Lymphangiogenesis Using D2-40 in Oral Squamous Cell and Mucoepidermoid Carcinoma and its Correlation with Clinicopathological parameters

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Abstract

Background: Oral squamous cell carcinoma (OSCC) and Mucoepidermoid carcinoma (MEC) are the most common malignant tumor of the oral cavity and salivary gland respectively, the extent of lymph node (LN) metastasis by lymphangiogenesis is a major determinant for the staging and the prognosis of these malignancies and often guides therapeutic decisions.

Objective: To correlate the lymphangiogenesis of OSCC and MEC with their clinicopathological parameters.

Material and Methods: Fifteen oral squamous cell carcinoma and eighteen mucoepidermoid carcinoma tissue samples collected during the period from 2008 till 2014. Age, gender, site of tumors and stage were reviewed. Lymphangiogenesis obtained by Immunohistochemical findings using D2-40 immunomarker.

Results: All cases of OSCC and MEC exhibited positive immunostaining for lymphangiogenic marker. There is a correlation between OSCC and MEC regarding the age group and site of tumors (P=0.001), while gender, lymphatic vessel density (LVD) and TNM stage have no correlation with OSCC and MEC. Lymphatic vessel density and showed no relation with stage of OSCC and MEC.

Conclusion: No statistical correlation was found between LVD which expressed by mean of lymphangiogenesis using D2-40 immunomarker and OSCC MEC stage.

Key word: Oral squamous cell carcinoma (OSCC), Mucoepidermoid carcinoma (MEC), D2-40, Lymphangiogenesis, lymph node (LN).

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Introduction

Oral squamous cell carcinoma (OSCC) is the most common malignant tumor of the oral cavity and one of the 10th most common causes of death. It arises from dysplastic oral squamous epithelium [1]. In previous studies [2]. Oral squamous cell carcinoma histologically confirmed the presence of nodal metastases by mean of lymphangiogenesis, Woolgar *et al.*, [2] found that the 3-year survival probability was similar in those with macroscopic or microscopic Extracapsular spread (ECS) (33% and 36%, respectively) and much worse than the rate of 72% for those with strict intranodal metastases. Additionally, it has been found that patients with multiple metastatic nodes have poorer prognosis [3].

Mucoepidermoid carcinoma (MEC) comprises approximately 10–15% of all salivary gland neoplasms and about 30% of salivary malignancies [4, 5]. It have; uniform

age distribution (20-70 years) [6, 7], one of the most common malignancies of the salivary glands in children's (8) (especially of the parotid gland) and usually appears as an asymptomatic swelling. Pain or facial nerve palsy may develop, usually in association with high-grade tumors. The minor glands constitute the second most common site [6, 9].

In order to establish lymph node (LN) metastasis, it is essential for tumor lymphangiogenesis to be induced and determined by mean of assessment the lymphatic vessel density (LVD) [10].

Metastasis; is the spread of cells from the primary neoplasm to the any other lymph nodes, distant organs, and it is the most serious aspect of cancer. Several pathways may contribute to the dissemination of primary malignant cancer cells, local invasion into the surrounding tissue, systemic metastasis via tumor-associated blood vessels to distant organs and lymphatic metastasis via tumor associated lymphatic vessels to draining LN, distal LN, and from there to distal organs [11].

The extent of LN metastasis is a major determinant for the staging and the prognosis of most human malignancies and often guides therapeutic decisions. Although the clinical significance of LN involvement is well documented, little is known about the molecular mechanisms that promote tumor spread via lymphatic vessels to sentinel and distal LN and beyond [11].

Research into the role of the lymphatic system in cancer metastasis has been hampered by the lack of specific markers that distinguish lymphatic vessels from blood vessels and by the lack of identified lymphatic-specific growth factors. However, recent discoveries have identified novel lymphatic specific markers, including podoplanin [11]

Lymphangiogenesis represents a dynamic process during embryogenesis, but largely absent after birth, under normal physiological circumstances [12]. Under pathological circumstances, such as inflammation, tissue repair and tumor growth, lymphangiogenesis has got a major contribution to the formation of new lymphatic vessels, by proliferating and germinating the endothelial cells within the pre-existent lymphatic vessels [13, 14].

The recent evidence suggests an active role of malignant tumors in the induction of intratumoral and peritumoral lymphangiogenesis [15]. D2-40 is a novel new selective immunomarker specific for lymphatic endothelium; it does not stain vascular endothelium [16,17].

The objective of the present study was to evaluate the immunohistochemical expression of D2-40 marker to evaluate the lymphangiogenic activity of OSCC and MEC and compare between them and correlate these findings with their clinicopathological parameters.

