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The Stem Cell Marker Bmi-1 Is Sensitive in Identifying Early Lesions of Carcinoma ex Pleomorphic Adenoma

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Abstract: In the present study, we evaluated and described the sensitivity of the stem cell marker B cell-specific moloney murine leukemia virus integration site 1 (Bmi-1) in identifying early lesions of carcinoma ex pleomorphic adenoma (CXPA). While invasive CXPAs are tumors with a prominent and easily recognizable malignant component, the identification of early carcinomatous changes in PA remains a diagnostic challenge due to the lack of objective morphological criteria. The immunohistochemical expression of Bmi-1 was assessed in both adenomatous and carcinomatous components of 9 CXPA cases at an early phase of histological progression (6 intracapsular and 3 minimally invasive) grouped according to the cellular differentiation as luminal (7 cases) or myoepithelial (2 cases). A selective nuclear expression of Bmi-1 was found exclusively in the malignant component of 8 cases (6 luminal type and 2 myoepithelial type), including intraductal carcinoma areas, except for 1 case in which scarce cells of the remnant PA were positive. Thus, Bmi-1 is expressed from the earliest morphologically detectable stages of PA malignant transformation. When faced with atypical features in PA, evaluation of Bmi-1 expression can provide more objective criteria for identification and diagnosis of early lesions of CXPA. This is applied to carcinomas with luminal or myoepithelial differentiation.

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Abbreviations: α-SMA = α-smooth-muscle actin, AJCC = American Joint Committee on Cancer, Bmi-1 = B cell-specific moloney murine leukemia virus integration site 1, CK = cytokeratin, CXPA = carcinoma ex pleomorphic adenoma, HER-2/neu = human epidermal growth factor receptor-type 2, PA = pleomorphic adenoma.

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INTRODUCTION

Carcinoma ex pleomorphic adenoma (CXPA) is defined as an epithelial malignancy that develops in association with a primary or recurrent pleomorphic adenoma (PA), the most common salivary gland tumor, and constitutes about 3.5% of all salivary gland neoplasms.[1,2] Most cases affect the parotid glands, but it can occur in any site where PA occurs, mainly in patients aged between the 6th and 7th decades of life.[2] The pathogenesis of CXPA is poorly understood and has been linked to the accumulation of genetic disturbances in long-standing PAs.[1,2]

Under microscopic examination, the diagnostic criteria for identification of carcinomatous areas in PA have not been fully established and are subjective. Besides this, PA may display cellular abnormalities or even intravascular tumor deposits, mainly after diagnostic procedures, and these findings alone are not sufficient for the diagnosis of malignancy.[3] Extensive stromal hyalinization, hypercellularity, frank cellular atypia, increased mitotic activity, necrosis, and capsular infiltration are commonly associated to malignant transformation in PA, although on histopathological examination alone it is sometimes difficult to distinguish ''innocent'' atypical features from true CXPA, especially early lesions.[1,2,4,5]

Recent evidences have shown the existence of subpopulations of neoplastic cells capable to maintain tumor growth and heterogeneity by its peculiar capacity of self-renewal.[6] The so-called cancer stem cells were firstly described in acute leukemia but have been identified in solid tumors.[7–9] Besides, cancer stem cells seem to be closely involved in tumor initiation and progression, as well as resistance to conventional chemotherapeutic and radiotherapeutic treatments.[10]

The expression of several markers has been associated with stem-cell-like properties in neoplastic cells. The B cell-specific moloney murine leukemia virus integration site 1 (Bmi-1) is a transcriptional receptor of the polycomb gene family involved in several cellular processes including cell-cycle regulation, apoptosis, and maintenance of adult and neoplastic stem cells by providing self-renewal capacity.[11,12] Further, Bmi-1 expression has been associated with malignant transformation, tumor progression, metastatic disease, and poor prognosis in human malignancies.[13–16] Therefore, we aimed to describe and evaluate the sensitivity of Bmi-1 expression as a diagnostic marker for malignant change in a case series of CXPA at an early phase of carcinomatous progression.

