J Oral Pathol Med (2004) 33: 574–80

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Expression patterns of integrins on pleomorphic adenoma and adenoid cystic carcinoma: study on specimens and *in vitro* investigation of the effects of extracellular matrix on the expression of these adhesion molecules

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BACKGROUND: Pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC) are neoplasms of distinct behaviour, showing similar origin, cell components and marked presence of extracellular matrix (ECM). Interactions between cells and ECM are important in the biology of tumours, being partially mediated by integrins. This study investigated these interactions on PA and ACC using paraffin-embedded tissue and an *in vitro* model of these conditions.

METHODS: Expression of integrins in paraffin-embedded samples was assessed by immunohistochemistry. Cells from PA and ACC were characterized using immunofluorescence, and integrin patterns of expression were investigated on cells cultivated on different ECM proteins.

RESULTS: Luminal cells of both PA and ACC were more intensely positive for integrins than myoepithelial cells. *In vitro* studies revealed that PA cells expressed more integrins than ACC cells regardless the ECM protein present.

CONCLUSIONS: This study revealed particular patterns of integrin expression in both specimens and *in vitro* models of PA and ACC. This might prove useful for a better understanding of the biology of these lesions.
J Oral Pathol Med (2004) **33**: 574–80

Keywords: adenoid cystic carcinoma; extracellular matrix; integrins; pleomorphic adenoma

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Accepted for publication February 10, 2004

Introduction

Pleomorphic adenoma (PA) and adenoid cystic carcinoma (ACC) are neoplasms of distinct behaviour, which show similar origin and cell components as well as marked presence of extracellular matrix (ECM). Interactions between cells and ECM are important in the biology of tumours, which are partially mediated by integrins. Integrins are a family of heterodimeric transmembrane molecules composed of two subunits – α and β. These molecules are subdivided into families according to the β-subunit and those of the β1 family play pivotal roles in epithelial cells adhesion. They bind chiefly to components of the ECM, broadly represented by collagen, laminin and fibronectin (1). Other dynamic biological mechanisms such as maintenance of intercellular contacts, cell signalling and regulation of gene expression leading to migration, proliferation and differentiation, either in normal or neoplastic processes have been related to the expression of integrins (2–4).

The expression of integrin receptors has been demonstrated by immunohistochemistry, in specimens obtained from diverse tissues and organs. However, we, and very few other workers have evaluated their presence and possible biological roles in salivary gland tumours (5–7).

The present study was undertaken to investigate the interactions between the stromal and cellular components of PA and ACC and relate those with tumour behaviour using paraffin-embedded tissue and cells cultivated from these neoplasms *in vitro*.

Material and method

Immunohistochemistry

Fifteen specimens of PA and 12 ACC were retrieved from the archives of the Oral Pathology Department of the Dental School of the University of São Paulo and from the Oral Pathology Department of Bart's and the

Table 1 Integrins, clone, source and dilution

Primary serum (Chemicon)	*Source*	*Clone*	*Dilution*
Integrin α2	P1E6	Chemicon	1:100
Integrin α3	P1B5	Chemicon	1:100
Integrin α5	NKI-SAM-1	Chemicon	1:100
Integrin β1	6S6	Chemicon	1:100

London School of Medicine and Dentistry (Local Ethics Committee Ref T/02/043). Briefly, 3 μm serial sections of these specimens were deparaffinized, exposed to a 0.3% solution of hydrogen peroxide in distilled water and submitted to antigen retrieval in 0.5% pepsin (pH 1.8) for 30 min at 37°C. Incubation with avidin- and biotin-blockers (Vector, Burlingame, CA, USA) was then performed for 15 min each, to block endogenous biotin from the specimens. Incubation with 1% bovine serum albumin (BSA) and 5% foetal bovine serum (FBS) in Tris-HCl (pH 7.4) for 60 min at room temperature followed to suppress non-specific binding of subsequent reagents. The sections were then incubated with the primary antibody overnight; source, clone and working dilutions are described in Table 1. The antibody-binding detection system Catalysed Signal Amplification System (CSA, HRP; Dako, Carpinteria, CA, USA) was employed to develop the reaction, which consisted of sequential 15 min incubations with biotinylated link, streptavidin complex, amplification reagent and streptavidin peroxidase (steps 4–9 of the Dako CSA System, HRP). Each of these 15 min incubation steps were preceded by a 5 min rinse with 1% Tween 20 in Tris-HCl (pH 7.4). Staining was completed with 3 min incubation with 3′3-diaminobenzidine tetrachloride (DAB; Sigma, St Louis, MO, USA), which resulted in a brown coloured precipitate at the antigen site. The specimens were then lightly counterstained with Mayer's haematoxylin, dehydrated and mounted with glass coverslip and xylene-based mountant.