Materials and Methods

Thirty three formalin-fixed, paraffin-embedded (FFPE) tissue blocks (fifteen squamous cell carcinoma and eighteen Mucoepidermoid carcinoma) obtained from the achieves of department of oral pathology/ college of Dentistry- Baghdad University, and some private laboratories were included in this study. Data concerning patient's age, gender, site, clinical presentation, clinical staging and histopathological grading were obtained from the associated reports. Tumor slides were reviewed by two histopathologists, and the representative paraffin blocks were selected.

Sections of 4-µm thickness was cut from each tissue block and mounted on positively charged slides (Esco, USA) to be stained with monoclonal antibodies to D2-40 lymphangiogenic marker (Abcam ab77854). Negative and positive tissue controls were included into each immunohistochemical run.

Immunohistochemical staining procedure

The slides were baked in hot air oven at 65°C overnight. Sections were sequentially de waxed through a series of xylene, graded alcohol and water immersion steps. For D2-40, drops of hydrogen peroxide block were added to slides were in a ready to use package (ab77854); All slides was followed by the application of the primary antibodies with a dilution of 1:40. The slides were incubated for 1 h at 37°C and then kept at 4°C in a humid chamber overnight. Next day, after washing with PBS (Phosphate Bupher Solution), biotinylated antimouse IgG were applied to the sections, incubated and rinsed with a stream of PBS. Conjugated antibodies were visualized with DAB chromogen. Sections were counterstained with Mayer's hematoxylin for 1–2 min, dehydrated and mounted, all this laboratory procedures done the college of dentistry/ Laboratory of department of oral pathology.

Lymphatic Vessel Density Determination (LVD)

Under low power (X10) all slides were scanned to select six fields with the highest number of stained lymphatic vessels that were identified as 'hotspots' (the area of greatest number of highlighted lymphatic vessels). In three intratumorally and three peritumorally (within an area of 1mm from the invasion front) the LVD was countered as the number of stained vessels per optical field (18)and the number of D2-40 positive vessels was calculated in each hotspot at a higher magnification (X40) and the average of them was obtained as total LVD (18,19).

All variables were compared using Chi-square test. While Pearson correlation coefficient was applied to plot a correlation .P values of less than 0.05 were considered statistically significant.

Results

The study samples consisted of fifteen FFPE blocks OSCC and eighteen MEC after diagnosis have been confirmed. The age range for OSCC was from (40-79) and the most predominant group was(50-59) affected 6 cases(40%) while for MEC, the age range was from (10-69) and the most predominant group was (40-49) affected 7cases (38.8%) with almost an equal mean±sd age for both OSCC and MEC (59.84±9.55) and (46.16±10.41) respectively, from statistical point, there is a significant relationship between age of OSCC and MEC .Table (1)

Within OSCC the male cases were 7 cases that constitute (53.8%) of the total OSCC samples while the female cases were 6 cases that constitute (46.2%) of the total OSCC samples, on the other hand with in MEC cases the male were 10 cases that constitute (55.6%) of the total MEC samples while the female cases were 8 cases that constitute (44.4%) of the total MEC samples and there is no significant relationship between gender of OSCC and MEC (Table 2).

Regarding the tumor site (Table 3) ,with in OSCC samples; the most effected site was lower lip 5 (35.7 %) then followed by tongue 4(28.6 %) , buccal mucosa 4 (28.6 %) and palate 1 (7.1 %) ,while the most effected site in MEC was submandibular gland 6 (33.3 %) followed by parotid gland 5 (27.8 %) and palate 4 (22.2 %), no expression have been seen with tongue and lower lip . from statistical point of view , there is a significant relationship between OSCC and MEC regarding site .

According to the clinical data , the TNM staging system have been assessed on 9 cases only of OSCC samples, 6(66.7%) were stage I,3 cases (33.3%) stage II, no cases found in stage III , IV ,while With MEC 15 cases were assessed, 5 (33.3%) cases were stage I, 3(20.0%) stage II ,4 (26.7%) cases were stage III ,2(13.3%) cases stage IV and 1 (6.7%) case was stage IVA and there is no

significant relationship between TNM stage of OSCC and MEC (P=0.202^{NS}) (Table 4).

Assessment of D2-40 immunostaining in OSCC and MEC

All cases of OSCC and MEC exhibited positive immunostaining for D2-40 lymphangiogenic marker (Fig. 1, 2) (Table 5,6) .

Generally lymphatic vessels in all samples were determined by brown immunostaining of endothelial cells lining the lymphatic vessels in both OSCC & MEC (Table 7,8)

The results of this study showed a higher mean±sd of ILVD (Intra-tumoral lymphatic vessel density) of OSCC in stage I (17.16±23.16) than stage II (2.70±2.68) while PLVD (peri-tumoral lymphatic vessel density) less in stage I (5.43±4.07) than stage II (7.20±5.21), but the TLVD was higher in stage I (22.59±7.63) than stage II (9.90±5.21) however, non-significant results revealed by ANOVA test between LVD and tumor stage (Table 5)

Concerning the MEC; this study showed a higher (mean±sd) of ILVD in stage I (15.14±18.02) than other stages, while PLVD was higher in stage III (8.22±7.19) than other stages, but the TLVD was higher in stage I (21.24±22.44) in comparisons to all other stages, however; nonsignificant results revealed by ANOVA test between LVD and tumor stage (Table 6).

