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Original contribution

Immunohistochemical expression of mammaglobin in salivary duct carcinomas de novo and salivary duct carcinoma ex pleomorphic adenoma☆,☆☆

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Summary Mammaglobin is expressed in breast and salivary gland secretory carcinomas; however, its expression in salivary duct carcinomas (SDCs) still not well established. Therefore, the aim of this study was to investigate the presence and distribution of mammaglobin immunoexpression in SDC ex-PA in different phases of the adenoma to carcinoma sequence evaluating its possible involvement in carcinogenesis and tumor progression, as well as to determine its expression in SDC de novo. Mammaglobin immunohistochemistry was performed in 84 SG tumors, including 41 pleomorphic adenomas (PA) without malignant transformation, 13 intracapsular SDC ex-PA, 5 frankly invasive SDC ex-PA, 25 SDC de novo and 10 secretory carcinomas. The reactions were qualitatively analyzed and digitally scored. Positive immunostaining for mammaglobin was observed in 37 out of 84 SG tumors evaluated (44.1%), but strong staining was consistently seen only in secretory carcinomas, SDC de novo and frankly invasive SDC ex-PA, while it was weaker in intracapsular SDC ex-PA and PA. In PA, mammaglobin expression was significantly associated with recurrence. This study has confirmed that the mammaglobin is commonly expressed in SDC de novo and secretory carcinomas. Its expression was higher in SDC ex-PA than in PA, suggesting that mammaglobin may play a role in its malignant transformation.

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1. Introduction

Mammaglobin is a protein of the secretoglobin superfamily that is frequently expressed in normal mammary glands and in most breast carcinomas, but its physiological function remains unknown [1,2]. Its role in cancer is also not fully clarified; some studies suggest that it can play a role inhibiting cell migration and invasion in breast cancer [3]. In addition, Picot et al have demonstrated that mammaglobin promotes cancer cell malignant features and it is associated with anticancer drug sensitivity [4]. Proteins of the secretoglobin family have also been correlated with apocrine secretion in submandibular glands [5], reinforcing that tumors with apocrine secretion may express mammaglobin.

Some salivary gland (SG) tumors share some overlapping morphologic and molecular features with breast tumors [6,7], in particular, salivary duct carcinomas (SDCs) and secretory carcinomas (SC). In this last one, mammaglobin expression is a well-known and important auxiliary tool for the diagnosis [8-10]. In contrast, in SDC, a tumor that can occur de novo or as a malignant component in pleomorphic adenoma, few studies have investigated the expression of mammaglobin so far [11,12]. These studies have particularly focused in de novo lesions showing that mammaglobin expression can be found in about 61.9% of them (13 cases considered positive of 21 studied). However, the frequency of mammaglobin expression in SDC arising in pleomorphic adenoma has yet to be determined. Other SG tumors have also been described to present variable mammaglobin expression, as follows: polymorphous adenocarcinomas (at least 50% of the tumor cells); focal expression in pleomorphic adenoma; adenoid cystic carcinoma and mucoepidermoid carcinoma [11,13,14]. In fact, the exact role of mammaglobin expression in SG tumors remains to be determined.

Given that mammaglobin has been reported to be involved in cancer progression and cellular phenotype [12,13]. The purpose of this study was to evaluate mammaglobin immunoexpression in SDC ex pleomorphic adenoma (SDC ex-PA) in different phases of the adenoma to carcinoma sequence in order to investigate its involvement (if any) in carcinogenesis and tumor progression. For comparison mammaglobin expression was also analyzed in SDC de novo, PA without malignant transformation and secretory carcinoma.

2. Material and methods

2.1. Tissue samples and Pathological analysis

Eighty-four salivary gland tumors including 41 pleomorphic adenomas (PA), 18 SDC ex-PA (13 intracapsular and 5 frankly invasive), 15 SDC de novo and 10 secretory carcinomas diagnosed in three pathology laboratories (University of Campinas, Brazil; Centro Clinico de Cabeza y Cuello, Guatemala; Private Service of Pathology, Brazil, and Rio Grande do Sul Federal University, Brazil) were reviewed. Clinical, demographic and follow-up information was collected from patient's medical records. All tumors were histologically classified according to the 2017 edition of the WHO/IARC Classification of Head and Neck Tumors [15]. Hematoxylin and eosin-stained slides and immunohistochemical reactions were performed in 3-μm paraffin sections. The present study was approved by the Ethics Committee of the Dentistry School of Piracicaba, University of Campinas, Brazil (Protocol no. #72075217.1.0000.5418).

