

Acinic cell carcinoma of the salivary glands: an immunohistochemical study of angiogenesis in 12 cases

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Abstract

Acinic cell carcinoma (ACC) is the third most common epithelial malignancy of the salivary glands in adults, exhibiting a low-grade malignancy that mainly occurs in the parotid gland and at a relatively younger age than other salivary gland tumors. We performed an immunohistochemically study regarding angiogenesis in ACC, by assessing the CD105+ tumor microvessels density and investigating the VEGF and its receptors VEGFR1 and VEGFR2 expression in tumor samples. The results indicated an active angiogenesis in ACC, with the highest CD105-MVD score recorded in the solid variant. This fact was supported by the reactivity of tumor cells and endothelial blood vessel cells for VEGF and its receptors (VEGFR1 and VEGFR2). Thus, we concluded that in ACC do exist autocrine and paracrine VEGF loops implicated in growth and progression of this kind of salivary gland tumors.

Keywords: acinic cell carcinoma, angiogenesis, CD105, VEGF, VEGFR1, VEGFR2.

Introduction

Acinic cell carcinoma (ACC) is a rare malignant epithelial tumor that accounts for about 1–6% of all salivary gland neoplasms [1]. It is a low-grade malignancy that occurs most often in the parotid gland with a predilection for females and presents at a relatively younger age than other salivary gland tumors [2]. Despite of low malignant behavior, ACC has a tendency to recur, to produce metastases (cervical lymph nodes and lungs), and may have an aggressive evolution with death rates ranging from 1.3% to 26% [2–5].

Angiogenesis represents neovascularisation derived from the pre-existing vasculature [6] and seems to play a crucial role in tumor growth and metastasis [7]. One of the most used methods to assess tumor angiogenesis is the "hot spot" method for counting microvessels density (MVD) introduced in 1991 by Weidner N *et al.* for breast cancer [8]. Since then, this method has been widely used for quantifying the neovascularisation in many human cancers, including salivary gland tumors [9–13]. Unlike pan-endothelial cell markers (*e.g.*, factor VIII-related antigen, CD31 or CD34), the CD105 is a specific marker for activated endothelial cells, being more specific for tumor angiogenesis [11, 13, 14].

Among the angiogenic cytokines involved in the angiogenic switch occurring in salivary gland tumors,

vascular endothelial growth factor (VEGF) is the principal factor involved. There are some reports of correlations between clinicopathological factors and VEGF in such tumors [15–17].

The biological activity of VEGF is dependent on its reaction with specific receptors, especially with VEGFR1 and VEGFR2. However, literature data concerning expression of these receptors in salivary gland tumors are few and limited [18–20].

Therefore, our study has aimed to investigate the angiogenesis in ACCs by assessing CD105-MVD, the expression of VEGF and its receptors VEGFR1 and VEGFR2, and to establish correlations between these angiogenic parameters and the major clinicopathological variables of these patients.

Materials and Methods

We reviewed medical records from the Laboratory of Pathology, Emergency County Hospital of Craiova, Romania, and identified those patients who had been operated for salivary ACC from 2000 through 2011. As clinical data, we noted each patient's sex, age and the site of the tumor and as pathological parameters, we look for histologic patterns, involvement at the surgical margins, presence of perineural and/or vascular invasion and pTNM (Table 1).

Table 1 – The major clinicopathological features and angiogenesis quantification results of the investigated ACC

No.	Age [years]	Gender	Site	Histological pattern	Surgical margins	CD105-MVD	VEGF scoring	VEGFR1 scoring	VEGFR2 scoring	pTNM
1.	43	F	Parotid	Solid	-	28±5	1	1	0	I (T1N0M0)
2.	47	F	Parotid	Solid	-	30.6±6.063	2	1	1	II (T2N0M0)
3.	52	F	Parotid	Microcystic	-	14.2±4.186	2	1	1	II (T2N0M0)
4.	32	M	Parotid	Papillary cystic	+	19.8±2.774	1	1	0	I (T1N0M0)
5.	55	F	Parotid	Mixed	-	17.4±4.774	2	1	0	II (T2N0M0)
6.	44	F	Parotid	Solid	-	31.2±3.492	2	2	1	III (T2N1M0)
7.	49	M	Buccal mucosa	Mixed	-	11.2±3.346	1	1	0	I (T1N0M0)
8.	54	M	Upper lip	Mixed	-	12±2.915	1	1	0	I (T1N0M0)
9.	28	F	Parotid	Papillary cystic	-	20.6±3.049	2	2	1	II (T2N0M0)
10.	59	M	Parotid	Solid	-	33.6±4.878	2	1	0	III (T2N1M0)
11.	63	M	Parotid	Mixed	-	17.8±3.42	2	2	1	II (T2N0M0)
12.	51	F	Parotid	Mixed	+	18±3.162	2	2	1	IV (T2N2M0)

Paraffin blocks from these patients were processed by classical histological techniques (HE stain) and for more detailed histopathological investigation were stained with Masson's trichrome kit (BioOptica, Albedo, Romania) and PAS stain after diastase digestion (PAS-D) using α -Amylase from porcine pancreas (Sigma-Aldrich, Albedo, Romania) according to the producers' protocols.

To illustrate more properly the acinar differentiation, we made an immunohistochemical detection for human Amylase (G-10, mouse anti-human, monoclonal, Santa Cruz, Redox, Romania) without antigen unmasking and incubating the slides overnight at 4°C with the primary antibody diluted as 1:2000.

