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ORIGINAL ARTICLE

Influence of Exposure to Chronic Persistent Low-Dose Ionizing Radiation on the Tumor Biology of Clear-Cell Renal-Cell Carcinoma. An Immunohistochemical and Morphometric Study of Angiogenesis and Vascular Related Factors

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Abstract Increased angiogenesis is related to boosted growth and malignancy in carcinomas. "Chronic Persistent Low-Dose Ionizing Radiation" (CPLDIR) exposure increases incidence and aggressive behavior of clear-cell renal-cell carcinoma (CCRCC). The aim was to study the biology of angiogenesis, including microvessel density (MVD), in human clear-cell renal-cell carcinomas (CCRCC) originating from a radio-contaminated geographical area (Ukraine) and to compare with similar tumors diagnosed in non-contaminated regions of Europe (Spain, Valencia) and Latin America (Colombia, Barranquilla). MVD was comparatively examined in 124 patients diagnosed with CCRCC from three geographical areas by means of digital micro-imaging and computerized analysis. Additionally, 50 adult normal kidneys were used for controls (autopsy kidneys from Valencia and Barranquilla). Furthermore, an immunohistochemical study of several vascular related growth factors was undertaken using a similar methodology. MVD as well as VEFG are the most discriminating factors associated with an aggressive behavior of CCRCC. Their expression increased in proportion to the level of exposure to chronic low-dose ionizing radiation in Ukrainian patients in the 25 years since the Chernobyl accident substantiated by comparison with the two control groups of renal carcinomas present in non-irradiated areas (Spain and Colombia). No major biological differences relating to angiogenesis appear to exist between the CCRCC diagnosed in two distant geographical areas of the world. HIF-1α expression was similar in all groups, with no statistical significance. Present findings demonstrate the existence of a significant relationship between MVD and VEGF in CCRCC: an increased expression of VEGF is associated with a high level of angiogenesis.

Keywords Clear-cell renal-cell carcinoma (CCRCC) · *Chronic persistent low-dose ionizing radiation* (CPLDIR) · Angiogenesis · Morphometry · Angiogenic factors · Microvessel density (MVD) · Immunohistochemistry

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Introduction

Clear-cell renal-cell carcinoma (CCRCC) constitutes the most frequent malignant epithelial carcinoma of this organ, and represents 3.8% of all neoplasms in adults [1]. Around 25 to 30% of cases will have already metastasized at diagnosis [1–3] displaying a poor survival rate of less than 10% at five

years [2]. Tumor angiogenesis is an essential factor for tumor growth and metastasis, but the process by which new intratumoral vessels develop from pre-existing ones remains controversial in malignant neoplasms [4, 5] including CCRCC [6, 7], which requires angiogenesis for the cancer cells to survive [8]. In addition, oxygen deficiency in the tumors generates a severe hypoxic environment promoting activation of hypoxia-related factors, which in turn to some extent become responsible for cell growth, invasion and metastasis [8–10].

It is well known that the biological effects of ionizing radiation on tissues are associated with carcinogenesis [11–14]. In this context, our group has already communicated [15–19] the presence of a higher incidence of CCRCC as a long-term outcome of exposure to "chronic persistent low-dose ionizing radiation" (CPLDIR) in the contaminated geographical regions of Ukraine attributed to a long-term after effect of the Chernobyl accident. In addition, Romanenko et al. [20] have observed increased intratumoral angiogenesis in CCRCC originating from high and low radio-contaminated areas of Ukraine.

The present study attempts to confirm previous investigations by performing a morphometric evaluation of both the intratumoral and the peritumoral microvessel density (MVD) observed with CD31 antibody staining and measured with a digital microimaging computerized analysis system. In addition, we have quantitatively evaluated the immunohistochemical expression of several angiogenic factors involved in vascular neogenesis and proliferation. The study included the previously analyzed group of CCRCC [20] and incorporated new neoplasms from non-irradiated areas of Spain (Valencia) and Colombia (Barranquilla). Normal kidneys were tested as controls. In addition, the present study included the evaluation of several tumor parameters comprising the histopathology grade (Fuhrman) and tumor stage (TNM).