2.2. Immunohistochemistry and digital quantification

Immunohistochemistry was performed for mammaglobin, cytokeratins (CKs)-7 and− 14, α-smooth muscle action, calponin, and p63 (Table 1). A heat-induced epitope retrieval was performed using citrate or EDTA/TRIS buffers. The sections were then incubated with the primary antibodies followed by the secondary antibody- and horseradish peroxidase-labeled polymer technique. Finally, the chromogenic substrate 3,3′-diaminobenzidene (DAB) was applied.

Table 1 Antibodies used in this study

Antibody	Clone	Antigen retrieval	Dilution	Source
Mammaglobin-A	31A5	EDTA-Tris solution (pH 9)	1:500	Abcam [a]
Pan-cytokeratin	AE1AE3	Citrate buffer (pH 6)	1:400	Dako [b]
Cytokeratin 7	OV-TL 12/30	Citrate buffer (pH 6)	1:300	Dako [b]
Cytokeratin 14	LL 002	EDTA-Tris solution (pH 9)	1:200	Novocastra [c]
Vimentin	Vim 3B4	Citrate buffer (pH 6)	1:400	Dako [b]
α-Smooth muscle actin (α-SMA)	1A4	Citrate buffer (pH 6)	1:400	Dako [b]
Calponin	CALP	Citrate buffer (pH 6)	1:600	Dako [b]
P63	4A4	Citrate buffer (pH 6)	1:300	Dako [b]
S100	Polyclonal	Citrate buffer (pH 6)	1:10000	Dako [b]

[a] Abcam (Cambridge, UK).
[b] Dako (Carpinteria, CA).
[c] Novocastra (Newcastle, UK).

Following the IHC reactions, the slides were scanned and digitally assessed for the establishment of digital IHC scores of mammaglobin expression scores with the Positive Pixel Count software (Aperio Technologies Inc, Vista, CA), as detailed in a previous study [16]. A visual qualitatively analysis considering only the tumor-positive cells proportion, scored from 0 to 4 (0 = none, 1 = 1%-25%, 2 = 26%-50%, 3 = 51%-75%, 4 = >75%) was also performed. For apocrine secretion, we scored it based on this scale (0 = no apocrine secretion; 1 = 1%-25%, focal apocrine secretion and 2 = >25%, diffuse apocrine secretion).

2.3. Statistical analysis

Differences in the mammaglobin expression between the digital IHC scores from different tumors were assessed with the ANOVA test. Recurrence rate curves for pleomorphic adenomas were performed with the Kaplan-Meier method, and the curves were compared with the log-rank test considering two groups: (1) mammaglobin-negative, and (2) positive focal mammaglobin expression. To evaluate if there was association between mammaglobin expression and apocrine secretion, a Spearman correlation test was applied. $P < .05$ was considered significant. All tests were performed with GraphPad Prism version 6.00 for Windows (GraphPad Software, La Jolla, CA).

Results

2.4. Clinicopathologic characteristics

We used on this study 84 salivary gland tumors, and the clinicopathologic characteristics are summarized in Table 2. Overall, the clinicopathologic features are similar to those previously described for these tumors, ie, occurring mainly in the parotid of adults, but 15 cases arose in minor salivary glands of the mouth. PA, frankly invasive SDC ex-PA, SDC de novo and SC were more common in women, while intracapsular SDC ex-PA showed no sex predilection. In the qualitatively analysis, staining for mammaglobin was considered positive in 37 out of 84 salivary gland tumors (44.1%) and negative in all normal salivary glands.

2.5. Mammaglobin IHC expression

The digital IHC analysis is presented in Fig. 1A and shows the mammaglobin positivity scores in the tumors. Invasive SDC ex-PA and SDC de novo exhibited significantly higher scores than intracapsular SDC ex-PA ($P < .01$, ANOVA). The scores for SC were similar to the ones found with invasive SDC ex-PA and SDC de novo. The values of correlation coefficients (r_s) for mammaglobin expression with apocrine secretion according to the tumor types were: PA (0.080; $P = .729$); CXPA (0.468; $P = .042$); SDC de novo (0.030; $P = .912$); SC (0.797; $P = .007$).

