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ORIGINAL PAPER

Presence of Myofibroblasts and Expression of Matrix Metalloproteinase-2 (MMP-2) in Ameloblastomas Correlate with Rupture of the Osseous Cortical

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Abstract Myofibroblasts are frequent in the stroma of neoplasm and by the expression of proteinases they can influence tumor infiltration and progression. In the present study, presence of myofibroblasts and expression of matrix metalloproteinase-2 (MMP-2) and urokinase plasminogen activator (uPA) were examined in intra-osseous solid multicystic ameloblastomas to determine their roles in the clinicopathological features of the tumors. Fifty seven ameloblastomas were analyzed immunohistochemically with antibodies against the isoform α of the smooth muscle actin (α-SMA), a specific marker of myofibroblasts, MMP-2 and uPA. Myofibroblasts were found in the stroma, in close contact with neoplastic cell islands, of ~58% (n=33) of the ameloblastomas. MMP-2 and uPA were found in the cytoplasm of both neoplastic and stromal cells. A significant correlation between presence of myofibroblasts and MMP-2 expression was observed. Abundant presence of myofibroblast in the stroma of the tumors and expression of MMP-2 in the neoplastic or stromal cells were significantly correlated with rupture of the osseous cortical, which has been considered an important prognostic marker of ameloblastoma aggressiveness. Ours results suggest that abundant presence of myofibroblasts and expression of MMP-2 in solid ameloblastomas may be associated with a more aggressive infiltrative behavior.

Keywords Ameloblastoma · Matrix metalloproteinase-2 · Myofibroblast · Prognosis · Urokinase plasminogen activator

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Introduction

Myofibroblasts are specialized stromal cells that exhibit a hybrid phenotype between fibroblasts and smooth muscle cells, and are characterized by expression of the specific isoform α of the smooth muscle actin (α-SMA) [1]. Several reports have shown the presence of cells with myofibroblastic features in normal tissues and in a variety of pathological situations, such as benign and malignant neoplasm [2–4]. Furthermore, myofibroblasts are the main cellular type involved in extracellular matrix deposition and remodelation during tissue repair and fibrotic diseases [5–7]. Through expression and secretion of cytokines, chemokines, growth factors, extracellular matrix molecules, adhesion molecules and receptors, myofibroblasts promote epithelial-mesenchymal interactions, including neoplastic

growth and invasion [4,8–13]. Myofibroblasts also secrete enzymes responsible for matrix remodeling, including matrix metalloproteinase-2 (MMP-2) [4,14–16] and urokinase plasminogen activator (uPA) [2,17–20]. In addition, activation of those enzymes can lead to release and activation of sequestered cytokines and growth factors, which may favor angiogenesis and promotion of cellular migration [4].

Solid ameloblastomas demonstrate an aggressive biological behavior by exhibiting a great infiltrative potential and a high recurrence rate [21]. Thus, identification of prognostic markers of the ameloblastoma's biologic behavior is of considerable importance to determine the most appropriate therapeutic approach and establish the prognosis of patient. Previous studies demonstrated the expression of MMPs and uPA in both neoplastic and stromal components of ameloblastomas, permitting the hypothesis that these matrix-degrading enzymes may contribute to the local invasiveness of this odontogenic tumor [22–25]. Since recent reports have demonstrated that identification of myofibroblast have a prognostic role in many neoplasm [2,12,26,27] and that those cells produce high levels of MMP-2 and uPA [2,4,14–20], the aim of the present study was to analyze the presence of myofibroblasts and the expression of MMP-2 and uPA in intra-osseous solid multicystic ameloblastoma to establish their prognostic significance.

Material and Methods

Samples

Fifty seven patients diagnosed with intra-osseous solid multicystic ameloblastoma and treated at the A. C. Camargo Cancer Hospital, São Paulo, Brazil were included in this study. The patients, 28 of which were males, ranged in age between 6 and 74 years, with a median of 28 years (mean 33.2±17.6 years). Patients had their symptoms for 1 to 240 months with a median of 12 months (mean 43.2± 64.1 months), and facial swelling was the most common presenting complain (92.9%) of the patients, followed by pain in 14 (25%). Clinical size of the tumor ranged between 2 and 25 cm, with a median of 5 cm (mean 5.63±3.72 cm). Mandible (87.7%), particularly in the posterior areas (92%), was more affected than maxilla. Twenty three patients (47.9%) had a unilocular radiolucent lesion, whereas 25 (52.1%) had a multilocular lesion. Rupture of the osseous cortical was observed in 17 (33%) of the patients. Plexiform histological type was observed in 33 (57.9%) patients, followed by follicular (29.8%), acanthomatous (8.8%), granular cell type (1.75%), and hemangiomatous (1.75%). Most patients (54.4%) had resection as treatment of the tumor, while 6 (10.5%) had enucleation and 20 (35.1%) had enucleation in association with cryotherapy. Recurrence, after a mean follow up of 149 months, was observed in 20 patients (35.1%), and only in those treated by enucleation or enucleation and cryotherapy. The study was carried out with approval of the Human Research Ethics Committee of the hospital.

Histological and Immunohistochemical Analysis

New sections were cut from the paraffin blocks and used for hematoxylin and eosin staining or immunohistochemistry. Immunohistochemical analysis was performed using the streptavidin-biotin peroxidase complex method. Following deparaffinization and hydration in graded alcohol solutions, the sections were treated with 3% H_2O_2 followed by antigen retrieval with 10 mM citric acid pH 6.0 in a microwave for 24 min for the antibody against α-SMA, or with 10 mM citrate buffer pH 6.0 in a pressure cooker for 4 min for MMP-2 and uPA antibodies. After washing with phosphate-buffered saline (PBS), the sections were treated with 1% bovine serum albumin (BSA, Sigma, St. Louis, MO, USA) in PBS for 1 h, and then incubated with primary antibodies for 16 h at 4°C. The primary antibodies used were as follows: monoclonal mouse anti-α-SMA (Dako Corp., Carpenteria, CA, USA) diluted 1:200, polyclonal rabbit anti-MMP-2 (NeoMarkers, Fremont, CA, USA) diluted 1:200, and monoclonal mouse anti-uPA (American Diagnostica, Greenwich, CT, USA) diluted 1:100. Subsequent incubations were with biotinylated IgG followed by streptavidin-biotin peroxidase complex (StrepABC Complex/HRP Duet kit, Dako). Reactions were developed by incubating the sections with 0.6 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB, Sigma) containing 0.01% H_2O_2 and counterstained with Mayer's hematoxylin. Positive and negative controls were included in all reactions.

