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Evaluation of CD44 Expression in Malignant Salivary Gland Tumors

Nermen S. Mohammed[1*], Amany M. Taha [2], Nadia A. Radi [3], Eman A. AboHager [4]

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azhardentj@azhar.edu.eg

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ABSTRACT

Purpose: The aim of this study was to assess the presence and correlation of CD44 in selected malignant salivary glands tumors (SGTs), carcinoma ex pleomorphic (Ca ex PA), mucoepidermoid carcinoma (MEC) and adenoid cystic carcinoma (ACC) histopathological variants. **Material and Methods:** Thirty-five formalin fixed paraffin embedded blocks of both normal and malignant salivary gland specimens were included. Immunohistochemical analysis was employed to assess the expression of CD44. **Results:** In the present study, comparing all groups revealed that the highest value was recorded in MEC, followed by ACC, then Ca ex PA, while the lowest mean was recorded in normal salivary tissue. ANOVA tests indicated that all groups had statistically significant differences (p=0,00). Comparing all subtypes of ACC revealed a significant difference between solid pattern and cribriform tubular patterns. Also, there is a significant difference between the high and low grades MEC. **Conclusion:** According to the current study, the pattern of expression of CD44 was correlated with the histopathological variants and serve as tumor behavior indicator.

KEYWORDS

Malignant salivary glands tumors; CD44; Cancer stem cells.

INTRODUCTION

Salivary gland tumors (SGTs) are infrequently encountered malignancies characterized by wide variabilities in their morphological characteristics and histological subtypes [(1)]. Previous epidemiological studies demonstrated that the SGTs accounts for nearly 3-6% of head and neck tumors, as well as less than 0.5% of all human malignancies [(2)]. The incidence of SGTs showed substantial geographic variation, with highest rate among Inuit; the global incidence of SGTs was reported to

1. Demonstrator of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.
2. Lecturer of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.
3. Associate Professor of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.
4. Professor of Oral and Dental Pathology. Faculty of Dental Medicine for Girls, Al Azhar University, Cairo, Egypt.

* Corresponding author email: nermenbastawesy.26@azhar.edu.eg

range from approximately 0.5 to 2 cases per 100,000 population [3,4]. SGTs affect mainly elderly population (highest incidence among patients aged more than 60 years old), with equal gender distribution [5]. The malignant SGTs make up just 20% of all SGTs; in addition, the malignant SGTs are both major and minor glands with almost 80% of the major gland tumors in the parotid gland[6]. Various risk factors were implicated in the development of malignant SGTs including radiation exposure, whether therapeutic or occupation, immunosuppression, EBV exposure, HIV infection, and diet low in Vitamin C [4]. Clinically, the presentation of malignant SGTs depends on their site and invasiveness, patients with malignant SGTs can present with facial mass, intraoral mass, or neurological impairment [7]. The prognosis of malignant SGTs is usually poor with high risk of recurrence and distant metastasis; the 5-year survival of spreading SGTs was reported to be 35-65%, with wide variations according to type of SGTs [6,8].

Malignant SGTs are historically categorized into a variety of subtypes with Mucoepidermoid (MEC) as the most common form comprising almost a third of all malignant parotid gland tumors [9]. MEC is classified according to morphological characteristics, extent of invasion to nearby structures, and lymphovascular/bony invasion into low, intermediate, and high-grade tumors[10]. Another type of malignant SGTs that mainly affects submandibular and minor glans is adenoid cystic carcinoma(ACC); the ACC is characterized by early invasion and distant metastasis [11]. Acinic cell carcinoma and carcinoma ex pleomorphic adenoma (CXPA) comprise other histologic subtypes of malignant SGTs [7]. CXPA is characterized as a malignant tumor of the salivary gland resulting from a primary or repeated pleomorphic adenoma (PA). It contains a number of histological sub groups, such as high-grade carcinoma, non-specific adenocarcinoma, sarcomatoid carcinoma and myoepithelial carcinoma [12]. Owing to the presence of heterogenous groups of malignant SGTs, the histopathological assessment and diagnosis of malignant SGTs is challenging; misdiagnosis of malignant SGTs is frequently association with poor survival of the affected patients [13].

