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ORIGINAL ARTICLE

Factors that may influence polymorphous low-grade adenocarcinoma growth

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Abstract There is mounting evidence on the importance of some biological processes in tumor growth, such as vascular supply, apoptosis, autophagy, and senescence. We have investigated these processes in polymorphous low-grade adenocarcinoma (PLGA), in an attempt to identify those that are relevant for this particular lesion. We analyzed 31 cases of PLGA using immunohistochemistry to antibodies against CD34 and CD105 to detect blood vessels; against D2-40 to detect lymphatic vessels; against Bax, Bcl-2, and survivin to explore cell apoptosis; and against Beclin and LCB3 to investigate autophagy and against p21 and p16 to assess senescence. Our results showed that PLGA growth does not depend on newly formed vessels but only on preexisting vasculature. Furthermore, PLGA is promoted by autophagy, sustained by both anti-apoptotic and anti-senescence signals, and stimulated by Bcl-2 and survivin.

Keywords Polymorphous low-grade adenocarcinoma · Angiogenesis · Apoptosis · Autophagy · Senescence · Bcl2 · Survivin · LC3B

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Background

Polymorphous low-grade adenocarcinoma (PLGA) is a malignant neoplasm almost exclusively of the minor salivary glands, characterized by cytologic uniformity, morphologic diversity, an infiltrative growth pattern, and low metastatic potential [1]. PLGA was first identified as a specific salivary gland adenocarcinoma almost simultaneously in 1983 by Freedman and Lumerman [2] and Batsakis et al. 1983 [3]. Investigations have since focused mostly on the clinical behavior of the disease which, despite a low risk of metastasis, presents a high risk of morbidity in addition to the potentially disfiguring nature of the treatment [4].

In order to elucidate how PLGA lesions grow, we assessed some factors deemed essential for tumor growth, such as vascularization, apoptosis, autophagy, and senescence.

Angiogenesis and lymphangiogenesis within the tumor microenvironment are processes that can influence neoplastic growth and metastatic dissemination. Investigating vascularization, imbalances between the mechanisms of cell regulation, proliferation, and death may shed some light on key features of PLGA and contribute to the current knowledge on its growth [5, 6].

Programmed cell death, which may occur through apoptosis, autophagy, or programmed necrosis, plays an important role in the maintenance of tissue homeostasis, including removal of damaged cells by controlling cell proliferation and death under physiological conditions. In cancer, however, this balance is lost and deregulation of programmed cell death is associated with various stages of carcinogenesis [7]. It has been recognized that apoptosis plays multiple roles in cancer development, growth, and response to treatment [8]. Loss of cell proliferation control and resistance to apoptosis are important mechanisms in tumor growth. Deregulated expression of proteins that control apoptosis can suppress elimination of

DNA-damaged cells, which in turn will contribute to accumulation of oncogenic mutations, thus facilitating malignant transformation and tumor development [9].

Autophagy is a critical cellular mechanism that can promote cell survival under many circumstances. Overactivation of autophagy pathways can, however, lead to cell death [7, 10, 11]. Several studies have demonstrated multidirectional communication between autophagy and carcinogenesis [12, 13]. Autophagy can slow or accelerate the development of tumors, depending on their type, microenvironment and stage [14].

It has recently been reported that autophagy has a dual role in carcinogenesis: (1) a supervising mechanism protecting normal cells from malignant transformation by recycling damaged organelles, aggregated proteins, mitochondrial abnormalities, damaged DNA, and (2) a mechanism supporting tumor growth by providing nutrients that are critical to tumor cell metabolism [13].

Senescence is a terminal proliferation arrest mechanism usually related to the aging process. Cumulative evidence, however, has shown that cellular senesence exerts a significant impact on tumourigenesis and degenerative disease [15–17]. Along with apoptosis, it is considered a failsafe mechanism that prevents cells from transforming into a malignant phenotype.

The aim of this study was to investigate the expression of a panel of biomarkers in PLGA by immunohistochemistry, based on the above-described processes that regulate cell death. Ultimately, the results might provide insight into the possible mechanisms involved in the growth of this particular entity.

Materials and methods

Immunohistochemistry

This study was approved by the Research Ethics Committee of the Sao Leopoldo Mandic Dental School and Research Institute, protocol number 42315715.1.0000.5374

Thirty-one cases of PLGA, previously characterized both morphologically and by immunohistochemistry, composed our sample population. Cases were included based on diagnosis and on a negative history of previous oncological treatment. In our series, no cases of cribriform adenocarcinoma of minor salivary glands (CAMSG) were included, as no cases in our series corresponded to the criteria for a diagnosis of CAMSG.

