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Original article

Microvascular density, macrophages, and mast cells in human clear cell renal carcinoma with and without bevacizumab treatment

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Abstract

Background: Clear cell renal cell carcinoma (ccRCC) represents a highly vascularized aggressive kidney cancer. Due to ccRCC chemotherapy resistance, antiangiogenesis is one of the most innovative targeted therapies for this tumor. The tumor microenvironment exerts important roles in tumor growth, angiogenesis, and metastatic escape.

Materials and methods: In this study, we investigated the composition of tumor cell microenvironment including mast cells, macrophages, and microvascular density in ccRCC tumor tissues collected from patients who underwent nephrectomy treated or not with bevacizumab as neoadjuvant therapy before surgery.

Results: The results of this study indicate that bevacizumab-treated ccRCC samples present reduced microvascular density as well as a lower number of CD68-positive macrophages and tryptase-positive mast cells in comparison with the untreated patients.

Conclusions: It follows that the antiangiogenic activity of bevacizumab may be due to a direct effect on angiogenic cytokines released by tumor cells and an indirect effect on the release of pro-angiogenic factors by inflammatory stromal cells.

Keywords: Angiogenesis; Bevacizumab; Antiangiogenesis; Renal cell carcinoma

1. Introduction

Renal cell carcinoma (RCC) represents the sixth most frequent cancer in men and the 10th in women, worldwide, accounting for 5% and 3% of all oncological diagnoses, respectively [1,2]. Clear cell RCC (ccRCC) accounts for 70%–80% of kidney cancer, and represents the most aggressive subtype [3]. There are a variety of risk factors associated to ccRCC including smoking, obesity, von Hippel-Lindau disease, and hypertension [2]. The ccRCC 5-year survival rate is ~92% as regards the localized and early detected cases, 67% in locally spreaded cases and 12% in systemically spreaded cases. Localized disease is curable by surgery; however, locally advanced or metastatic disease is not curable in most cases due to the limited response to drug treatment. Due to ccRCC high resistance to most of the current cytotoxic drugs and its high vascular supply, novel agents targeting angiogenesis have been introduced in its treatment. Vascular endothelial growth factor (VEGF) and VEGF receptor-2 are highly expressed in renal cell in RCC [4–6]. The association between the increased microvessel density and poor prognosis in RCC date back to more than 20 years ago [7].

The antiangiogenic agents used in the treatment of RCC including sorafenib, sunitinib, pazopanib, axitinib, and bevacizumab, are generally well tolerated and are considered first– and second–line treatment options for patients with

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advanced ccRCC [8–10]. These drugs are approved standard of care treatments options in first-line and second-line, after showing improved progression-free survival (PFS) in randomized phase III trials compared with interferon-α, placebo, or other targeted agents [11–13]. Response rates and duration show high variability, and adverse effects have a major influence on patient's quality of life [14].

These antiangiogenic agents cause initial tumor responses but in most of the cases, it has been observed the disease recurrence is due to the development of treatment resistance. Acquired resistance to VEGF pathway inhibitors is thought to be mediated through the angiogenic escape consisting in the activation of alternative signaling pathways that restore tumor perfusion. Identification of these pathways may enable better treatment of VEGF-driven disease and ultimately be the key to the development of combination regimens with more durable clinical benefit [15]. Moreover, antiangiogenic treatment has been shown to select for hypoxia-tolerant or vessel-independent tumor cells or a tumor vasculature that arises in the absence of angiogenesis. Thus, blocking angiogenesis may lead to the occurrence of more aggressive tumor cells and increased metastasis [16–19]. In recent years, many investigators have suggested that interactions between cancer cells and the surrounding stromal microenvironment exert important roles in the malignant aggressiveness in many types of solid tumors, including RCC [20–22].The tumor microenvironment contains a heterogeneous and complex mixture of stromal cells including fibroblasts, pericytes, endothelial, mesenchymal, and cells of the hematopoietic system including lymphocytes, mast cells, and macrophages that actively support tumor growth, angiogenesis and are associated with resistance to anti-VEGF therapy [23].

In this study, we investigated the composition of tumor cell microenvironment including mast cells, macrophages, and microvascular density in ccRCC primary renal tumor tissues collected from patients who underwent nephrectomy treated or not with bevacizumab as neoadjuvant therapy before surgery.

2. Materials and methods

2.1. Patients

A total of 48 primary renal tumor tissues were collected from patients who underwent nephrectomy for ccRCC. Twenty four patients with locally advanced disease were treated with bevacizumab as neoadjuvant therapy at a dose of 10 mg/kg IV every 2 weeks for 8 weeks before surgery. All patients were preoperatively staged by thoracoabdominal computed tomography or magnetic resonance imaging. Drug-induced primary tumor response was assessed by computed tomography scans according to the Response Evaluation Criteria in Solid Tumors. Tumor staging was reassigned according to the seventh edition of the AJCC-UICC TNM classification. The 2016 World Health Organization and Fuhrman classifications were used to attribute histological type and nuclear grade, respectively. Written informed consent to take part was given by all participants. The protocol for the research project has been approved by the local Ethics Committee

Table 1
Clinical and pathological data.

