Original paper

Heterogeneous vascular patterns in renal cell carcinomas

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The present study proposes a classification of renal cancer tumor blood vessels according to their morphology and maturation grade. We identified four vascular patterns: reticular, diffuse, fasciculated and trabecular. The reticular pattern was present in 63% of cases, being characterized by the predominance of mature CD34+/SMAct+ tumor vessels, highly interconnected. For this pattern, 74% of cases had vascular invasion, and a significant correlation was observed between tumor grade and immature state of tumor vessels ($p = 0.022$). The diffuse pattern was observed in 23% of cases and was characterized by non-interconnected vessels predominantly of mature CD34+/SMAct+ type and vascular invasion in 64% of cases. Only 8% of cases, had a fasciculate model of vessels distribution, all of them being of mature type, located in the connective axis of papillary renal tumors. For this pattern vascular invasion was found in 50% of cases. In 6% of cases a trabecular pattern was observed and the lowest rate of vascular invasion was registered. We defined here four distinct vascular patterns in renal cell carcinomas showing a strong impact on vascular invasion. A complete morphological and molecular characterization of tumor vessels would be beneficial in elucidating the mechanisms that underlie the ineffectiveness of antiangiogenic/antitumor therapies.

Key words: renal cell carcinoma, CD34, SMAct, vascular pattern.

Introduction

Several antiangiogenic and antivascular therapies have been applied for renal cell carcinomas in both experimental models and clinical trials, but their efficiency was questionable and did not improve patient survival and prognosis [1, 2]. Most likely, the reduced response rate to antivascular/antiangiogenic therapy is probably due to the lack of a more accurate evaluation of blood neovessel morphology and phenotype in renal tumors.

Tumor blood vessels mature by the acquisition of perivascular cells. This process influences the endothelial cells, which return to their quiescent state and become less sensitive to anti-angiogenic agents, particularly to anti-VEGF and VEGFR2 therapeutics, commonly used in clinical practice [3, 4].

Most malignant tumors tend to have immature type vessels without perivascular cells around them and therefore with activated endothelial cells sensitive to anti-angiogenic/antivascular therapies [5, 6].

Renal cell carcinomas are particular malignancies because of the predominance of mature type tumor blood vessels inside the neoplastic tissue but, at this time, the factors that induce early maturation of the intratumoral vessels, and the pathogenic substrate of this early maturation are completely unknown. Scattered data have been reported regarding the impact of tumor blood vessels maturation on prognosis and therapy efficiency in renal cell carcinomas [7, 8], but these reports were usually controversial [9, 10]. One should also add that vessels of normal renal tissue, adjacent to the tumor, currently, are not fully characterized in terms of the distribution of perivascular cells.

The complexity and heterogeneity of renal cell carcinomas tumor vasculature were previously recognized by a few researchers and were focused on the differentiation of endothelial cells [11] or microvessel area [12] and to a lesser extent pericyte involvement.

Based on these controversial data, we considered that the evaluation of tumor blood vessels in renal cell carcinomas is not complete and an accurate assessment of their maturation degree is necessary. Thus, we propose here the classification of renal cell carcinoma blood vessels in four main types according to pericytes coverage and their impact on tumor invasion and prognosis.

Material and methods

Fifty archival paraffin-embedded specimens of renal carcinomas were selected for this retrospective study. We retrospectively selected paraffin-embedded specimens from 30 male and 20 female patients aged between 29 and 78 years old who were admitted to the hospital with left or right tumor masses accidentally detected or diagnosed by CT or MRI. No associated significant disease was reported for our study group. All cases were re-submitted for routine morphologic techniques (sectioning and staining with hematoxylin and eosin method) to reevaluate the histopathology and to select cases for immunohistochemistry. After reevaluation, one case was excluded because of improper primary processing which was not compatible with a valuable interpretation of subsequent immunohistochemical stainings. We finally selected 49 cases for immunohistochemistry. Colocalization of endothelial and perivascular markers was based on a double stain immunohistochemical method by using CD34 (clone QBEnd 10, DAKO Carpinteria, CA, USA) for endothelial cell assessement and smooth muscle actin (SMAct, clone 1A4, DAKO Carpinteria, CA, USA) for perivascular cells. Immunohistochemistry used a biotin-free visualization system (Bond Refine Detection System, DAB, Leica, Microsystems, UK) and the final product was stained brown, highlighting cytoplasmic expression of CD34 antigen, followed by additional use of Bond Refine Detection System Red (from the same manufacturer) for the visualization of SMA as a red signal with cytoplasmic distribution also, inside perivascular cells surrounding tumor blood vessels. Immunohistochemistry was fully automated, being performed with the Bond Max System (Leica Microsystems, UK). Microscopic data and image acquisition was performed by using an AxioZoom Imager A2 research microscope (Zeiss, Germany).