2.6. Pleomorphic adenomas (PA)

Morphologically, PAs presented double-layered ducts with variable amounts of mesenchymal, chondroid and adipose tissue. The luminal cells were positive for CK-7, whereas myoepithelial cells showed positivity for vimentin, CK-14, α-SMA, calponin and S-100. PAs were negative except 8 cases (19.5%) which were considered focally positive (score 1), with few mammaglobin-positive ductal cells (Fig. 1). Scores for normal salivary glands and PA were lower than the other studied groups. Considering the recurrence rate in patients with PA, 6 patients had local recurrence and 5 of them (83.3%) were positive for mammaglobin in a focal pattern ($P = .03$, log-rank, Fig. 1B). We also calculated the values of positive predictive value (0.8333; 95% CI 0.8424-0.9992), negative predictive value (0.9143; 95% CI 0.7694-0.9820), sensitivity (0.6250; 95% CI 0.2449-0.9148) and specificity (0.9697; 95% CI 0.8424-0.9992). All recurrent PA but 2 had free surgical margins. The median time between the primary surgery and the recurrence was 24.6 months.

2.7. Intracapsular SDC ex-PA

Intracapsular SDC ex-PA was considered positive in 38.5% of the cases with score 2 (Fig. 2). The expression occurred exclusively in the malignant cells, and the residual areas of PA were negative. A particular case of intracapsular SDC ex-PA has drawn our attention due to its unusual morphological features, showing a microcystic pattern with areas of secretion

Table 2 Clinicopathologic characteristics of the tumors included in this study

	PA (n = 41)	Intracapsular SDC-CXPA (n = 13)	Invasive SDC-CXPA (n = 5)	SDC de novo (n = 15)	SC (n = 10)
Age (y)					
Range (mean)	19-92 (52.5)	36-70 (54.6)	34-72 (53)	22-74 (49)	28-62 (45.1)
Sex					
Male/female	18:23	7:6	1:4	7:8	2:8
Location					
Parotid	22	7	5	8	8
Submandibular	12	2	-	5	-
Minor	7	4	-	2	2

Abbreviations: PA, pleomorphic adenoma; CXPA, carcinoma ex pleomorphic adenoma; SDC, salivary duct carcinoma; SC, salivary gland secretory carcinoma.

