Immunohistochemical clue for the histological overlap of salivary adenoid cystic carcinoma and polymorphous low-grade adenocarcinoma

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Abstract: It remains difficult to distinguish adenoid cystic carcinoma (ACC) from polymorphous low-grade adenocarcinoma (PLGA). Although these neoplasms exhibit nearly similar histologic patterns, their biologic behavior is significantly different. This study was carried out in an attempt to overcome the histological overlap between these tumors using immunohistochemical method for c-kit and galectin-3 proteins on twenty cases of salivary gland tumors including twelve ACC and eight PLGA. Results revealed positive cytoplasmic reactivity for c-kit in 100% of ACC cases and only in 25% of PLGA. On the other hand, galectin-3 expression was observed in 100% of both ACC and PLGA cases. Moreover, solid variant of ACC showed overexpression of both proteins than cribriform and tubular subtypes. Significant positive correlation between the two studied proteins in ACC and PLGA was also observed ($p < 0.05$). Upon these results, over expression of c-kit and galectin-3 in ACC cases supports the concept of solid variant as a high-grade tumor. Moreover, c-kit may be used as a helpful marker to distinguish ACC from PLGA in cases where the diagnosis can be challenging.

Keywords: c-kit, galectin-3, adenoid cystic carcinoma (ACC), polymorphous low-grade adenocarcinoma (PLGA)

Introduction

Salivary gland tumors (SGTs) constitute an important area in the field of oral and maxillofacial pathology. Their incidence around the world ranges from about 1.0 to 6.0 cases per 100,000 people per year, and they represent 2–4% of head and neck neoplasms [1]. Adenoid cystic carcinoma (ACC) and polymorphous low-grade adenocarcinoma (PLGA) are malignant salivary gland tumors that represent diverse clinical behavior [2].

ACC is the third most common malignant salivary gland tumor overall (after mucoepidermoid carcinoma and PLGA). It represents 28% of malignant submandibular gland tumors, making it the single most common malignant salivary gland tumor in this region [3]. ACC occurring in the minor salivary glands of the head and neck tends to spread along nerves [4]. ACC is a malignant tumor with a deceptively benign histologic appearance. Numerous studies have attempted to elu-

cidate accurate histologic prognostic features but have often yielded conflicting results [5–7].

PLGA is the second most common type of malignant neoplasm in minor salivary glands, primarily those in the palate. Its origin in major salivary glands is considered exceedingly rare [8]. PLGA was first reported in 1983 by two different groups under the terms lobular carcinoma of the salivary glands and terminal duct carcinoma and since that it has become an established clinicopathologic entity [9, 10]. It has unique clinical, histomorphologic and behavioral aspects [11]. Owing to its location and its distinct cribriform pattern, this lesion was initially diagnosed as an unusual variant of ACC [12].

Both ACC and PLGA are believed to originate from the intercalated duct, as both are composed of both luminal epithelial and abluminal myoepithelial cells [13]. The overlapping clinicopathological features of PLGA and ACC may result in a diagnostic pitfall. ACC has a

much worse prognosis than PLGA, making differentiation important for therapeutic and prognostic purposes [14–16]. The biologic behavior of ACC and PLGA is significantly different. ACC is characterized by slow relentless growth, with late recurrences, extensive bony and soft tissue invasion, a high rate of eventual metastasis and overall poor long-term survival [13, 17–21]. In contrast to ACC, PLGA, has a much lower risk of recurrence, a good local control after a wide local excision and rarely metastasizes [5, 6, 13, 22–24]. Because of the aggressive behavior of ACC, it is important to distinguish it from other benign and malignant neoplasms of the head and neck, including those that can mimic ACC in small biopsies [25].

