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SHORT COMMUNICATION

Angiogenesis and Lymphangiogenesis in the Adrenocortical Tumors

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Abstract Adrenocortical tumors (ACT) are common adrenal tumors. The majority of ACTs are non-functioning and benign, while adrenocortical carcinomas (ACC) are rare, usually very aggressive and often metastasized when first diagnosed. Our aim was to assess whether blood and lymph vessel density within ACTs correlate with the malignancy character or tumor functionality. For that, the microvascular distribution was evaluated by immunohistochemistry staining with D2–40 antibody, for lymph vessels and CD-31 antibody, for blood vessels, in ACCs (n = 15), adenomas with Cushing syndrome (n = 9) and non-functioning adenomas (n = 10). The percentage of stained area was quantified by computerized morphometric analysis. D2–40 expression was significantly lower in ACC as compared to adenomas with Cushing syndrome ($p < 0.01$) and correlated positively with the expression of the steroidogenic acute regulatory protein (StAR) ($R^2 = 0.553$, $p < 0.001$). CD31 expression was found to be significantly higher in ACC as compared to adenomas with Cushing syndrome ($p < 0.05$). Our results show that angiogenesis is increased in ACC, suggesting that this phenomenon may have an important role in ACT biological behavior, while lymph vascular density seems to be more closely related to the tumor functional status than malignancy.

Keywords Adrenocortical tumors · Adrenocortical carcinoma · Lymphangiogenesis · Angiogenesis

Introduction

Adrenocortical tumors (ACTs) are common tumors affecting 3% to 10% of the human population. ACT can be classified according to their biological behavior in benign or malignant, and according to their functionality in non-functioning or functioning, depending on their capacity of autonomous steroid secretion. The majority of ACTs are benign, non-functioning and discovered incidentally during imaging studies performed for unrelated conditions [1]. In contrast, adrenocortical carcinomas (ACCs) are rare malignant tumors with an annual incidence of 1 to 2 cases per million persons worldwide. The majority of ACCs are in advanced stages when diagnosed, leading to a poor prognosis. The most common metastatic sites for ACC are the lung (46–79%), the liver (44–93%) and the lymph nodes (18–73%) [1].

Cancer cell dissemination occurs mostly through the vascular system, either through blood or lymph vessels [2]. Angiogenesis or lymphangiogenesis, are complex processes leading to the formation of new blood or lymph vessels, which are regulated by a high number of signal transduction pathways that were described to be altered in tumors and to contribute to their dissemination [2, 3].

A diversity of molecular markers that allow the evaluation of the vascular system are now available. Factor VIII-related antigen, CD31, and CD34 are the most commonly targeted antigens used to identify blood

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vessels by immunohistochemistry [4], while lymphatic endothelial hyaluronan receptor-1 (LYVE-1) and podoplanin (D2–40) are specific markers for the lymphatic endothelium [3, 4].

Since angiogenesis and lymphangiogenesis have been poorly characterized in ACTs particularly in correlation with the tumor functionality and malignant character [5–7], our aim was to assess whether blood and lymph vessel density within ACT were correlated with the biological behavior of these tumors.

Material and Methods

Adrenal Tissues

Adrenal tissues were obtained during elective surgical procedures from patients with ACT (n = 34), comprising ACCs (n = 15) and adrenocortical adenomas (ACA) (n = 19). Benign tumors included non-functioning adenomas (ACAn) (n = 9) and cortisol secreting adenomas with Cushing syndrome (CUSH) (n = 10).

Immunohistochemistry Analysis

Immunohistochemistry was performed in 3 μm formalin-fixed paraffin embedded tissue sections mounted on adhesive microscope slides. Antigen retrieval was performed by microwave treatment in 0.01 M-citrate buffer at pH 6.0, during 20 min. Endogenous peroxidase inhibition was performed with hydrogen peroxide (MERK, Germany) at 0.3%, for 15 min, before incubation with the primary antibodies against D2–40 (mouse, 1:200, ref.: M3619, Dako, Denmark) or CD31 (mouse, 1:100, ref. M0823, Dako, Denmark) for 1 h at room temperature. The detection of the immune reaction was performed by incubation for 60 min with the commercial Dako REAL™ EnVision™ Detection System (ref.: K5007, Dako, Denmark), which includes a dextran backbone with peroxidase (HRP) molecules coupled to goat secondary antibody molecules against rabbit immunoglobulins. DAB (3,3′-Diaminobenzidine), also included in the Dako System, was used as chromogen. Normal thyroid tissue was used as positive control for D2–40 and normal tonsil tissue for CD-31. The omission of primary antibody was used as negative control.

