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ORIGINAL ARTICLE

Tumor biology of non-metastatic stages of clear cell renal cell carcinoma; overexpression of stearoyl desaturase-1, EPO/EPO-R system and hypoxia-related proteins

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Abstract Clear cell renal cell carcinoma (ccRCC) is the most common subtype of renal carcinomas. There is great interest to know the molecular basis of the tumor biology of ccRCC that might contribute to a better understanding of the aggressive biological behavior of this cancer and to identify early biomarkers of disease. This study describes the relationship among proliferation, survival, and apoptosis with the expression of key molecules related to tumoral hypoxia (hypoxia-inducible factor (HIF)-1α, erythropoietin (EPO), vascular endothelial growth factor (VEGF)), their receptors (EPO-R, VEGFR-2), and stearoyl desaturase-1 (SCD-1) in early stages of ccRCC. Tissue samples were obtained at the Urology Unit of the J.R. Vidal Hospital (Corrientes, Argentina), from patients who underwent radical nephrectomy for renal cancer between 2011 and 2014. Four experimental groups according to pathological stage and nuclear grade were organized: T1G1 (n = 6), T2G1 (n = 4), T1G2 (n = 7), and T2G2 (n = 7). The expression of HIF-1α, EPO, EPO-R, VEGF, VEGFR-2, Bcl-x_L, and SCD-1 were evaluated by immunohistochemistry, Western blotting, and/or RT-PCR. Apoptosis was assessed by the TUNEL in situ assay, and tumor proliferation was determined by Ki-67 immunohistochemistry. Data revealed that HIF-1α, EPO, EPO-R, VEGF, and VEGF-R2 were overexpressed in most samples. The T1G1 group showed the highest EPO levels, approximately 200 % compared with distal renal tissue. Bcl-x_L overexpression was concomitant with the enhancement of proliferative indexes. SCD-1 expression increased with the tumor size and nuclear grade. Moreover, the direct correlations observed between SCD-1/HIF-1α and SCD-1/Ki-67 increments suggest a link among these molecules, which would determine tumor progression in early stages of ccRCC. Our results demonstrate the relationship among proliferation, survival, and apoptosis with the expression of key molecules related to tumoral hypoxia (HIF-1α, EPO, VEGF), their receptors (EPO-R, VEGFR-2), and SCD-1 in early stages of ccRCC.

Keywords Clear cell renal cell carcinoma (ccRCC) · Erythropoietin (EPO) · EPO receptor (EPO-R) · Apoptosis · Stearoyl desaturase-1 (SCD-1)

Introduction

Renal cell carcinomas (RCCs) are the most frequent tumors of the adult kidney, and they account for about 3 % of all human adult malignant diseases [1].

Clear cell renal cell carcinoma (ccRCC), the predominant subtype of RCC (75–80 %) is usually associated with severe prognosis and underlying gene mutations [2]. At the time of diagnosis, 30 % of symptomatic patients have overt metastasis, and the response to conventional therapy has been shown to be poor. On the other hand, the frequency of asymptomatic incidental diagnosis of this cancer is still growing and

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although there are treatments available for advanced ccRCC, none are curative since drug resistance occurs in the majority of cases [3, 4]. Therefore, there is great interest to know the molecular basis of the aggressive biological behavior of ccRCC and to identify early biomarkers of disease, prognosis, or responsiveness to therapy.

The most remarkable phenotypic feature of the ccRCC is its clear cell morphology, which has been linked to an enhanced rate in lipid biosynthesis and glycogen accumulation. However, the molecular mechanism underlying this process is an important component of renal carcinogenesis that needs to be clarified [5].

As with numerous solid tumors, ccRCC frequently presents hypoxic areas as a consequence of tumor growth that exceeds the capacity of its vascular network. The hypoxia-inducible factor (HIF)-1 is the master regulator of oxygen homeostasis triggering metabolic adaptations to hypoxia [6]. HIF isoforms (1α and 2α) are important mediators of hypoxic adaptation and also control several genes involved in tumor growth. Between the two isoforms, HIF-1α is directly involved in metabolic adaptations to hypoxia and is thought to have a role in glucose metabolism by increasing glucose transporter expression and glycolytic enzymes [7, 8].

The erythropoietin (EPO) gene is under the direct control of hypoxia through HIF-1α, a *trans*-acting factor that binds to *cis*-acting DNA hypoxia-responsive elements of the EPO gene promoter [9, 10]. EPO, a widely known growth factor for erythropoiesis, can also stimulate angiogenesis [11], as well as tumor cell proliferation and survival [12]. It has been reported that EPO is expressed in ccRCC and that its expression is mainly regulated by hypoxia via HIF [13]. In addition, it has been demonstrated that human RCC expresses EPO receptor (EPO-R) and that its activation stimulates the proliferation of renal carcinoma cells in vitro [14]. Nevertheless, there are controversial reports about the degree of the EPO and EPO-R coexpression in ccRCC and whether this may lead to cell proliferation via autocrine stimulation, an essential step in renal carcinoma tumorigenesis [15]. Consequently, the complex biology of EPO and EPO-R signaling in ccRCC requires further investigation [12].

