Lysozyme Expression Can be Useful to Distinguish Mammary Analog Secretory Carcinoma from Acinic Cell Carcinoma of Salivary Glands

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Abstract Lysozyme is an enzymatic marker of acinar and intercalated duct cells of normal salivary glands. The aim of this study was to verify whether lysozyme expression could be useful to distinguish acinic cell carcinoma (ACC) from its main mimic, mammary analog secretory carcinoma (MASC). For comparison, DOG1 expression was analyzed as well. Seventeen cases of ACC, 15 MASC, and 125 other salivary tumors were studied. Lysozyme expression was found in tumor cells as well as in secreted material of MASC (86.6 % of cases) and in ductal cells of epithelial–myoepithelial carcinoma (EMC–53.8 %), pleomorphic adenoma (PA–29.1 %) and polymorphous low-grade adenocarcinoma (PLGA–23.8 %). However, in ACC, lysozyme was not expressed. Three patterns of DOG1 staining were seen: apical–luminal, cytoplasmic, and mixed cytoplasmic/membranous. The apical–luminal pattern was detected in ductal cells of ACC (58.8 % of cases), EMC (38.4 %), adenoid-cystic carcinoma (AdCC–35.3 %), PA (8.3 %), and PLGA (4.8 %). These tumors also showed mixed membranous/cytoplasmic staining for DOG1. MASC, mucoepidermoid, and salivary duct carcinomas

exhibited only DOG1 cytoplasmic staining. In conclusion, lysozyme cannot be used as a marker of acinar differentiation in salivary tumors. However, lysozyme expression can be helpful to distinguish MASC from ACC due to its high frequency in the former and absence in ACC. It is likely that in MASC, lysozyme expression may reflect a lactational-like secretory differentiation since lysozyme belongs to breast milk proteins. Regarding DOG1 expression, the apical–luminal pattern is related to acinar and intercalated duct differentiation whereas the cytoplasmic staining does not seem to be associated with a specific cellular phenotype.

Keywords Lysozyme · DOG1 · Acinic cell carcinoma · Mammary analog secretory carcinoma

Introduction

Salivary gland tumors frequently share architecture and cytologic features so their histopathological classification can be a challenging task. Mammary analog secretory carcinoma (MASC) of salivary glands, a new diagnostic entity, and acinic cell carcinoma (ACC) belong to the group of tumors with morphological overlap, which may be quite remarkable in some lesions [1]. Both tumors can present microcystic, follicular, and/or papillary-cystic architecture and encompass a variety of cells, such as intercalated duct-like, vacuolated, non-specific glandular, and clear [2]. Serous acinar cells containing zymogen granules are only found in ACC and thus characterize this tumor. However, the zymogen granules can be infrequent and difficult to detect (zymogen granule-poor ACC) even when PAS staining after diastase digestion is used for granule identification [3]. This type of ACC, i.e. zymogen

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granule-poor, is the main mimic of MASC [4]. Regarding the architectural patterns of ACC, the microcystic and papillary microcystic variants are those that pose a significant difficulty distinguishing from MASC [5].

Both tumors, ACC and MASC, are considered low-grade neoplasms but they present some clinical differences. The frequency of metastasis is higher in MASC and there is a predominance of male in MASC and female in ACC [5, 6]. Moreover, MASC and ACC differ in relation to molecular alterations. The former harbors a key molecular event that is not observed in ACC, the ETV6–NTRK3 gene fusion similar to secretory carcinoma (SC) of the breast [7]. The ETV6 translocation detected by fluorescence in situ hybridization (FISH) is the criterion standard for the definitive diagnosis of MASC. As this method is not available in many laboratories, immunohistochemical markers have been used to distinguish MASC from ACC in an attempt to reduce the dependence on molecular genetic tests [2–4, 7–15]. Expression of mammaglobin and S-100 along with absence of reactivity for DOG1 (a molecular component of Ca^{2+} -activated Cl^- channels) have been considered useful for detecting MASC [1, 3, 9]. However, mammaglobin and S-100 do not have high specificity since these proteins were found to be expressed in up to one-third of ACC cases [3]. On the other hand, DOG1 has recently been described as a marker of salivary acinar and intercalated duct differentiation, but a variable number of ACC cases can be negative for this protein [3, 9, 13, 16].

