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Immune microenvironment and evasion mechanisms in adenoid cystic carcinomas of salivary glands

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ABSTRACT

Objectives: The objective of the present study was to investigate the expression of immune checkpoints (PD-L1, PD-L2, PD-1 and CTLA-4), immune inhibitory molecule HLA-G, markers of tumor-infiltrating lymphocytes (TIL) and dendritic cells (DC), as well as its association with clinicopathological features of adenoid cystic carcinomas (ACC) of the salivary glands.

Materials and methods: Thirty-six samples from patients with ACC were analyzed immunohistochemically for the expression of PD-L1, PD-L2, PD-1, CTLA-4, HLA-G, CD8, GrB, CD1a and CD83. Positivity of HLA-G, PD-L1 and PD-L2 expression was defined by cut-offs values. $CD8^+$ TIL was measured semiquantitatively and also using cut-off values obtained by the ROC curve considering recurrence of the lesion.

Results: ACC showed low $CD8^+$, GrB^+ TIL, CD1a and CD83 populations, as well as scarce positivity for CTLA-4 and PD-1. In contrast, PD-L2 and HLA-G expression was increased, while no PD-L1 expression was detected. Interestingly, cases with lower $CD8^+$ TIL density presented greater recurrence rates.

Conclusion: Our findings suggest that the ACC microenvironment exhibits low immunogenicity, represented by low TIL and DC density. Moreover, there seems to be activation of the immune inhibitory proteins/PD-L2 and HLA-G, a scenario that may favor tumor escape from the immune system and partially explain the poor prognosis of ACC.

Introduction

Adenoid cystic carcinoma (ACC) is a rare salivary gland malignancy characterized by the term “wolf in a sheep’s skin” because of its indolent but unrelenting growth and dissemination [1–5]. In general, the prognosis of head and neck ACC is rather poor [4,6]. Regional lymph node and hematogenous metastases, especially to liver, bone and lung have been documented [3,4,7,8]. Few changes in treatment strategies for ACC have occurred over the last four decades, with radical surgery followed by postoperative radiotherapy and/or chemotherapy being indicated for most patients [4,9].

An effective antitumor response involves the participation of $CD8^+$ tumor infiltrating lymphocytes (TIL) that release perforin and granzyme B (GrB). These enzymes are responsible for the apoptotic death of neoplastic cells [10]. These events depend on recognition and capture of tumoral neoantigens by dendritic cells (DC) that transport these antigens to the regional lymph nodes, presenting them to T lymphocytes [11]. Failure of this process occurs due to the tumor ability to inhibit the immune activation of antigen-presenting cells, $CD8^+$ TIL and natural killer (NK) cells [12–15]. This mechanism involves the expression of molecules such as PD-1, PD-L1 and CTLA-4 [12,16]. Interference with these pathways has emerged as a potential cancer immunotherapy target [12].

The expression of immune inhibitors such as the anti-programmed death-1 (PD-1) receptor and its ligands (PD-L1 and PD-L2) and human leucocyte antigen-G (HLA-G) has been reported in several malignancies

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[15,17–20], including head and neck squamous cells carcinomas (SCC) [19,20] and, more recently, salivary gland neoplasms (e.g., ACCs, mucoepidermoid carcinomas, salivary duct carcinomas, adenocarcinomas, and others) [21–25]. Some investigators have demonstrated that ACC exhibits low numbers of CD8 TIL, PD-1, and cytotoxic T lymphocyte antigen 4 (CTLA-4; also known as CD152) positive cells, as well as overexpression of immune suppressive mediators like PDL-2 [21,22]. In contrast, only a few ACC cases have shown positivity for PD-L1 [21–23]. In a series of 70 malignant salivary gland tumors including 15 ACCs, it was shown that low PD-L2 expression was independently associated with shorter relapse-free survival [22]. Currently, immunotherapy can be achieved by blocking PD-1, PD-L1 or CTLA-4 [12], opening new possibilities of adjuvant therapies for salivary gland tumors.

Considering the prognostic value and immunotherapeutic potential of these proteins, there is a need to characterize their expression as well as the profile of immune cells and other mediators involved in the antitumor response. Thus, the objective of the present study was to evaluate the presence of the immune cells (CD8, GrB, CD1a and CD83) and regulatory/inhibitory pathways (CTLA-4, PD-1/PD-L1/PD-L2 and HLA-G) in ACCs of the major and/or minor salivary glands. These data might contribute to the definition of potential therapeutic targets in ACC.

Materials and methods

Study design and ethical approval

This cross-sectional study was approved by the Ethics Committee of Universidade Federal de Goiás (Goiânia, GO, Brazil) (Approval No. 1.460.804). Paraffin blocks were obtained from 36 patients surgically treated for ACC of the major and/or minor salivary glands who had undergone a biopsy between 2005 and 2015 at the Service of Head and Neck Surgery of Hospital Araújo Jorge (Goiânia, GO, Brazil). In seven patients, tissue was obtained from both primary and local recurrent lesions. All tissues were formalin fixed and paraffin embedded before storage. The patient's identity remained anonymous according to the Declaration of Helsinki.