Immunohistochemical reactivity was evaluated blindly by two independent investigators without knowledge of the clinicopathological information, and any disagreement was settled by discussion. The presence of α-SMA-positive cells was classified as negative (0), scanty (1) or abundant (2) as described previously [27]. Immunohistochemical reactivity for MMP-2 and uPA was evaluated separately in neoplastic and stromal (fibroblastic-like) cells, and graded semi-quantitatively in negative, (+) lower than 50% of positive cells, and (++) number of positive cells higher than 50%.

Statistical Analysis

For statistical purposes, tumors with lack or scanty presence of myofibroblast were combined and compared with tumors with abundant presence of myofibroblasts. Similarly, MMP-2 positive samples (graded as + and ++) were combined and compared with negative samples. Correla-

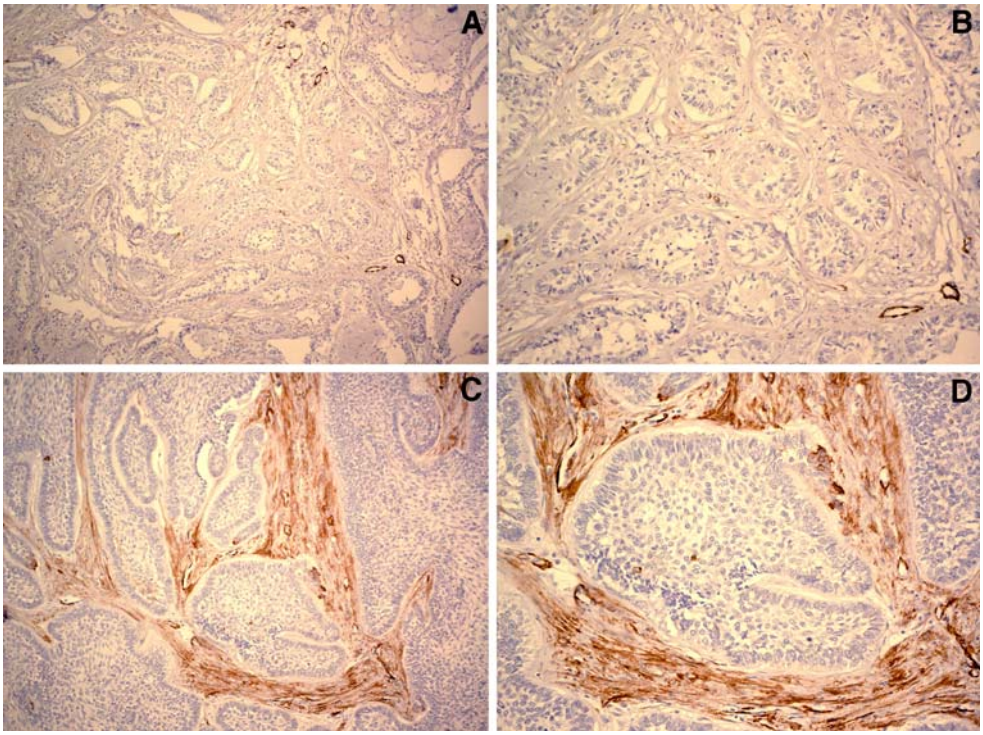

Fig. 1 Presence of myofibroblasts in intra-osseous solid multicystic ameloblastomas. α-SMA immunohistochemical expression of representative samples of this study classified as negative (**a** and **b**) and abundant presence of myofibroblasts (**c** and **d**). Myofibroblasts were found immediately adjacent to the islands of ameloblastic cells, whereas areas of tumor-free stroma demonstrated a complete lack of α-SMA-positive cells. The smooth muscle of the blood vessel walls was positive in all samples. (Original magnification: A and C x100, B and D x200)

tions were evaluated by Fisher's exact test (for frequency data in contingency tables) or Mann-Whitney U-test. Statistical significance was at $p \leq 0.05$.

Results

Presence of Myofibroblasts and Expression of MMP-2 and uPA in Ameloblastomas

All samples demonstrated positivity for α-SMA in the smooth muscle of the blood vessel walls, which worked as an internal positive control Immunohistochemical reaction for α-SMA showed positive cells in the stroma of 57.9% (n=33) of the ameloblastomas. Scanty presence of myofibroblasts was observed in 20 (60.6%) out of ameloblastoma-positive samples, whereas 13 (39.4%) samples demonstrated an abundant distribution of myofibroblasts. Myofibroblasts were located in the stroma in close contact to neoplastic islands (Fig. 1), and areas of tumor-free stroma demonstrated a complete lack of myofibroblasts. Myofibroblasts were found in the tumor stroma in a reticular or fascicular pattern of distribution (data not shown). Twenty samples (60.6%) demonstrated a reticular pattern, and 13 (39.4%) demonstrated a fascicular pattern of myofibroblast distribution. Interestingly, 10 out of 13 samples with abundant myofibroblasts showed a fascicular pattern of distribution.

Immunohistochemical reactivity for MMP-2 was observed in the cytoplasm of the neoplastic and stromal cells. Eventually, a weak immunostaining was observed in the endothelial cells. In neoplastic cells, expression of MMP-2 was mainly detected in the peripheral columnar cells, with few positive cells in the central island area (Fig. 2). Many fibroblast-like cells in the intralesional fibrous stroma were strongly reactive for the antibody anti-MMP-2 (Fig. 2).

