Immunolocalization of IP3R and V-ATPase in Ameloblastomas

Allan Fernando Giovanini¹ · Thaynara Fernanda Priesnitz² · Bruna Til² · Gisele Reisdoerfer² · Tuanny Carvalho de Lima do Nascimento¹ · Bernardo Sobreiro¹ · Adriane Sousa de Siqueira² · João de Jesus Viana Pinheiro³

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Abstract

The goal of this study was to investigate the immunolocalization of inositol 1,4,5-trisphosphate receptor (IP3R) and vacuolar ATPase (V-ATPase) in ameloblastomas with special attention to the invasive front. Thirty-seven cases of previously diagnosed formalin-fixed paraffin-embedded (FFPE) human ameloblastoma samples were selected for this study. The samples were grouped according to the predominant histologic pattern and comprised twelve plexiform, eighteen follicular, and seven unicystic ameloblastomas. Of the unicystic variants, six demonstrated purely luminal and intraluminal growth, and one displayed mural extension. One granular cell variant was included in the follicular ameloblastoma group. All specimens were evaluated for IP3R and V-ATPase expression by immunohistochemistry (IHC). IP3R was positive in columnar cells, similar to ameloblasts, and non-peripheral cells in all samples. In the area of tumor protrusion and front of invasion, membranous and cytoplasmic IP3R expression was observed. In contrast, areas adjacent to tumoral protrusion demonstrated only membranous staining patterns. V-ATPase was not expressed in peripheral columnar cells of the unicystic and granular cell variants of ameloblastoma; however, strong staining was present in these cells in plexiform ameloblastomas, follicular ameloblastomas, and areas of mural growth of unicystic ameloblastomas. In areas of tumor protrusion, reactivity for V-ATPase was observed with both membranous and cytoplasmic staining, while other areas showed only membranous V-ATPase. These findings suggest that concomitant immunolocalization of IP3R and V-ATPase, with both cytoplasmic and membranous expression in the peripheral columnar cells, may indicate the invasive potential of ameloblastomas. Furthermore, these results suggest the tumoral spread of ameloblastomas may be correlated with the autophagy process and channelopathy. The expression of these proteins could establish a baseline for future research and provide therapeutic targets for treatment of ameloblastomas.

Keywords Ameloblastoma · IP3R · V-ATPase · Invasiveness

Introduction

Ameloblastoma is an ectodermal tumor arising from odontogenic epithelium. It is characterized by basilar palisaded columnar cells, similar to ameloblasts, that demonstrate hyperchromatic nuclei polarized away from the basement

membrane. In one of the most commonly recognized histologic variants, the follicular pattern, the palisaded basal layer surrounds a second population of freely arranged cells that resembles the stellate reticulum of the enamel organ [1, 2]. Although deemed benign, the infiltrative/invasive potential of ameloblastomas leads to local aggression, increased frequency of recurrence and, in turn, morbidity [3]. It is noteworthy that aggressive behavior seems to be closely associated with the histologic architecture. Whereas the solid/multicystic types, to include plexiform and follicular patterns, infiltrate the marrow spaces, a unicystic ameloblastoma usually demonstrates expansile growth and a more indolent course [4].

One study attributed the local invasiveness of ameloblastoma to expression of proteins inducing invadopodia [5]. More recently, Sharp et al. inferred that the progression and invasive potential of an ameloblastoma could be associated with

✉ Allan Fernando Giovanini
afgiovanini@gmail.com

¹ Medical School, Positivo University Curitiba, R Pedro Viriato Parigot de Souza, 5300 Campo Comprido, Curitiba, Paraná 81280-330, Brazil

² Dentistry School, Positivo University Curitiba, Curitiba, Paraná, Brazil

³ Department of Oral Pathology, School of Dentistry, Federal University of Pará, Belem, Brazil

the autophagy process [6]. Autophagy is a catabolic process whereby autophagic vesicles sequester injured organelles and proteins for degradation through fusion with the lysosomes [7]. It should be highlighted that several proteins, including ion channels, contribute to autophagy simultaneously [8].

