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Immunoexpression of Integrins in Ameloblastoma, Adenomatoid Odontogenic Tumor, and Human Tooth Germs

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The expression of integrins α2β1, α3β1, and α5β1 in 30 ameloblastomas (20 solid and 10 unicystic tumors), 12 adenomatoid odontogenic tumors (AOTs), and 5 human tooth germs in different stages of odontogenesis was analyzed. The distribution, location, pattern, and intensity of immunohistochemical expression were evaluated. Intensity was analyzed using scores (0 = absence, 1 = weak staining, and 2 = strong staining). No difference in the immunoexpression of the integrins was observed between solid and unicystic ameloblastomas. When these two ameloblastoma types were pooled into a single group, the following significant differences were found: immunoexpression of integrin α2β1 was stronger in ameloblastomas than in AOTs and tooth germs, and the expression of integrin α5β1 was stronger in ameloblastomas than in AOTs. The lack of detection of integrin α3β1 in tooth germs and its detection in the odontogenic tumors studied suggest that this integrin might be used as a marker of neoplastic transformation in odontogenic tissues.

Keywords: ameloblastoma; adenomatoid odontogenic tumor; human tooth germs; integrins

Introduction

Odontogenic tumors comprise a complex group of lesions characterized by diverse biological behaviors and histological types that present, as observed for odontogenesis, various inductive interactions between the epithelium and the ectomesenchyme.[1]

Ameloblastomas are the most common epithelial odontogenic tumors[2] and the second most frequent neoplasms among odontogenic tumors.[3] Based on clinical behavior, histopathology, and prognosis, three types of ameloblastomas can be distinguished—solid or multicystic ameloblastoma, unicystic ameloblastoma, and peripheral ameloblastoma.[4] The latest classification of the World Health Organization[5] considers desmoplastic ameloblastoma to be a variant with specific clinical, radiographic, and histological features. Among the intraosseus types, unicystic ameloblastomas show a more favorable biological behavior than the solid type.[6] Adenomatoid odontogenic tumors (AOTs) have been shown to be the third most frequent odontogenic tumor.[3,7] These tumors are characterized by a slow and progressive growth, showing a favorable prognosis in most cases.[5]

Extracellular matrix (ECM) proteins have been demonstrated to play an important role in epithelial–mesenchymal interactions in odontogenic tumors.[8,9] Many types of cells are unable to proliferate in the absence of an anchor to an extracellular substrate.[10] This binding to EMC proteins is mediated by integrins, including in tumors,[11] and studies have demonstrated a correlation between the expression of integrins and the biological behavior of some tumors.[12-14]

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Integrins are transmembrane receptors formed by the noncovalent association of two glycoprotein subunits (an α subunit and a β subunit), which modulate cell–cell and cell–matrix binding and have been implicated in the growth, adhesion, migration, proliferation, apoptosis, and morphology of cells.[15,16]

The objective of the present study was to analyze the immunohistochemical expression of integrins α2β1, α3β1, and α5β1 in ameloblastomas, AOTs, and human tooth germs to better understand the role of these integrins in cellular events and in the cell–matrix interaction in odontogenic tumors and during odontogenesis.

Material and Methods

The following cases were selected: 30 ameloblastomas (20 solid and 10 unicystic tumors) and 12 AOTs obtained from the archives of the Laboratory of Oral Pathology, Dental School, Federal University of Rio Grande do Norte (UFRN) and 5 fetal tooth germs in different stages of odontogenesis (one in the dental lamina stage, two in the bud stage, one in the cap stage, and one in the late bell stage in which the initial stages of dentin deposition is observed) obtained from the archives of Laboratório de Patologia e Citopatologia Ltda., Aracaju, Sergipe, Brazil. Among the 20 solid ameloblastoma cases, the predominant histological types were the plexiform type in 6 cases, the follicular type in 4 cases, the basal cell type in 1 case, the desmoplastic type in 1 case, and cases concomitantly exhibiting more than one histological pattern: follicular and acanthomatous (3 cases), desmoplastic and plexiform (1 case), desmoplastic and follicular (1 case), follicular and granular (1 case), plexiform and follicular (1 case), and 1 case showing a combination of the plexiform, acanthomatous, and basal cell patterns. The unicystic ameloblastoma cases were selected according to the criteria proposed by Philipsen and Reichart.[4] The present study was approved by the Research Ethics Committee of UFRN.