CD44 is one of the most commonly identified clusters of differentiation markers, whose gene is located on chromosome 11. CD44 is transmembrane, adhesion, molecule that was previously correlated with the degree of malignancy and therapeutic resistance in various solid cancers[14,15]. In normal tissues, CD44 exerted many physiological functions that range from regulation of hematopoiesis and wound repair to regulation of embryogenesis [16]. Previously, CD44 was found to be a strong biomarker of CSCs and CD44hi cells in a variety of tumors. In tumor development, CD44 is thought to stem from its antiapoptotic properties, which preserve the tumor cells form destruction; through its binding to hyaluronan in cell membrane, the CD44 enables a variety of pathways for signaling that contribute to cell proliferation and survival. Furthermore CD44 has been found to participate in cell signaling, epithelial transition to Mesenchymal cell invasion and metastasis subsequently [17,18]. Overexpressed CD44 was reported to be associated with extensive malignant features and poor prognosis in many cancers [19].

To date, few studies reveal the role of interactive function of CD44 in malignant SGTs. This study was conducted to investigate and compare CD44 expression with the histopathological variants of selected malignant SGTs.

MATERIALS AND METHODS

Case selection

The specimens obtained for this study were collected from the archives of the Department of Oral and Dental Pathology, Faculty of Dental Medicine for Girls, Al-Azhar University and the Department of Oral Pathology, Faculty of Dentistry, Alexandria University as a paraffin embedded block. The cases were divided into two groups: group I; the control

group included five cases of normal salivary gland tissue. Group II was divided into malignant salivary gland tumors, including 30 cases, 10 cases of Ca ex PA, 10 cases of MEC with different grades, and 10 cases of ACC with different patterns.

Immunohistochemical analysis

Four micron (4μ) sections were cut on electrically positive charged slides for immunohistochemical analysis and deparaffinized by overnight xylene incubation, then rehydrated in a gradual descending ethanol concentration followed by phosphate-buffered saline (PBS) wash. The endogenous peroxidase activity was blocked by 3 percent hydrogen peroxide (H_2O_2) at room temperature for 5 minutes. Tissue sections were placed in glass jars containing 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval and boiled twice in a microwave oven for 5 minutes each to increase immunoreactivity (reserve the antigenicity loss that occurred with some formalin-fixed paraffin-embedded tissue epitopes). It was allowed to cool the slides and rinsed with PBS, pH 7.2. Immunohistochemical staining for the CD44 antibody was performed using the strept-avidin-biotin peroxidase method, as directed by the manufacturer. In phosphate buffered saline, the dilution used was 1:50.

The detection was performed by washing slides in PBS in 5 minutes with a universal kit (DAKO, Denmark) and incubated with an antimicrobial biotinylation of goat serum combined with rabbit and mouse sera for 30 minutes. Sections were then washed in PBS for 5 minutes and a diaminobenzidine [DAB] diaminobenzidine visualization in PBS with 40% H_2O_2 was developed. Sections have been washed for 10 minutes under running tap water, then countered with the hematoxylin of Mayer, and mounted.

Histomorphometric analysis:

Immunoreactivity for CD44 was already assessed using the Leica image analyzer computer system (Germany) by estimating the percentage of positive immunostained cells within the region examined in each test. In order to convert the measuring units (pixels) produced by the image analyzer program, the image analyzer is calibrated automatically into actual microns. The CD44 reactive area percentage was determined using magnification (x200) with reference to a standard measurement frame of 11434.9 μm2. Reactive areas with positive immunostaining are masked with a blue binary color by means with color detection. Each of the patients was taken and evaluated histomorphometrically in five fields each slide segment. Mean values for each specimen were then obtained.

Statistical analysis

Mean and standard deviation (SD) values were confirmed by the results, and the ANOVA test was used to compare means of more than two classes. In the procedure of pair comparisons between the groups when the ANOVA test is important, Tukey-Kramer multiple comparisons were used. The P value, if less than or equal to 0.05 ($P \leq 0.05$), is important. Using instate graph pad version 3.10 and Microsoft ® excel 2007, statistical analysis was performed.