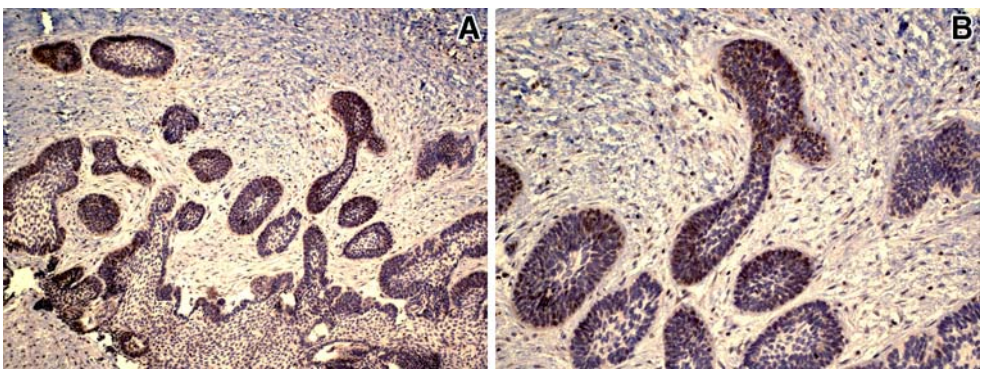

Fig. 2 Immunohistochemical expression of MMP-2 in ameloblastomas. Neoplastic islands showed strong reactivity in the cytoplasm of the peripheral columnar cells and weak reactivity in the central cells. Many fibroblastic-like cells in the stroma were also positive for MMP-2. (Original magnification: **a** x100, **b** x200)

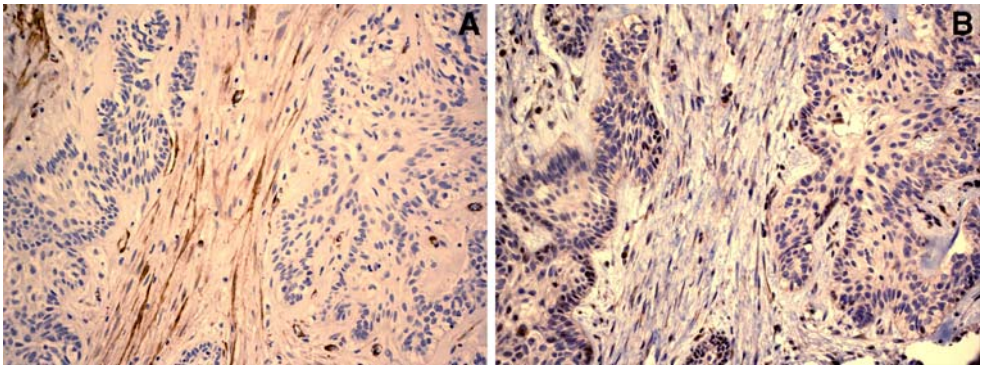

Fig. 3 Expression of α-SMA and MMP-2 in serial sections of one representative sample of this study. Myofibroblasts, as revealed by the immunopositivity for α-SMA is showed in (**a**), whereas MMP-2 expression is demonstrated in (**b**). Note that MMP-2-positive cells in the stroma are myofibroblasts. (Original magnification: A and B x200)

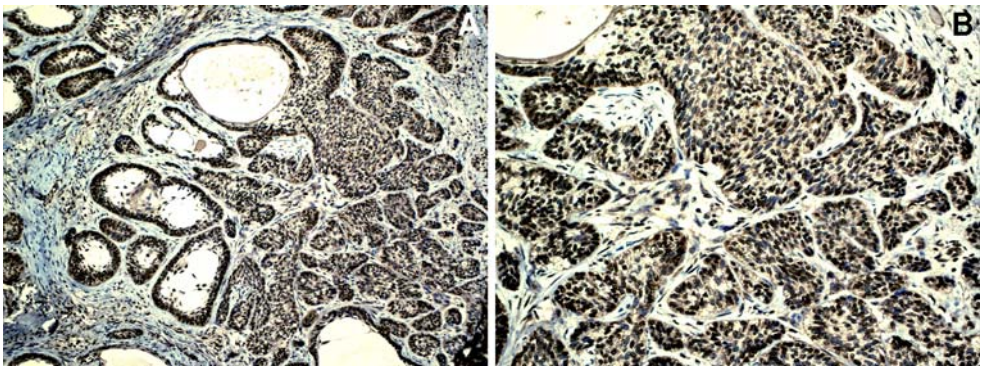

Fig. 4 Expression of uPA in ameloblastomas. uPA immunopositivity was observed in the cytoplasm of the neoplastic and stromal cells. Endothelial cells were also positive. In the neoplastic islands, both peripheral columnar cells and central polyhedral cells demonstrated uPA expression. (Original magnification: A x100, B x200)

