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Immunophenotypical alterations with impact on the epithelial–mesenchymal transition (EMT) process in salivary gland adenoid cystic carcinomas

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Abstract

Adenoid cystic carcinoma (ACC) is one of the most common malignant salivary glands neoplasms with an indolent clinical course, slow-growing but locally aggressive and quite often with delayed recurrence and distant metastasis. In order to elucidate this tumoral behavior, we conducted an immunohistochemical study investigating the alterations of epithelial phenotype with anti-cytokeratin (CK) AE1/AE3 and anti-E-cadherin antibodies, and the acquisition of mesenchymal phenotype with vimentin, fibronectin, N-cadherin and P-cadherin in salivary ACCs. Thus, we recorded a reduction of CK AE1/AE3, E-cadherin, P-cadherin and fibronectin reactivity in the solid variant and especially in the cells from the periphery of invasive neoplastic proliferations, regardless histological type. These phenotypical alterations suggest the involvement of the epithelial–mesenchymal transition (EMT) process in the progression of salivary ACCs.

Keywords: adenoid cystic carcinoma, epithelial–mesenchymal transition, immunohistochemistry, invasiveness, salivary gland.

Introduction

Adenoid cystic carcinoma (ACC) is one of the most common malignant salivary glands neoplasms with an indolent clinical course, slow-growing but locally aggressive and quite often with delayed recurrence and distant metastasis [1]. According to the literature, the local recurrences rate varies from 10.3% to 74% [2–4], with a 65% recurrence rate at five years after surgery [5]. Recently, for distant metastasis was observed a tendency to become more common than regional recurrences [6], with values ranging from 17.2% to 68.5% [2–4]. Although the prognosis of such tumors seems to be good with a five-year overall survival in patients with ACC without recurrences ranging from 62.9% [7] to 100% [5], these results dropped to 56.2% for those cases with recurrences [7], and even to 46% in those cases with distant metastasis [8]. In order to improve the prognosis of these patients, in-depth studies on tumor progression are required with special attention to the epithelial–mesenchymal transition (EMT) process. It is well known that EMT process plays a major role in the epithelial tumor invasion and metastasis [9, 10]. The key event of the EMT process consists in loss of E-cadherin and increase of N-cadherin expression. This cadherin switch is followed by acquisition of a mesenchymal morphology, which confers tumor cells the ability of migration and invasion, and thus metastatic properties [9]. Few and relatively recent studies proved that EMT process is involved in tumor progression of salivary gland ACC [11–14]. Thus, we investigated by immunohistochemistry this process by highlighting the alterations of epithelial phenotype with anti-cytokeratin (CK) AE1/AE3 and anti-E-cadherin antibodies and the acquisition of mesenchymal phenotype with vimentin, fibronectin, N-cadherin and P-cadherin in salivary ACCs.

Materials and Methods

In this study were include 32 patients diagnosed with salivary gland ACC, between 2010 and 2019, in the Department of Oral and Maxillo-Facial Surgery from Emergency County Hospital, Craiova, Romania. For immunohistochemistry, we used the paraffin blocks archived in the Laboratory of Pathology of the same Hospital. From these, 4 μm thick seriate sections were cut, which were further dewaxed, clarified and hydrated. Then, endogenous peroxidase was blocked with hydrogen peroxide (0.3%) for 15 minutes, at room temperature, and as antigen retrieval, we used microwaving for 20 minutes in 0.1 M citrate buffer of pH 6 (with the exception of fibronectin for which we used enzymatic retrieval with proteinase K, for 15 minutes, at 37°C). To avoid the nonspecific binding, the slides were covered with 2% bovine serum albumin (BSA) for one hour, at room temperature. Subsequently, the slides were incubated overnight, at 4ºC, with the primary antibodies whose characteristics are presented in Table 1. Then, we used an amplification based on labeled Streptavidin–Biotin 2 (LSAB2) enzyme detection system and the correspondent Dako kit (Redox, Romania – K0675).

As chromogen, we used the 3,3'-Diaminobenzidine

(DAB, Dako, K3468) and the Mayer's Hematoxylin kit (Tunic, Bio-Optica, Romania – M06002) for counter-staining. As negative internal controls, we used the same slides and procedures, but omitting the primary antibody.

Table 1 – ***The antibodies and immunostaining protocol***

Antibody	Clone / Producer, Catalog No.	Dilution	Antigen retrieval	External positive control
CK AE1/AE3	Cocktail of two mouse monoclonal antibodies AE1 and AE3 / Novocastra, PA0909	RTU	0.1 M Citrate, pH 6	Skin
E-cadherin	Mouse monoclonal – 36B5 / Novocastra, PA0387	RTU	0.1 M Citrate, pH 6	Skin
Vimentin	Mouse monoclonal – V9 / Novocastra, PA0640	RTU	0.1 M Citrate, pH 6	Skin
Fibronectin	Rabbit polyclonal / Dako, A024502-2	1:400	Proteinase K	Kidney
N-cadherin	Mouse monoclonal – IAR06/ Novocastra, NCL-L-N-Cad	1:50	0.1 M Citrate, pH 6	Testis
P-cadherin	Rabbit polyclonal / Sigma-Aldrich, HPA001767	1:100	0.1 M Citrate, pH 6	Placenta

CK: Cytokeratin; RTU: Ready-to-use.

The slides were examined by two authors (BIC and MC) and the reactions were evaluated using the immuno-reactivity score (IRS) given by Remmele & Stegner [15]. According to this score, the slides were assessed with values between 1 and 12.

For statistical analysis, we utilized the Statistical Package for the Social Sciences (SPSS) 10 software package. In order to assess the association between different categorical classes in this study, data was organized in contingency tables, and a χ^2 (*chi*-square) test was utilized, with $p<0.05$ being considered statistically significant. The analysis of variance (ANOVA) test was used for comparisons of more than two groups of continuous data variables.

Results

Patients included in this study had a median age of 57.5 years, men being more common affected then women (with about 1.46:1 gender ratio). In half of the cases, tumors developed from major salivary gland with parotid as the most common affected (28.12%). In the other half of the cases, the minor salivary gland from hard palate mucosa was more commonly involved (18.75%). The solid variant was the most encountered morphological subtype (46.88%), followed by the tubular variant (34.37%) and with cribriform variant as the least observed subtype (18.75%). More than two thirds of the cases had peri-neural invasion (71.87%) and almost a fifth of cases have been diagnosed with lymph nodes metastases. Most of patients presented at the stage II – pTNM (71.87%), and 40.62% of them had positive surgical margins.

In the residual salivary gland parenchyma was noticed an intense and diffuse cytoplasmic CK AE1/AE3 reaction. This reactivity was more obvious at the excretory salivary gland structures both in luminal and abluminal cells. The CK AE1/AE3 reactivity was also observed in acinar cells, especially in the serous type and with much lower intensity in the mucous type. Moreover, reactivity was also noticed even in the oral epithelium adjacent to the salivary glands, and for those tumors developed in lips, reactions were present even in epidermis, hair follicles and sweat glands.

Regarding salivary ACC reactivity, we noticed a cytoplasmic positive reaction in the tumoral cells but with a certain degree of heterogeneity dictated mainly by tumor histological subtype. The median IRS for the all casuistry was 8.5±3.255, with nominal values ranging between 1 and 12. The maximum reactivity was recorded in the solid histological ACC subtype (IRS 9±1.667). The reaction pattern was generally intense and diffuses, but focally in the centre of large tumoral islands the intensity was obviously lower (Figure 1A). At the cellular level, the reactivity was more obvious at the luminal cells that outline the few remaining lumens from the tumor islands with prevalent intensity at the apical and lateral borders (Figure 1B). An intermediate reactivity, we recorded in those cases with the tubular ACC subtype (IRS 8±1.293) (Figure 1C). In these cases, the CK AE1/AE3 reactivity was more obvious in the luminal cells, with a prevalent enodomembranous cytoplasmic pattern, especially at the luminal border (Figure 1D). The reactivity was reducing in the abluminal cells, and even absent in these cells from the invasive tubular neoplastic proliferations (Figure 1E). This reactivity pattern was also recorded at the neoplastic proliferations, which surround or infiltrate the nerve fibers. The lowest reactivity was recorded in the cribriform ACC subtype (IRS 2±0.983), which was obvious only at the level of the tubular proliferations present in this type of ACC (Figure 1F). The intensity of the reaction was greatly reduced at the invasion front.