Immunohistochemical staining was performed using the following antibodies (Table 1): anti-CD34 and anti-CD105 to detect blood vessels; anti-D2-40 to detect lymphatic vessels; anti-Bax, anti-Bcl-2, and anti-survivin to detect cell apoptosis; anti-Beclin and anti-LCB3 to detect autophagy; and anti-p21 and anti-p16 to detect senescence.

From formalin-fixed paraffin-embedded tumor tissue samples, 5 μm sections were cut and mounted, dewaxed, and rehydrated and endogenous peroxidase activity was quenched by immersing slides in 3% hydrogen peroxide. Antigen retrieval (AR) was achieved by immersing the slides in boiling citrate buffer (pH 6.0) for staining for CD34, Bax, BCl-2, survivin, Beclin, LC3B, p21, and p16. For CD105 staining, AR was performed using 0.4% pepsin, while for D2-40, Tris-EDTA was used (Table 1). Only the sections for CD105 staining were incubated at 37 °C with a serum-free protein blocking solution (code x0909, Dako, SA, Denmark) for 30 min. Subsequently, the sections were incubated with the primary antibody for 60 min (anti-CD34, anti-Bax, anti-Bcl-2, anti-survivin, anti-Beclin, anti-LC3B, anti-P21) or overnight (anti-Cd105, anti-D2-40, anti-P16) at 4 °C followed by EnVision polymer HRP and Envision+ (code K1491, DAKO, SA, Denmark) for 1 h at 37 °C. The sections were developed for 5 min at 37 °C with 3.3′- diaminobenzidine tetrahydrochloride (DAB) and counter-stained with hematoxylin. A negative control was obtained by omitting the primary antibody. No staining was observed on this section.

As incision biopsies only provide a limited amount of tissue for analysis, some paraffin blocks did not contain sufficient material for all immunohistochemistry reactions, hence the different numbers reported in the results.

Table 1 Details of the antibodies used for immunohistochemistry

Specificity	Clone	Dilution	Source	Buffer (AR)
CD34	QBEnd 10	1:50	Dako[a]	Citrate
CD 105	SNG	1:10	Dako[a]	Pepsin
D2-40	D2-40	1:200	Dako[a]	Tris-EDTA
VEGF	Sc-7269	1:100	Santa Cruz[b]	Citrate
BAX	6A7	1:150	Abcam[c]	Citrate
Bcl2	124	1:150	Dako[a]	Citrate
Survivin	Ab24479	1:50	Abcam[c]	Citrate
Beclin	EPR1733Y	1:50	Abcam[c]	Citrate
LC3B	Ab51520	1:100	Abcam[c]	Citrate
p21	Ab18209	1:100	Abcam[c]	Citrate
p16	G175-405	1:50	Zeta Corporation[d]	Citrate

[a] Dako Corporation, Glostrup, Denmark

[b] Santa Cruz Biotechnogy, Inc., Santa Cruz, CA, USA

[c] Abcam, Cambridge, UK

[d] Zeta Corporation, Sierra Matre, CA, USA

Density of blood and lymphatic microvessels

For all 31 cases, immunohistochemical staining for CD34, CD105, and D2-40 was interpreted by two experienced pathologists (VCA and ABS) using a double-headed microscope. Vascularization was assessed on all specimen, though only the highest cellular areas were selected for blood vessel quantification. In order to achieve this, images were obtained from 5 fields (hotspots) per case (×40 objective, 0.44 mm field

diameter) using a digital camera (Infinity 1, Canada) attached to an Olympus CX30 microscope. The images were evaluated using the Imagelab analysis software (version 2.4), which allowed manual segmentation of the target vessels. Blood and lymphatic microvascular densities obtained from CD34, CD105, and D2-40 immunostaining were expressed as the mean number of intratumoural microvessels counted. There was no restriction regarding the size of a countable microvessel, although vessels presenting muscle walls were not counted and necrotic areas were excluded.