MATERIALS AND METHODS

Patients and Tissue Samples

This study was approved by the local Ethics Committee. Nine cases of CXPA of the head and neck region at an early phase of carcinomatous progression (early CXPAs) were retrieved from the files of Anatomical Pathology Diagnostic

TABLE 1. Details of the Primary Antibodies

Antibody	Clone	Immunohistochemical Dilution	Antigenic Retrieval	Incubation (h)
Bmi-1	D20B7	1:300	Citrate	18
CK7	OV-TL12130	1:50	Citrate	1
CK14	LL02/1	1:500	Citrate	1
α-SMA	1A4	1:200	Citrate	1
Vimentin	Vim 3B4	1:800	Citrate	1
p63	4A4	1:400	Tris–EDTA	1

α-SMA = α-smooth muscle actin; CK = cytokeratin.

Service at the School of Dentistry of the University of São Paulo and of the Department of Anatomic Pathology at the School of Medicine of the State University of Campinas. Were considered as early CXPAs those cases in which the carcinoma was confined to the PA capsular boundaries (intracapsular) or with up to 1.5 mm of extracapsular infiltration (minimally invasive), according to the World Health Organization classification system.[1] All diagnosis were reviewed and confirmed by 3 pathologists (A.A., B.T.S., and S.S.) using slides routinely stained in hematoxylin and eosin. Cases were also subdivided according to cellular component of the carcinomatous areas into 2 groups: early CXPAs with luminal differentiation and early CXPAs with myoepithelial differentiation. Clinical information was obtained from the patient's medical records and cases were staged according to the American Joint Committee on Cancer (AJCC).[17]

Immunohistochemistry

One representative paraffin block was chosen for immunohistochemical examination and the anti-Bmi-1 antibody was used. In order to accurately classify the carcinomas according to the cellular differentiation (ie, luminal/epithelial or abluminal/myoepithelial) and to identify areas of intraductal carcinoma, the following antibodies were used: cytokeratins 7 (CK7) and 14 (CK14), α-smooth-muscle actin (α-SMA), vimentin, and p63. The details of the antibodies used are summarized in Table 1.

Briefly, sections of 3-μm thickness were obtained from formalin-fixed and paraffin-embedded tissues. Dewaxed sections were subjected to antigen retrieval for 30 minutes by boiling in a steamer at 95°C (citrate pH 6.0 or Tris–EDTA pH 9.0). Endogenous peroxidase was quenched by incubation with 3% hydrogen peroxide and methanol. Sections were incubated with primary antibody and then with EnVision polymer (DakoCytomation, Carpinteria, CA) for 1 hour, followed by staining with 3,3′-diaminobenzidine tetrahydrochloride (DAB) and then counterstaining with Mayer's hematoxylin. Appropriate positive controls were used in all immunohistochemical reactions. Negative control was obtained by omitting the primary antibody.

Immunostaining Evaluation

The labeled sections were evaluated by 2 observers without knowledge of patient's clinicopathological data. Immunoexpression of CK7, CK14, α-SMA, vimentin, and p63 was classified qualitatively as positive or negative. For Bmi-1 expression, the percentage of tumor cells was assessed semiquantitatively in both adenomatous and carcinomatous areas, according to the following previously described score system[18]:

(1) Score 0—no positive cell;
(2) Score 1—up to 30% of positive cells;
(3) Score 2—30–50% of positive cells;
(4) Score 3—50–80% of positive cells;
(5) Score 4—over 80% of positive cells.

RESULTS

Clinical Data

The studied group of patients consisted of 5 women and 4 men with mean age of 56 years (range 41–74). The most common localization was the parotid gland, followed by intraoral salivary glands and lacrimal gland. All cases consisted of T_1, T_2, or T_3 tumors without nodal or distant metastasis at the time of diagnosis and treated by surgical excision. No patient received adjuvant therapy.

