EGYPTIAN DENTAL JOURNAL
www.eda-egypt.org
VOL. 70, 1399:1409, APRIL, 2024
PRINT ISSN 0070-9484 • ONLINE ISSN 2090-2360
ORAL MEDICINE, X-RAY, ORAL BIOLOGY AND ORAL PATHOLOGY
Submit Date : 29-02-2024 • Accept Date : 16-03-2024 • Available online: 05-04-2024 • DOI : 10.21608/EDJ.2024.273732.2965

CLINICAL-PATHOLOGICAL CORRELATION OF CD68 AND CD8 IN TUMOR IMMUNE MICROENVIRONMENT OF SALIVARY GLAND NEOPLASMS

Shaimaa O. Zayed *, Yasmine Ahmed Fouad** *and* Basma Abdelrahman Ahmed***

ABSTRACT

Background: Salivary gland neoplasms represent a diverse entity of tumors in the head and neck. Tumor-associated macrophages (TAMs) and tumor-infiltrating lymphocytes (TILs) were proven to have diverse roles in the carcinogenesis of head and neck neoplasms including salivary gland tumors.

Objectives: To detect and compare the expressions of CD68 and CD8 in benign and malignant salivary gland neoplasms and unveil the diagnostic ability of the markers in detecting malignancy. Besides detecting any correlation with clinical parameters, lymph node metastasis (LNM) and with each other.

Methods: We measured the immunohistochemical area % of CD68, and CD8 at Intra-tumor (IT) and tumor front (TF) sites in pleomorphic adenoma (PA), mucoepidermoid carcinoma (MEC), and Adenoid cystic carcinoma (ACC). Then the data was statistically analyzed for detection of any further correlations.

Results: A statistically significant increase in area % of CD68 and CD8 was observed in MEC and ACC when compared to PA. Both markers revealed accuracy in detecting malignancy with CD68 being more significantly accurate. IT CD8 area % significantly correlated with LNM. IT area % of CD68 negatively correlated with age while CD8 correlated positively with the patient's age. A statistically significant correlation between CD68 and CD8 at TF was observed.

Conclusions: Both CD68 and CD8 presented accuracy in the diagnosis of malignant salivary neoplasms. CD8 may help predict nodal metastasis in malignant salivary tumors. CD68 and CD8 might be associated with age changes. A cross-talk at the TF between TAM and TIL might be present, affecting the tumor invasive potential.

KEYWORDS: Salivary gland neoplasms; CD68; CD8; TAM; TIL

* Assistant Professor Oral Maxillofacial Pathology Department, Cairo University
** Lecturer of Oral Medicine, Periodontology and Oral Diagnosis, Faculty of Dentistry, Ain Shams University,
*** Lecturer of Oral Pathology, Faculty of Dentistry, Ain Shams University

INTRODUCTION

Salivary gland neoplasms constitute a divergent group of neoplasms as demonstrated by the World Health Organization (WHO) which presented more than 30 benign and malignant salivary tumors affecting major and minor glands (El-Naggar et al., 2017). These neoplasms are commonly present with complex clinicopathological characteristics and variable biological behavior. Understanding the surrounding micro-environment paves the way for developing more specific treatment modalities (Lavareze et al., 2022)

Many researchers have illustrated the importance of the host immune system in tumor progression management (Dutsch-Wicherek et al., 2011; Yang 2015). Tumor microenvironment (TME) has shown a diversity of molecular modifications and immune-mediated pathways, which have been lately considered crucial predictors of patients' prognosis in head and neck cancer (Henke et al., 2020).

A significant component of the innate immune cells inside the tumor tissue was formed of tumor-associated macrophages (TAMs). It has been reported that TAMs remarkably control tumor growth and progression. TAMs may have a role in salivary gland cancer progression due to their ability to increase intra-tumor blood vessel formation and promote tumor growth and metastasis. Their various histological entities and special features have been proven to affect the cross-talk between the neoplasm and the immune system (Zhang et al., 2012)

The importance of immune checkpoint blockade as an effective therapy has been identified to be widely linked to the amount of cytotoxic cluster of differentiation 8-positive (CD8+) tumor-infiltrating lymphocytes (TILs) as it was proposed that the tumors of more CD8+ cells had a better response to immune checkpoint inhibitors (Ribas and Wolchok, 2018). Few reports examined the prevalence of CD8+ cells in various tumors including salivary gland tumors (Mosconi et al., 2019; Blessin et al., 2020; De Virgilio et al., 2023).