In the normal residual salivary parenchyma, the E-cadherin reactivity was present both in acinar cells with membranous pattern, that was more obvious in the serous type and also in excretory salivary ducts, where the reaction pattern was both cytoplasmic and membranous. Moreover, in the specimens with oral mucosa topography, E-cadherin reactivity was also noticed at the level of the oral epithelium, with membranous pattern, especially at the level of cells from the intermediate layer. The salivary ACC specimens proved a heterogeneous E-cadherin reactivity with both cytoplasmic and membranous patterns regardless of the tumoral histological subtype. Overall, the tumoral E-cadherin reactivity (IRS 8±1.934) was slightly smaller compared to CK AE1/AE3 reactions. We also observed differences in reactivity depending on the tumor histological subtype. Thus, the most reactive specimens were those from the patients with salivary ACC tubular subtype (IRS 9±1.566) (Figure 2A). The subcellular pattern reactivity it was in almost equal proportions both membranous and cytoplasmic. This reactivity was obvious at the luminal cells and less in the abluminal cells (Figure 2B). Next, in what it regarded the E-cadherin reactivity, were those cases with salivary ACC of solid type (IRS 8±1.907) (Figure 2C). The reactivity was heterogeneous, with areas with intense reactivity alternating with weak reactive areas (Figure 2D). The dominant pattern was membranous but

focally a cytoplasmic pattern was also observed. In some areas, the neoplastic cells were negative to E-cadherin immunostaining. The lowest reactivity was for the cases of salivary ACC of cribriform type (IRS 7±1.095) (Figure 2E). In these cases, the reactivity was also heterogeneous, both cytoplasmic and membranous, especially in those neoplastic cells that outlined the lumen of few scattered neoplastic tubes. Some reactivity was also recorded in the neoplastic cells from the thickness of the neoplastic cords (Figure 2F). Moreover, regardless the histological subtype we noticed that in the neoplastic cells, especially in those from the periphery of tumor proliferations at the invasive front and also from those that surround and infiltrate the nerve fibers the intensity of E-cadherin was reduce and the pattern changed from a membranous to a predominantly cytoplasmic one.

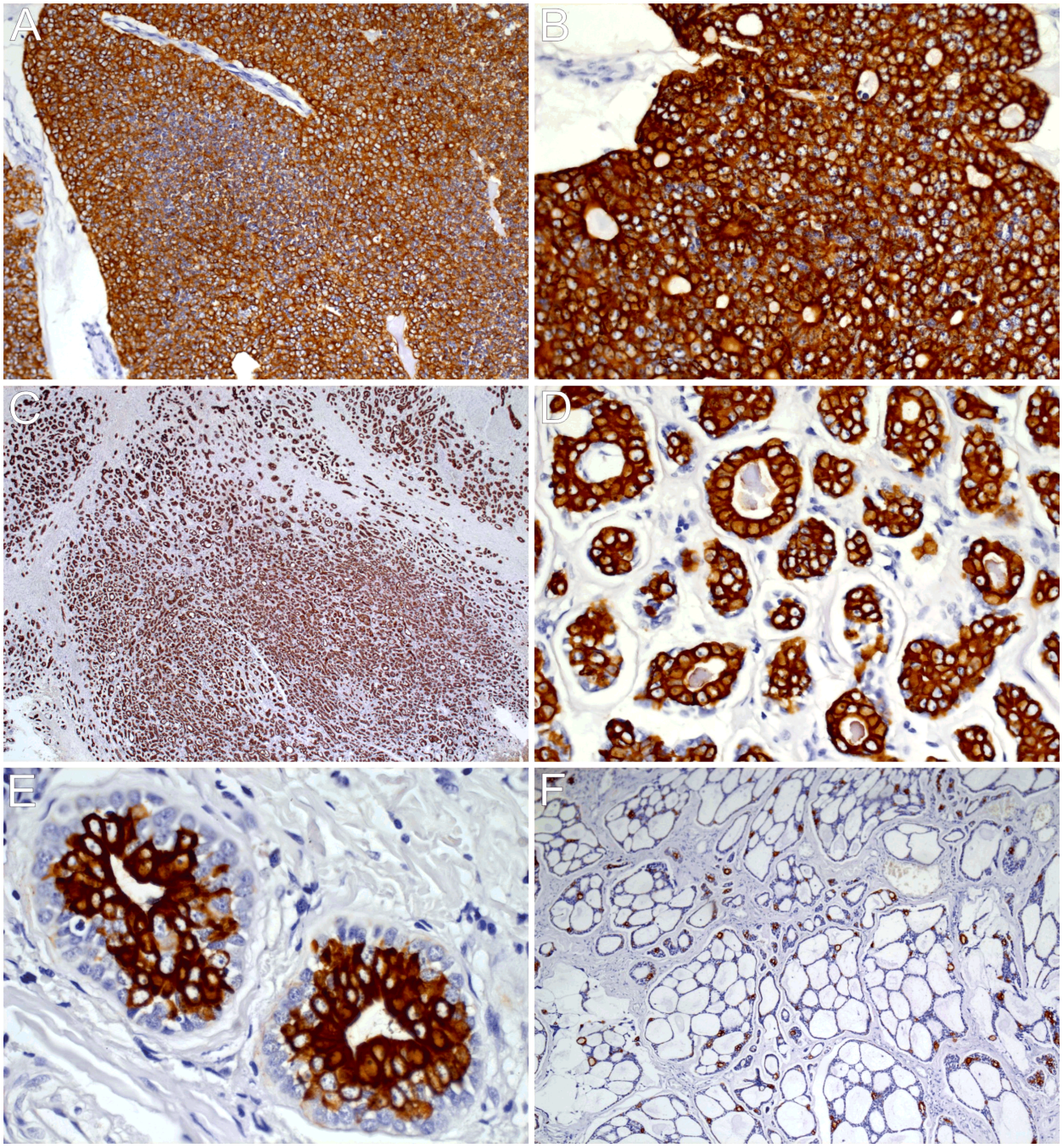

Figure 1 – ***CK AE1/AE3 reactivity in salivary ACC: (A) Solid type with intense and diffuses reactivity, but focally in the centre of large tumoral islands the intensity was obviously lower; (B) The reactivity was more obvious at the luminal cells that outline the few remaining lumens from the tumor islands with prevalent intensity at the apical and lateral borders; (C) An intermediate reactivity was recorded in the case of salivary ACC with tubular pattern; (D) In the tubular type, the reaction was more obvious in the luminal cells, with a prevalent enodomembranous cytoplasmic pattern, especially at the luminal border; (E) Also, we noticed a reduce CK AE1/AE3 reactivity in the abluminal cells, and even absent in these cells from the invasive tubular neoplastic proliferations; (F) The lowest reactivity was recorded in the cribriform ACC subtype, which was obvious only at the level of the tubular proliferations. Anti-CK AE1/AE3 antibody immunomarking: (A) ×200; (B) ×400; (C) ×25; (D and E) ×600; (F) ×100. CK: Cytokeratin; ACC: Adenoid cystic carcinoma.***