Residual Pleomorphic Adenoma

Residual PA areas consisted of occasional double-layered ductiform structures associated with cords and islands of epithelioid to plasmacytoid myoepithelial cells embedded in a myxochondroid stroma, often with hyalinization (Figure 1A). There was no cellular pleomorphism. Luminal cells expressed CK7 and the modified myoepithelial component strongly stained to CK14, vimentin, p63, and focally to α-SMA. In only 1 luminal-type CXPA case, scarce cells in residual PA were positive to Bmi-1 (Figure 1B).

Early CXPAs With Luminal Differentiation

This group was composed by 5 intracapsular and 2 minimally invasive CXPAs. In such cases, the neoplastic cells were strongly positive to CK7 and focally to CK14, lacking any staining against myoepithelial markers. Intraductal carcinoma (transitional) areas were present in 6 cases and were characterized by duct-like structures expanded by transformed luminal cells with marked pleomorphism and associated with central necrosis, positive to CK7 and focally to CK14. Those structures were externally bounded by a rim of benign oval to spindled compressed myoepithelial cells positive to CK14, α-SMA, vimentin, and p63 (Figure 1C). In these areas, the atypical luminal cells diffusely expressed Bmi-1 (Figure 1D). In frequent regions, the reminiscent myoepithelial layer was not evident characterizing invasion of the carcinomatous component into the PA stroma, but still respecting the PA capsule (Figure 2A). Such areas were hypercellular with numerous irregular ductiform spaces lined by large and pleomorphic cells, with similar immunoprofile to luminal cells in intraductal areas, replacing PA structures (Figure 2B). In 2 cases, the carcinoma

