Immunohistochemical Expression of CD34 as Biological Marker of Angiogenesis and Expression of D2-40 as Marker of Lymphangiogenesis in Mucoepidermoid Carcinoma of Salivary Glands

Mostafa G. Taher[1], Bashar H. Abdullah[2], Luay E. Al-Khuri[3]

[1]Assistant Lecturer, Department of Pathology, College of Medicine, Diyala University, Diyala, Iraq
[2]Assistant Professor, Department of Oral Pathology, College of Dentistry, University of Baghdad, Baghdad, Iraq
[3]Assistant Professor, Department of Pathology, College of Medicine, University of Baghdad, Baghdad, Iraq

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Correspondence to: Mostafa G. Taher

Address: College of Medicine, Diyala University, Diyala, Iraq

Email: mostafa.ghany@hotmail.com

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ABSTRACT

BACKGROUND: Mucoepidermoid carcinoma (MEC) is a malignant epithelial neoplasm characterized by the proliferation of epidermis, mucous, and intermediate cells in various proportions. This study evaluates the immunohistochemical expression of CD34 and D2-40 as marker of angiogenesis and lymphangiogenesis of MEC respectively and their correlation with the tumor grade and stage.

MATERIALS AND METHODS: We studied 22 salivary gland MEC tissue samples collected between 1972 and 2010. Age, sex, site, stage and histologic grades were reviewed. The samples were immunohistochemically stained with monoclonal antibodies against CD34 and D2-40.

RESULTS: The stage of MEC had a significant relationship with Brandwein grading system (P=0.039). The mean of microvessel density count by CD34 immunomarker was (10.74±5.97) with no significant relation to tumor grade or stage (P=0.579, P=0.438).The lymphatic vessel density expressed by D2-40 immunomarker was (18.15±15.92) which was also not significantly related to tumor grade or stage (P=0.573, P=0.773).

CONCLUSIONS: Microvessel density and lymph vessel density were not associated with tumor grade or stage. Thus, we found no correlation between the histological grade or tumor stage of MEC and angiogenesis or lymphangiogenesis.

Keywords: Mucoepidermoid Carcinoma; CD34 Antigen; D2-40 MAb; Lymphangiogenesis

INTRODUCTION

Salivary gland tumors (SGTs) constitute an important area in the field of oral and maxillofacial pathology. Its incidence around the world ranges from about 1.0 to 6.5 cases per 100,000 people per year, and it represents 2-4% of head and neck neoplasms [1]. Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumors, with uniform distribution between the ages of 20 and 70 years [1, 2]. It is the most common malignant salivary gland tumors in children [3, 4]. Histopathologically, MEC is composed of a mixture of mucus-producing, intermediate and squamous (epidermoid) cells [5, 6, 7, 8].

Due to their high metabolic needs, malignant tumors have to induce formation of new blood and lymphatic vessels [10, 11, 12, 13, 14, 15]. Angiogenesis is the process of forming new blood vessels [9]. Immunohistochemical staining for CD34 is a sensitive and well-studied marker of vascular epithelium and a useful tool to determine microvessel density within tumors. CD34 staining has been established to be useful in predicting tumor relapse or metastasis [16].

Microvessel density evaluated by CD34 immunostaining had been studied as prognostic marker relevant in MEC [17]. Lymphangiogenesis, the formation of new lymphatic vessels, has also been implicated in metastasis. Recent evidence suggests an active role of malignant tumors in the induction of intratumoral and peritumoral lymphangiogenesis [18]. D2-40 is a novel new selective immunomarker specific for lymphatic endothelium; it does not stain vascular endothelium [19].

While tumors induce both angiogenesis and lymphangiogenesis, their metastatic potential may be related to the extent of these two processes within tumors. While angiogenesis within MEC tumors has been studied with CD34, lymphangiogenesis with D2-14 has not been examined. Only after examining both processes in a tumor, the true metastatic potential of a tumor can be predicted. Thus, we hypothesized that the differences in the malignant and metastatic behavior of MECs can be explained by the differences in the extent of angiogenesis and lymphangiogenesis. To test this hypothesis, we examined the association of MEC tumor grade and stage with its CD34 and D2-40 staining.

MATERIALS AND METHODS

Twenty two formalin-fixed paraffin-embedded tissue blocks of salivary gland MEC were collected from the Department of Oral Diagnosis, College of Dentistry, University of Baghdad from the period of 1972 to 2010. Four micrometer thick sections were cut from each paraffin tissue block and stained with hematoxylin and eosin for diagnostic confirmation and histological grading. Tumors were classified into low, intermediate and high grade MEC according to Brandwein grading system [20]. TNM staging was applied to 18 cases only in which the required clinical data relevant to tumor stage were properly mentioned in the case sheet. Another 4µm thick section was cut from each tissue block and mounted on positively charged slides (Esco, USA) to be stained with CD34 monoclonal antibodies (USBiological-C2386-10). Negative and positive tissue controls were included into each immunohistochemical run.

