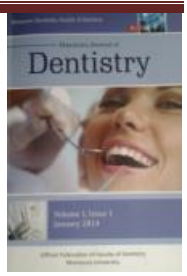
Immunohistochemical Expression of podoplanin and VEGFA in oral precancerous lesions and Oral Squamous Cell Carcinoma



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Abstract:

Oral premalignant lesions OPL are lesions that can potentially transform into malignancy in a variety of tissues, including the oral cavity. Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers. Podoplanin (D2-40), a newly reported monoclonal antibody that recognizes human podoplanin, was introduced as an excellent immunohistochemical marker of lymphatic endothelial cells in investigations of neoplasms of lymphatic origin within neoplastic tumors. The vascular endothelial growth factor A (VEGFA) is a strong positive regulator of angiogenesis that stimulates endothelial cell functions needed for new blood vessel formation, such as endothelial migration, differentiation and endothelial cell proliferation. The present study investigated the immunohistochemical expressions and correlations of podoplanin and VEGFA with studied cases. The expressions of podoplanin and VEGFA in 20 cases of OPL and 20 cases of OSCC studied cases were determined immunohistochemically. The findings of the present study revealed high statistically significant correlation between degree of dysplasia as well as clinical forms of OPL studied cases, tumor differentiation and lymph node involvement of OSCC studied cases with podoplanin expressions. VEGFA expression revealed high significant correlation with clinical forms of OPL studied cases, tumor differentiation and lymph node involvement of OSCC studied cases. These results suggest that podoplanin and VEGFA may be predictor of early oral tumorigenesis and for malignant transformation risk assessment of OPL and as biomarkers for advanced grades of OSCC may be useful to identify OSCC patients at risk of a more unfavorable clinical outcome.

Introduction

Oral cancer is the sixth most common cancer worldwide, representing about 5.5% of all malignancies.[1] Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers.[2] Oral premalignant lesions OPL are lesions that can potentially transform into malignancy in a variety of tissues, including the oral cavity.[3] Early detection of potentially malignant disorders can decrease the morbidity and mortality associated with oral cancer.[4]

The incidence of OSCC differs in different regions of the world varying from over 20/100,000 in India to 10/100,000 in the USA, and < 2/100,000 in the Middle East.[5] Within the Middle East, rates of smoking are high although alcohol consumption is limited.[6,7] This is especially true for Egypt where smoking rates are increasing for both cigarettes and water-pipe.[7] However, there have been very few studies depicting the magnitude of head and neck cancer HNC in the Middle East and Egypt. Previous hospital-based studies from Egypt showed that HNC constitutes about 17-20% of all malignancies.[8,9]

Recently, Podoplanin (D2-40), a newly reported monoclonal antibody that recognizes human podoplanin, was introduced as an excellent immunohistochemical marker of lymphatic endothelial cells in investigations of neoplasms of lymphatic origin, and lymphatic architectures within neoplastic tumors. In a number of studies, the D2-40 antigen unexpectedly became immunolocalized in tumor cells, including those of squamous cell carcinomas (SCCs) in different organs.[10-18] It was noted that podoplanin was expressed in some hyperplastic and dysplastic lesions adjacent to the primary oral cancers, suggesting that expression of podoplanin may occur in early oral tumorigenesis and may play a role in the malignant transformation.[19]

The vascular endothelial growth factor A (VEGFA) is a strong positive regulator of angiogenesis that stimulates endothelial cell functions needed for new blood vessel formation, such as endothelial precursor cell migration, differentiation and endothelial cell proliferation.[20] In tumor angiogenesis, VEGFA is produced by neoplastic cells and stroma, including fibroblasts and inflammatory cells.[20] High VEGFA expression stimulates new blood vessel formation from pre-existing vessels, whereas lower VEGFA expression induces endothelial cell apoptosis.[21]

However, there has been few detailed investigation of the characteristics of podoplanin and VEGFA expressions in oral precancerous lesions and oral squamous cell carcinoma and tumor margins, though it would be of great interest to determine such expressions and to assess the correlations between the podoplanin and VEGFA with the studied cases in details in the present study.