The reactions were amplified with LSAB2 (Dako, Redox, Romania) and visualized with 3,3'-Diaminobenzidine (DAB) (Dako, Redox, Romania). For counterstaining, we used Mayer's Hematoxylin. The resection margins that include normal salivary gland parenchyma were used as internal control.

For illustrating the angiogenesis, we use double immunohistochemical reactions, using as first primary antibodies: CD105 (rabbit anti-human, polyclonal, Santa Cruz, Redox, Romania, in dilution 1:50), VEGF (rabbit anti-human, polyclonal, Santa Cruz, Redox, Romania, in dilution 1:50), VEGFR1 (rabbit anti-human, polyclonal, Sigma-Aldrich, Redox, Romania, in dilution 1:50) and VEGFR2 (rabbit anti-human, polyclonal, Abcam, MST solution, Romania, in dilution 1:50), and as second primary antibody the Amylase, with the above-mentioned specifications.

For the first antibody developing, we used citrate pH 6 heat-induced antigen retrieval, LSAB2 System-HRP and DAB as chromogen, according to the manufacturing protocols.

After blocking the endogenous biotin with Avidin/Biotin Blocking Kit (Dako, Redox, Romania) the second antibody was visualized with LSAB2 System-AP (Dako, Redox, Romania) and Vulcan Fast Red (Biocare, Rotest, Romania) as chromogen, according to the manufacturing protocols. As external control for these angiogenesis markers, were used section of human placenta.

The histopathological criteria for ACC diagnosis were those established by WHO (2005) and the images were acquired by utilizing a Nikon Eclipse 55i microscope (Nikon, Apidrag, Bucharest) equipped with a 5-megapixel cooled CCD camera and the Image ProPlus AMS7 software (Media Cybernetics Inc., Buckinghamshire, UK).

Immunohistochemical assessment

MVD assessment

Slides were scanned at low-power magnification ($\times 40$) to identify five areas with the greatest number of blood vessels (hotspots).

Microvessels were counted under $\times 200$ magnification ($0.8305 \text{ mm}^2/\text{field}$), considered the mean number of vessels in these areas in each sample.

Single endothelial cells or clusters of these cells, with or without lumen, were considered individual vessels. Vessels with muscular walls were excluded.

Evaluation of VEGF, VEGFR1, and VEGFR2 immunohistochemical expression

Protein expression was quantified in the various samples examined using a semi-quantitative scoring method. A mean percentage of positive tumor cells was determined in at least five areas at a magnification of $400\times$, and assigned to one of the three following scores: 0 (no reaction), 1 (positive in less than 10% of the total tumor cells), and 3 (positive in more than 10% of the total tumor cells).

Statistical analysis

Statistical analysis was done in SPSS version 16.0 for Windows, using the χ^2 test for dependence assessment, and ANOVA testing for multiple inter-group comparisons, the results being considered statistically significant for a p -value < 0.05 .

Results

Clinico-epidemiological data

According to the data presented in Table 1, the

majority of ACCs developed in the fifth decade of life (five cases, respectively 41.66%), with an average of 45.75 years. The youngest patient had 28-year-old and the oldest was 63 years. Women were affected more frequently than men in a ratio of 1.4 to 1, with no age predilection.

Almost 84% of all ACCs occurred in the parotid gland, and only in two cases, the tumors involved the intraoral minor salivary glands, respective the right buccal and the upper lip mucosa groups [21].

In 75% of the investigated cases, the pTNM stage was I/II, with no cases of perineural or vascular invasion, but with lymph node dissemination presented in only three cases.

Histopathological aspects

The most frequently encountered histological pattern was the solid form present in all investigated cases, but extensively in only four (33.33%) cases. In almost 42% of the cases, we noticed a mixture of two or more growth patterns with the solid/lobular and microcystic patterns more frequently associated [21].

CD105 expression and MVD assessment

CD105-positive vascular endothelial cells were clearly identified by their brown staining. In the residual normal salivary gland parenchyma from the resection tumor edges microvessels were rarely expressed CD105 and staining was faint and weak (Figure 1A).