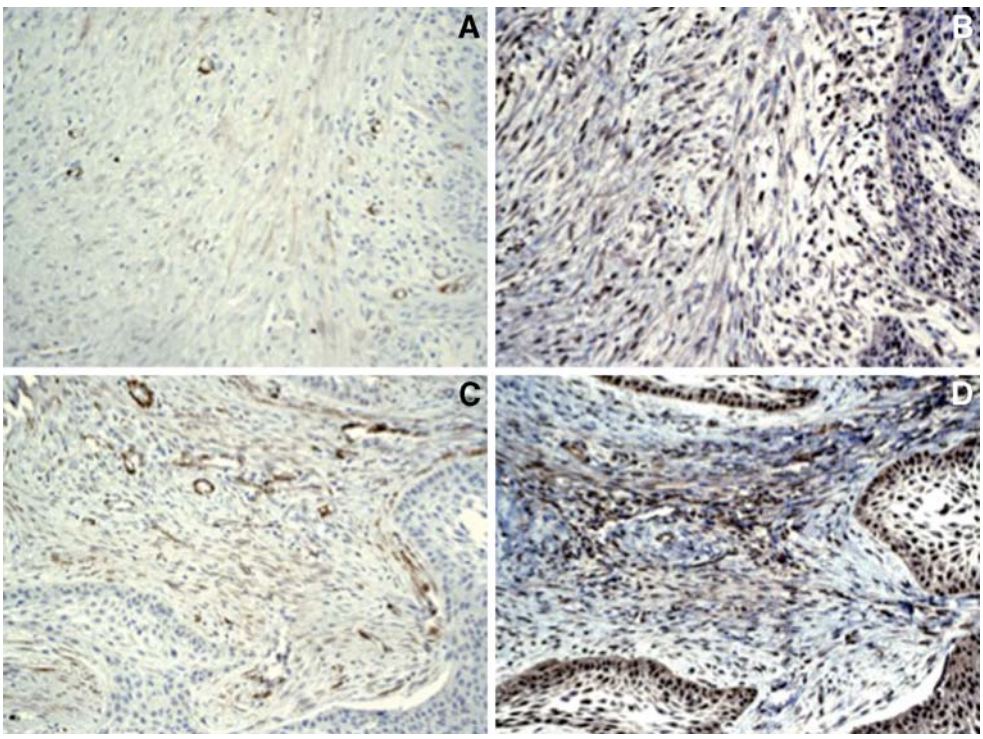

Fig. 5 Expression of α-SMA and uPA in serial sections of representative samples of this study. Immunoreactions for α-SMA (**a** and **c**) and uPA (**b** and **d**). Sample in A and B demonstrated scanty presence of myofibroblasts but a high expression of uPA in the stroma, whereas sample in C and D showed elevate number of myofibroblasts and strong uPA expression. There was no correlation between presence of myofibroblasts and expression of uPA in the stroma of ameloblastomas. (Original magnification: A and C x100, B and D x200)

Interestingly, most of the MMP-2-positive cells in the stroma were also stained for α-SMA, revealing a myofibroblastic phenotype (Fig. 3). Expression of MMP-2 was observed in the neoplastic cells of 26 (47.28%) ameloblastomas, with half of those (n=13, 26.64%) showing more than 50% of MMP-2-positive cells. In 29 (52.72%) ameloblastomas, MMP-2 immunoreactivity was not found in the neoplastic cells. Twenty one (38.18%) ameloblastomas demonstrated MMP-2 immunostaining in the fibroblastic-like cells located in the stroma, with 13 (23.64%) showing more than 50% of positive cells. A significant correlation was observed between abundant presence of myofibroblasts and expression of MMP-2 in both neoplastic cells (p=0.03) and stromal cells (p=0.000004).

Expression of uPA was detected in the cytoplasm of neoplastic cells, fibroblastic-like cells, endothelial cells and inflammatory cells. Neoplastic islands showed strong uPA expression in the peripheral columnar cells and weak expression in the central polyhedral cells (Fig. 4). In opposite of MMP-2, uPA was expressed by both fibroblasts and myofibroblasts (Fig. 5). All ameloblastoma samples showed uPA-positive cells in the neoplastic and stromal component. Fourth eight (85.72%) of the tumors demonstrated uPA immunoreactivity in more than 50% of the neoplastic cells, whereas 34 (60.72%) of the ameloblastomas showed strong reactivity (more than 50% of positive cells) for uPA in the stroma, excluding endothelial and inflammatory cells. No correlation between presence of myofibroblasts and uPA or MMP-2 and uPA was observed.

Clinicopathological Correlations Among Myofibroblast, MMP-2 and uPA

The results of the clinicopathological correlations with the presence of myofibroblasts and expression of MMP-2 and uPA are summarized in Tables 1, 2 and 3, respectively. Abundant presence of myofibroblast in the stroma of the solid ameloblastomas was significantly correlated with rupture of the osseous cortical (p=0.001). Presence of myofibroblasts was not associated with age, gender, ethnic, symptoms or duration of it, anatomic location, radiographic pattern, and histological type. There was also no association between presence of myofibroblasts and treatment modality or recurrence. Similarly, immunoreactivity for MMP-2 in the neoplastic cells or stromal cells significantly correlated with rupture of the osseous cortical (p=0.027 for neoplastic cells and p=0.006 for stromal cells). Presence of MMP-2-positive cells in the stroma of plexiform ameloblastomas tended to be more frequent than in other variants (p=0.054). Expression of uPA in stromal cells was significantly correlated with plexiform ameloblastomas (p=0.04). No significant correlations were observed between the expression of MMP-2 and uPA and other clinicopathological parameters.

Discussion

Activation of myofibroblasts in the stroma is predicted to be a critical step in tumor growth and progression [28,29]. Furthermore, recent reports demonstrated that increased presence of myofibroblasts in the stroma of several cancers, including those of the oral cavity, is correlated with a worse prognosis for the patients [2,11–13,30]. In oral squamous cell carcinomas, increased amount of myofibroblasts was significantly correlated with lymph node metastasis, vascular, lymphatic and perineural invasion of the tumor cells, and patient's shorter overall survival [30]. Moreover, α-

Table 1 Clinicopathological correlation of the presence of myofibroblasts in ameloblastomas

Parameters	Myofibroblasts[1]		P value
	0/1 n (%)	2 n (%)	
Age			
< 28 years	21 (47.72)	6 (46.15)	0.92
≥ 28 years	23 (52.28)	7 (53.85)	
Gender			
Male	24 (54.55)	4 (30.77)	0.13
Female	20 (45.45)	9 (69.23)	
Symptoms			
Without pain	31 (72.09)	11 (84.61)	0.36
With pain	12 (27.91)	2 (15.39)	
Duration of the symptoms			
< 12 months	14 (34.14)	6 (46.15)	0.43
≥ 12 months	27 (65.86)	7 (53.85)	
Tumor size			
< 5 cm	12 (33.34)	7 (58.34)	0.12
≥ 5 cm	24 (66.66)	5 (41.66)	
Localization			
Maxilla	4 (9.09)	3 (23.07)	0.17
Mandible	40 (99.91)	10 (76.93)	
Radiographic presentation			
Unilocular	19 (50)	4 (33.34)	0.57
Multilocular	19 (50)	6 (66.66)	
Rupture of the osseous cortical			
No	31 (79.48)	2 (18.18)	0.0001
Yes	8 (20.52)	9 (81.82)	
Histological type			
Plexiform	23 (52.27)	10 (76.92)	0.11
Others[2]	21 (47.73)	3 (23.08)	
Treatment			
Resection	23 (52.27)	8 (61.54)	0.60
Enucleation	6 (13.62)	0	
Enucleation + Cryotherapy	15 (34.09)	5 (38.46)	
Recurence			
No	28 (63.64)	9 (69.23)	0.71
Yes	16 (36.36)	4 (30.77)	

[1] Presence of myofibroblast: (0) negative, (1) scanty, and (2) abundant.
[2] Others: follicular (n=17), acanthomatous (n=5), granular cell (n=1), and hemangiomatous (n=1) histological variants.