Materials and methods

1) The present retrospective study was carried out on twenty archival paraffin blocks previously diagnosed as oral precancerous lesions (20), and twenty archival paraffin blocks oral squamous cell carcinoma cases (20). These specimens were collected from archival files of Oral Pathology Department, Faculty of Dentistry, Mansoura University. Cases were retrieved from January 2016 to December 2017.
2) Haematoxylin and eosin stain: to confirm diagnosis based on histological classification of OPL and OSCC of the studied cases.
3) Immunohistochemical staining: for podoplanin was performed using (Dako cytomation, California Inc. USA). Slides were incubated with mouse monoclonal antihuman podoplanin primary antibody (D2-40).
4) Immunohistochemical staining: for VEGFA was performed using (DAB) (DAKO Corporation, Carpinteria, CA). Sections were incubated for 2 h with VEGFA antibody (VG1 clone).
5) **Immunostaining evaluation and statistical analysis:**

For podoplanin:

The podoplanin expression was observed in cell membrane and cytoplasm of the tissue sections. In oral leukoplakia, immunostaining was assessed using scoring system as stated by Kawaguchi et al.[14]

0—if no expression was observed in any part of the epithelium.
1—if expression was restricted to the basal layer of the epithelium.
2—if expression was observed in the basal and suprabasal layers at one area.
3—if the suprabasal layer expression was observed at two or three areas.
4—if the suprabasal layer expression was observed at more than three areas.
Score was calculated in 10 high power fields in each slide and mean was calculated per slide. Calculation of cancer risk (according to De Vicente et al.[22] and Kawaguchi et al.[14]):
Score 0-1: low risk or negative expression.
Score 2 or more: high risk or positive expression.

In OSCC podoplanin expression was scored as described by Rodrigo et al.[23] by quantity of positive tumor cells on a scale of 0 to 5 as follows:
0: negative, 1: less than 10%, 2: more than 10% and less than 30%.
3: more than 30% and less than 50%, 4: more than 50% and less than 80%, 5: more than 80% positive staining.
Based on the staining intensity, positive specimens were classified into 4 categories:
3 = strong—dark brown staining of cells.
2 = moderate—staining between 2 extremes (dark brown and weak staining). 1 = weak—faint staining. 0 = negative—no staining.

German Immunoreactive Score (IRS) was calculated by multiplying quantity score and staining intensity scores. Scores could range from 0 to 15:
7 or higher = high reactivity and 0 to 6 = weak reactivity.[23]
Score was calculated in 10 high power fields in each slide and mean was calculated per slide.

For VEGFA:

VEGFA expression was estimated semiquantitatively as the propor- tion of stained cells and the percentage of immunostained cells was scored as follows: 0 (<5%), 1 + (5–25%), 2 +(25–50%), 3 + (50–75%), and 4 + (>75%).[24]

Results

The analysis of 20 patients with OPL studied cases revealed youngest patient was 38 years old and the oldest was 60 years old, with mean age (49.2) years. There was a male predilection among OPL studied cases. The most common site was the tongue and the buccal mucosa (50% of OPL studied cases for each one). Regarding the clinical form, it was found that leukoplakia was the most represented among current cases by 65%. Meanwhile, the least frequency was oral submucous fibrosis by 5 % of OPL studied cases. The heamatoxylin and eosin stained sections of the studied cases were examined and classified according to criteria and signs of epithelial dysplasia. The most common type of the current studied cases was the mild dysplastic leukoplakia (40%) of OPL studied cases.

Among 20 patients of OSCC studied cases revealed youngest patient was 28 years old and the oldest was 75 years old, with mean age (53.65) years. There was a male predilection among OSCC studied cases. The most common site was the tongue by (40%), while the least frequency site was the mandible by (5%) of OSCC studied cases. Tumor size (T1) was the most size form recognized (65%) of the OSCC studied cases. Regional lymph node involvement was observed in (55 %) of the OSCC studied cases. Clinical stage III was the most clinical stage observed in (50%) of the OSCC studied cases. The heamatoxylin and eosin stained sections of the studied cases were examined and classified according to the traditional histopathological grading (tumor differentiation) WHO of OSCC patients. The most common types of the current studied cases were well differentiated OSCC and moderate differentiated OSCC (40% for each type).The least common type of the current studied cases was poorly differentiated OSCC which detected in only (20%) of OSCC studied cases.

The evaluation of podoplanin expression was performed in 20 cases of OPL and 20 cases of OSCC. Among 20 cases of OPL, immunohistochemical analysis revealed high risk positive reaction in 17 cases (85%) and low risk reaction in 3 cases (15%). Non significance difference revealed between podoplanin in relation to age, gender and sites of OPL studied cases. As regard to clinical form of oral precancerous lesions, podoplanin was more expressed in carcinoma in situ and oral submucous fibrosis cases. High significant relation revealed between podoplanin expression and clinical forms of OPL cases (P=0.007). High significant differences revealed

between podoplanin over expression and degree of dysplasia of OPL cases (P=0.003).