Currently, there is little and contradictory data regarding the clinicopathological correlation and prognostic role of TAMs (CD68+) and TIL (CD8+) cells in cancer development including salivary gland neoplasms (Dutsch-Wicherek, 2011; Linxweiler et al., 2020; Sato et al., 2021). Thus, the purpose of the present study was to perform a comparative analysis and correlation of CD68 and CD8 expressions in benign and malignant salivary gland neoplasms, explore the diagnostic accuracy of the markers in detecting malignancy, and define any association with the clinical parameters and lymph node involvement.

MATERIALS AND METHODS

A. Study Design

The study design involved a double-center cohort retrospective study. The study was carried out at the Department of Oral Maxillofacial Pathology, Faculty of Dentistry, Cairo University, and the Oral Pathology Department, Ain Shams University after approval by the ethical committee (approval number: FDASU-Rec-ER012428). The study was carried out following principles of the Declaration of Helsinki, and waiver from consent was applied as all the blocks were coded and the patient's personal information was anonymous.

The study cases involved 42 benign and malignant salivary gland neoplasms representing the all salivary neoplasms diagnosed at both Oral Pathology Departments from 2015 to 2022. The inclusion criteria were as follows: (1) diagnosis of salivary gland neoplasms (2) surgical treatment with elective or therapeutic neck dissection. The exclusion criteria were as follows: (1) defects in slides and blocks, (2) insufficient tissue (3) patients without incomplete records, and (4) previous treatments (e.g., radiotherapy). The clinical reports of the patients were reviewed to collect the following clinical data: age; gender; tumor site; lymph node status; adjuvant treatments; and follow-up data.

B. Histopathological Analysis

The wax blocks and slides of the cases in the current study were retrieved from the department archives. New H&E stained slides were prepared to be re-examined by an experienced pathologist to choose the most representative tumor sample which had to contain both tumor and peritumoral salivary gland tissues. Then, consecutive 4μm slices were used to create slides for immunohistochemical (IHC) staining.

C. Immunohistochemical Staining and Analysis

Slides were stained using the BOND-III Staining System (Leica Biosystems) along with anti-CD68 (514H12, Leica Biosystems, Germany) and anti-CD8 (ab33786, Abcam, United States) primary monoclonal antibodies, following the manufacturer's instruction. After IHC staining, the slides were used for further analysis. The field of interest was divided into two different regions represented by the intra-tumor (IT), and the tumor front (TF) considered as 500μm outward and 500μm inward of the tumor border (De Virgilio et al., 2023). Five different fields of each region were captured using Canon camera (EOS 650D) and each tumor area was analyzed using Image J software, (version, 1.4.3.67) to obtain the area%.

D. Statistical analysis

Categorical data were introduced as frequency and percentage values. Numerical data was represented as mean and standard deviation (SD) values.They were tested for normality and variance homogeneity by viewing data distribution and using Shapiro-Wilk's and Levene's tests respectively. They were found to be normally distributed with homogenous variances and were analyzed for different comparisons and associations using a one-way ANOVA test followed by Tukey's post hoc test. Diagnostic accuracy was determined using ROC curve analysis. The best cutoff values were determined based on the highestYouden index. ROC curves were compared using DeLong'stest (DeLong et al., 1988). Correlations were analyzed using Spearman's rank-order correlation coefficient. The significance level was set at $p<0.05$ within all tests. Statistical analysis was performed with R statistical analysis software version 4.3.1 for Windows (R Core Team, 2023).