Like DOG1, lysozyme is a protein that has been detected in acinar and intercalated duct cells of normal salivary glands [17, 18]. This enzyme has a nonspecific antibacterial activity in the saliva [19]. Immunohistochemical expression of lysozyme has been described as helpful to identify intercalated duct lesions [20, 21], but its utility to detect acinar differentiation in salivary tumors has yet to be analyzed. Thus, the aim of this study was to verify whether pattern and intensity of lysozyme expression could be useful to distinguish ACC from MASC and other salivary tumors. For comparison, DOG1 expression was investigated as well.

Materials and Methods

Tumors/Tissues

The present study was approved by the Committee of Ethics of the University of Campinas, Brazil and was performed in 17 ACC samples (all with discrete unequivocal serous acinar differentiation by either hematoxylin and eosin (H&E) stain or periodic acid Schiff reaction after diastase treatment (PAS–D) and 15 MASC. In relation to MASC, 9 cases had ETV6 translocation detected by FISH

and such detection was performed for a previous study of our group [15]. In the remaining 6 MASC cases, the diagnosis was based on the histologic findings highly suggestive of MASC along with strong staining for mammaglobin, S-100, and adipophilin [2, 15]. For comparison, 125 cases of salivary tumors classified as pleomorphic adenoma (PA), myoepithelioma (M), Warthin tumor (WT), polymorphous low-grade adenocarcinoma (PLGA), epithelial–myoepithelial carcinoma (EMC), adenoid cystic carcinoma (AdCC), mucoepidermoid carcinoma (MEC), salivary duct carcinoma (SDC) and myoepithelial carcinoma (MC) were retrieved from the surgical pathology archives of the Hospital of the UNICAMP. Clinical details were obtained from medical records.

Immunohistochemistry

Immunohistochemical studies were performed on sections from representative formalin fixed paraffin embedded blocks. All cases were stained with DOG1 (Abcam, clone DOG1.1, prediluted) and lysozyme (DAKO, clone EC32117, 1:8000). For immunohistochemical staining, 5 μm sections from each paraffin block were deparaffinized, hydrated, and endogenous peroxidase activity was quenched by immersion of the slides in 3 % hydrogen peroxide. The antigen retrieval (AR) was achieved by boiling in a steamer immersed in citrate buffer (pH 6.0) for lysozyme and in TrisEDTA (pH 9.0) for DOG1. Subsequently, for all antibodies, the sections were incubated overnight at 4 °C with the primary antibody and afterwards with the EnVision peroxidase system, dual link (K4061, DAKO, Carpinteria, CA, USA) for 1 h at 37 °C. After washing, sections were stained for 5 min at 37 °C with 3,3'-diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. Appropriate positive and negative controls were used throughout. Immunoreactivity for each antibody was assessed separately according to the tumor-cell types: ductal/epithelial and basal/myoepithelial and classified as absent (0–5 %), focal (>5–50 % of cells) and diffuse (>50 %). For DOG1, the pattern of staining was classified as: (a) apical–luminal, (b) mixed membranous and cytoplasmic, (c) cytoplasmic. In the mixed pattern, the membranous component did not exhibit the apical–luminal staining.