C-kit (CD117); is a proto-oncogene located on the long arm of chromosome 4 [26, 27]. C-kit is also known as kit, stem cell factor receptor, mast cell growth factor receptor, steel factor receptor, and p145. It encodes a transmembrane receptor-type protein tyrosine kinase that is structurally related to the platelet-derived growth factor receptor and the colony stimulatory factor receptor [28]. In addition to hematopoiesis, c-kit has been shown to play a role in normal migration and development of germ cells and melanocytes. Moreover, it plays an important role in cell proliferation, differentiation, apoptosis and adhesion [29]. In human neoplasia, the c-kit tyrosine kinase receptor pathway has been implicated in tumor development and progression. The c-kit is thought to promote cell transformation and tumorigenicity via auto-/paracrine stimulation in a variety of human tumors, either with or without activating c-kit mutations [30]. In a number of various tumors, c-kit expression has recently been implicated as an adverse prognostic indicator, including adenocarcinoma and squamous cell carcinoma of the lung [31] and endometrial adenocarcinoma [32]. In neuroblastoma, c-kit expression has been associated with lower stage at presentation and found to be an independent predictor of event free survival [33]. In advanced serous carcinoma of the ovary, c-kit expression has been associated with chemotherapy resistance [34].

Galectins are a growing family of proteins defined by their affinity for β -galactosides and by conserved sequence elements [35]. Galectin-3 is one of the most extensively investigated members of this family concerning with cancer. Galectin-3 was shown to be involved in various biological events, including cell growth, adhesion, differentiation, apoptosis [36], angiogenesis [36, 37], tumorigenesis [38], and metastasis [39]. Unlike many galectins, such as galectin-1, -7, and -9, galectin-3 was reported to be an antiapoptotic molecule [40]. Overexpression of galectin-3 has been shown to correlate with tumor progression and metastasis in renal [41], tongue [42], thyroid [43], and prostatic cancers [44]. In contrast, galectin-3 down-regulation has been reported during the progression of cancers of the breast [45] and

uterus [46]. There are several studies investigating the expression of galectin-3 in head and neck squamous cell carcinomas and most of these studies demonstrated the relationship between the galectin-3 expression and the biological behavior in tumors from the various head and neck sites [42, 47, 48].

PLGA and ACC have several overlapping histological patterns, including cribriform, tubular and solid patterns. Therefore, histopathological distinction of PLGA and ACC in minor salivary glands is a problem especially in small biopsies [15]. Numerous studies have utilized several immunohistochemical markers in attempts to distinguish between PLGA and ACC [15, 49]. This study examines the potential use of c-kit and galectin-3 as ancillary markers for distinguishing PLGA from ACC.

Material and Methods

Tissues

Tissue samples were all archival material. Twenty cases were collected from the Pathology Department in Oncology Center, Mansoura University Mansoura, Egypt. They were diagnosed as PLGA (8 cases) and ACC (12 cases). The present study protocol was reviewed and approved by the Institutional Review Board, Faculty of Dentistry, Mansoura University, Mansoura, Egypt. Evaluations were done for all patients after they granted informed consents.

Four-micron thick sections were cut on positive charged slides for hematoxylin and eosin (H & E) and immunohistochemistry. All H & E stained slides were reviewed and the diagnoses were confirmed.

Immunohistochemistry

Tissue sections were deparaffinized in xylene, rehydrated in graded alcohols, and washed with phosphate-buffered saline (PBS) (pH 7.4). Endogenous peroxidases were then blocked with 3% hydrogen peroxide for 10 min. Heat-induced antigen retrieval was performed in 10 mM citrate buffer for 2 min at 100 °C. To reduce nonspecific binding, block solution (ScyTek, Utah, USA) was applied for 5 min. The sections were incubated with either mouse monoclonal antibody to galectin-3 (clone 9C4, dilution 1:80, Thermo Fisher, Fremont, USA) or the primary antibody (CD117/c-kit/SCF Receptor clone: Ab-6; ready-to-use; rabbit polyclonal antibody, NeoMarkers, Labvision, USA) and the sections were incubated overnight at 4 °C. The final reaction product was developed in diaminobenzidine tetra-hydrochloride (DAB) mixture for 10 minutes. Finally, the slides were counter stained with hematoxylin.