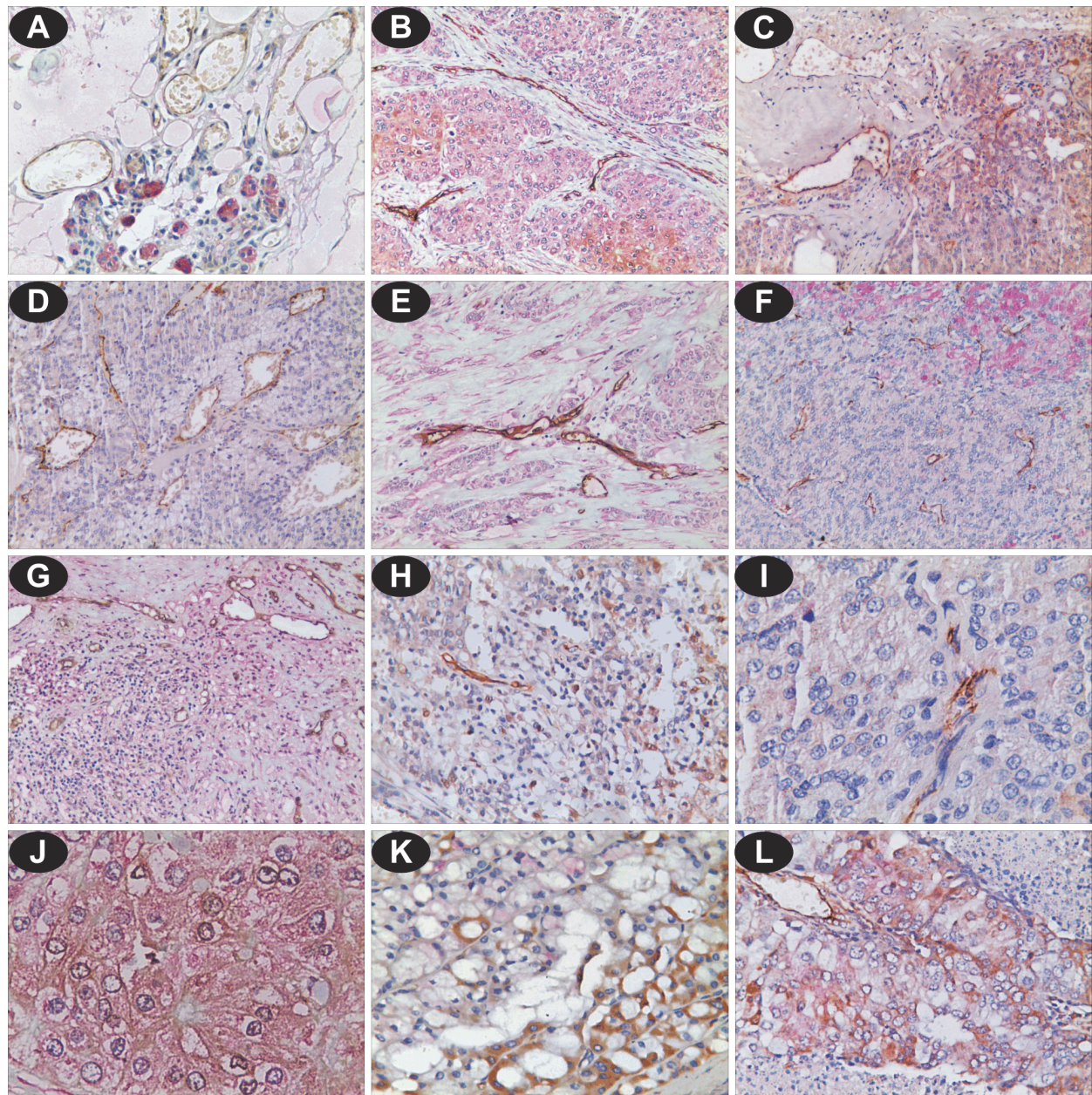


Figure 1 – ACC: Angiogenesis, IHC staining, CD105 (brown)/Amylase (red). (A) Residual normal salivary gland parenchyma vessels CD105+, ×100. (B and C) Intratumoral and peritumoral vessels CD105+, ×100. (D and E) Tumor vessels CD105+ with aberrant morphology, tortuous, with or without clear lumen, ×200. (F) Tumor vessels CD105+ predominantly of small-caliber, ×100. (G) The highest density appears to be in the inflammatory tumor stromal areas, ×100. (H and I) Tumor areas with individual endothelial CD105+ cells, ×100/×400. (J) Tumor cells from solid variant positive to CD105, ×400. (K) Tumor cells from microcystic variant positive to CD105, ×200. (L) Tumor cells from papillary variant positive to CD105, ×200.

These vessels were regularly distributed and had regular courses and cross-sectional shapes. We also notice a moderate CD105 staining in skeletal muscle cells on specimens with muscle invasion.

CD105 stained intensively intratumoral and peritumoral (at the tumor advancing edge) microvessels (Figure 1, B and C).

The tumor vessels were mostly of aberrant morphology, tortuous, with or without clear lumen (Figure 1, D and E).

In addition, they varied greatly in size, predominating microvessels of small-caliber (smaller than 15- μ m diameter) (Figure 1F).

The highest density appears to be in the inflammatory tumor stromal areas (Figure 1G).

In many tumoral areas, we identified individual endothelial CD105 positive cells (Figure 1, H and I).

Regardless of tumor pattern, we observed a weak cytoplasmic reactivity in tumor cells, the highest intensity being observed in intercalated duct-like tumor cells (Figure 1, J and L).

Microvessels density varied among tissue samples from seven to 41 (median 20). According to the histological pattern the highest MVD values were recorded in solid variant of ACC with 30.85 ± 4.97 , while at the opposite was the microcystic variant with 14.2 ± 4.81 MCD value (Table 1) (Figure 2).

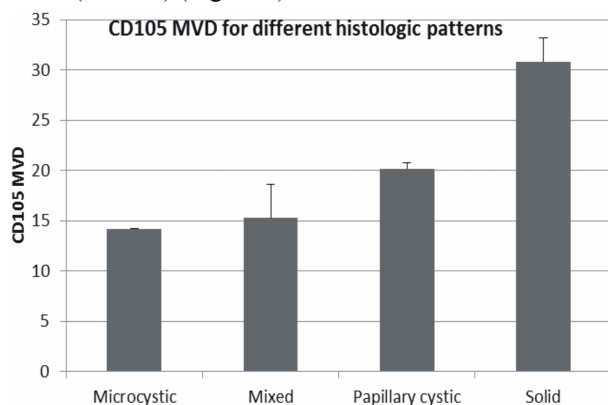


Figure 2 – Distribution of CD105-MVD in relation to tumor histological subtypes.

Intermediate MVD values were obtained in mixed (15.28 ± 4.49) and papillary (20.2 ± 2.78) variants. The statistical analysis proved the existence of significant difference between the CD105 MVD values for different histological patterns, $F(3,8)=25.81$, $p<0.001$.

We did not observe any statistical correlation between CD105-MVD and VEGF, VEGFR1 and VEGFR2 scores. In addition, we did not reveal any correlation of CD105-microvessels density with gender, sex or pTNM stage.