RESULTS

A. Patient's Characteristics

This study included a total of 42 patients (28 males and 14 females) with a mean age of 47.47 years. 16 cases were benign pleomorphic adenoma cases (PA) and 26 cases were malignant divided into 15 mucoepidermoid carcinoma (MEC) and 11 adenoid cystic carcinoma (ACC). Details of the patient's demographic data are presented in Table 1

TABLE (1) Patient's clinical-pathological characteristics

Parameter		Value
Gender [n (%)]	***Male***	28 (66.67%)
	Female	14 (33.33%)
Age (Mean±SD) (years)		47.47±9.31
Lesion site [n (%)]	***Lip***	5 (11.90%)
	Buccal mucosa	5 (11.90%)
	Palate	23 (54.76%)
	Parotid gland	7 (16.67%)
	Submandibular gland	2 (4.76%)
Lymph node metastasis (LNM) of malignant cases [n (%)]	***No***	16 (61.54%)
	Yes	10 (38.46%)
Pain sensation [n (%)]	***Painless***	34 (80.95%)
	Painful	8 (19.05%)
Diagnosis [n (%)]	***PA***	16 (38.10%)
	MEC	15 (35.71%)
	ACC	11 (26.19%)

B. Immunohistochemical Expressions and Inter-group Comparisons of CD68 and CD8

Both TAM (CD68 +) and TIL (CD8 +) cells revealed nuclear and cytoplasmic immuno-stain (Fig.1, 2). Results of inter-group comparisons detected a statistically significant increase of IT and TF CD68 area % in both MEC and ACC when compared to PA, however, no statistically significant difference was detected between MEC and ACC. Moreover, a higher statistically significant IT and TF CD8 area % was found in malignant tumors relative to benign PA, with only a significant difference between MEC and ACC in the TF Table (2).