The staining condition was adjusted using gastro intestinal stromal tumor to express c-kit and breast carcinoma to express galectin-3 as positive controls. Negative controls were obtained by omitting the primary antibodies. Both positive and negative controls were included in all runs.

Digital image analysis

Slides were photographed using Olympus® digital camera installed on Olympus® microscope with 1/2 X photoadapter, using $\times 40$ objective. The images were analyzed on Intel® Core I3®-based computer using VideoTest Morphology® software (Russia) with a specific built-in automated object counting routine for immunohistostain analysis and stain density.

Evaluation of c-kit immunostaining

The evaluation system for c-kit immunostaining proposed by Miliaras et al. [50] was followed. It included evaluation of the intensity as well as the extent of staining. The intensity of staining was graded as either absent, weak, moderate, or strong (0 to 3 scale). Tissue mast cells, which stain 3+, served as a positive control and were used as an internal scoring guide [51]. The extent of staining was categorized as 0%, less than 10%, between 10% and 50%, between 50% and 80%, and more than 80% (0 to 4 scales). Aggregate total scores of both intensity and extent of staining were then calculated and recorded for each case (range: 0–7).

Evaluation of galectin-3 immunostaining

Galectin-3 cytoplasmic expression was considered to be positive. Tumor cell immunoreactivity was scored as fol-

lows: (–): 100% of cells were negative, mild (+): 0% – >25%, moderate (++) : 25% – 50%, and intense (+++): >50% of the cells were positive.

Statistical analysis

Data are expressed as mean value $\pm$ SD and median. For comparisons between two groups, Mann–Whitney test was used while for more than two groups Kruskal–Wallis test was used for comparison followed by Wilcoxon multiple comparison test, using SPSS for Windows (15.0 Version). Kendall's Tau-b Correlation was used to assess relations between variables. Differences were considered statistically significant when $p < 0.05$.

Results

In the present study, all the tumors were intraoral, and the palate was the most common site ($n = 10$). Our study included twenty cases of ACC and PLGA with a ratio of 3:2. Twelve patients (60%) were women and 8 (40%) were men, with an average age of 52 years. The age range was 22 to 75 years. Of these tumors, five (25%) arose in major salivary glands; fifteen (75%) in the minor salivary glands. The most common ACC subtype was the cribriform pattern (50%). There were three tumors (25%) with solid pattern. The remaining ACC cases were tubular pattern (25%) (Table I).

Table I Shows the numbers and percentages of the different histological subtypes of ACC studied cases

ACC variants	Number	Percent (%)
Tubular	3	25.0
Cribriform	6	50.0
Solid	3	25.0
Total	12	100.0

Fig. 1. PLGA exhibited different growth patterns within the same tumor. The cells of these tumors were arranged in solid nests, cribriform, cystic, and tubular patterns $\rightarrow$ pseudo-adenoid cystic appearance. The stroma was mucoid in nature in some cases (A) or slightly eosinophilic hyalinized in other cases (B). (H&E $\times 100$)

Microscopical results

Histologically, PLGAs were characterized by the presence of monotonous cells with a wide spectrum of growth patterns within the same tumor. These tumor cells were arranged in solid nests, trabeculae, cribriform, papillary, cystic, and tubular patterns. Two of these tumors showed perineural invasion. All these microscopical features provided pseudo-adenoid cystic appearance. Tumor cells were round to polygonal in shape, of small to medium size, with indistinct cellular borders and with abundant pale eosinophilic cytoplasm. The nuclei were round to ovoid or spindled and containing vesicular nuclear chromatin in most of cases. The nuclei were more basophilic in some areas. The stroma was mucoid in nature in some cases and slightly eosinophilic hyalinized in other cases (*Fig. 1*).