Negative controls were treated as above, but a solution of 1% BSA in Tris-HCl (pH 7.4) replaced the primary antibody. Additionally, further controls were used by employing monoclonal antibodies with irrelevant specificity, but with the same isotype (mouse monoclonal antibody, clone SY5, isotype IgG1; Novocastra Laboratories Ltd, Newcastle, UK) and E-selectin (mouse antihuman monoclonal antibody, clone P2H3, isotype IgG1; Chemicon International, Inc., Temecula, CA, USA). Staining patterns of the basal layer of epithelium, when present, were considered as internal positive control.

Specificity of the antibodies used was verified in our previous publications (5–7).

Culture and characterization of human PA and ACC cells

Surgical specimens of PA and ACC were finely minced with a scalpel under sterile conditions and the explants were incubated in 25 cm^2 culture flasks with minimum essential medium (DMEM) supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS, at 37°C in a humidified 5% CO_2 atmosphere. After reaching confluence of 75–90%, cells were subcultured two to five times before cell line characterization was performed using immunofluorescence. The panel of monoclonal antibodies employed for cell characterization is described in Table 2. Briefly, passage 6 cells were plated on glass slides and incubated in DMEM supplemented with 100 U/ml penicillin, 100 mg/ml streptomycin and 10% FBS. After 48 h cells were fixed in 3.7% buffered paraformaldehyde, permeabilized with 1% Triton X100 in phosphate-buffered saline (PBS) and incubated overnight with the monoclonal primary serum at room temperature. Cell monolayers were rinsed with PBS then incubated with sheep antimouse fluorescein isothiocyanate (FITC)-conjugated immunoglobulin (Novocastra Laboratories Ltd) diluted 1:100 in BSA-PBS followed. The glass slides containing the cells monolayer were then mounted with Vecta Shield (Vector Laboratories, Burlingame, CA, USA) and observed using Zeiss microscope (Carl Zeiss, Göttingen, Germany) equipped with epi-illumination and filters for fluorescein. Negative controls were performed by omitting the primary antibody.

Table 2 Antibodies used for cell characterization

Primary serum	*Clone*	*Source*	*Dilution*
Keratin	AE1/AE3	Boehringer-Mannheim (Indianapolis, IN, USA)	1:50
Vimentin	V9	Dako	1:50
SMA	1 A4	Biogenex (San Ramon, CA, USA)	1:50
Calponin	CALP	Dako	1:50
h-Caldesmon	TD107	Novocastra	1:50

Effect of extracellular matrix protein on integrin expression of PA and ACC cells

To assess integrin expression on cells derived from PA and ACC, the cells were grown on glass coverslips coated with collagen I (Sigma), collagen IV (Becton Dickinson Labware, Bedford, MA, USA), laminin (Sigma) and fibronectin (Sigma). Cells grown on glass coverslips without matrix coating were used as controls. Briefly, glass coverslips were coated with the ECM proteins according to manufacturer's instructions, under sterile conditions. Coverslips were then placed into wells of 24-well plates and seeded at 10^4 cells/well and plates were incubated for 48 h. After this period, cells were fixed with paraformaldehyde at (3.7% in PBS) and immunofluorescence reactions against integrin subunits α2, α3, α5 and β1 were performed following the protocol described above. Antibodies were used at dilutions of 1:20 and their specifications are described in Table 1.

Results

Immunohistochemistry

The PA specimens consisted of sheets, cords and nests of neoplastic myoepithelial cells, intermingled with neoplastic ductal structures composed of two cell layers. Presence of stroma was a marked feature.