In addition to EPO, the vascular endothelial growth factor (VEGF) is included among the protein products of HIF-responsive genes which are upregulated in many human malignancies. VEGF stimulates endothelial cell mitogenesis and cell migration, as well as vasodilatation and vascular permeability in RCC [16]. Furthermore, VEGF expression and its correlation with HIF-1α expression in ccRCC has been previously reported [17].

Tumor-associated lipogenesis appears to be found in most human cancers. It has been communicated that lipids, in addition to their classical structural and bioenergetics roles, contribute to several aspects of tumor biology, such as growth, redox homeostasis, and metastasis. Thus, numerous studies have confirmed that tumoral tissues show aberrant activation of the novo lipogenesis and that inhibition of different enzymes involved in fatty acid biosynthesis can block cancer cell growth [18, 19]. Moreover, emerging literature suggests that lipid biosynthesis and desaturation is a requirement for tumor cell survival [20]. Stearoyl desaturase-1 (SCD-1), the main isoform of human SCD, is the rate-limiting enzyme catalyzing the synthesis of monounsaturated fatty acids, predominantly oleate (18:1) and palmitoleate (16:1). SCD-1 inhibition has been recently shown to limit the growth and proliferation of cancer cells and, as a result of this observation, several studies have focused on the inhibition of SCD-1 as a novel target for cancer therapy [21, 22].

HIF-1α, EPO, and VEGF, key markers of tumor hypoxia, as well as EPO-R and VEGFR-2, have been studied separately in ccRCC [6, 8, 13, 23–27]. However, a global and simultaneous analysis of all of them related to SCD-1 expression in early stages of ccRCC is described here for the first time, on our best understanding. In particular, a statistical association among all these markers and SCD-1 has not been previously communicated.

Thus, the aim of present work was to study whether a relationship exists between these variables in patients who underwent radical nephrectomy for localized RCC (T1-2N0M0) with Fuhrman nuclear grade 1 and 2 (G1 and G2) in order to identify potential early biomarkers of this disease.

Materials and methods

Patients, clinicopathological data, and sampling procedures

Surgical specimens were obtained from 28 patients with ccRCC. Patients were treated by radical nephrectomy at the Urology Unit of the J.R. Vidal Hospital (Corrientes, Argentina) between 2011 and 2014. Of the total of 28 patients that underwent nephrectomy, 18 (64.3 %) were detected incidentally and 10 (35.8 %) had symptoms pertaining to RCC. Among symptomatic patients (n = 10), 5 (50.0 %) presented hematuria, 3 (30.0 %) asthenia, and 2 (20.0 %) adinamia.

Surgically removed ccRCC and counterpart normal tissues were collected from specimens and quickly frozen in liquid nitrogen.

Each tumor underwent pathological staging according to the TNM system of classification and Fuhrman nuclear grading [28]: 13 (46.4 %), 11 (39.3 %), 3 (10.7 %), and 1 (3.6 %) cases were staged T1, T2, T3, and T4, respectively. Fuhrman grades of G1, G2, and G3 were reported for 10 (35.7 %), 16 (57.2 %), and 2 (7.1 %) cases. Specimens were fixed for histopathology, immunohistochemistry procedures, and terminal deoxynucleotidyl transferase-mediated deoxyuridin triphosphate nick end labeling (TUNEL) in situ assays.

Additionally, samples of each patient were divided and tissue homogenates were prepared for Western blotting.

In the present study, 24 tumors were selected according to their pathological stage and nuclear grade: T1, T2, G1, and G2. Four experimental groups were organized as follows: T1G1 ($n = 6$), T2G1 ($n = 4$), T1G2 ($n = 7$), and T2G2 ($n = 7$).

The design and methods of this research have been approved by the Bioethics Committee of the School of Medicine of the Northeastern National University and by the Department of Medical Research of the J.R. Vidal Hospital from Corrientes, Argentina.

Immunohistochemistry (IHC)

Paraffin-embedded sections were deparaffinized and rehydrated in graded alcohols using routine protocols. Briefly, sections (4 μm) were stained with the following antibodies: rabbit polyclonal anti-EPO and anti-EPO-R (Epo: H-162, sc-7956; Epo-R: H-194, sc-5624, Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-VEGF (C-1, sc-7269; Santa Cruz Biotechnology); rabbit polyclonal anti-Ki-67 (H-300, sc-15402; Santa Cruz Biotechnology); goat polyclonal anti-SCD (E-14, sc-30435, Santa Cruz Biotechnology), rabbit monoclonal anti-VEGFR-2 and anti-Bcl-x_L (VEGFR-2: #2479; Bcl-x_L #2764; both from Cell Signaling Technology, Beverly, MA, USA).