The paraffin-embedded specimens were cut into 3-μm-thick sections, and the sections were submitted to immunohistochemistry by the streptavidin–biotin–peroxidase method optimized with the Dako CSA system (catalyzed signal amplification system for mouse primary antibodies) and incubated with the following primary antibodies for 60 minutes: anti-α2β1 integrin (clone $BHA_{2,1}$, Chemicon, Temeluca, Calif; diluted 1:1000 in Tris–HCl), anti-α3β1 integrin (clone $M\text{-}KD_{102}$, Chemicon; diluted 1:500 in Tris–HCl), and anti-α5β1 integrin (clone JBS_5, Chemicon; diluted 1:1000 in Tris–HCl).

Antigen retrieval was carried out in 0.5% pepsin, pH 1.8, at 37°C for 30 minutes. Before incubation with the primary antibodies, the sections were incubated with 1% bovine serum albumin and 5% fetal bovine serum in Tris–HCl, pH 7.4, for 60 minutes to block reactions with nonspecific tissue proteins. After incubation with the primary antibodies, the specimens were washed twice in 1% Tween 20 solution for 5 minutes each. Next, the sections were incubated for 15 minutes with the sequential reagents of the Dako CSA kit, with the specimens being washed twice in 1% Tween 20 solution between steps. The reaction was developed using diaminobenzidine (Sigma, St. Louis, Mo) as the chromogen, and the sections were counterstained with Mayer's hematoxylin. Specimens in which the primary antibody was replaced with 1% bovine serum albumin in Tris–HCl were used as negative controls.

For immunohistochemical analysis, the distribution, location, pattern, and intensity of immunohistochemical staining were evaluated. Staining intensity was analyzed by selecting a cut-off value per specimen for each marker and scored on a scale from 0 to 2, where 0 = absence of staining, 1 = weak staining, and 2 = strong staining, because of which nonparametric tests were performed. To compare independent groups the Kruskal–Wallis test was applied, followed by Dunn's multiple comparison posttest, because the first test showed a significant difference. In addition, the comparison between the two clinicopathologic types of ameloblastoma (solid and unicystic) was done by means of the Mann–Whitney test. The level of significance was established at 95% ($P < .05$).

Results

In solid ameloblastomas, immunohistochemical staining was observed at the intercellular contacts and at the connective tissue interface. Staining was diffuse for integrins α2β1 and α5β1 (Figure 1) and focal for integrin α3β1 (Figure 2), with the observation of a linear and granular expression pattern. In the plexiform type, immunohistochemical staining was more evident in epithelial sheets and cords where the cells were loosely arranged, resembling the stellate reticulum of the enamel organ. In follicular ameloblastomas, the central and peripheral cells of the follicles were equally immunoreactive, with a strong tendency toward the focal expression

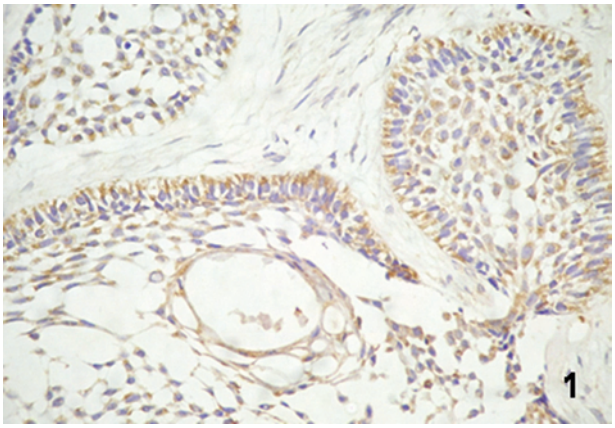

Figure 1. Diffuse labeling for integrin α2β1 in the central and peripheral cells of the follicles in solid ameloblastoma (streptavidin–biotin, magnification ×400).