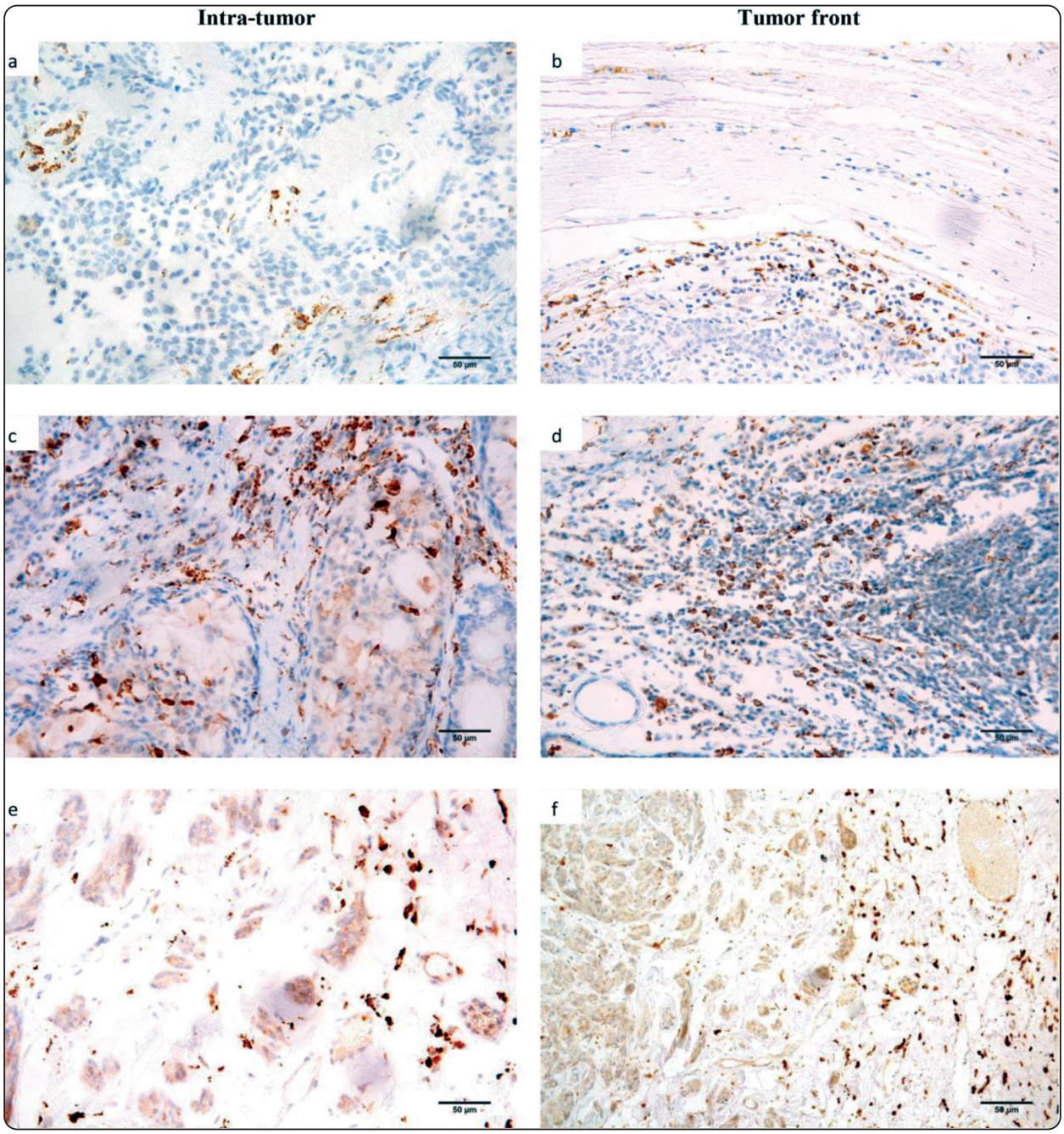

Fig. (1): A photomicrograph showing intra-tumor and tumor front expressions of CD68 in pleomorphic adenoma (a,b), mucoepidermoid (c,d), and adenoid cystic carcinoma carcinoma (e,f) (magnification, 400X).