To evaluate the percentage of the stained area for each marker a computerized image analysis was performed. First, slides were scanned using the image acquisition Olympus VS110 virtual slide scanning system and captured using the image acquisition software VS-ASW (version 2.3 for Windows). Then, the images obtained were analyzed using the FIJI color deconvolution plugin (HDab), which allowed the separation of the stained area from the initial image, based in the RGB system. The stained area with the D2–40 or CD31 antibodies in the total tumor areas was quantified as previously described [8].

Statistical Analysis

The continuous variables are represented as mean ± standard error of the mean (SEM). The variables normality was evaluated using the D'Agostinho & Pearson test. For variables that passed this test, the one-way ANOVA test with the post-hoc Tukey was used to compare the means of the three groups. For the variables that did not pass the normality test, the Kruskal Wallis with a Post-hoc Dunn's was used. The correlations between continuous variables were evaluated using the Pearson Test. Statistical analysis was performed using the GraphPad Prism (version 7.00 for Windows). A $p < 0.05$ was considered statistical significant.

Results

D2–40 Expression

Lymph vessels in ACTs were immunohistochemically stained for D2–40, an O-linked sialoglycoprotein found on the lymphatic endothelium (Fig. 1a–c). Lymph vessels density was significantly lower in the ACC (0.129 ± 0.046%) compared with the CUSH (0.933 ± 0.268%, $p < 0.01$) (Fig. 1d). No differences were observed between the two groups of adenomas and the ACC vs ACAn (0.557 ± 0.255%).

CD31 Expression

ACT tumoral blood vessel density was evaluated by immunohistochemistry staining for CD31. Blood vessel density was significantly higher in ACC than in CUSH (ACC: 1.927 ± 0.391% vs CUSH: 0.698 ± 0.113%, $p < 0.05$) (Fig. 2).

Correlation between D2–40, CD-31 Expression with a Steroidogenesis Marker

The steroidogenic acute regulatory protein (StAR) is responsible to the acute regulation of steroid hormone biosynthesis since it mediates the cholesterol transfer to the inner mitochondrial membrane. A significant correlation was observed between D2–40 expression StAR protein expression ($R^2 = 0.553$, $p < 0.001$) (data from previous published results from the same tumors) [8]. No correlation was found between the CD-31 and StAR expression (Table 1).