of integrin α3β1 being observed in this pattern. Acanthomatous ameloblastomas showed strong immunoreactivity mainly to integrin α5β1 in cells undergoing squamous metaplasia (Figure 3). The desmoplastic and basal cell patterns exhibited predominantly strong staining both at intercellular contacts and at the epithelial–mesenchymal interface. Strong immunoreactivity to integrins α2β1 and α5β1 and weak reactivity to integrin α3β1 were observed in one tumor of the granular cell type, with the immunohistochemical staining being more evident in cuboidal cells at the periphery of epithelial nests and scarce in central granular cells. Analyzing solid ameloblastomas in general, no significant difference in staining intensity was observed among the integrins studied (Tables 1 and 2).

In unicystic ameloblastomas, immunohistochemical staining occurred in a diffuse manner at the epithelial–connective tissue interface and at intercellular contacts in all layers of the epithelial component, with a linear and granular expression pattern for integrins α2β1 and α5β1 (Figure 4) and a focal and granular pattern for integrin α3β1. In these ameloblastomas, a significant difference in the intensity of immunohistochemical expression was observed between integrins α3β1 and α5β1, with stronger staining being detected for the latter (Tables 1 and 2).

No significant difference in the intensity of integrin expression was observed between solid and unicystic ameloblastomas (Table 3).

In AOT specimens, immunohistochemical staining tended to show a focal distribution for integrins

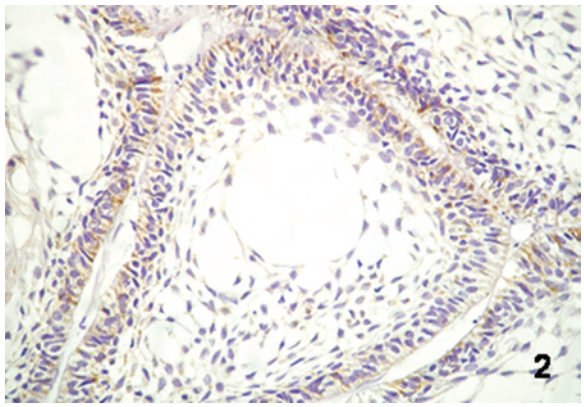

Figure 2. Focal labeling for integrin α3β1 in the follicular type of ameloblastoma (streptavidin–biotin, magnification ×400).

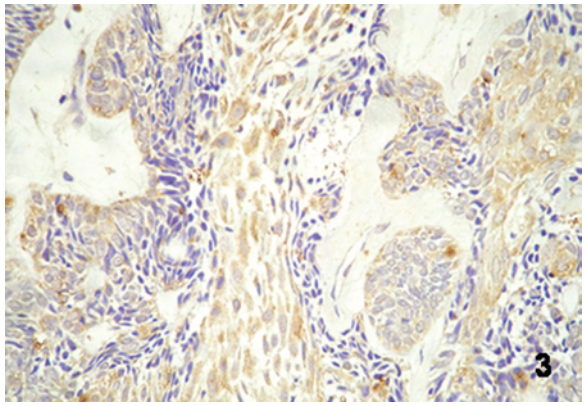

Figure 3. Strong immunoreactivity to integrin α5β1 in cells undergoing squamous metaplasia in acanthomatous ameloblastomas (streptavidin–biotin, magnification ×400).