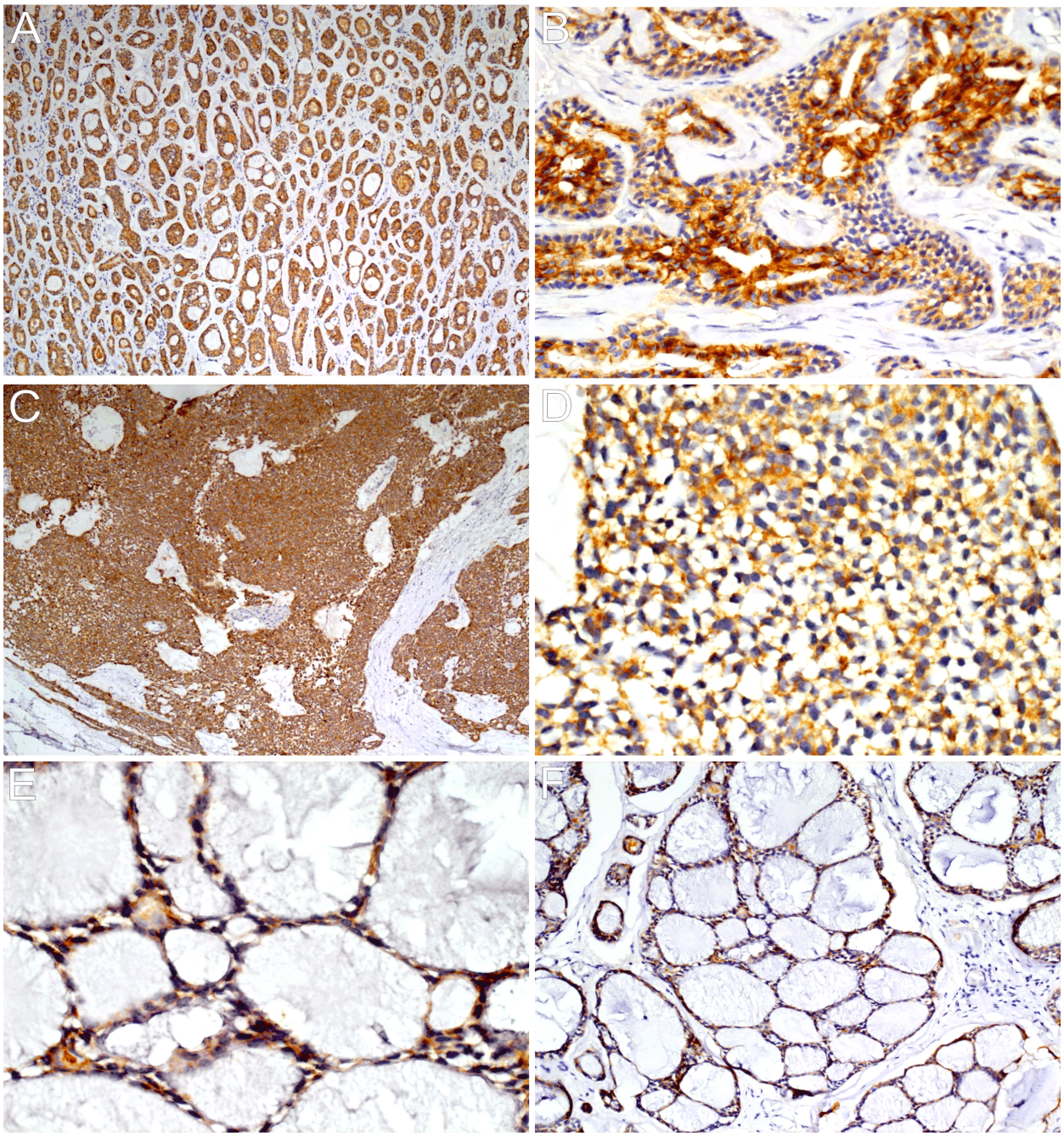

Figure 2 – ***E-cadherin reactivity in salivary ACC: (A) The most reactive specimens were those from the patients with salivary ACC tubular subtype; (B) The subcellular pattern reactivity it was in almost equal proportions both membranous and cytoplasmic; (C) An intermediate reactivity was recorded in salivary ACC cases of solid type; (D) The reactivity was heterogeneous with areas with intense reactivity alternating with weak reactive areas; (E) The lowest reactivity was observed in the cases of salivary ACC of cribriform type; (F) The reactivity was most obvious in the neoplastic cells from the thickness of the neoplastic cords. Anti-E-cadherin antibody immunomarking: (A, C and E) ×100; (B) ×400; (D and F) ×600. ACC: Adenoid cystic carcinoma.***

The residual salivary parenchyma from the tumoral specimens presented positive vimentin reaction at the level of myoepithelial cells, endothelial blood cells, nerve fibers and stromal and inflammatory cells. Overall, the median reactivity for vimentin (median IRS 8±3.614) in tumor samples was around the values obtained for E-cadherin reactivity with the highest values in the solid ACC type (median IRS 9±1.569), followed by the cribriform (median IRS 7±1.966) and finally the tubular type (median IRS 3±1.549). In the solid ACC variant, the reactivity was heterogeneous with poorly reactive and even negative areas alternating with highly reactive areas (Figure 3A). The pattern reaction was cytoplasmic with more intensity near the membrane (Figure 3B). Inside the solid tumor proliferations, the reaction was lower and even absent, but it was always present in the neoplastic cells from the periphery. Regardless of the histological type, the vimentin reaction was more obvious at the invasion front, in the neoplastic proliferations that surround or infiltrate the nerve fibers and at the level of neoplastic emboli (Figure 3, C and D). In the cribriform pattern, the vimentin reactivity was present in the neoplastic cells that outlined the cribriform spaces and from the periphery of tumor islands and less in outer cell layers, between the cribriform spaces

(Figure 3E). In the tubular ACC type, the vimentin reactivity was recorded in the cytoplasm from the non-luminal cell layers and was absent in the luminal cells (Figure 3F), with the exception of those cells composing the neoplastic proliferation of the invasion front, namely those around and that infiltrate the nerve fibers.