Patients ccRCC treated		*n*	%	Patients ccRCC untreated		*n*	%
		24	100.00			24	100.00
Sex	Male	18	75.00	Sex	Male	18	75.00
	Female	6	25.00		Female	6	25.00
Age	40–49	14	58.33	Age	40–49	4	16.67
	50–59	0	0.00		50–59	8	33.33
	60–69	6	25.00		60–69	2	8.33
	70–80	4	16.67		70–80	10	41.67
Bevacizumab neoadjuvant therapy	8 wks (10 mg/kg every 2 wks)			Bevacizumab neoadjuvant therapy	no		
Isotype	ccRCC	24	100.00	Isotype	ccRCC	24	100.00
Max. diameter (cm)	<5	4	16.67	Max. diameter (cm)	<5	8	33.33
	6–10	10	41.67		6–10	12	50.00
	11–15	4	16.67		11–15	4	16.67
	>15	6	25.00		>15	0	0.00
G	1	0	0.00	G	1	2	8.33
	2	10	41.67		2	11	45.83
	3	12	50.00		3	8	33.33
	4	2	8.33		4	3	12.50
pT	1	4	16.67	pT	1	10	41.67
	2	2	8.33		2	10	41.67
	3	14	58.33		3	4	16.67
	4	4	16.67		4	0	0.00

ccRCC = clear cell renal cell carcinoma; G = grade.

and conforms to the provisions of the Declaration of Helsinki in 1995. Tissue samples were fixed in formalin and embedded in paraffin according to standard procedures. Four-micrometer-thick sections were cut and mounted on glass slides. Clinical and pathological data of patients are reported in Table 1.

2.2. Tryptase, CD68, CD163, and CD31 immunohistochemistry

Histological sections, collected on poly-L-lysine-coated slides (Sigma Chemical, St Louis, MO), were deparaffinized. The sections were rehydrated in a xylene-graded alcohol scale and then rinsed for 10 minutes in 0.1M PBS. Sections were pretreated with sodium citrate pH 6.1 (Dako Corporation, Milan, Italy) in Dako PT Link for antigen retrieval solution for 30 minutes at 98˚C and then incubated with mouse monoclonal antitryptase (NB-100-64820, Novus Biologicals, Littleton, CO), rabbit polyclonal anti-CD31 (ab28364, Abcam, Cambridge, UK) to stain endothelial cells, mouse monoclonal anti-CD68 (NCL-CD68-KP1, Novocastra Laboratories Ltd, Newcastle, UK) to stain total tumor associated macrophages (TAMs), and mouse monoclonal anti-CD163 (NCL-L-CD163, Novocastra Laboratories Ltd, Newcastle, UK) to stain M2 TAMs subpopulation, diluted according to the respective datasheet indications. Thereafter, the sections were counterstained with Mayer hematoxylin and mounted in synthetic medium. Specific preimmune serum (Dako), replacing the primary antibodies, served as negative control. Sections from both experimental groups, were scanned using the whole-slide morphometric analysis scanning platform Aperio Scanscope CS (Leica Biosystems, Nussloch, Germany). All the slides were scanned at the maximum available magnification (40×) and stored as digital high resolution images on the workstation associated with the instrument. Digital slides were inspected with Aperio ImageScope v.11 software (Leica Biosystems, Nussloch, Germany) at 20× magnification and 10 fields with an equal area were selected for the analysis at 40× magnification. The protein expression was assessed with the Positive Pixel Count algorithm embedded in the Aperio ImageScope software and reported as positivity percentage, defined as the number of positively stained pixels on the total pixels in the image. Data related