Results

Seventeen tumors were classified as ACC and all showed unequivocal serous acinar differentiation (Fig. 1a). In the ACC group, the age range was 20–71 years (mean 46.2 year); seven patients were female, two were male and

Fig. 1 Expression of lysozyme (**b, e, h, k**) and DOG1 (**c, f, i, l**) in salivary tumors. Acinic cell carcinoma (**a**): there is no lysozyme expression in the tumor cells (note the presence of expression in the normal salivary gland surrounding the tumor) (**b**); DOG1 is expressed in an apical–luminal pattern in tumor cells (**c**). Mammary analog secretory carcinoma (**d**): lysozyme is expressed in tumor cells as well as in secreted material (**e**); DOG1 stained tumor cells in a granular

cytoplasmic pattern (see detail above) (**f**). Epithelial–myoepithelial carcinoma (**g**): lysozyme is expressed in inner ductal cells (**h**); DOG1 is expressed in an apical–luminal pattern in ductal cells (*left*) and in a mixed membranous cytoplasmic staining in myoepithelial cells (*right*) (**i**). Polymorphous low-grade adenocarcinoma (**j**): lysozyme is expressed in ductal cells (**k**); DOG1 is expressed in a luminal–apical pattern (**l**)

gender was not available for eight. All tumors occurred in major salivary glands. All showed solid pattern with some microcystic or papillary–microcystic area.

In the MASC group, the age range was 19–65 years (mean 36.3 year); seven patients were male, four were

female and gender was not available for three. All tumors but one occurred in major salivary glands. The predominant growth pattern was papillary–cystic in four tumors, and a mixture of microcystic, follicular, papillary–cystic, and solid patterns was observed in the remaining cases. In

all cases, PAS-positive secretion within the microcystic and tubular spaces was observed and neoplastic cells exhibited low-grade nuclei, and granular pink vacuolated cytoplasm (Fig. 1d). The other 125 cases of salivary tumors were distributed as follows: PA—10 cases, M—10 cases, WT—17 cases, PLGA—21 cases, EMC—14 cases, AdCC—17 cases, MEC—11 cases, SDC—4 cases, and MC—7 cases.

Immunohistochemical Findings

Lysozyme Expression

In the normal salivary gland surrounding the tumor, lysozyme expression was observed in most intercalated ducts (luminal cells) and in small quantity of serous acinar cells (Fig. 2a). Lysozyme expression showed a granular cytoplasmic pattern in all cases. In the salivary tumors, lysozyme expression was found in MASC, EMC, PA, and PLGA (Table 1; Fig. 1e, h, k). However, in ACC, lysozyme was not expressed. In MASC, the majority of cases (86.6 %) exhibited lysozyme expression, which was present in the tumor cells as well as in the secreted material. In EMC, PA, and PLGA, the frequency of positivity for lysozyme was smaller (53.8, 29.1, and 23.8 %, respectively) and in most of them in a focal pattern (<50 % of cells). In EMC and PA, lysozyme expression was seen in luminal cells of the ductal structures, but not in myoepithelial cells.

DOG1 Expression

In normal salivary glands, serous acini and luminal cells of intercalated duct showed membranous staining for DOG1 in an apical–luminal pattern (Fig. 2b); no cytoplasmic staining for DOG1 was observed. In salivary tumors, DOG1 exhibited three patterns of staining: (a) apical–luminal, (b) mixed membranous and cytoplasmic, (c) cytoplasmic (Table 2). Apical–luminal pattern was detected in ductal cells of ACC (58.8 % of cases), EMC (38.4 %),

AdCC (35.3 %), PA (8.3 %) and PLGA (4.8 %) (Fig. 1c, i, l). This pattern was seen in more than 50 % of cells (diffuse expression) in 35.2 % of ACC cases (6/17), 23 % of EMC (2/13) and 5.8 % of AdCC (1/17–5.8 %). Mixed membranous and cytoplasmic staining was also found in ACC (41 % of cases). However, in the ACC group, this pattern of staining was present in a lower amount of cells than the apical–luminal one and in 3 cases, two or more patterns of staining (apical–luminal, mixed cytoplasmic/membranous and only cytoplasmic) were seen. Mixed membranous and cytoplasmic staining was also observed in ductal as well as myoepithelial cells of EMC (30.7 %), PLGA (14.3 %), and PA (8.3 %) (Table 2). MASC, MEC, and SDC presented granular cytoplasmic staining for DOG1; the intensity of staining was weak in all cases (Table 2; Fig. 1f).