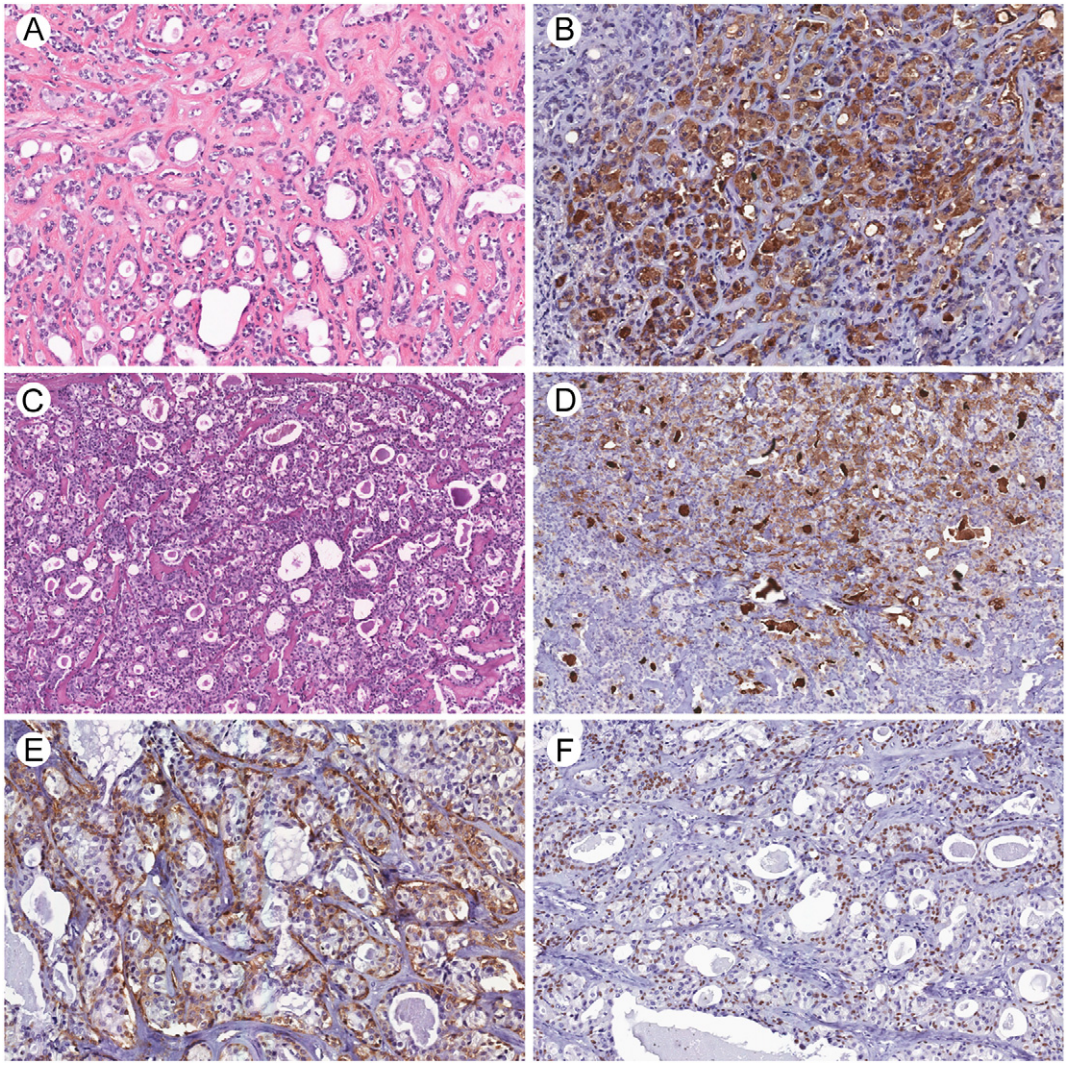

Fig. 1 Mammaglobin expression in intracapsular SDC ex-PA. A and B, Positive moderate to strong immunostaining in malignant epithelial cells of intracapsular SDC ex-PA of the parotid gland. C-F, Intracapsular SDC ex-PA of the minor salivary gland showing a microscopic pattern with microcysts, which resembles a secretory carcinoma (C), malignant cells were positive for mammaglobin (D); the epi-myoepithelial double-layered ducts were highlighted by expression of calponin (E), and P63 (F) on myoepithelial cells.

PAS-positive. As tumor cells were diffusely positive for CK-7, S-100, and mammaglobin, SC was considered in the differential diagnosis; however, it was tested for *ETV6-NTRK3* fusion by fluorescent in situ hybridization (FISH), which was consistently negative. In addition, immunohistochemistry for CK-14, calponin, α-SMA, vimentin and p63 showed areas containing ductal structures composed of two types of cells, ie, epithelial and myoepithelial cells, leading to the diagnosis of intracapsular (early stage) SDC ex-PA. This case is illustrated in Fig. 2 C-F.

2.8. Frankly invasive SDC ex-PA

Regarding invasive SDC ex-PA, mammaglobin-positivity was consistently present (score 4, 5 of 5, 100%), with strong pattern in about 50% of the tumor cells (Table 3, Fig. 3). Tumor cells were also diffusely positive for CK-7, S-100 and pan-cytokeratin. CK-14, α-SMA, calponin and vimentin was positive in the myoepithelial cells of the PA.

2.9. SDC de novo

In the SDC de novo, 60% of the cases (score 3-4 cases and score 4-5 cases) showed strong and diffuse positivity for mammaglobin (Table 3, Fig. 3). These SDCs were also positive for CK-7 and pan-cytokeratin; negative for CK-14, α-SMA, calponin and P63.

2.10. Secretory carcinomas

All secretory carcinomas were diffusely positive for CK-7, S-100 and mammaglobin (in a strong pattern). CK-14, α-SMA, calponin and P63 and calponin were negative.