Materials and Methods

Selection of Cases

Tumors and normal tissue samples were collected in 298 paraffin blocks, corresponding to CCRCC and peritumoral tissues of 124 patients (25 from Spain, 24 from Colombia, 50 from the highly contaminated area and 25 from the low contaminated area of Ukraine). All tumors were obtained by nephrectomy between 2005 and 2007 in previously non-treated patients submitted to surgery at the Ukrainian Urological Institute Kiev, Hospital Clínico Universitario of Valencia and Hospital Universidad del Norte in Barranquilla. The patients from Ukraine were resident in both the more "contaminated areas" close to Chernobyl (less than 80 km) and the "less contaminated or clean areas" more distant from the site of the nuclear power station (more than 80 km and mainly resident in Kiev). The 50 normal renal tissue samples which served as control (25 from Spain and 24 from Colombia) were obtained from autopsies performed between 2005 and 2010 in adults free of any kind of renal pathology.

The tumor stage of all CCRCC was evaluated according to the TNM system and the AJCC 2002 [21]. Tumor grade was determined based on the Fuhrman grading system [22] and tumor histology was classified according to WHO criteria 2004 [23]. The histology was performed on well-preserved CCRCC (Fuhrman grades I-IV), excluding samples with large necrosis and/or with huge inflammatory infiltration. The specimens were reviewed by two pathologists (ALLB, AR); normal control tissue samples were obtained from the cortical region of the kidneys free of any post-mortem degeneration or comorbidities. All clinical information such as age, gender, presence of regional lymph node or distant metastases was obtained from the databases of the home institutions.

TMA Production

The tissue specimens were fixed in buffered formalin and embedded in paraffin. Sections, 4 μm thick, were cut and stained with Hematoxylin and Eosin for control. Well-preserved tumor areas from two different fields were selected for TMA production. Cores of 1.2 mm in diameter were obtained using a Beecher Instrument (Silver Springs, MD, USA) handset. Two cores of tumor tissue and two of surrounding kidney with normal histology were placed in each slide.

Immunohistochemistry

The following antibodies related to endothelial cell biology were used: CD 31, VEGF, FGF-2, HIF-1α, VE-cadherin, PDGFR, VEGFR-1, 2, and 3 (DAKO Envision and Santa Cruz Biotechnology) using the avidin-biotin immunoperoxidase procedure as recommended by the manufacturer. A standardized DAKO Autostainer Universal Staining System was used. Antigen retrieval was performed by autoclaved incubation for 3 min at 1.5 atm with citrate buffer pH 6.1. The sections were then washed three times with Tris buffered Saline (TBS). The peroxidase activity was visualized using 3,3 diaminobenzidine (DAB) and imidazole (0.01 M) as chromogen. CD31 and VE-cadherin were counterstained with hematoxylin-eosin and methyl green respectively.

Quantification by Morphometry

Quantitative morphometric studies were performed using image analysis with two different semiautomatic methods: MVD value with the Image Pro Plus 7.0 (Infaimon, Media Cybernetics), and expression of angiogenic factors with the Scan Panoramic Viewer 1.15.

Quantification of Microvessel Density (MVD)

Intratumoral MVD was assessed according to previously described criteria [24, 25]. The slides were examined and photographed using a DMD 108 Leica microscope. The observation was blind with respect to the clinical and histological outcomes. Samples were immunostained with anti CD-31 antibody. The slides were first examined at low magnification (40×) to identify highly vascularized areas (hot spots). The 6 most representative hot spots were selected and images acquired at 200× magnification, the MVD was calculated by counting the number of microvessels in all 6 fields with tissue area measured in mm^2 by a semiautomatic method. The mean value of the vessel counts in the selected hot spots was taken as the final MVD value. Any positively-stained individual endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells or other connective tissue elements was considered as a countable microvessel. The presence of lumina was not a prerequisite for microvessel counting, and large vessels with a muscle wall or a lumen larger than 50 μm were excluded. In each case the diameter of the vessels was of 7.3 to 15 μm.