Table I. Cases distribution according with histopathology and Fuhrman grade

RCC	Fuhrman			
	1	2	3	4
Clear cell	5	31	7	0
Papillary	0	1	0	1
Cromophobe	0	1	1	1
Unclassified	0	0	0	1

Microscopic evaluation included the histopathologic diagnosis with evaluation of tumor grade by original Fuhrman score use and the assessment of double stained blood vessels inside and around the tumor and also in the normal renal tissue adjacent to the malignant tissue. Histopathologic types and Fuhrman grade are summarized in Table I.

Based on the smooth muscle cell distribution and vascular morphology, we defined four types of tumor blood vessels which were correlated with tumor grade and vascular invasion. Tumor blood vessels assessment included the evaluation of CD34 and SMAct colocalization and, based on this aspect, we defined tumor blood vessels as immature (CD34+/SMAct–) and mature (CD34+/SMAct+). The presence of SMA discriminate between immature (active) and mature (dormant) tumor blood vessels. Four vascular patterns were defined according to the intercapillary distance and microscopic arrangement of tumor blood vessels, these patterns being described in the Results section. Statistical analysis used SPSS software, version 17. A p value < 0.05 was considered statistical significant.

Results

Expression of CD34 and smooth muscle actin (SMAct) in normal renal parenchyma

Significant differences were observed in the expression of CD34/SMAct between the renal cortex and medulla blood vessels. At the cortical level we observed differences in expression between glomerular and stromal capillaries. Glomerular capillaries had an expression pattern defined as CD34+/SMAct–. Efferent and afferent arterioles showed the expression pattern of CD34+/SMAct+.

The perivascular SMAct+ cells surrounding the stromal capillaries from the renal cortex were special

Table II. Results summary of the four vascular patterns described in the present study regarding distribution of CD34+ and SMA+ cells in the tumor blood vessels

	Reticular Both CD34+/SMA+ CD34+/SMA– Small intercapillary distance	Diffuse CD34+/SMA+ exclusively Large intercapillary distance	Fasciculate CD34+/SMA+ exclusively	Trabecular CD34+/SMA+ exclusively Large intercapillary distance
Clear cell	predominant	rare	–	–
Papillary	–	rare	predominant	–
Cromophobe	–	predominant	–	rare
Unclassified	–	predominant presence of particular structures as glomeruloid bodies	–	rare

in morphology, in that they presented a series of extensions connected with extensions of other similar cells, surrounding neighboring vessels. Most of the renal medulla stromal vessels were CD34+/SMAct–. In the renal hilum the vessels were of arterial and venous type CD34+, with a strong, well-organized periendothelial smooth muscle cell layer, SMAct+.

Evaluation of CD34/SMAct expression in renal tumor blood vessels

The overall assessment of the 49 cases of renal tumors showed intratumoral vascular heterogeneity, found in the different areas of the same tumor. For this reason we considered useful the definition of the distribution patterns of intratumoral blood vessels and trying to identify a link between histopathological shape, degree of tumoral differentiation and the vascular network architecture. Due to the increased vascular density observed in some types of kidney tumors, which made it impossible to quantify the number of vessels, we considered that microvascular density assessment is not appropriate for our study, being surpassed by the distribution patterns of blood vessels.

In terms of the expression of CD34/SMAct in renal tumors, we encountered immature vessels (CD34+/SMAct–) and also vessels of mature type (CD34+/SMAct+). The two types of vessels, with different immunohistochemical profiles, were observed within the same tumor mass but with a heterogeneous distribution in the tumor area.

The intercapillary distance seems to influence the degree of tumor vessel maturation of renal carcinomas. If the intercapillary distance was small, most of the vessels were of mature (CD34+/+SMAct) type. Despite their mature appearance, CD34+ endothelial cells lining such blood vessels tended to exhibit the phenomenon of sprouting, but in most cases these cells were accompanied by SMAct+ cells.