Immunohistochemical staining procedure

Slides were baked in hot air oven at 65°C overnight. Sections were sequentially dewaxed through a series of xylene, graded alcohol, and water immersion steps. For CD34, endogenous peroxidase activity was blocked with 0.03% hydrogen peroxide followed by blocking the nonspecific antibody binding with normal goat serum (USBiological-I7506A). Primary CD34 antibodies at a dilution of 1:40 were applied to all slides. The slides were then incubated for 1 hour at 37°C and kept at 4°C in a humid chamber overnight. Next day, after washing the sections with phosphate buffer solution (PBS), biotinylatedantimouse IgG was applied to slides followed by incubation and rinsing with a stream of PBS. Conjugated antibodies were visualized with diaminobenzidine (DAB) chromogen stain. Sections were counterstained with Mayer's hematoxylin for 1–2 minutes, dehydrated and mounted.

Assessment of immunohistochemical results

Microvessel Density Determination (MVD)

To determine the microvessel density, the stained normal and cancer tissue sections were initially screened microscopically at low power (X10) to identify the areas of highest vascularization ("hotspots"). Five intratumoral and peritumoral high power (X40) fields were then chosen randomly, and the number of microvessels in each high power field was counted for each sample. MVD for each sample was taken as the mean of the five values obtained. Both peritumoral and intratumoral MVD were counted separately, and total MVD was obtained [21, 22].

Lymphatic Vessel Density Determination (LVD)

All MEC slides were scanned at low power (X10) 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 intratumoral and three peritumoral (within an area of 1mm from the invasion front), the LVD was countered as the number of stained vessels per optical field 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. In addition, the whole tumor area was scanned to determine lymphatic vessel invasion LVI (the presence of tumor cells within a lymph vessel) in each case. Intratumoral or peritumoral LVI was considered evident if at least one tumor cell cluster was clearly visible inside a D2-40 positive vessel. [23,24]. D2-40 expression was also evaluated in

tumor cells. Only the positivity of staining was assessed. Cytoplasmic and/or membranous immunoreactivity were considered a positive. The positivity was evaluated as follows; - <10%, + 10-25%, ++ 26-50%, +++51-100% [25].

The data was analyzed with SPSS (Statistical Package for the Social Sciences) statistical software (Version 17). Chi square and ANOVA tests were applied to compare variables as needed. Pearson's correlation coefficient was applied to plot a correlation matrix among the different immunohistochemical markers expression values. P values of less than 0.05 were considered significant, and less than 0.01 were considered highly significant.

RESULTS

The sample comprised 14 males and 8 females. The age range of the patients with MEC was between 19 and 65 years (mean=45.9±10.53). Submandibular gland was most affected (7 cases) followed by palate (6 cases), parotid gland (5 cases) and the buccal mucosa (4 cases). According to Brandwein grading system, 7 cases were found as low grade, 8 were intermediate and 7 cases were high grade. TNM staging system of MEC (only 18 cases) showed 7 cases being stage I, 3 cases stage II, 4 cases stage III and 4 cases stage IV.

The stage of MEC had a highly statistically significant relation with Brandwein grading system (P=0.039). There was no significant relationship between the predominant cell type and grade or stage of the tumor (Table 3). Data showed that the stage of MEC had a significant relationship with Brandwein grading system (P=0.039). The mean of microvessel density count measured in all cases by CD34 immunomarker was 10.74±5.97 (Figure 1, 2); however, no significant relationship was found with tumor grade or stage; P=0.58, P=0.48, respectively (Table 1).

DISCUSSION

In this study, we showed that there is no association between angiogenesis or lymphangiogenesis and tumor grade or stage in

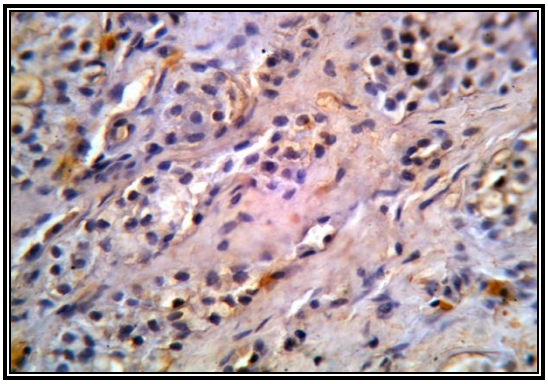

Figure 1: Photomicrograph showing CD34 immunostaining - positive blood vessels (High-grade MEC) (Original magnification X400)