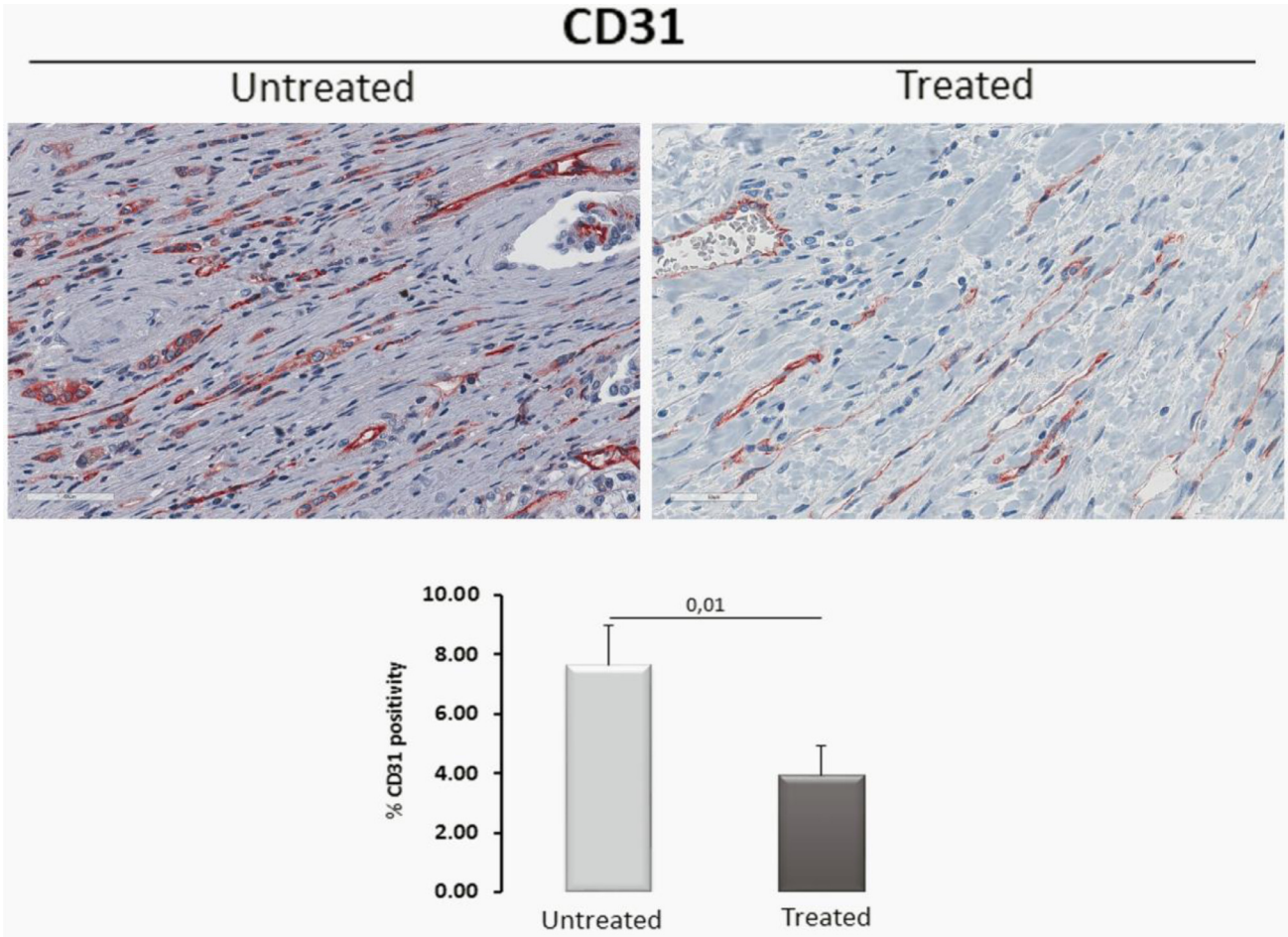

Fig. 1. Immunohistochemical staining of CD31 microvessels in bevacizumab treated and untreated ccRCC tumor samples. The percent of CD31 positive microvessel resulted decrease in treated specimens as compared to untreated. Scale bar: 60 μm. ccRCC = clear cell renal cell carcinoma.

to the 2 experimental groups, treated and untreated samples are reported as means ± SEM. Newman–Keuls multiple comparisons post-test was used to compare all treatment groups after 1-way ANOVA. The Graph Pad Prism 5.0 statistical package (GraphPad Software, San Diego, CA) was used for analyses and the limit for statistical significance was set at $P < 0.05$.

As concerns the evaluation of the association between CD31, tryptase, and CD68 expression and patient survival in bevacizumab-treated group, statistical calculations were performed with MedCalc 9.2.0.1 (MedCalc software, Mariakerke, Belgium) and PASW 18 software (PASW 18, SPSS, Chicago, IL). Comparisons of median values between different groups were evaluated by Mann–Whitney *U* test. Receiver operating characteristic curve analysis was performed to identify the CD31, CD68, and tryptase expression cut-offs for survival stratification. Spearman's correlation was applied to evaluate associations between these markers and primary tumor size reduction (%) in treated patients. A *P* value of < 0.05 was considered statistically significant.

3. Results

3.1. CD31 expression

The vascular density in histological specimens derived by bevacizumab treated and untreated ccRCC patients has been evaluated through the CD31 immunostaining. It has found the significant lower number of $CD31^+$ microvessels in bevacizumab treated specimens (3.94% ± SE 0.9%) as compared to the untreated (7.67% ± SE 1%) (Fig. 1).

3.2. Tryptase, CD68, and CD163 expression

Bevacizumab treated and untreated ccRCC specimens were immunostained for tryptase (Fig. 2), CD68 (Fig. 3), and CD163 (Fig. 4) to estimate mast cells, total TAMs, and M2 TAMs subpopulation. As concerns tryptase-positive mast cells present in the tissue, their number resulted significantly lower in treated specimens (0.08% ± SE 0.01%) as compared to the untreated (0.87% ± SE