The distribution of SMAct+ cells around the mature vessels considered by us was different from that found in the normal renal parenchyma vessels. If SMAct+ vessels of normal renal parenchyma showed pericytes closely attached to the external surface of the vascular wall, SMAct+ perivascular cells from tumor vessels were disposed adjacent to the vascular wall but not completely attached. Perivascular cells from tumor vessels had a distinct morphology. If in the normal renal parenchyma the pericytes had an extra thin body and were tightly attached to the vascular endothelium, in the tumor blood vessels they showed elongated body and cytoplasmic extensions that were incompletely detached from the vascular wall and tended to be organized into SMAct+ networks bridging neighboring vessels. We observed vessels with perivascular cells completely attached to the vascular wall and perivascular cells that were partially attached. Different morphology of tumor blood vessels and distribution heterogeneity of SMAct+ perivascular cells helped us to define four main patterns of tumor vasculature in renal cell carcinomas, according also with intercapillary distance: reticular, diffuse, fasciculate and trabecular.

The reticular pattern was characterized by reduced intercapillary distance, high vascular density and common capillary interconnections. In most cases this pattern was characterized by the presence of CD34+/SMAct+ vessels.

The diffuse pattern had no interconnected tumor blood vessels lined by CD34+ endothelial cells which defined a distinct, perfused lumen. Also, CD34+ isolated endothelial cells were frequently observed. Most of these tumor blood vessels were of immature type, SMAct+ cells being present but unattached to isolated CD34+ cells or the capillary wall.

The fasciculate pattern was specific for papillary type renal carcinoma, tumor blood vessels being

restricted to the connective tissue core of papillary structures. The vessels of the fascicular pattern were of mature type, with a thin lumen, often collapsed. The cross sections through a tumoral papilla showed the clear presence of a lumen, well defined and perfused.

Note that, unlike the exception of the fasciculate pattern, the reticular and diffuse patterns coexisted inside the same tumor, the reticular distribution model being observed in the peripheral zone, immediately beneath the capsule, while the diffuse pattern was predominantly found in the center of the tumor.

The trabecular variant was composed of CD34+ tumor blood vessels surrounded by a thick area of SMAct+ perivascular cells. Because of these large SMAct+ areas the vessels could not be classified as mature or immature. Most of them were small with a perfused lumen. It was hard to say exactly where the vessels showed perivascular SMAct+ cells.

In one case, we found glomeruloid bodies in various stages of evolution, complex structures consisting of CD34+ vessels and disorganized smooth muscle cells, more or less attached to the CD34+ vessels (mostly with a lumen). This case was considered by histopathology as an unclassified variant. It also showed heterogeneity in the vascular patterns, presenting predominantly the diffuse variant of tumor vessel distribution.

Based on the four previously described vascular patterns, we observed that 63% had a reticular pattern (Fig. 1A), 23% had a diffuse pattern (Fig. 1B), 8% had a fasciculated pattern (Fig. 1C) and and 6% had a trabecular pattern (Fig. 1D).

About 87% of cases with a reticular pattern had coexpression of CD34+/SMAct+, suggesting the predominance of mature vessels. Despite the fact that the diffuse pattern was seen in about a quarter of cases, 91% of them had vessels of mature type; this seems to reduce the vascular invasion. All cases with trabecular and fasciculated patterns had mature vessels only. Vascular invasion was observed in a small percentage of cases, compared with the first