Histological evaluation of ACC

Histologically, ACC were categorized into the following patterns according to the 2017 classification of the World Health Organization (WHO) [8]: cribriform pattern, characterized by nests of tumor cells interrupted by sharply punched-out spaces filled with basophilic matrix. Tubular pattern composed of bilayered tubules with true lumina and tumor cells presenting scant cytoplasm and typically small angulated and hyperchromatic nuclei. Solid pattern, characterized by largely basaloid tumor cells growing in sheets without lumina formation. Also, samples were scored using a simplified grading scheme proposed by van Weert et al. [2], i.e., the presence or absence of solid type ACC in the specimen was considered, regardless of its amount.

The material selected was sectioned with a microtome (Leica 2165 model RM Microsystems, Inc., Bannockburn, IL, USA) with each block yielding consecutive 5 µm sections that were graded using hematoxylin and eosin (H&E). The histopathological data of these ACC were reviewed independently by four oral and maxillofacial pathologists (A.C.B., E.F.M., R.A.M., and F.P.F.). Disagreements between the evaluators were solved upon discussion and consensus between them.

Immunohistochemical (IHC) analysis

Serial sections approximately 3 µm thick were obtained with a microtome, mounted on polarized slides (Surgipath® X-tra® Clipped Corner Leica Biosystems Richmond, Inc., Richmond, IL, USA) and subjected to immunohistochemistry.

IHC analyses were performed using monoclonal antibodies against anti-HLA-G (clone MEM-G/2; Exbio, Vestec, PRG Czech Republic; 1:100), anti-PD-L1 (clone E1L3N®; Cell Signaling Technology, Danvers, MA, USA; 1:600), anti-PD-L2 (clone 176611; R&D Systems, Inc., Minneapolis, MN, USA; 1:800), anti-CD8 (clone C8/144B; Dako, Carpinteria, CA, USA; 1:200), anti-GrB (clone GrB-7, Dako, Carpinteria, CA, USA; 1:100), anti-CD1a (clone MA1-80170; Thermo Scientific, Barrington, IL, USA; 1:50), anti-CD83 (clone HB15a; Santa Cruz, Biotechnology, Santa Cruz, CA, USA; 1:50), anti-CTLA-4 (clone F-8, Santa Cruz Biotechnology, Santa Cruz, CA, USA, 1:200) and anti-PD-1 (clone NAT105, Cell Marque, Rocklin, CA, USA; ready to use, overnight). The antigen-retrieval step was performed using the TRILOGY™ Concentrate (Cell Marque, Rocklin, CA, USA; 1:100) at a temperature of

Table 1
Demographic data and clinicopathological features of the sample.

Variable	Number (%)
Gender	
Male	12 (33.3)
Female	24 (66.7)
Age (years)	
10–19	1 (2.8)
20–29	3 (8.3)
30–39	4 (11.1)
40–49	4 (11.1)
50–59	9 (25)
60–69	10 (27.8)
70–79	4 (11.1)
80–89	1 (2.8)
Anatomic location	
Major salivary gland	
Parotid	10 (27.8)
Sublingual	9 (25)
Submandibular	5 (13.9)
Minor salivary gland	
Palate	4 (11.1)
Tongue	4 (11.1)
Jugal mucosa	4 (11.1)
Histology/grade	
I	25 (69.4)
II	5 (13.9)
III	6 (16.7)
Solid	
Yes	14 (38.9)
No	22 (61.1)
*Primary tumor (T)**	
T1 and T2	13 (36.1)
T3 and T4	23 (63.9)
*Regional lymph node involvement (N)**	
N0	24 (66.7)
N1	12 (33.3)
Distant metastasis (M)[*,§]	
M0	31 (86.1)
M1	5 (13.9)
Local recurrence	
No	25 (69.4)
Yes	11 (30.6)
Survival (DFS)[#]	
< 12 months	11 (36.7)
≥ 12 months	19 (63.3)
Outcome	
Dead	6 (16.7)
Alive	30 (83.3)

DFS, disease-free survival.
* Based on the Union for International Cancer Control (UICC, 8th Edition).
§ Distant metastasis was not available in some cases.
# Some patients did not attend a follow-up appointment.