All primary antibodies were assayed using a 1:100 dilution with an overnight incubation at 4 °C. For EPO and EPO-R, slides of adult kidney [29] and placenta [30] were used as positive controls.

Immunostaining was performed using a DAKO LSAB+/HRP kit (Dako Cytomation) followed by the application of a chromogene DAB (DAKO kit) according to manufacturer's instructions. All negative controls were obtained by excluding the primary antibody from the reaction. Samples were then counter-stained with hematoxylin and visualized under a light microscope. Images were taken using an Olympus Coolpix-microdigital camera fitted on a CX-35 microscope (Olympus, Japan).

Evaluation of immunostaining

Slides stained with EPO, EPO-R, Bcl-x_L, VEGF, and VEGFR-2 were analyzed using light microscopy by two independent investigators who were blinded to the patient data. The immunohistochemical expression was evaluated and categorized in three groups: no (0), weak (+), and strong (++) IHC expression.

In situ cell death detection (TUNEL assay)

TUNEL assay was performed using an in situ Cell Death Detection Kit (Roche, Indianapolis, IN, USA) according to the manufacturer's instructions. For positive controls, sections were treated with 0.7 mg/ml DNAse I (Sigma-Aldrich) for 15 min before treatment with TdT. This enzyme was replaced with the same volume of distilled water in negative controls. Slides were examined under a fluorescence microscope (Olympus CX-35 equipped with a Coolpix Digital camera), and images were processed using Adobe Photoshop 14.0 (Adobe System, San Jose, CA).

Proliferative and apoptotic indexes

The Ki-67 index (KI) and apoptotic index (AI) were expressed as the percentage of Ki-67-positive and TUNEL-positive cells per 1000 examined cancer cells, respectively. These percentages were obtained from observations done in histologically proven cancer areas without necrosis, hemorrhages, or mononuclear infiltrations. Nuclei of apoptotic cells exhibit bright green fluorescence while proliferative cancer cells have brownish nuclei due to Ki-67 immunostaining.

Western blot analysis

Expressions of SCD-1, EPO-R, and EPO were determined by immunoblotting of cytosolic extracts. Samples from ccRCC patients and a distal section of renal normal tissue of each patient were homogenized and lysed in an ice-cold buffer [10 mM HEPES pH 7.4, 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.1 % IGEPAL (Sigma Co, MO, USA)], supplemented with a protease inhibitor cocktail. Cell lysates were centrifuged at 14,000*g* for 20 min, and the supernatant (cytosolic fraction) was used for different assays.

The nuclear pellets were gently resuspended with ice-cold wash buffer (20 mM HEPES pH 7.4, 1.5 mM $MgCl_2$, 420 mM ClNa, 25 % glycerol, 0.2 mM ethylene glycol-bis (beta-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetra acetic acid (EDTA), 0.5 mM DDT, 0.2 mM PMSF with protease inhibitors) and incubated for 1 h at 4 °C. Samples were centrifuged at 16,000*g* at 4 °C for 30 min, and supernatants were collected as nuclear extracts for HIF-1α immunoblotting. Positive controls for HIF-1α expression were performed using nuclear extracts from kidneys of mice submitted to 6 h of hypoxia in a hypobaric chamber (0.40 atm).

Cytosolic proteins (40 μg) were separated by a 12 % SDS-PAGE, blotted on nitrocellulose membranes (Bio-Rad, CA, USA). Eight percent SDS-PAGE was used for nuclear proteins. Membranes were treated with 1:500 dilutions of primary antibodies purchased in Santa Cruz Biotechnology, Santa Cruz, CA, USA: anti SCD (E-14: sc-30435), EPO-R (M-20: sc-697), and anti EPO (H-162: sc-7956 sc-5624). Identical dilution was used with anti HIF-1α (Novus Biologicals, Littleton, CO) and anti-βactin (Sigma-Aldrich). Membranes were incubated with horseradish peroxidase-conjugated secondary

antibodies (Jackson Immunoresearch Inc., USA). Immunocomplexes were detected by an Opti4CN kit (Bio-Rad, CA, USA). Band optical density (OD) was determined using NIH Image software, and the results were expressed as the ratio: (protein of interest OD/β-actin OD) × 100. All experiments have been performed at least three times, and representative results of one experiment are shown.