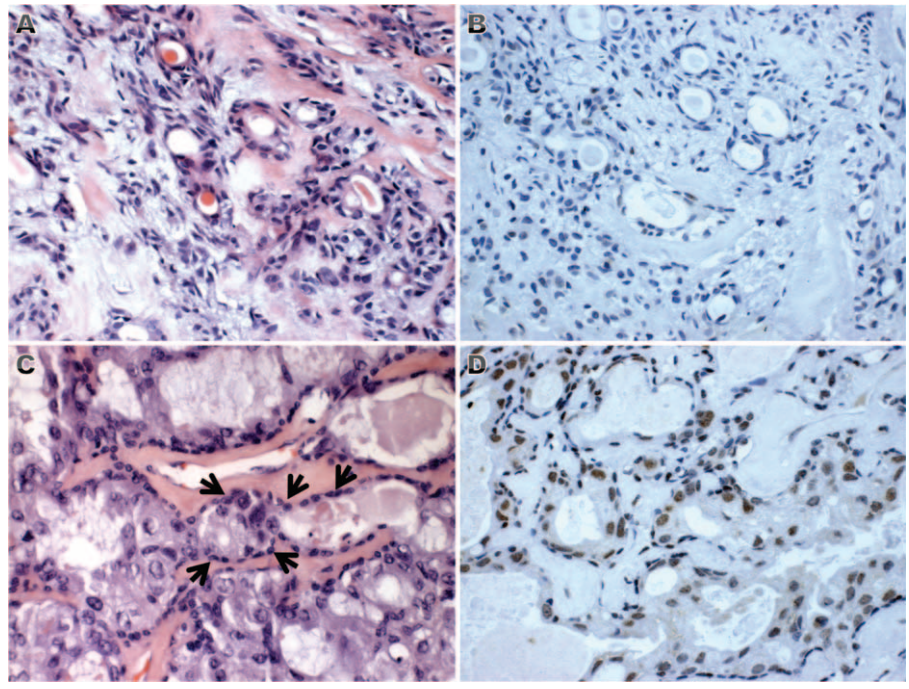

FIGURE 1. (A) Occasional double-layered ductiform structures and cords of modified myoepithelial cells in a myxoid stroma compose residual pleomorphic adenoma areas. No cellular pleomorphism is present (hematoxylin and eosin (H&E), original magnification ×400). (B) Scarce Bmi-1-positive cells in remnant pleomorphic adenoma areas (original magnification, ×400). (C) Intraductal carcinoma area characterized by expanded ductiform structures lined by highly pleomorphic transformed ductal cells, occasionally with individual necrotic cells, externally bounded by a rim of bland-looking myoepithelial cells (arrows) (H&E, original magnification, ×400). (D) Bmi-1 expression in luminal transformed cells (original magnification, ×400).

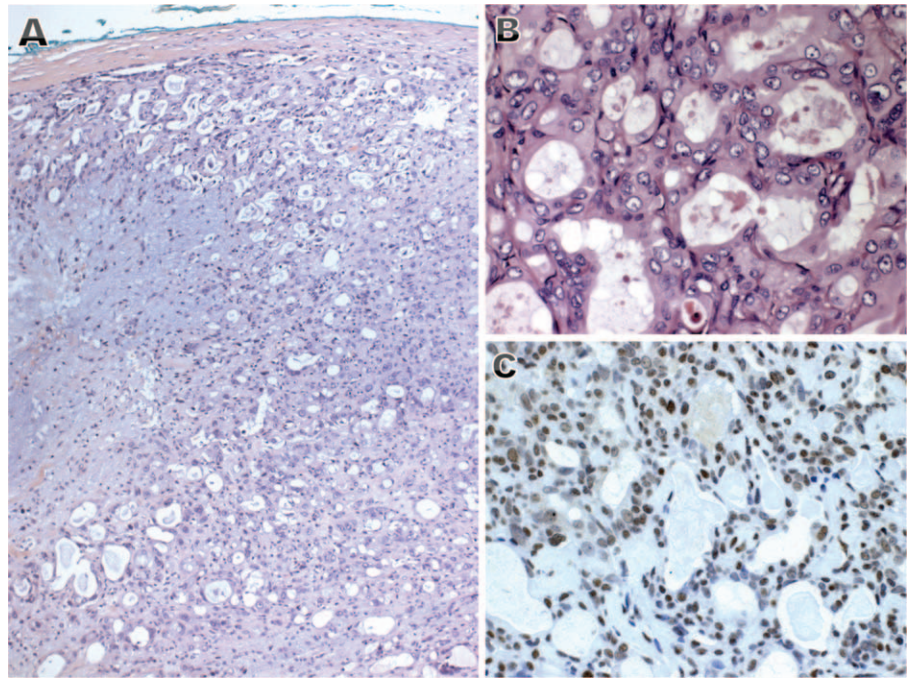

FIGURE 2. Intracapsular luminal-type carcinoma ex pleomorphic adenoma with extraductal component. (A) Numerous irregular duct-like structures replacing the ''maternal'' pleomorphic adenoma (myxoid area), but respecting the capsule (hematoxylin and eosin (H&E), original magnification ×100). (B) In a high-power view, juxtaposed duct-like structures lined by pleomorphic cells (H&E, original magnification ×400). (C) Nuclear Bmi-1 expression in carcinomatous cells (original magnification, ×400).

