51 ± 7.01), and well differentiated OSSC which recorded the lowest vessels count mean area (23.51 ± 6.15) (Table 1 and Figures 1 A, 1B, 1C). There was statistically significant difference found between the three studied groups regarding CD31 level; the post hoc analysis shows that there was statistically significant increase in the level of CD31 in moderate differentiated OSCC than well differentiated group and also there was statistically significant increase in the level of CD31 in poorly differentiated OSCC than moderate differentiated group where P value was< 0.001 (Table 1 and Figure 2).

Table 1
Relation of CD31 level in different types of OSSC

CD31	Well differentiated OSCC	Moderate differentiated OSCC	Poorly differentiated OSCC	Test value	P-value	Sig
	No. = 5	No. = 5	No. = 5			
Mean ± SD	23.51 ± 6.15	34.51 ± 7.01	45.36 ± 8.57	11.155 •	0.002	HS
Range	16.44 – 30.14	27.43 – 43.73	33.25 – 54.91			
Post hoc analysis by LSD						
Well Vs moderate		Well Vs poorly		Moderate Vs poorly		
0.035		< 0.001		0.037		

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant.
CD31

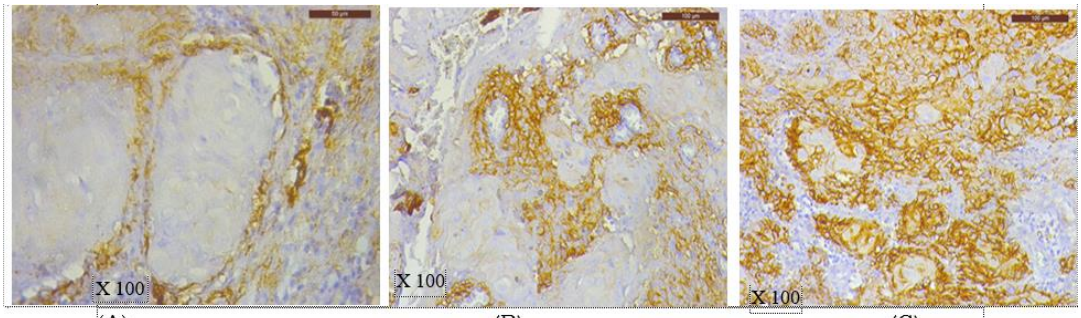


Figure 1. CD31- staining hotspot blood vessels in a peritumoral and intratumoral stroma subjacent to the malignant invading nest in (A) well; (B) moderately; and poorly differentiated OSSC (C)