Cell apoptosis, autophagy and senescence

Immunohistochemical staining for Bax yielded an assessable result in 17 cases, for Bcl-2 in 29 cases, and for survivin in 29 cases. A ratio of Bax and Bcl2 staining was calculated on the 17 cases in which both Bax and Bcl2 were expressed. Expression of the autophagy-associated marker Beclin and LC3B was evaluated in 23 and 20 cases, respectively. Expression of cell senescence-associated markers p21 and p16 was analyzed in 23 and 30 cases, respectively. Immunostained sections were evaluated qualitatively and semi-quantitatively. Qualitative analysis was performed based on the proportion of positive neoplastic cells relative to all neoplastic cells throughout the tissue section, with 10% as cut-off between negative (<10%) and positive cases. Semi-quantitative analysis was performed by assigning cases with less than 10% positive cells score 0, 10–25% positive cells score 1, 25–50% score 2, and higher than 50% positive cells score 3.

Results

The 31 patients included in this study had a mean age of 57.8 years and were mostly female (71%). Data regarding patient demographics and site of the lesions are listed in Table 2.

Table 2 Clinicopathological findings of PLGA

Case	Age (year)	Gender	Site
1	72	M	Palate
2	50	F	Palate
3	62	F	Buccal mucosa
4	48	F	Palate
5	51	F	Palate
6	35	F	Palate
7	83	F	Palate
8	48	F	Buccal mucosa
9	57	M	Palate
10	68	F	Upper buccal sulcus
11	66	M	Buccal mucosa
12	73	F	Upper lip
13	51	F	Lower buccal sulcus
14	67	F	Upper lip
15	70	M	Palate
16	52	F	Palate
17	69	F	Palate
18	58	M	Palate
19	77	M	Alveolar ridge and palate
20	41	M	Maxilla
21	36	F	NA
22	65	F	Palate
23	55	F	Palate
24	70	F	Palate
25	68	M	Alveolar ridge
26	45	F	Alveolar ridge
27	64	F	Palate
28	41	F	Upper lip
29	51	F	Palate
30	33	F	Upper buccal sulcus
31	67	M	Palate

F female, *M* male, *NA* not available

Assessed of microvascular density and lymphatic vessel density using anti-D34, CD105, and D2-40 staining

High vascular density was observed in PLGA, with vessels distributed throughout the tumor, both in tumor cell masses and in the stroma. Immunohistochemical staining showed that these vessels were mostly CD34 positive (mean = 12.62), whereas few if any were CD105 positive (mean = 0.34) (Fig. 1a, b). Only a small number of D2-40-positive (lymphatic) vessels were observed (mean = 0.96) (Fig. 1c) (Fig. 2a).

Expression of apoptosis markers Bax, Bcl2, and survivin

Bax expression by immunohistochemistry was granular cytoplasmic but remarkably heterogeneous, varying from weak or negative to positive or intense. Six cases (35%) scored 0, three cases (18%) scored 1, six cases (35%) scored 2, and two cases (12%) scored 3 (Fig. 1d). Strong membrane and cytoplasmic expression of Bcl2 was noted in tumor cells in all cases, with more than 50% of tumor cells positive (score 3) (Fig. 1e). Survivin expression was observed in both the nucleus and cytoplasm. Twenty-five cases (86%) showed strong diffuse staining for survivin, with more than 50% of tumor cells staining (score 3). Score 2 was observed in two cases (7%) and the remaining two cases (7%) scored 1 (Fig. 1f) (Fig. 2b).

The calculated Bax/Bcl2 ratio ranged from 0 to 1, because all Bcl2-positive cases scored 3. The Bax-Bcl2 ratio for most cases ($n = 15$) was less than 1, suggesting that virtually no apoptosis was taking place. For the remaining two cases, the ratio was 1.