Quantification of Expression of Angiogenic Factors

The immunostained microarray (TMA) images were assembled, and following calibration the results were automatically compiled using Densito-Quant software. The findings were reviewed manually and compared to the original image, and the final results transferred to an Excel spreadsheet. The immunostaining was evaluated using automatic quantification software that provided the percentage of stained cells for each intensity, and quantified as 3 (+ + +) or strongly positive; 2 (+ +) moderately positive; 1 (+) weakly positive and 0 (−) negative.

Statistical Analysis

For the statistical analysis the ANOVA or Pearson correlation tests were made, using SPSS for Windows software (IBM SPSS statistics version 19), taking $p \leq 0.01$ as indicative of a statistically significant value.

Results

Table 1 summarizes the clinical parameters of the different groups: control groups from Colombia and Spain, and the cases from low and high contaminated areas of Ukraine, including gender, age, tumor size, Fuhrman tumor grade and TNM stage. Figure 1 shows TMA histology for CCRCC, 10X magnification.

Assessment of Neoangiogenesis

Quantification of MVD: Mean Vascular Parameters in Relation to the Groups

Positive staining for CD31 was detected in the membrane of the endothelial cells within the tumor. (Fig. 2). The MVD values ranged from 41 to 516 with a median of 198.75 microvessels/mm^2. Statistical analysis showed that MVD was significantly related to the tumor stage of RCC ($p < 0.01$).

The MVD presented a statistically significant progression ($p < 0.01$) when comparing the non-irradiated and irradiated groups: (Colombia and Spain: 152.77 ± 61.34 microvessels/mm^2); (Ukraine low contaminated: 211.15 ± 80.15 microvessels/mm^2; Ukraine high contaminated: 241.09 ± 103.58 microvessels/mm^2). No statistically significant findings were seen when comparing the two (high and low radiation) groups from Ukraine (ANOVA test $p = 0.37$). (Fig. 3).

Normal kidney tissue provided the following results: Spain and Colombia: 52.44 ± 14 μ vessels/mm^2; while in peritumoral fields the MVD was: Spain and Colombia: 56.25 ± 17.1 μ vessels/mm^2, Ukraine low contaminated: 62.50 ± 25.9 microvessels/mm^2, Ukraine high contaminated: 63 ± 29.7 μ vessels/mm^2 (Table 2). Based upon the present findings, we conclude that a statistically significant difference (ANOVA test $p < 0.01$) exists between normal kidney tissue and tumor groups (non-radiated and radiated), but not between normal kidney tissue and peritumoral tissue.

An evaluation of the relationship between MVD and the clinical parameters of CCRCC is summarized in Table 3. Analyzing the correlation between MVD and Fuhrman grade, the highest number of microvessels was seen in Fuhrman 3 tumors from high and low contaminated areas of Ukraine, while the highest number of microvessels in Fuhrman 1 tumors was seen in from Spain and Colombia. Similar results were found with respect to TNM.

No statistically significant differences were found when comparing patients, gender and age in any of the groups.

Evaluation of Endothelial Cell Immunomarkers

Tumor cells stained with the following vascular expression immunomarkers: VEGF, VEGFR-1,2,3, FGF-2, PDGFR-α, HIF-1 and VE-cadherin were quantitatively measured and evaluated in all CCRCC cases. Most of the groups and factors analyzed had over 90% positive staining, except HIF-1α which displayed a lower percentage in all cases (66.5%) (Table 4). All normal kidney tissue were positive.

Considering positive cells by tumor groups, we found that VEGF expression in tumors from the contaminated area of Ukraine was significantly higher than in the rest of the tumors, ($p < 0.001$). In contrast, no statistically significant differences

Table 1 Clinical findings of the 4 groups including Fuhrman tumor grade and TNM stage