Discussion

Based on the expression of lysozyme in normal salivary glands, where this enzyme can be observed in acinar and intercalated duct cells, our findings in the current study could be considered unexpected. Lysozyme was found in MASC but not in ACC. Indeed, in MASC, the great majority of cases was positive (86.6 %) and lysozyme was detected in tumor cells as well as in the secreted material. Recently, our group hypothesized that MASC might present differentiation toward milk-secreting mammary epithelial cells since this tumor shows abundant large lipid droplets covered by adipophilin, which is a component of milk lipid globule membranes [15]. Interestingly, lysozyme belongs to breast milk host defense proteins [22]. Therefore, the high frequency of lysozyme in MASC reinforces our previous idea that such tumors may display a lactational-like secretory differentiation.

In mammary tumors, lysozyme expression has been detected in a significant percentage of carcinomas (70 %), including the ACC subtype, where this protein has been reported to be intensely positive [23, 24]. In contrast, in

Fig. 2 Expression of lysozyme (a) and DOG1 (b) in normal salivary gland. **a** Lysozyme shows a granular cytoplasmic pattern of staining and is observed in both acinar and ductal cells of intercalated duct. **b** DOG1 presents a luminal–apical pattern of staining that is stronger in acinar than ductal cells of the intercalated duct

Table 1 Lysozyme expression in salivary gland tumors

Histopathological diagnosis	Number of positive cells (%)	Type of cell stained	Qt. positive cells	
			+	++
Pleomorphic adenoma	7/24 (29.1)	Epithelial	4	3
Myoepithelioma	0/10 (0)	–	–	–
Warthin tumor	0/17 (0)	–	–	–
Polymorphous low grade adenocarcinoma	5/21 (23.8)	Epithelial	5	–
Epithelial–myoepithelial carcinoma	7/13 (53.8)	Epithelial	5	2
Adenoid cystic carcinoma	0/17 (0)	–	–	–
Mammary analogue secretory carcinoma	13/15 (86.6)	Epithelial	5	8
Acinic cell carcinoma	0/17 (0)	–	–	–
Mucoepidermoid carcinoma	0/11 (0)	–	–	–
Salivary duct carcinoma	02/4 (50)	Epithelial	2	–
Myoepithelial carcinoma	0/7 (0)	–	–	–

Qt. positive cells quantity of positive cells: + (<50 % of positive cells), ++ (>50 % of positive cells)

Table 2 DOG1 expression in salivary gland tumors

Histopathological diagnosis	Total cases	Staining localization (no of cells/%)				Qt. positive cells	
		Epithelial/ductal		Myoepithelial	Positive cases	+	++
		AL	C/M				
Pleomorphic adenoma	24	2 (8.3)	–	2 (8.3)	4 (16.6)	4	–
Myoepithelioma	10	–	–	–	–	–	–
Warthin tumor	17	–	–	–	–	–	–
Polymorphous low grade adenocarcinoma	21	1 (4.8)	3 (14.3)	–	4 (19)	4	–
Epithelial–myoepithelial carcinoma	13	5 (38.4)	–	4 (30.7)	6 (46.1) ^a	2	4
Adenoid cystic carcinoma	17	6 (35.3)	–	–	6 (35.3)	5	1
Mammary analogue secretory carcinoma	15	–	6 (40) ^c	–	6 (40)	1	5
Acinic cell carcinoma	17	10 (58.8)	7 (41.1)	–	14 (82.3) ^b	6	8
Mucoepidermoid carcinoma	11	–	3 (27.3) ^c	–	3 (27.3)	3	–
Salivary duct carcinoma	4	–	1 (25) ^c	–	1 (25)	1	–
Myoepithelial carcinoma	7	–	–	1 (14.3)	1 (14.3)	–	1