Among 20 cases of OSCC, immunohistochemical analysis of podoplanin revealed high risk positive reaction in 6 cases (30%) and low risk reaction in 14 cases (70%). Non significance difference revealed between podoplanin in relation to age, gender and sites of OSCC studied cases. Statistically significant difference was observed between podoplanin expression and lymph node involvement in studied cases of OSCC (P= 0.02). As regard to clinical staging, high risk positive expression of podoplanin was observed more among advanced clinical stages III and IV (88.2%) of OSCC studied cases, although Non significance difference between clinical staging of OSCC studied cases in relation to podoplanin expression. Statistically there was a high significant difference between podoplanin expression and tumor differentiation of the studied cases of OSCC (P= 0.002).

The evaluation of VEGFA expression was performed in 20 cases of OPL and 20 cases of OSCC. Among 20 cases of OPL, immunohistochemical analysis of VEGFA revealed very high reaction in (15%), high reaction in (10%), moderate reaction in (25%), mild reaction in (40%), and weak reaction in (10%) of OPL studied cases. Non significance difference revealed between VEGFA in relation to age, gender and sites of OPL studied cases. As regard to clinical form of oral precancerous lesions, VEGFA expression was very high reaction in 2 cases of carcinoma in situ and one case of oral submucous fibrosis cases. High significant relation between VEGFA expression and clinical forms of OPL cases (P=0.008). Not significance differences between VEGFA over expression and degree of dysplasia of OPL cases.

Among 20 cases of OSCC, immunohistochemical analysis of VEGFA revealed very high reaction in (10%), high reaction in (10%), moderate reaction in (60%), and mild reaction in (20%) of OSCC studied cases. Non significance difference was formed between VEGFA expressions in relation to age, gender and sites of the OSCC studied cases. As regard to clinical staging, very high reactions of VEGFA expressions were in one case of clinical staging IV and one case of clinical staging III (50% and 10% respectively), high reactions were in one case of clinical staging IV and one case of clinical staging III (50% and 10% respectively). Statistically significant difference was observed between VEGFA expression and lymph node involvement in studied cases of OSCC (P=0.04).

Discussion:

Oral cancer is a leading cause of cancer death and oral squamous cell carcinoma is the most common type of oral cancer.[25] Carcinogenesis is a sequential and multi-step process.[26] Although several markers have been proposed for diagnosing and predicting the behavior of dysplastic lesions, only few can be used as a biomarker for cancer risk assessment. Thus, identifying a new biomarker is of great clinical interest.

The present work was an effort to demonstrate the immunohistochemical expressions and correlations of podoplanin and VEGFA with studied cases. In the present study, overall expression of podoplanin in OPL cases revealed high risk positive reaction in (85%) of OPL studied cases. These in accordance with study done by Deepa et al. 2017, Patil et al. 2015 and study done by Kawaguchi et al. 2008.[14,27,28] As regard to clinical form of oral precancerous lesions, podoplanin was more expressed in carcinoma in situ and oral submucous fibrosis cases while the least expression was in leukoplakia cases. High significant relation revealed between podoplanin expression and clinical forms as well as degree of dysplasia among OPL cases. These in accordance with Deepa et al. 2017 and Patil et al. 2015 studies.[27.28] These may be represent tumor initiating cells and may represent upward clonal expansion of stem cells during carcinogenesis and oral premalignancy with such clonal expansion may imply significantly higher risk of malignant transformation. Overall expression of podoplanin in OSCC cases revealed high risk positive reaction in (30%) of OSCC studied cases. These findings in agreement with Patil et al. 2015 and Logeswari et al. 2015.[28,29] Significant difference was observed between podoplanin expression and lymph node involvement in studied cases of OSCC. Podoplanin expression was revealed in the cytoplasm and membranous of the tumor cells and increase from well to poorly differentiated OSCC. High significant difference between podoplanin expression and tumor differentiation of the studied cases of OSCC. These suggest that podoplanin may play some role in the regulation of differentiation, growth, and tumor progression of OSCC.[22,23]