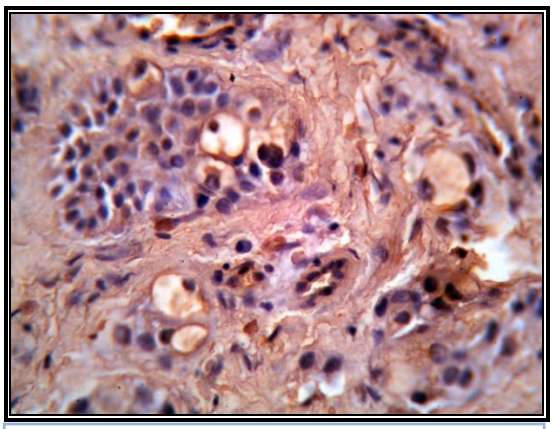

Figure 2: Photomicrograph showing CD34 immunostaining - positive blood vessel (Original magnification X400)

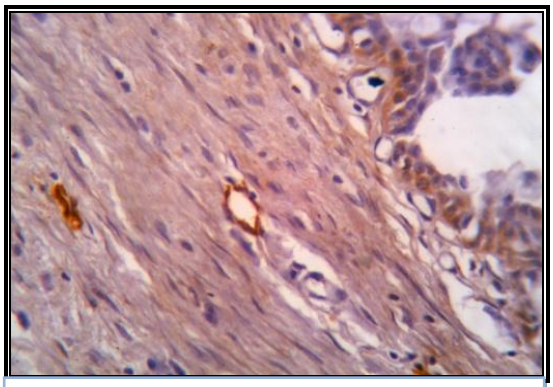

Figure 3: Photomicrograph showing D2-40 immunostaining - positive lymphatic vessel (Low-grade MEC) (Original magnification X400)

		Grade				Stage				
		Low N=7	Inter-mediate N=8	High N=7	P Value ANOVA	I N=7	II N=3	III N=4	IV N=4	P Value ANOVA
MVD (CD34) *mean± sd*	IMvD	5.88±3.57	6.12±3.80	7.31±4.73	0.780 ^{NS}	5.91±2.88	9.33±7.48	5.90±4.38	6.25±1.44	0. 637 ^{NS}
	PMvD	4.54±1.93	3.22±1.96	5.34±3.92	0.336 ^{NS}	2.94±1.85	6.86±6.32	5.25±1.93	4.20±1.57	0. 281 ^{NS}
	TMvD	10.42±3.94	9.35±4.98	12.65±8.57	0.579 ^{NS}	8.85±3.64	16.2±13.81	11.15±4.48	10.45±2.6	0. 428 ^{NS}

Table 1: Immunohistochemical findings of CD34 in relation to tumor grade and stage
^{NS} Non-significant relation (P>0.05)

MEC tumors. We used two well-established methods of studying angiogenesis and lymphangiogenesis in tumors. Most blood vessels were properly stained with the anti-CD34 antibody in our study, which is consistent with findings from other studies [30]. Similarly, D2-40 also stained most of the new lymphatic vessels, although this marker has not been studied in MEC tumors before.

Several studies have examined the association between angiogenesis and MEC tumor grade and staging with inconsistent results. Etemad et al. (2010) found that angiogenesis may have an important role in the pathogenesis of salivary gland MEC and may be useful in the prediction of its biologic behavior depending on the grade and the method of tumor grading. Similarly, studies have found inconsistent association between lymphangiogenesis and tumor grade and stage in head and neck tumors.

We also found that 22% of the tumors had invading cells in the lymphatic system which may explain the ability of MEC to form carcinomatous emboli. This behavior of tumor cells is widespread and other tumors, such as gastric carcinoma, cervical carcinoma, malignant melanoma, oral squamous cell carcinoma, breast carcinoma, and pleomorphic adenoma have also

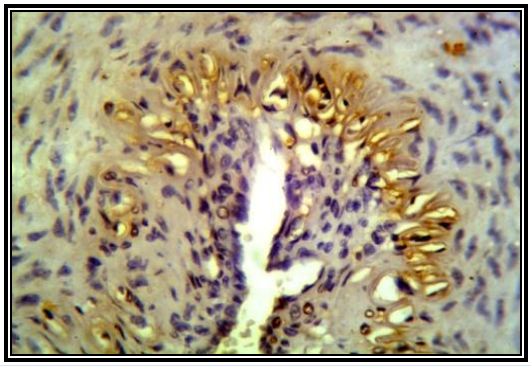

Figure 4: Photomicrograph showing D2-40 immunostaining - positive lymphatic vessels surrounding a negatively stained blood vessel (High-grade MEC) (Original magnification X400)

been reported to have tumor emboli [23, 24, 33-39]. Probably the invasion of tumor cells into the lymphatic vessels may reflect a significant route for the spread of tumor cells to regional lymph nodes [33].