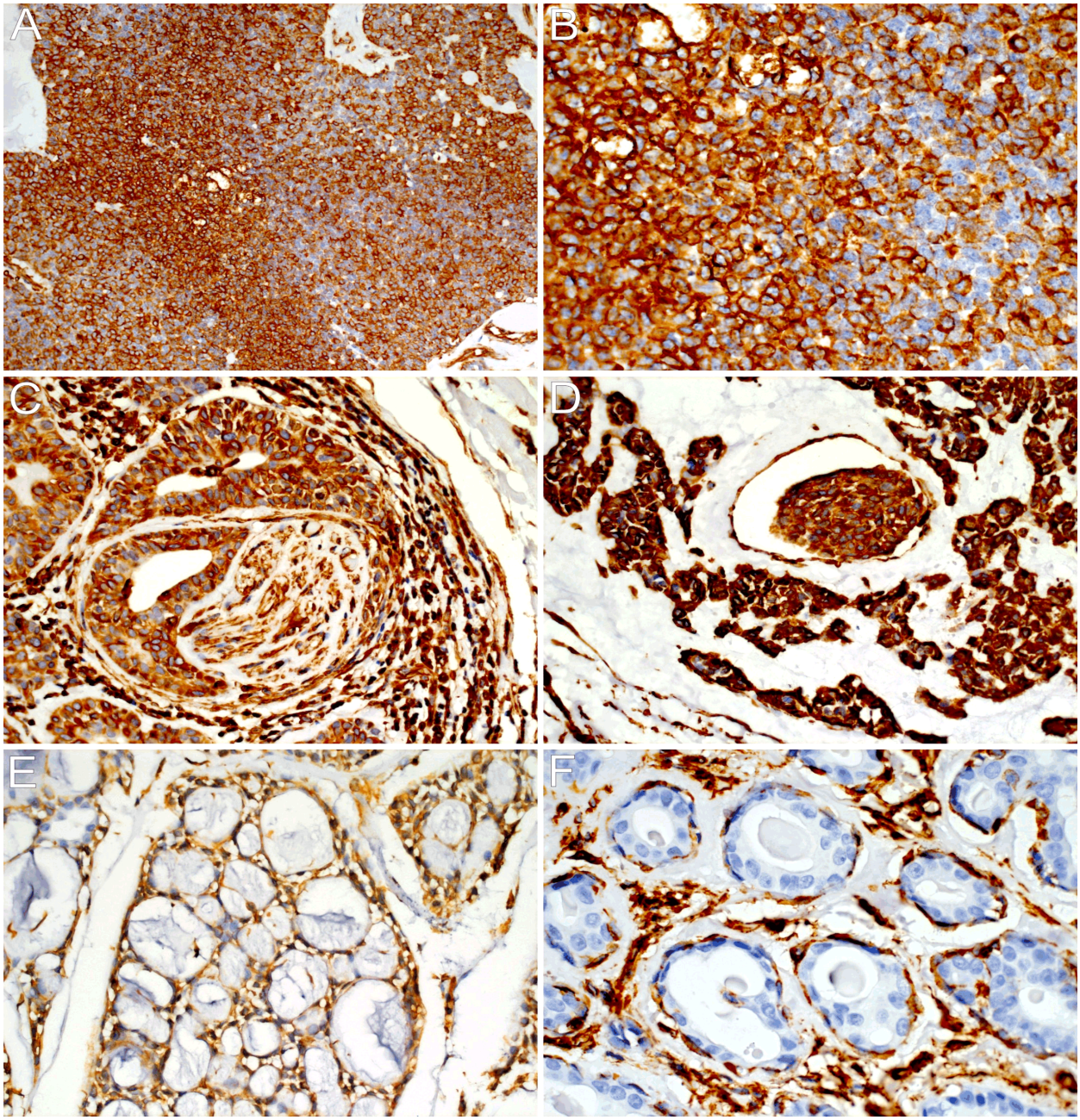

Figure 3 – ***Vimentin reactivity in salivary ACC: (A) In the solid ACC variant, the reactivity was heterogeneous with poorly reactive and even negative areas alternating with intensively reactive areas; (B) The pattern reaction was cytoplasmic with more intensity near the membrane; (C) Regardless of the histological type, the vimentin reaction was more obvious in the neoplastic proliferations that surround or infiltrate the nerve fibers; (D) An intense reaction was also recorded in the neoplastic emboli; (E) In the cribriform pattern, the vimentin reactivity was present in the neoplastic cells that outlined the cribriform spaces and from the periphery of tumor islands; (F) In the tubular ACC type, the vimentin reactivity was recorded in the cytoplasm from the non-luminal cell layers and was absent in the luminal cells. Anti-vimentin antibody immunomarking: (A) ×200; (B and F) ×600; (C–E) ×400. ACC: Adenoid cystic carcinoma.***

At the level of the residual salivary parenchyma, the fibronectin reactivity was recorded in the acinar cells, especially the serous type with cytoplasmic and membranous pattern, with weak to moderate intensity. Also, a low cytoplasmic reactivity and moderate membranous at luminal pole was noticed in the luminal cell of the excretory salivary ducts. In addition, reactivity was observed in adipocytes, endothelial blood cells, fibroblasts and smooth muscle fibers. In tumor specimens, the reactivity was obvious especially in the matrix between neoplastic proliferations and inside pseudocyst and tumor tubular lumina (Figure 4A). The pattern reactivity was filamentous or as homogeneous solid masses of variable sizes and shapes. As intensity, the highest reactivity we recorded in the tubular subtype at the level of the material from the tubular lumina (Figure 4B), followed by the cribriform subtype in the material from the pseudocysts (Figure 4A) and finally in the solid subtype in the matrix between compact neoplastic

proliferations (Figure 4C). Also, we noticed a fibronectin reactivity at the level of basement membranes that surround the neoplastic proliferations from the tubular and cribriform subtype, while in the solid variant the reactivity was either absent, or with irregular pattern at the edges of tumor proliferations. This reactivity was evident in full tumor mass, but was reduce and even absent in the periphery of invasive tumor nests, especially for the solid subtype salivary ACC cases. In addition, at the level of pseudocyst, we also recorded a high intense reactivity at the periphery of these spaces in close contact with neoplastic proliferations similar to basement membrane, which outline these spaces (Figure 4D). Regarding the neoplastic cell reactivity to this marker, the highest immunohistochemical (IHC) score was recorded in the tubular subtype (IRS 4±1.337), followed by the cribriform subtype (IRS 3±1.169) and finally by the solid one (IRS 1±0.703). The neoplastic cell reactivity was observed more obvious in the cytoplasm of that cells that outline the lumina (Figure 4E). We also noticed a high reactivity in those neoplastic proliferations that surround and infiltrate the nerve fibers (Figure 4F).