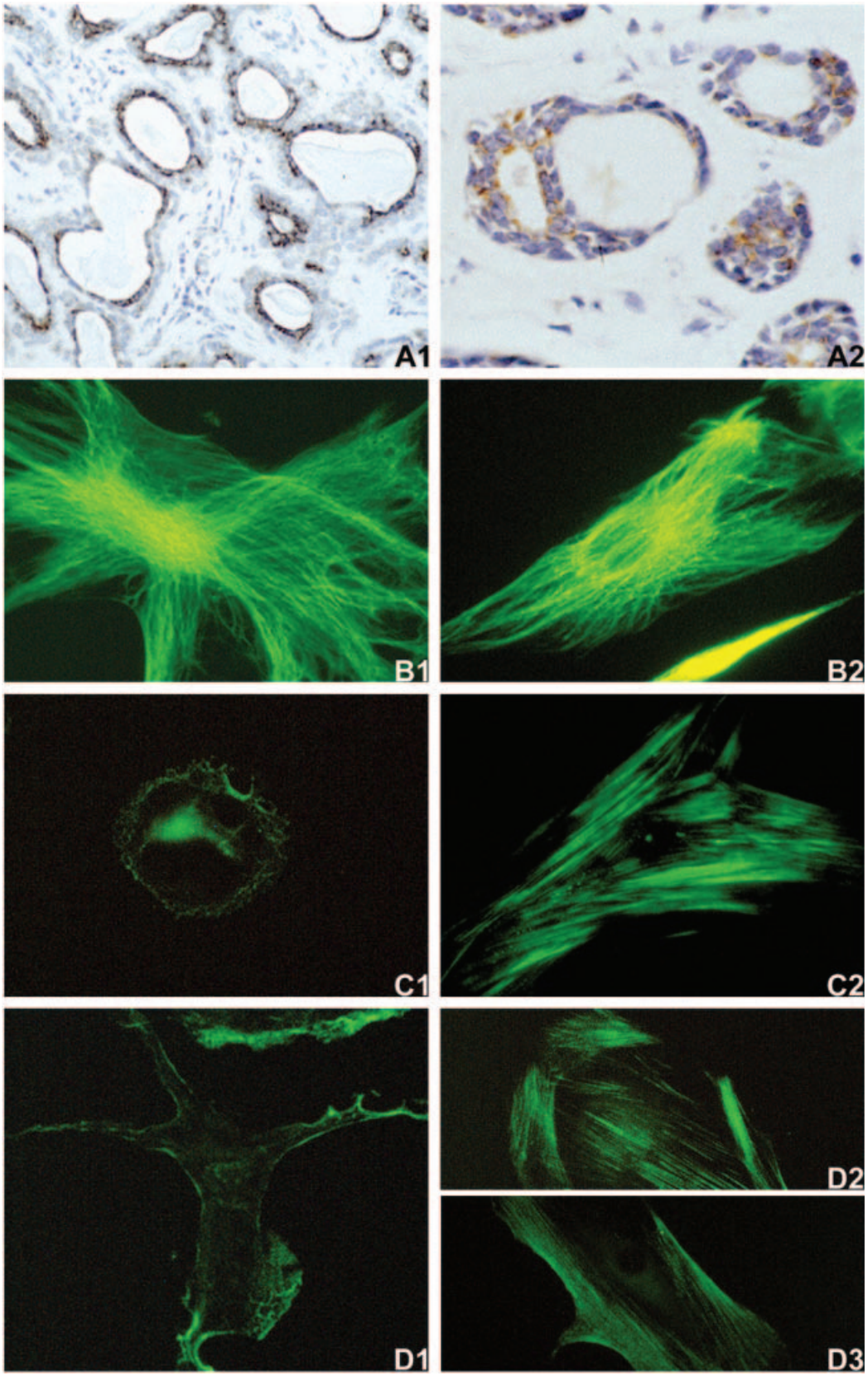

Figure 1 (A1) Pleomorphic adenoma: immunoexpression of integrin β1 mainly on luminal structures (original magnification ×200). (A2) Adenoid cystic carcinoma: expression of integrin α2 on luminal cells of cribriform structures (original magnification ×400). (B1, C1, D1) Expression of vimentin, calponin and caldesmon, respectively in cells derived from pleomorphic adenoma. (B2, C2, D2, E2) Expression of vimentin, smooth muscle actin, calponin and caldesmon in cells derived from adenoid cystic carcinoma.