Reverse transcription-polymerase chain reaction analysis

SCD-1 messenger RNA (mRNA) expression was determined by RT-PCR. Total RNA was extracted using the TRIzol reagent method (Invitrogen) according to the manufacturer's protocol. First-strand cDNA was obtained by using the Moloney murine leukemia virus reverse transcriptase (Promega) from 2 μg of RNA. PCR was then performed using specific primers for SCD-1 as follows: 5′-TTCCTACCTGCAAGTTCTACACC-3′ (forward), 5′-CCGAGCTTTGTAAGAGCGGT-3′ (reverse) with a product of 116 bp. GAPDH were used as housekeeping gene: 5′-ATGGGGAAGGTGAAGGTCG-3′ (forward); 5′-GGGGTCATTGATGGCAACAATA-3′ (reverse) with a product of 108 bp. All primers were tested for specificity using the Blast program available at the National Center for Biotechnology Information web site (http://www.ncbi.nlm.nih.gov/). Cycling conditions were as follows: 1 cycle at 95 °C for 10 min, 35 cycles at 95 °C for 30 s, 58 °C for 45 s, 72 °C for 1 min, and a final extension at 72 °C for 10 min. The relative mRNA abundance for a given gene was calculated using the NIH ImageJ for Mac OS X application software.

Statistical analysis

Statistical analysis and graphic presentation were performed using the GraphPad PrismR 6.0 and the Instat R 5.0 packages (GraphPad, San Diego, CA, USA). Results were expressed as mean ± standard error of mean (SEM) or ± standard deviation (SD) according to the statistical analysis employed. Comparisons between groups were analyzed using two-tailed paired Student's *t* test.

The Mann-Whitney *U* test was used to compare the apoptotic and proliferative indexes between ccRCC and normal renal tissue. Pearson correlation was used to determine the association between the percentage of SCD-1 and HIF-1α increment as well as between the percentage of Ki-67 index and SCD-1 enhancement. All results with a P level <0.05 were considered statistically significant, whereas a P value between 0.05 and 0.10 was considered of borderline significance.

Results

Patient's characteristics and tumor classification

Patient characteristics and clinicopathological data are summarized in Table 1.

In the present study, the results obtained for T1, T2, G1, and G2 were analyzed in four experimental groups according to pathological stage and nuclear grade as previously described in Materials and methods.

Hypoxic microenvironment in ccRCC: expression of EPO, EPO-R, VEGF, and VEGFR-2

The expression of HIF-1α was determined by immunoblotting in nuclear homogenates of ccRCC samples and compared with normal renal distal tissue samples from the same patient. HIF-1α overexpression was detected in the majority of samples when compared to normal distal kidney tissue (Fig. 1a). ccRCC samples showed a significant increase of HIF-1α expression of approximately 50 % (Fig. 1b).

The percentage of increase in HIF-1α expression was analyzed with the pathological stage and the nuclear grade of the tumors' samples as it is shown in Fig. 1c. These data revealed that HIF-1α significant overexpression was observed in all experimental groups.

The expression of EPO/EPO-R in ccRCC and normal renal tissue was assessed by immunoblotting (Figs. 2 and 3) and immunohistochemistry (Fig. 4).

Western blots revealed that EPO (Fig. 2a, b) and EPO-R (Fig. 3a, b) exhibited a significant increase in immunostaining from ccRCC when compared to bands obtained from control distal tissue homogenates. Tumoral samples from T1G1 group showed the highest EPO expression (approximately 200 % of increase as compared with control distal tissue homogenates). The immunohistochemistry of EPO and EPO-R confirmed their enhanced coexpression in the majority of ccRCC samples. EPO-R was detected in 87.5 % of ccRCC revealing membranous and cytosolic staining patterns, whereas EPO immunostainings were more evenly distributed than EPO-R in ccRCC samples (Fig. 4a, b).

The VEGF and its receptor, VEGFR-2, are responsible for the angiogenic phenotype of ccRCC. The majority of the ccRCC samples were positive for VEGF expression (83.3 %; $n = 24$) showing a perimembranous and diffuse cytoplasmic immunohistochemical staining. However, VEGFR-2 expression exhibited mainly a membranous pattern in most ccRCC samples (Fig. 5a). The VEGFR-2 protein-positive phenotype of ccRCC was frequently observed (75.0 %; $n = 24$) (Fig. 5b).