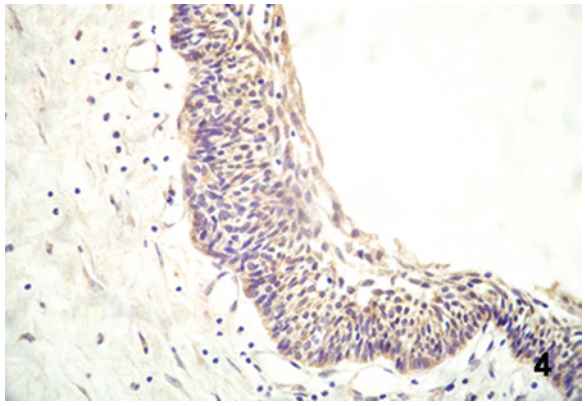

Figure 4. Diffuse immunohistochemical labeling for integrin α2β1 detected at the epithelial–connective tissue interface and in the intercellular contacts in all layers of the epithelial component of unicystic ameloblastoma (streptavidin–biotin, magnification ×400).

Table 1. Distribution and Variation in the Intensity of the Immunohistochemical Expression of Integrins in the Cases Studied

Tissue	Integrin	n	Median	Minimum	Maximum
Solid ameloblastoma	α2β1	20	1.50	1.00	2.00
	α3β1	20	1.00	0.00	2.00
	α5β1	20	2.00	1.00	2.00
Unicystic ameloblastoma	α2β1	10	2.00	1.00	2.00
	α3β1	10	1.00	1.00	2.00
	α5β1	10	2.00	1.00	2.00
Adenomatoid odontogenic tumor	α2β1	12	1.00	1.00	1.00
	α3β1	12	1.00	0.00	1.00
	α5β1	12	1.00	1.00	2.00
Tooth germs	α2β1	5	0.00	0.00	1.00
	α3β1	5	0.00	0.00	0.00
	α5β1	5	1.00	1.00	2.00

Table 2. Nonparametric Analysis of the Differences Observed Among the Integrins Studied

Tissue	Integrin	n	Mean of Ranks	Sum of Ranks	Kruskal–Wallis KW	*P* value
Solid ameloblastoma	α2β1	20	31.75	635.0	4.216	.1215
	α3β1	20	25.05	501.0		
	α5β1	20	34.70	694.0		
Unicystic ameloblastoma	α2β1	10	17.50a,b	175.0	8.136	.0171
	α3β1	10	10.00a	100.0		
	α5β1	10	19.00b	190.0		
Adenomatoid odontogenic tumor	α2β1	12	18.00	216.0	4.595	.1005
	α3β1	12	16.58	199.0		
	α5β1	12	20.92	251.0		
Tooth germs	α2β1	5	6.30a	31.50	11.089	.0039
	α3β1	5	5.00a	25.00		
	α5β1	5	12.70b	63.50		

Note: KW and *P* values for the Kruskal–Wallis test. Letters "a" and "b" express the results obtained with Dunn's posttest, considering $P = .05$.

Table 3. Immunoexpression of Integrins in the Different Types of Ameloblastoma

Integrin	Ameloblastoma	n	Mean of Ranks	Sum of Ranks	Mann–Whitney *U*	*P* value
α2β1	Solid	20	14.5	290.0	80.0	.3840
	Unicystic	10	17.5	175.0		
α3β1	Solid	20	15.8	316.0	94.0	.8049
	Unicystic	10	14.9	149.0		
α5β1	Solid	20	14.5	290.0	80.0	.3818
	Unicystic	10	17.5	175.0		

Note: Nonparametric Mann–Whitney test.

α2β1 and α3β1 (Figure 5) and a diffuse distribution for integrin α5β1 (Figure 6). In solid areas and cords, staining was observed at intercellular contacts, conferring a reticular pattern. In duct-like structures, granular or linear expression was observed at the luminal pole of cells in some areas and a tendency toward bipolar expression in others. In these tumors, the immunostaining intensity did not differ significantly among the integrins studied (Tables 1 and 2).

