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CD34 immunostaining enhances a distinct pattern of intratumor angiogenesis with prognostic implications in clear cell renal cell carcinoma

JOSÉ I. LÓPEZ,[1,2] ASIER ERRAMUZPE,[3] ROSA GUARCH,[4] JESÚS M. CORTÉS,[3,5,6] RAFAEL PULIDO,[2,5] ROBERTO LLARENA[7] and JAVIER C. ANGULO[8]

[1]Department of Pathology, Cruces University Hospital, University of the Basque Country (UPV/EHU); [2]Biomarkers in Cancer Unit, BioCruces Health Research Institute; [3]Quantitative Biomedicine Unit, BioCruces Health Research Institute, Barakaldo, Bizkaia; [4]Department of Pathology, Complejo Hospitalario B de Navarra, Pamplona, Navarra; [5]Ikerbasque, Basque Foundation for Science, Bilbao; [6]Department of Cell Biology and Histology, University of the Basque Country (UPV/EHU), Leioa; [7]Department of Urology, Cruces University Hospital, University of the Basque Country (UPV/EHU), Barakaldo, Bizkaia; and [8]Department of Urology, Hospital de Getafe, Universidad Europea de Madrid, Madrid, Spain

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Clear cell renal cell carcinoma is an aggressive neoplasm related to *VHL* gene inactivation. The molecular events derived from this initial alteration lead to a permanent intracellular pseudo-hypoxic status that stimulates vascular proliferation. The resulting increased intratumor angiogenesis is the target of most modern therapies. Although intratumor angiogenesis has received full attention in the last years, few studies have focused on its potential importance from a strict morphological approach. Intratumor angiogenesis has been analyzed in a retrospective series of clear cell renal cell carcinomas (n = 208) with long-term follow-up (n = 177). Two different patterns of angiogenesis have been highlighted with CD34 at the front of tumor invasion, termed continuous and discontinuous, respectively. The continuous pattern of angiogenesis showed a complete microvascular network surrounding totally tumor nests. Conversely, the discontinuous pattern displayed an incomplete network around tumor nests. The continuous pattern was associated to shorter 5-year (p = 0.00064, hazard ratio = 2.8) and 15-year (p = 0.014, hazard ratio = 1.7) survivals. Cox regression multivariate analysis also showed that the continuous pattern (p = 0.016373) remains a significant variable when considered together with grade (p = 0.001755) and stage (p = 0.000952). These findings support the notion that a continuous $CD34^{+}$ pattern of intratumor angiogenesis may be useful for pathologists in predicting tumor behavior in clear cell renal cell carcinomas.

Key words: Clear cell renal cell carcinoma; angiogenesis; CD34; prognosis; survival.

José I. López, Department of Pathology, Hospital Universitario Cruces, Plaza de Cruces s/n, 48903 Barakaldo, Bizkaia, Spain. e-mail: joseignacio.lopez@osakidetza.eus

Renal cell carcinoma is a common neoplasm in Western Countries, with more than 62 000 new cases expected in the United States in 2016 (1). Clear cell renal cell carcinoma (CCRCC) is the most frequent histological subtype, accounting for 70–80% of the cases (2). CCRCC is an aggressive radio- and chemo-resistant neoplasm and only surgery has demonstrated a significant impact in patient survival, with a 5-year overall mortality still reaching around 40% (3).

Truncal mutations in *VHL* gene is the hallmark of this disease, but therapies against this target have obtained only partial results. The intrinsic high intratumor heterogeneity displayed by CCRCC is a major obstacle to implement efficient therapies with significant impact in survival (4).

VHL-associated neoplasms, such as hemangioblastoma and CCRCC, are characterized by a high vascularity (5). When the tumor-suppressor

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gene *VHL* is inactivated, its resulting defective VHL protein does not participate in the degradation of the hypoxia-inducible factor (HIF), which accumulates in the nucleus leading to the transcription of several genes involved in tumor angiogenesis, among them the *VEGF* gene (6). The effective blockade of VEGF and mTOR pathways leads to a reduced angiogenesis and are fundamentals of current therapies in CCRCC. However, the high temporal and spatial intratumor heterogeneity (7), the wide variety of genes upregulated through the maintenance of HIF activity (8), and the development of resistance to these drugs (6) explain why the therapeutic results obtained so far are somehow disappointing.