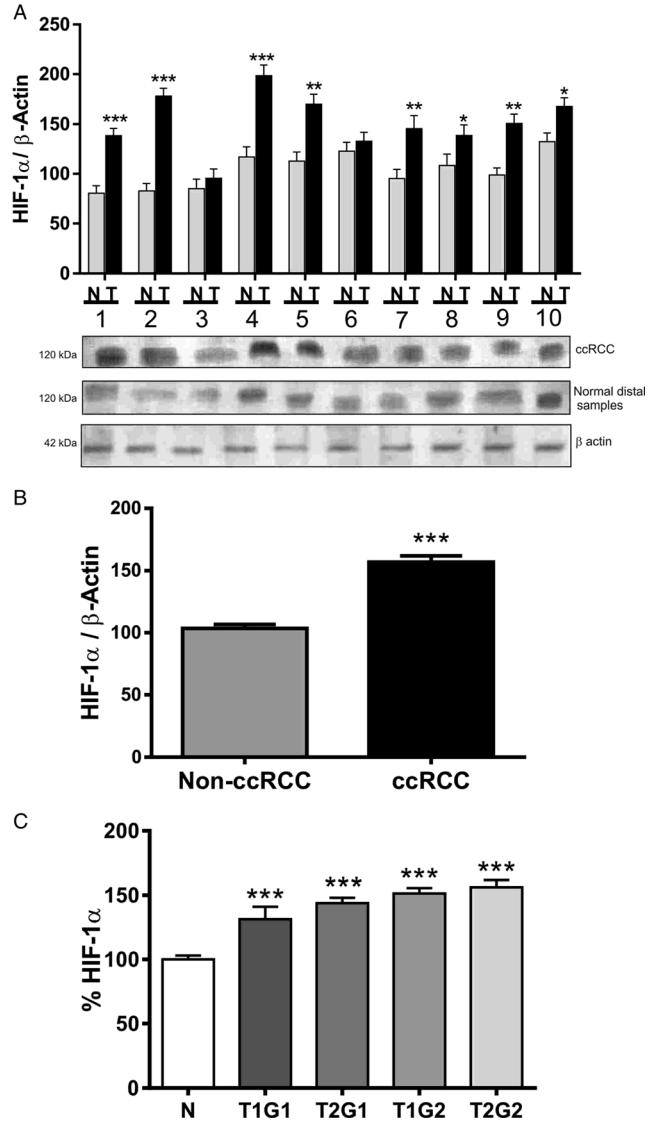


Fig. 1 HIF-1α expression in ccRCC samples. **a** Western blotting of HIF-1α. Ten representative normal distal (N) and ccRCC (T) samples are shown. Immunoblottings were performed by triplicate from a single sample. Data were normalized to β-actin used as loading control. **b** HIF-1α/β-actin ($n = 24$). **c** Percentage of increment of HIF-1α expression related to the pathological stage and the nuclear grade of the tumors' samples compared with the matched adjacent non-tumor tissues, respectively. Values are mean ± SEM.* $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ indicate significant differences