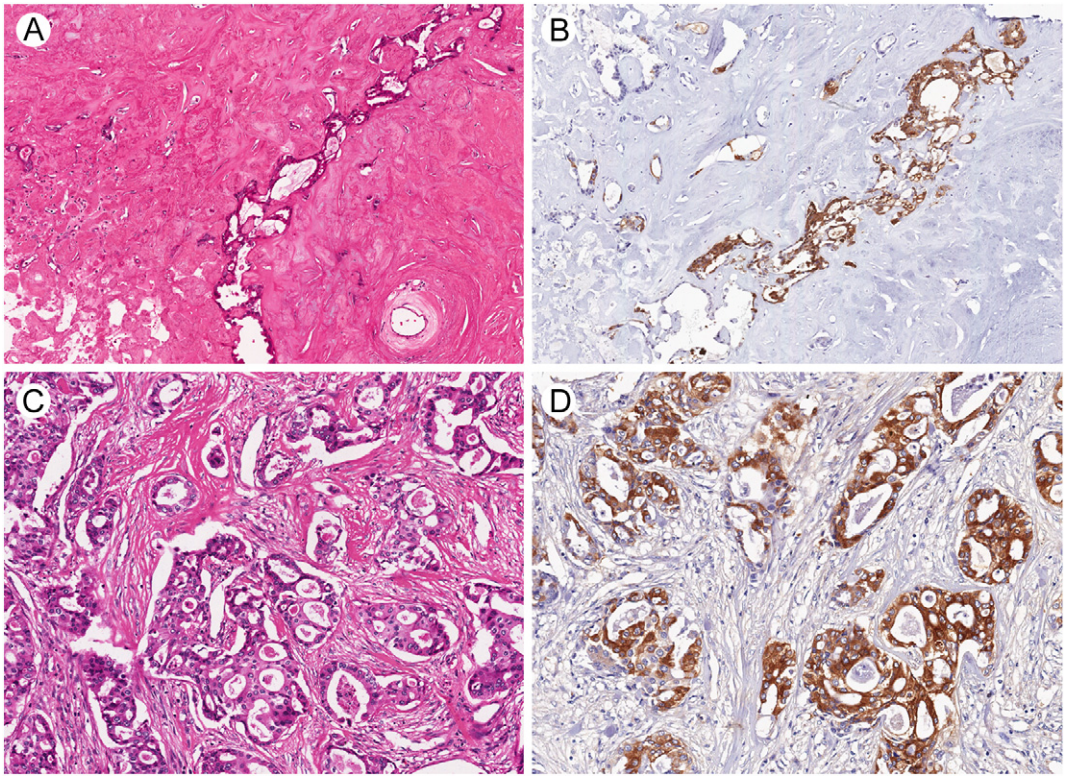

Fig. 2 Mammaglobin expression in frankly invasive SDC ex-PA and de novo SDC. A and B, Strong mammaglobin staining was seen in all invasive SDC ex-PA. C and D, Diffusely and strong staining in de novo SDC.

3. Discussion

The PA is the most common benign salivary gland (SG) tumor and it may suffer occasional malignant transformation into carcinoma ex-PA (CXPA). A wide spectrum of histological types of carcinoma can arise in PA but most of them present SDC phenotype [15]. The early phase of the malignant transformation (intraductal CXPA) is characterized by ductal structures composed of transformed malignant luminal cells, (which present abnormal morphology with enlarged eosinophilic cytoplasm, nuclei with dispersed chromatin, and evident nucleoli), lined by myoepithelial (nonluminal) cells. Following this step, the ductal structures show progressive loss of the myoepithelial cells, and epithelial cells blocks and islets without the myoepithelium invade adjacent glands and tissues (invasive CXPA). These morphologic events underlying PAs and CXPAs represent a well-recognized model of carcinogenic process of the SG tumors [17-19]. Our study shows for

Table 3 Immunoexpression of mammaglobin in breast analogue-salivary gland (SG) tumors based on the qualitative analysis

Tumor type	Mammaglobin staining (%)	Notes
Pleomorphic adenoma	8/41 (19.5)	Very focal immunostaining in isolated ductal structures All cases: score 1
Salivary duct carcinoma-ex-pleomorphic adenoma		
Intracapsular	5/13 (38.5)	Strong immunostaining in the ductal malignant cells and negative or very focal in residual PA areas All cases: score 2
Frankly invasive	5/5 (100)	Diffuse and strong immunostaining in invasive cells, and focal expression was detected in residual PA areas All cases: score 4
Salivary duct carcinoma	9/15 (60)	Diffuse and strong immunostaining in most of the malignant cells Score 3: 4 cases Score 4: 5 cases
Secretory carcinoma	10/10 (100)	Diffuse and strong immunostaining in most cases Score 3: 3 cases Score 4: 7 cases
Total	39/86 (45.3)	