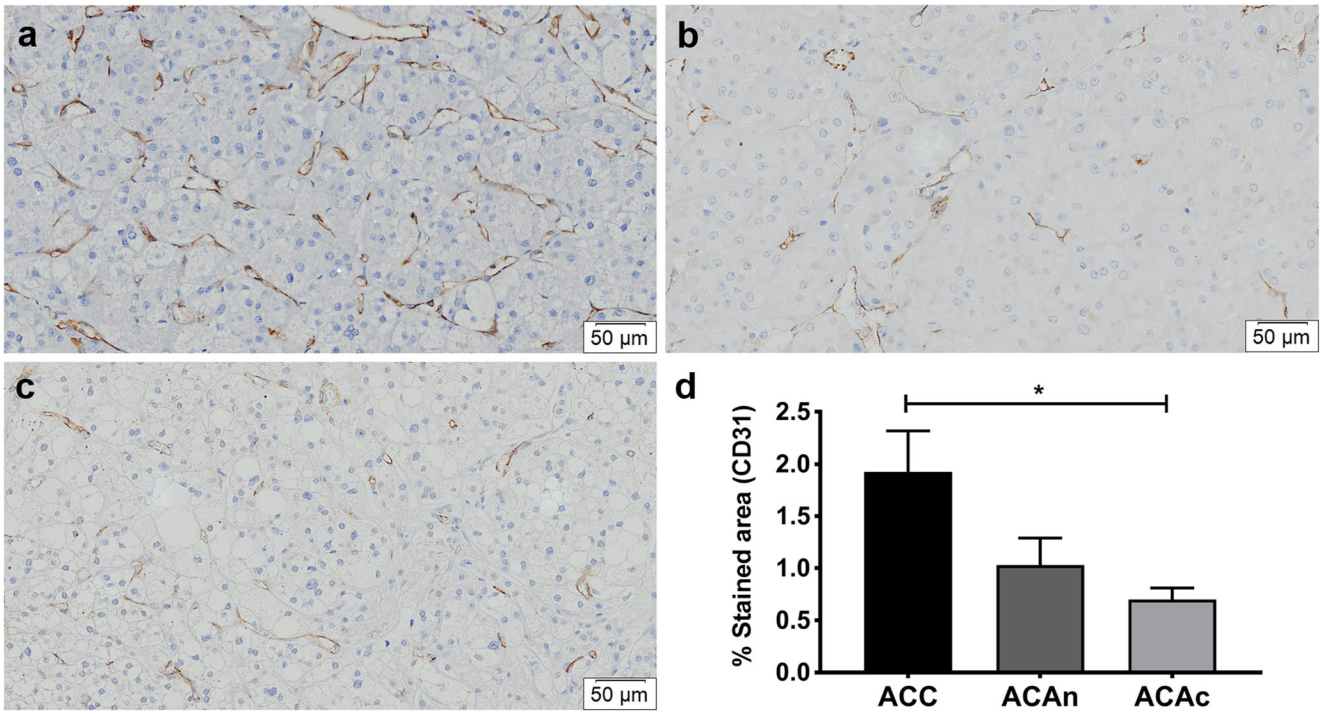

Fig. 1 Immunohistochemistry staining for D2–40 (Scale = 50 μm). **a** Adrenocortical carcinoma (ACC); **b** Adrenocortical adenoma with Cushing Syndrome (CUSH); **c** Non-functioning adrenocortical adenoma (ACAn); **d** Graphic representation of the percentage of the stained area for D2–40 in the different studied groups (ANOVA: ** $p < 0.01$)

Discussion

Angiogenesis and lymphangionesis are complex processes of substantial importance in cancer biology as these mechanisms may contribute to nurturement of the tumoral cells, dissemination of their humoral secretions as well as to the spread of the neoplastic cells. The majority of the ACCs are very aggressive tumors, most of them already being metastasized when first diagnosed [1].

Our aim was to study the expression of the D2–40 lymph vessel marker and the CD31 endothelial marker in the different ACT and to assess whether these were correlated with the malignant character of the tumors or their functionality.

In our study, lymph vessel density was found to be lower in the ACCs compared to CUSH. Besides that, as a positive correlation between D2–40 expression and StAR protein expression was found. So, we concluded that lymphangiogenesis in ACTs seems to be more related to the production of steroids than to the carcinogenesis process. StAR is an enzyme involved in the transport of cholesterol to the inner mitochondrial membrane, which is the first and limiting step of steroidogenesis [8]. The association of StAR expression with lymph vessel density could be hypothetically related with the needs of cholesterol supplying to the adrenal. Alternatively it could be connected to the effluent distribution of secreted adrenal steroids from functioning tumors. These two hypothesis were not tested, although certainly deserve further consideration.

Lymphangiogenesis in ACTs is a poorly characterized phenomena. In a previous study, D2–40 immunostaining was reported to be strong and diffuse but similar in both adrenocortical adenomas ($n = 5$) and carcinomas ($n = 3$). No information about functionality was provided [5].

Blood vessel density, on the contrary, was found to be significantly higher in ACC compared with functioning ACA. ACCs are aggressive tumors that frequently metastasize to the lung and the liver, and so the high blood vessels density found in this type of tumors is not unexpected. Besides that, the lower blood vessels density found in CUSH could be due the prior described anti-angiogenic actions of cortisol [9]. The use of CD31 to analyze the blood vessels may be influenced by lymphatic vessels cross staining. However, as the expression pattern of the CD31 and D2–40 was found to be opposite among the different studied groups, the differences in CD31 expression between the adrenocortical carcinomas and the adrenocortical adenomas could only be even stronger, and thus the cross reactivity associated with CD31 did not influence the results observed or challenge the conclusions.

The previous studies that addressed angiogenesis in ACT yielded inconsistent results. Bernini et al. analyzed the vascular density in benign and malignant ACT by CD34 immunostaining and showed that ACCs had a significantly lower vascular density as compared with ACAs [6]. Contrarily, Zhu et al., using the same marker and the same methodologic