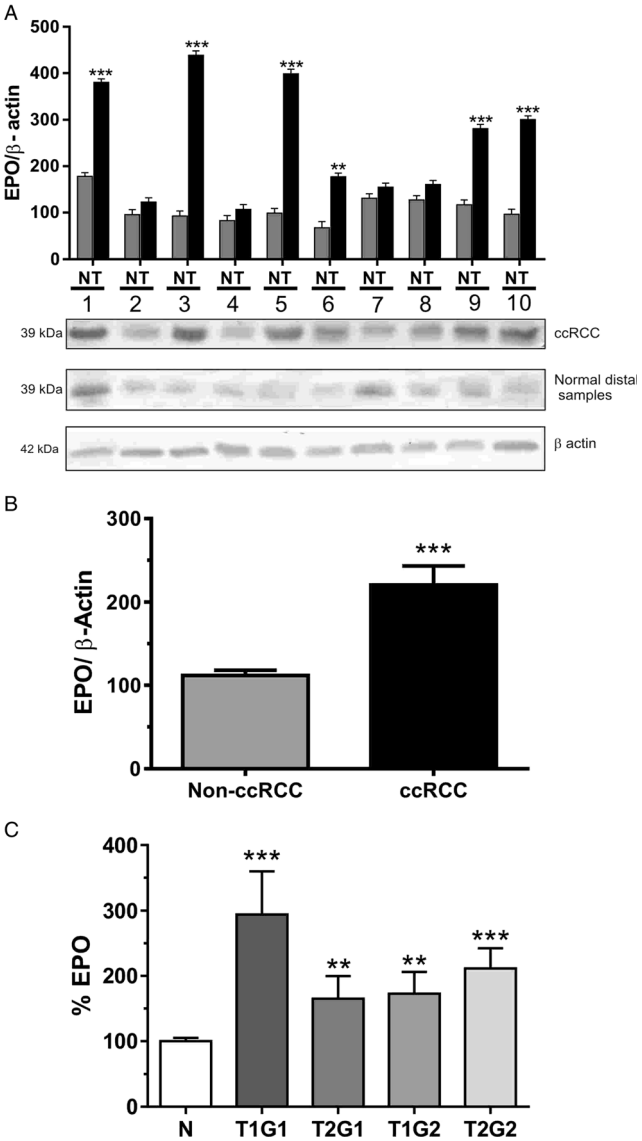


Fig. 2 EPO expression in ccRCC. **a** Western blottings of EPO in ccRCC. EPO (39 kDa) was overexpressed in most of the homogenates of ccRCC (T) compared to distal normal renal samples (N) from the same patient. Ten patients were selected for illustration. Data were normalized to β-actin used as loading control. **b** EPO/β-actin (n = 24). **c** Percentage of increment of EPO expression related to the pathological stage and the nuclear grade of the tumors' samples compared with the matched adjacent non-tumor tissues, respectively. Values are mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences

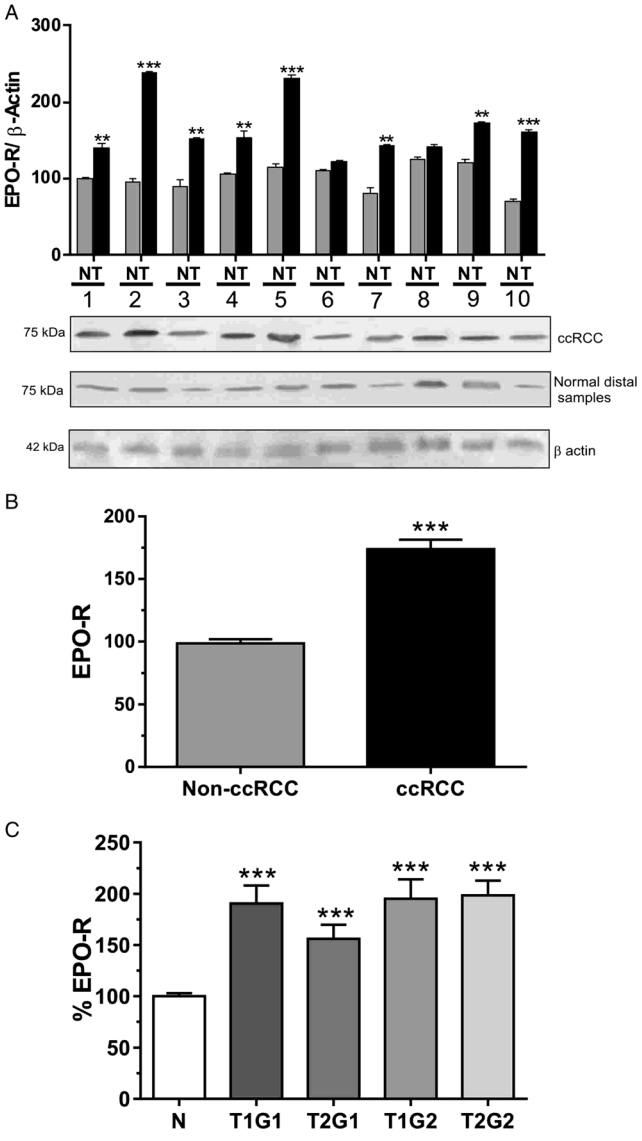


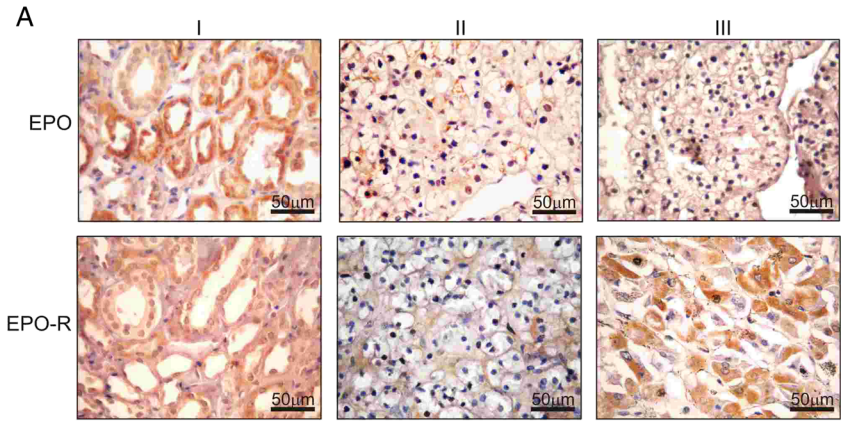
Fig. 3 EPO-R expression in ccRCC. **a** Western blottings of EPO-R in ccRCC. EPO-R (75 kDa) was overexpressed in most of the homogenates of ccRCC (T) compared to distal normal renal samples (N) from the same patient. Immunoblottings were performed by triplicate from a single sample. Ten patients were selected for illustration. Data were normalized to β-actin used as loading control. **b** EPO-R/β-actin (n = 24). **c** Percentage of increment of EPO-R expression related to the pathological stage and the nuclear grade of the tumors' samples compared with the matched adjacent non-tumor tissues, respectively. Values are mean ± SEM. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences

1α ($r^2 = 0.97$; $p = 0.0018$) as it is shown in Fig. 9a. Moreover, the proliferative index (Ki-67) was positively correlated with SDC-1 protein levels ($r^2 = 0.97$; $p = 0.0018$) (Fig. 9b).