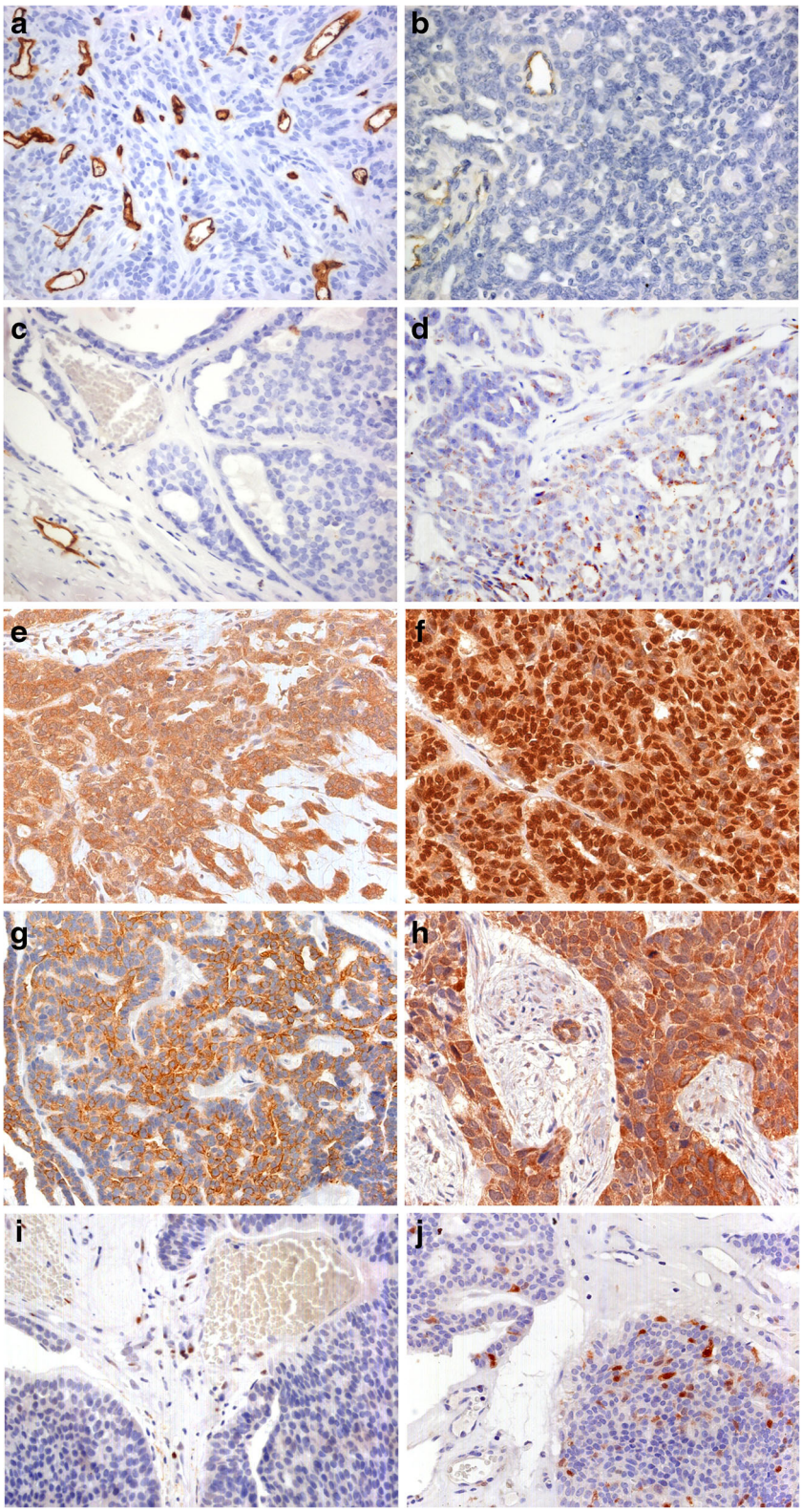
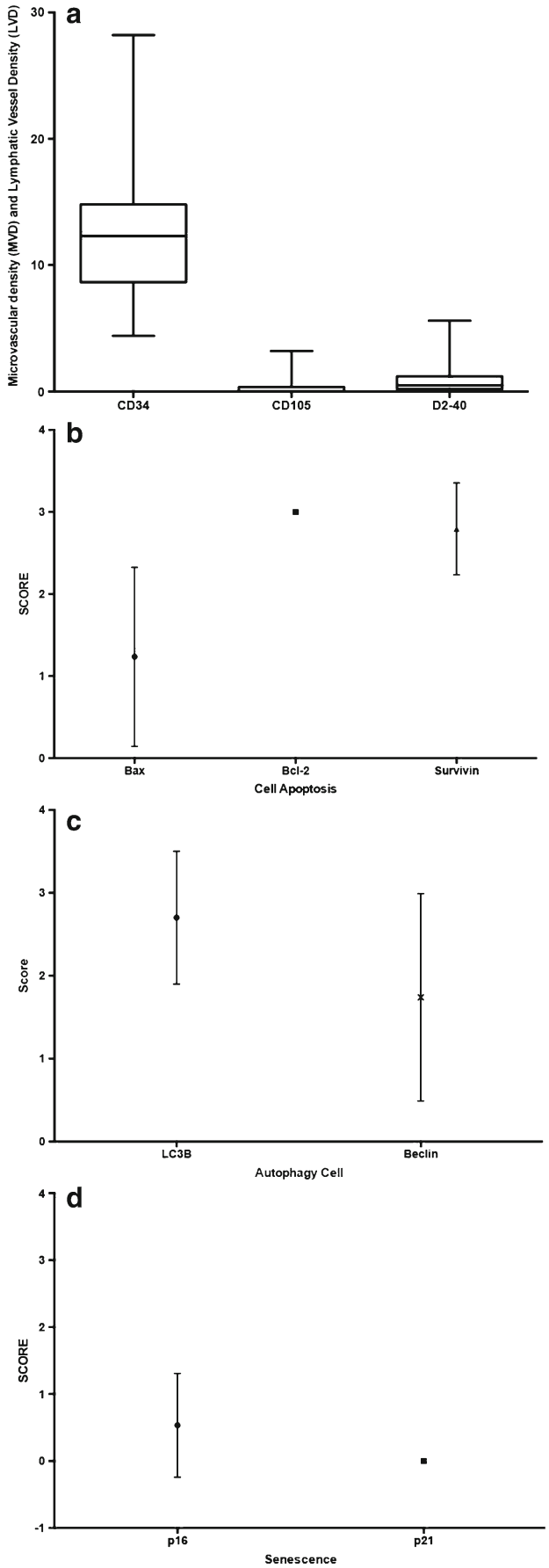