A retrospective series of CCRCC has been analyzed with the focus in the intratumor angiogenesis. We, here, define a pattern of intratumor angiogenesis highlighted with CD34 immunostaining which correlates with patient survival.

MATERIALS AND METHODS

The authors declare that all the experiments carried out in this study comply with current Spanish and European Union legal regulations. Samples and data from patients included in this study were obtained from the medical records and archives of Pathology Lab. All patients were informed about the potential use for research of their surgically resected tissues and accepted this eventuality by signing a specific document approved by the Ethical and Scientific Committees (CEIC 2015/060, CEIC-E PI2015101).

A total of 208 CCRCC were included in the study in a retrospective way. The series included 179 total nephrectomies and 29 partial nephrectomies of patients without previous tyrosine-kinase inhibitors treatment. Follow-up was obtained from the clinical histories and was closed at December 31, 2014. Cases were reviewed by two pathologists (JIL, RG), who assigned Fuhrman grade (9) and 2010 AJCC Staging System (10) on hematoxylin–eosin sections from tumor samples obtained following standard protocols.

Tissue microarrays (TMA) were performed for the evaluation of CD34 expression in intratumor microvessels. Two distant cores (2.5 mm in diameter) of well-preserved tumor tissue obtained at the front of invasion into the renal parenchyma were selected for TMA in each case to take into consideration the possibility of intratumor heterogeneity at this specific area of the tumor. CD34 antibody (Ventana, QBEnd/10, catalog number 790–2927, ready to use) was evaluated in the capillary network surrounding neoplastic nests. Immunohistochemical stainings were performed in an automated immunostainer (BenchMark Ultra, Ventana Medical Systems, Tucson, AZ, USA) following routine methods. Tris-EDTA was used for antigen retrieval. Negative controls were slides not exposed to the primary antibody, and these were incubated in PBS and then processed under the same conditions as the test slides. The analysis was performed using a Nikon Eclipse 80i microscope (Tokyo, Japan).

R software was used for statistical analysis. Kaplan–Meier (KM) curves and log-rank p values are displayed after performing a univariate analysis to compare 5-, 10-, and 15-year survivals. Hazard ratio (HR) was also computed. R's 'survplot' module was used to create the KM curves and 'coxph' function to calculate the HR and log-rank p values. Cox regression multivariate analysis was performed to identify which variables will remain significant if all variables were considered altogether. Classic histopathological parameters (Fuhrman grade, tumor staging) and vascular architecture (continuous/discontinuous) were included in this analysis. Grade was grouped as low (G1/G2) and high (G3/G4) and Stage as low (pT1/2) and high (≥pT3).

RESULTS

Males predominated in the series (157 M/51 F) with an average age of 66 years (range, 25–93 years). Follow-up data were obtained in 177 (85%) patients and oscillated between 1 and 240 months (median, 89.7 months). A total of 80 patients died of disease (38.5%). Average tumor diameter was 6.22 cm (range, 1–19 cm), 67 (32.2%) cases being ≤4 cm and 141 (68.8%) cases >4 cm. Fuhrman's grade distribution was as follows: 35G1 (16.8%), 99G2 (47.6%), 47G3 (22.6%), and 27G4 (13%), 134 cases (64.4%) being low grade (G1/2) and 74 (35.6%) high grade (G3/4).

This study focused specifically on intratumor angiogenesis in the front of invasion in two distant regions of the tumor. For a more precise analysis of this phenomenon, the angiogenesis pattern within the tumors was evaluated in CD34-stained slides. The microscopic study revealed a distinct pattern of angiogenesis surrounding the tumor nests with a continuous disposition in 82 cases (Fig. 1). This peculiar CD34 vascular arrangement was detected with difficulties on hematoxylin–eosin-stained sections. The remainder cases in the series displayed several degrees of microvascular density, but always the pattern of distribution of the vessels around the tumor nests was discontinuous (Fig. 2).