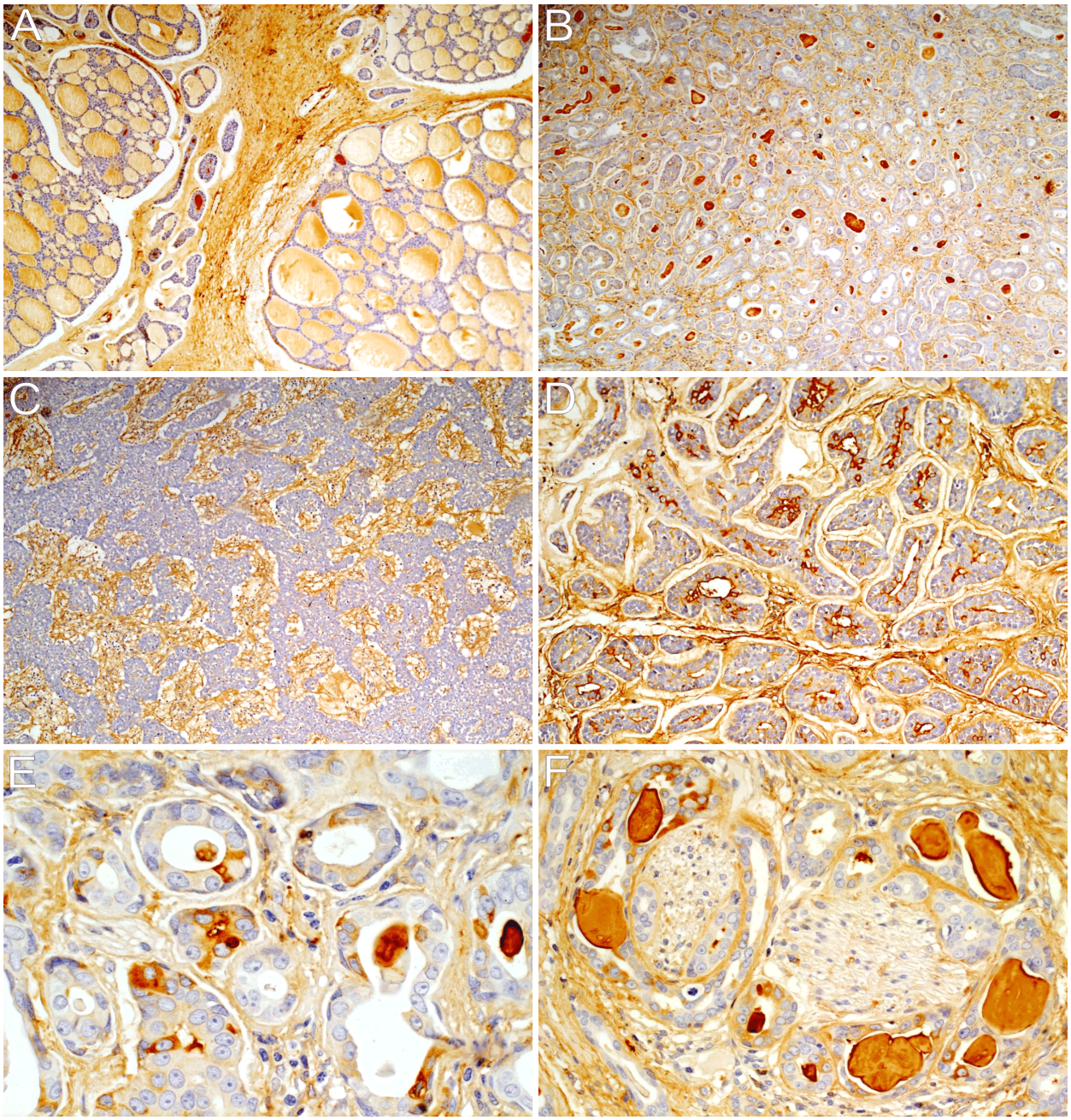

Figure 4 – ***Fibronectin reactivity in salivary ACC: (A) Tumor reactivity especially in the matrix between neoplastic proliferations and inside pseudocyst and tumor tubular lumina; (B) The highest reactivity we recorded in the tubular subtype, at the level of the material from the tubular lumina; (C) In the solid subtype, the reactivity was reduced and present mainly in the matrix between compact neoplastic proliferations; (D) At the level of pseudocyst, we also recorded a high intense reactivity at the periphery of these spaces, in close contact with neoplastic proliferations, similar to basement membranes which outline these spaces; (E) The neoplastic cell reactivity was observed more obvious in the cytoplasm of that cells that outline the lumina; (F) A high reactivity was also recorded in those neoplastic proliferations that surround and infiltrate the nerve fibers. Anti-fibronectin antibody immunomarking: (A–C) ×100; (D) ×200; (E) ×600; (F) ×400. ACC: Adenoid cystic carcinoma.***

In the residual normal salivary gland, we noticed a positive N-cadherin reaction predominant with cytoplasmic pattern in the interlobular salivary ducts. The tumor reactivity was present only in 11 cases, representing about 35% of all investigated salivary ACCs. Regarding the histological subtype, the N-cadherin reactivity was recorded in six cases of solid type, three cases of tubular type and two cases of cribriform variant. The semi-quantitative analysis revealed the following IRS scores: 4±1.214 for the solid type, 3±0.577 for the tubular type, and 2.5±0.707 for the cribriform variant. Regardless histological type, the N-cadherin reaction was more obvious in cases with perineural and lymphovascular invasion, at the invasion front (Figure 5A) and in the neoplastic cells from the periphery of the tumor proliferations (Figure 5B). In the solid variant, we identify the most numerous neoplastic cells positive to N-cadherin, the reactivity being present both inside and at the periphery of tumor proliferation (Figure 5C). The reactivity was more obvious at the invasion front in the small neoplastic islands, especially at their periphery (Figure 5D). In the tubular variant, the reactivity was low and prevailing in the neoplastic luminal cells with a cytoplasmic and membranous pattern (Figure 5E).

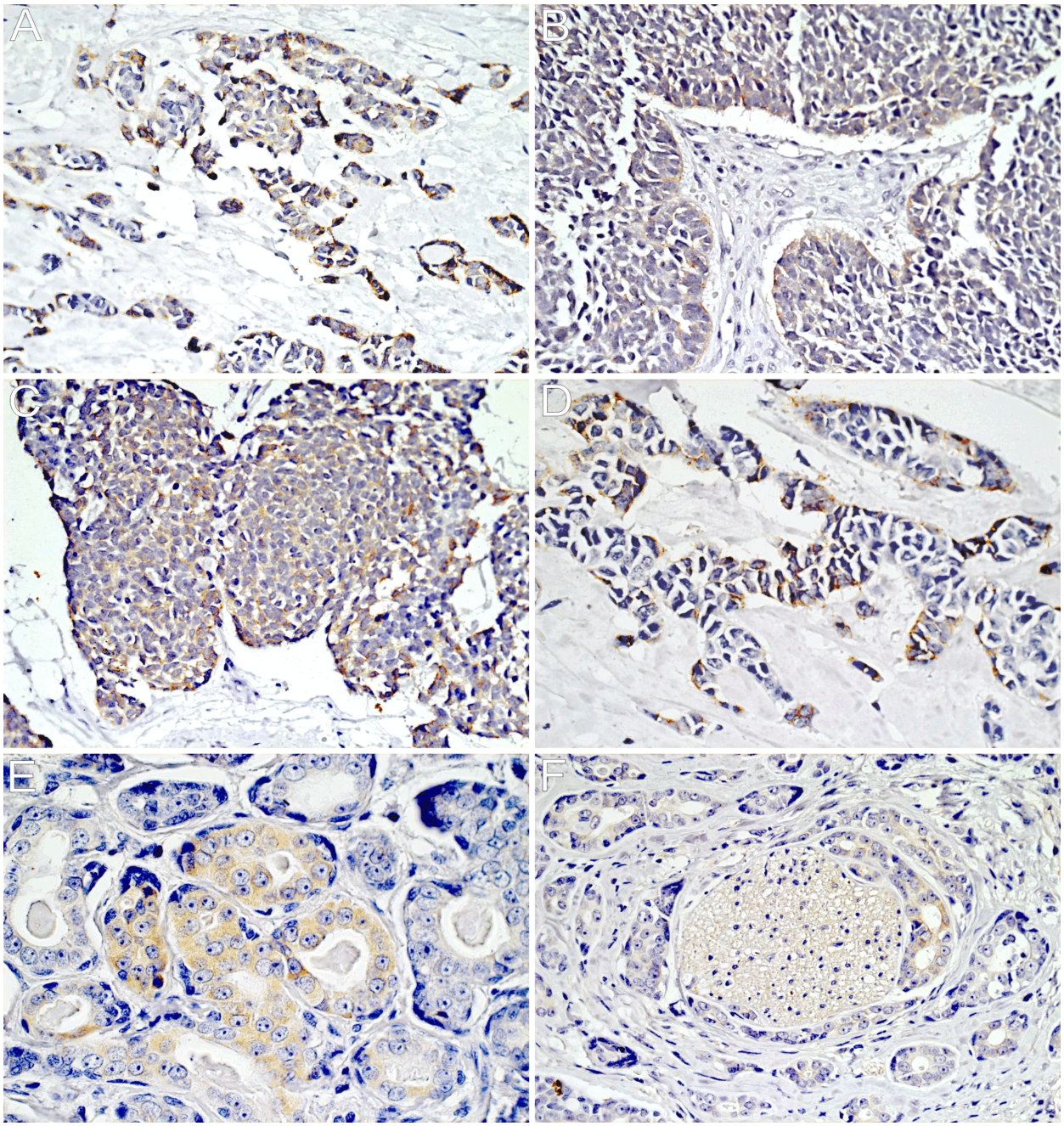

Figure 5 – ***N-cadherin reactivity in salivary ACC: (A) N-cadherin reaction was more obvious at the invasion front; (B) N-cadherin reaction in the neoplastic cells from the periphery of the tumor proliferations; (C) In the solid variant, the reactivity was present both inside and at the periphery of tumor proliferation; (D) The reactivity was more obvious at the invasion front in the small neoplastic islands, especially at their periphery; (E) In the tubular variant, the reactivity was low and prevailing in the neoplastic luminal cells with cytoplasmic and membranous pattern; (F) N-cadherin reactivity was also observed in the neoplastic cells that surround and infiltrate the nerve fibers. Anti-N-cadherin antibody immunomarking: (A–C) ×400; (D and E) ×600; (F) ×400. ACC: Adenoid cystic carcinoma.***

In the cribriform variant, we recorded the lowest reactivity, limited to the tubular neoplastic cells that were present in the composition of these tumors. Regardless the histological subtypes, the N-cadherin reactivity was also observed in the neoplastic cells that surround and infiltrate the nerve fibers (Figure 5F).