Our study adds information to the MEC literature

in other ways as well. In our study, we found a preponderance of males with MEC, which is in contrast to the reported sex ratio in the literature [7, 28, 29]. This discrepancy may stem from the fact that we studied a different population and the distribution of risk factors between two sexes may be quite different in our population. Consistent with published literature [43], we found a significant association between the MEC tumor stage and the Brandwein grading system, suggesting that the grading criteria are relevant for tumor staging system as well. Also, we did

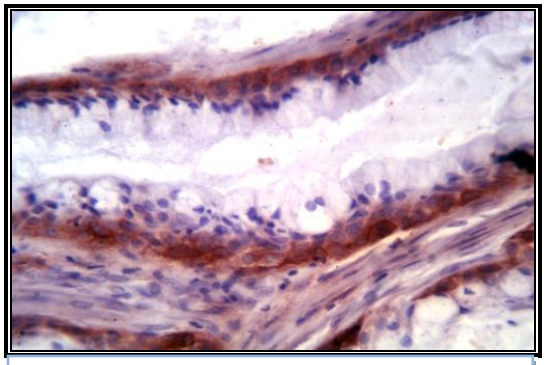

Figure 5: Photomicrograph showing D2-40 positive squamous cells and negative mucous cells (Low-grade MEC) (Original magnification X400)

not find an association between the predominant tumor cell type (squamous, intermediate, or mucous) and tumor grade or stage, in contrast to other studies [36, 43]. This discrepancy may be due to the predominance of low and intermediate grade tumors in our sample; only one case showed a squamous cells predominance and was found to have high tumor stage.

Immunohistochemical findings

Assessment of angiogenesis (CD34 immunostaining)

CD34 was expressed in all 22 cases of MEC (peritumoral and intratumoral). This is in conjunction with a previous study [17] that found the same result in MEC; however, 6 (27.27%) cases showed stromal positivity and cross reaction with perivascular stromal cells and other stromal elements. Similar cross reactivity findings have been reported by a previous study (Figure 1, 2) [31]. Therefore, careful examination is required in assessing and differentiating positively stained CD34 microvessels from positively stained background and stromal cells. The mean value of MVD in the current study increased with higher grade; however, it did not demonstrate a significant relation with Brandwein grading system. Similarly, no relation was seen with TNM stage while a previous study concluded that MVD expressed by CD34 could be considered as biological markers for invading behavior in salivary gland tumors [32]. On the other hand, Kuo et al. [17] found that there was no significant correlation between peritumoral and intratumoral MVD in MECs and the

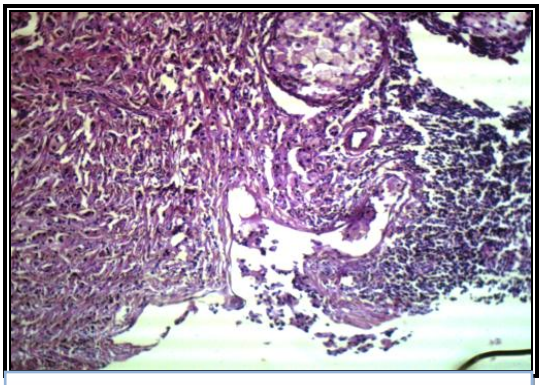

Figure 6: Photomicrograph showing intra lymphatic invasion with tumor emboli (H&E stain) (Original magnification X100)

histological grade, but there was a correlation between intra MVD with its stage. These conflicting results may be due to different method of assessment and differences in tumor grades, one using Brandwein system and the other using Aucliar system. The grading system by Auclair et al. [7] is based on a scoring system of five histologic features: intracystic component <20% (+2), neural invasion (+2), necrosis (+3), four or more mitoses per 10 high power (+3), anaplasia (+4); and based on the total score a case is categorized as low grade (score 0-4), intermediate (score 5-6) and high grade (score 7-14). On the other hand, Brandwein et al. (20) grading system adds three additional histological features: invasion in the form of small nests or islands (+2), lymphovascular invasion (+3) and bony invasion (+3); and based on the total score a case is categorized as low grade (score 0), intermediate (score 2-3) and high grade (score 4 or more).