Expression of integrin subunits in this tumour varied between the various integrin subunits tested and the tumour cell types. For example, integrin α2 and α5 were intensely stained on neoplastic luminal cells, being present mainly in the luminal pole of these cells. A bipolar expression pattern could also be observed. Neoplastic myoepithelial cells showed only a discrete positivity for α2 integrin at intercellular contacts and were negative for the integrin subunit α5. The same structures that stained integrin α2-positive, were also α3-positive, but presented a disk-like pattern of expression. β1-Integrin was widely expressed on luminal cells of neoplastic ducts, showing dots, discs or clusters concentrated at the luminal pole (Fig. 1A1). Myoepithelial cells were positive for all integrins used in the study. The positivity was always seen at points of intercellular contacts.

The ACC specimens comprised tubular, cribriform and solid histological types. Luminal cells of neoplastic ductal structures present in tubular and cribriform ACC were positive for all integrin subunits included in our study. They were present around the membrane of the neoplastic luminal cells, and mainly concentrated at their apical pole in a cluster-like pattern (Fig. 1A2). In myoepithelial cells, integrin α2 showed only a discrete positivity as dots at the intercellular contacts, α3 demonstrated a discoid expression pattern in the same localization, α5 was weakly positive in dots, mainly at the baso-lateral portion of these cells and β1 integrin expression was displayed as discrete dots at intercellular contacts. Neoplastic cells surrounding pseudocystic spaces were mostly negative for all integrins or showed weak staining restricted to the baso-lateral contacts of some myoepithelial cells. All subunits studied were present in a bipolar fashion in neoplastic cells situated at the periphery of tumour islands and lobules, which are in direct contact with the stroma.

Culture and characterization of human PA and human ACC cells

Culture of PA and ACC specimens resulted in cell growth from the explants within 48 h. After 5–10 days, large polyhedral, stelate and spindle cell phenotypes was observed intermingled by a few spindle cells, verified as fibroblasts (data not shown). The growth of fibroblasts was inhibited by selective trypsinization and addition of Geneticin to the cell cultures until passage 6. Cells of both tumours were positive for vimentin, calponin and caldesmon (Fig. 1B1–D1 and B2,D2,E2). A few ACC cells, especially in the first passages, revealed the presence of smooth muscle actin (SMA) (Fig. 1C2). The cells cultured from PA and ACC were then categorized as benign and malignant neoplastic myoepithelial cells, respectively.

Effect of extracellular matrix protein on integrin expression of PA and ACC cells

Generally speaking, PA cells expressed higher level of integrins than ACC cells, under all culture conditions. Briefly, in PA cells, α2 integrin was present mainly at the intercellular contacts as fine lines, staining became more intense when these cells were cultivated on collagen I and IV. In ACC cells, the presence of this integrin was more discrete, but followed similar patterns of expression and distribution. Expression of α3 integrin was fine, granular and diffuse over the cell membrane in both cell types, with more prominence in PA cells. Expression of this integrin was more intense when cells were cultivated on collagen I, IV and laminin. Integrin α5 presented as fine lines and discs over the cell membrane, being more intensely expressed when cells were grown on fibronectin. Finally, β1 integrin was markedly positive on PA cells, mainly concentrated at the cell periphery, cell prolongations and cell contacts, and sometimes radiating out from the centre to the cell periphery. In ACC cells, the expression of this integrin was extremely weak. The intensity of integrin expression is shown in Tables 3 and 4 and some examples of integrin expression patterns are illustrated in Fig. 2.

Table 3 Intensity of integrin expression of pleomorphic adenoma (PA) cells grown on different extracellular matrix proteins

	Glass coverslips	*Collagen I*	*Colagen IV*	*Laminin*	*Fibronectin*
α2	+ +	+ + +	+ + +	+ +	+
α3	+ +	+ + +	+ + +	+ + +	+ +
α5	+ +	+ +	+ +	+ +	+ + +
β1	+ + +	+ + +	+ + +	+ + +	+ + +

+, low intensity; + +, medium intensity; + + +, high intensity.

Table 4 Intensity of integrin expression of adenoid cystic carcinoma (ACC) cells grown on different extracellular matrix proteins

	Glass coverslip	*Collagen I*	*Collagen IV*	*Laminin*	*Fibronectin*
α2	+	+	+ +	+ +	+
α3	+ +	+ +	+ + +	+ +	+
α5	+	+	+	+	+ + +
β1	+	+	+	+	+

+, low intensity; + +, medium intensity; + + +, high intensity.

Discussion

The present study showed that all integrin subunits investigated here were present in PA and ACC, with peculiar patterns of expression and distribution.