It has been suggested that inositol 1,4,5-trisphosphate receptor (IP3R), a calcium ion channel, is correlated with invasive behavior and progression of several types of neoplasia including breast cancer, prostate cancer, uterine cancer, ovarian cancer, and pulmonary adenocarcinoma [9–12]. Its expression is also associated with poor prognosis in colorectal carcinoma [13].

Another protein linked to infiltrative tumor properties is vacuolar (V)-ATPase. V-ATPases are ATP-driven proton pumps that are present in intracellular membranes, such as lysosomes, endosomes, and secretory vesicles. They create the acidic environment required for degradation of proteins by acid-dependent proteases, including metalloproteinases (MMPs) [14, 15]. These properties have caused V-ATPase to be regarded as an indicator of infiltration and metastasis of some cancers, and its presence leads to a poor prognosis in carcinomas of the breast, pancreas, and oral cavity [16, 17].

Based on this evidence, we evaluated the immunolocalization of IP3R and V-ATPase in formalin-fixed paraffin-embedded (FFPE) samples of several histologic patterns of ameloblastomas.

Materials and Methods

Immunohistochemistry

Thirty-seven ameloblastoma samples were used in this study. The specimens were retrieved from the files of the Department of Oral Pathology, School of Dentistry, Federal University of Para (Belem, PA, Brazil) and Histopathology Laboratory of Positivo University (Curitiba, PR, Brazil) and classified by their dominant histologic pattern. The specimens comprised seven unicystic variants (six with purely luminal and intraluminal lining epithelium and 1 with mural extension), 12 plexiform, and 18 follicular ameloblastomas. One granular cell ameloblastoma was grouped under the follicular variant.

FFPE tissues were studied by immunohistochemistry. Sections of 3 µm were obtained and mounted on 3-aminopropyltriethoxysilane-coated slides (Sigma Chemical Corp., St Louis, MO, USA).

Sections were dewaxed in xylene and hydrated in graded ethanol. Antigen retrieval was performed using citrate (pH 6.0) in a microwave oven for three cycles of 5 min. Sections were immersed in 3% H₂O₂ in methanol for 20 min for inhibition of endogenous peroxidase activity and then blocked with 1% bovine serum albumin (Sigma) in

phosphate-buffered saline (PBS) for 1 h. Slides were incubated with primary antibodies anti-IP3R (1.0 mg/ml, Abcam, Cambridge, UK, ab5804; with a dilution factor of 1:1000, incubated overnight) and V-ATPase (200 µg/ml, Santa Cruz Biotechnology Inc., sc-166848, CA, USA; with a dilution factor of 1:150, incubated overnight). Subsequently, sections were incubated for 30 min with biotin-free horseradish peroxidase enzyme labelled polymer of the EnVision Plus Detection System (Dako, Carpinteria, CA, USA). Diaminobenzidine (Sigma) was used as a chromogen, and sections were counterstained with Harris hematoxylin (Sigma). A negative control was performed for all samples, using rabbit polyclonal isotype IgG (2 g/ml, Abcam ab27472) for 10 min at room temperature as a primary antibody. We simultaneously used 3 bone fragments with osteoclasts for V-ATPase⁺ and 2 biopsies of brain tissue containing Purkinje cells to evidence the presence of IP3R⁺ as positive controls.

Immunohistochemical Analysis and Design for Labeling Index

Sections were analyzed using light microscopy at a final magnification of 400×, either in areas of front of invasion, with evident projection of epithelial cells toward connective tissue, or in non-infiltrative areas that exhibited a smooth line between epithelial neoplasm and stroma. In this study, peripheral columnar cells and non-peripheral cells were evaluated and analyzed separately. The expression of IP3R and V-ATPase was scored according to the number of immunopositive cells without consideration of differences in intensity of immunoreactivity. Protein expression was characterized as only membranous or membranous and cytoplasmic. The labeling index was determined by observing 1000 cells and counting positive cells. All results obtained were converted into a percentage.