Qt. positive cells quantity of positive cells: + (<50 % of positive cells), ++ (>50 % of positive cells)

AL apical luminal, C/M cytoplasm and/or membrane

^a 3 cases with epithelial/ductal and myoepithelial cells stained by DOG1

^b 3 cases with AL and C/M patterns of staining

^c Only cytoplasmic pattern of staining

salivary tumors, the present study showed that lysozyme expression seems to be restricted to some histological subtypes. In fact, we found that: (a) lysozyme could be helpful to distinguish MASC from ACC (86 vs. 0 % positivity, respectively) and (b) this enzyme could be considered a marker of intercalated duct but not of acinar differentiation in salivary tumors. In addition to MASC, lysozyme was also expressed in EMC, PA, PLGA, and SDC, which usually do not enter into the differential

diagnosis of MASC. EMC and PA present structures that recapitulate the phenotype of salivary intercalated ducts, i.e. a bilayered arrangement of inner ductal and outer basal and/or myoepithelial cells. In these tumors, like in the intercalated ducts, we found lysozyme expression in the cytoplasm of inner ductal cells. In EMC, a tumor that has been considered closely related to intercalated duct lesions, ductal lysozyme expression was particularly high—57.1 % of cases [23]. Although PLGA does not exhibit a biphasic

Table 3 Studies on DOG1 expression in acinic cell carcinoma

Author	Clone/source	Materials and methods (pattern of staining)	Figure (pattern of staining)	ACC positive cases no. (%)
Chênevert et al. [13]	1.1/Zeta Corporation	Membranous (apical–luminal, basolateral, and complete) and cytoplasmic	Apical/luminal, membranous and cytoplasm	28/28 (100 %)
Hemminger and Iwenofu [27]	K9/Leica	Cytoplasmic, membranous and luminal	Luminal	4/5 (80 %)
Jung et al. [9]	Spring science	–	Weak apical and apico-luminal membranous	3/6 (50 %)
Hsieh et al. [16]	SP31/Roche Ventana	–	Luminal and cytoplasm	20/21 (95 %)
Raboh and Hakim [30]	1.1	Membrane (apical–luminal, basolateral and complete), cytoplasmic or both	Apical–luminal, membranous and cytoplasm	9/9 (100 %)
Stevens et al. [26]	SP31/Dako	–	Canalicular, membranous and cytoplasm	13/13 (100 %)
Thompson et al. [31]	SP31/Cell Marque Corp	Luminal	Luminal and weak cytoplasmic	8/13 (62 %)
Urano et al. [3]	SP31/Nichirei Corporation	–	Membranous	3/6 (50 %)

ACC acinic cell carcinoma

differentiation, it is also believed to reproduce the phenotype of distal intercalated duct region, which could explain our findings of lysozyme expression in few tumors (23.8 % of cases). Regarding SDC, the importance of lysozyme reactivity (if any) should be confirmed in further studies since our series contains only a small number of SDC cases, which showed a focal pattern of expression.

MASC and ACC can present morphological overlap with adenocarcinoma/cystadenocarcinoma, not otherwise specified (NOS) and mucoepidermoid carcinoma as well [2, 7]. In the latter, lysozyme expression was not identified in any of the cases but in adenocarcinoma/cystadenocarcinoma, not otherwise specified (NOS) no test was done.