ACC exhibited the growth patterns identified in PLGA. In contrast with PLGA, the cells of ACC were small and cuboidal exhibiting deeply basophilic nuclei and little cytoplasm (*Fig. 2*).

Immunohistopathological results

Cytoplasmic immunoreactivity for c-kit was detected in all studied cases of ACC (100%) (5+: 3 cases; 4+: 6 cases; 3+: 3 cases) and in 25% of PLGA (2+: 2 cases). A distinct pattern of c-kit immunoreactivity was noted among the tubular and cribriform subtypes of ACC cases where the inner epithelial cells, but not the outer myoepithelial cells, showed strong staining. This pattern was particularly pronounced in the tubular subtype. In contrast, in the solid subtype of ACC, tumor cells exhibited immunohistochemical evidence of c-kit expression in a diffuse pattern (*Fig. 3A*). Staining intensity of c-kit was diffuse and weak in the cribriform variant (6 tumors; 50%), diffuse and strong in solid form (3 tumors; 25%) and focal and weak in tubular type (3 tumors; 25%). Meanwhile, in PLGA c-kit immunoreactivity was weak (*Fig. 3B*).

Galectin-3 was expressed in cytoplasm of all studied ACC (*Fig. 4A*) and PLGA (*Fig. 4B*) cases. The lesional cells of both tumors exhibited diffuse and strong galectin-3 immunostaining.

Fig. 2. (A) Solid ACC: this pattern exhibited large sheets of tumor cells with very little or no cystic or ductal spaces. (B) Tubular ACC: the tumor exhibited multiple small duct-like structures or tubules within a hyalinized stroma. The tubular lumina can be lined by one to several layers of cells. (H&E $\times$ 250)

Fig. 3. (A) Solid variant of ACC shows strong and diffuse c-kit immunoreactivity (*ABC $\times$ 250). (B) Weak c-kit immunostaining in PLGA (ABC $\times$ 100). *ABC = avidin-biotin complex

Fig. 4. Diffuse and strong expression of galectin-3 in tubular variants of ACC (A) and in PLGA (B). (ABC $\times$ 250)

Fig. 5. Histogram shows the mean $\pm$ SD of c-kit and galectin-3 immunoreactivity in ACC and PLGA

The mean $\pm$ standard deviation (SD) as well as the median of expression of c-kit and galectin-3 were higher in ACC cases than PLGA cases (Fig. 5). In this study, there was a significant difference between ACC and PLGA regarding c-kit ($p = 0.001$) and galectin-3 ($p = 0.006$) expressions (Table II).

The median of c-kit and galectin-3 expressions were lower in tubular ACC compared with cribriform type

while the highest median was detected in solid ACC variant (Table III). Comparative tests (Kruskal–Wallis test for comparison and Wilcoxon multiple comparison test) revealed a statistically significant difference between ACC variants in relation to expression of c-kit and galectin-3. Among the subtypes of ACC, the solid variant had the highest staining score for c-kit and galectin-3. This showed that high grade tumors had a statistically significant higher total score of c-kit expression ($p = 0.012$) and galectin-3 ($p = 0.014$). There was no significant difference between tubular and cribriform ACC subtypes in relation to c-kit and galectin-3 expressions (Table III).

By Kendall's τ_b correlation test, there was a statistically positive correlation between the immunohistochemical staining of c-kit and galectin-3 in the studied cases of ACC and PLGA (Fig. 6). In relation to the subtypes of ACC, there was a significant correlation between the expression of c-kit and the different histological patterns. Similarly, the same correlation existed between the expression of galectin-3 and the different histological variants of ACC (Table IV).