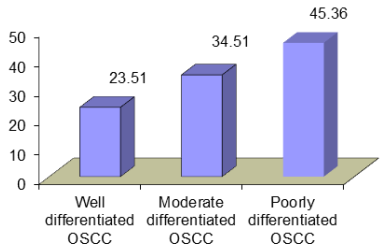


Figure 2. Bar chart representing relation of CD31 level with OSCC groups

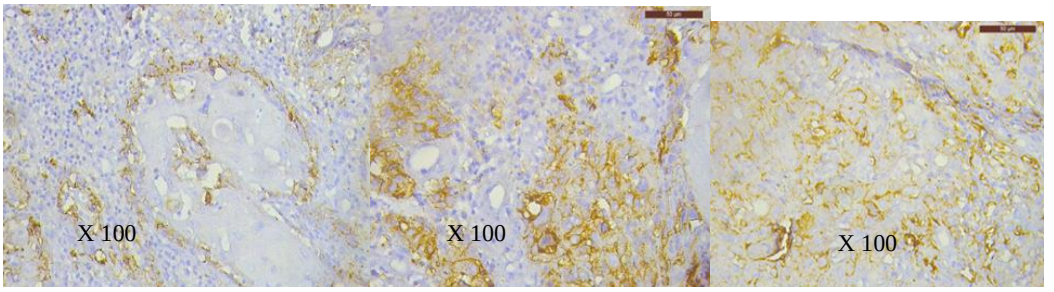
D2-40 protein expression results

D2-40 expression was positive in lymphatic vessel and did not stain the endothelium of blood vessels. Lymph vessels were identified both in the peritumoral area and intratumoral area of malignant invading nest of poor differentiated OSSC tissue sections recorded the highest vessels count mean area (11.30 ± 3.56), when compared to moderate differentiated OSSC (7.22 ± 1.50), and well differentiated OSSC had recorded the lowest vessels count mean area (3.17 ± 1.16) (Table 2 and Figures 3 A, 3 B, 3 C). There was statistically significant difference found between the three studied groups regarding D2-40 level; the post hoc analysis shows that there was statistically significant increase in the level of D2-40 in moderate differentiated OSCC than well differentiated group and also there was statistically significant increase in the level of D2-40 in poorly differentiated OSCC than moderate differentiated group where P value was< 0.05 (Table 2 and Figure 4). Also there was statistically significant positive correlation found between CD31 level and D2-40 level and vice versa (Table 3 and Figure 5).

Table 2
Relation of D2-40 in different types of OSSC

D2-40	Well differentiated OSCC	Moderate differentiated OSCC	Poorly differentiated OSCC	Test value	P-value	Sig
	No. = 5	No. = 5	No. = 5			
Mean ± SD	3.17 ± 1.16	7.22 ± 1.50	11.30 ± 3.56	15.262 •	0.001	HS
Range	2.18 – 5.04	5.41 – 9.05	7.2 – 16.75			
Post hoc analysis by LSD						
Well Vs moderate		Well Vs poorly		Moderate Vs poorly		
0.017		0.000		0.017		

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant.
D2-40



A B C

Figure 3. D2-40 - staining hotspot lymph vessels with relatively wide lumen, thin wall and slightly irregular contour in a peritumoral and intratumoral stroma subjacent to the malignant invading nest in (A) well; (B) moderately; and (C) poorly differentiated OSSC

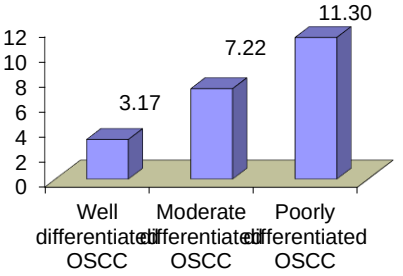


Figure 4. Bar chart representing relation of D2-40 level with OSCC groups

Table 3
Correlation between CD31 level and D2-40 level

	CD31	
	r	P-value
D2-40	0.643**	0.009

P-value > 0.05: Non significant; P-value < 0.05: Significant; P-value < 0.01: Highly significant.

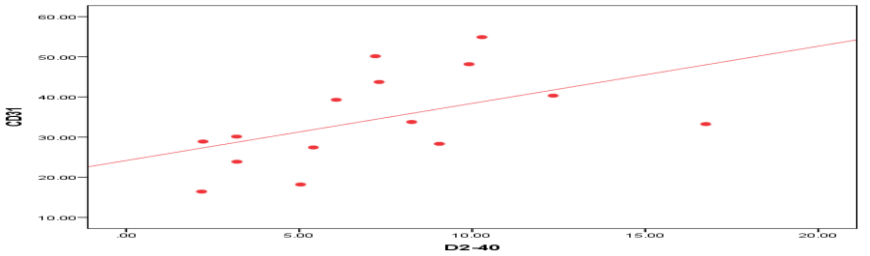


Figure 5. Correlation between CD31 level and D2-40 level

Discussion

In many solid tumors, the presence of neovascularization around neoplastic tissue is a typical finding. Angiogenesis appears to be a key biological factor in

tumor development, metastasis, and progression [26]. HNSCC's lymphangiogenesis, vascular mimicry, and mosaic blood vessel aspect must all be taken into account. Tumor cells were less angiogenesis dependent as a result of these mechanisms, and they grew and metastasized using these alternative vessels. Vascularization is necessary but not sufficient for the primary tumor's rapid growth and metastasis of its cells to distant organs [27].