Results

The immunohistochemical expression of IP3R and V-ATPase for each histopathological pattern of ameloblastoma is briefly described below. The quantitative data for the immunolocalization profile of the proteins, as well as its correlation to histologic type of ameloblastoma, are summarized in Fig. 1.

Unicystic Ameloblastoma

In all six unicystic ameloblastomas that demonstrated purely luminal and intraluminal growth, expression of IP3R was observed. IP3R was present in both peripheral columnar cells and non-peripheral cells surrounding the lumen. In non-peripheral cells, the presence of this protein

Fig. 1 Immunolocalization of IP3R and V-ATPase in several histologic variants of ameloblastoma. **a** reveals the percentage (mean and standard deviation) of distribution of IP3R and V-ATPase in the membrane and membrane and cytoplasm simultaneously. It also dem-

onstrates the lack of this protein in peripheral ameloblastoma cells (columnar). **b** shows the same criteria for luminal cells. Unicystic with follicular differentiation = ameloblastoma with mural extension

was cytoplasmic and membranous. In peripheral columnar cells, immunoexpression of IP3R was only observed in the basolateral membrane (Fig. 2a1, a2). V-ATPase occurred occasionally in peripheral columnar cells and exuberantly in luminal cells (Fig. 2b1, b2). In columnar cells, expression was restricted to the membrane of a subset of cells. On the contrary, both the membranous and cytoplasmic immunoexpression for V-ATPase was present in luminal cells. The immunolocalization of these proteins differed in the ameloblastoma with mural extension. Specifically, in the front of invasion, cytoplasmic and membranous expression of both IP3R (Fig. 2c1, c2) and V-ATPase (Fig. 2d1, d2) was observed in the peripheral columnar cell population.

Follicular Ameloblastoma

In the classic follicular ameloblastoma pattern, IP3R and V-ATPase were observed in both columnar and luminal cells (Fig. 3a, b, respectively). The columnar cells with

membranous and cytoplasmic positivity for both proteins were localized to regions of evident tumor projections. Meanwhile, in areas with a smooth interface between the tumor epithelium and connective tissue, these proteins were observed only in the apical and basolateral poles of the plasmatic membrane. For the luminal cells, both IP3R and V-ATPase showed membranous and cytoplasmic expression. However, IP3R was expressed in the majority of cells whereas V-ATPase was observed in approximately half of the cells. In the tumor with granular cells, strong membranous and cytoplasmic IP3R positivity was observed in all cells (Fig. 3c). Few cells exhibited positivity for V-ATPase (Fig. 3d).

Plexiform Ameloblastoma

In all plexiform ameloblastomas, cytoplasmic and membranous immunopositivity for IP3R (Fig. 3e) and V-ATPase

Fig. 2 Micrographs **a1** and **a2** reveal the immunopositivity for IP3R in unicystic ameloblastomas with purely luminal and intraluminal lining epithelium. The strong positivity for IP3R is verified in all layers of tumors; however, the predominant immunoexpression in only the membrane in ameloblast-like cells (notched arrows) (**a2**). Meanwhile, in luminal cells, both membranous and cytoplasmic positivity is observed. **b1** and **b2** show the lack of immunolocalization for V-ATPase in ameloblast-like cells (notched arrows) on a unicystic ameloblastoma. **c1** and **c2** (IP3R) and **d1** and **d2** (V-ATPase) show

a specific case of unicystic ameloblastoma with mural growth. The same pattern for IP3R can be seen, as well as strong membranous and cytoplasmic immunopositivity for IP3R and V-ATPase (arrow) and scarce expression only in the membrane (notched arrows) (in detail in **c2**). **d2** shows lack of positivity for V-ATPase in an area that does not show tumor protrusion (chevron). (Original magnification **a1**, **b1** $\times 100$, **c1** and **d1** $\times 40$; **a2**, **b2**, **c2**, and **d2** $\times 200$; Bars in $\times 40 = 800 \mu\text{m}$; Bars in $\times 100 = 260 \mu\text{m}$; bars in $\times 200 = 100 \mu\text{m}$)

(Fig. 3f) was observed in the majority of both peripheral columnar cells and luminal cells.