No immunohistochemical expression of integrin α3β1 was observed in tooth germs. Expression of integrin α2β1 was only detected in the late bell

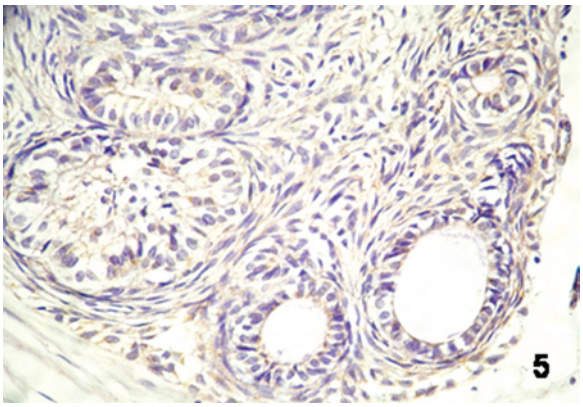

Figure 5. Focal immunoreactivity for integrin α3β1 in the intercellular contacts of adenomatoid odontogenic tumor (streptavidin–biotin, magnification ×400).

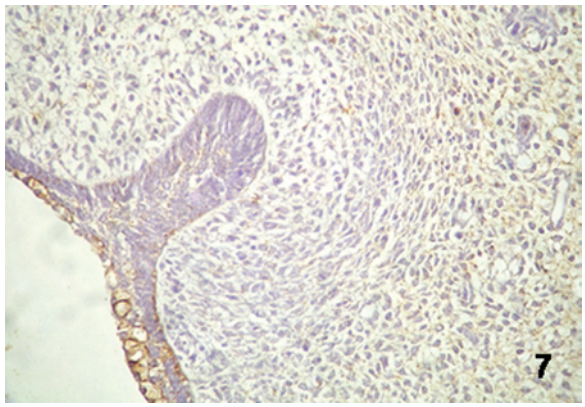

Figure 7. Immunopositivity, focal and granular, for integrin α5β1 in dental lamina of human tooth germs (streptavidin–biotin, magnification ×400).

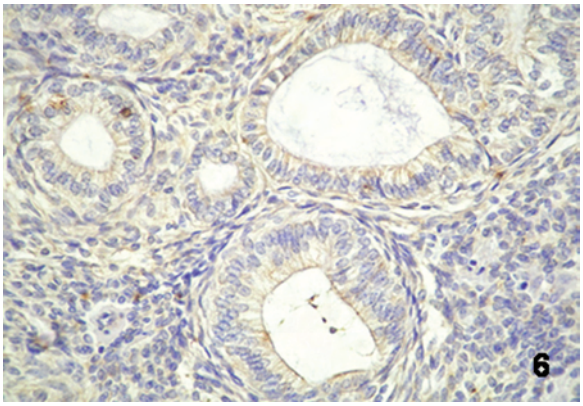

Figure 6. Diffuse labeling for integrin α5β1 in adenomatoid odontogenic tumor (streptavidin–biotin, magnification ×400).

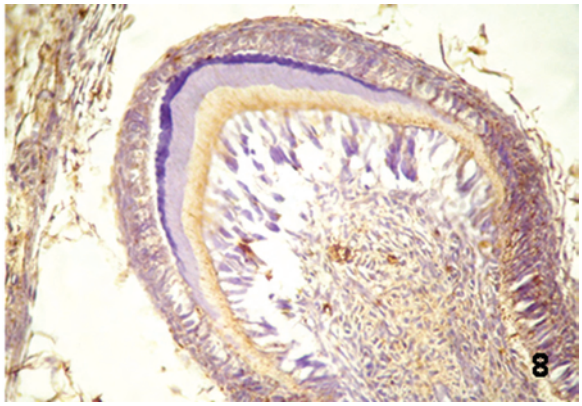

Figure 8. Expression pattern for integrin α5β1 in late bell stage human tooth germs (streptavidin–biotin, magnification ×400).

stage, with the observation of granular staining in the dentin layer being formed. Expression of integrin α5β1 was detected in all tooth germs. In the dental lamina stage, focal and granular staining was observed in some central epithelial cells (Figure 7). In the bud stage, granular and focal immunostaining was detected in the central cells of the epithelial component and in some condensed ectomesenchymal cells. In the cap stage, granular and diffuse staining was noted in the central cells of the enamel organ, at the interface with the basement membrane of the inner epithelium, and in some condensed ectomesenchymal cells. In the late bell stage, a granular and diffuse expression pattern was observed in the cells of the inner epithelium (calcification area), stellate reticulum, outer epithelium, dentin layer, and dental papilla (Figure 8). Comparison of the immunostaining intensity in this group showed a significant difference between integrins α2β1 and α3β1 and integrin α5β1, which exhibited a more intense staining (Tables 1 and 2).