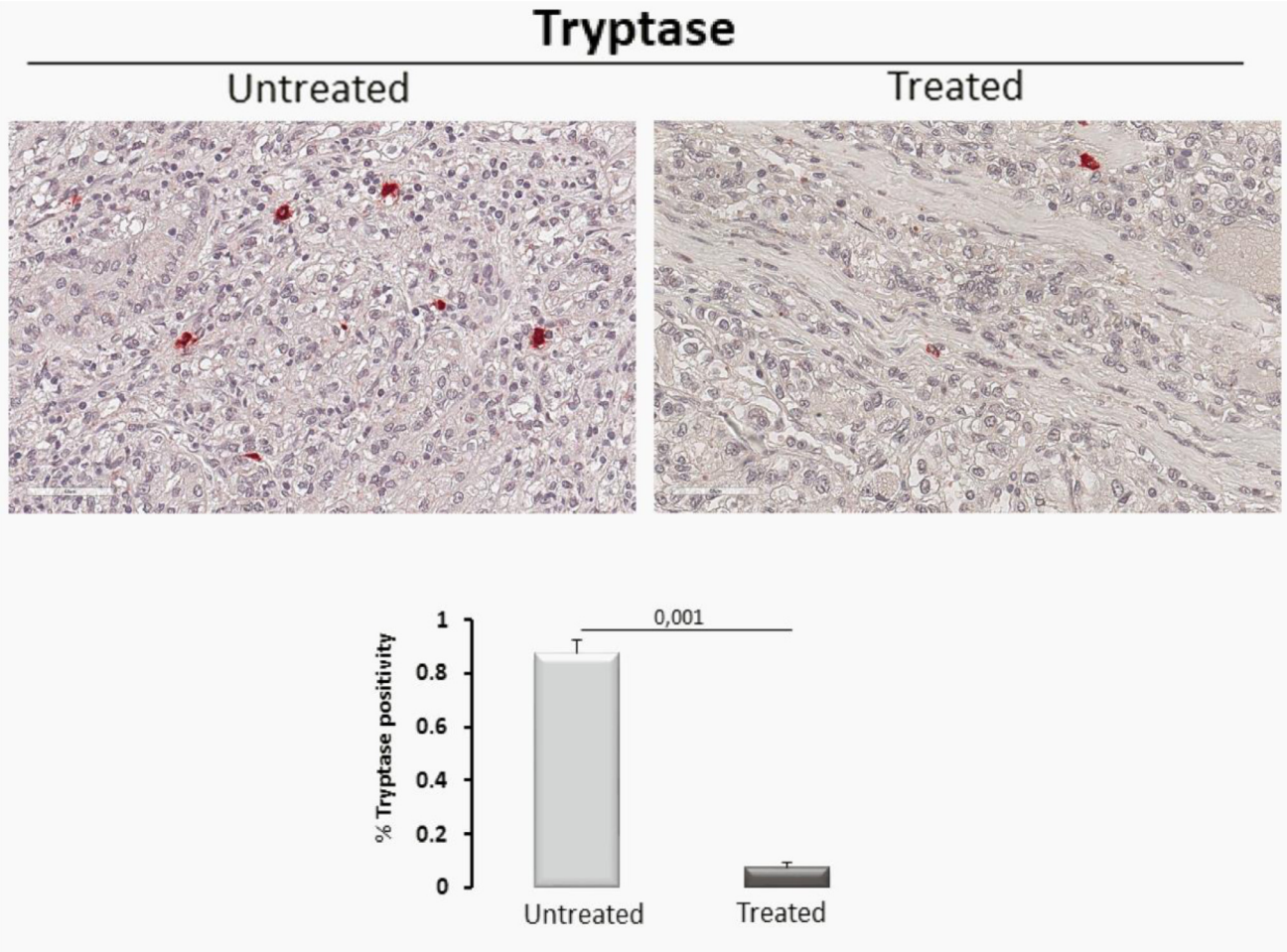

Fig. 2. Immunohistochemical staining of tryptase in bevacizumab treated and untreated ccRCC tumor samples. The percentage of tryptase positive mast cells decreases in treated specimens as compared to untreated. Scale bar: 60 μm. ccRCC = clear cell renal cell carcinoma.