RESULTS

Immunohistochemical findings:

CD44 immunostaining was observed in the cell membrane and cytoplasm of the ductal and serous acini cells in normal salivary gland tissue. In membranous staining, basolateral CD44 is more intense than the apical membrane **(Fig. 1-A)**. Regarding malignant neoplasm, In Ca ex PA, CD44 was seen in cell membrane and cytoplasm of some malignant epithelial cells and myoepithelial cells present in the myxoid area of Ca ex PA **(Fig. 1-B).** For MEC, CD44 was observed in the cell membrane of low grade MEC epidermoid cells and mucous secretion cells **(Fig. 1- C)**. It was observed in the cell membrane and cytoplasm of most high-grade MEC epidermoid cells **(Fig. 1-D)**. In ACC,

cribriform pattern showed, positive immunostained cells in both the luminal and non-luminal cells with the intensity of membranous staining in non-luminal cells **(Fig. 1- E)**. CD44 was found in the two cell layered ducts in the tubular pattern, but numerous outer cells show greater immunoreactivity to CD44 **(Fig. 1- F)**. In solid pattern CD44 was detected in most of neoplastic cells **(Fig. 1- G)**.

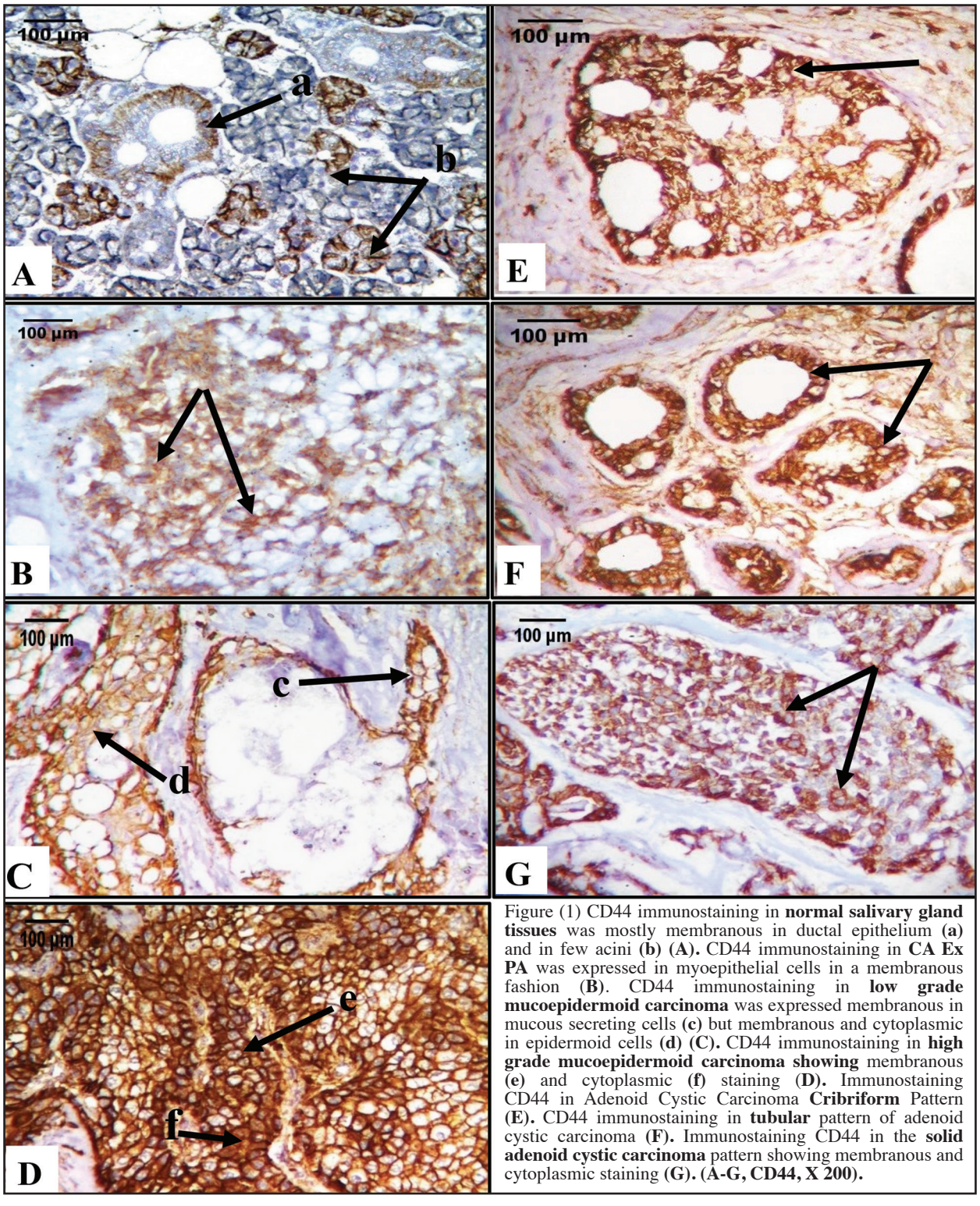

Figure (1) CD44 immunostaining in **normal salivary gland tissues** was mostly membranous in ductal epithelium **(a)** and in few acini **(b) (A).** CD44 immunostaining in **CA Ex PA** was expressed in myoepithelial cells in a membranous fashion **(B)**. CD44 immunostaining in **low grade mucoepidermoid carcinoma** was expressed membranous in mucous secreting cells **(c)** but membranous and cytoplasmic in epidermoid cells **(d) (C).** CD44 immunostaining in **high grade mucoepidermoid carcinoma showing** membranous **(e)** and cytoplasmic **(f)** staining **(D).** Immunostaining CD44 in Adenoid Cystic Carcinoma **Cribriform** Pattern **(E).** CD44 immunostaining in **tubular** pattern of adenoid cystic carcinoma **(F).** Immunostaining CD44 in the **solid adenoid cystic carcinoma** pattern showing membranous and cytoplasmic staining **(G). (A-G, CD44, X 200).**