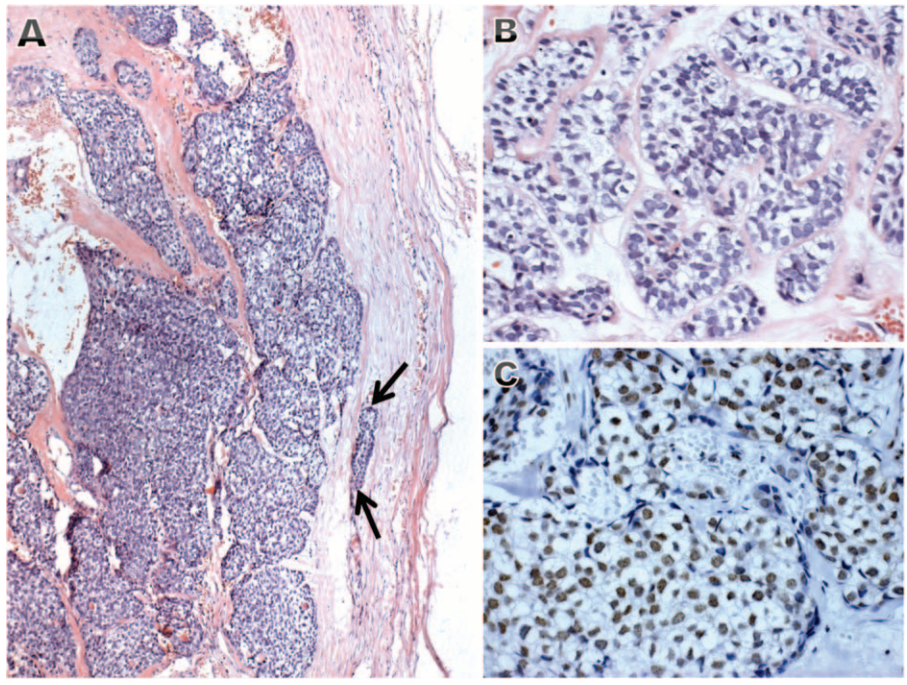

FIGURE 3. Intracapsular myoepithelial-type carcinoma ex pleomorphic adenoma. (A) Blocks of clear to eosinophilic cells pushing the capsule. A neoplastic islet is infiltrating the capsular connective tissue (arrows) (hematoxylin and eosin (H&E), original magnification, ×100). (B) In detail, myoepithelial carcinoma composed by compact nests of clear cells lacking pleomorphism, ductal formation, and mitotic activity (H&E, original magnification, ×400). (C). Diffuse nuclear Bmi-1 expression in malignant myoepithelial cells (original magnification, ×400).

penetrated into the extracapsular tissues in up to 1.5 mm (minimally invasive CXPAs). Similar to intraductal carcinoma areas, the extraductal carcinomatous component exhibited positive nuclear expression against Bmi-1 while residual PA areas were negative, except for 1 case (Figure 2C).

Early CXPAs With Myoepithelial Differentiation

One intracapsular and 1 minimally invasive CXPA composed this group. In both cases, intraductal carcinoma areas were not identified and the transition from the adenoma to carcinoma was abrupt. The carcinomatous component was characterized by invasive solid blocks composed predominantly by clear to plasmacytoid cells in a hyalinized and hemorrhagic background with occasional foci of coagulative necrosis (Figure 3A). Those cells lacked pleomorphism, brisk mitotic activity, or ductal formation (Figure 3B) and were strongly positive to CK14, vimentin, p63 and focally to α-SMA. The 2 cases displayed a diffuse and selective expression of Bmi-1 in the carcinomatous component (Figure 3C).

All clinicopathological and immunohistochemical results are summarized in Table 2. Statistical analysis was not performed due to the small number of cases and marked differences between benign and malignant areas seen in all cases.

DISCUSSION

In the present study, we have demonstrated Bmi-1 protein, a stem cell marker, as a helpful tool in identifying areas of malignant transformation in PA. CXPA is an uncommon tumor, representing the 6th most common malignancy of the salivary glands according to Armed Forces Institute of Pathology data.[2] The real incidence of malignization in PA is difficult to estimate, but it seems to be inwardly associated with accumulation of genetic disturbances in long-standing tumors, occurring in about 6.2% of patients with PA.[1,2,19] The risk of malignant transformation increases from 1.6% for PAs with clinical duration of 5 years or less to 9.5% in cases with over than 15 years of duration.[2]