Expression of integrins in specimens of PA revealed that these molecules are expressed in luminal cells. Integrin expression on myoepithelial cells was restricted to the intercellular contacts. These findings are in accordance with the study of Sunardhi-Widyaputra and Van Damme (7) who demonstrated similar patterns of integrin expression in this tumour. The presence of integrins at the apical surface of luminal neoplastic cells adds information to the findings of other authors who speculated that formation of integrin-cytoskeletal linkages provided new dockings for vesicles (8). These are responsible for transporting membrane proteins to the cell surface, relating integrin expression patterns with secretory functions.

Only subtle differences could be observed between the patterns of expression pattern of the different integrins

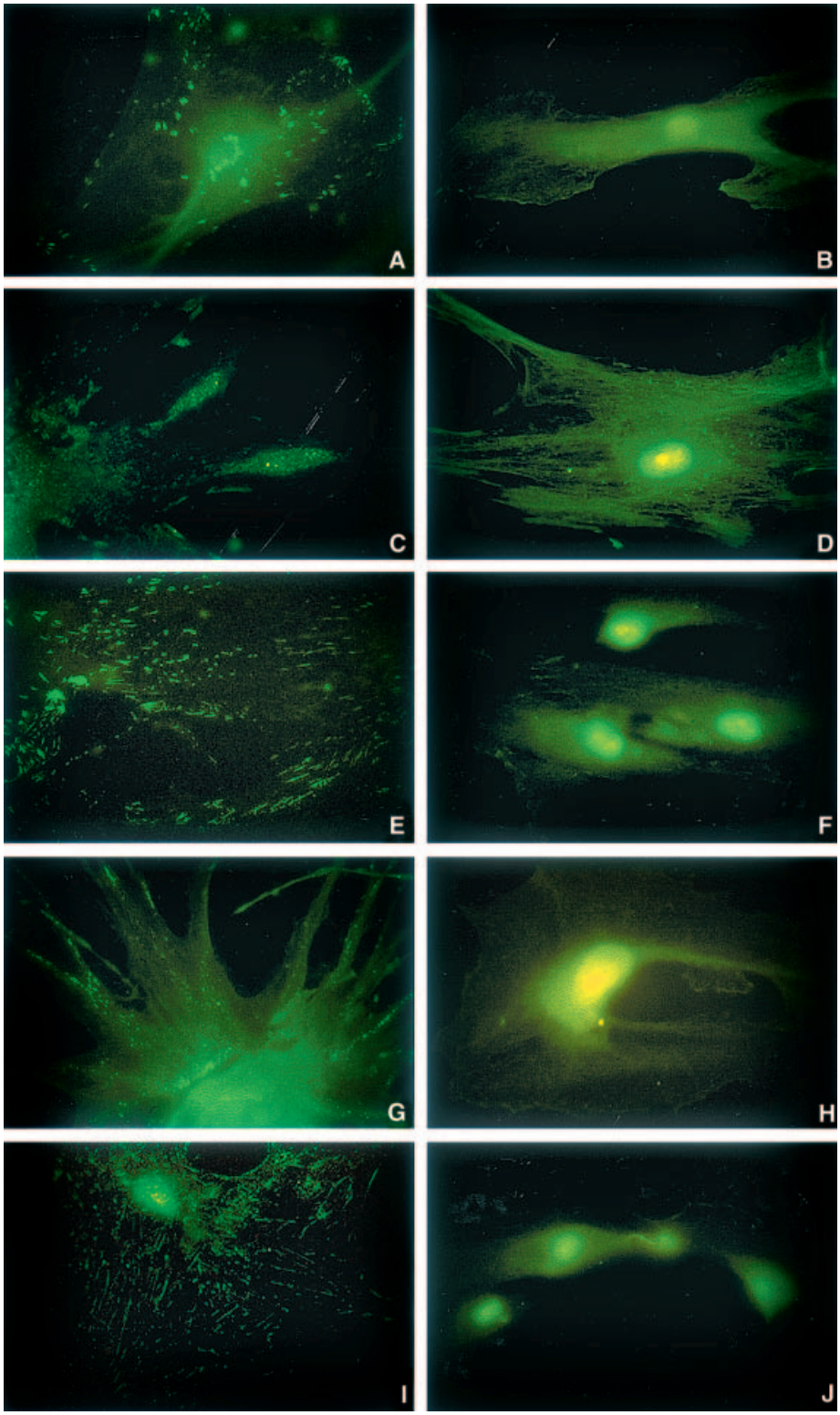

Figure 2 Examples of integrin β1 expression on cells derived from pleomorphic adenoma and adenoid cystic carcinoma. (A, B) PA and ACC cells, respectively, grown on plain glass coverslip; (C, D) PA and ACC cells, respectively, grown on collagen I; (E, F) PA and ACC cells, respectively, grown on collagen IV; (G, H) PA and ACC cells, respectively, grown on laminin; (I, J) PA and ACC cells, respectively, grown on fibronectin.