Fig. 1 PLGA Immunohistochemistry panel. Microvascular and lymphatic vessels (**a**, **b**, **c**): **a** Expression of CD34 showing numerous vessels distributed throughout the tumor. **b** CD105 positive in rare newly formed vessels. **c** D2-40 positivity in the peripheral area of the tumor; note the absence of lymphatic vessels in the bulk of the tumor. Markers of cell apoptosis (**d**, **e**, **f**): **d** Relatively low immunoexpression of Bax, **e** intense Bcl2 expression, **f** survivin expression; note the strong nuclear pattern of immunoexpression. Autophagy (**g**, **h**): **g** Beclin is expressed in nearly all cells, **h** strong cytoplasmic expression of LC3B. Senescence (**i**, **j**): **i** p21 was rarely expressed in stromal cells and not at all in the parenchyma, **j** p16 is seldom observed in parenchymal cells

Expression of autophagy markers Beclin and LC3B

Membrane and cytoplasmic Beclin expression was remarkably heterogeneous, varying between weak or negative and positive or intense. Five cases (22%) scored 0, six cases (26%) scored 1, two cases (9%) scored 2, and 10 cases (43%) scored 3 (Fig. 1g). Cytoplasmic LC3B expression was strong and diffuse in 17 (85%)



◀ **Fig. 2** Distribution of positive cell counts (mean ± SD) or scores (median) associated to each set or immunohistochemical markers. **a** Box-plot of microvessel density (MVD cell counts) and lymphatic vessel density (LVD cell counts). **B** Median distribution of cell apoptosis based on Bax, Bcl2, and survivin. **C** Median distribution of the autophagy-related markers Beclin and LC3B. **D** Median distribution of the senescence-related markers p21 and p16

cases (score 3), one case (5%) each scored 0, 1, and 2 (Fig. 1h) (Fig. 2c).

Expression of senescence markers p21 and p16

In all cases, nuclear expression of p21 was entirely negative or in less than 10% of tumor cells (score 0) (Fig. 1i). Most cases were weakly positive for p16, all expression exclusively nuclear. Eighteen (60%) cases scored 0, nine cases (30%) scored 1, two cases (7%) scored 2, and only one case (3%) was strongly positive (score 3) (Fig. 1j) (Fig. 2d).

Discussion

There is controversy as to whether or not PLGA is a single pathological entity, as recent evidence shows that similar lesions with worse prognosis (notably cribriform adenocarcinoma of the tongue [18] and cribriform adenocarcinoma of the tongue and minor salivary glands (CAMSG) [19]) may present histologically with features overlapping those of PLGA. CAMSG is considered a unique entity, as it usually arises from the base of the tongue, features cervical lymph node metastases at presentation, and exhibits optically clear nuclei, reminiscent of papillary thyroid cancer [20]. Moreover, in CAMSG, myoepithelial cells are focally present [18, 19]. We included only PLGA and excluded lesions with features of CAMSG or with lymph node metastasis at diagnosis.

Our findings shed some light on the possible route through which PLGA lesions grow. As the name suggests, this tumor is of low grade of malignancy, because very few cases with metastatic or recurrent disease have been reported. PLGA may, however, grow to cause considerable local destruction, especially as it affects the palate more than any other intraoral site. Mutilating treatment may contribute to local destruction.