Discussion

It remains difficult to distinguish ACC from PLGA. Although these neoplasms exhibit nearly similar histologic patterns, their biologic behavior is significantly differ-

Table II Shows the mean $\pm$ SD with the median of the immunohistochemical expression of c-kit and galectin-3 in ACC and PLGA cases. Mann–Whitney test was used for comparison

Comparison		ACC	PLGA	<i>p</i> value
C-kit	Mean $\pm$ SD	3.92 $\pm$ 0.996	0.63 $\pm$ 1.188	0.001*
	Median	4.00	0.00	
Galectin-3	Mean $\pm$ SD	50.83 $\pm$ 16.214	33.75 $\pm$ 5.175	0.006*
	Median	47.50	35.00	

*Significant at $p < 0.05$

Table III Shows the median of the immunohistochemical expression of c-kit and galactin-3 for ACC subtypes. Kruskal–Wallis test was used for comparison followed by Wilcoxon multiple comparison test

Comparison	ACC	Tubular	Cribriform	Solid	<i>p</i> value
C-kit	Median	3	4	5 ^{ab}	0.012
Galactin-3		35	47	75 ^{ab}	0.014

Significant at $p < 0.05$

^a Significance between tubular and cribriform or tubular and solid groups for c-kit and galactin-3

^b Significance between cribriform and solid groups for c-kit and galactin-3

Fig. 6. Shows the positive correlation between c-kit and galactin-3 in ACC and PLGA

Table IV Kendall's τ_b correlations

Comparison	<i>r</i>	<i>p</i>
C-kit & galactin-3	0.63	0.000
C-kit & types of ACC	0.83	0.002
Galactin-3 & types of ACC	0.81	0.001

Significance when $p < 0.05$

ent [11, 13]. The present study demonstrated c-kit immunoreactivity in all studied ACCs. This finding is in agreement with Jeng et al. [25] and Holst et al. [52] who mentioned that ACCs of major and minor salivary glands have been reported to express c-kit protein. Other studies indicate that c-kit is expressed in most ACC cases (34/36), raising the possibility that c-kit might be useful as an ancillary marker for this tumor [20]. Moreover, Arber et al. [53] and Albers et al. [54] found that

c-kit expression was present in 80% to 100% of ACC. In accordance, Edwards et al. [55] mentioned that c-kit immunoreactivity was uniformly positive in the cytoplasm of neoplastic cells in ACCs (15/15, 100%).

Like previous reports, the pattern and intensity of c-kit immunostaining in ACC in this study varied with the different histological subtypes, with the finding that more aggressive tumors showed greater staining intensity [24, 56]. The solid type showed diffuse reactivity compared to luminal expression in the cribriform and tubular variants. C-kit expression primarily in the inner cell layer with no expression in the outer myoepithelial cells within the tubular and cribriform variants indicates that the myoepithelial cells do not express c-kit [24]. The variable patterns and intensity of staining observed in ACC may be the result of a loss of cellular heterogeneity in the solid variant, with the differentiation primarily along the line of the luminal cell layer, and may correlate with the worse clinical course of the solid variant of

ACC [57]. Meanwhile, the cribriform pattern exhibited higher c-kit reactivity than the tubular pattern alone, others found c-kit expression to be higher among solid and tubular subtypes than the cribriform subtype [20]. In contrast, Freier et al. [57] showed staining intensity of the solid type to be significantly lower than the cribriform and tubular subtypes, an unexpected finding since solid ACC is clinically more aggressive and may inherit a higher number of cytogenetic aberrations. Moreover, the studies conducted by Seethala et al. [58] and Meer et al. [59] suggested that c-kit staining is more frequently found in well-differentiated ACC tumors and is possibly lost during dedifferentiation.

In the current work, indistinct and low expression of c-kit in PLGAs was found. This has been confirmed in other studies. Schwarz et al. [60] showed that c-kit expression was virtually lacking in PLGAs. In addition, Penner et al. [24] suggested that in histologically ambiguous salivary gland tumors, or in the setting of a limited biopsy, the absence or weak immunoreexpression of c-kit may serve as additional evidence for the diagnosis of PLGAs. On the other hand, Edwards et al. [55] identified positive c-kit immunoreactivity in the majority of PLGAs (16/17, 94%), with at least 25% of the tumor cells being positive.