SMA myofibroblasts were not found in the stroma of normal oral mucosa and pre-malignant leucoplakias with dysplasia histologically confirmed, but approximately 60% of the oral squamous cell carcinomas contained a significantly proportion of myofibroblasts [30]. Similarly, Lewis et al [31]. demonstrated that myofibroblasts were not found in the adjacent stroma of invasive tumors of the oral cavity and in benign oral mucosal polyps, which contained a hyperplastic squamous epithelium, while 14 out of 15 oral squamous cell carcinomas contained myofibroblasts independent of the tumor differentiation. Interestingly, highly polymorphic ductal carcinoma in situ of breast and non-invasive urothelial carcinoma of bladder induced myofibroblast appearance in the stroma surrounding the cancer cells [32,33], demonstrating that factors derived from aggressive tumor cells are able to diffuse through basement membrane stimulating myofibroblast transformation. Indeed, we have previously demonstrated that oral squamous carcinoma cells induced transdifferentiation of normal fibroblasts to myofibroblasts via secretion of transforming growth factor-beta1 (TGF-β1), and in turn, myofibroblasts secreted factors that stimulated tumor cell proliferation, which favor tumor growth [27]. Since increased expression of TGF-β1 is found in ameloblastomas [34], further investigation should be done to elucidate whether myofibroblast emergency occurs through TGF-β1-released by ameloblastoma-neoplastic cells.

Table 2 Clinicopathological correlation of the MMP-2 immunohistochemical expression in ameloblastomas

Parameters	MMP-2 in neoplastic cells[1]		P value	MMP-2 in stromal cells		P value
	Negative n (%)	+/++ n (%)		Negative n (%)	+/++ n (%)	
Age						
< 28 years	12 (41.38)	14 (53.84)	0.35	14 (41.17)	12 (57.14)	0.25
≥ 28 years	17 (58.62)	12 (46.16)		20 (58.83)	9 (42.86)	
Gender						
Male	16 (55.17)	11 (42.31)	0.34	17 (50)	10 (47.62)	0.86
Female	13 (44.83)	15 (57.69)		17 (50)	11 (52.38)	
Symptoms						
Without pain	24 (82.75)	16 (64)	0.12	24 (72.72)	16 (76.19)	0.77
With pain	5 (17.25)	9 (36)		9 (27.28)	5 (23.81)	
Duration of the symptoms						
< 12 months	20 (68.96)	21 (87.5)	0.11	23 (71.87)	18 (85.71)	0.24
≥ 12 months	9 (31.04)	3 (12.5)		9 (28.13)	3 (14.29)	
Tumor size						
< 5 cm	13 (56.52)	13 (56.52)	1.00	14 (51.85)	12 (63.15)	0.44
≥ 5 cm	10 (43.48)	10 (43.48)		13 (48.15)	7 (36.85)	
Localization						
Maxilla	3 (10.34)	4 (15.38)	0.57	3 (8.82)	4 (19.04)	0.27
Mandible	26 (89.66)	22 (84.62)		31 (91.18)	17 (80.96)	
Radiographic presentation						
Unilocular	12 (48)	11 (50)	0.89	15 (50)	8 (47.06)	0.85
Multilocular	13 (52)	11 (50)		15 (50)	9 (52.94)	
Rupture of the osseous cortical						
No	20 (80)	12 (50)	0.027	24 (80)	8 (42.1)	0.006
Yes	5 (20)	12 (50)		6 (20)	11 (59.9)	
Histological type						
Plexiform	17 (58.62)	16 (61.54)	0.82	17 (50)	16 (76.19)	0.054
Others[2]	12 (41.38)	10 (38.46)		17 (50)	5 (23.81)	
Treatment						
Resection	15 (51.71)	11 (42.31)	0.75	17 (50)	9 (42.85)	0.51
Enucleation	4 (13.81)	5 (19.23)		4 (11.76)	5 (23.81)	
Enucleation + Cryotherapy	10 (34.48)	10 (38.46)		13 (38.24)	7 (33.34)	
Recurence						
No	18 (62.07)	17 (65.38)	0.79	20 (58.83)	15 (71.42)	0.34
Yes	11 (37.93)	9 (34.62)		14 (41.17)	6 (28.58)	

[1] MMP-2 immunohistochemical expression: (+) number of positive cells is lower than 50%, and (++) number of positive cells is higher than 50%.
[2] Others: follicular (n=17), acanthomatous (n=5), granular cell (n=1), and hemangiomatous (n=1) histological variants.