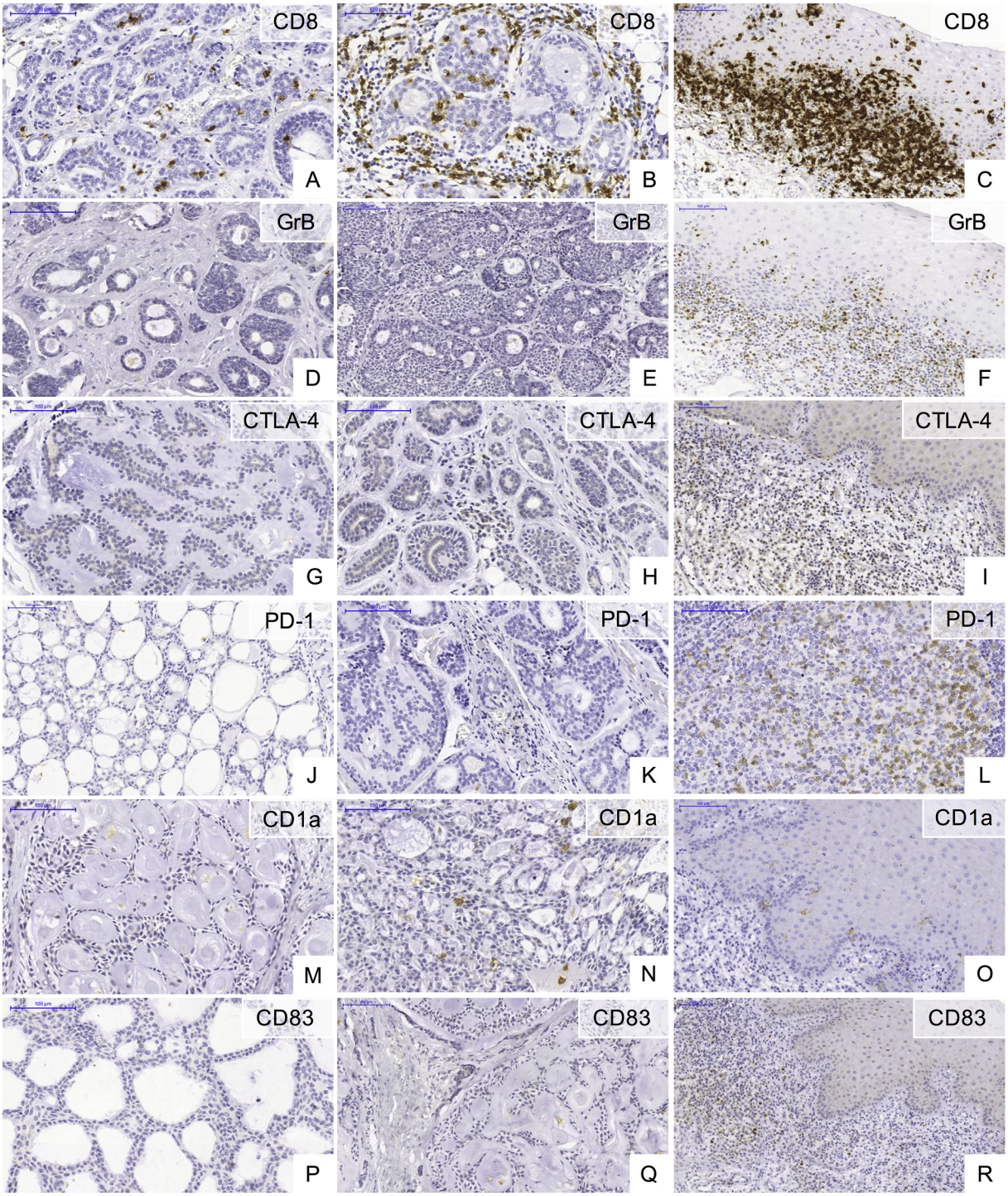

Fig. 1. Adenoid cystic carcinomas of the salivary glands exhibited low TIL in A, D, G, J, M, and P and high TIL in B, E, H, K, N, and Q. Photomicroscope images C, F, I, O, and R indicate positive oral lichen planus for CD8, GrB, CTLA-4, CD1a, and CD83, respectively, and L indicates a positive lymph node for PD-1. Immunohistochemistry: A-R = 20× (100 μm scale).

96 °C in a digital water bath (DeLeo, Porto Alegre, RS, Brazil) for 30 min. Next, the sections were treated with the Kit Novolink™ Max Polymer Detection System (Novocastra, Leica Biosystems Gmb, Wetzlar, HE, Germany) and the reactions were developed with 3.3′-diaminobenzidine (DAB, Dako, Carpinteria, CA, USA).

The trophoblast was used as a positive control for HLA-G, PD-L1 and PD-L2. Oral lichen planus or lymph nodes were used as a positive control for CD8, GrB, CD1a, CD83, CTLA-4 and PD-1.

$CD8^+$ TIL were measured semiquantitatively by the method of Ward et al. [26], i.e., 1 = 20%, 2 = 20–80% and 3 ≥ 80% of stroma, and also using cut-off value (49.9 cells/mm^2) obtained by the ROC curve considering recurrence of the lesion. The density (per mm^2) of GrB^+, $CD1a^+$, $CD83^+$, $CTLA\text{-}4^+$ and $PD\text{-}1^+$ in the intratumoral and stromal regions was evaluated in the samples with the aid of an integration graticule network (4740680000000-Netzmikrometer 12.5x, Carl Zeiss, Göttingen, Germany) connected to a light microscope (AxioScope, Carl Zeiss) in 10 alternating fields at 40x magnification. At this magnification, the area of one field corresponds to 0.0961 mm^2.

Table 2
Median and interquartile range (IQR) of density/mm^2 of GrB, CD1a and CD83 positive cells infiltrating ACC in the presence of high and low CD8$^+$ TIL counts.

