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Angiopoietin-2 Expression in Relation To Angiogenesis And Vessel Maturation In Oral Squamous Cell Carcinoma.

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ABSTRACT

Angiogenesis has an essential role in tumor growth, progression, and metastasis. Angiopoietin-2(Ang-2) may over express in oral squamous cell carcinoma (OSCC) and it always associated with aggressive tumor and poor prognosis. This study conducted to determine the expression of Ang-2 in OSCC and assess its correlations with clinic opatho logical parameter, angiogenesis, and vessel maturation. Retrospective 36 formalin- fixed, paraffin-embedded tissue blocks his to logically diagnosed as OSCC were examined for Ang-2 expression, micro vessel density (MVD) and vessel maturation index (VMI) calculations were assessed by conventional immune his to chemistry staining using anti Ang-2, anti-CD105 and anti α-SMA antibodies respectively. The proportion of OSCC samples positive for Ang-2 was significantly associated with histological grade, MVD, and VMI (P value=0.011, 0.029, 0.003 respectively), the association between VMI and the histological grade was highly significance (P value=0.0001). There is no significant association between Ang-2 expressions with the clinic opatho logical parameters analyzed in OSCC patients. Expression of Ang-2 was significantly associated with angiogenesis and vessel maturation in OSCC.

Keywords: angiopoetin-2, angiogenesis, OSCC.

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INTRODUCTION

Head and neck malignant tumor is the sixth most common kind of malignancy around the world and oral squamous cell carcinoma (OSCC) characterizes about more than 90% of all oral cancers [1].There are various etiological factors associated with OSCC development include those endogenous factors as: mutated genes[2]and nutrient insufficiency[3],or exogenous factors as: Tobacco, alcohol, snuff, and human papilloma virus [4].The development and progression of the tumor are strictly related to an effective angiogenesis[5]Angiogenesis means newly formed blood vessels (BVs) from preexisting one [6].Angiogenesis has an essential role in different physiological situations, such as embryonic development, chronic inflammation, and wound repair [7]. In addition, it' s significant role in tumor growth, progression, and metastasis. [8]

Angiopoietin- 2 (Ang-2), a member of the angiopoietins (vascular growth factors), is found to have an essential role in angiogenesis[9].In various studies, it is thought that malignancies which are hyper vascularized, showed higher expression of Ang-2 [10]Ang-2 was obviously highly expressed in OSCC tissues [11]. Up regulation of Ang-2leads to increase the migration and invasion of the OSCC cell line [12].

Enderlin (CD105): is a powerful pleiotropic angiogenic factor expressed on activated endothelial cells (ECs) during angiogenesis procedure, so it suggested to be a particular marker to detect tumor angiogenesis.[13,14]Alpha smooth muscle actin (α-SMA) is actin isoform that prevails within vascular smooth muscle cells (v SMCs), in arteries and veins, or within pericytes in capillaries and venues. These mural cells play a great role in keeping the blood vessels (BVs) survive and stable [15, 16].
Micro vessel density (MVD): A quantification of tissue angiogenesis can be made through measuring micro vessels number in a given area by immunohistochemical staining with one of endothelial marker [17].Vessel Maturation Index (VMI): could be defined as the ratio of the number of the BVswhich positively stained with α-SMA antibody to CD 105-positive vessels in the same vascular hot spot region in which micro vessel density were calculated [18].

The aims of the current study wereimmunohistochemical evaluation of Ang-2 expression with angiogenesis and vessel maturation, in addition to the evaluation of Ang-2, MVD and VMIin relation to clinic pathological parameters of OSCC.