Discussion

Autophagic mechanisms may play a role in the behavior of ameloblastomas [6]. Autophagy is a well-coordinated process involving the sequestration of damaged proteins in double-membraned vesicles known as autophagosomes [18]. The lysosomal-derived metabolites and degradation products are then recycled back into the cell to support survival

Fig. 3 **a** and **b** demonstrate the pattern of strong expression of IP3R and V-ATPase, respectively, in the usual follicular ameloblastoma. The expression was both membranous and cytoplasmic in areas of infiltration (arrow) of columnar cells, while areas without tumor projection exhibited positivity for IP3R and V-ATPase only in the membrane (notched arrow). Micrographs **d** and **e** reveal the pattern of expression for IP3R and V-ATPase, respectively, in an ameloblastoma with granular cells. The presence of IP3R in granular cell

ameloblastomas is observed, while **e** demonstrates scarce cells positive for V-ATPase in both the membrane (notched arrow) and cytoplasm (arrow). Micrographs **f** and **g** show the strong positivity for ameloblastic cells (arrow) as well as intraluminal cells for IP3R and V-ATPase in plexiform ameloblastomas. (Original magnification **a**, **b**, **c**, and **d** $\times 200$; **e** and **f** $\times 100$; Bars in $\times 100 = 260 \mu\text{m}$; bars in $\times 200 = 100 \mu\text{m}$)

and sustain biosynthesis during stress. At the same time, the trafficking of ubiquitinated protein to the lysosome impacts cell migration [19].

It is well established that the intracellular calcium (Ca^{2+}) storage organelles play a role in basal autophagy initiation and progression. This phenomenon is related to the Ca^{2+} -dependent energizing of the mitochondria through basal and constitutive Ca^{2+} release events from the endoplasmic reticulum. This is an event mediated by IP3R channels, which are also involved in sustaining mitochondrial bioenergetics. These channels drive NADH production and subsequent ATP synthesis by continuously providing Ca^{2+} to the mitochondria [20].

It is also noteworthy that the accumulation of cytosolic Ca^{2+} through the IP3R channel acts as a second messenger. This pathway leads to the activation of proto-oncogenes,

inactivation of tumor-suppressor genes, and alteration of signal transduction [10]. These conditions are important for development of neoplasia. At the same time, the presence of Ca^{2+} promotes cytoskeletal remodeling, deformation of the plasma membrane, and actin polymerization. In combination, these effects contribute to pseudopodia and favor cellular migration and invasiveness [21].

The intracytoplasmic presence of IP3R indicates the occurrence of a Ca^{2+} channel in the intracellular microenvironment and the influx of this ion into the cellular compartment. The cytoplasmic immunopositivity for IP3R in columnar cells localized to the front of invasion suggests that the effect of autophagy and alteration in Ca^{2+} -dependence is associated with ameloblastoma progression.

We also demonstrated an association between the expression of V-ATPase by ICH and the front of invasion of an

ameloblastoma. Immunoexpression of this protein occurred in these specific areas in the cellular membrane or membrane and cytoplasm.

V-ATPase is a proton ion channel that generates energy for cells and maintains the acidic luminal pH necessary for zymogenic activation within the organelle [22]. V-ATPase is also present in the cytoplasmic membrane and intracellular organelles for active proton (H⁺) transport to the extracellular microenvironment. Acidification of the osseous tissues demineralizes bone and contributes to the degradation of adjacent cells and extracellular matrix. Furthermore, V-ATPase is associated with the activation of MMPs [17]. Through these mechanisms, the presence of V-ATPase contributes to posterior autophagy and neoplastic migration.