	Intratumoral region						Stromal region					
	GrB	*p-value*	CD1a	*p-value*	CD83	*p-value*	GrB	*p-value*	CD1a	*p-value*	CD83	*p-value*
Low CD8$^+$ TIL counts	0.1 (0.94)	0.80	2.04 (4.16)	0.94	2.08 (4.16)	0.41	0.1 (0.71)	0.87	0 (0.52)	0.20	0 (2.08)	0.44
High CD8$^+$ TIL counts	0.1 (1.98)		1.56 (5.05)		1.04 (2.08)		0.1 (1.72)		0 (1.30)		0 (4.78)	
Min-Max	0.1–6.24		0–24.97		0–8.32		0.1–23.93		0–19		0–19.17	

Cut-off for dichotomization of CD8$^+$ TIL counts (low/high) = 49.9 cells/mm^2.

Table 3
Relationship between the clinicopathological data of ACC and stromal CD8$^+$ TIL, CD83$^+$ and intratumoral CD8$^+$ TIL, CD1a$^+$ cell density.

Variable	Total	Stromal region						Intratumoral region					
		CD8$^+$ TIL			CD83$^+$			CD8$^+$ TIL			CD1a$^+$		
		Low	High	*p*-value	Low	High	*p*-value	Low	High	*p*-value	Low	High	*p*-value
Gender													
Male	12	8	4	0.72	8	4	1.00	9	3	0.08	7	5	0.31
Female	24	14	10		14	9		10	14		9	14	
Age													
≤57	19	12	7	1.00	12	6	0.73	9	10	0.52	10	9	0.50
> 57	17	10	7		10	7		10	7		6	10	
Anatomic location													
Minor salivary gland	12	7	5	1.00	8	3	0.47	6	6	1.00	7	4	0.27
Major salivary gland	24	15	9		14	10		13	11		9	15	
Histology/grade													
I	25	15	10	**0.05**	12	12	0.05	13	12	0.11	12	12	0.73
II	5	1	4		5	0		1	4		2	3	
III	6	6	0		5	1		5	1		2	4	
Solid component													
No	22	13	9	1.00	11	10	0.16	13	9	0.49	12	10	0.29
Yes	14	9	5		11	3		6	8		4	9	
Primary tumor (T)[*]													
T1 and T2	13	11	2	**0.05**	8	5	1.00	10	3	0.05	4	9	0.29
T3 and T4	23	11	12		14	8		9	14		12	10	
Regional lymph node involvement (N)[*]													
N0	24	17	7	0.14	14	9	1.00	12	12	0.73	8	15	0.09
N1	12	5	7		8	4		7	5		8	4	
Distant metastasis (M)[*]													
M0	31	20	11	0.35	18	12	0.63	14	17	**0.04**	11	19	**0.01**
M1	5	2	3		4	1		5	0		5	0	
Local recurrence													
No	25	12	13	**0.02**	15	10	0.70	10	15	**0.03**	12	13	0.72
Yes	11	10	1		7	3		9	2		4	6	
Survival (DFS)[#]													
< 12	11	10	1	**0.02**	9	1	0.20	7	4	0.70	5	5	1.00
≥12	19	9	10		12	7		10	9		10	9	
Outcome													
Dead	6	5	1	0.37	4	2	1.00	5	1	0.18	2	4	0.66
Alive	30	17	13		18	11		14	16		14	15	

[§]DFS: Disease free survival.
[*] Based on Union for International Cancer Control (UICC, 8th Edition).
[#] Some patients did not return to follow-up appointment.

HLA-G, PD-L1 and PD-L2 expression was defined as membranous and cytoplasmic immunoreactivity using the cut-off values described by Ilie et al. [27] (PD-L1: ≥1% of positive tumor cells), Sridharan et al. [21] (PD-L2: ≥10% of positive tumor cells) and Mosconi et al. [25] (HLA-G: ≥50% of positive tumor cells). PD-1 was analyzed by the method of Taube et al. [28], i.e., cut-off value of ≥5% of positive tumor cells, and CTLA-4 was analyzed by the method of Karpathiou et al. [29], i.e., cut-off value of ≥5% of positive tumor cells. PD-L1, PD-L2 and HLA-G stromal cells (fibroblasts, endothelial cells, immune-inflammatory cells) were classified as percentage of positive cells.

Data analysis

Data analysis was performed using the Statistical Package for the Social Sciences (SPSS) software, version 25.0 (SPSS Inc., Armonk, NY, USA). Associations between nominal variables were analyzed using the Fisher exact test. After evaluating the distribution of numerical data using the Shapiro-Wilk test, comparison was performed using the nonparametric Mann-Whitney test. Survival time was calculated from surgical resection to the last follow-up appointment or the death of the patient. Thus, the samples were dichotomized as follows: 1 = absent or < 50% of HLA-G, PD-L1 and PD-L2, and 2 = ≥50% of positive cells,

Table 4
Expression of immunotherapy targets PD-L1, PD-L2, HLA-G, PD-1, and CTLA-4 in intratumoral and stromal regions of ACCs (number and %).