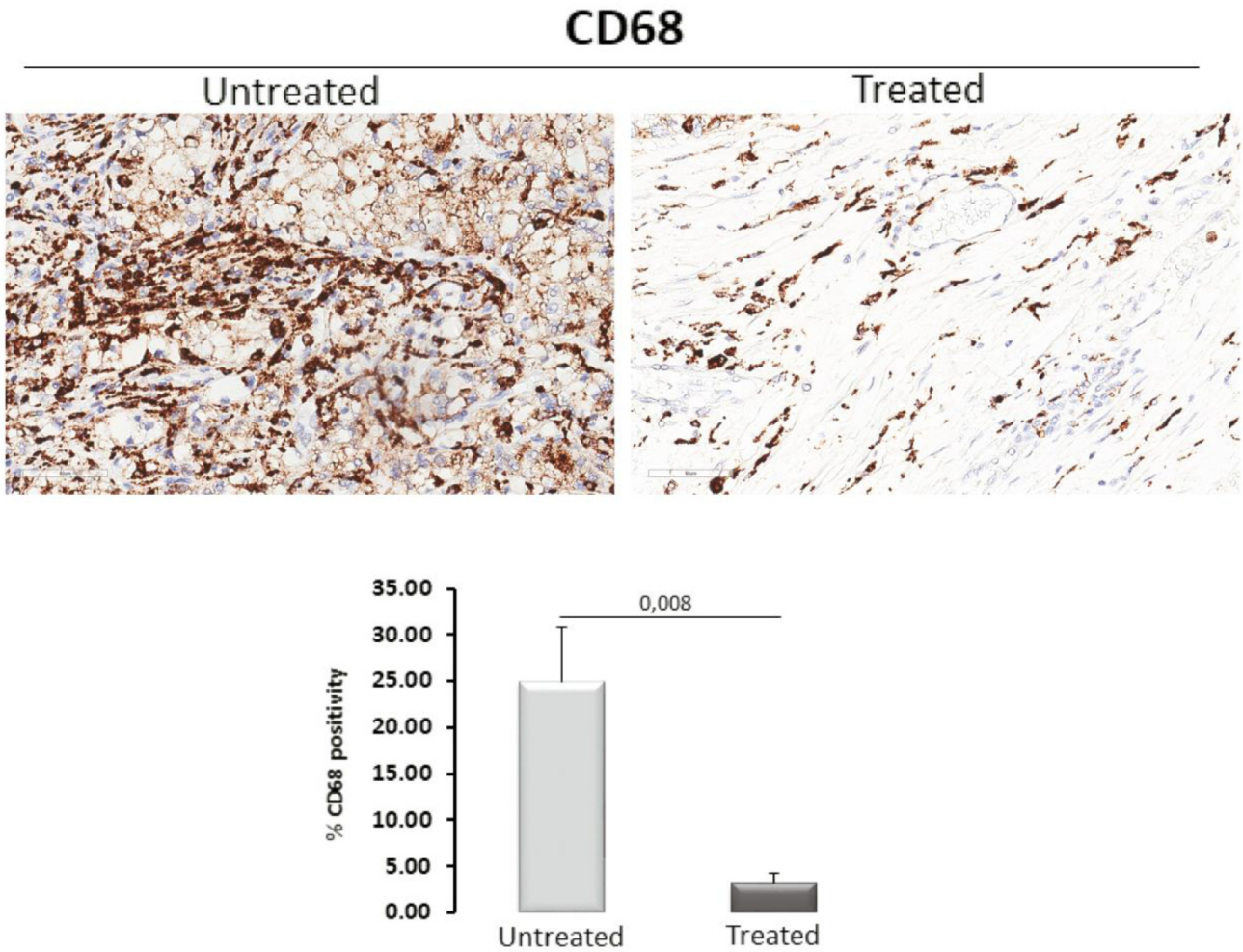

Fig. 3. Immunohistochemical staining of CD68 in bevacizumab treated and untreated ccRCC tumor samples. The percent of CD68 positive macrophages decreases in treated specimens as compared to untreated. Scale bar: 60 μm. ccRCC = clear cell renal cell carcinoma.

0.05%) specimens (Fig. 2). As concerns $CD68^{+}$ macrophages, their number in untreated patient samples is very high (24.9% ± SE 3.2%) but it revealed significant decreas in bevacizumab treated specimens (5.9% ± SE 1.1%) (Fig. 3). The number of CD163-positive TAMs in both bevacizumab treated (3.16% ± SE 0.8%) and untreated (3.4% ± SE 0.2%) samples does not show any variation (Fig. 4).

The tumor size showed a significant reduction after treatment with bevacizumab for 8 weeks (Fig. 5). Moreover, the patients with shorter cancer-specific survival and PFS, showed higher number of CD68-positive macrophages and tryptase-positive mast cells, in association with an increased microvascular density (Fig. 6). Finally, in the bevacizumab-treated group, we found a statistically significant inverse correlation between primary tumor size reduction and CD31 ($rs = -0.81$, $P = 0.0001$), CD68 ($rs = -0.44$, $P = 0.01$), and trypatse ($rs = -0.61$, $P = 0.001$) expression values.

4. Discussion

Inflammation is strongly correlated with cancer, implying a role for the inflammatory infiltrate to enhance the development of malignances. Inflammatory cells establish a crosstalk with tumor cells, inflammatory cells, and endothelial cells to create a complex microenvironment essential for the survival and development of the malignancy [24–26]. Specifically, TAMs and mast cells have been demonstrated to have effects on both tumor growth and angiogenesis, producing several pro-angiogenic cytokines [27,28].

TAM infiltration in RCC microenvironment contributes to cancer progression and metastasis by stimulating angiogenesis, tumor growth, and cellular migration and invasion [29].

The role of mast cells in ccRCC and their relation to the tumor angiogenesis in renal carcinoma has not yet been well-defined. In normal human kidney, there are few mast cells located in the interstium of the renal cortex between