In the residual salivary parenchyma, P-cadherin reactivity was observed only in the salivary duct units with the most obvious reaction in the interlobular ducts. The pattern reaction was both membranous and cytoplasmic. In tumor specimens, we recorded the highest IRS scores in tubular salivary ACC subtype (median IRS 12±1.544), followed by the cribriform subtype (IRS 7±1.505), and finally the solid type with lowest IRS (4±1843) scores. Regardless histological subtype, the reaction pattern was both membranous and cytoplasmic, with small differences between these and inside *versus* invasion front. In the tubular subtype, P-cadherin was positive both in the luminal and abluminal cells, but with more intensity and membranous predominance for the luminal cells (Figure 6A). In addition, the reactivity decreased in the invasion front (Figure 6B). In the cribriform subtype, the reaction was heterogeneous, with more intensity inside the tumor compared with the invasion front. The membranous reaction was prevalent at the level of the cells that outline the pseudocystic spaces at the cellular side that borders these spaces (Figure 6C).

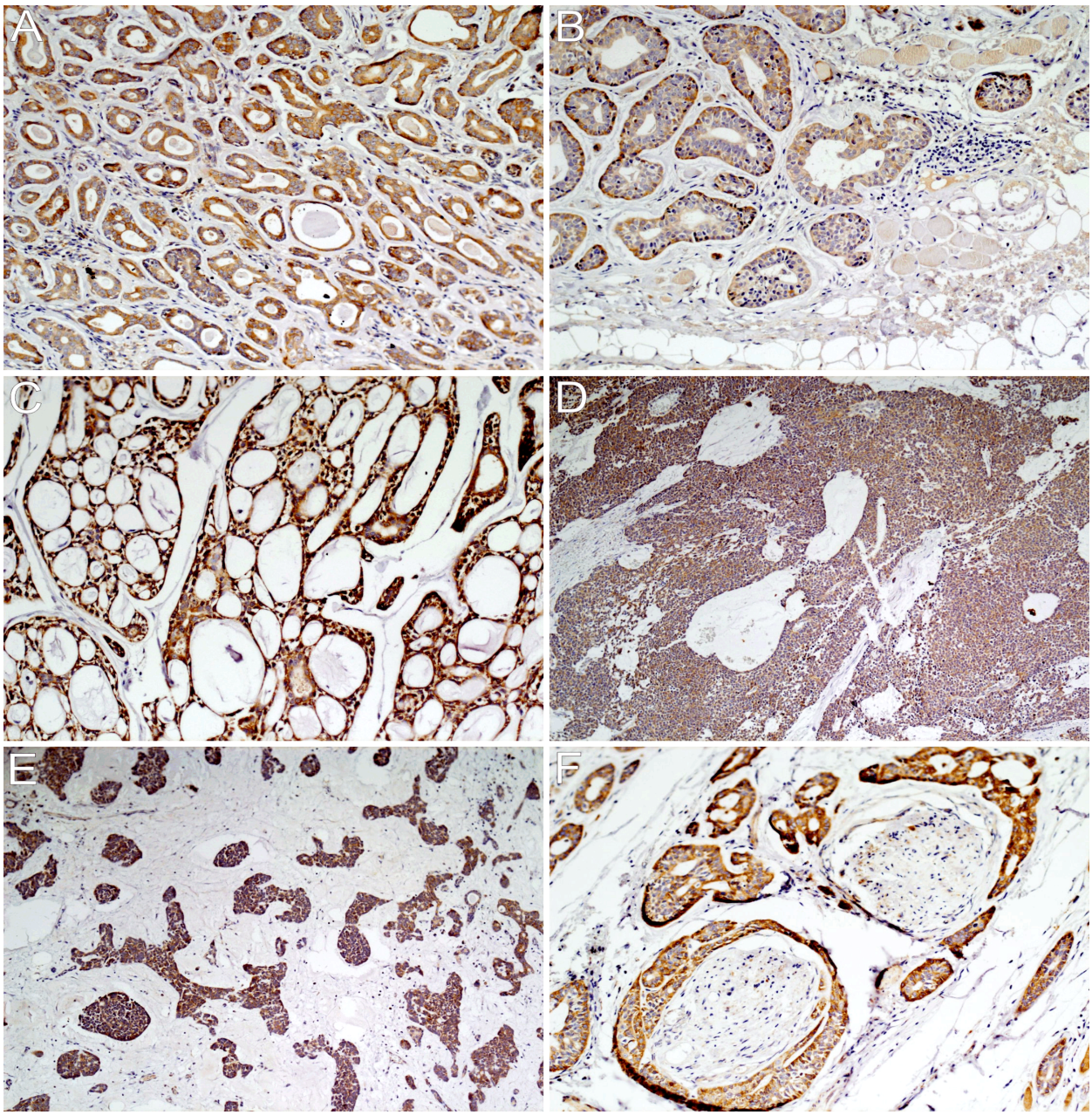

Figure 6 – ***P-cadherin reactivity in salivary ACC: (A) In the tubular subtype, P-cadherin was positive both in the luminal and abluminal cells, but with more intensity and membranous predominance for the luminal cells; (B) A decreased reactivity was recorded at the invasion front; (C) In the cribriform subtype, the membranous reaction was prevalent at the level of the cells that outline the pseudocystic spaces; (D) In the solid variant, some tumor islands were negative or with reduced P-cadherin reactivity; (E) A P-cadherin intense reaction was noticed in the small islands or neoplastic trabeculae from solid type; (F) A high P-cadherin reactivity was present in the tubular or cribriform structures that surround or infiltrate the nerve fibers. Anti-P-cadherin antibody immunomarking: (A–C) ×200; (D and E) ×100; (F) ×400. ACC: Adenoid cystic carcinoma.***

In the solid variant, the reactivity was heterogeneous with some tumor islands that were negative or with reduced reactivity (Figure 6D) and with other areas, mainly in small islands or neoplastic trabeculae were the reactivity was obvious (Figure 6E). In these solid neoplastic proliferations, the predominant pattern was the cytoplasmic, present mainly in the tumor cells from inside the proliferations (Figure 6E). In the solid type, at the invasion front was recorded the lowest P-cadherin reactivity. When we investigate the perineural invasion, we had found a positive reaction especially in those tubular or cribriform structures that surround or infiltrate the nerve fibers (Figure 6F). Both subcellular patterns (membranous and cytoplasmic) were present with a high membranous reaction at the cellular side that borders these structures.

Statistically, we did not find significant correlations between most of the morphoclinical variables and the IHC scores. The strongest correlation was noticed between E-cadherin and vimentin scores (r=0.999, p=0.027), proving the existence of the tendency that in invasive cases the decrease of E-cadherin reactivity is followed by the increase of vimentin reactivity (Figure 7A). An inverse correlation was observed between the CK AE1/AE3 and P-cadherin reactivity, but this correlation was weak (r=-0.375, p=0.047) (Figure 7B). Weaker to moderate correlations were also noticed for the CK AE1/AE3 and vimentin (r=0.242, p=0.042), CK AE1/AE3 and fibronectin (r=0.361, p=0.034), and E-cadherin and P-cadherin (r=0.3, p=0.011) IHC scores.