	Colombia	Spain	Ukraine LC	Ukraine HC	Total
Age (mean)	55	64	57	58	58
Range	27–82	29–83	41–73	30–80	27–83
Sex					
Female	11 (46%)	14 (56%)	13 (52%)	31 (38%)	69 (55.6%)
Male	13 (54%)	11 (44%)	12 (48%)	19 (62%)	55 (44.35%)
TNM Stage					
I	7 (29.2%)	10 (40%)	6 (24%)	6 (12%)	29 (23.4%)
II	3 (12.5%)	11 (44%)	15 (60%)	26 (52%)	55 (44.4%)
III	8 (33%)	4 (16%)	0 (0%)	15 (30%)	27 (21.8%)
IV	6 (25%)	0 (0%)	4 (16%)	3 (6%)	13 (10.4%)
Fuhrman Grade					
1	8 (33%)	7 (28%)	9 (36%)	5 (10%)	29 (23.4%)
2	10 (42%)	9 (36%)	2 (8%)	16 (32%)	37 (29.8%)
3	3 (12.5%)	7 (28%)	7 (28%)	17 (34%)	34 (27.4%)
4	3 (12.5%)	2 (8%)	7 (28%)	12 (24%)	24 (19.4%)
Mean Size (cm)	7.7	5.6	5.1	6.7	6.4

LC = Low Contamination; HC = High Contamination; TNM Stage = (T: size of the primary Tumor; N: regional lymph Nodes; M: distant Metastasis)

existed between tumors from Spain and Colombia compared to those from the low contaminated area in Ukraine (Fig. 4). In addition, regarding HIF1-α expression no statistically significant differences could be found among the three groups (Fig. 5).

The remaining factors showed very similar positivity in all groups (Table 4).

Correlations between MVD and the Tumor Cell Expression of Endothelial Markers

A Pearson correlation test revealed a significant association ($\rho = 0.4$ $p < 0.001$) between MVD in CCRCC from the CPLDIR highly contaminated area (241.09 capillaries/mm2), and high VEGF expression (100%) and HIF-1alfa (72%). The Ukrainian group with low CPLDIR showed an increase in MVD (211.15 capillaries/mm^2) associated with a simultaneous increase in expression of VEGF (92%) and HIF-1α (64%). Controls from Spain and Colombia displayed lower MVD (152.77 capillaries/mm^2) as well as low staining for VEGF (67%) and HIF-1α (65%).

When we correlated MVD and positivity of tumor cells for VEGF and HIF1-α with the TNM and Fuhrman staging scales, we found that grade III tumors from the Ukraine highly contaminated area expressed a higher percentage of VEGF, HIF-1 and most MVD.

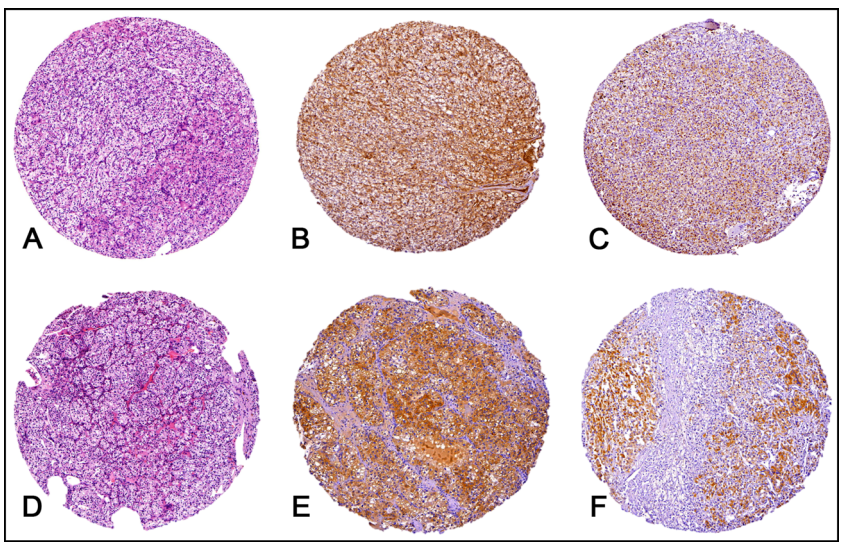

Fig. 1 TMA from Clear-cell renal-cell carcinoma (CCRCC) 10X magnification. In **a**, **b** and **c** from Kiev High contaminated area and in **d**, **e** and **f** from Valencia (Spain). In A and D: H-E stained, in **b** and **e** immunohistochemical analysis of VEGF expression and in **c** and **f**, immunohistochemical analysis of HIF-α expression