Interestingly, the continuous microvascular pattern in the front of tumor invasion, detected at least in one of the two regions sampled, was associated to a poorer survival in CCRCC. KM survival curves and multivariate regression analysis were performed in 177 patients with available clinical follow-up, 66 (37.2%) of them displaying continuous and 111 (62.8%) discontinuous $CD34^+$ angiogenic patterns. A significant difference in survival between the two groups after 5 ($p = 0.00064$, $HR = 2.8$) and 15 ($p = 0.014$, $HR = 1.7$) years of follow-up (Fig. 3). As expected, tumor diameter (cut-off, 4 cm), grade (low/high), and stage (T1/2 vs ≥pT3) also displayed significant differences in survival with $p = 0.000028$,

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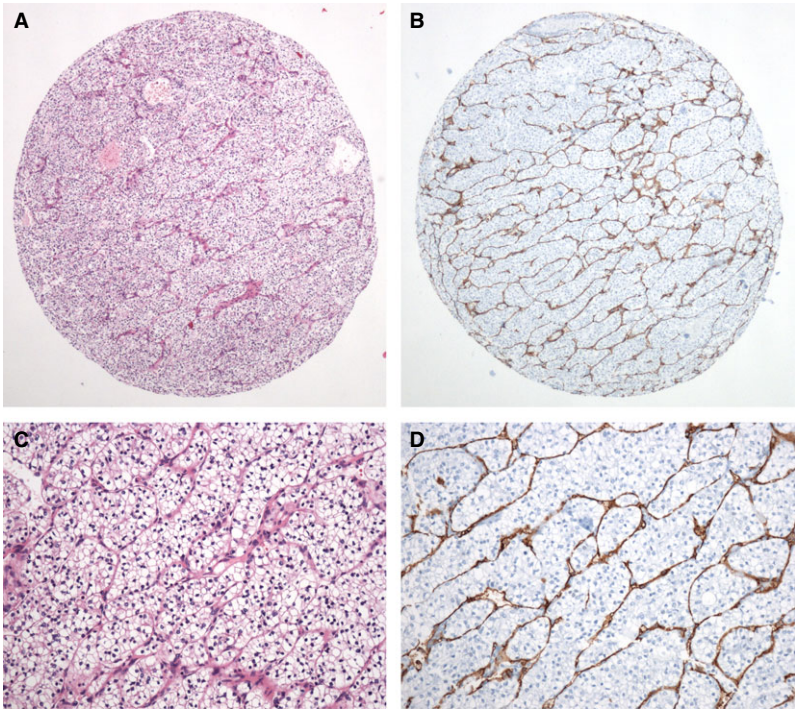

Fig. 1. Continuous pattern of angiogenesis in clear cell renal cell carcinoma stained with hematoxylin–eosin and CD34. The vascular network on hematoxylin–eosin sections both on low (A) and high-power (C) views is unremarkable. However, CD34 (B and D) staining highlights a continuous pattern of angiogenesis surrounding completely the tumor nests.