		Positive (≥10%)	Negative (< 10%)	Positive (≥50%)	Negative (< 50%)
Intratumoral region	PD-L1	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)
	PD-L2	36 (1 0 0)	0 (0)	28 (77.8)	8 (22.2)
	HLA-G	36 (1 0 0)	0 (0)	27 (75)	9 (25)
	PD-1	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)
	CTLA-4	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)
Stromal region	PD-L1	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)
	PD-L2	29 (80.5)	7 (19.5)	17 (47.2)	19 (52.8)
	HLA-G	29 (80.5)	7 (19.5)	14 (38.9)	22 (61.1)
	PD-1	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)
	CTLA-4	0 (0)	36 (1 0 0)	0 (0)	36 (1 0 0)

and CD8 TIL cells were dichotomized according to the ROC curve value. The level of significance was set at $p < 0.05$.

Results

The general data of the 36 individuals with ACC are shown in Table 1. The sample consisted of 24 females (66.7%) and 12 males (33.3%), with a female-to-male ratio of 2:1. The mean age of the sample as a whole was 53.5 years (range: 19 to 80 ± 13.3 years), and the mean age of men and women was 45.8 and 57.3 years, respectively. The major salivary glands were the most affected site (66.7%), with greater occurrence in the parotid glands (27.8%).

Microscopically, ACC samples were classified as cribriform (n = 25, 69.4%), solid (n = 6, 16.7%), and tubular (n = 5, 13.9%). The presence of a solid component was evident in 14 cases (38.9%). Primary tumor size was T1 and T2 in 36.1% of patients, while 63.9% were categorized as T3 and T4. The mean survival of affected subjects was 46.4 months (range: 1.0 to 104 ± 28.1 months). Eleven patients (30.6%) developed local recurrence, 12 (33.3%) had cervical lymph node metastases, five (13.9%) developed distant metastasis, and six died (16.7%) (Table 1).

ACC showed a microenvironment with low $CD8^+$ TIL (n = 22 cases, 61%) and most samples were negative for GrB (n = 20 cases, 55%), CTLA-4 (n = 32 cases, 88%) and PD-1 (n = 33 cases, 91%). Significant negativity for CD1a (n = 15 cases, 41%) and CD83 (n = 18 cases, 50%) was also observed (Fig. 1). Table 2 displays the median and interquartile range densities of positive cells per mm^2 for GrB, CD1a and CD83 quantified in the intratumoral and stromal regions for different $CD8^+$ TIL profiles (low/high). No significant association was observed between $CD8^+$ TIL and GrB^+, $CD1a^+$ or $CD83^+$ cells ($p > 0.05$), i.e., $CD8^+$ TIL density was not associated with positivity for GrB, CTLA-4, PD-1, CD1a and CD83.

Individuals that developed recurrences showed low density of $CD8^+$ TIL cells in the stromal ($p = 0.02$) and intratumoral ($p = 0.03$) regions. Patients with longer survival (≥ 12 months) demonstrated high densities of $CD8^+$ TIL cells in the stromal region ($p = 0.02$). Despite the low number of cases with distant metastasis (n = 5), an association was observed between this clinical variable and low density of $CD8^+$ TIL ($p = 0.04$) and $CD1a^+$ cells ($p = 0.01$) in the intratumoral region (Table 3). No association of $CD8^+$ TIL, CD83 and CD1a with gender, age, anatomical location, solid component, regional lymph node involvement, or outcome was observed (Table 3).

The expression of PD-L1, PD-L2, PD-1 and CTLA-4 in intratumoral and stromal regions is presented in Table 4. The intratumoral region showed positivity (≥10% tumor cells) for PD-L2 and HLA-G in about 100% of cases. When positivity was considered to be ≥50% tumor cells for PD-L2 and HLA-G, 77.7% and 75% were expressed, respectively. In the stromal region, PD-L2 and HLA-G showed similar expression (80.5%). Since positivity for PD-L2 and HLA-G was considered to be ≥50% of tumor cells, 47.2% and 38.9% of cases showed positivity, respectively (Fig. 2). Additionally, seven individuals with both a primary tumor and local recurrence of ACC showed a similar immune cell profile and presence of inhibitory proteins. The density values (per mm^2) were $CD8^+$: 23.4, GrB: 1.1, CD1a: 1.8, CD83: 0.4, PD-1: 0, and CTLA-4: 0 for the primary tumor, and: $CD8^+$: 22.9, GrB: 4.7, CD1a: 1.4, CD83: 0.2, PD-1: 0, and CTLA-4: 0 for local recurrence ($p > 0.05$).