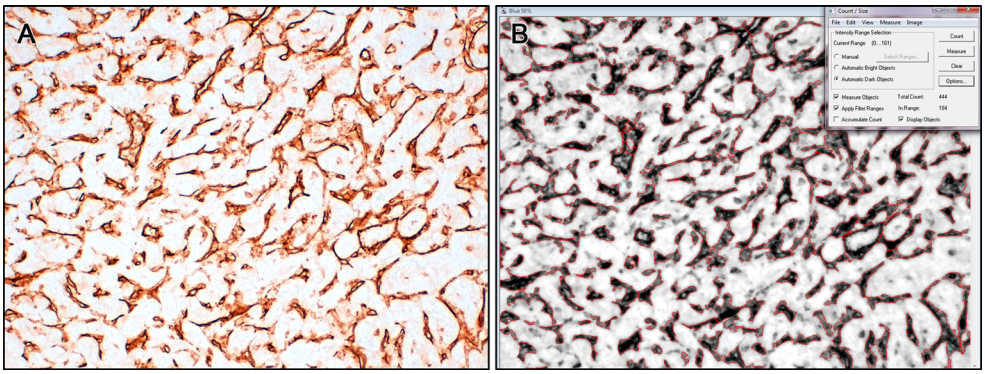

Fig. 2 **a** Section of CCRCC tissue stained with CD31 which revealed the capillary distribution. **b** Section of CCRCC with capillaries measured by the image analyzer

Discussion

Clear-cell renal-cell carcinoma represents the most frequent malignant tumor in the adult kidney, with a high incidence worldwide [26]. This incidence has increased in the geographical areas that suffered a sudden increase in contamination due to high or low doses of radiation detected as a long-term effect after the Chernobyl accident in Ukraine [27, 28]. The influence of "Chronic Persistent Low-Dose Ionizing Radiation" (CPLDIR) exposure on the biology of cancer growth has been demonstrated in several publications which show the existence of high proliferative activity and major biological aggressive behavior in CPLDIR-related neoplasms when compared to similar carcinomas detected in non-radiation-contaminated regions. [19, 20].

In many malignant tumors, increased MVD is associated with poor prognosis; however, in clear cell renal cell carcinoma (CCRCC) this association is controversial, since as many studies have been published that associate increased MVD with a better prognosis (Xin Yao [29], Imao T [30], Rious-Leclercq N [31], Sabo E [32], Schrami P [33]), as those that associate MVD with a worse prognosis (Iakolev V [34], Nativ O [35], Joo HJ [36]). In this study we have analyzed 124 renal tumors with different degrees of aggressiveness to observe the behavior of the blood vessels and provide more data to help clarify this controversial topic. Furthermore, we offer further evidence on the effect of chronic exposure to low dose ionizing radiation on MVD, and the correlation with different vascular factors directly related to the growth of blood vessels, such as VEGF, Cathepsin D and HIF 1alfa.

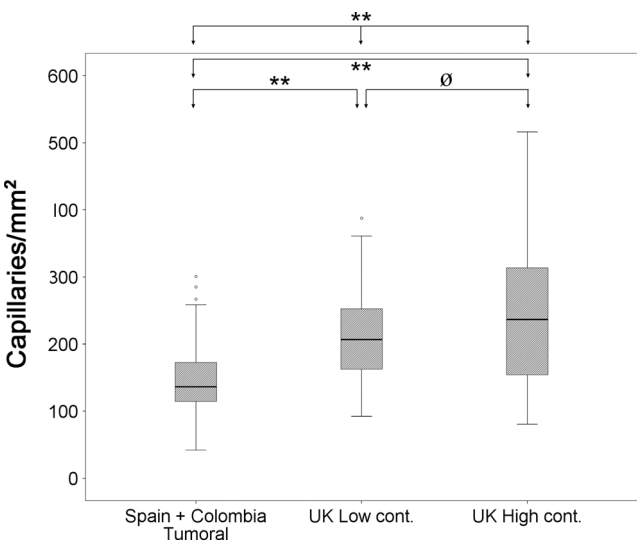

Fig. 3 Box plot diagram illustrating distribution of capillaries/mm2 by geographical areas. Spain and Colombia uncontaminated area, Ukraine low contaminated area and Ukraine high contaminated area. ** $p < 0.01$. ∅ not statistically significant