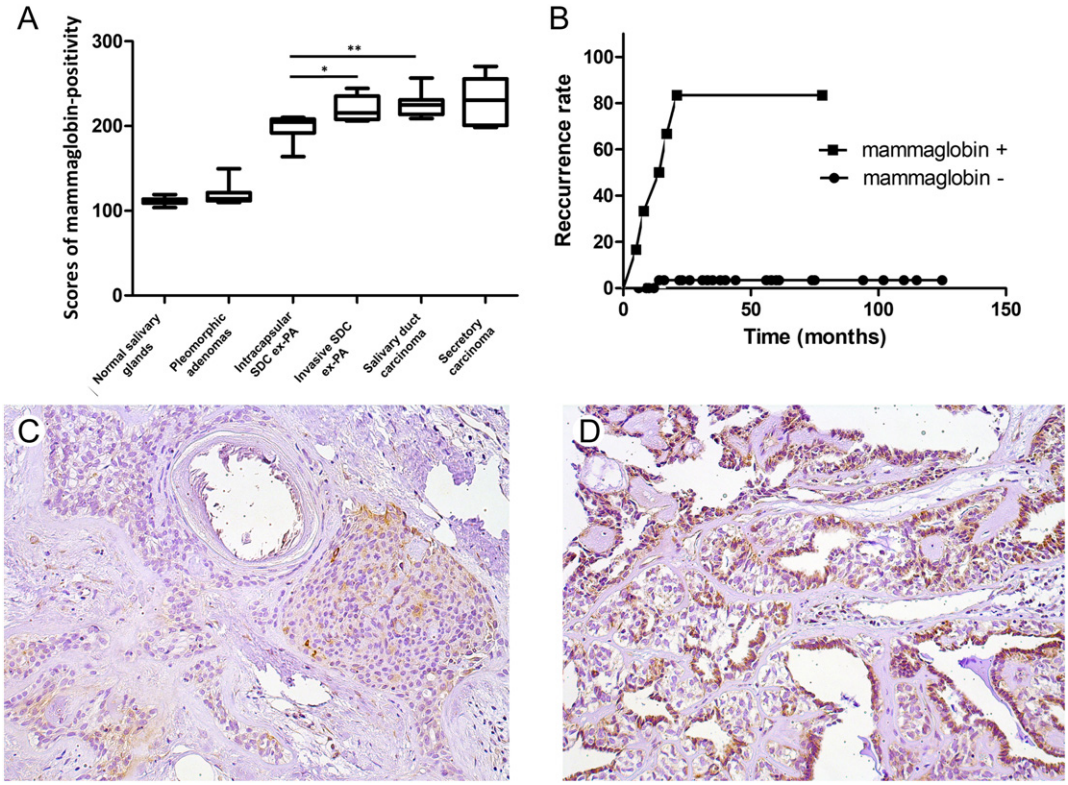

Fig. 3 Quantitative IHC scores for mammaglobin expression and recurrence rate of pleomorphic adenomas. A, Box-plot with the digital IHC scores for mammaglobin expression in normal salivary glands (SG), pleomorphic adenomas, intracapsular and invasive salivary duct carcinoma ex-pleomorphic adenoma (SDC ex-PA), de novo SDC, and secretory carcinomas of the SG. B, Recurrence rate curves for patients with pleomorphic adenomas according to the mammaglobin expression status. C and D, Examples of mammaglobin expression in recurrent pleomorphic adenomas.

the first-time increasing mammaglobin expression by malignant epithelial cells during the carcinogenic process of SDC arising in PA, ie, in its early (intracapsular) as well as advanced (frankly invasive) phase.

The expression of mammaglobin in SDC was previously studied by Stevens et al [12], demonstrating a strong positivity. However, the authors analyzed only two cases of SDC ex-PA. We examined a larger series of SDC ex-PA and showed that mammaglobin expression was significantly higher in invasive SDC ex-PA and SDC de novo than in intracapsular SDC ex-PA. Furthermore, in the latter, the protein expression was restricted to ductal malignant cells. In contrast, PA and normal salivary gland presented rare and low expression of mammaglobin. Therefore, our findings show that mammaglobin expression is mostly induced during SDC carcinogenesis lending support to the hypothesis that such protein could play a role in the SDC progression into an advanced phase. Interestingly, in ovarian carcinomas, similar phenomenon has been found; mammaglobin expression was significantly increased in invasive ovarian carcinoma than in borderline tumors and normal ovarian tissue. However, in breast carcinomas, mammaglobin is overexpressed in most lesions regardless of its phase of progression (early or advanced); 90% of invasive ductal carcinomas and 80% of intraductal carcinomas have been found to be positive for this marker [20,21]. Of interest, unlike normal salivary and ovarian tissues, mammary glands typically express mammaglobin leading us to speculate that the site of tumor origin and cellular phenotype could influence mammaglobin expression.