An interesting observation noted in the present study was that ACC showed a significantly higher immunohistochemical expression of c-kit compared with PLGA ($p \leq 0.001$). These results are supported by previous results. Beltran et al. [49] demonstrated a statistically significant difference in c-kit expression between the tumors, with 100% c-kit immunoreactivity in ACC and no expression in 80% of PLGA. Others reported greater positive cytoplasmic c-kit expression in ACCs (>50% in 80–83% of cases), with no or minimal staining in PLGA (<50% in 41% of cases) [56, 61]. Moreover, PLGA showed a significantly weaker expression of c-kit when compared to ACC showing better prognosis than ACC [49].

These differences may be due to the use of different antibodies and the lack of standardized c-kit immunostaining and a scoring protocol. The differences may also be partly associated with the distribution of solid and cribriform variants in different series, because solid ACC have shown more diffuse and intense staining, whereas cribriform ACC showed staining in the lumenally differentiated cells only [55, 62]. The discrepancies in the specificity of c-kit staining are reported to be due to the variability of the primary antibodies selected and the influence of factors such as differences in immunohistochemical protocols (including deparaffination, epitope retrieval methods, dilutions, detection reagents used, and immunohistochemical methods), the varying methods of evaluating immunoreactivity, and the limited number of cases reviewed [49, 56, 61].

Recent researches showed that galectin-3 can be a useful and reliable marker for predicting the aggres-

siveness of different tumors due to its involvement in cell growth, adhesion, differentiation, angiogenesis, apoptosis, tumorigenesis, and metastasis [48, 63, 64]. Honjo et al. [42] observed that enhanced expression of galectin-3 in the cytoplasm was associated with a reduced level of disease-free survival in patients with tongue cancers. Piantelli et al. [65] found a significant correlation between galectin-3 tumor positivity and longer relapse-free periods and overall survival in patients with node-negative laryngeal squamous cell carcinomas.

This study revealed that galectin-3 was expressed in all studied cases of ACC and PLGA. ACC showed a significantly higher immunohistochemical expression of galectin-3 compared with PLGA ($p \leq 0.006$). The distinction between ACC and PLGA is crucial as ACC has more aggressive course. This coincides with other previous reports, Sunil et al. [11] indicated that the high immunostaining of galectin-3 in ACC may be responsible for their aggressive nature; while PLGA expressed galectin-3 with a significantly lower level compared with ACC and this may be responsible for their indolent nature. In contrast, Xu et al. [66] have showed that galectin-3 was expressed in low-grade salivary gland tumors and reduced in higher grade salivary gland malignancies. In accordance with previous studies, solid ACC expressed galectin-3 with higher levels than other ACC patterns. This could be interpreted by Sunil et al. [11] who suggested that galectin-3 has a potential role in aggressive behavior of solid ACC.

The findings of this study demonstrated a statistically significant positive correlation between the immunohistochemical reactivity for c-kit and galectin-3 in the studied cases of ACC and PLGA as well as between these expressions in ACC subtypes, further studies with increased number of cases will be recommended to clarify this observation.

Conclusion

From this study we can conclude that our finding of increased c-kit and galectin-3 expressions in solid than in tubular and cribriform variants of ACC supports the concept of solid variant ACC as a high-grade tumor, this suggests that the overexpression of these markers may be an excellent indicator of biologically more advanced tumors. Moreover, the statistical results support the potential use of c-kit, but not galectin-3, as a meaningful tool to distinguish PLGA from ACC in cases where the diagnosis can be challenging. In addition, the strong uniform expression of c-kit in ACC may suggest a potential role for c-kit inhibitors in the management of ACC. Further researches will be recommended to clarify this suggestion.

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