Statistically comparing all groups revealed that the highest mean value was recorded in high grade MEC, followed by low grade MEC, then solid ACC, cribriform ACC, then tubular ACC, then carcinoma ex pleomorphic adenoma, while the lowest mean was recorded in normal salivary tissue. The ANOVA test revealed that all groups had a statistically significant difference (p=0.00). No significant difference between low grade mucoepidermoid carcinoma and solid adenoid cystic carcinoma was found in Tukey's post hoc test. In addition, cribriform and tubular adenoid cystic carcinoma did not differ significantly from carcinoma of pleomorphic adenoma (**Table 1, Fig. 2**).

Table (1) *Descriptive statistics of area percent measurements of immunoexpression in different groups.*

	M	SD	Std. Error	95% Mean Confidence Interval		Min	Max	P
				Lower Bound	Upper Bound			
Normal salivary gland	9.07[e]	3.68	1.64	4.51	13.64	5.45	13.13	
Ca ex PA	32.10[d]	3.25	1.46	28.06	36.14	27.29	35.16	
Cribriform ACC	39.11 [c,d]	8.82	3.94	28.16	50.05	28.29	49.93	
Tubular ACC	35.25 [c,d]	6.62	2.96	27.04	43.46	25.34	42.60	0.00*
Solid ACC	44.06 [b,c]	4.80	2.15	38.11	50.02	39.93	52.10	
MEC (Low grade)	54.15[b]	8.18	3.66	43.99	64.32	43.16	61.18	
MEC (High grade)	66.11[a]	1.29	.58	64.51	67.72	64.57	67.86	

**significant when p≤0.05. Tukey's post hoc test: when mean shares the same superscript letter isn't significantly different*

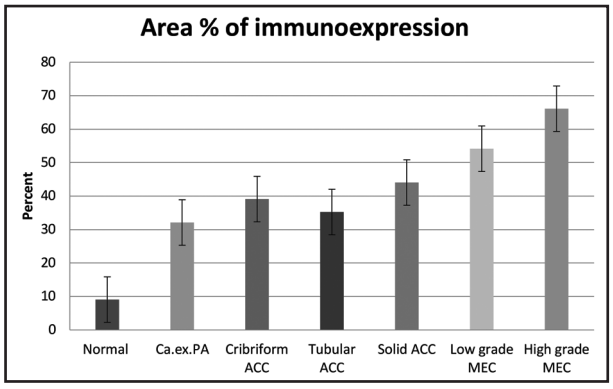

Figure (1) Bar chart illustrating mean value of area percent in different groups.

DISCUSSION

In recent years , the role of CD44 in malignant SGTs has been characterized by a growing body of evidence; previous studies showed overexpression of CD44 in many malignant subtypes of SGTs, highlighting CD44's potential role as a biomarker for these malignancies and their aggressive behavior[20].