MATERIALS AND METHODS

Tissue sample

Thirty six formalin- fixed, paraffin-embedded tissue blocks diagnosed histopathological as OSCC which obtained from the archives of the Oral Maxillofacial Pathology Department / Dentistry CollageofBaghdad University were enrolled in this study. Demographic and clinical data: patients name, age, gender, clinical presentation, tumor site, and histological grade), were obtained from the archives. The positive tissue controls (formalin fixed, paraffin-embedded tissue blocks): normal placental, normal lung, human breast adenocarcinoma for immunohistochemical evaluation of Ang-2, CD105, and α-smooth muscle actin antibodies respectively were obtained from the archives ofAl-Shaheed Ghazi Hospital, Teaching Laboratory Department/ Baghdad Medical City.

Conventional immune his to chemistry

All studied blocks were cut into 5µm serial sections. Ordinary Haematoxylin and Eosin staining (H&E) to confirm diagnosis was be done first. Ang-2, CD105, and α-SMAexpression were evaluated using abcam, expose mouse and rabbit specificHRP/DAB detection IHC kit (ab80436, Lot: GR288328-11). From the beginningdeparaffinization and rehydration were done, then blocking of endogenous peroxidase by incubation the histological sections with 3 % hydrogen peroxide for 10 min. The samples were then blocked for nonspecific antigens binding with normal goat serum incubated for 1 hour. Antigen retrieval procedure was carried out by incubating the samples in Citrate Buffer Saline (CBS) PH 6.0, and heating in water bath (95^{o}C) for 10 min. This procedure was carried out only for sections those got immune his to chemical staining with α-SMA antibody. Sections were then incubated with one of primary antibody, an anti Ang-2 monoclonal antibody(1/100) dilution; (abcam company [MM0020-1F29] ab56301), an anti CD105 poly clonal

antibody(1/200) dilution; (abcam company [ab49228]), and an anti α-SMA monoclonal antibody(1/200) dilution; (abcam company[ab125057])overnight at 4°C. After that the sections were washed with phosphate-buffered saline (PBS, pH = 7.0). Then using the previous mentioned IHC detection kit, the slides were incubated with secondary antibody (rabbit anti mouse antibody unconjugatedthen Gout anti –rabbit HRP conjugated) 10min.for each followed by incubation with 3′-3′-diaminobenzidine chromones (DAB) for 1min at room temperature.

All sections then stained with Haematoxylin as a counter stain, dehydrated through graded alcohols, and at last, mounted. For each IHC run a positive control tissue sample consisting of either normal placenta , normal lung, human ducal adenocarcinoma were included in addition to negative control consisted of sample in which the primary antibody were replaced by PBS.

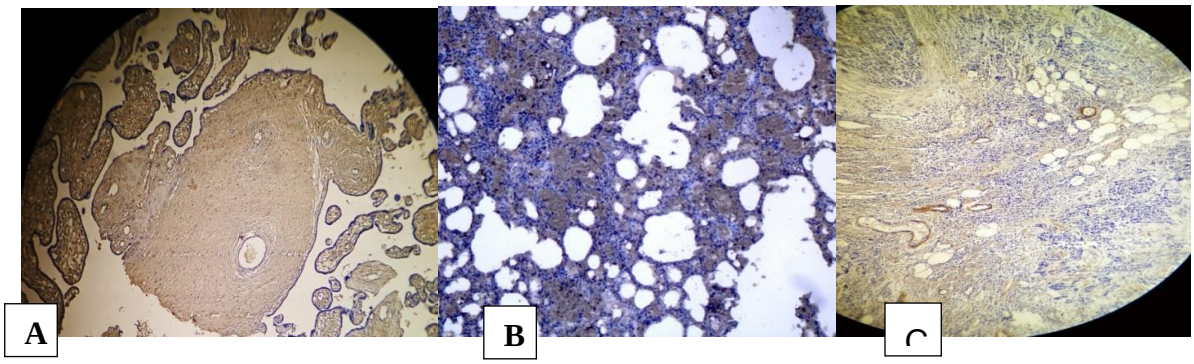

Figure1: Positive expression of (A: Ang-2 in normal placental tissue, B: CD105 in normal human lung tissue, C: α- SMA in human breast ducal adenocarcinoma(X100).