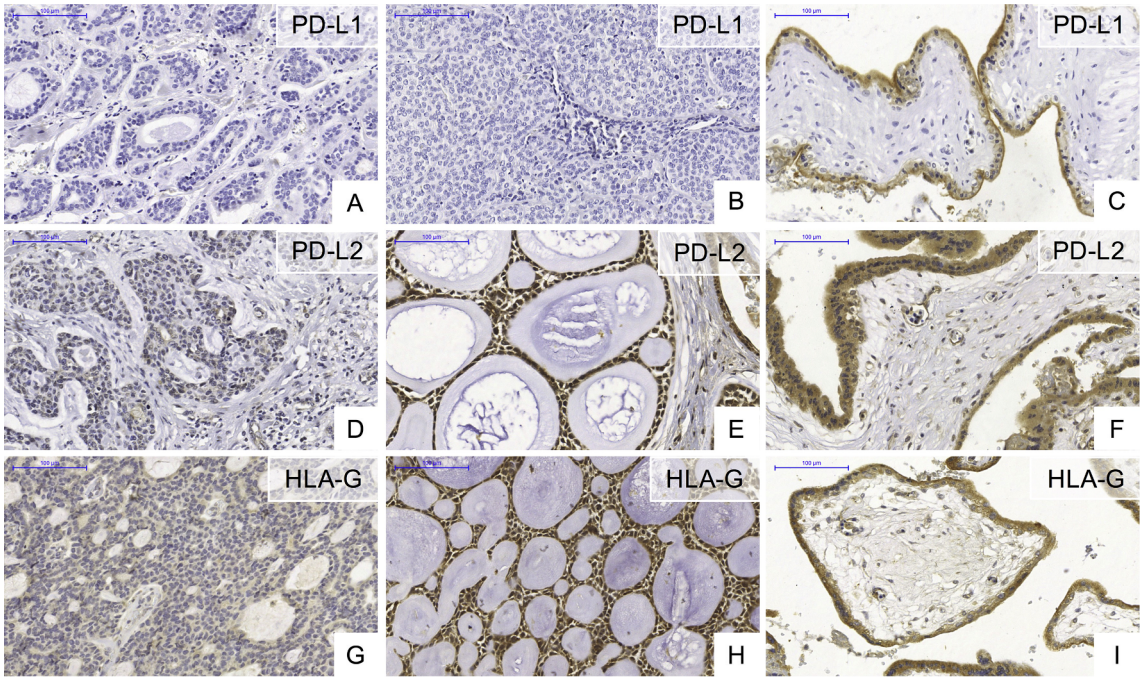

Fig. 2. Adenoid cystic carcinomas of the salivary glands showed negative expression of PD-L1 in A and B; for PD-L2 in D, as well for HLA-G in G, whereas they showed high positivity for PD-L2 in E, and for HLA-G in H. Photomicroscope images (C, F, and I) indicate positivity of trophoblasts PD-L1, PD-L2 and HLA-G, respectively. Immunohistochemistry: A-I = 20× (100 µm scale).

Discussion

The aim of this study was to characterize the expression of the immune-related markers in ACCs of salivary glands. Our main findings were: (1) ACC exhibits a tumor microenvironment with few cells related to antitumor immune response; (2) Cases with high densities of $CD8^+$ TIL cells showed low quantities of GrB^+ cells; (3) Low densities of CTLA-4 and PD-1 positive cells were observed in most samples; (4) Most cases were positive for HLA-G and PD-L2, whereas all cases were negative for PD-L1; (5) Finally, we observed a significant association between larger numbers of $CD8^+$ and $CD1a^+$ cells in the tumoral microenvironment and less recurrence and longer survival.

Taken together, our results suggest that the ACC microenvironment exhibits low immunogenicity, represented by low TIL and DC densities. This low infiltration of antitumor immune cells might be explained by the low tumor mutation burden or neoantigen load, which have been previously documented in ACCs [30,31]. In contrast to ACC, in head and neck SCC, increased infiltration of $CD8^+$ TIL cells was associated with a broader mutation landscape [17]. The relationship between low immunogenicity and low mutation rates has been demonstrated in several malignancies [17], including salivary gland neoplasms (e.g., myoepithelial carcinomas, acinic cell carcinomas, and polymorphous low-grade adenocarcinomas) [30]. Sridharan et al. [21] evaluated the association between intratumoral immune infiltrate and differential expression of 700 genes in 21 subjects with ACC. According to their findings, a lack of immune cell infiltrate was associated with expression of genes in the β-catenin/Wnt and PI3K pathways. However, some genes such as Syk, IL2RB, and TGFβ were correlated with immune infiltration.

The effectiveness of the antitumor immune response depends on CD8 activation via secretion of perforin and GrB [10]. Herein, scarce GrB^+ cells were demonstrated independently of low or high numbers of $CD8^+$ TIL cells. This finding can be associated with the expression of immune inhibitory molecules in the tumor microenvironment, such as PD-L2 and HLA-G [18,31]. In line with our data, some authors have observed that the majority of ACC cases express PD-L2, although they also present few infiltrating immune cells [21,22]. Similarly, the association between high expression of HLA-G and a reduced number of GrB^+ cells has been demonstrated in intraoral mucoepidermoid carcinomas [25]. To our knowledge, this is the first study investigating expression patterns of immune-related markers such as HLA-G and immune checkpoints in salivary gland ACC. The expression of PD-L2 and HLA-G was not strictly associated with clinicopathological parameters. Similarly, in salivary duct carcinomas, PD-L2 expression was not associated with prognosis [24]. On the other hand, despite the reduced CD8/GrB ratio, the ACC group with increased $CD8^+$ density showed association with less recurrence and longer survival. Indeed, there is accumulating evidence that number of $CD8^+$ TIL cells has a significant impact on the prognosis of individuals with oral SCC, as well as on the prognosis of individuals with ACCs [22,32].