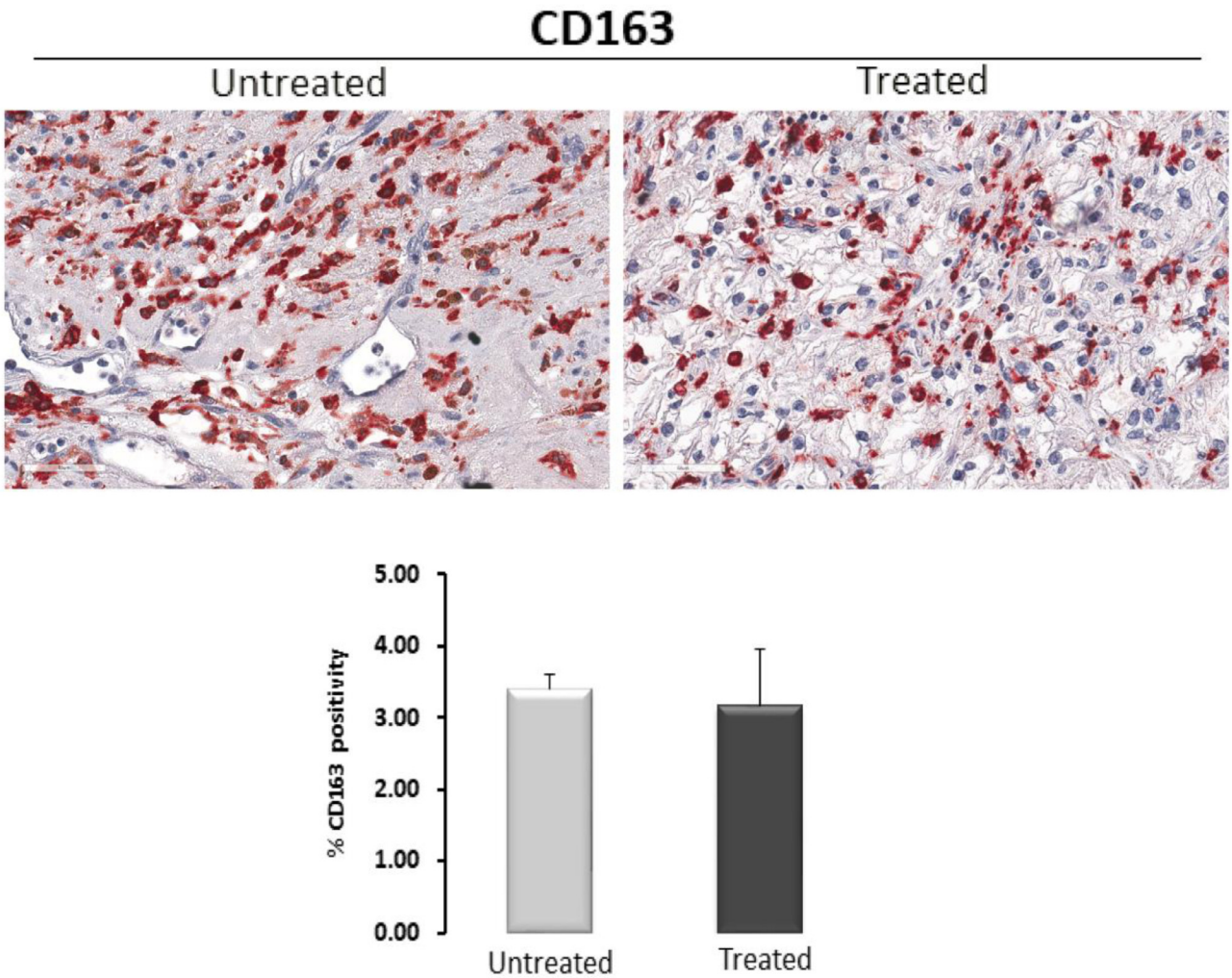

Fig. 4. Immunohistochemical staining of CD163 in bevacizumab treated and untreated ccRCC tumor samples. The percent of CD163 positive macrophages remains unchanged in both treated and untreated specimens. Scale bar: 60 μm. ccRCC = clear cell renal cell carcinoma.

blood vessels and renal tubules [30]. Tuna et al. [31] reported a significant correlation between mast cells with microvessel density in RCC [31]. On the contrary, Mosheni et al. [32] and Guldur et al. [33] reported lack of correlation between mast cells and microvessel density in RCC. Moreover, Guldur et al. found that mast cells were more numerous in ccRCC than in non-ccRCC, and they found that mast cells were concentrated in tumoral tissue and peritumoral inflammatory renal tissue compared to the nontumoral renal parenchyma [33].

The results of this study indicate that histological sample of bevacizumab treated ccRCC patients present a lower number of CD68-positive macrophages and tryptase-positive mast cells in comparison with the untreated patients. The reduction of tryptase- and CD68-positive cells is associated to the reduction of microvascular density. In this context, it has been confirmed the antiangiogenic activity of bevacizumab, but also its effects on the number of mast cells and macrophages in tumor microenvironment. Bevacizumab alone is efficacious also in the treatment of metastatic diseases, as it has been demonstrated in a randomized trial showing that inhibition of VEGF using single agent bevacizumab led to prolonged PFS [34].

TAMs are generally categorized in 2 types, M1 (classically activated) and M2 (alternatively activated). M1 TAMs are able to kill microorganisms as well as tumor cells and secrete high levels of pro-inflammatory cytokines and tumoricidal agents. M2 TAMs have poor attitude to destroy tumor cells, are better adapted to promoting angiogenesis, repairing, and remodeling wounded or damaged tissues, and suppressing adaptive immunity, and are a target for anticancer therapy [35].