Immune his to chemical evaluation of Ang-2 antibody:

To evaluate immune his to chemical expression of Ang-2 was performed by two pathologists, who were blinded to the patients' information. Immune-re activity of Ang-2 was primarily detected in the cytoplasm of OSCC cells. Both mean value score (MVS) of the percentages of positively stained cells and staining intensity scale (SIS) value were determined [19].

MVS was calculated using the following scale: 0= 0–10 %; 1=10–30 %; 2= 30–50 %; and 3= 50–100 %. The SIS was graded using the following scale: 0, no detectable staining; 1, faint staining; 2, moderate staining; or 3, strong staining .Positive immunohistochemical expression of Ang-2 if MVS + SIS ≥ 3 [20].

Quantification of MVD and VMI

Weidner ET al.In 1991, discovered a new procedure to perform (MVD) within tumors. Any browned stained (endothelial lined lumen)micro vessels, stained ECs clusters or even single stained ECs that separated obviously from neighboring micro vessels, neoclassic cells or other connective tissue components was measured as a single, countable micro vessel[21].

In current study, MVD was evaluated by defining the number of CD105- positively stained endothelial cells using (Pro. Way, China) light microscopy. The stained sections were screened at X40 magnification to recognize three regions which showed the maximum number of browned stained micro BVs, called vascular hot spots. The number of these vessels was counted in each vascular hotspot at X200magnification (X20objective lens and X10ocular lens).The mean of the number in those three hot spot regions was recorded and demarcated as MVD.

The VMI was defined as the ratio of α-SMA-positive vessels to CD105-positive vessels in the same vascular hot spot regions in which MVD was calculated. The VMI achieved as a quantity assessment of vessel maturation [18].

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Statistical analysis

Analysis of data was carried out using the available statistical package of SPSS-22(Statistical Package for Social Science-version 22). Data were presented in simple measures of mean, standard deviation, degree of freedom, and range "minimum and maximum values" for continuous variables. Using independent student-t-test for difference between two means, ANOVAs test (the one-way analysis of variance was used to compare the difference between means of several groups), categorical variables were represented by number(n) and percentage (%) and the different percentages were tested using Chi-square test(x^2).
Statistical significance was considered whenever the P value was less than 0.05.

RESULTS

This study included thirty six of histopathological confirmed OSCC cases, males were 20 (55.6%) while female were 16 (44.4%). Patient's age ranged between 22-83 years, and the mean age was 52.4 years. The most predominant age group was (70-79) years (27.78%).Ulcer was the most predominant clinical presentation in the study sample as 21 cases(58.33%).The most predominant effected site with tumor was tongue of 17 cases (47%), followed by floor of mouth of 7 cases (19%).Most predominant histological grade was moderately differentiated as 14 cases (38.89%).

Immune his to chemical Evaluation of Ang-2 antibody:

The expression of Ang-2 considered positive only when (MVS+SIS≥ 3), so 27 cases (75%) were positive while 9 cases (25%) were negative.

Table1: showed the association between Ang-2 expression and each of (age, gender, site, clinical presentation) which were found to be statistically non significance (P value>0.05).

Table 2: The association between Angio-2 expression &age, gender, site, and clinical presentation

Clinical Parameter		Ang-2						
		negative		Positive		Total		
		No	%	No	%	No	%	P-Value
AGE	<0r=39	1.0	11.11	6.0	22.22	7.0	19.44	0.415
	40-49	1.0	11.11	4.0	14.81	5.0	13.89	
	50-59	3.0	33.33	2.0	7.41	5.0	13.89	
	60-69	2.0	22.22	7.0	25.93	9.0	25.00	
	>0r=70	2.0	22.22	8.0	29.63	10.0	27.78	
Gender	Male	6.0	66.67	14.0	51.85	20.0	55.56	0.439
	Female	3.0	33.33	13.0	48.15	16.0	44.44	
SITE	Tongue	5.0	55.56	12.0	44.44	17.0	47.22	0.325
	Floor of mouth	.0	.00	7.0	25.93	7.0	19.44	
	Buccal mucosa	1.0	11.11	3.0	11.11	4.0	11.11	
	Man dib	2.0	22.22	1.0	3.70	3.0	8.33	
	Soft palate	1.0	11.11	1.0	3.70	2.0	5.56	
	Hard palate	.0	.00	2.0	7.41	2.0	5.56	
	Alveolar ridge	.0	.00	1.0	3.70	1.0	2.78	
Presentation	Ulcer	7.0	77.78	14.0	51.85	21.0	58.33	0.172
	Mass	2.0	22.22	13.0	48.15	15.0	41.67	