Presence of myofibroblasts in odontogenic tumors has been reported in few studies. However, those were isolated case reports [35,36] or studies comparing the presence of myofibroblasts in aggressive and non-aggressive odontogenic tumors [37,38]. Vered et al.[38] demonstrated, in a small group of tumors, that the number of myofibroblasts in solid ameloblastomas is significantly higher than in unicystic variants, suggesting that myofibroblasts can contribute to the biological behavior of aggressive lesions. In the present study, we found an elevate number of solid ameloblastomas contained myofibroblasts. Interestingly, there were no α-SMA-positive myofibroblasts in the tumor-free stroma of the samples, suggesting that a close contact is needed for ameloblastoma cells induction of myofibroblast transdifferentiation. More importantly, we have demonstrated that abundant presence of myofibroblasts leads to a more aggressive behavior of the ameloblastomas, as revealed by the significant correlation with rupture of the osseous cortical. Some clinical parameters that could influence the rupture of the bone cortex, including size of the tumor and time of disease evolution, showed no significant correlation with bone cortical status, demonstrating that presence of myofibroblasts is an independent factor for bone infiltration. This is, to our knowledge, the first evidence that elevated myofibroblast may be useful to predict the prognosis of ameloblastoma

Table 3 Clinicopathological correlation of the uPA immunohistochemical expression in ameloblastomas

Parameters	uPA in neoplastic cells[1]		P value	uPA in stromal cells		P value
	+ n (%)	++ n (%)		+ N (%)	++ n (%)	
Age						
< 28 years	6 (75)	20 (41.67)	0.08	10 (45.45)	16 (47.06)	0.91
≥ 28 years	2 (25)	28 (58.33)		12 (54.55)	18 (52.94)	
Gender						
Male	4 (50)	24 (50)	1.00	12 (54.55)	16 (47,06)	0.58
Female	4 (50)	24 (50)		10 (45.45)	18 (52.94)	
Symptoms						
Without pain	5 (62.5)	37 (78.72)	0.32	15 (71.43)	27 (79.41)	0.49
With pain	3 (37.5)	10 (21.28)		6 (28.57)	7 (20.59)	
Duration of the symptoms						
< 12 months	5 (62.5)	36 (80)	0.27	15 (78.95)	26 (76.47)	0.83
≥ 12 months	3 (37.5)	9 (20)		4 (21.05)	8 (23.53)	
Tumor size						
< 5 cm	5 (62.5)	21 (55.26)	0.65	13 (68.42)	13 (46.43)	0.13
≥ 5 cm	3 (37.5)	18 (44.74)		6 (31.58)	15 (53.57)	
Localization						
Maxilla	1 (12.5)	6 (12.5)	1.00	2 (9.09)	5 (14.7)	0.53
Mandible	7 (87.5)	42 (87.5)		20 (90.91)	29 (85.3)	
Radiographic presentation						
Unilocular	4 (50)	18 (46.15)	0.84	7 (38.89)	15 (51.72)	0.39
Multilocular	4 (50)	21 (53.85)		11 (61.11)	14 (48.28)	
Rupture of the osseous cortical						
No	5 (62.5)	28 (68.29)	0.75	11 (61.11)	22 (70.96)	0.47
Yes	3 (37.5)	13 (31.71)		7 (38.89)	9 (29.04)	
Histological type						
Plexiform	5 (62.5)	27 (56.25)	0.74	9 (42.85)	23 (67.64)	0.04
Others[2]	3 (37.5)	21 (43.75)		13 (57.15)	11 (32.36)	
Treatment						
Resection	1 (12.5)	27 (56.25)	0.07	12 (54.54)	16 (47.06)	0.84
Enucleation	2 (25)	7 (14.58)		3 (13.63)	6 (17.64)	
Enucleation + Cryotherapy	5 (62.5)	14 (29.17)		7 (31.83)	12 (35.3)	
Recurence						
No	7 (87.5)	29 (60.42)	0.14	17 (77.27)	19 (55.88)	0.10
Yes	1 (12.5)	19 (39.58)		5 (22.73)	15 (44.12)	

[1] MMP-2 immunohistochemical expression: (+) number of positive cells is lower than 50%, and (++) number of positive cells is higher than 50%.
[2] Others: follicular (n=17), acanthomatous (n=5), granular cell (n=1), and hemangiomatous (n=1) histological variants.

patients, since rupture of the osseous cortical is one of the major prognostic factors of aggressiveness in patients with ameloblastomas [39,40]. Patients with cortical rupture show significantly higher recurrence rates than those with preserved cortical [41]. However, we did not find any association between myofibroblasts and disease recurrences. The reason for that is probably the type of surgery performed. All patients in this study with rupture of the osseous cortical were treated by dentoalveolar segmental resection of the lesion.

The results presented here also revealed that expression of MMP-2 by ameloblastic cells or stromal cells, excluding endothelial and inflammatory cells, was correlated with rupture of the osseous cortical. Importantly, a strong correlation was observed between presence of myofibroblasts and expression of MMP-2 in the stroma of ameloblastomas. Indeed, our observations demonstrated that the main source of MMP-2 in the stroma of ameloblastomas is myofibroblasts. This is in line with previous reports that demonstrated myofibroblasts as the main source of MMP-2 in fibrotic diseases [42,43] and some types of cancers [44]. Kumamoto et al.[24] and Pinheiro et al.[25] analyzed MMP-2 expression in ameloblastomas and found that both neoplastic and stromal cells were strongly positive. In addition, Pinheiro et al.[25] found MMP-2 in stromal cells close to the bone interface, suggesting that this proteinase digests bone matrix and contributes for tumor invasion. Growth factors, including TGF-β1 and tumor necrosis factor alpha (TNFα), which are produced in elevate amounts by myofibroblasts, induce MMP secretion by myofibroblasts, contributing to bone resorption [45]. Moreover, a recent study demonstrated that adenovirus-mediated expression of tissue inhibitor of MMPs (TIMP-1 or TIMP-2) in prostate cancer cells inhibits MMP-2 activity and, subsequent, bone resorption [46]. Taken together, these findings indicate an important role of MMP-2 synthesized by tumor cells and myofibroblasts in the infiltrative expansion of ameloblastomas.

uPA is a serine proteinase that catalyze the activation of plasminogen into plasmin by cleaving the arginine–valine bond [47]. In turn, plasmin degrades most extracellular matrix proteins and facilitates the release of several proteolytic enzymes, including MMPs, facilitating cellular migration and invasion [48,49]. uPA also aids in antithrombolytic activities to remove blood clots and helps stimulate angiogenesis in tumor cells [47]. This enzyme is expressed by many cellular types, including normal and tumor cells [50,51]. In ameloblastomas, uPA was found in more that 95% of the tumors in the cytoplasm of ameloblastic cells and stromal cells, including fibroblast-like and endothelial cells [23]. In the current series, all samples demonstrated immunoreactivity for uPA in both neoplastic and stromal cells. In addition, a significant correlation was seen between uPA expression in stromal cells and plexiform histologic variant. Curiously, uPA was expressed by fibroblast and myofibroblasts, and no correlation between uPA expression and myofibroblasts was observed. However, it is well acceptable that myofibroblasts produce higher amounts of uPA compare to fibroblasts [2,17–20]. Furthermore, uPA is now considered to be implicated in myofibroblast transdifferentiation and proliferation via maintenance of full-length uPA receptor on the cell surface [52]. Although uPA expression did not show any significant clinicopathological correlation, an evaluation of other components of the uPA system, including the receptors and the inhibitors, warrant investigation.