		Grade				Stage				
		Low N=7	Inter-mediate N=8	High N=7	P Value ANOVA	I N=7	II N=3	III N=4	IV N=4	P Value ANOVA
LVD (D2-40) *mean±sd*	ILVD	3.72±2.27	12.71±14.18	13.05±11.52	0.210 ^{NS}	11.72±15.97	8.40±1.70	7.95±8.05	15.22±14.26	0. 850 ^{NS}
	PLVD	9.62±7.91	5.68±4.64	9.60±9.66	0.515 ^{NS}	5.21±5.22	6.86±2.57	8.30±7.14	12.07±12.80	0. 567 ^{NS}
	TLVD	13.35±7.45	18.4±17.6	22.65±20.4	0.573 ^{NS}	16.94±19.96	15.26±2.02	16.25±9.94	27.30±26.82	0. 773 ^{NS}

Table 2: Immunohistochemical findings of D2-40 in relation to tumor grade and stage
^{NS} Non-significant relation (P>0.05)

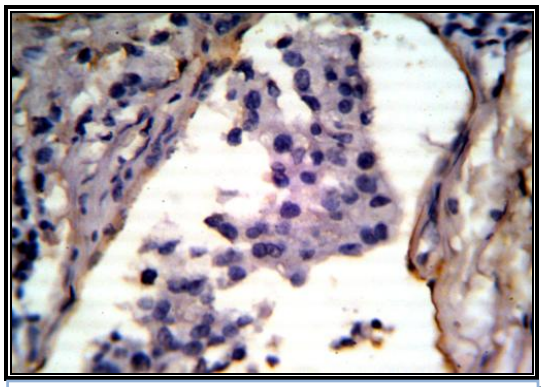

Figure 7: Photomicrograph showing intra lymphatic invasion with tumor emboli, D2-40 positive lymphatic vessel (High-grade MEC) (Original magnification X400)

Assessment of lymphangiogenesis (D2-40 immunostaining)

Epidermoid variety of tumor cells were stained by D2-40 marker in all study samples while mucous cells were not stained. The intermediate cells showed a variable staining pattern. This staining pattern of the intermediate cells may reflect their undifferentiated state with features of both epidermoid and mucous cells. Another implication of this finding may be in differentiating MEC epidermoid cells from cells originating from other tumors. However, studies of D2-40 staining pattern in other tumors are needed before concluding the specificity of D2-40 to MEC epidermoid cells (Figure 3) [16, 29].

The major limitation of our study is its small sample size and its retrospective nature. However, MEC is a relatively rare tumor and we utilized all the samples available to us for this study. Moreover, a prospective study would be prohibitively long. This is the first study of its kind in this population group, which is the strength of this study.

Our results show that angiogenesis and lymphangiogenesis are very frequent in MEC and present in all tumor grades. One corollary of the widespread presence of new vessel formation is that MEC tumors may have high metastasis potential irrespective of the tumor grade.

CONCLUSION

In conclusion, we found no association between histological grade or stage of MEC tumors and angiogenesis or lymphangiogenesis. Additional immunohistochemical characteristics of the tumors must be sought that are predictive of tumor grade and/or stage.

		Predominant cells					Chi square Test
		Intermediate	Squamous	Mucous	Mixed	Total	
Grading	Low	2 (28.6%)	0 (0.0%)	2 (28.6%)	3 (42.9%)	7 (100.0%)	0.139^{NS}
	Intermediate	5 (62.5%)	0 (0.0%)	3 (37.5%)	0 (0.0%)	8 (100.0%)	
	High	1 (14.3%)	1 (14.3%)	1 (14.3%)	4 (57.1%)	7 (100.0%)	
	Total	8 (36.4%)	1 (4.5%)	6 (27.3%)	7 (31.8%)	22 (100.0%)	
TNM Stage	I	4 (57.1%)	0 (0.0%)	2 (33.3%)	1 (25.0%)	7 (38.9%)	0. 279^{NS}
	II	0 (0.0%)	0 (0.0%)	2 (33.3%)	1 (25.0%)	3 (16.7%)	
	III	2 (28.6%)	0 (0.0%)	2 (33.3%)	0 (0.0%)	4 (22.2%)	
	IV	1 (14.3%)	1 (100.0%)	0 (.0%)	2 (50.0%)	4 (22.2%)	
	Total	7 (100.0%)	1 (100.0%)	6 (100.0%)	4 (100.0%)	18 (100.0%)	

Table 3: Predominant cells in relation with tumor grading and TNM stage ^{NS} Non-significant relation (P>0.05)

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