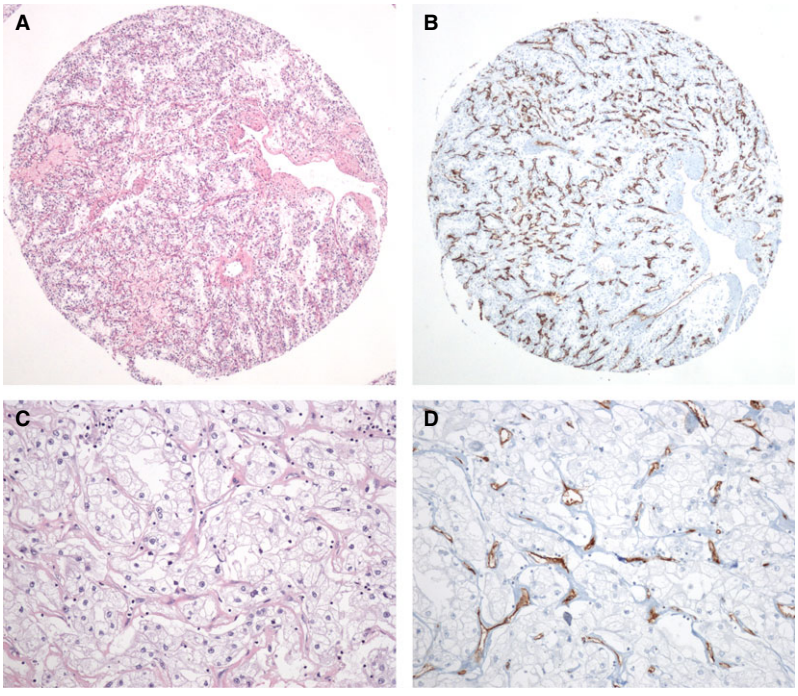

Fig. 2. Discontinuous pattern of angiogenesis in clear cell renal cell carcinoma stained with hematoxylin–eosin and CD34. The vascular network on hematoxylin–eosin sections both on low (A) and high-power (C) views is unremarkable. However, CD34 (B and D) staining shows a discontinuous pattern of angiogenesis surrounding incompletely the tumor nests.

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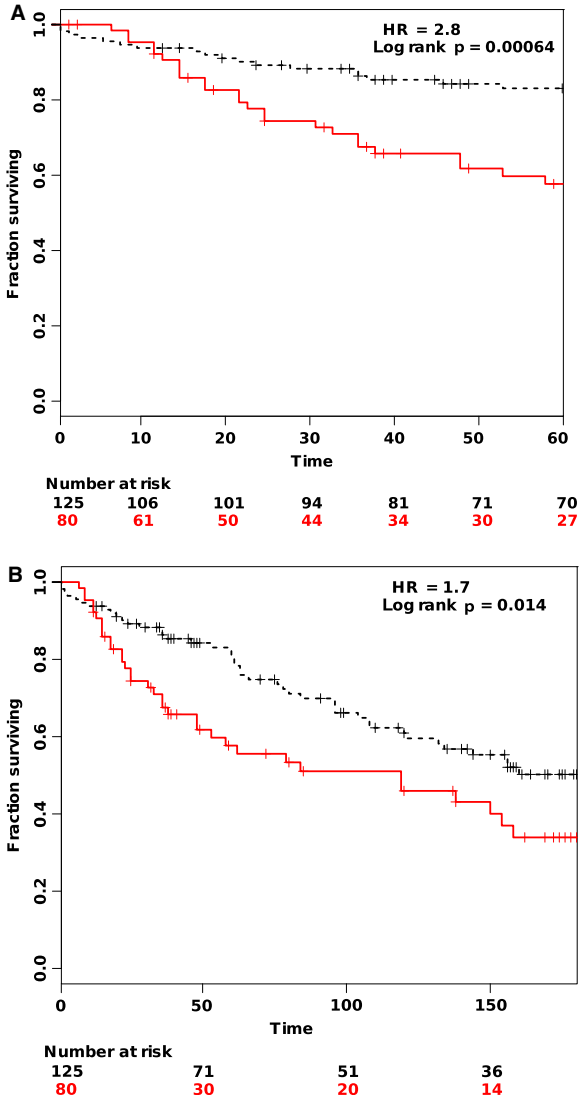

Fig. 3. Five (A) and 15-year (B) Kaplan–Meier survival curves. In both cases, the continuous pattern of angiogenesis (continuous red line) shows significant poorer survival numbers compared with discontinuous pattern (discontinuous black line).

p = 0.0000012, and p = 0.0000000000085 values at 15 years, respectively.

Finally, Cox regression multivariate analysis showed that the continuous microvascular pattern (p = 0.016373) remained a significant variable when considered together with grade (p = 0.001755) and stage (p = 0.000952) (Table 1).

Table 1. Univariate regression analysis

Variable	p-value
Grade (low/high)	0.000000339
Stage (T1/2 vs ≥T3)	0.0000000181
Vascular pattern (continuous/ discontinuous)	0.0154
Multivariate regression analysis	
Grade (low/high)	0.001755
Stage (T1/2 vs ≥T3)	0.000952
Vascular pattern (continuous/ discontinuous)	0.016373

DISCUSSION

Decades before knowing the intricate molecular mechanisms underlying intratumor angiogenesis in CCRCC and their importance for treatment, pathologists had already recognized – based only on hematoxylin–eosin sections – that this neoplasm was characterized by a particular and profuse vascular supply (11, 12). Later on, the clinical prominence of the vascular network in CCRCC has received specific attention (13–21).