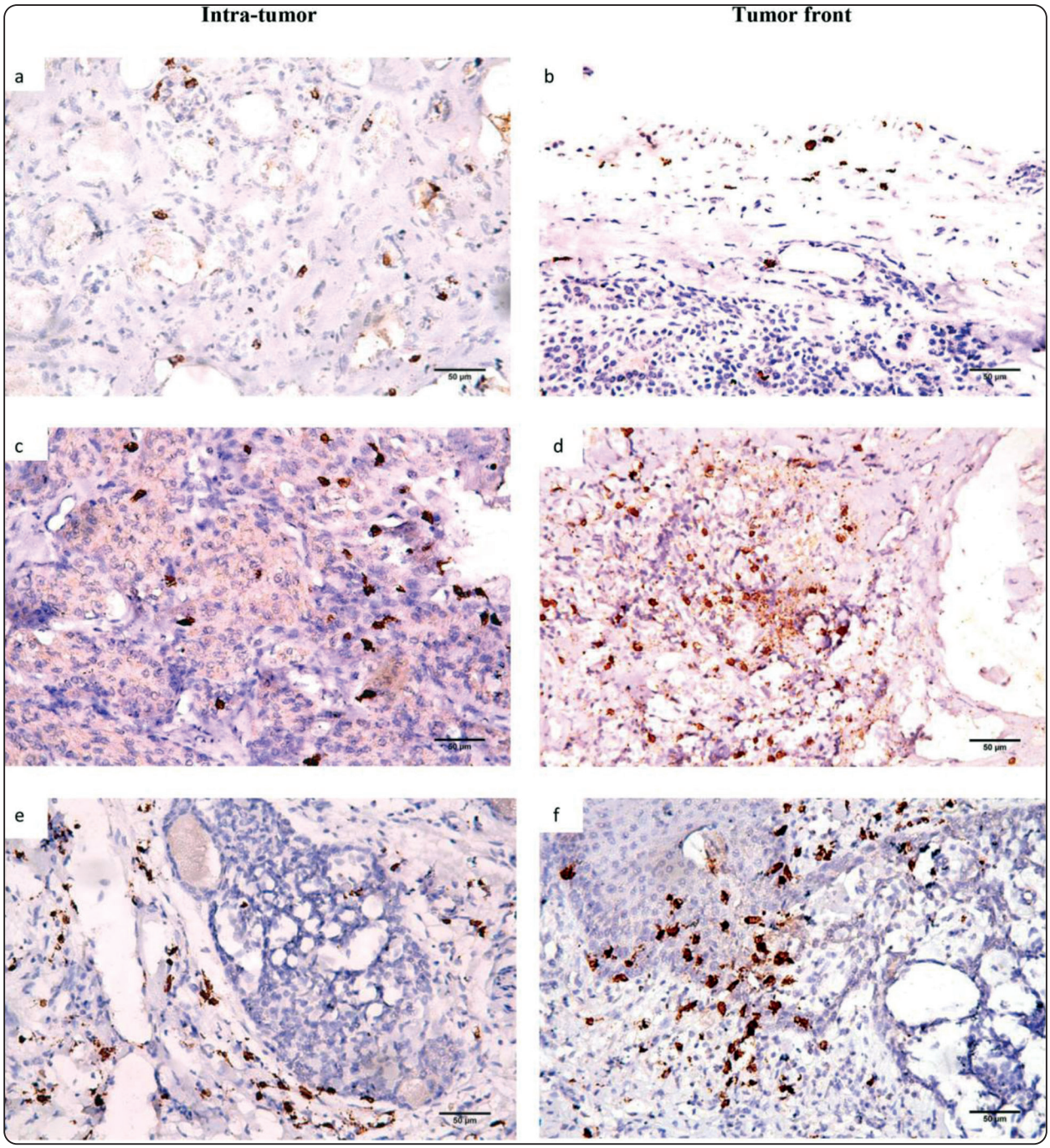

Fig. (2): A photomicrograph showing intra-tumor and tumor front expressions of CD8 in pleomorphic adenoma (a,b), mucoepidermoid (c,d), and adenoid cystic carcinoma (e,f) (magnification, 400X)

TABLE (2) Inter-group comparisons of CD68 and CD8 area (%)

IHC Marker	Measurement	Area (%) (Mean±SD)			p-value
		PA	*MEC*	*ACC*	
CD68	***IT***	0.52±0.24^{B}	1.97±0.68^{A}	1.51±0.32^{A}	**<0.001***
	TF	0.22±0.08^{B}	1.69±0.27^{A}	1.35±0.43^{A}	**<0.001***
CD8	***IT***	0.35±0.13^{B}	0.72±0.17^{A}	0.99±0.33^{A}	**<0.001***
	TF	1.08±0.57^{C}	2.85±0.15^{A}	2.31±0.35^{B}	**<0.001***

Values with different superscript letters within the same horizontal row are significantly different, *significant (p<0.05)

IHC: Immunohistochemical, IT: Intra –tumor, TF: Tumor front

C. Accuracy of CD68 and CD8 in Detecting Malignancy

The ROC curve revealed a statistically significant accuracy of both CD68 and CD8 in detecting malignancy. CD68 showed a 100.00% sensitivity and 96.88% specificity in detecting malignancy while CD8 exhibited 90.38% sensitivity and 68.75% specificity.The discriminating ability of CD68 in detecting malignancy was significantly higher than CD8 (p<0.001)(Table 3, Fig.3).

TABLE (3) Diagnostic Accuracy of CD68 and CD8 in Detecting Malignancy

Parameter	CD68	CD8	AUC difference (95%CI)	z-value	p-value
Sensitivity (95%CI)	100.00% (90.38%:100.00%)	90.38% (48.08%:100.00%)			
Specificity (95%CI)	96.88% (90.62%:100.00%)	68.75% (46.80%:100.00%)			
Accuracy (95%CI)	97.62% (94.05%:100.00%)	80.95% (67.86%:88.10%)			
Cut off point	>=0.80	>=0.68	**0.154 (0.068:0.240)**	**3.50**	**<0.001***
NPV (95%CI)	100.00% (86.49%:100.00%)	81.25% (54.24%:100.00%)			
PPV (95%CI)	98.11% (94.55%:100.00%)	82.54% (74.29%:100.00%)			
AUC (95%CI)	0.996 (0.988:1.000)	0.842 (0.757:0.927)			

Significant (p<0.05).

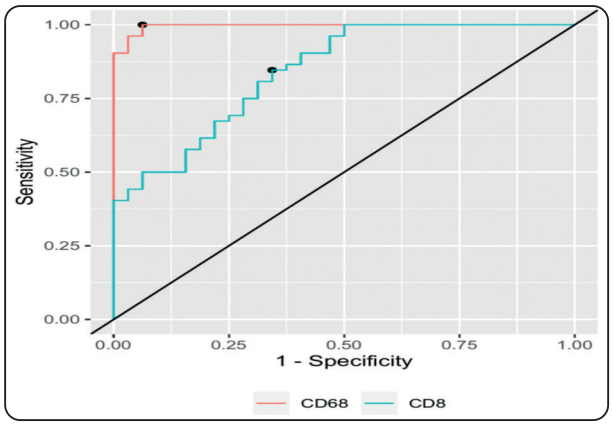

Fig.(3): ROC curve.