Neo-angiogenesis has been extensively investigated in many tumor types, aiming to identify those that might benefit from anti-angiogenic treatment [21]. Our PLGA cases were well vascularized, containing numerous CD34-positive blood vessels surrounding the tumor epithelial cell masses. CD105 immunostaining, however, revealed that most of these vessels were preexisting rather than neo-angiogenic, suggesting that PLGA does not induce neo-angiogenesis. PLGA may instead capitalize on other sources of nutrition to maintain a steady growth.

Metastatic dissemination through the lymphatic system represents one of the major routes through which neoplastic

disease reaches distant organs, and it is also a key element in determining prognosis [6]. Our results show that lymphatic vessels are scarce and whenever present, confined to the periphery of the lesions. The combination of low neoangiogenesis and poor lymphatic vascularization may explain why metastasis is a rare finding in PLGA.

We found Bcl2 to be strongly and uniformly expressed throughout the PLGA biopsies, whereas Bax expression was weaker and heterogeneous, often within the same lesion. Bax is a pro-apoptotic protein, which is inhibited by Bcl2 in order to bypass cell death. This suggests that rather than the expressed quantity of either protein, the balance between them will determine whether a cell will live or die [22]. As a consequence, the ratio between the level of expression of Bax and Bcl2 might be more informative of the biological behavior of the tumor than that of each marker alone. This is corroborated by the findings from our study, which strongly suggest a shift towards anti-apoptosis. In support of this notion, a recent study showed that Bcl2 does not only prevent apoptosis but, in some tumors, also promotes cell migration and invasion [23].

We also studied cell survival through expression of survivin, which appeared to be strongly expressed in both the nucleus and cytoplasm of PLGA cells. In the cytoplasm, survivin acts as an inhibitor of apoptosis, whereas in the nucleus it is an effector of mitosis and promotes tumor cell proliferation [24]. Only two studies reporting survivin expression in salivary gland tumors have been published, one by Ko et al. [25] focusing on adenoid cystic carcinoma and the other by Ettl et al. [26] on survivin expression in 286 salivary gland carcinomas, including 11 cases of PLGA. Both concluded that nuclear expression of survivin is associated with high-grade malignancy. Since the latter study identified survivin expression in the nucleus, it is conceivable that the location of expression of a protein depends upon the tumor type on which the study was performed. This implies that data on immunohistochemical expression should be cautiously interpreted, and should include other biological mechanisms that stimulate tumor growth. For PLGA, our findings strongly suggest that apoptosis is properly regulated while cell proliferation might be stimulated by Bcl2 and nuclear expression of survivin.

We also investigated cell death via autophagy, using Beclin and LC3B as biomarkers. Autophagy is an important mechanism of cell survival under nutrient and energy starvation and hypoxia [7, 27]. Our results show that both autophagy markers are strongly expressed in PLGA cells, suggesting that in PLGA autophagy is an important mechanism to support tumor growth. Therefore, therapeutic strategies designed to control or inhibit autophagy ought to be considered, as has been proposed for other tumors [13].

Several studies have focused on cross-talk between apoptosis and autophagy, as comprehensively discussed in the review by El-Khattouti et al. [28]. Suppression of apoptosis induces autophagy and inhibition of autophagy leads to apoptosis, key aspects of tumor behavior to be taken into account when planning treatment.

Senescence has been suggested as the resultant of tumor suppressor pathways. Physiologically, in aging cells, shortening telomeres activate the p53-p21-pRB pathway, whereas in neoplasia this pathway is activated through p16-pRB. Conceivably, p21 may transiently inhibit pRB, with p16 inducing a state of permanent pRB hypophosphorylation [29, 30]. This provided the rationale for our investigation on expression of p21 and p16 in PLGA, even though they are not regarded as specific markers of senescence. Our results show that PLGA cells do not express p21 and only a small proportion of lesions express p16, though weak at best (<50%). These findings suggest that abrogation of senescence pathways may be an additional route to PLGA growth.

We investigated vascularization of PLGA and found it to be well vascularized but not dependent on newly formed vessels. PLGA appear to grow at the expense of autophagy, sustained by anti-apoptotic and anti-senescence signals and stimulated by Bcl2 and survivin, the former providing a link to cell migration and invasion and the latter to proliferation.

In summary, the findings from this study provide new insight into the natural history of PLGA, which ought to be taken into consideration when designing novel therapeutic strategies. These should reduce mutilating approaches, which will improve the quality of life of patients diagnosed with this disease.

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Conflict of interest The authors declare that they have no conflict of interest.

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