VEGF expression and its assessment

In the residual normal salivary gland parenchyma the VEGF reactivity was confined to duct epithelial cells and some myoepithelial cells (Figure 3, A and B). In addition, an acinar VEGF reactivity was observed especially in serous acini (Figure 3C).

In addition, we recorded a VEGF positive reaction in

the skeletal muscle fibers invaded or not by tumors, and in the blood vessels, and inflammatory cell infiltrate both from tumors and normal residual salivary gland parenchyma.

However, VEGF immunoreactivity was mainly located in the cytoplasm of the tumor cells (Figure 3, D and E) in all investigated ACC cases (100%).

In five (41.67%) cases, the VEGF staining was present in less than 10% of tumoral cells, while in the remaining seven cases, the VEGF scoring was 2 (Table 1).

As regarding the histological pattern, it seems that the microcystic, solid and mixed variants were the most reactive to VEGF and at the opposite pole were the papillary variants (Figure 3, F–I).

Related to tumor cell types, the most reactive were intercalated duct-like cells and non-specific glandular cells (Figure 3J), followed by the tumor cells with acinar differentiation (Figure 3K) and last ranged were the vacuolated tumor cells (Figure 3L).

We did not observe any statistical correlation of VEGF expression either with CD105-MVD neither with the major clinicopathological parameters. However, it seems that VEGF score 1 predominated in pTNM stage I, while score 2 prevailed in stage II and III [$\chi^2(3, N=12)=12$, $p=0.007$].

VEGFR1 expression and its assessment

In the residual normal salivary gland parenchyma, the VEGFR1 reactivity was mostly confined to duct epithelial cells and some myoepithelial cells (Figure 4, A and B).

We did not observe any acinar VEGF reactivity (Figure 3C) but a weak reaction was recorded in some blood vessels (Figure 4D), and inflammatory cells infiltrate (Figure 4B), both from tumors and normal residual salivary gland parenchyma.

In tumor specimens, we noticed a weak granular cytoplasmic reaction in all investigated ACC cases (Figure 4, E and F).

The semiquantitative VEGF assessment revealed that in most cases (eight cases, representing 66.66%) less than 10% of tumor cells were positive to this marker (Table 1).

In only four cases, we recorded a more intense reaction with more than 10% of positive tumor cells. This immunohistochemical score 2 was recorded in two cases with mixed pattern (Figure 4G), one case of ACC with solid pattern (Figure 4H) and one case with papillary pattern (Figure 4I).

Cytologically, the most intense reaction was noted in intercalated duct-like and non-specific glandular cells (Figure 4J).

In the tumor cells with acinar differentiation the VEGFR1 reaction was weak (Figure 4K), and it was absent in vacuolated tumor cells (Figure 4L).

The statistical analysis did not reveal any significant correlations of VEGFR1 reactivity with any clinical or histological investigated parameters.