DOG1 (also known as ANO1, TMEM16 A) is a transmembrane anion channel, which mediates Ca^{2+} -dependent Cl^{-} secretion in glands and flat epithelia [24]. Structurally, DOG1 protein consists of eight transmembrane segments and cytosolic N- and C-termini [25]. In normal salivary glands, this protein is expressed in acinar and intercalated ductal cells in an apical–luminal pattern, which is in agreement with its channel function with secretory role [13]. Cytoplasmic staining of DOG1 has not been observed in normal constituent of salivary glands [13]. However, in salivary tumors, DOG1 has been described to present a more complex pattern of staining, which includes apical–luminal, cytoplasmic, and mixed cytoplasmic and membranous [13]. In the current series, these three patterns of DOG1 staining were seen. ACC was the tumor subtype where apical–luminal expression of DOG1 was more often observed—58.8 % of cases. Besides

ACC, this pattern of expression (apical–luminal) was mainly detected in ductal cells of tumors morphologically related to the phenotype of salivary intercalated ducts: EMC—38.4 % of cases, AdCC—35.3 %, PA—8.3 %, and PLGA—4.8 %. These findings reinforce those of Chênevert et al. [13], showing that apical–luminal expression of DOG1 can be considered a marker of salivary acinar and to a less extent of intercalated duct differentiation. Thus, this specific pattern of staining of DOG1 (apical–luminal) could be useful to separate ACC from MASC since in the latter, we observed cytoplasmic staining without membranous expression of the protein. However, the sensitivity of apical–luminal expression of DOG1 for ACC detection was not high (approximately 40 % of ACC cases were negative). In the literature, the frequency of DOG1 expression in ACC has varied from 100 to 50 % (Table 3). This variability of frequency may be due to different clones of DOG1 used in the studies and/or to the patterns of staining evaluated. Of note, in our series of ACC, when all patterns of staining were considered (apical–luminal, cytoplasmic, and mixed cytoplasmic/membranous), the frequency of DOG1 expression was markedly higher—82.3 %. A literature search (MEDLINE) reveals that in several studies assessing DOG1 expression in ACC (Table 3), there is no explanation on methods of analysis of DOG1 expression (i.e. patterns of staining). However, all describe and/or illustrate strong apical–luminal along with membranous and/or cytoplasmic expression of DOG1 in ACC. On the other hand, in MASC, some authors also found luminal

expression of DOG1 but unlike ACC, such expression was limited to a few areas [13, 26].

In general, DOG1 has been studied on two aspects: (a) as a marker with membranous pattern of expression that helps the diagnosis of certain tumors (such as gastrointestinal stromal tumors) and (b) the relationship of its channel function with tumorigenesis as well as with proliferation, migration, and apoptosis resistance of cancer cells [27–29]. In salivary tumors, DOG1 has only been analyzed in relation to its utility as a diagnostic marker. In this sense, DOG1 has been described particularly in ACC and less frequently in biphasic tumors [13, 21]. In biphasic tumors, DOG1 is expressed in a combined staining profile: apical–luminal pattern in ductal cells and membranous/cytoplasmic in myoepithelial ones. As normal myoepithelial cells do not express DOG1, it was suggested that this protein might be a marker of a transformed myoepithelial phenotype [13]. Our findings reinforce that neoplastic myoepithelial cells frequently express DOG1, which usually presented a mixed cytoplasmic and membranous pattern of staining. Nevertheless, regarding only cytoplasmic expression of DOG1 (without membranous component), in our series, we observed such expression in most tumor types: ACC, MASC, MEC and SDC, suggesting that this pattern of reactivity could not be used as marker of a specific cellular phenotype. Therefore, the role (if any) of cytoplasmic reactivity of DOG1 still needs to be better clarified.

In conclusion, lysozyme can be used as a marker of intercalated duct but not of acinar differentiation in salivary tumors. However, lysozyme expression can be helpful to distinguish MASC from ACC due to its high frequency in the former and absence in ACC. In MASC, lysozyme expression might reflect a lactational-like secretory differentiation since lysozyme is a milk protein. Regarding DOG1 expression, the apical–luminal pattern is related to acinar and intercalated duct differentiation whereas cytoplasmic staining does not seem to be associated with a specific cellular phenotype.

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