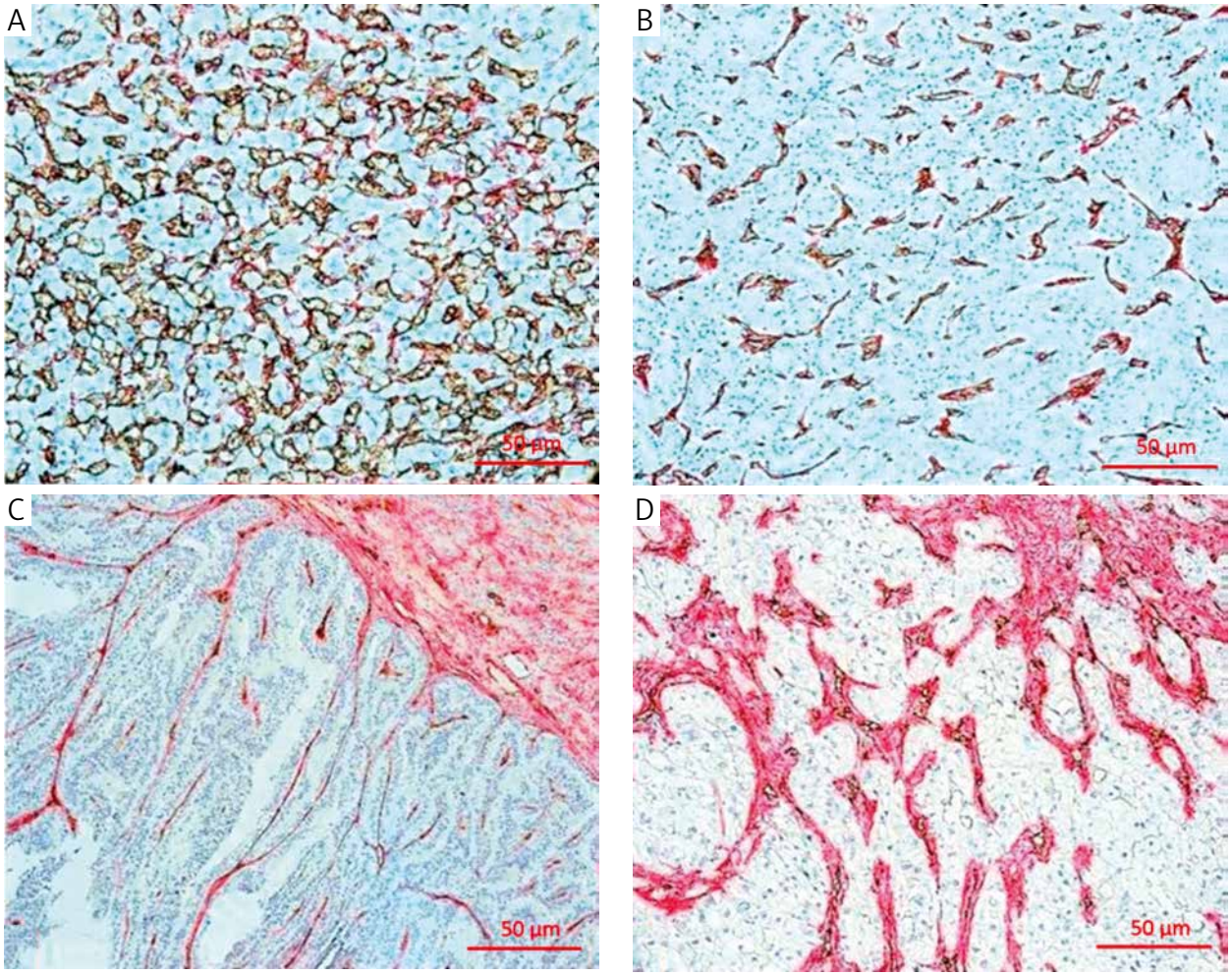

Fig. 1. Four microscopic vascular patterns identitfied by colocalisation of endothelial and perivascular cells markers (CD34 and SMAct). Networks of small interconnected blood vessels defined reticular patters (A; magnification 100×). No connections between tumor blood vessels from diffuse vascular pattern (B; magnification 100×). Fasciculate pattern was specific papillary type renal cell carcinoma (C; magnification 100×). Thick trabeculae of SMAct positive cells surrounding tumor blood vessels (D; magnification 100×)