Reviewing the literature, we observed that the majority of studies that use CD31 or Factor VIII as endothelial cell markers find that higher MVD is associated with a worse prognosis and shorter survival (Xin Yao [29], Ofer Nativ [35], Yoshino S [37]), while studies that use CD34 or CD105 find that higher MVD is related with a better prognosis and longer survival (Xin Yao [29], Imao T [30], Rious-Leclercq N [31], Sabo E [32], Schrami P [33]). Our results confirm the first hypothesis, finding that MVD is associated with a worse prognosis when using CD31 as endothelial marker.

Also, Romanenko et al. [38] using CD31 as endothelial cell marker, have documented increased angiogenesis in tumors subjected to irradiation at low doses over a prolonged time in patients living for more than 20 years in radio-contaminated (^{137}Cs) areas of Ukraine, finding a close relationship between the amount of MVD and CPLDIR in CCRCC.

In the present study we have confirmed the statistically significant increase of MVD in tumors originating in the radio contaminated areas of Ukraine when compared to cases diagnosed both in Spain and Colombia, supporting the hypothesis of a different molecular pathway, as described in bladder cancer in Ukraine, before and after the Chernobyl disaster [39]. In addition, no major differences could be found between the two radio-contaminated geographical regions of Ukraine, confirming previous findings on this subject [20, 28, 40]. This observation also supports the prevailing suspicion [20] that in Ukraine the radiation contamination levels were similar

Table 2 Mean of capillaries/mm^2 by groups. In brackets typical deviation values of each group

	Tumor area (Capillaries/mm^2)	Peritumoral area (Capillaries/mm^2)	No tumoral area (Capillaries/mm^2)
Total cases	124	124	49
Ukraine HC	241.09 [103.58]	63.00 [18.15]	
Ukraine LC	211.15 [80.15]	62.50 [21.25]	
Spain/Colombia	152.77 [61.34]	57.83 [17.31]	52.44 [14.00]

LC = Low Contamination; HC = High Contamination

within and beyond the officially-established 80-km extent of radiation contamination around Chernobyl [27]. Factors such as obesity, smoking and race may be implicated in increased MVD. However, although an association does exist between cigarette smoking and RCCs among white and black smokers [41], no such relationship has been described in relation to increased MVD, and these factors were not included in our study.

We also studied, by comparison with normal kidneys, the expression of several angiogenic factors, VE-cadherin, VEGF, FGF-2 and HIF-1α, the receptors VEGFR-1, VEGFR-2, VEGFR-3 and PDGFR-α, known to be involved in tumor growth [42, 43], in order to discern any possible role in the angiogenic process within the tumor. Moreover, the search also focused on comparatively determining what level of CPLDIR influences the angiogenic process as measured by MVD in relation to the expression of the above-mentioned factors.

VEGF is known to be an active endothelial growth factor [33, 35]. Furthermore, in RCC the level of serum VEGF has been shown to be closely related to tumor stage and grade of RCC, and the expression of VEGF to be significantly associated with tumor stage [37]. In agreement with these findings, our study demonstrated a close relationship between VEGF expression and tumor stage in CCRCC. It has recently been suggested that VEGF may play an important role in the vascular biology of renal cancer and in particular as a mediator in angiogenesis. VEGF expression has been closely related to MVD in a range of cancers [44]. A previous study revealed a positive relationship between the expression of VEGF mRNA and MVD in a total of 47 cases of RCC [45]. In agreement with these data, our study demonstrated a significant relationship between MVD and VEGF expression in CCRCC, indicating that increased expression of VEGF is coincidental with a high level of angiogenesis in CCRCC.