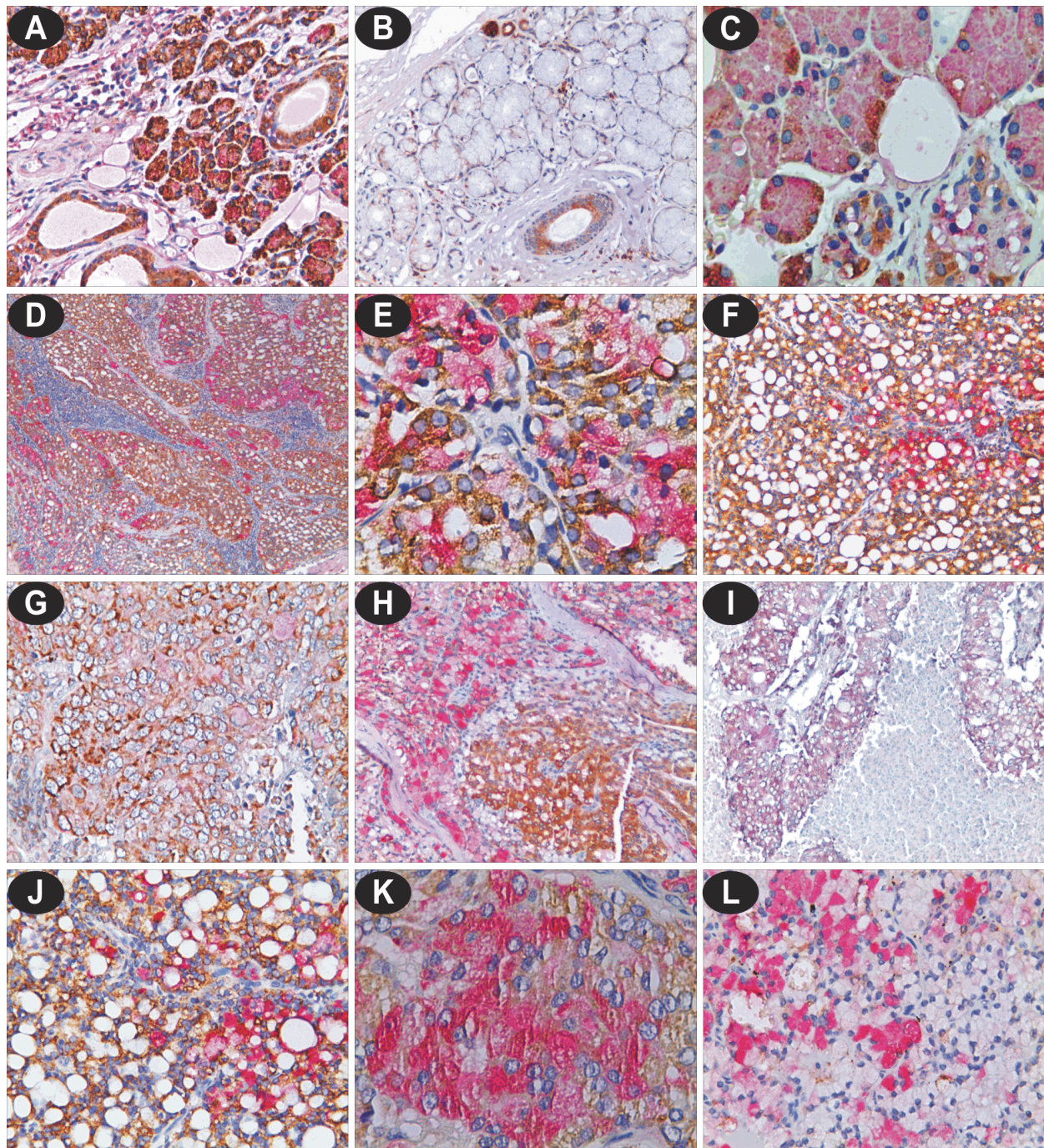


Figure 3 – ACC: VEGF reactivity, IHC staining, VEGF (brown)/Amylase (red). (A and B) Residual normal salivary gland parenchyma VEGF reactivity confined to duct epithelial cells and some myoepithelial cells, $\times 200/\times 100$. (C) Acinar VEGF reactivity in serous acini, $\times 400$. (D and E) Microcystic variant of ACC with VEGF immunoreactivity mainly located in the cytoplasm of the tumor cells, $\times 40/\times 400$. (F) Microcystic variant of ACC with VEGF reactivity, $\times 200$. (G) Solid variant of ACC with VEGF reactivity, $\times 200$. (H) Mixed variant of ACC with VEGF reactivity, $\times 100$. (I) Papillary variant of ACC with VEGF reactivity, $\times 100$. (J) Intercalated tumor duct-like cells positive to VEGF, $\times 200$. (K) Non-specific tumor glandular cells and acinar tumor cells positive to VEGF, $\times 200$. (L) Vacuolated tumor cells with weak or negative VEGF reactivity, $\times 200$.

VEGFR2 expression and its assessment

In normal specimens, the VEGFR2 reactivity has overlapped on the VEGFR1 reactivity but was much weaker. Thus, we noticed a granular cytoplasmic reaction in duct epithelial cells and some myoepithelial cells (Figure 5, A and B).

As a characteristic, we noticed a higher VEGFR2 reactivity on vascular endothelium in both residual

salivary parenchyma and in tumor specimens (Figure 5, C and D).

In the tumoral samples, VEGFR2 reactivity was weaker than VEGFR1 and it was observed in only 50% of the ACC investigated cases.

The semiquantitative VEGF assessment revealed that in these positive cases less than 10% of tumor cells were reactive for this marker (Table 1).

We could not establish correlations of VEGFR2

reactivity with tumor histological pattern. The intercalated duct-like and non-specific glandular tumor cells were the most reactive (Figure 5E) and at the opposite pole were the acinar tumor cells with a weak reaction (Figure 5F), and vacuolated tumor cells, which were mainly negative for this marker.

The only significant correlations regarding VEGFR2 tumor reactivity were:

- the immunohistochemical VEGFR2 score 0 was mostly associated with the VEGF score 1, while VEGFR2 score 1 status was mostly associated with the VEGF score 2, $\chi^2(1, N=12)=6, p=0.014$;
- the VEGFR2 score 0 was mostly associated with the VEGFR1 score 1, while VEGFR2 score 1 status was mostly associated with the VEGFR1 score 2, $\chi^2(1, N=12)=6, p=0.014$.