In its natural history of tumor progression, CXPA develops within the confines of the parent PA and invades beyond the capsule with time. Irrespective of the histological subtype, CXPA can be subclassified into 2 main prognostically related groups regarding the capsular invasion: invasive and noninvasive/early CXPAs.[20] The former are the most common histological subtype of CXPA, often associated with metastatic disease and poor prognosis.[1,2] In such cases, the histological malignant component is usually evident and easily recognizable.[1,2,20] On the other hand, early CXPAs, the least common form of CXPA which justifies the limited number of cases here studied, generally have good prognosis but its diagnosis remains a challenge once the morphologic criteria for detecting early lesions of CXPA have not been fully established.[21] Despite its benign clinical behavior, these tumors are malignancies not completely devoid of metastatic potential.[22] Thus, recognition of at least small foci of malignant transformation in PA has clinical relevance since patients need to be carefully monitored. No patient here presented had metastatic disease at the time of diagnosis or was diagnosed at an advanced clinical stage, reaffirming the favorable clinical behavior of early CXPA.

Both luminal and myoepithelial components of PA may undergo malignant transformation and the carcinoma seems to arise from the former in most cases.[23] Morphological evidence that support this theory is the identification in some CXPA cases of the so-called intraductal/transitional carcinoma areas,

TABLE 2. Clinical Data, Histopathological Aspects, and Bmi-1 Expression in Early Carcinoma ex Pleomorphic Adenoma Cases

Case	Sex	Age	Localization	Histological Component	Degree of Capsular Invasion	TNM*	Stage*	Treatment	Bmi-1 Expression in PA Areas†	Bmi-1 Expression in CXPA Areas†
1	Female	49	Buccal mucosa	Myoepithelial	Intracapsular	$T_2N_0M_0$	II	Ex	Score 0	Score 4
2	Male	60	Palate	Luminal	Intracapsular	$T_2N_0M_0$	II	Ex	Score 0	Score 4
3	Male	56	Upper lip	Luminal	Intracapsular	$T_1N_0M_0$	I	Ex	Score 0	Score 3
4	Female	62	Parotid	Luminal	Intracapsular	$T_2N_0M_0$	II	Ex	Score 0	Score 2
5	Male	54	Parotid	Luminal	Intracapsular	$T_3N_0M_0$	III	Ex	Score 1	Score 3
6	Female	59	Parotid	Luminal	Intracapsular	$T_3N_0M_0$	III	Ex	Score 0	Score 1
7	Male	41	Parotid	Luminal	Minimally invasive	$T_3N_0M_0$	III	Ex	Score 0	Score 4
8	Female	57	Parotid	Luminal	Minimally invasive	$T_2N_0M_0$	II	Ex	Score 0	Score 3
9	Female	74	Lacrimal gland	Myoepithelial	Minimally invasive	$T_2N_0M_0$	II	Ex	Score 0	Score 4

CXPA = carcinoma ex pleomorphic adenoma; Ex = excision; PA = pleomorphic adenoma; TNM = tumor-node-metastasis.

* According to the American Joint Committee on Cancer (AJCC).[17]

† Percentage of positive neoplastic cells: score 0 (no positive cell), score 1 (up to 30% of positive cells), score 2 (30–50% of positive cells), score 3 (50–80% of positive cells), and score 4 (over 80% of positive cells).[18]

characterized by ductal structures expanded by pleomorphic transformed luminal cells confined by a rim of bland-looking benign myoepithelial cells.[2,20,23] The identification of Bmi-1 expression in such areas of our luminal-type cases seems to indicate that this is probably an early event in malignant transformation of PA. Bmi-1 is a key epigenetic regulator that mediates gene silencing by regulating chromatin structure and it is known to enhance self-renewal in cancer stem cells.[12,24] The selective expression of Bmi-1 in transformed cells of CXPA also suggests the acquisition of stem cell-like properties by these cells. These characteristics may contribute to the biological advantages necessary for tumor progression and invasion. Furthermore, Bmi-1 expression has been associated with malignant transformation and tumor progression in carcinomas of the upper aerodigestive tract, prostate, lung, and stomach.[7,13,14,16,25,26]