The association was found to be statistically significance between Ang-2 expression and the histological grade of the study sample (P value<0.05).

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Table2: The association between the Angio-2 expression& Grade

Ang-2 Expression	GRADE							
	W.D		M.D		P.D		Total	
	N	%	N	%	N	%	n	%
Negative	7	53.85	1	7.14	1	11.11	9	25.00
Positive	6	46.15	13	92.86	8	88.89	27	75.00
Total	13	100.00	14	100.00	9	100.00	36	100.00

χ^2=9.078 df=2 p=0.011*
Ang-2: Angiopoietin-2 antibody.
W.D: Well differentiated grade.
M.D: Moderately differentiated grade.
P.D: Poorly differentiated grade.
*: significant association.

Quantification of MVD

Quantitative analysis of angiogenesis was done by measuring MVD using anti CD105 antibody. MVD ranged between (8.8-29), the average is 18.9. There is no statistical association between MVD and clinic pathological parameters (age, gender, clinical presentation, sites and histological grades of OSCC sample; P value>0.05).

The highest mean value of MVD (18.04±4.72) was in (40-49) age group, in female (17.19±5.56), in the floor of the mouth (18.62±4.22), and in ulcer (16.93±3.91).

The association between Angio-2 expression and MVD was found to be statistically significance (P value< 0.05). (Table 4).

Quantification of VMI

There is no significant association between VMI and clinic pathological parameter(P value>0.05), the highest mean values of VMI were appeared in(40-49)age group as(0.36±0.7), in male as(0.34±0.07), in the mandible as(0.37±0.03), and in ulcers as (0.34).

The association between VMI and histological grade of the studied tumor was found to be statistically highly significance (P value<0.05).

The association between VMI and Ang-2 expression was highly significant (P value<0.01).

Table3: The distribution of study sample according to MVD, VMI and grade

		Mean±SD	ANOVA test	P-value
MVD	**Well differentiated**	15.18±2.29	F=0.728	0.490
	Moderately differentiated	17.17±3.87		
	Poorly differentiated	17.05±4.55		
VMI	**Well differentiated**	0.46±0.06	F=21.422	0.0001*
	Moderately	0.28±0.09		

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	differentiated			
	Poorly differentiated	0.23±0.09		

*: Significance (P value<0.05).

Table4: the distribution of study sample according to MVD, VMI & Angio-2 expression.

	Angio-2	N	Mean ± SD	Test	P-value
MVD	Positive	27	19.098±4.54	t=2.284	0.029*
	Negative	9	15.42±2.54		
VMI	Positive	27	0. 29±.06	t=-3.151	0.003*
	Negative	9	0.44±.09		

.*: Significance (P value<0.05).