It is worth noting that the interaction between IP3R and V-ATPase occurs in usual situations during cell migration or osteoclast activity. As soon as extracellular H⁺ and posterior Ca²⁺ bioavailability increases due to bone degradation, these ions exert a negative feedback signal to suppress V-ATPase. This minimizes resorptive and/or migratory actions [23]. Conversely, the simultaneous cytoplasmic expression of IP3R and V-ATPase suggests a likely feedforward effect in ameloblastoma cells. Ca²⁺ influx and H⁺ secretion seem to occur simultaneously, thus suppressing negative feedback and favoring autophagy and tumor invasiveness. This effect seems to occur in columnar neoplastic cells, in areas of pseudopodia, and in projections toward the mesenchyme where concomitant immunolocalization of IP3R/V-ATPase is evident in the membrane and cytoplasm simultaneously. Furthermore, the high score for positive cells that exhibit co-immunolocalization of IP3R and V-ATPase may help to explain the increased aggressive behaviors of follicular and plexiform ameloblastomas, and unicystic ameloblastomas with mural growth. It should be noted, however, that limited evidence can be established regarding ameloblastomas with granular cells due to the small sample size (one in this study). In this case, we identified low V-ATPase expression despite the strong staining pattern of cytoplasmatic IP3R.

Unicystic ameloblastomas with purely luminal and intraluminal lining epithelium demonstrate a lack of V-ATPase in columnar cells while exhibiting membranous immunohistochemical expression for IP3R. These results may explain their expansive and cystic growth. In these cases, the peripheral columnar cells seem to shield against frank invasion. The absence of V-ATPase in the front of the invasion not only seems to reduce the metabolic effect in the columnar cells, but also prevents extracellular acidification and decreases the area for migration.

Despite the limitation of immunohistochemical analysis, our results provide new understanding of the infiltrative and aggressive behavior of each histopathological pattern of ameloblastoma. The present study could establish a premise for future research. There is potential that these proteins may

be used as therapeutic targets. V-ATPase inhibitors concanamycin A and bafilomycin have been used in breast tumor cytorreduction [24]. Similarly, some studies have demonstrated the inhibition of IP3R with arrest of neoplasia using 2-amino-ethoxy diphenyl borate [25]. Our findings suggest similar methods may become applicable in the treatment of ameloblastomas.

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Compliance with Ethical Standards

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

Ethical Approval All the procedures performed in our study were in accordance with the ethical standards for human research set by institutional and/or national research committees and with the 1964 Helsinki declaration, including its later amendments or comparable ethical standards. This study was approved by the Research Ethics Committee for Analysis of Research Project under protocol number # 877.322.

References

1. Wright JM, Vered M. Update from the 4th edition of the World Health Organization classification of head and neck tumours: odontogenic and maxillofacial bone tumors. *Head Neck Pathol.* 2017;11(1):68–77.
2. Gruica B, Stauffer E, Buser D, Bornstein M. Ameloblastoma of the follicular, plexiform, and acanthomatous type in the maxillary sinus: a case report. *Quintessence Int.* 2003;34(4):311–4.
3. Siqueira AS, Carvalho MR, Monteiro AC, Freitas VM, Jaeger RG, Pinheiro JJ. Matrix metalloproteinases, TIMPs and growth factors regulating ameloblastoma behaviour. *Histopathology.* 2010;57(1):128–37.
4. McClary AC, West RB, McClary AC, Pollack JR, Fischbein NJ, Holsinger CF, et al. Ameloblastoma: a clinical review and trends in management. *Eur Arch Otorhinolaryngol.* 2016;273(7):1649–61.
5. Pinheiro JJ, Nascimento CF, Freitas VM, de Siqueira AS, Junior SM, Jaeger RG. Invadopodia proteins, cortactin and membrane type I matrix metalloproteinase (MT1-MMP) are expressed in ameloblastoma. *Histopathology.* 2011;59(6):1266–9.
6. Sharp RC, Effiom OA, Dhingra A, Odukoya O, Olawuyi A, Arotiba GT, et al. Enhanced basal autophagy supports ameloblastoma-derived cell survival and reactivation. *Arch Oral Biol.* 2019;98:61–7.
7. Petibone DM, Majeed W, Casciano DA. Autophagy function and its relationship to pathology, clinical applications, drug metabolism and toxicity. *J Appl Toxicol.* 2017;37(1):23–37.
8. Kondratskyi A, Kondratska K, Skryma R, Klionsky DJ, Prevarskaya N. Ion channels in the regulation of autophagy. *Autophagy.* 2018;14(1):3–21.
9. Monteith GR, McAndrew D, Faddy HM, Roberts-Thomson SJ. Calcium and cancer: targeting Ca²⁺ transport. *Nat Rev Cancer.* 2007;7(7):519–30.
10. Akl H, Bultynck G. Altered Ca(2+) signaling in cancer cells: proto-oncogenes and tumour suppressors targeting IP3 receptors. *Biochim Biophys Acta.* 2013;1835(2):180–93.