Regarding TNM staging system of both OSCC and MEC and application of Chi-Square Test; it appears that the relation values; did not reached the statistical point of view between the two tumors (Table 4).

Table (1): Case distribution of OSCC and MEC according to age groups and their relation.

Age groups	MEC		Age groups	OSCC	
	NO.	%		NO.	%
10-19	1	5.5	40-49	2	13.3
20-29	0	0	50-59	6	40
30-39	3	16.6	60-69	3	20
40-49	7	38.8	70-79	4	26.6
50-59	6	33.3			
60-69	1	5.5			
Total	18	99.7%	Total	15	99.9%

	Type	N	Mean	Std. Deviation
Age	MEC	18	46.16	10.41
	SCC	13	59.84	9.55

	* t-test for Equality of Means		
	t	df	Sig. (2-tailed)
	-3.734-	29	.001

* Significant relation (p≤0.05)

NS Non-significant relation (p> 0.05)

Table (2): Case distribution according to sex.

Gender	TYPE		
	OSCC	**MEC**	**Total**
F	6 (46.2%)	8 (44.4%)	14 (45.2%)
M	7 (53.8%)	10 (55.6%)	17 (54.8%)
Total	13 (100%)	18 (100%)	31 (100%)
	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	.009	1	.925 ^{NS}

* Significant relation (p≤0.05)
^{NS} Non-significant relation (p> 0.05)

Table (3): Case distribution according to Site

	Site						Total
	Parotid	**Palate**	**Sub mandibular**	**Buccal mucosa**	**Tongue**	**Lower Lip**	
OSCC	0 (0.0%)	1 (7.1%)	0 (0.0%)	4 (28.6%)	4 (28.6%)	5 (35.7%)	14 (100%)
MEC	5 (27.8%)	4 (22.2%)	6 (33.3%)	3 (16.7%)	0 (0.0%)	0 (0.0%)	18 (100%)
*Pearson Chi-Square	Value	df	Asymp. Sig. (2-sided)				
	21.783	5	.001				

* Significant relation (p≤0.05)
^{NS} Non-significant relation (p> 0.05)

Table (4): Correlation between TNM stage of OSCC and MEC samples.

	TNM Stage					
	I	**II**	**III**	**IV**	**IVA**	**Total**
OSCC	6 (66.7%)	3 (33.3%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	9 (100$)
MEC	5 (33.3%)	3 (20.0%)	4 (26.7%)	2 (13.3%)	1 (6.7%)	15 (100%)
Pearson Chi-Square	Value	df	Asymp. Sig. (2-sided)			
	5.964	4	.202 ^{NS}			

* Significant relation (p≤0.05)
^{NS} Non-significant relation (p> 0.05)

Table (5): Correlation between ILVD, PLVD, TLVD of OSCC and TNM stage.

OSCC		*TNM Stage*					
		I N=6	**II N=3**	**III N=0**	**IV N=0**	**Test**	**P Value**
LVD (D2-40) mean±sd	ILVD	17.16±23.16	2.70±2.68	-	-	**ANOVA**	0. .332 ^{NS}
	PLVD	5.43±4.07	7.20±5.21	-	-	**ANOVA**	0. 591^{NS}
	TLVD	22.59±7.63	9.90±5.21	-	-	**ANOVA**	0. 503^{NS}

* Significant relation ($p \leq 0.05$)
^{NS} Non-significant relation ($p > 0.05$)

Table (6): Correlation between ILVD, PLVD, TLVD of MEC and TNM stage.

MEC		*TNM Stage*					
		I N=5	**II N=3**	**III N=4**	**IVA N=3**	**Test**	**P Value**
LVD (D2-40) mean±sd	ILVD	15.14±18.02	8.13±1.90	7.72±8.03	8.50±6.55	**ANOVA**	0. 765^{NS}
	PLVD	6.10±5.99	6.73±2.53	8.22±7.19	5.73±2.61	**ANOVA**	0. 923^{NS}
	TLVD	21.24±22.44	14.86±2.04	15.95±7.09	14.23±8.86	**ANOVA**	0. 891 ^{NS}

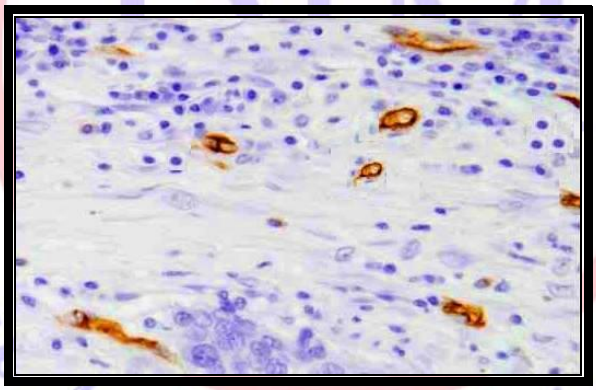

Figure (1): Photomicrograph showing OSCC with D2-40 immunostaining-positive lymphatic vessel. (Original magnification X40).