With the intent to minimize subjectivity in evaluating the presence of early carcinomatous changes in PA, some biomarkers have been demonstrated as helpful tools in distinguishing innocent atypical features from true CXPA lesions. Besides Bmi-1, other immunohistochemical markers can help in identifying malignant change in PA. Membranous expression of human epidermal growth factor receptor-type 2 (HER-2/neu) or its amplification by fluorescent in situ hybridization has been exclusively associated with luminal-type malignant transformed cells in CXPA, even in noninvasive cases, as well as a high proliferative index of Ki-67 or less reliably p53 expression.[27–29] Still, HER-2/neu does not seem to be sensitive in identifying CXPA cases with myoepithelial differentiation or those cases with low-grade histology.[27]

It is estimated that 50% of myoepithelial carcinomas arise in association with previous PA.[30] Further, these tumors use to lack cellular pleomorphism and identification of an invasive growth is mandatory for the diagnosis of malignancy.[2,30] This fact makes the recognition of myoepithelial-type CXPAs more difficult when compared to those cases with luminal phenotype due to the fact that the contrast between the malignant myoepithelial component and the PA is less evident. Added to this, PA may emit ''pseudopods'' to the capsule and cases of the intraoral region commonly are cellular with an exuberant myoepithelial component and ill-defined limits due to the incomplete or even lack of encapsulation.[2,3,31] These peculiarities may raise suspicion for malignancy in some cases. Interestingly, the 2 cases of myoepithelial-type CXPAs here presented showed a diffuse and selective Bmi-1 expression in malignant myoepithelial cells. Despite the small number of cases, our data indicate that Bmi-1 may be a useful marker to recognize myoepithelial carcinoma associated with PA, even in intracapsular cases or those in early phases of invasion.

In 1 intracapsular CXPA case of our series, the PA component displayed a slight positivity to Bmi-1. Such areas were devoid of cellular pleomorphism on histopathological examination, but were closely associated with carcinomatous foci. This finding could be partially explained by the fact that Bmi-1 is part of a protein complex and it may be involved in tumorigenesis of either, benign and malignant tumors. Besides, morphology is the final product of multiple genetic and environmental events in association and those positive cells in residual PA areas may display an intrinsic genotype associated with malignant transformation but, at that time, still not translated into a carcinomatous phenotype. Cases in which Bmi-1 expression is inconclusive, an accurate correlation of clinical history and morphological aspects is crucial to the definitive diagnosis. Knowledge of previous diagnostic manipulation is helpful for the evaluation of atypical features in PA. Fine needle aspiration

cytology, incisional biopsies or trauma may result in necrosis, extensive hemorrhage and mucosal ulceration often associated with reactive cytologic atypia, metaplastic elements, epithelial proliferation, or even intravascular tumor deposits.[2,3,32] Such areas use to be focal and isolated by fibrosis and inflammation.[2] On the other hand, histological findings of frank cytologic atypia with mitotic activity, including atypical forms, necrosis of individual cells or in foci, extensive stromal hyalinization and capsular violation are highly indicative of malignant change instead of a reactive process, mainly in the absence of clinical information that support these findings.

Thus, in conclusion, PA without malignant transformation may share overlapping morphological aspects with early lesions of CXPA, making microscopic distinction a challenge even for experienced pathologists. Added to this, the histopathological finding of cellular atypia alone is not sufficient to define malignant transformation in PA. The selective Bmi-1 expression from the earliest morphologically identifiable stages of CXPA indicates that this protein is important to the process of PA malignant transformation. Ultimately, in a clinicopathological context, Bmi-1 expression can provide more objective criteria, alone or associated with other markers, to distinguish atypical features in PA from true lesions of CXPA. This is applied to CXPAs with luminal or myoepithelial differentiation.

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