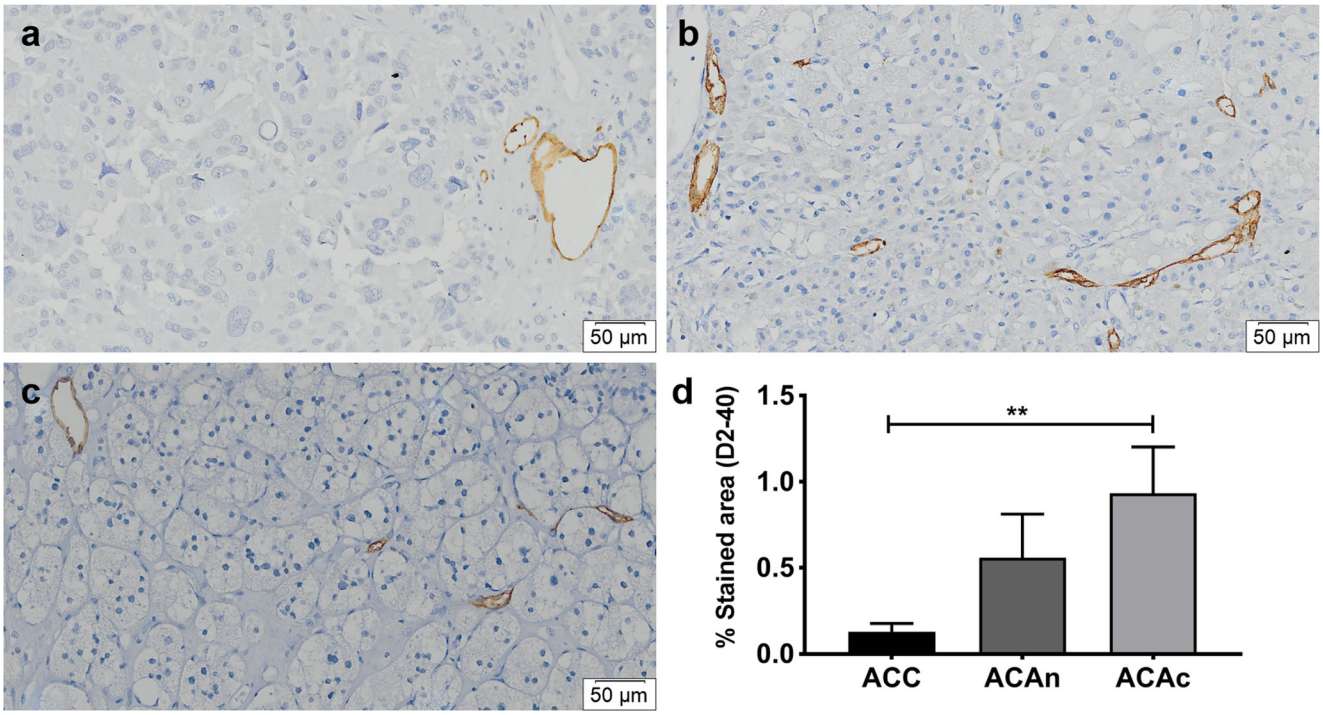

Fig. 2 Immunohistochemistry staining of CD31 (Scale = 50 μm). **a** Adrenocortical carcinoma; **b** Adrenocortical adenoma with Cushing Syndrome; **c** Non-functioning adrenocortical adenoma; **d** Graphic representation of the percentage of the CD31 in the studied groups (ANOVA: * $p < 0.05$)

approach to assess the angiogenesis in ACT demonstrated that CD34 expression was higher in ACCs than in ACAs [7]. In support of our finding vascular endothelial growth factor (VEGF) plasma levels and VEGF immune staining of the tumors was also found to be significantly higher levels in ACCs when compared to ACAs [6, 10].

All the previous studies that analyzed the vascular density have used the classical assessment method of semi-quantitative hotspot examination that restricts the analysis to representative tumor areas selected at the discretion of the observer to assess vascular density, in contrast to our current study in which the entire tumor tissue available was analyzed using a computerized morphometric method that is less prone to bias. Before conducting the herein study, the use of this computerized quantification method was validated in our group by performing a direct comparison with the traditional manual vessel counting to evaluate lymphatic vessels density in prostate tumors induced in mice models. Using this dual morphometric approach has allowed us to confirm that the results obtained through computerized analysis were accurate and comparable to those achieved after using more conventional counting methods, while in a more advantageous way [11]. In addition, this computerized method to access the vessels density was also used in other studies [12, 13].

The evaluation of the vessels density in the tumor periphery was not possible since adjacent adrenal gland was not present in the majority of the adrenocortical carcinomas, rendering a comparative analysis virtually impossible to perform. Besides that, atrophy of the adrenal tissue adjacent to cortisol secreting tumors is a well-known phenomenon [14]. In these atrophic adrenal glands the staining is extremely concentrated in the capsule, what makes the analysis not feasible.

In conclusion, blood vessel density is increased in ACC suggesting that angiogenesis could have an important role in the ACT biological behavior, while lymph vessel density seems to be more closely related to the tumor functional status than with malignancy.

Table 1 Correlation results between the CD-31, D2–40 and the marker of steroidogenesis StAR

Correlation	Coefficient correlation (R^2)	p
D2–40 and StAR expression	0.553	$p < 0.001$
CD-31 and StAR expression	0.049	non-significant

StAR steroidogenic acute regulatory protein

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Compliance with Ethical Standards

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Conflict of Interest The authors declare that they have no conflict of interest.

Informed Consent The participants provided written informed consent for adrenal tissue samples deposited at our institutional Tumor Bank to be used for future research.

Ethical Approval The study was approved by the local Ethics Committee.

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