CD68 positive cells include both M1 and M2 subpopulations, while CD163 marks selectively M2 subpopulation. In this study, we have demonstrated that the number of CD68-positive cells is significantly decreased in bevacizumab treated as compared to untreated bioptic ccRCC samples, while the number of CD163-positive cells corresponding to M2 subpopulation is unchanged in both bevacizumab treated and untreated bioptic ccRCC samples. Our evidence

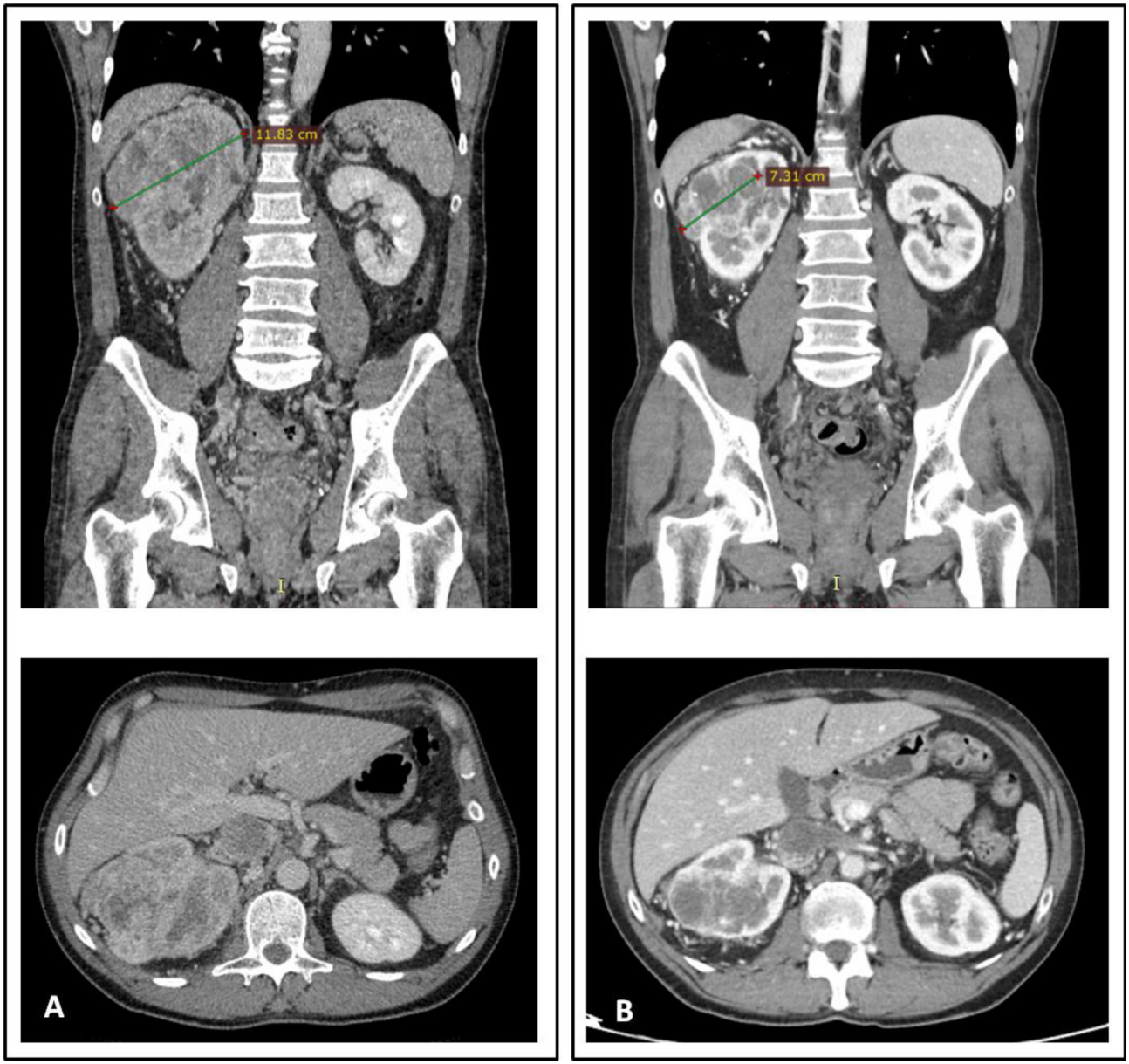

Fig. 5. CT scan showing that primary renal tumor (A) significantly reduces its size after treatment with bevacizumab for 8 weeks (B). CT = computed tomography.

showing an unvaried number of CD163-positive cells in histological samples of bevacizumab treated ccRCC patients and untreated ones might be related to the evidence that in ccRCC CD163 marks macrophages and also cancer cells [36].

It follows that the antiangiogenic activity of bevacizumab might be due to a direct effect on angiogenic cytokines released by tumor cells and an indirect effect on the release of pro-angiogenic factors by inflammatory stromal cells.