Discussion

In this work, we have studied cell proliferation, apoptosis/survival, the expression of SCD-1, HIF-1α, and their regulated proteins (VEGF, EPO), as well as EPO-R and VEGFR-2 in tumor samples from ccRCC patients. This is, to our knowledge, the first study describing the relationship among proliferation, apoptosis/survival and the expression of key molecules involved in ccRCC tumoral behavior as a function of early pathological stage and nuclear grade.

Tumor cells within a growing lesion often need to adapt and survive in hypoxic conditions. The HIFs are important mediators in the hypoxic adaptation of cancer cells, and they control several genes that have been implicated in tumor



B

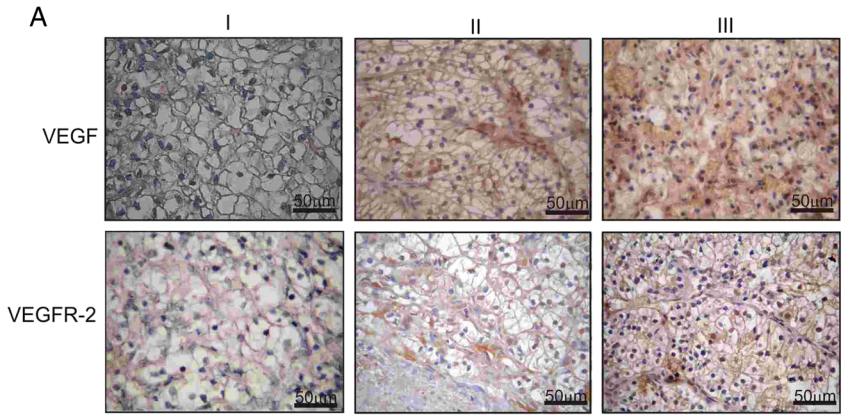
EPO and EPO-R expression in ccRCC

	0		+		++		Total
	n	%	n	%	n	%	
EPO	10	41.7	8	33.3	6	25.0	24
EPO-R	3	12.5	14	58.3	7	29.2	24

ccRCC (Clear cell renal cell carcinoma)

Fig. 4 Immunohistochemistry of EPO and EPO-R in ccRCC. **a** EPO and EPO-R immunoreactions in normal distal sections denote the constitutive expression of these proteins in the cytoplasm of tubular renal cells (*I*). Weak immunoreactivity of EPO and EPO-R with membranous pattern in ccRCC sections (*II*). Strong EPO and EPO-R immunostainings with cytoplasmic and membranous patterns respectively in ccRCC sections (*III*). Original magnification × 400. **b** Semiquantitative evaluation of EPO and EPO-R immunoreactivity in ccRCC: (0) no, (+) weak, and (++) strong immunohistochemical expression of EPO and EPO-R ($n = 24$)

phenotype and progression [31, 32]. The two best-characterized HIF-α isoforms are HIF-1α and HIF-2α. Although most of their functions are overlapping, their functions in tumor cells are controversial [8, 33]. We have studied the expression of HIF-1α since Wiesener et al. provided in vivo evidence of upregulation of HIF-1α linked to



B

VEGF and VEGFR-2 expression in ccRCC

	0		+		++		Total
	n	%	n	%	n	%	
VEGF	4	16.7	8	33.3	12	50.0	24
VEGFR-2	6	25.0	9	37.5	9	37.5	24

ccRCC (Clear cell renal cell carcinoma)

Fig. 5 Immunohistochemistry of VEGF and VEGFR-2 in ccRCC. **a** VEGF and VEGFR-2 immunoreactions in early stages of ccRCC: no (*I*), weak (*II*), and strong (*III*) immunoreactivity. VEGF expression exhibited a perimembranous and diffuse cytoplasmic pattern. The immunodetection of VEGFR-2 was mainly membranous with some tumor cells containing immunopositive cytoplasmic pattern. Original magnification × 400. **b** Semiquantitative evaluation of VEGF and VEGFR-2 immunoreactivity. (0) no, (+) weak, and (++) strong immunohistochemical expression in ccRCC samples ($n = 24$)