Overall expression of VEGFA in OPL cases revealed positive reaction in 90 % of studied cases. The expression of VEGFA was revealed in the cytoplasm of the basal and parabasal cells of OPL studied cases. Carcinoma in situ and oral submucous fibrosis cases revealed the high positive reaction staining in the dysplastic cells. High significant relation revealed between VEGFA expression and clinical forms of OPL cases. VEGFA expression was intense and increased throughout the entire thickness of epithelium in severe epithelial dysplasia of OPL studied cases. Although, statistically revealed not significant differences between VEGFA over expression and degree of dysplasia of OPL cases. These findings in agreement with work reported by Varma et al 2014.[26] This may be due to the fact that as cells transform from normal to dysplastic, the balance between proangiogenic and antiangiogenic factors is altered and the epithelial tumor cells themselves acquire transient angiogenic properties.[30,31] Overall expression of VEGFA in OSCC cases revealed very high reaction in (10%) and high reaction in (10%). These findings in accordance with Sales et al. 2016 and Varma et al. 2014 studies.[24,26] Significant difference was observed between VEGFA expression and lymph node involvement in studied cases of OSCC. It was very high reaction expression for VEGFA in four cases (all of poor differentiated cases) 20 % of OSCC studied cases, and the expression was revealed in the cytoplasm of the tumor cells. Significant difference between VEGFA expression and tumor differentiation of the studied cases of OSCC. [194] Joo YH et al., 2009 observed clear correlation between VEGF over expression and both of tumor angiogenesis as well as advanced lymph node involvement in HNSCC through its possible role in facilitating the growth of blood and lymphatic vessels and also by increasing the vascular permeability.[32]

Generally, elevated levels of podoplanin and VEGFA expression in OPL and OSCC studied cases associated and increase with severity of dysplasia, advanced clinical stages, lymph node involvement and poorly differentiated OSCC. These reinforce that podoplanin and VEGFA expressions together may predict malignant transformation of OPL and may useful to identify OSCC patients at risk of a more aggressive clinical outcome.

Conclusion

The expressions of podoplanin and VEGFA correlated with severe dysplasia of OPL cases, lymph nodes involvement and poorly differentiated OSCC cases. Podoplanin can be used as a biomarker for early oral tumorigenesis and for evaluating malignant transformation risk assessment in patients with OPL in addition to may be useful to identify OSCC patients at risk of a more unfavorable clinical outcome. VEGFA may play an important role in tumor progression during malignant transformation of OPL cases beside VEGFA may be a biomarker for prediction of the progression and prognosis of OSCC cases.

Table 1: Relation between podoplanin expression and clinical parameters, degree of dysplasia, histological grading of OPL and OSCC studied cases:

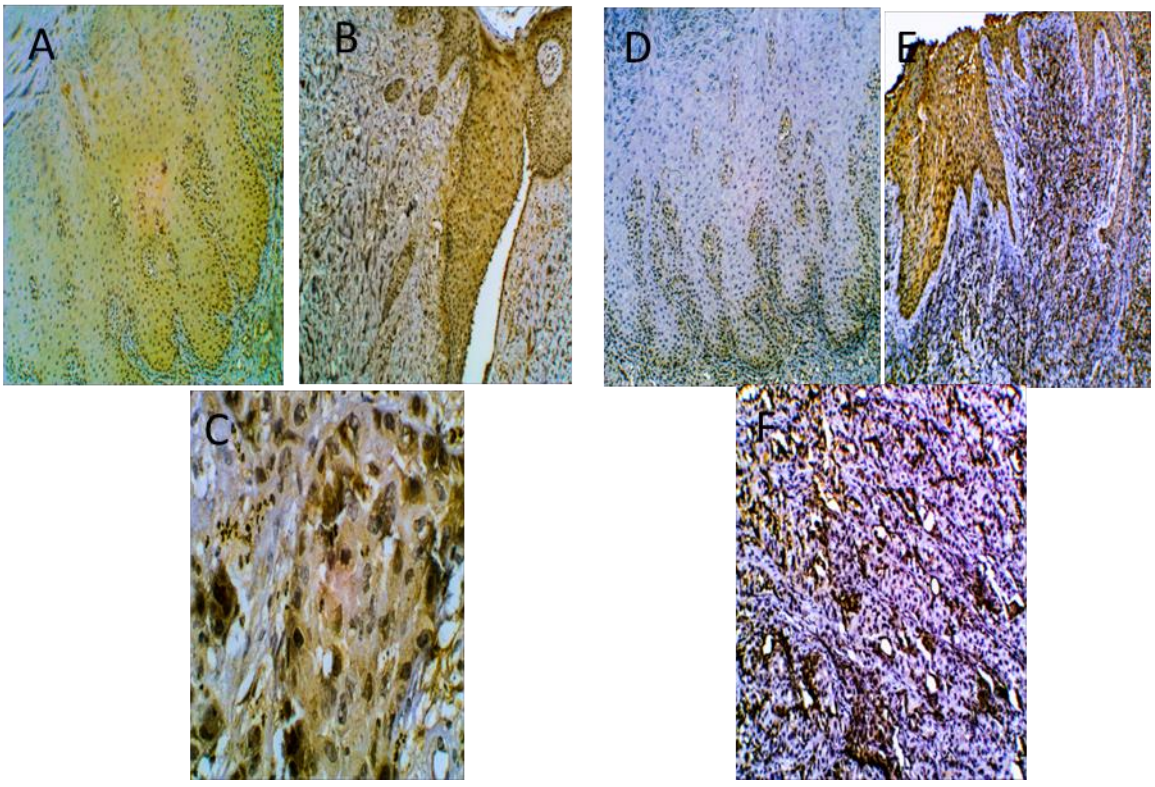
		Podoplanin score(0-4)				
		Weak reaction (0-1) score		High reaction (2-4) score		
		No	%	No	%	
Clinical forms of OPL	Leukoplakia		3.1%	0	6.9%	.007
	CIS		.0%		00.0%	
	OSF		.0%		00.0%	
	Erythroplakia		.0%		00.0%	
Degree of dysplasia OPL	Mild		7.5%		2.5%	.003
	Moderate		.0%		00.0%	
	Severe		.0%		00.0%	
Lymph node involvement OSCC	Negative	0	1.4%		6.7%	.02*
	Positive		8.6%		3.3%	
Clinical staging OSCC	Stage I		8.6%		6.7%	.1
	Stage II		1.4%		.0%	
	Stage III		0%		0%	
	Stage IV		.0%		3.3%	
Histological grading OSCC	Well		2.9%		3.3%	.002*
	Moderate		7.1%		.0%	
	Severe		.0%		6.7%	

Data expressed as frequency(Number-percent) P:Probability *:significance <0.05
Test used: Chi-square

Table 2: Relation between VEGFA expression and clinical parameters, degree of dysplasia, histological grading of OPL and OSCC studied cases:

			VEGFA IH scoring (0-4)					
			Weak reaction	Mild reaction	Moderate reaction	High reaction	Very high reaction	
Clinical forms OPL	Leukoplakia	No						.02
		%	5.4%	3.8%	0.8%	.0%	.0%	
	OSF	No						
		%	.0%	.0%	.0%	.0%	00.0%	
	CIS	No						
		%	.0%	.0%	.0%	0.0%	0.0%	
	Erythroplakia	No						
		%	.0%	0.0%	0.0%	.0%	.0%	
	Mild	No						.2

		%	2.5%	2.5%	5.0%	.0%	.0%	
Degree of dysplasia OPL	Moderate	No						
		%	.0%	00.0%	.0%	.0%	.0%	
	Severe	No						
		%	0.0%	.0%	0.0%	.0%	0.0%	
Clinical staging OSCC	Stage I	No						.96
	Stage II	%						
	Stage III	No						
	Stage IV	No						
Lymph node involvement OSCC	Negative	No						.04*
		%	.0%	6.4%	3.6%	.0%	.0%	
	Positive	No						
		%	.0%	.0%	5.6%	.2%	.2%	
Histological grading OSCC	Well	No						.04*
		%	.0%	7.5%	2.5%	.0%	.0%	
	Moderate	No						
		%	.0%	2.5%	5.0%	.0%	2.5%	
	Poor	No						
		%	.0%	.0%	5.0%	0.0%	5.0%	



D2-40 **VEGFA**

Figure.1 **(A)** Mild leukoplakia shows score (2) positive cytoplasmic immunoreaction for D2-40 **(B)** OSF shows score (4) positive cytoplasmic immunoreaction for D2-40 **(C)**Poorly differentiated OSCC shows score (15) positive cytoplasmic immunoreaction for D2-40 **(D)** Mild leukoplakia shows score (1) positive cytoplasmic immunoreaction for VEGFA **(E)** OSF shows score (4) positive cytoplasmic immunoreaction for VEGFA **(F)** Poorly differentiated OSCC shows score (3) positive cytoplasmic immunoreaction for VEGFA (ABC, DAB ×100).

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