D. Association of CD68 and CD8 Area (%) of Malignant Tumors with Clinical Parameters

Comparing the relation of CD68 and CD8 area % of malignant tumors with different clinical parameters revealed no correlation of both markers with gender and lesion site. Only the IT CD8 area% was associated with LNM (p value<0.001) (Table 4).

TABLE (4) Association CD68 and CD8 area (%) of malignant tumors with clinical parameters

			CD68		CD8	
Measurement	**Parameter**		**Area (%) (Mean±SD)**	**p-value**	**Area (%) (Mean±SD)**	**p-value**
IT	*Gender*	*Male*	1.76±0.46	**0.871**	0.82±0.27	**0.806**
		Female	1.81±0.83		0.86±0.32	
	Lesion site	*Lip*	2.05±0.61	**0.315**	0.60±0.12	**0.148**
		Buccal mucosa	1.36±0.21		0.68±0.11	
		Palate	1.90±0.70		0.89±0.28	
		Parotid gland	1.57±0.39		0.71±0.18	
		Submandibular gland	1.52±0.01		1.26±0.27	
	Lymph node metastasis (LNM)	*No*	1.91±0.72	**0.082**	0.66±0.11	**<0.001***
		Yes	1.56±0.19		1.11±0.23	
TF	*Gender*	*Male*	1.56±0.36	**0.76**	2.70±0.33	**0.186**
		Female	1.51±0.43		2.48±0.41	
	Lesion site	*Lip*	1.59±0.25	**0.373**	2.97±0.08	**0.089**
		Buccal mucosa	1.14±0.28		2.23±0.48	
		Palate	1.60±0.38		2.54±0.34	
		Parotid gland	1.67±0.17		2.85±0.14	
		Submandibular gland	1.45±0.86		2.83±0.33	
	Lymph node metastasis (LNM)	*No*	1.45±0.35	**0.116**	2.65±0.36	**0.186**
		Yes	1.70±0.39		2.58±0.40	

Significant (p<0.05)** ***IT: Intra –tumor, TF: Tumor front

E. Correlation of CD68 and CD8 area (%) with Age and CD68-CD8 Correlation with Each Other

There was a negative moderate correlation between CD68 IT area% and age, while for CD8, the correlation was strong and positive (pvalue=0.044, <0.001 respectively) (Table 5).

For tumor front measurements, there was a moderate positive correlation between CD68 and CD8 (p value=0.029). However, no detection of any correlation of the IT expression of markers was observed (Table.5)

Table (5): Correlations of CD68 and CD8 area (%) with age and with each other

Variables	Measurement	Correlation coefficient (95%CI)	p-value
Age -CD68	***IT***	-0.397 (-0.680:-0.012)	**0.044***
	TF	0.239 (-0.164:0.573)	**0.240**
Age -CD8	***IT***	0.700 (0.428:0.855)	**<0.001***
	TF	-0.293 (-0.611:0.106)	**0.146**
CD68 -CD8	***IT***	-0.091 (-0.462:0.308	**0.660**
	TF	0.432 (0.053:0.702)	**0.029***

Significant (p<0.05)** **IT: Intra –tumor, TF: Tumor front

DISCUSSION

There is a continuing conflict about the prognostic role of immune cells in TME with both favorable and unfavorable outcomes being reported in various malignancies of the head and neck (Furgiuele et al., 2022). Immunotherapy targeting certain types of tumor-immune cells may have potential applications in salivary gland cancers (Mosconi et al., 2019). The prognostic relevance of our study was related to patients with benign and malignant salivary gland neoplasms (pleomorphic adenoma, mucoepidermoid carcinoma, adenoid cystic carcinoma). Thus, we aimed to explore the inter-relation of tumor immune cells CD8 and CD68, besides correlating their area % values with the demographic data of the patients, LNM, and predicting the accuracy of the markers in detecting malignancy.