Because no significant difference in the intensity of the immunohistochemical expression of the integrins studied was observed between solid and unicystic ameloblastomas (Table 3), these two clinical–pathological types were pooled into a single group for comparison with AOTs and tooth germs, which revealed a significant difference for all the integrins studied. A difference was observed between ameloblastomas and AOTs and between ameloblastomas and tooth germs for integrin α2β1, which showed a stronger immunostaining in ameloblastomas;

Table 4. Distribution and Variation in the Intensity of the Immunohistochemical Expression of Integrins in Ameloblastomas, Adenomatoid Odontogenic Tumors (AOTs), and Tooth Germs

Integrin	Tissue	n	Median	Minimum	Maximum
α2β1	Ameloblastoma	30	2.00	1.00	2.00
	AOT	12	1.00	1.00	1.00
	Tooth germs	5	0.00	0.00	1.00
α3β1	Ameloblastoma	30	1.00	0.00	2.00
	AOT	12	1.00	0.00	1.00
	Tooth germs	5	0.00	0.00	0.00
α5β1	Ameloblastoma	30	2.00	1.00	2.00
	AOT	12	1.00	1.00	2.00
	Tooth germs	5	1.00	1.00	2.00

Table 5. Nonparametric Analysis of the Differences Observed in Immunoexpression of Integrins in Ameloblastomas, Adenomatoid Odontogenic Tumors (AOT), and Tooth Germs

Integrin	Tissue	n	Mean of Ranks	Sum of Ranks	Kruskal–Wallis KW	*P* value
α2β1	Ameloblastoma	30	29.68a	890.0	21.648	<.0001
	AOT	12	17.50b	210.0		
	Tooth germs	5	5.50b,c	27.5		
α3β1	Ameloblastoma	30	28.18a	845.0	20.281	<.0001
	AOT	12	21.87a	262.0		
	Tooth germs	5	4.00b	20.0		
α5β1	Ameloblastoma	30	27.67a	830.0	8.661	.0132
	AOT	12	15.92b	191.0		
	Tooth Germs	5	21.40a,b	107.0		

Note: KW and *P* values for the Kruskal–Wallis test. Letters "a", "b," and "c" express the results obtained with Dunn's posttest, considering $P = .05$.

between ameloblastomas and tooth germs and between AOTs and tooth germs for integrin α3β1, whose expression was stronger in tumors; and between ameloblastomas and AOTs for integrin α5β1, which exhibited a stronger staining in ameloblastomas (Tables 4 and 5).

Discussion

The binding of cells to ECM proteins mediated by integrins can lead to changes in the cytoplasmic domains of these proteins, which are associated with different postbinding events such as cell differentiation, migration, proliferation, and gene induction.[17]

Various inductive interactions between the epithelium and the ectomesenchyme have been shown to occur both during odontogenesis and in odontogenic tumors,[1] and studies have demonstrated a correlation between the expression of integrins and the biological behavior of some tumors.[12-14] In view of these findings, we investigated the expression of integrins α2β1, α3β1, and α5β1, known ligands for ECM proteins (collagen, laminin, and fibronectin), in ameloblastomas and AOTs, two odontogenic tumors presenting different biological behaviors,[4,6] and in tooth germs in different stages of odontogenesis. Among ameloblastomas, integrin expression was compared between the solid and unicystic types because they are clinical types of the same tumor but with different biological behaviors.[6] In view of the diversity and functional versatility of integrins[15,16] and the lack of studies on these receptors in odontogenic tumors, immunohistochemical staining for these molecules was analyzed in the present study in terms of location, distribution, expression pattern, and intensity to better understand the role of integrins in these tumors.