HIF-1α is involved in several cell processes related to cellular homeostasis, particularly as a transcription factor involved in the cellular response to hypoxia [46, 47]. This factor has traditionally been linked to an aggressive tumor phenotype by promoting processes essential for tumor growth, such as angiogenesis. In the present study, HIF-1α stained all CCRCC, independently of their geographical location, finding that staining intensity was higher in the neoplasms originating in Ukraine, but without statistical significance. Several authors have communicated that both CPLDIR [20, 48] and a high expression of HIF-1α [49, 50] are consistent with higher tumor aggressiveness. Our study confirms this finding in CCRCC, nevertheless these differences are without statistical significance. Possibly this finding is due to the complex interplay of regulatory factors involved in angiogenesis existing between the tumor and the local environment, which may lead to a rapid activation of a full hypoxic response by adaptive

Table 3 Comparison between MVD and clinical parameters of CCRCC. In brackets typical deviation values

Grade	Ukraine HC (Capillaries/mm^2)	Ukraine LC (Capillaries/mm^2)	Spain/Colombia (Capillaries/mm^2)
TNM			
I	195.78 [49.52]	261.42 [71.71]	170.12 [67.99]
II	227.89 [111.50]	180.55 [57.48]	158.50 [59.21]
III	290.77 [102.57]	- -	135.90 [57.95]
IV	205.44 [43.38]	250.47 [124.90]	123.00 [44.83]
Fuhrman			
1	174.68 [110.44]	220.14 [83.35]	159.91 [72.45]
2	227.48 [86.56]	191.64 [80.05]	155.44 [60.89]
3	273.49 [121.97]	254.24 [80.91]	151.93 [57.78]
4	233.19 [86.71]	162.08 [58.53]	122.35 [34.79]

LC = Low Contamination; HC = High Contamination. TNM = (T: size of the primary Tumor; N: regional lymph Nodes; M: distant Metastasis)

Table 4 Immunohistochemical expression of vascular factors in CCRCC cells. Expressed as the number of cases with the percentage in parentheses

	VEGF %	VEGFR-1%	VEGFR-2%	VEGFR-3%	HIF-1α %	FGF-2%	PDGFR-α%	VE-Cad %
Ukraine HC	50(100)	48(96)	49(98)	49(98)	36(72)	50(100)	50(100)	47(94)
Ukraine LC	23(72)	25(100)	25(100)	24(96)	16(64)	25(100)	25(100)	23(92)
Spain	22(88)	25(100)	25(100)	24(96)	16(64)	25(100)	25(100)	25(100)
Colombia	11(46)	24(100)	24(100)	24(100)	16(66)	24(100)	24(100)	24(100)

LC = Low Contamination; HC = High Contamination; VEGF = Vascular Endothelial Growth Factor; VEGFR-1 = Vascular Endothelial Growth Factor Receptor-1; VEGFR-2 = Vascular Endothelial Growth Factor Receptor-2; VEGFR-3 = Vascular Endothelial Growth Factor Receptor-3; HIF-1α = Hypoxia Inducible Factor-1α; FGF-2 = Fibroblast Growth Factor-2, PDGFR-α = Platelet Derived Growth Factor Receptor-α; VE-Cad = Vascular Endothelial Cadherin

measures that increase cell survival when oxygen levels suddenly drop [51].

Regarding the expression of other endothelial related growth factors such as FGF-2 and PDGFR- α, the staining was similar in all tumors, independently of geographical location and degree of CPLDIR. Nonetheless, VE-cadherin staining became more intense in the Ukrainian tumors, when compared to those from Spain and Colombia. These results are in agreement with those of Markovic-Lipkovski J who found that decreased VE-Cadherin expression is related to higher aggressiveness in neoplasms [52].

VEGF receptors, VEGFR-1, VEGFR-2 and VEGFR-3, have been found not only in endothelial cells, but also in the cytoplasm of tumor cells [53]. However, in the CCRCC from Ukraine, these three receptors displayed no relationship to Fuhrman grade or TNM stage independently of the degree of radiation.