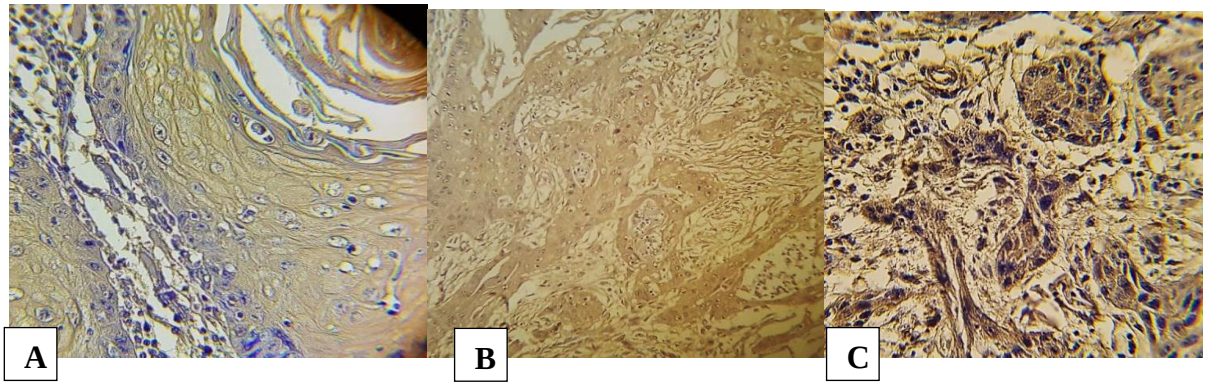

Figure 2: Expression of Ang-2 inOSCC tissue sample (A. in well differentiated OSCC, B in moderately differentiated OSCC, and D. in poorly differentiated OSCC.

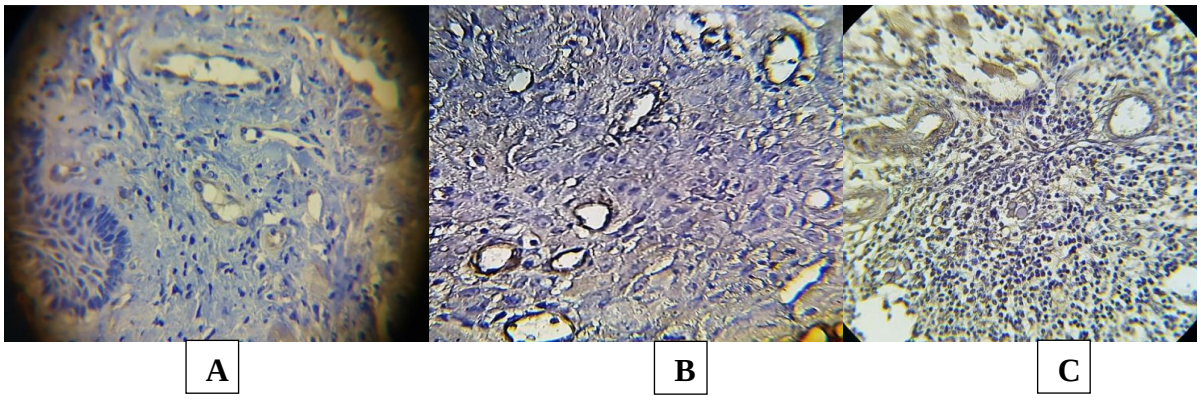

Figure3: Expressionof CD105 in A-Well, B-Moderately, C- Poorly differentiated OSCC tissue sample(X 200).

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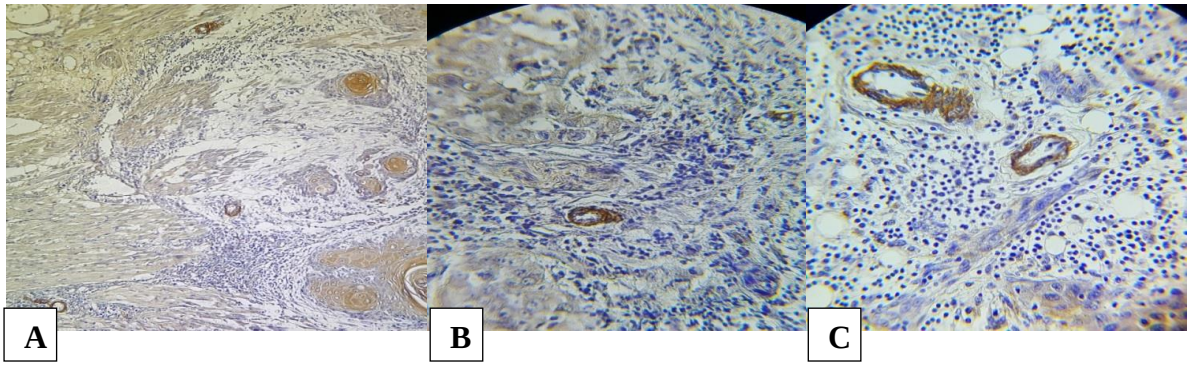

Figure4: Expression of α-SMA in Well, Moderately, and poorly differentiated OSCC tissue sample (A: X100, B and C: X200).