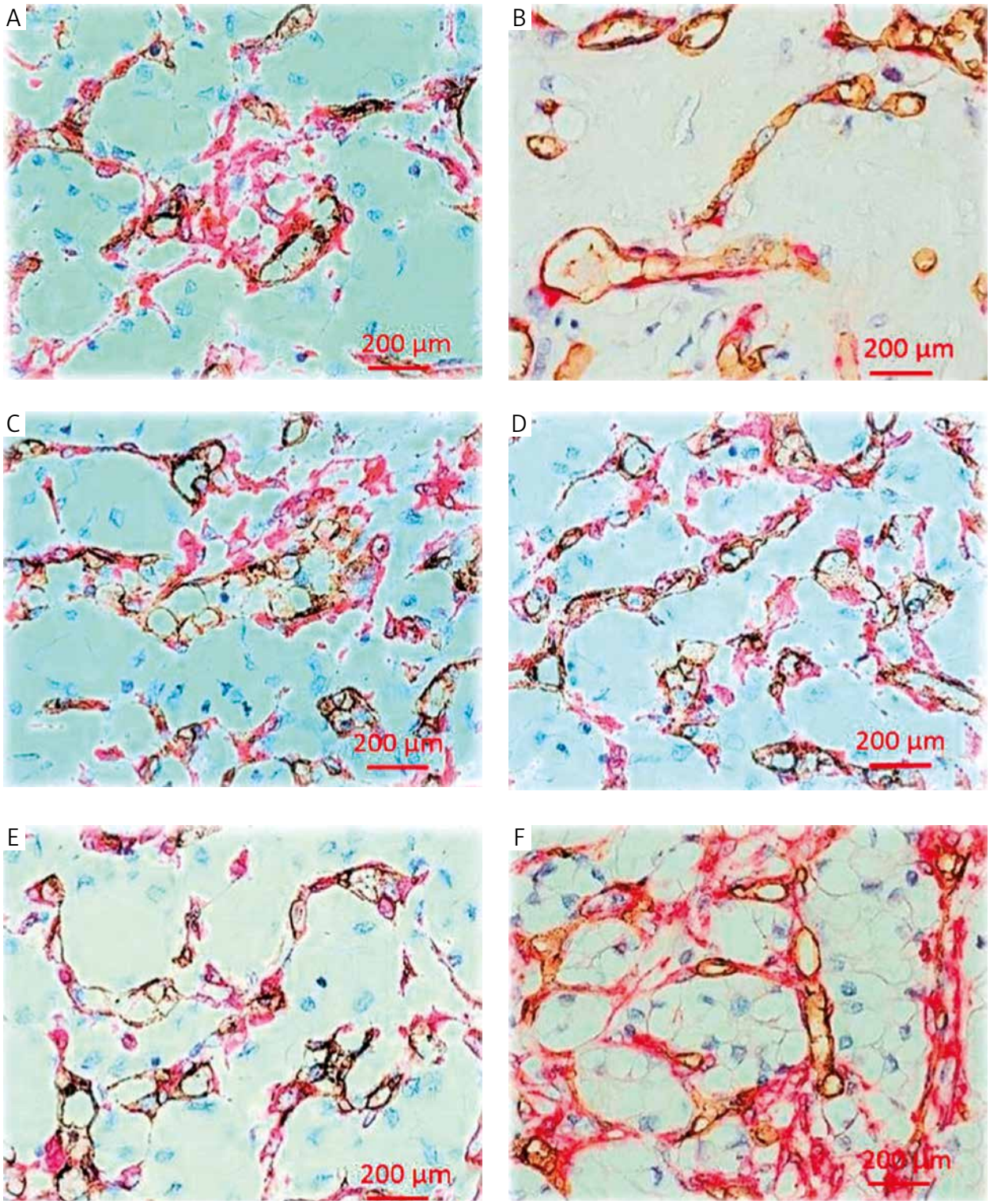

Fig. 2. Distribution heterogeneity of SMA positive perivascular cells (red) related to CD34 positive endothelial cells (brown) from tumor blood vessels of RCC. Perivascular cells with a chaotic attachements to the tumor blood vessels (A; magnification 400×) disposed as clusters of SMA positive perivascular cells giving branches for several tumor blood vessels in a disordered fashion. Well defined lumen with closely apposed SMA+ perivascular cells (B) were specific for peritumor stroma and normal adjacent renal tissue while CD34 positive "cord like" structures with no lumen and incomplete attachement of SMA+ perivascular cells were specific for reticular pattern (C,D; magnification 400×). Sprouting CD34+ endothelial cells were interconnected with SMA+ perivascular cell and not surrounded by them as in normal blood vessels (E; magnification 400×). Instead of surrounding tumor blood vessels with lumen, SMA+ cells have tendency to interconnect them by a well defined network of branches partially attached to the blood vessels (F; magnification 400×)

two patterns described, although a small difference was observed, vascular invasion being lower in the trabecular pattern (33%). This difference can be explained by the presence of blood vessels in trabeculae rich with myofibroblast-like cell type, trabeculae with variable thickness, intensely positive for smooth muscle actin, which clearly delineated the areas of tumor cells from the vessels.