in both cell types – myoepithelial and luminal cells. This is probably related to common integrin localization at focal contacts and with their functions as both receptors and signalling molecules at these sites (9).

The cases of ACC included in our study were tubular, cribriform and solid histological types. Tubular structures of ACC presented strong expression of integrins in luminal neoplastic cells and discrete integrin expression

in neoplastic myoepithelial cells, restricted to dots at intercellular contacts. These results add to the literature on the expression of cytoskeleton proteins by this tumour and confirm the histological composition of ACC, which shows two cell types – luminal and myoepithelial (10–13).

The pattern of integrin expression in neoplastic cells surrounding pseudocystic spaces of ACC was intriguing. Although pseudocystic spaces are filled with replicated basement membrane, rich in collagen and laminin (14), only a discrete presence of integrins was observed. This might occur due to the absence of a relevant receptor, which stimulates the formation of a defective reduplicated basement membrane, as described by other workers (15–17). Yet, the sparse integrin expression in this case associated with abundant ECM components may be related to invasive mechanisms of ACC cells. Integrins are possibly being relocated on cell surface, promoting lamelipodia formation, which then facilitates cell migration (5, 18).

The expression of integrins in solid ACC was discrete, probably reflecting the aggressive nature of this tumour, imitating the integrin expression patterns of other aggressive malignant tumours such as breast and lung adenocarcinomas (19). Loss of homophilic adhesion, followed by acquisition of receptors that give invasive and migratory properties to neoplastic cells are common mechanisms described in the development of a malignant tumours. These mechanisms result from impairment of cadherins and action of integrins (20). However, this observation is not conclusive, as down-regulation of integrin expression has not been reported as a requirement for malignant growth or invasion of cells in other malignant tumours such as melanomas (21).

In all situations of cell culture used in our study we observed that PA cells expressed more integrin subunits than ACC cells. This is in accordance with other authors who showed an inverse relationship between integrin expression and malignancy (22). Yet, decreased expression of integrins has been correlated with dedifferentiation and tumour progression in the mammary gland. For example, a loss of $\alpha2\beta1$ expression has been linked with a less differentiated phenotype of murine mammary glands (23). It is important to highlight the discrete expression of $\alpha2$ integrin in ACC cells. It is known that tumour progression and cell transformation are associated with modification in the expression/function of integrins. Loss of $\alpha2$ and reduction of $\alpha3$ subunits has been reported in transformed salivary gland cells and in metastatic clones (22).

Independent of the matrix protein present, all integrin subunits were expressed in the cells, but different patterns and intensity were observed in each situation. This redundancy in integrin expression *in vitro*, may occur due to the involvement of integrins in other biological functions, such as signalling through their cytoplasmic tails as well as a compensatory mechanism of integrin expression, which regulate proliferation and cell differentiation (24). Integrin signalling is not well-understood, but it is closely related with individual ligands interaction, inasmuch as conformational changes lead to different cell events (24, 25). For example, it is known that binding to fibronectin or to collagen via integrins $\alpha5\beta1$ or $\alpha2\beta1$, respectively, stimulates cell cycle progression, decreasing the growth factor requirement for DNA synthesis up to 1000-fold (26). On the contrary, unoccupied integrin receptors will perform a reverse function, and, in this way, transformed cells derived from malignant neoplasms, will show decreased level of integrins, which might facilitate their motility and proliferation through other mechanisms such as growth factors stimulation (27, 28).

Finally, our results showed a marked decrease or absence of integrin $\beta1$ on ACC cells, compared with PA cells. This deserves a special consideration, as $\beta1$ integrin pairing with α subunits is necessary to form an active integrin receptor (29, 30).

Based on the present work, we are carrying out functional studies in order to understand the roles of each integrin subunit in these tumours.

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Acknowledgements

Authors thank FAPESP grants 03/02676-7 and 03/00450-4 for the financial support.