Several immunotherapies have been approved by the Food and Drug Administration for use in advanced kidney cancers, including the anti-PD-1 checkpoint inhibitor nivolumab, the combination of nivolumab (Opdivo) and the anti-CTLA-4 checkpoint inhibitor ipilimumab (Yervoy). The combination of antiangiogenic therapy with immunotherapy has been proposed as a potential new therapeutic approach for the treatment of RCC [37]. De Liu et al. [38] have demonstrated that in RCC the antiangiogenic therapy is associated with an increased infiltration of T lymphocytes and of Tregs, suggesting that PD-L1 blockade might improve the antitumor activity of T lymphocytes and the efficacy of antiangiogenic therapy in RCC.

Acquired resistance to antiangiogenic therapy also involves alterations of the tumor microenvironment. Castro et al. [39] have demonstrated that in bevacizumab-resistant glioblastoma patients, the number of TAMs is increased with a M2 polarization which, in turn, promote tumor cell proliferation and invasive growth as an expression of

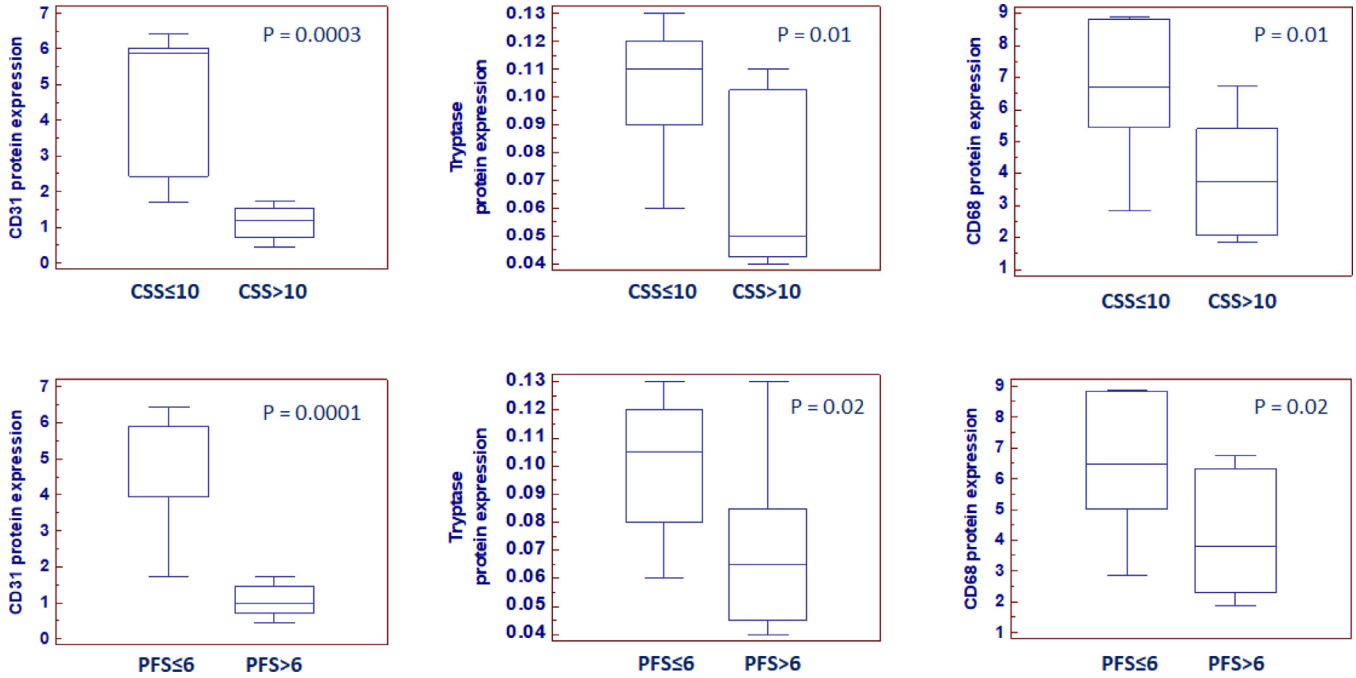

Fig. 6. Association between CD31, tryptase, and CD68 protein expression and patient survival in bevacizumab-treated group. Patients with cancer-specific survival ≤10 months and with progression-free survival ≤6 months showed higher expression level of CD31, tryptase, and CD68 as compared with patients with better survivals.

bevacizumab-resistance. The results of our study, showing that the number of CD68-positive cells is significantly decreased in bevacizumab treated as compared to untreated bioptic ccRCC samples, while the number of CD163-positive cells corresponding to M2 subpopulation is unchanged in both the condition suggest that bevacizumab treatment in RCC is not associated to the development of resistance.

Knowledge of the interaction between tumor microenvironment cells and cancer cells, may open the way to innovative therapeutic strategies for ccRCC patients. Macrophages has become an appealing target for antiangiogenic therapies through 2 approaches: compounds that suppress secretion of angiogenic molecules by macrophages or compounds that inhibit macrophage infiltration into the tumor mass. As concerns mast cells, they might act as a new target for the adjuvant treatment of ccRCC through the selective inhibition of angiogenesis, tissue remodeling, and tumor promoting molecules, allowing the secretion of cytotoxic cytokines and preventing mast cell mediated immunesuppression.

Conflict of interests

All authors have seen and approved the manuscript being submitted and have no conflict of interest to declare.

Competing interests

The authors of the current manuscript submitted for evaluation declare that they have no competing interest.

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