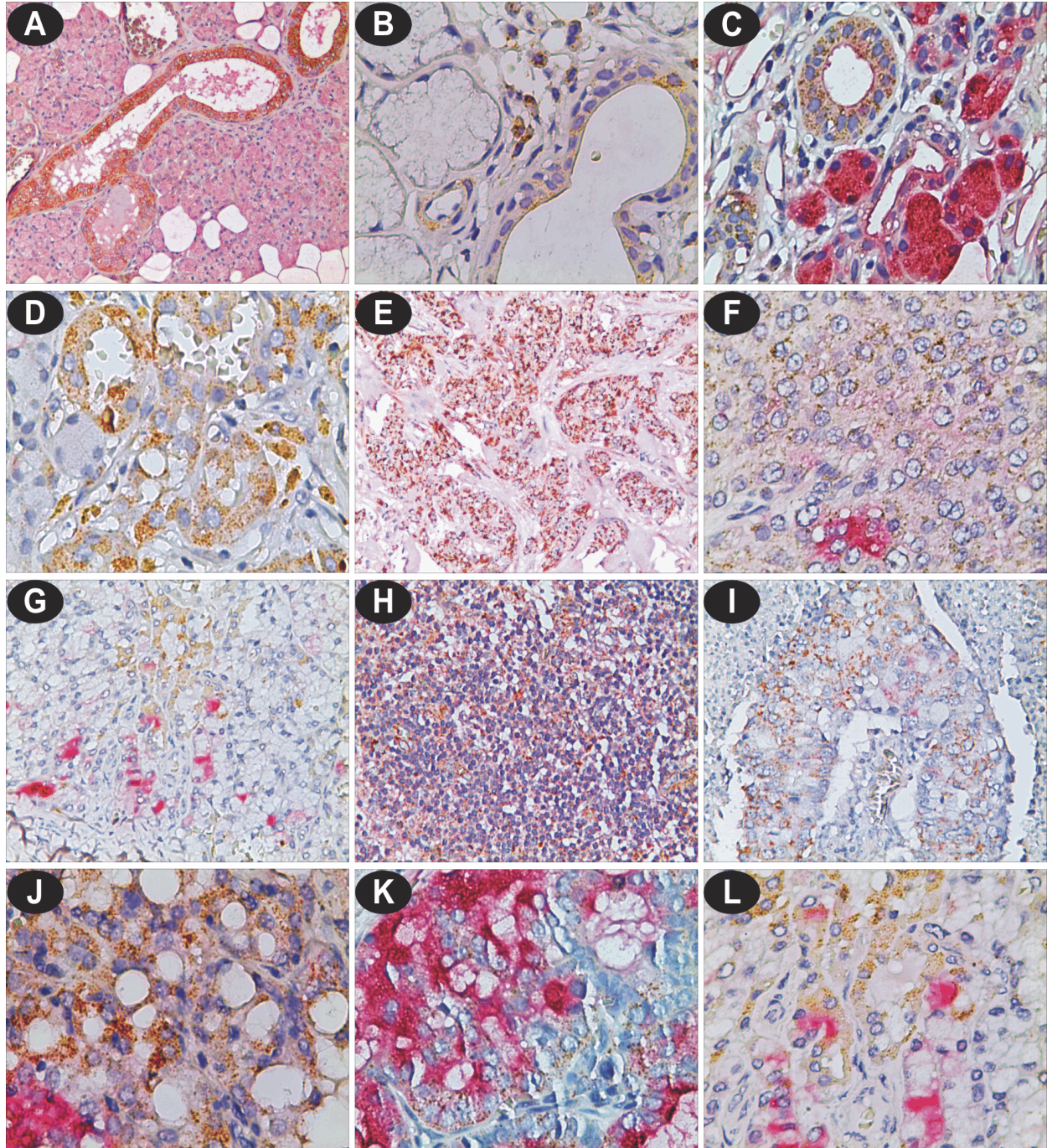


Figure 4 – ACC: VEGFR1 reactivity, IHC staining, VEGFR1 (brown)/Amylase (red). (A and B) Residual normal salivary gland parenchyma VEGFR1 reactivity confined to the duct epithelial cells and some myoepithelial cells, $\times 100/\times 200$. (C) No acinar VEGFR1 reactivity in serous acini, $\times 400$. (D) Mixed variant of ACC with VEGFR1 reactivity both in vessels and tumor cells, $\times 400$. (E) Mixed variant of ACC with granular cytoplasmic VEGFR1 immunoreactivity in the tumor cells, $\times 100$. (F) Solid variant of ACC with granular cytoplasmic VEGFR1 immunoreactivity in the tumor cells, $\times 400$. (G) Mixed variant of ACC with VEGFR1 reactivity, $\times 200$. (H) Solid variant of ACC with VEGFR1 reactivity, $\times 200$. (I) Papillary variant of ACC with VEGFR1 reactivity, $\times 200$. (J) Intercalated duct-like and non-specific glandular cells positive to VEGFR1, $\times 400$. (K) Acinar tumor cells weakly positive to VEGFR1, $\times 400$. (L) Vacuolated tumor cells negative VEGFR1 reactivity, $\times 400$.