Focal distribution of integrin α3β1, an important receptor for laminin,[18] was observed in the two clinical–radiographic types of ameloblastoma (solid and unicystic). This finding agrees with the study of Souza et al,[19] who demonstrated discontinuous

laminin staining in the basement membrane of ameloblastomas. Integrin α3β1 plays an important role in the maintenance and integrity of the basement membrane,[20] and focal expression of this integrin by neoplastic ameloblastoma cells may therefore result in basement membrane defects or failure in the anchorage of these cells to the basement membrane, thus facilitating cell migration. However, the possibility that ameloblastoma cells use another integrin, such as integrin α6β4, for binding to laminin cannot be ruled out.

In unicystic ameloblastomas, a significant difference in immunostaining intensity was observed between integrins α5β1 and α3β1, with the intensity being stronger for the former. However, no significant difference in intensity among the integrins studied was observed for solid ameloblastomas. Integrin α5β1 plays an important role in cell growth, migration, and tumorigenicity, and its expression inactivates cell proliferation genes such as *c-fos*, *c-jun*, and *junB*, decelerating cell growth and reducing tumorigenicity.[21] Taking together the staining intensity results obtained for the two clinical types of ameloblastoma and the focal expression of integrin α3β1, which, as discussed earlier, facilitates cell migration and tumor invasiveness, we may suggest a compensatory mechanism of this invasiveness in unicystic ameloblastomas involving a stronger expression of integrin α5β1, which was not observed in solid ameloblastomas. This fact might explain the more favorable biological behavior of unicystic ameloblastomas compared with the solid type. However, this hypothesis cannot be completely confirmed in the present study because no significant difference in the intensity of integrin staining was observed between solid and unicystic ameloblastomas (Table 3).

Some variations in the location and intensity of immunohistochemical staining were observed among the different histological types of solid ameloblastomas, which reflect the various functions attributed to these molecules.[15,16]

In the acanthomatous type, strong expression of integrin α5β1 was observed in cells undergoing squamous metaplasia, which were located in the center of tumor islands where the amount of ECM for anchorage of these cells is lower than at the periphery. Excessive production of this integrin in such a way that these molecules are not bound to ECM has been suggested as a mechanism of tumor suppression and promotion of apoptosis induced by the *p16* gene.[22] Studies on ameloblastomas have shown the absence of staining for cyclin D1 and protein p27, in addition to the presence of apoptotic bodies in cells undergoing squamous metaplasia.[23,24] Thus, the presence of intensive staining for integrin α5β1 in the central cells of acanthomatous ameloblastomas observed in this study suggests a mechanism of terminal differentiation or apoptosis of these cells. Supporting this hypothesis, a significantly lower recurrence rate has been reported for acanthomatous ameloblastomas.[6]

In the present study, strong staining for all the integrins analyzed was observed in basal cell ameloblastomas. Studies using cell proliferation markers have demonstrated a higher proliferative rate in this histological type,[23,25,26] as well as poor reactivity to apoptosis markers.[24] Furthermore, studies have shown an integrin-mediated signaling pattern in cell cycle promotion and that the interaction of these molecules with growth factors regulates various steps during the G1 to S phase transition of the cell cycle.[27] Our findings regarding integrins in basal cell ameloblastomas suggest an important role of these molecules in the survival and cell proliferation of this type of tumor.

In duct-like structures of AOTs, expression of the integrins studied showed a linear or granular pattern at the luminal pole of cells or even a bipolar pattern. The presence of these integrins in cells that surround duct-like structures might be related to the regulation of cell polarization in these structures because the participation of β1 integrins in this regulation has been demonstrated in cell cultures.[28] In addition, expression of these integrins at the luminal pole of cells that surround these duct-like structures might be necessary for the activation of ECM protein synthesis or for anchorage of these cells to ECM proteins whose secretion by AOT cells, associated with their retention in the lumen of duct-like structures, has been demonstrated.[29]