The vascular invasion decreased from the reticular to the trabecular pattern. This demonstrates that the perivascular cells and/or the myofibroblast-like cells found in the study, represent a protective factor against tumor invasion by using the vascular route. For this reason we considered the evaluation of the perivascular cells of the renal tumors useful.

Thus, the most heterogeneous morphology and distribution of perivascular cells was observed in the reticular pattern. Even if they were present, the perivascular cells formed a discontinuous layer, being incompletely attached. Frequently, there were observed perivascular spaces between the perivascular cell body and the vascular wall. Also, SMAct+ perivascular cells showed several extensions that apparently were distributed to several tumor vessels (Fig. 2A–E).

For the diffuse pattern, perivascular cells formed a continuous layer, but unlike normal media of blood vessels, we observed several gaps between the perivascular cells (Fig. 3).

A particular issue was encountered in the case of glomeruloid bodies, when depending on their degree of maturation, the perivascular cells were arranged in groups separated from the blood vessels, but in close proximity to them mixed with areas where the perivascular cells tended to be attached to the CD34+ endothelial cells.

Statistical analysis included tumor vessel maturation degree, tumor degree of differentiation and vascular invasion. The overall analysis showed no significant correlations between vascular pattern, vessels types and vascular invasion. The vascular pattern was significantly correlated with Fuhrman score of 2 and 3 ($p = 0.01$).

For cases having a reticular pattern tumor grade was correlated with immature vessels ($p = 0.022$) and between the immature vessels and vascular invasion ($p = 0.00$). For the diffuse pattern tumor grade did not correlate with immature or mature vessels or with vascular invasion. For the fasciculated and trabecular pattern types a statistical analysis could not be performed due to the small number of cases included in the study.

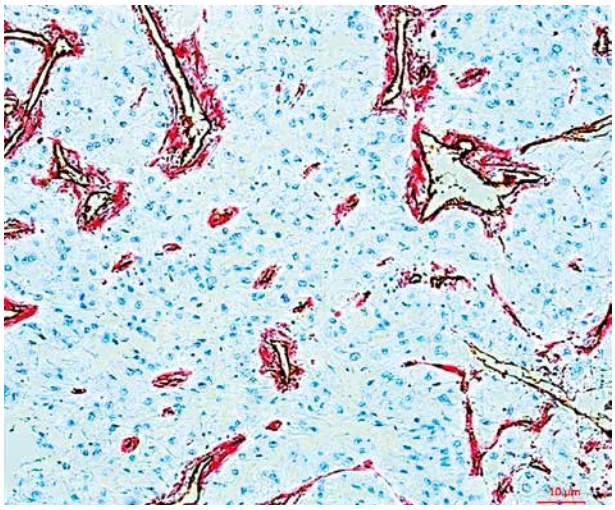

Fig. 3. Tumor blood vessels distribution in the diffuse pattern. Note that tumor blood vessels apparently looks like having a continous layer of SMA+ perivascular cells (red) around them but if we look carefully we are able to identify small spaced between these cells

Discussion

The architecture of the vessels in the renal carcinoma is different from that found in other types of malignancies. Architectural differences are seen not only between tumors but also between the different regions of the same tumor. Microvascular density [13, 1] and VEGF expression [15, 16] are the most studied topics regarding renal cell carcinomas vasculature together with other growth factors [17, 18] to a lesser extent.

Frequently used markers for the assessment of tumor vasculature in renal carcinomas are CD34, CD31 and to a lesser extent endoglin (CD105). Regardless of the markers used, so far microvascular density has-not demonstrated a prognostic or therapeutic impact in renal tumors. Although observational studies were performed on the vascular architecture of the renal tumors, in the literature there are currently no more than 20 articles describing this vascular complexity. Moreover, some of them were carried out using Dynamic Contrast Enhanced Ultrasound and to a lesser extent on paraffin specimens [19].

Isolated data indicated that the stromal architecture of the renal tumors makes them different regarding their therapeutic response to VEGF inhibitors used as a single therapeutic option [20].