In the present study, inter-group comparisons detected a statistically significant increase of IT and TF CD68 and CD8 area % in both MEC and ACC when compared to PA with only a significant difference between MEC and ACC in CD8 TF. In accordance with our results, the role of CD68+ and M2 macrophages as inducers of the malignant behavior of different salivary tumors like MEC, adenocarcinoma, ACC, and salivary duct carcinoma was previously affirmed (Shieh et al.,2009;Dutsch-Wicherek et al., 2011; Linxweiler et al., 2020). Moreover, Blessin et al., 2020 claimed that PA showed the lowest median number of CD8+ lymphocytes compared to the different studied tumors with a cell count of 6 cells/mm^2. Additionally, Sato et al., 2020 detected a high expression of CD8+ cells in malignant salivary gland tumors, and Kesar et al., 2020 found a statistically significant correlation between IT and TF CD8 scores and the

histological type of malignant salivary tumors. To further confirm the accuracy of CD68 and CD8 in detecting salivary gland malignancy, ROC curve analysis was performed and revealed a statistically significant accuracy of both CD68 and CD8 with 100.00% sensitivity and 96.88% specificity of CD68 and 90.38% sensitivity and 68.75% specificity of CD8 with cut off points of >=0.80 for CD68 and >=0.68for CD8.

Although previous studies showed an association between CD68+ TAMs and LNM in patients with salivary gland adenocarcinoma and other malignant salivary gland tumors (Dutsch-Wicherek et al., 2011; De Virgilio et al., 2023), our results did not find any correlation between IT and TF CD68 area % and LNM. Going along with our results, Aikian et al., 2019 did not find any relation between CD68+TAM and LNM in breast cancer. The discrepancy in results between different studies may be attributed to the variability in the histological tumor types.

Comparing the relation of CD8 area % of malignant tumors with different clinical parameters revealed that IT CD8 expression was associated with LNM. The same observation was recorded by Kesar et al. who found that tumors with increased total CD8+ cells revealed an aggressive behavior as regards lymph node involvement (Kesar et al., 2020). Moreover, De Virgilio et al., 2023 claimed that CD8 expression at tumor interior and invasive margins correlated with LNM. Mosconi et al., 2019 found that a high CD8+ cell density in the TF of ACC was related significantly to poor prognosis. Similarly, a recent study by Hirai, et al, 2023 detected that elevated expressions of CD8, FOXP3, programmed cell death protein 1(PD1), cytotoxic T-lymphocyte associated protein 4 (CTLA4), and lymphocyte activating 3 gene (LAG3) were associated with more advanced lymph node involvement, metastasis, and adverse prognosis. On the other hand, Sato et al., 2021 found no significant correlation of CD8 + density with patient prognosis.

The results of this study also revealed a negative correlation between CD68 IT area% and age, while CD8 showed a positive correlation with age, a finding that predicts the association between CD68+ cells and salivary gland malignancy in young age while the role of CD8 might be mainly related to tumors in old age patients. No previous reports were found correlating CD68 and CD8 with patient's age in salivary gland neoplasms, however, Zhang et al., 2013 and Xu et al., 2019 did not find any association of CD68 and CD8 with age in breast cancer and hepatocellular carcinoma respectively.

Finally, we detected a statistically significant correlation of both CD68 and CD8 at TF indicating a possible cross-talk between TAMs and TILs at the time of invasion of the malignant salivary tumors.

The main limitations of this study involved the inability to present multiple types of salivary gland neoplasms as the different histopathological variants harbor heterogeneity with variable clinical behavior and prognosis, in addition to the retrospective nature of the analysis.

CONCLUSIONS

CD68 and CD8 could be regarded as accurate markers in the diagnosis of malignant salivary gland tumors with CD68 having a higher accuracy, especially at young age, while CD8+ cells role in salivary malignancy might be associated with the increase in age. IT CD8 may represent an efficient predictor for LNM in malignant salivary neoplasms. CD68+ TAM and CD8+ TIL might have an interactive role at TF affecting tumor invasion.

Ethics statement

A double center retrospective study was accomplished at the Department of Maxillofacial Pathology, Faculty of Dentistry, Cairo University and Ain Shams University after approval by the ethical committee (FDASU-Rec-ER012428)

Author contributions

SH.O.Z, B.A.A: Study concept, design, data acquisition. B. A.A AND Y.A.F: data analysis. SH.O.Z, B.A.A, Y.A.F: manuscript preparation, revision, and approval.

Funding

Self-funding

Conflict of interest

All authors declare no conflict of interest.

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