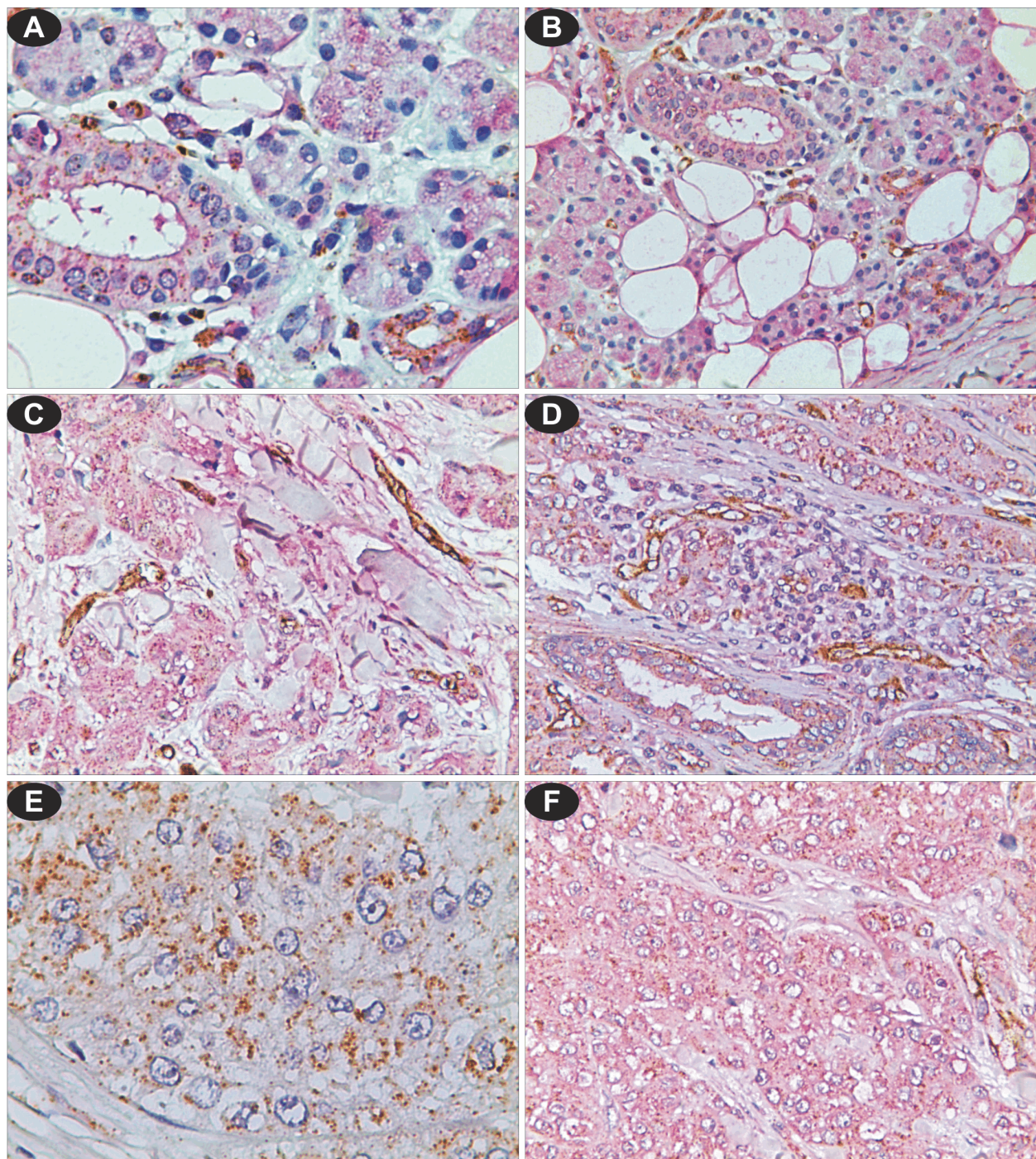


Figure 5 – ACC: VEGFR2 reactivity, IHC staining, VEGFR2 (brown)/Amylase (red). (A and B) Residual normal salivary gland parenchyma VEGFR2 reactivity confined to duct epithelial cells and some myoepithelial cells, $\times 200/\times 400$. (C and D) Intense VEGFR2 reactivity in endothelial cells from ACC vessels, $\times 400$. (E) Solid variant of ACC with granular cytoplasmic VEGFR2 immunoreactivity in the intercalated duct-like and non-specific glandular tumor cells, $\times 400$. (F) Mixed variant of ACC with weak granular cytoplasmic VEGFR2 immunoreactivity in the acinar tumor cells, $\times 200$.

Discussion

So far, angiogenesis of acinic salivary gland tumors has received limited attention. The reported MVD measurements in such salivary tumors were very few. Therefore, when we revised the English *PubMed* data, we discovered only one paper that discusses this problem [12] analyzed using automated image analysis and CD34 as marker of tumor vessels the MVD for 34 patients with ACC [12]. The authors found that patients who died of this kind of salivary gland cancer had significant

differences in vessel size, irregularity, staining intensity, and density compared with patients who survived during the five-year follow-up. Thus, they concluded that computer-assisted analysis of CD34-stained microvessels in such tumors might have prognostic value [12]. In our study, the CD105 reaction identified an active angiogenesis in the investigated ACC cases, the tumor vessels being of small-caliber, with aberrant morphology, tortuous, with or without a clear lumen. The highest density appears to be in the inflammatory

tumor stromal areas. Also, we established a significant difference between the CD105-MVD values for different histological patterns, with the highest MVD values recorded in solid variant and the lowest in microcystic variant. In addition, we observed a weak cytoplasmic reactivity in tumor cells, with the highest intensity noticed in intercalated duct-like tumor cells.

Other studies regarding MVD in salivary gland tumors have showed that tumors without myoepithelial cells had higher MVD than those containing myoepithelial cells [9–11, 13]. It was suggested that myoepithelial cells express and secrete high levels of angiogenic inhibitors, but very low levels of proangiogenic and angiogenic factors [9, 22, 23]. To compensate for the low angiogenesis level, it seems that salivary gland tumors with myoepithelial cells develop bigger blood vessels than those without myoepithelial cells [13]. However, Costa AF *et al.* (2008) [10] pointed out that the anti-angiogenic phenotype of myoepithelial cells may not play a pivotal role in the lower angiogenesis in salivary carcinomas with myoepithelial differentiation [10].

Unlike other types of cancer, in salivary carcinomas the process of angiogenesis seems to be most active in intratumoral regions, most likely due to tissue-specific tumor features and/or growth rate of the tumors within this location [10].

According to Dhanuthai K *et al.* (2012) [11], although malignant salivary gland tumors showed higher MVD than their correspondent benign neoplasm, MVD itself could not be an indicator to distinguish between benign and malignant salivary gland tumors [11]. Moreover, even if the *in vitro* study of Zhang J and Peng B (2007) [24] has suggested that adenoid cystic carcinomatous cells with higher metastatic potential could present a greater stimulus to angiogenesis, Costa AF *et al.* (2008) [10] comparing adenoid cystic carcinomas with and without distant metastases did not observe an increase in CD34-MVD nor in CD105-MVD in the group with metastases [10]. In addition, Cardoso SV *et al.* (2009) [9] recorded that the mean intratumoral MVD assessed by CD105 was not significantly different between the entire samples of malignant tumors that did or did not metastasize, and even all of the polymorphous low-grade adenocarcinomas and more than half of the adenoid cystic carcinomas that metastasized did not present CD105 positive vessels. Therefore, the authors suggested that angiogenesis is neither an absolute determinant nor required for acquisition of metastatic phenotype in these salivary gland tumors. One possible explanation for this variation in the metastatic potential of salivary gland tumors might be the differences in intrinsic properties of cancer cells themselves and/or of the tumor micro-environment [25].