Anti-immune pathways such as PD-L1/PD-1 and CTLA-4 have been studied because they can be used by the tumor to overcome the immune system. Nowadays, they are being used as immunotherapy targets [12–14,16,33]. A previous study [23] associated PD-L1 expression with poor disease-free survival in salivary gland carcinomas.

In our study, the ACC showed an immune microenvironment with the absence of PD-L1 cells and scarce $PD\text{-}1^+$ and $CTLA\text{-}4^+$ cells. According to the classification proposed by Teng and colleagues [14], cancers have been categorized into four different tumor microenvironments based on the presence of TIL and PD-L1 expression, i.e., type I: immune resistance, type II: immune ignorance, type III: intrinsic induction, and type IV: immune tolerance. Therefore, the majority of ACC cases reported here would be classified as type IV ($CD8^+$ TIL/$PD\text{-}L1^-$, i.e., tolerance). Considering these data as a whole, immunotherapeutic strategies against PD-L1, PD-1 and CTLA-4 may not be useful for all cases of ACC. Therefore, further studies analyzing the set of these molecules are encouraged in order to design an immunotherapeutic strategy against ACCs.

The present study has strengths and limitations. Although we did analyze individuals over a period of 10 years, only 36 patients were retrieved. However, the rarity of ACC and the long sampling period contribute to representativeness of the sample. The second limitation refers to missing data because some patients did not attend a follow-up appointment, a fact inherent to the retrospective nature of the study.

Conclusion

In summary, our findings suggest that the ACC microenvironment exhibits low immunogenicity, represented by low $CD8^+$, GrB^+ TIL and DC densities. Moreover, there seems to be activation of the immune inhibitory proteins/PD-L2 and HLA-G. This immune scenario may favor tumor escape from the immune system and may partially explain the poor prognosis of ACC. In addition, a better understanding of the immunoprofile of the tumor may provide potential targets for immunotherapy.

Conflict of interest

None.

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References

[1] Ellington CL, Goodman M, Kono SA, Grist W, Wadsworth T, Chen AY, et al. Adenoid cystic carcinoma of the head and neck: incidence and survival trends based on 1973–2007 surveillance, epidemiology, and end results data. Cancer 2012;118:4444–51.
[2] van Weert S, van der Waal I, Witte BI, Leemans CR, Bloemena E. Histopathological grading of adenoid cystic carcinoma of the head and neck: analysis of currently used grading systems and proposal for a simplified grading scheme. Oral Oncol 2015;51:71–6.
[3] Coca-Pelaz A, Rodrigo JP, Bradley PJ, Vander Poorten V, Triantafyllou A, Hunt JL, et al. Adenoid cystic carcinoma of the head and neck – an update. Oral Oncol 2015;51:652–61.
[4] van Weert S, Bloemena E, van der Waal I, de Bree R, Rietveld DH, Kuik JD, et al. Adenoid cystic carcinoma of the head and neck: a single-center analysis of 105 consecutive cases over a 30-year period. Oral Oncol 2013;49:824–9.
[5] Fonseca FP, Carvalho Mde V, de Almeida OP, Rangel AL, Takizawa MC, Bueno AG, et al. Clinicopathologic analysis of 493 cases of salivary gland tumors in a Southern Brazilian population. Oral Surg Oral Med Oral Pathol Oral Radiol 2012;114:230–9.
[6] Ciccolallo L, Licitra L, Cantú G, Gatta G. EUROCARE Working Group. Survival from salivary glands adenoid cystic carcinoma in European populations. Oral Oncol 2009;45:669–74.
[7] Marcinow A, Ozer E, Teknos T, Wei L, Hurtuk A, Old M, et al. Clinicopathologic predictors of recurrence and overall survival in adenoid cystic carcinoma of the head and neck: a single institutional experience at a tertiary care center. Head Neck 2014;36:1705–11.
[8] El-Naggar AK, Chan JKC, Grandis JR, Takata T, Slootweg PJ. World health organization classification of head and neck tumours. 4th ed. Lyon: IARC Press; 2017.
[9] Cordesmeyer R, Kauffmann P, Laskawi R, Rau A, Bremmer F. The incidence of occult metastasis and the status of elective neck dissection in salivary adenoid cystic carcinoma: a single center study. Oral Surg Oral Med Oral Pathol Oral Radiol 2018;125:516–9.
[10] Afonina IS, Cullen SP, Martin SJ. Cytotoxic and non-cytotoxic roles of the CTL/NK protease granzyme B. Immunol Rev 2010;235:105–16.
[11] Tran Janco JM, Lamichhane P, Karyampudi L, Knutson KL. Tumor-infiltrating dendritic cells in cancer pathogenesis. J Immunol 2015;194:2985–91.
[12] Ribas A, Wolchok JD. Cancer immunotherapy using checkpoint blockade. Science 2018;359:1350–5.