The molecular function of mammaglobin in cancer has been a matter of debate [20,21]. In breast cancer, elevated mammaglobin mRNA levels were correlated with clinicopathologic parameters and high-grade tumors [22,23]. In ovarian cancer, mammaglobin expression was significantly associated with tumor stage, high-grade tumor and high mitotic index [2]. In contrast, in salivary adenocarcinomas, mammaglobin expression can be present in both high-grade and low-grade tumors. In this study, mammaglobin was correlated with apocrine secretion in SC and SDC ex-PA, indicating that in these tumors, its expression may be associated with increased apocrine secretion. However, mammaglobin was not associated with apocrine secretion in SDC de novo, indicating a possible role in carcinogenesis and aggressiveness of these tumors. SDC and SC tumor cells have in common the apocrine appearance and, interestingly, it has been demonstrated that the apocrine secretion is mediated by secretoglobulin proteins

[5], and mammaglobin is one of them. In line with this idea, mammaglobin has also been found to be expressed by secretory cells of low-grade mucoepidermoid carcinoma, ie, globet cells, reinforcing the relationship of this protein with secretory activity [12]. Therefore, further investigations are required to verify the specific role of mammaglobin in salivary gland tumors and particularly in the carcinogenesis of SDC ex-PA and de novo lesions.

Another point that deserves comments in our series was the association between mammaglobin expression in PA and tumor recurrence. As expected, PA occasionally recurred but, interestingly, most recurrent cases (5/6 cases) were positive for mammaglobin. Studies have shown that recurrent PAs can present a different immunoprofile from non-recurrent tumors [24,25]. In recurrent PA, expression of PDGF-A, PDGF-B, PDGF-Rα, FGF-2, BEK, Flg and MUC1 have been found to be stronger than that seen in non-recurrent lesions and, in addition, the level of expression of this group of proteins is higher in PA with malignant transformation [19,26]. Our results showing that 83% of PA that had local recurrence were positive for mammaglobin leads to hypothesis that tumors prone to recur may present modification of its immunoprofile from the very beginning and not only in the recurrent lesion. However, further studies are needed to evaluate if mammaglobin would have a role in PA recurrence and if this protein could have predictive value for tumor recurrence.

The idea to study the mammaglobin in a series of salivary gland tumors arose from a diagnostic pitfall, ie, an initial misinterpretation of an intracapsular SDC ex-PA of minor salivary glands as secretory carcinoma (Fig. 1C-F). In fact, the tumor had a microcystic pattern with several ductal structures, which demonstrated abundant amorphous extracellular secretion, apart from the positivity for mammaglobin and S100. The differential diagnosis of secretory carcinoma include SG tumors of microcyst appearance that resemble salivary cystadenomas, intraductal, and mucoepidermoid carcinomas and, as stated here, intracapsular SDC ex-PA [27]. The association of the morphology with IHC reactions was mandatory to establish proper diagnosis. In this particular case, the detection of myoepithelial cells (positive for CK-14, calponin, α-smooth muscle actin, and p63) surrounding the ductal neoplastic cells (strongly positive for CK-7, S-100, and mammaglobin), was fundamental for the correct diagnosis of early stage (intracapsular) SDC ex-PA. In addition, the tumor was negative for *ETV6-NTRK3* fusion. Altogether, these observations are important to highlight a pitfall diagnosis of SDC ex-PA of minor salivary glands, particularly in small biopsies, and the mammaglobin and S-100 immunostainings are not sufficient to diagnose secretory carcinomas.

In conclusion, the mammaglobin immunoexpression is relatively common in breast analogue-salivary gland tumors, including salivary duct carcinoma de novo or ex pleomorphic adenoma besides secretory carcinomas. Thus, considering that mammaglobin expression is regarded as a secretory carcinoma characteristic, its expression must be interpreted with caution, particularly in those tumors which may mimic secretory carcinomas. Lastly, the results of the present study indicate that mammaglobin expression may be associated with recurrence in patients with PA and that this protein probably plays a role in malignant transformation of PA to CXPA; and therefore, mammaglobin expression may be a helpful diagnostic marker in the early stage of PA malignant transformation.

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