The aim of this study was to provide new information about the relation between angiogenesis, tumor stage OSCC, In the present study there were significant difference among the mean of CD31 level between different grade of OSCC ($p \leq 0.001$), The results of the present study showed increased CD31 level related to stage of OSCC and are consistent with angiogenesis in tumor progression which was in accordance with the previous studies, Ashkavandi et al (2010) [28], Minhajat R (2006) [29] and Elpek GO (2001) [30], MVD was the most often utilized parameter to assess vascularity in most studies of angiogenesis in OSCC [31-33]. According to Shieh et al., increased vascularity at the tumor's periphery is seen during the initiation of oral SCC. Intratumoral vascularity rises as the tumor progresses. They showed that peritumoral vascular density differed from that of the intra-tumoral area, which helps to explain why angiogenesis in oral SCC is still debated [34].

Williams et al. detected that tumors with significant angiogenesis have a greater regional recurrence rate, which they used as an independent prognostic predictor [35]. Pignataro et al., also stated that patients with tumors that were inadequately vascularized had a better prognosis. In the latter stages of the tumor, no correlation between microvessel density and clinical pathological characteristics or prognosis was found, which is almost similar to our findings. Angiogenesis may be an early stage in the development of laryngeal tumors, according to the authors [36]. The lymphatic system is the primary pathway for tumor dissemination, and lymph node metastasis is the most important independent prognostic factor [37]. The prognosis is good when lymph nodes are clear of malignancy (10% -20% mortality), and the effect of adjuvant chemotherapy is less pronounced than in lymph node-positive cases. As a result, defining a practical reason for neoadjuvant therapy is crucial, and a positive prognostic therapeutic impact should outweigh the discomfort, side effects, and cost of adjuvant therapy [38]. Lymphatic vessel invasion (LVI) has an independent prognostic value, and it is routinely assessed as part of the tumor pathology report [39].

In our study, we used both H&E and D2-40 IHC methods for the evaluation of LVI. Based on the results, there was statistically significant increase in the level of D2-40 in moderate differentiated OSCC than well differentiated group and also there was statistically significant increase in the level of D2-40 in poorly differentiated OSCC than moderate differentiated group where P value was< 0.05, and the results of the present study showed increased D2-40 level related to stage of OSCC which was in accordance with the previous studies [40-42]. Partu et al., [43] reported a progressive increase in D2-40 reactivity from normal epithelium to dysplastic epithelium and then to SCC, implying that these immunomarkers may be involved in the early stages of squamous cell carcinogenesis in the palate. D2-40 is a monoclonal antibody that recognizes podoplanin, a family of mucin-like transmembrane glycoproteins found on the endothelium of lymphatic vessels [44].

Furthermore, the authors speculated that this expression might support a greater likelihood of loco-regional lymph node metastases for OSCCs with such localizations. Most of authors found a robust link between podoplanin expression and the rate of lymph node metastases [45–47]. In current study, there was statistically significant positive correlation found between CD31 level and D2-40 level and vice versa. This result could be due to the tumor embolism, which completely filled the lumen of the lymphatic vessel. In addition, with the H&E method, vascular invasion, either lymphatic or blood, could be detected and it can't be decided whether it is a lymph vessel or a blood vessel. In agreement with our findings Dileep A and his co-worker found D2-40 as a useful marker for lymphatic invasion and CD31 for blood vessel invasion [48].

Conclusion

In our study we concluded that CD31 and D2-40 related to stage of OSCC and are consistent with angiogenesis in tumor progression.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Institutional Review Board Statement: Not applicable.
Informed Consent Statement: Not applicable.

Data Availability Statement

The data sets used during current study are available from the corresponding author on reasonable request.

Conflicts of Interest

The authors declare no conflict of interest. All authors have read and agreed to the published version of the manuscript.

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