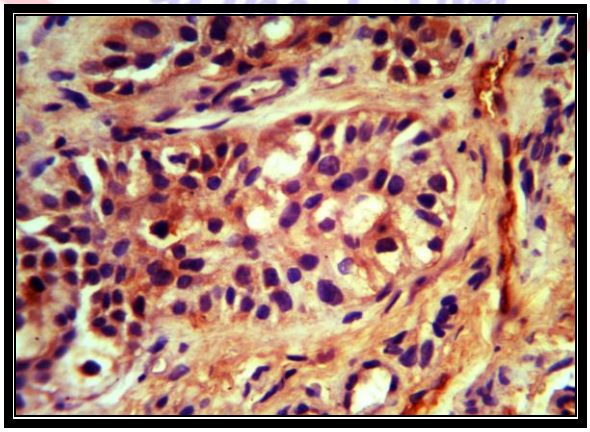

Figure (2): Photomicrograph showing MEC with D2-40 immunostaining-positive lymphatic vessel. (Original magnification X40)

Discussion

Tumors metastasis to regional lymph nodes is a crucial step in the progression of cancer. Detection of tumour cells in the lymph nodes is an indication of the spread of the tumour, and is used clinically as a prognostic tool and a guide to therapy [20].

This present study showed 6 (40 %) of patients with OSCC were over 50 years of age. Similar findings were reported in Iraqi study [21, 22]. And studies in other part of the world [23, 24].

While other studies show the majority of age were above 40 years [25, 26, 27, 28, 29]. on other side the MEC cases showed that 7(38.8%) of cases over 40 years of age which is similar to international study [30] ,this study showed that there is a significant relationship between the age of both OSCC and MEC .

The OSCC.; is predominantly a disease of men in all age groups [31,32,33,34] as we noticed in our study where 7 (53.8%) cases male, but some studies revealed a female predilection in young patients with OSCC [35,36] , also in MEC the male is the most affected cases 10 (55.6%) as found in other studies (4,37) .This distribution may due to that the males is more exposed themselves to carcinogenic factors such as cigarette smoking, alcohol consumption and others.

Regarding the site distribution of the tumor , with in OSCC the majority of cases were in lower lip 5 (35.7%) and this disagree with several studies [25,38,39], this demonstrated that buccal mucosa were the most effected site while other studied [28,40,41,42] demonstrated that the tongue were the most effected site , on other hand in MEC the submandibular glands were the most effected site and this disagree with international article in the world [4], however the significant statistical relation could be credit with the fact that the current study and some of the others are not an epidemiological type of studies, therefore the small sample size exclude for definitive clinical findings.

Depending on the clinical data that collected for both OSCC and MEC cases, the TNM stag for both were assessed on 9 cases of OSCC and 15 cases of MEC and the statistical results that appeared show that there is no correlation between the stages of OSCC and MEC respectively .this results may related to the facts of the variation in the methods of the data collection and the samples size (P=0.202NS) (Table 4).

Regarding the OSCC , a study showed that lymphangiogenesis was associated with metastasis in OSCC patients [43], In addition, the presence of lymph node metastasis was significantly associated with a higher intratumoral lymphatic density [44], however this study showed a higher mean±sd of ILVD in stage I for both OSCC (17.16±23.16) and MEC (15.14±18.02) respectively and in spite of that there is no significant relation between ILVD and stage I of OSCC and MEC , this is also found in other international study regarding OSCC [45], on other hand the TLVD was higher in stage I in both OSCC (22.59±7.63) and MEC (21.24±22.44) respectively , but in spit of this results , there is no significant statistical relation between TNM stage of OSCC and MEC (P=0.202 *NS*) . Table (4). This is may be due to differences in sample size and clinical data collected.

Conclusion

Only significant relations were found between age, site of OSCC and MEC, on other side no relation found among sex, TNM stage and TLVD with each OSCC and MEC respectively.

This study shoewd immunohistochemical findings obtained by D2-40 considering the lymphangiogenesis have no relation with TNM stage of OSCC and MEC in spite of that, lymph node

involvement that depend on ability of tumor cells to reach the LN by mean of lymphangiogenesis, since lymph node involvement cannot predict truly the tumor prognosis, an additional criteria should be studied, and added to the old standard criteria.

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