[13] Chae YK, Chung SY, Davis AA, Carneiro BA, Chandra S, Kaplan J, et al. Adenoid cystic carcinoma: current therapy and potential therapeutic advances based on genomic profiling. Oncotarget 2015;6:37117–34.
[14] Teng MW, Ngiow SF, Ribas A, Smyth MJ. Classifying Cancers Based on T-cell Infiltration and PD-L1. Cancer Res 2015;75:2139–45.
[15] Carosella ED, HoWangYin KY, Favier B, LeMaoult J. HLA-G-dependent suppressor cells: diverse by nature, function, and significance. Hum Immunol 2008;69:700–7.
[16] Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. Nat Rev Cancer 2012;12:252–64.
[17] Ock CY, Keam B, Kim S, Lee JS, Kim M, Kim TM, et al. Pan-cancer immunogenomic perspective on the tumor microenvironment based on PD-L1 and CD8 T-cell infiltration. Clin Cancer Res 2016;22:2261–70.
[18] Carosella ED, Ploussard G, LeMaoult J, Desgrandchamps F. A systematic review of immunotherapy in urologic cancer: evolving roles for targeting of CTLA-4, PD-1/PD-L1, and HLA-G. Eur Urol 2015;68:267–79.
[19] Kansy BA, Concha-Benavente F, Srivastava RM, Jie HB, Shayan G, Lei Y, et al. PD-1 status in CD8(+) T cells associates with survival and anti-PD-1 therapeutic outcomes in head and neck cancer. Cancer Res 2017;77:6353–64.
[20] Pai SI, Jack Lee J, Carey TE, Westra WH, Ferrone S, Moore C, et al. HLA class I antigen processing machinery (APM) component expression and PD-1:PD-L1 pathway activation in HIV-infected head and neck cancers. Oral Oncol 2018;77:92–7.
[21] Sridharan V, Gjini E, Liao X, Chau NG, Haddad RI, Severgnini M, et al. Immune profiling of adenoid cystic carcinoma: PD-L2 expression and associations with tumor-infiltrating lymphocytes. Cancer Immunol Res 2016;4:679–87.
[22] Chang H, Kim JS, Choi YJ, Cho JG, Woo JS, Kim A, et al. Overexpression of PD-L2 is associated with shorter relapse-free survival in patients with malignant salivary gland tumors. Onco Targets Ther 2017;10:2983–92.
[23] Mukaigawa T, Hayashi R, Hashimoto K, Ugumori T, Hato N, Fujii S. Programmed death ligand-1 expression is associated with poor disease free survival in salivary gland carcinomas. J Surg Oncol 2016;114:36–43.
[24] Sato F, Akiba J, Kawahara A, Naito Y, Ono T, Takase Y, et al. The expression of programed death ligand-1 could be related with unfavorable prognosis in salivary duct carcinoma. J Oral Pathol Med 2018. [Epub ahead of print].
[25] Mosconi C, Arantes DAC, Gonçalves AS, Alencar RCG, Oliveira JC, Silva TA, et al. Immunohistochemical investigations on the expression of programmed cell death ligand 1, human leukocyte antigens G and E, and granzyme B in intraoral mucoepidermoid carcinoma. Arch Oral Biol 2017;83:55–62.
[26] Ward MJ, Thirdborough SM, Mellows T, Riley C, Harris S, Suchak K, et al. Tumour-infiltrating lymphocytes predict for outcome in HPV-positive oropharyngeal cancer. Br J Cancer 2014;110:489–500.
[27] Ilie M, Hofman V, Dietel M, Soria JC, Hofman P. Assessment of the PD-L1 status by immunohistochemistry: challenges and perspectives for therapeutic strategies in lung cancer patients. Virchows Arch 2016;468:511–25.
[28] Taube JM, Klein A, Brahmer JR, Xu H, Pan X, Kim JH, et al. Association of PD-1, PD-1 ligands, and other features of the tumor immune microenvironment with response to anti-PD-1 therapy. Clin Cancer Res 2014;20:5064–74.
[29] Karpathiou G, Casteillo F, Giroult JB, Forest F, Fournel P, Monaya A, et al. Prognostic impact of immune microenvironment in laryngeal and pharyngeal squamous cell carcinoma: Immune cell subtypes, immuno-suppressive pathways and clinicopathologic characteristics. Oncotarget 2017;8:19310–22.
[30] Ross JS, Gay LM, Wang K, Vergilio JA, Suh J, Ramkissoon S, et al. Comprehensive genomic profiles of metastatic and relapsed salivary gland carcinomas are associated with tumor type and reveal new routes to targeted therapies. Ann Oncol 2017;28:2539–46.
[31] Leng C, Li Y, Qin J, Ma J, Liu X, Cui Y, et al. Relationship between expression of PD-L1 and PD-L2 on esophageal squamous cell carcinoma and the antitumor effects of $CD8^+$ T cells. Oncol Rep 2016;35:699–708.
[32] Cho YA, Yoon HJ, Lee JI, Hong SP, Hong SD. Relationship between the expressions of PD-L1 and tumor-infiltrating lymphocytes in oral squamous cell carcinoma. Oral Oncol 2011;47:1148–53.
[33] Hodi FS, O'Day SJ, McDermott DF, Weber RW, Sosman JA, Haanen JB, et al. Improved survival with ipilimumab in patients with metastatic melanoma. N Engl J Med 2010;363:711–23.