More recently, apart from the histopathological differences between different types of renal cancers, occasionally architectural differences in the renal vasculature are described.

Based on indirect evidence that vascular architecture may influence the prognosis and therapy of renal tumors, we considered it useful to define vascular patterns that may have a clinical, prognostic and therapeutic impact. Therefore, based on the study of co-localization of CD34 and SMAct, we identified four vascular patterns with demonstrated statistically

significant correlations with histopathological data or tumor grade and the presence of vascular invasion.

The reticular pattern predominantly characterized clear cell carcinoma. If for some tumors the interconnections were made by capillaries directly (through interconnections between endothelial cells, as evidenced by continuous CD34 staining for endothelial cells), for the others the reticular appearance was given by actin-positive perivascular cells forming bridges between the capillaries of the vascular network or by sending extensions from these cells to several adjacent capillaries.

In the diffuse pattern the degree of attachment of perivascular cells, where they were present, was much higher than that found in the reticular type. As evidence of the involvement of perivascular cells in the prognosis of renal tumors, the vascular invasion detected in the diffuse type was significantly lower than that found in the reticular pattern. The mature type vessels were characterized by reduced vascular invasion.

The four patterns could explain the differences in response to antiangiogenic/antivascular therapies reported in the literature for renal tumors [21].

The impact of the identification of these four vascular patterns is supported by the statistically significant correlations found between the tumor grade and vascular invasion. The correlation with tumor grade suggests a close interrelation between the tumor cells and blood vessel phenotype. The correlation obtained between Fuhrman grade 2 and 3 and the vascular pattern supports active angiogenesis in these stages, when the endothelial cells and also the perivascular cells could be more sensitive to anti-angiogenic/antivascular therapy.

Our study revealed that the maturation of blood vessels decreases the proportion of vascular invasion in the renal tumors by microscopic evaulation. Vessels stabilization in renal tumors using stimulation therapies with perivascular cells could be an adjuvant therapy in reducing distant metastases from renal carcinomas. This is supported by the fact that the trabecular pattern, which we observed in our study, had the lowest rate of vascular invasion, only one third of cases presented vascular invasion.

Our results suggest that the reticular vascular pattern is the most sensitive pattern to antiangiogenic/antivascular therapy, supported by the fact that it was correlated with the number of immature vessels on the one hand and on the other hand with the extent of tumor invasion.

The prognostic and therapeutic impact of the study is that it could help to stratify the patients who may receive adjuvant therapy, not only on the basis of tumor type and angiogenic factor assessment, but also based on tumor vessel maturation. The definition of the vascular groups of this study partly explains the resistance to antiangiogenic therapy in renal tumors, on the one hand, by the presence of different degrees of maturation of blood vessels and, on the other hand, by combining the heterogeneity of the vascular patterns in the same tumor.

Existing data show partial overlapping of CD105/CD34 expression in renal carcinoma and suggest that coexpression of CD105 with actin (absent in most other tumor types) suggests a fast degree of maturation of vessels in renal carcinomas, maturation that currently cannot be explained because of the lack of identification of the tumor and vascular factors that influence this process [22, 23]. The definition of the four vascular patterns and also the observation of decreased tumoral invasion in the fasciculated and trabecular patterns supports myofibroblast stimulation or an increased ability of myofibroblasts cell differentiation into perivascular cells in renal tumors, not necessarily as a mechanism of maturation, but more as a mechanism of tumor growth and neovascular expansion limitation.

Conclusions

Our study defines four vascular patterns in renal cell carcinomas, based on morphology and co-expression of CD34 and SMAct: reticular, diffuse, fasciculated and trabecular. Our statistical analysis sustain the influence of these patterns on tumor vascular invasion and also a direct interrelation with tumor grade.

Each pattern can be predominantly, but not exclusively, in the same tumor, something that could be an exhaustive aspect of our classification. Chromophobe and unclassified types seem to have a special vascular architecture, but the limited number of cases prevents us from achieving correlation with prognostic or therapeutic purpose.

The presence of glomeruloid bodies along with the described patterns, suggests an active angiogenesis, sequential in renal tumors, that can not be characterized in terms of time of evolution and development of the renal tumors, because most tumors included in the study were diagnosed in advanced stages.

The authors declare no conflict of interests.

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