In summary, the results presented here demonstrate that abundant presence of myofibroblasts and MMP-2 expression on solid ameloblastomas lead to a more aggressive behavior, such as rupture of the osseous cortical. In addition, our data demonstrated that myofibroblasts are the main source of MMP-2 in the stroma of ameloblastomas. Further investigations are needed to confirm the potential prognostic value of myofibroblasts in solid ameloblastomas, as well as in other locally aggressive odontogenic tumors. However, the clinical behavior of ameloblastomas will be better understood when clinical and microscopic data are considered in conjunction with new molecular techniques that are becoming available.

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References

1. Desmouliere A, Chaponnier C, Gabbiani G (2005) Tissue repair, contraction, and the myofibroblast. Wound Repair Regen 13:7–12
2. Desmouliere A, Guyot C, Gabbiani G (2004) The stroma reaction myofibroblast: a key player in the control of tumor cell behavior. Int J Dev Biol 48:509–517
3. Kuroda N, Shimasaki N, Miyazaki E et al. (2006) The distribution of myofibroblasts and CD34-positive stromal cells in normal renal pelvis and ureter and their cancers. Histol Histopathol 21:1303–1307
4. Powell DW, Adegboyega PA, Di Mari JF et al. (2005) Epithelial cells and their neighbors I. Role of intestinal myofibroblasts in development, repair, and cancer. Am J Physiol Gastrointest Liver Physiol 289:G2–G7
5. Darby IA, Hewitson TD (2007) Fibroblast differentiation in wound healing and fibrosis. Int Rev Cytol 257:143–179
6. Bitu CC, Sobral LM, Kellermann MG et al. (2006) Heterogeneous presence of myofibroblasts in hereditary gingival fibromatosis. J Clin Periodontol 33:393–400
7. Gabbiani G (2003) The myofibroblast in wound healing and fibrocontractive diseases. J Pathol 200:500–503
8. Kunz-Schughart LA, Knuechel R (2002) Tumor-associated fibroblasts (part II): Functional impact on tumor tissue. Histol Histopathol 17:623–637