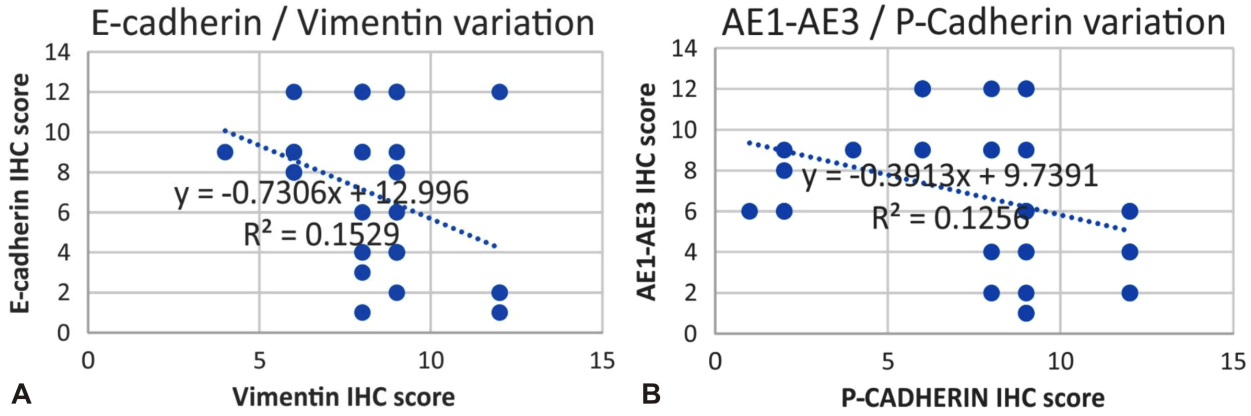

Figure 7 – ***(A) Statistical analysis proving a strong correlation between E-cadherin and vimentin IHC scores (r=0.999, p=0.027). (B) A weak correlation between CK AE1/AE3 and P-cadherin IHC scores (r=-0.375, p=0.047). IHC: Immunohistochemical; CK: Cytokeratin.***

Discussions

ACC is the most frequent malignancy of minor salivary glands, but with an annual global incidence of only 3–4.5 cases per million people [16]. Although this type of tumor is rare, it has an indolent and variable biological behavior, being highly invasive and with a high potential for metastasis along the nerve fibers and through lymphovascular invasion [17]. Thus, patients with such tumors have poor prognosis that has not been improved over the past years mainly due to the lack of powerful biomarkers for prediction of invasion and metastasis [18]. For these reasons are required the development of novel approaches to advance research especially concerning the process of proliferation, migration and metastasis in salivary ACC. The process of salivary ACC metastasis seems to be regulated by the expression and modification of extracellular matrix, which in turn are responsible for the promotion of EMT process in tumor cells [19]. Basically, during this process the epithelial cells is transdifferentiated towards a mesenchymal cell type, by the loss of epithelial markers, such as E-cadherin or some CKs, and also by gain of mesenchymal markers, such as vimentin, fibronectin, N-cadherin or P-cadherin [20]. In view of the foregoing, the aim of our study was to investigate the expression of these representative EMT markers in our salivary ACC casuistry.

In our study, CK AE1/AE3 reactivity was maximum in solid histological ACC subtype, followed by the tubular ACC subtype and finally by the cribriform ACC subtype. Regardless of the histological subtype, the reactivity was more obvious at the luminal cells, and decreased in the abluminal cells and even was absent in the cells from the periphery of invasive neoplastic proliferations. Such an immunophenotype suggests on one hand that the solid type is the most aggressive, and on the other that at the invasion front the neoplastic epithelial cells lose some of their differentiation.

In 2007, Meer & Altini investigating the CKs profile of salivary gland tumors found that in ACC the neoplastic cells were intense and diffuse positive for CK AE1/AE3 similar with the normal major or minor residual parenchyma reactivity [21]. Moreover, the same authors revealed that ACC had a CK7-positive/CK20-negative profile, results that are similar with those recorded by Lee *et al.* [22]. Ben Salha *et al.* (2016) reported in a case of solid ACC a positive and diffuse reaction for CK AE1/AE3 and CK7, but a focal neoplastic cells reactivity for CK5/6 and CAM5.2 [23]. In the paper of Pidorodeski Nagano *et al.* (2016) was recorded an alteration of the CKs profile from the primary ACC to the lung metastatic lesions which, although they did not have prognostic value, may be relevant in determining the primary origin of metastatic lesions [24]. Gao *et al.*, found that presence of CK14-positive cells in the invasive front of salivary ACC promoted collective cell invasion of salivary ACC and may be a significant prognostic marker in these patients [25].

Regarding the E-cadherin reactivity, in our casuistry we observed a reduced reactivity in the solid and cribriform salivary ACC subtypes compared with the tubular type. Also, regardless the histological subtype, the neoplastic cells from the tumor proliferations by the invasive front and those from proliferations that surround and infiltrate the nerve fibers had a reduced E-cadherin reactivity and the subcelullar pattern changed from a membranous to a predominantly cytoplasmic one.

Some studies showed that E-cadherin expression was downregulated in salivary ACC specimens than that from the residual normal adjacent salivary parenchyma and this event seems to be responsible for nerve invasion, lymph node and regional recurrences and distant metastases [26, 27]. Also, Ge *et al.* (2012) recorded some correlation between E-cadherin expression and different histopathological types and T-stage [26]. Moreover, Zhang *et al.* (2000) noticed a correlation between E-cadherin expression and the degree of salivary ACC cell differentiation [28]. More recently, Xie *et al.* (2016) recorded that cadherin 4 (*CDH4*) gene, responsible for retinal cadherin (R-cadherin) encoding, could inhibit the growth and metastasis of salivary ACC *via* co-expression with E-cadherin [29]. Zhao *et al.* (2013) [30], similar to other authors [31, 32], reported that reduced E-cadherin expression in salivary ACC correlated with solid type, advanced stages, perineural invasion, recurrences and distant metastasis. The same authors suggested that integrin linked-kinase (ILK) overexpression may be responsible for inducing of EMT *via* E-cadherin/N-cadherin dysregulation, which would facilitate the invasion and metastasis in salivary ACC. Unlike all of the above, Cavalcante *et al.* (2017) noticed a slightly higher E-cadherin expression in solid salivary ACC [33]. It would seem that this fact would be necessary to maintain the intercellular adhesion at the tumor islands, but the cadherin–catenin complex might not be functioning. Moreover, the authors concluded that E-cadherin expression in salivary ACC and pleomorphic adenoma would be subject to their cellular composition and the degree of differentiation of myoepithelial cells from these two tumors [33].

In our study, the vimentin reactivity was at the maximum intensity in the solid type, followed by the cribriform and finally by the tubular subtype. Regardless histological subtype, the abluminal neoplastic cells were more reactive to vimentin than the luminal neoplastic cells. Also, the vimentin reactivity was more obvious in the neoplastic cells from periphery of tumor proliferations by the invasive front and in those that infiltrate or surround the nerve fibers. Such phenotype suggests a more prominent EMT process in the solid salivary ACC, the mesenchymal phenotype being more evident in the neoplastic cells from the invasive front and in those that infiltrate or surround the nerve fibers.

Caselitz *et al.* (1984) found vimentin reaction in neoplastic cells from salivary ACC, reactivity being observed in all histological subtype, but with more intensity in the outer cell layers compared with the inner cell layers of the tumor sheets [34]. Moreover, the authors reported the co-expression of the vimentin and keratin in neoplastic cells from salivary ACC. In the study conducted by Cavalcante *et al.* (2007) was proved that vimentin was expressed only in 62.5% of cases, while calponin in 100% of cases with a predominantly diffuse pattern [35]. The authors concluded that neoplastic cells from solid salivary ACC may have the ability to differentiate ductal/luminal and myoepithelial cells. Chomette *et al.* (1991) reported the co-expression of vimentin and keratin in the neoplastic myoepithelial and basaloid cells from salivary ACCs, and they suggest that these tumors may develop from progenitor intercalated duct reserve cells [36]. Recently, Xu *et al.* (2019), proved that *MYB* proto-oncogene to *NFIB* (the gene that encodes nuclear factor I B-type) tended to be negatively correlated with *CDH1* [the gene that encodes cadherin-1 (E-cadherin)] and positively correlated with *VIM* (the gene that encodes vimentin) [37]. Moreover, the authors observed that in neoplastic cells from salivary ACC, *MYB* upregulated the EMT-associated markers, including vimentin. Thus, the authors suggested that *MYB* regulate salivary ACC metastasis by promoting the EMT.