Intratumor microvessel density has been associated with tumor aggressiveness, high stage at clinical presentation, greater incidence of metastases, and poorer survival in many neoplasms, including renal cancer (14). By the end of the last century, however, results were contradictories. While MacLennan and Bostwick (13) did not find in 1995 any correlation between microvessel density and tumor-specific survival in renal cell carcinomas, Nativ et al. in 1998 (15) showed that angiogenesis could be a useful parameter in predicting survival. Later, this controversy still continues (17, 18, 20). A step ahead in the knowledge of the influence of intratumor vascularity in the prognosis of CCRCC consisted in recognizing its architectural angiogenic complexity (16), the intrinsic characterization of these new vessels (19, 21), and the co-influence of some stromal components (22).

A fractal analysis of tumor microvasculature complexity was performed by Sabo et al. (16) in a series of 49 low-grade CCRCC. These authors concluded that a complex intratumor vasculature correlates with a better prognosis, supporting the idea that an increased microvascular density correlates with an improved patient survival (16). However, other authors have focused on the diverse quality of these vessels. Yao et al. (19) recognized two different types of blood vessels with prognostic implications in CCRCC. The authors distinguished undifferentiated ($CD31^+/CD34^-$) and differentiated ($CD34^+$) intratumor blood vessels and concluded that a higher undifferentiated vascularization correlates with higher grade and shorter survival (19). In the same sense, Sato et al. (21) distinguished between immature ($CD34^+$/smooth-muscle $actin^-$) and mature ($CD34^+$/smooth-muscle $actin^+$) intratumor blood vessels and found that areas with

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immature vessels seem to be associated with CCRCC aggressiveness.

In this study, however, only the architectural disposition of the intratumor vessels stained with CD34 has been taken into account, as this morphological feature can be easily recognized under routine diagnostic conditions. Aside from the classical parameters i.e., stage and grade, the identification of a continuous $CD34^+$ vascular pattern in the routine practice may help the pathologist in assigning the prognosis of CCRCC since this finding is an independent factor in the multivariate analysis.

Stromal tumor components have shown a critical influence in the biology of CCRCC. We have shown very recently the prognostic importance of fibroblast-associated protein (FAP) immunostaining in the stromal fibroblasts of CCRCC (23), and others have identified a similar role of tumor-associated macrophages with respect to tumor angiogenesis (22). These findings stress the importance of tumor microenvironment in tumor development and open a promising field for future research.

The relationship of prognosis and survival with angiogenesis in CCRCC is also being approached by molecular techniques (24–28). *VHL* gene inactivation is a major driver in the CCRCC pathogenesis. The resulting loss of function of VHL protein promotes a pseudo-hypoxic intracellular environment that lead to a constant HIF activation. This cascade of intracellular events provokes tumor cell proliferation and accelerated angiogenesis via the stimulation of VEGFR (24, 27). Recent studies have stressed the heterogeneous effect of the hypoxia pathway depending on the activation of HIF-1α or HIF-2α isoforms, associated to favorable and adverse prognoses, respectively (28). mRNA expression profiling in 150 CCRCC has shown important differences in both microvascular density and endothelial cell proliferation (26). The authors conclude that high-grade CCRCC display higher endothelial cell proliferation and lower microvascular density than low-grade CCRCC, a finding that may have potential implications in anti-VEGFR therapies (26).

This study describes a continuous $CD34^+$ immunostaining pattern of angiogenesis at the tumor invasion front that is associated with high grade, high stage, and short survival in CCRCC. This continuous $CD34^+$ pattern consists of a delicate vascular network surrounding completely the tumor nests. This pattern is grade and stage independent (Table 1), and it has been detected in up to 37% of the cases. We consider that this vascular pattern may provide valuable information for pathologists when assigning criteria of aggressiveness in CCRCC.

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