9. Kunz-Schughart LA, Knuechel R (2002) Tumor-associated fibroblasts (part I): Active stromal participants in tumor development and progression? Histol Histopathol 17:599–621
10. Scotton CJ, Chambers RC (2007) Molecular targets in pulmonary fibrosis: the myofibroblast in focus. Chest 132:1311–1321
11. Mukaratirwa S, Koninkx JF, Gruys E et al. (2005) Mutual paracrine effects of colorectal tumour cells and stromal cells: modulation of tumour and stromal cell differentiation and extracellular matrix component production in culture. Int J Exp Pathol 86:219–229
12. Offersen BV, Nielsen BS, Hoyer-Hansen G et al. (2003) The myofibroblast is the predominant plasminogen activator inhibitor-1-expressing cell type in human breast carcinomas. Am J Pathol 163:1887–1899
13. Orimo A, Weinberg RA (2006) Stromal fibroblasts in cancer: a novel tumor-promoting cell type. Cell Cycle 5:1597–1601
14. Helary C, Ovtracht L, Coulomb B et al. (2006) Dense fibrillar collagen matrices: a model to study myofibroblast behaviour during wound healing. Biomaterials 27:4443–4452
15. Soini Y, Satta J, Maatta M et al. (2001) Expression of MMP2, MMP9, MT1-MMP, TIMP1, and TIMP2 mRNA in valvular lesions of the heart. J Pathol 194:225–231
16. Pender SL, MacDonald TT (2004) Matrix metalloproteinases and the gut - new roles for old enzymes. Curr Opin Pharmacol 4:546–550
17. Hatane T, Yoshida E, Kawano J et al. (1998) Prostaglandin I2 analog enhances the expression of urokinase-type plasminogen activator and wound healing in cultured human fibroblast. Biochim Biophys Acta 1403:189–198
18. Dubuisson L, Monvoisin A, Nielsen BS et al. (2000) Expression and cellular localization of the urokinase-type plasminogen activator and its receptor in human hepatocellular carcinoma. J Pathol 190:190–195
19. Monvoisin A, Neaud V, De Ledinghen V et al. (1999) Direct evidence that hepatocyte growth factor-induced invasion of hepatocellular carcinoma cells is mediated by urokinase. J Hepatol 30:511–518
20. Plekhanova OS, Stepanova VV, Ratner EI (2006) Urokinase plasminogen activator in injured adventitia increases the number of myofibroblasts and augments early proliferation. J Vasc Res 43:437–446
21. Reichart PA, Philipsen HP, Sonner S (1995) Ameloblastoma: biological profile of 3677 cases. Eur J Cancer B Oral Oncol 31B:86–99
22. Kumamoto H, Ooya K (2006) Immunohistochemical detection of MT1-MMP, RECK, and EMMPRIN in ameloblastic tumors. J Oral Pathol Med 35:345–351
23. Kumamoto H, Ooya K (2007) Immunohistochemical detection of uPA, uPAR, PAI-1, and maspin in ameloblastic tumors. J Oral Pathol Med 36:488–494
24. Kumamoto H, Yamauchi K, Yoshida M et al. (2003) Immunohistochemical detection of matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) in ameloblastomas. J Oral Pathol Med 32:114–120
25. Pinheiro JJ, Freitas VM, Moretti AI et al (2004) Local invasiveness of ameloblastoma. Role played by matrix metalloproteinases and proliferative activity. Histopathology 45:65–72
26. Suh KS, Crutchley JM, Koochek A et al. (2007) Reciprocal modifications of CLIC4 in tumor epithelium and stroma mark malignant progression of multiple human cancers. Clin Cancer Res 13:121–131
27. Kellermann MG, Sobral LM, da Silva SD, Zecchin KG, Graner E, Lopes MA, Kowalski LP, Coletta RD (2008) Mutual paracrine effects of oral squamous cell carcinoma cells and normal oral fibroblasts: induction of fibroblast to myofibroblast transdifferentiation and modulation of tumor cell proliferation. Oral Oncol 4:509–517
28. Mahadevan D, Von Hoff DD (2007) Tumor-stroma interactions in pancreatic ductal adenocarcinoma. Mol Cancer Ther 6:1186–1197
29. Casey TM, Eneman J, Crocker A et al (2007) Cancer associated fibroblasts stimulated by transforming growth factor beta1 (TGF-beta1) increase invasion rate of tumor cells: a population study. Breast Cancer Res Treat Aug 3. doi:10.1007/s10549-007-9684-7
30. Kellermann MG, Sobral LM, da Silva SD et al. (2007) Myofibroblasts in the stroma of oral squamous cell carcinoma are associated with poor prognosis. Histopathology 51:849–853
31. Lewis MP, Lygoe KA, Nystrom ML et al. (2004) Tumour-derived TGF-beta1 modulates myofibroblast differentiation and promotes HGF/SF-dependent invasion of squamous carcinoma cells. Br J Cancer 90:822–832
32. Chauhan H, Abraham A, Phillips JR et al. (2003) There is more than one kind of myofibroblast: analysis of CD34 expression in benign, in situ, and invasive breast lesions. J Clin Pathol 56:271–276
33. Shimasaki N, Kuroda N, Miyazaki E et al. (2006) The distribution pattern of myofibroblasts in the stroma of human bladder carcinoma depends on their invasiveness. Histol Histopathol 21:349–353
34. Takata T, Miyauchi M, Ogawa I et al. (2000) Immunoexpression of transforming growth factor beta in desmoplastic ameloblastoma. Virchows Arch 436:319–323
35. Smith SM, Bartov SA (1986) Ameloblastoma with myofibroblasts: first report. J Oral Pathol 15:284–286
36. Rothouse LS, Majack RA, Fay JT (1980) An ameloblastoma with myofibroblasts and intracellular septate junctions. Cancer 45:2858–2863
37. Lombardi T, Morgan PR (1995) Immunohistochemical characterisation of odontogenic cysts with mesenchymal and myofilament markers. J Oral Pathol Med 24:170–176
38. Vered M, Shohat I, Buchner A et al. (2005) Myofibroblasts in stroma of odontogenic cysts and tumors can contribute to variations in the biological behavior of lesions. Oral Oncol 41:1028–1033
39. Carlson ER, Marx RE (2006) The ameloblastoma: primary, curative surgical management. J Oral Maxillofac Surg 64:484–494
40. Ueno S, Mushimoto K, Shirasu R (1989) Prognostic evaluation of ameloblastoma based on histologic and radiographic typing. J Oral Maxillofac Surg 47:11–15
41. Sampson DE, Pogrel MA (1999) Management of mandibular ameloblastoma: the clinical basis for a treatment algorithm. J Oral Maxillofac Surg 57:1074–1077; discussion 78–9
42. McKaig BC, McWilliams D, Watson SA et al. (2003) Expression and regulation of tissue inhibitor of metalloproteinase-1 and matrix metalloproteinases by intestinal myofibroblasts in inflammatory bowel disease. Am J Pathol 162:1355–1360
43. Czochra P, Klopcic B, Meyer E et al. (2006) Liver fibrosis induced by hepatic overexpression of PDGF-B in transgenic mice. J Hepatol 45:419–428
44. Seomun Y, Kim J, Lee EH et al. (2001) Overexpression of matrix metalloproteinase-2 mediates phenotypic transformation of lens epithelial cells. Biochem J 358:41–48
45. Porter KE, Turner NA, O'Regan DJ et al. (2004) Tumor necrosis factor alpha induces human atrial myofibroblast proliferation, invasion and MMP-9 secretion: inhibition by simvastatin. Cardiovasc Res 64:507–515
46. Deng X, He G, Levine A et al. (2008) Adenovirus-mediated expression of TIMP-1 and TIMP-2 in bone inhibits osteolytic degradation by human prostate cancer. Int J Cancer 122:209–218
47. Dass K, Ahmad A, Azmi AS et al. (2008) Evolving role of uPA/uPAR system in human cancers. Cancer Treat Rev 34:122–136
48. Fisher JL, Mackie PS, Howard ML et al. (2001) The expression of the urokinase plasminogen activator system in metastatic murine osteosarcoma: an in vivo mouse model. Clin Cancer Res 7:1654–1660

49. Stillfried GE, Saunders DN, Ranson M (2007) Plasminogen binding and activation at the breast cancer cell surface: the integral role of urokinase activity. Breast Cancer Res 9:R14
50. Nielsen BS, Rank F, Illemann M et al. (2007) Stromal cells associated with early invasive foci in human mammary ductal carcinoma in situ coexpress urokinase and urokinase receptor. Int J Cancer 120:2086–2095
51. Ji F, Chen YL, Jin EY et al. (2005) Relationship between matrix metalloproteinase-2 mRNA expression and clinicopathological and urokinase-type plasminogen activator system parameters and prognosis in human gastric cancer. World J Gastroenterol 11:3222–3226
52. Bernstein AM, Twining SS, Warejcka DJ et al. (2007) Urokinase receptor cleavage: a crucial step in fibroblast-to-myofibroblast differentiation. Mol Biol Cell 18:2716–2727