DISCUSSION

Oral squamous cell carcinoma (OSCC) characterizes about more than 90% of all oral cancers [1]. Numbers of studies have showed that angiogenic activators play a great role in the development and metastasis of tumors. May be responsible for the high percentage occurrence of OSCC in the current study (more than 25% of the cases).

In the current study, there is a remarkable up regulation of Ang-2 expression (75% of cases showed positive expression of Ang-2). In addition, there is a statistical association between Ang-2 expression and histological grade(out of 9 cases of poorly differentiated OSCC grade, only one case was negatively expressed for Ang-2)this agreed with some authors stated that, s in OSCC, Ang-2 over expression comes along with increased malignancy and poor prognosis. [11, 20].

The association between the expression of Ang-2 and both MVD, VMI were found to be statically significant (P value=0.029, 0.003 respectively) which were agreed with previous studies reporting that the Ang-2 was an effective marker in angiogenesis [19, 22]. Over expression of Ang-2, leads to block the binding of Ang-1 with Tie-2 tyrosine kinase receptor expressed on ECs (stopped phosphorylation) instead Ang-2 bind to Tie-2 receptors which lead to enhance the ECs to proliferate and promote angiogenesis with less recruitment of mural cells in newly formed BV [12].

Assessment of CD 105 immunohistochemical expression

CD105, Enderlin, is a glycoprotein expressed on the cell membrane of ECs considered as a component of the transforming growth factor (TGF-β) receptor and involved in TGF-β signaling pathway [23]. It plays an important role in modulation of the angiogenesis because it regulates proliferation, differentiation, and migration of ECs [24]. In the current study, There is a statistical association between MVD with Ang-2 (P value=0.029) which in consent with previous studies that showed CD105 was highly expressed in tumor BVs [25, 26]. Some authors proposed that using anti-CD105 antibodies in measuring MVD has advantages upon other endothelial markers in determine the pathological behavior of the tumor and predict the outcome of the disease because that CD105 expression was mainly found to be restricted on activated ECs which involved in angiogenesis [27, 28].

Assessment of immunohistochemical expression of α-smooth muscle actin

MVD is limited to indicate the number of newly formed BVs without reflection of the functional status of them[22], so the assessment of α -SMA expression within the same hot spots in which quantification of BVs occurred may indicates the level of maturity of the angiogenic BVs in the tumor. In the current study, VMI was calculated as a ratio between α-SMA+ to MVD. SMA Immunostaining has been identified in the cytoplasm of the smooth muscle fibers, pericytes [29].

In the current study, there is a high significant association between VMI and histological grade (p value=0.0001) this finding is agreed with some authors [30] and disagreed with others [11].

The most important elements in the mediation of vascular stabilization are mural cells "pericytes and v SMCs"[31]. Both of them are providing an essential mechanical and physiological maintenance to ECs [32, 33] which is performed by the possible interactions between them [34].Pericytes on micro vessel of cancerous tissues are In contrast to those of normal capillaries, in which they are lightly connected toECs and show an irregular shape, occasionally their processes leave the endothelium toward the tumor cells [35], so the resulted BVs are immature and able for continuous angiogenesis [36].

CONCLUSION

Ang-2 was found to be highly expressed in OSCC, and significantly associated with histological differentiation of the tumor. It could be act as an angiogenic marker in enhancement new BVs formation and decrease their levels of maturation.

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