When analyzing the tumors based on their TNM classification and Fuhrman grade, and in regard to the three factors more directly involved in the mechanisms of angiogenesis (MVD, VEGF and HIF-1α), we found that grade 3 tumors from Ukraine CPLDIR, in both low and high contaminated areas, presented an increased expression of MVD, VEGF and HIF-1α. Nevertheless, in the uncontaminated areas (Spain and Colombia) greater expression of MVD, VEGF and HIF-1α was found in grade I tumors, but without statistically significant differences when compared with grade II and III. These findings support the hypothesis that the highest expression of these three factors occurs in the more aggressive tumors present in the low and high contamination areas of Ukraine.

Recently, Dorderic et al. [54] and Minardi et al. [44] clinically sub-classified a group of more aggressive CCRCC based upon their high expression of VEGF-A and HIF1α. Considering the present findings, we propose that the tumors expressing a high MVD should also be included within this subset of more aggressive CCRCC.

In conclusion, the present findings confirm that a high intratumoral MVD determined by CD31, together with high expression of VEGF are two discriminating factors associated with the aggressiveness of CCRCC. We also found that a higher density of undifferentiated microvessels was an independent

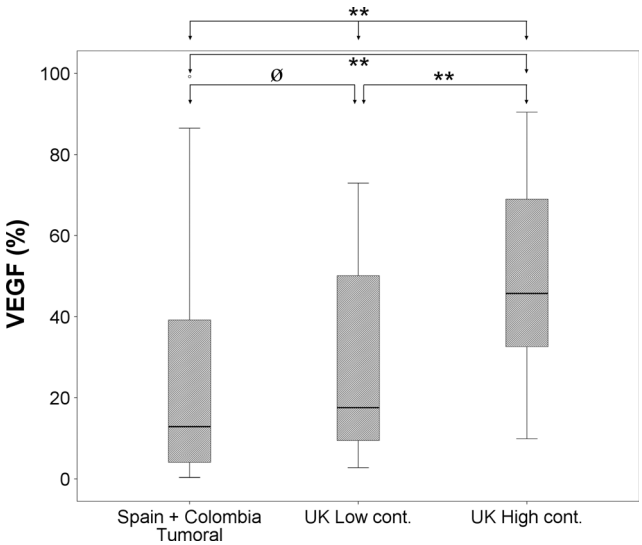

Fig. 4 Box plot diagram illustrating distribution of Vascular Endothelial Grow Factor, expressed in % of positive cells, by geographical areas. Spain and Colombia uncontaminated area, Ukraine low contaminated area and Ukraine high contaminated area. ** $p < 0.01$. ∅ not statistically significant

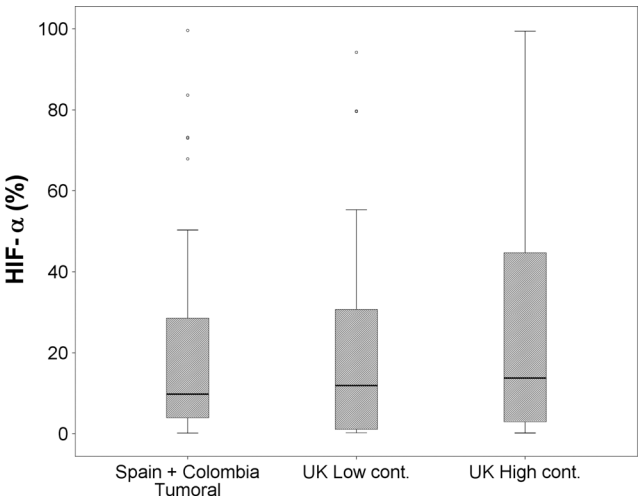

Fig. 5 Box plot diagram illustrating distribution of Hypoxia Inducible Factor 1 , expressed in % of positive cells, by geographical areas. Spain and Colombia uncontaminated area, Ukraine low contaminated area and Ukraine high contaminated area. ** $p < 0.01$. ∅ not statistically significant

prognostic factor of higher histopathologic grade. Moreover, these factors differentiate between tumors from CPLDIR regions of Ukraine and the tumors diagnosed in radiation-free countries such as Spain and Colombia. Further studies are necessary to confirm the role of angiogenesis in these highly heterogenous, yet distinct renal tumors, in order to improve the design of therapeutic protocols.

Compliance with Ethical Standards

Conflict of Interest We declare no conflict of interest

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