With regard to the results of the current study, CD44 was expressed in the cell membrane and cytoplasm of studied salivary gland specimens. CD44 is an adhesion molecule which is expressed in normal and tumor tissues. the presence of CD44 within the cytoplasm is attributed to the intracellular fragment domain of the CD44 which is translated into the nucleus leading to immunoreactivity of CD44 staining in the cytoplasm [21].

Regarding the expression of CD44 in normal SGT, our results demonstrated that the CD44 immunostaining was detected in the cell membrane and cytoplasm of ductal and serous acini cells. CD44 is more intense in basolateral than apical membrane. According to our findings, it was shown that CD44 expression was more prevalent in the basolateral cells and in myoepithelial cells of the ducts than the apical membrane in the normal salivary tissues [22]. The presence of CD44 expression in normal salivary gland specimens is clearly explained by the physiological function of CD44; it is an adhesion molecule that involved in regulation of hematopoiesis, wound repair, and leucocyte activation [16]. The predominance of CD44 in basolateral aspect and scarcity of CD44 expression in the apical surface can be explained by the lack of CD44 ligands in the luminal surface [22].

Regarding Ca ex PA specimens of the present study demonstrated that CD44 was noticed in cell membrane and some malignant epithelial cells cytoplasm and myoepithelial cells present in the chondroid area. In agreement with our findings, membranous CD44 stain was observed in myoepithelial and ductal cells in ex PA specimens[21]. Similarly, CD44 expressions were seen in myoepithelial Ca ex PA specimens; however, there was no expression of the ductal component [23]. Our findings, as well as the above-mentioned findings, confirm the crucial role of myoepithelial cells in growth control within the SGTs.

In consideration of the MECs, CD44 was seen in epidermoid cells' cell membrane and in mucous cells of low-grade MEC in this study. It was seen in cell membrane and cytoplasm in the majority of high grade MEC epidermoid cells. The lowest and highest grade MEC revealed a high mean value in high grade MEC, whereas the lowest mean was in low grade MEC, with a statistically significant difference. In agreement with our findings, it was reported that the highest mean value was recorded in MEC high grade, while the lowest value was recorded in MEC low grade, with a statistically significant difference [20]. In accordance with our findings CD44 was demonstrated in high grade MEC as compared to low grade MEC, which was significantly over-expressed, with significant correlation with proliferative activity and motility[19]. In comparison to low-grade tumors, strong CD44 expression was already reported in high-grade MEC. Notably, the authors reported significant associations between CD44 expressions with recurrences/metastases [20].

Similarly, a comparable finding was reported. Higher expression of CD44 in MEC high grade possibly stems from the presence of overexpressed growth-factors in the high grade MEC, leading to subsequent overexpression of CD44 [8]. CD44 and the CD44 variants in MEC may be associated in relation to other adenomas with an abundance of extracellular matrices in these tumors, whereas the loss of CD44v3 and CD44v6 associated with the occurrence of MEC may contribute to the growth of remote metastases and eventually to the development of stromal invasion. In line with our findings, it was demonstrated a high expression of CSCs in high grade MEC, the CSCs strongly express CD44 [24,25]. Notably, The current study reported that CD44 immunostaining was positive in mucous cells, despite they are not subpopulations of stem cells; such findings could be explained by the previous reports demonstrating normal expression of CD44 in mucous membrane and epithelium [25].

Regarding ACC the present study showed that the CD44 was seen mostly in cell membrane in some tumor cells of cribriform, tubular, and solid patterns. The highest mean value was recorded in solid adenoid cystic carcinoma and the lowest in tubular adenoid cystic carcinoma followed by cribriform, and statistically significant differences were not recorded in tubular adenoid cystic carcinoma. High expression of CD44 in solid than cribriform ACC was found to be significantly correlated with worse prognosis and malignant clinicopathological features in the tumor. However, on the contrary to our findings, it was reported that the highest expression of CD 44 was noted in pleomorphic adenoma. These variations in results could be due to methodological differences [26].

CONCLUSION

From previous results, we can conclude that the pattern of expression of CD44 is correlated with the histopathological grading and serves as tumor behavior indicator.

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