11. Wang L, Xu MM, Li Z, Shi M, Zhou X, Jiang X, et al. Calcium and CaSR/IP3R in prostate cancer development. *Cell Biosci.* 2018;8:16.
12. Szatkowski C, Parys JB, Ouadid-Ahidouch H, Matifat F. Inositol 1,4,5-trisphosphate-induced Ca²⁺ signalling is involved in estradiol-induced breast cancer epithelial cell growth. *Mol Câncer.* 2010;9:156.
13. Shibao K, Fiedler MJ, Nagata J, Minagawa N, Hirata K, Nakayama Y. The type III inositol 1,4,5-trisphosphate receptor is associated with aggressiveness of colorectal carcinoma. *Cell Calcium.* 2010;48(6):315–23.
14. Pamarthy S, Kulshrestha A, Katara GK, Beaman KD. The curious case of vacuolar ATPase: regulation of signaling pathways. *Mol Câncer.* 2018;17(1):41.
15. Chung C, Mader CC, Schmitz JC, Atladottir J, Fitchev P, Cornwell ML, et al. The vacuolar-ATPase modulates matrix metalloproteinase isoforms in human pancreatic cancer. *Lab Investig.* 2011;91(5):732–43.
16. Whitton B, Okamoto H, Packham G, Crabb SJ. Vacuolar ATPase as a potential therapeutic target and mediator of treatment resistance in cancer. *Cancer Med.* 2018;7(8):3800–11.
17. Stransky L, Cotter K, Forgac M. The function of V-ATPases in cancer. *Physiol Rev.* 2016;96(3):1071–91.
18. He L, Zhang J, Zhao J, Ma N, Kim SW, Qiao S, Ma X. Autophagy: the last defense against cellular nutritional stress. *Adv Nutr.* 2018;19(11):3466.
19. Kenific CM, Wittmann T, Debnath J. Autophagy in adhesion and migration. *J Cell Sci.* 2016;129(20):3685–93.
20. Kania E, Roest G, Vervliet T, Parys JB, Bultynck G. IP₃ receptor-mediated calcium signaling and its role in autophagy in cancer. *Front Oncol.* 2017;7:140.
21. Wei C, Wang X, Zheng M, Cheng H. Calcium gradients underlying cell migration. *Curr Opin Cell Biol.* 2012;24(2):254–61.
22. Chan CY, Dominguez D, Parra KJ. Regulation of vacuolar H⁺-ATPase (V-ATPase) reassembly by glycolysis flow in 6-phosphofructo-1-kinase (PFK-1)-deficient yeast cells. *J Biol Chem.* 2016;291(30):15820–9.
23. Sakai H, Kawawaki J, Moriura Y, Mori H, Morihata H, Kuno M (2006) pH dependence and inhibition by extracellular calcium of proton currents via plasmalemmal vacuolar-type H⁺-ATPase in murine osteoclasts. *J Physiol.* 2006;576(Pt 2):417–25.
24. Pérez-Sayáns M, Somoza-Martín JM, Barros-Angueira F, Rey JMG, García-García A. V-ATPase inhibitors and implication in cancer treatment. *Cancer Treat Rev.* 2009;35(8):707–13.
25. Singh A, Chagtoo M, Tiwari S, George N, Chakravarti B, Khan S, et al. Inhibition of inositol 1,4,5-trisphosphate receptor induce breast cancer cell death through deregulated autophagy and cellular bioenergetics. *J Cell Biochem.* 2017;118(8):2333–46.

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