Regarding fibronectin salivary ACC reactivity, we noticed an intense extracellular reaction especially in the material secreted in the neoplastic tubular lumina, followed by the reactivity of the material present in the pseudocysts and finally in the matrix that surround and cleave the neoplastic proliferations. Within the tumor mass, we also observed a continuous basement membrane-like reactivity at the periphery of tubular or pseudocystic neoplastic proliferations, which was in contrast with no reactivity or with irregular pattern at the edges of solid tumor proliferations. This reactivity reduces and even disappears in the periphery of invasive tumor nests, especially for the solid subtype salivary ACC cases. Such immunoprofile certify the fibronectin as a key player in process of salivary ACC invasiveness. Moreover, a high reactivity was recorded in those neoplastic proliferations that surround and infiltrate the nerve fibers, suggesting a possible role of this marker in the process of perineural invasion.

Most of the authors revealed a positive fibronectin reaction in all investigated specimens of salivary ACC, most prevalent with filamentous pattern but also as irregular forms both in matrix and inside pseudocyst [38–40]. Wegner *et al.* (2007) found a lower fibronectin expression in the tubular variant of salivary ACC compared with two other types solid and cribriform [40]. Also, the authors speculated that this expression might have prognostic role. However, other two studies revealed that fibronectin expression was lower in the solid salivary ACC compared with the non-solid types [41, 42]. In the non-solid type, the fibronectin expression was recorded mainly in the basement membrane, while in the solid type ACC, the expression was either absent, or with irregular pattern at the margins of tumor proliferations, as a reminiscent of incomplete basement membranes [41]. The same authors found that the BTBD7 (a spliced variants of BTBD, containing binding sites for notable transcription factors) expression were significantly associated tumor type and metastasis in patients with salivary ACC, while the fibronectin expression was significantly associated with low tumor stages, non-solid type and non-metastatic ACC cases. Thus, they concluded that in salivary ACCs, between BTBD7 and fibronectin exist an inverse relationship and the BTBD7 may have prognostic role for invasion and metastasis in such salivary malignancies [41]. On the other hand, Shintani *et al.* (1997) reported that in the central areas of salivary ACC, fibronectin was positive as a continuous band-like pattern along cell boundaries at the

periphery of tumor islands, but no reactivity was observed in the periphery of invasive tumor nests [43]. In the study conducted by Toyoshima *et al.* (1999) was proved that fibronectin isolated from the ACC3 cell culture may act as a substrate for attachment of these neoplastic cells, or that ACC3 cells trap and retain fibronectin in their pericellular space [44]. Thus, the authors suggested that this isoform of fibronectin play a key role in the ACC invasiveness and in the stromal pseudocyst formation that are peculiar to the cribriform ACC variant.

In our study, the N-cadherin reactivity was present in only 35%, but generally with low intensity and with cytoplasmic pattern regardless histological subtypes. As number of positive cases, the most numerous reactive cases were from the solid subtype, followed by the tubular and finally the cribriform variant. Also, we recorded a high reactivity in those cases with perineural and lymphovascular invasion, at the invasion front and in the neoplastic cells from the periphery of the tumor proliferations. As subcellular pattern, we observed the presence of both membranous and cytoplasmic but with the prevalence of the latter. In addition, regardless histological subtypes, the N-cadherin reactivity was also observed in the neoplastic cells that surround and infiltrate the nerve fibers.

No N-cadherin expression was recorded in the normal salivary gland, with the exception of the neural tissue, endothelial and smooth muscle cells of vessels and mast cells in the stroma [45]. Six of the 14 investigated cases of salivary ACC were positive to N-cadherin with membranous pattern and different intensity. There was no difference between the three histological ACC subtypes and peculiar the reaction was more intense in those neoplastic cells that invade the neural tissue and vessels. Zhao *et al.* (2013) found positive N-cadherin reaction in 68.1% of the investigated cases of salivary ACC [30]. The pattern reaction was membranous and/or cytoplasmic, with no difference between the three histological subtypes of salivary ACC. The strongest expression was recorded in those cases associated with perineural invasion. Also, the authors showed that N-cadherin expression was inversely correlated with E-cadherin expression and positive associated with the expression of Snail and ILK [30]. Having in mind that other authors have proved that ILK overexpression transforms epithelial cells into mesenchymal cells through down-regulation of E-cadherin or up-regulation of N-cadherin, Zhao *et al.* suggested that ILK overexpression may induce EMT by dysregulation of the expression of these two cadherins, and thus facilitating the invasion and metastasis of salivary ACC. Also, Jiang *et al.* (2015) noticed that specimens of salivary ACC cases with metastasis had high expression of thioredoxin 1 (TXN) and thioredoxin reductase 1 (TXNRD1), which were correlated with high expression of the N-cadherin and low expression of the E-cadherin [46]. Thus, the authors concluded that TXN overexpression promote an EMT-like phenotype, facilitating cell migration and invasion, and consequently the process of metastasis from salivary ACC.

In our study, the P-cadherin reactivity was more obvious in the tubular and cribriform subtypes with a predominant membranous pattern. The most reactive neoplastic cells were the luminal cells and the cells that outline the pseudocystic spaces. In the solid variant, we recorded the lowest P-cadherin, which was present mainly at the level of neoplastic cells from inside neoplastic proliferations and the dominant subcellular patterns was the cytoplasmic one. Regardless histological subtype, the lowest P-cadherin reactivity was recorded at the invasion front. In addition, we found P-cadherin reactivity at the level of tubular or cribriform neoplastic proliferations that surround or infiltrate the nerve fibers.

So far, no study has been done on the P-cadherin expression both in normal human salivary gland or their tumors. In one study, conducted by Amano *et al.* (2011), among other things was investigated the expression of P-cadherin in salivary gland epithelial cells of klotho (kl)-deficient mice [47]. The authors found that granular ducts were filled with P-cadherin, and the amount of this cadherin was larger in the wild type mouse submandibular glands than in the sublingual and parotid glands of wild-type mouse, and in the submandibular glands of kl/kl mouse. Thus, the authors concluded that granular duct could by an organ that secretes soluble P-cadherin into the saliva. However, in one study that investigated the P-cadherin expression in the oral squamous cell carcinoma was noticed that only 55.2% of cases were positive to this marker with membranous/cytoplasmic pattern [48]. P-cadherin overexpression was observed only in well-differentiated tumors, while in the poor differentiated cases, the reaction was reduced or absent and in the positive cases, the pattern reaction was predominant cytoplasmic. No statistical differences were recorded between P-cadherin expression and other variables, but its expression was correlated with prognosis (positive cases with membranous pattern have a good prognosis) and the authors suggested that this marker could be an early marker of poor prognosis in oral squamous cell carcinomas. It was showed, in some tumor models, including oral squamous cell carcinoma that P-cadherin acts as a tumor suppressor, since its absence is associated with a more aggressive cancer cell phenotype [49–51]. Some others recorded an underexpression of P-cadherin in high-grade oral squamous cell carcinomas and patients with reduced or no P-cadherin membranous expression had poorer overall and disease-free survival rates than the group of patients with P-cadherin-expressing tumors [52, 53].

All this data suggest the key role played by EMT in the acquisition of an invasive and metastatic potential by the salivary ACC [54]. This fact can lead to the development of targeted therapeutic approaches in patients with salivary ACC with the possibility of increasing their survival rate.

Conclusions

Our investigation recorded a reduction of CK AE1/AE3, E-cadherin, P-cadherin and fibronectin reactivity in the solid variant and especially in the cells from the periphery of invasive neoplastic proliferations, regardless of the histological type. A high expression of vimentin and N-cadherin was observed in the neoplastic cells from the invasive front and in those that infiltrate or surround the nerve fibers. Such immunophenotypical changes suggest the existence of an active EMT process in the progression of salivary ACCs with the solid variant as the most invasive histological type. In addition, we noticed that regardless histological type these alterations were more obvious in those cases with locoregional invasiveness and with lymphovascular and perineural invasion.

Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper. All authors read and approved the final manuscript.

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