In tooth germs, integrin α3β1 was not observed and integrin α2β1 was detected only in the late bell stage. The weak expression or lack of expression of integrins α2β1 and α3β1, respectively, during odontogenesis might be a necessary mechanism for cell survival after the degradation of the basement membrane, which is an important event in the differentiation of inner epithelial cells into ameloblasts, as demonstrated in other studies.[30] The results we obtained for integrin α5β1, the main receptor of fibronectin, showing a more diffuse distribution in the cap and bell stages, reflect the important role of

fibronectin in the events of morphogenesis and differentiation during odontogenesis, as described in other studies.[31,32] Furthermore, the wide distribution of integrin α5β1 during the various stages of odontogenesis may facilitate cell migration in a fibronectin-rich matrix.

The intensity of staining for integrin α2β1 was significantly stronger in ameloblastomas than in AOTs or tooth germs ($P < .0001$). Integrin α2β1 acts as a receptor for collagen in platelets and epithelial cells and also activates collagen synthesis and induces the expression of collagenases and matrix metalloproteinases (MMPs) 1 and 13.[33] MMP-1 binds to domain 1 (domain A) of the α2 subunit of integrin α2β1, and the association integrin–MMP locates the activity of ECM degradation in the same domain that is responsible for the plasma membrane adhesion, thus promoting cell migration.[34] MMP-2 secretion is increased in human cervical tumor cells through a mechanism mediated by integrin α2β1.[35] Furthermore, keratinocytes use this integrin for migration to collagen I,[36] an ECM protein widely distributed in the stroma of ameloblastomas.[9] A stronger expression of integrin α2β1 in ameloblastomas might therefore be associated with a greater cell migration in the collagen-rich matrix, or even with the formation of a complex between this integrin and MMPs whose presence and activity has been demonstrated in ameloblastomas,[37] a fact suggesting an important role of this integrin in the local invasiveness of these tumors.

The intensity of α3β1 integrin staining did not differ significantly between ameloblastomas and AOTs, whereas this integrin was not detected in tooth germs. Integrin α3β1 has been suggested to mediate the migration of tumor cells.[38] Giannelli et al[39] demonstrated that this integrin is not present in normal hepatic parenchyma but is abundantly expressed in primary and metastatic hepatic carcinomas, a fact that renders this integrin to be a potential marker for the development of hepatic carcinomas and metastases. Similar results were obtained in the present study in which this integrin was detected in neoplastic odontogenic epithelium but not in tooth germs.

The intensity of α5β1 integrin staining was significantly stronger ($P < .05$) in ameloblastomas than in AOTs. Integrin α5β1 is widely expressed in tissues involved in the active deposition of pericellular fibronectin and in matrix remodeling.[21] Extensive expression of fibronectin has been demonstrated during odontogenesis[31,32] and in ameloblastomas.[9] Thus, tooth germ cells and ameloblastomas use integrin α5β1 for migration and binding to a matrix rich in fibronectin. In addition, binding of integrin α5β1 to fibronectin increases the expression and secretion of MMPs involved in tumor invasion.[21] Therefore, the extensive expression of fibronectin in ameloblastomas demonstrated in another study[9] and the stronger expression of integrin α5β1 in these tumors than in AOTs suggest the participation of this integrin in the local invasiveness of ameloblastomas, probably mediated by a mechanism of induction of MMP synthesis.

In the present study, analyzing the immunohistochemical expression of integrins α2β1, α3β1, and α5β1 in ameloblastomas and AOTs (odontogenic tumors) and during the different stages of odontogenesis, important findings were obtained when evaluating the intensity of integrin staining, with the expression of these molecules tending to be stronger in ameloblastomas. The lack of detection of integrin α3β1 in tooth germs and its similar staining intensity in the odontogenic tumors studied suggest that this integrin might be used as a possible marker of neoplastic transformation in odontogenic tissues. In addition, blockade of integrins α2β1 and α5β1, which are more strongly expressed in ameloblastomas, may become an important strategy in the treatment or control of the local invasiveness of this tumor.

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