All these aspects of salivary gland tumors angiogenesis must be recognized especially in the light of their implication in the field of anti-angiogenic therapy. It is well known that CD105 promoter is predominantly active in proliferating endothelial cells, and this molecule is currently being evaluated as an ideal target for antiangiogenic therapies that aim to prevent the development of neovasculature [26]. Thus, of this therapy

could mainly benefit patients with salivary gland tumors with high MVD, that express CD105 [10, 11].

Vascular endothelial growth factor (VEGF) is the main factor promoting angiogenesis and its expression may therefore be an indicator for the angiogenic potential and biological aggressiveness of a tumor [27]. The main salivary gland screening of the VEGF expression was conducted especially on mucoepidermoid carcinoma, adenoid cystic carcinoma and pleomorphic adenoma. Swelam W *et al.* in their study proved that salivary gland cells are capable of producing and secreting VEGF under normal conditions. Even more, the authors showed an increased VEGF expression in pleomorphic adenoma cells, given the fact that these tumors have a poor blood supply. Therefore, it was suggested that the up-regulation of VEGF due to the hypoxia might compensate for the poorly vascularized pleomorphic adenoma stroma [18]. Regarding adenoid cystic carcinoma VEGF expression, Lim JJ *et al.* (2003) [16] reported correlations with age, size, lymph node metastasis, clinical stage, perineural and vascular invasion, recurrence, and survival, but no correlation with histologic types. Also, Zhang J *et al.* (2005) [24] showed that VEGF expression and MVD had significant correlations with the clinicopathologic factors, such as tumor size, clinical stage, vascular invasion, recurrence, and distant metastasis in adenoid cystic carcinomas. Moreover, the authors found that VEGF expression was significantly higher in solid type tumors than that from tubular and cribriform types, and a tendency towards higher VEGF staining was recorded with more cases with perineural invasion [24]. Thus, the authors concluded that VEGF and MVD might play an important role in the prognosis of adenoid cystic carcinomas and that they could represent important therapeutic targets.

Our study proved that VEGF reactivity is mainly located in the cytoplasm of the tumor cells with the highest intensity in intercalated duct-like cells and non-specific glandular cells, and in the microcystic and solid ACC variants. Statistically we observed that the intensity of VEGF reaction correlated with pTNM stage with the score 2 prevailing in stage II and III. However, we could not establish any other correlations.

Lequerica-Fernández P *et al.* (2007) [15] suggested that VEGF can contribute to the progression of salivary gland carcinomas and seems to be associated with neck node metastasis, worse survival and poor local control of the disease [15]. Moreover, it seems that VEGF and angiogenesis could be used in the prevention or treatment of salivary cancer metastasis. Such supposition is partially supported by the investigation undertaken by Younes MN *et al.* (2006) [20], which treated salivary adenoid cystic carcinomas with AEE788, a dual inhibitor of EGF and VEGF receptor tyrosine kinases, and they found a reduced microvessels density and metastasis [20].

Data regarding VEGFR-1 and VEGFR-2 expression in salivary gland tumors are scarcer. Thus, in normal salivary gland it seems that both receptors had the same immunolocalization as the VEGF, namely in duct epithelial cells and some myoepithelial cells [18]. In pleomorphic adenoma tissues, the same authors revealed that while

VEGFR-2 and VEGFR-1 were immunolocalized in stellate cells, tumor cells forming tubular structures were more intensively positive for VEGFR-2. The simultaneously immunopositivity for VEGF, VEGFR-1, and VEGFR-2 of tumor cells in pleomorphic adenomas, proved that these tumor cells seem to survive due to an autocrine mechanism through both VEGFR-1 and VEGFR-2 signaling pathways [18]. Tampouris AI *et al.* (2012) [19] have also supported the existence of an autocrine loop implicated in survival, growth and progression of salivary gland tumors with myoepithelial differentiation, and more particularly, in pleomorphic adenomas [19]. In adenoid cystic carcinomas, Younes MN *et al.* (2006) [20] reported a predominantly VEGFR-2 expression in the tubular structures of tubular tumor variant and in the duct-like structures of the large cribriform pattern of this salivary gland tumor. Moreover, the authors proved that blockade of EGFR and VEGFR by AEE788 had a significant antitumor effects on human salivary adenoid cystic carcinomas cell xenografts in nude mice.

Our investigation regarding the expression of VEGF receptors in ACC samples revealed that their reactivity was overlapped on the VEGF reactivity. Overall, the VEGFR2 tumor reactivity was lower than that of VEGFR1 and at the cellular level the most reactive were intercalated tumor duct-like and non-specific tumor glandular cells. Statistics showed that the lowest values of VEGFR2 reactivity corresponded to scores 1 of VEGF and VEGFR1 reactivity while the highest VEGFR2 reactivity corresponded to the highest VEGF and VEGFR1 expression. For both receptors, we did not establish correlations with other clinical or histological investigated parameters, most probable due to the low number of cases. The highest VEGFR2 reactivity was present mainly in the vascular endothelial cells.

✉ Conclusions

Our investigation ascertained the existence of active angiogenesis in ACC, which is dependent on the histological variant, with the highest CD105-MVD scores in solid tumors. Responsible for this angiogenesis seems to be the VEGF and its receptors that were expressed in almost all of the investigated cases. In addition, their co-expression on tumor cells supported the existence of autocrine and paracrine loops implicated in the growth and progression of this type of salivary gland tumors.

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