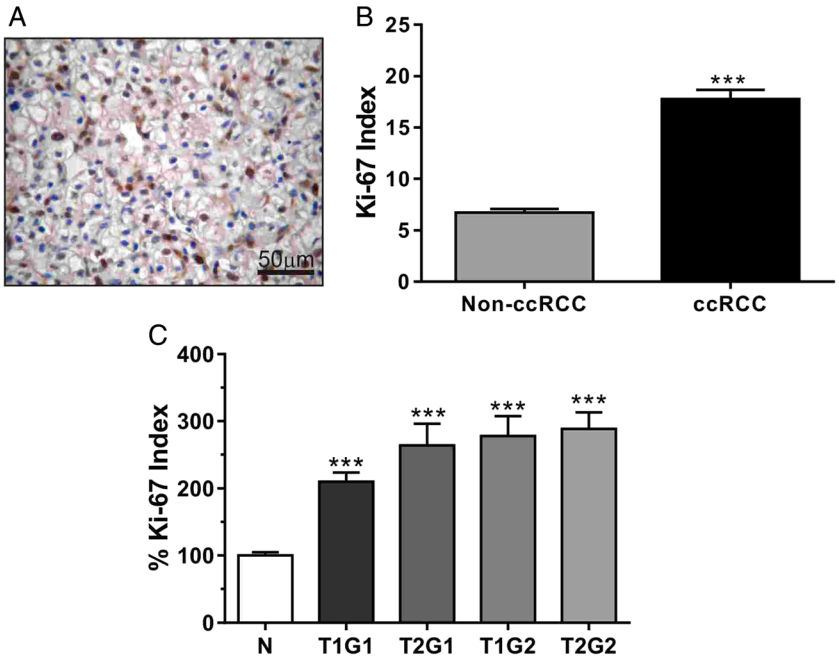


Fig. 6 Ki-67 detection in ccRCC. **a** Ki-67 antigen labeling was localized with a strong and homogeneous brownish granularity in ccRCC nuclei. Original magnification ×400. **b** Proliferative index (Ki-67 antigen) from tumors' samples ($n = 24$) compared with the matched adjacent non-tumor tissues. **c** Percentage of increment of Ki-67 index related to the pathological stage and the nuclear grade of the tumors' samples compared with the matched adjacent non-tumor tissues, respectively. Values are mean ± SEM. ***$P < 0.001$ indicates significant differences

functional inactivation of the von Hippel-Lindeau (VHL) gene product in ccRCC [34]. Moreover, Chuang and collaborators have described that HIF-1α has an important role in the epithelial-mesenchymal transition in renal cancer cells [33]. It is well known that the epithelial-mesenchymal transition is a process in which polarized epithelial cells are converted into motile mesenchymal cells by alterations in adhesion, morphology, cellular architecture, and migration capacity. The present results showed HIF-1α overexpression in most of ccRCC samples and that its enhancement was related with the non-metastasic stages of this cancer.

Larger tumors exhibit hypoxic conditions, and this triggers HIF action, which further increases the production of VEGF [26]. Additionally, it has been demonstrated that the expression of these proteins are involved in tumor growth and metastasis [35], and therefore, provides targets for antiangiogenic therapies [16, 17, 36]. Conflicting results have been communicated on the significance of VEGF overexpression in relation to nuclear grade, tumor stage, and neovascularization in RCC [27, 37, 38]. To date, only a few reports have described the expression of the pair VEGF/VEGFR-2 in early stages of ccRCC. In the current study, we observed coexpression of

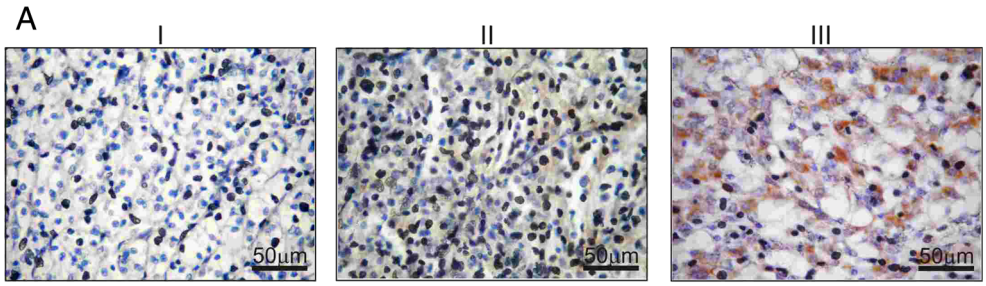


Fig. 7 Immunohistochemistry of Bcl-x_L in ccRCC. **a** No (*I*), weak (*II*), and strong (*III*) immunoreactivity of Bcl-x_L in early stages of ccRCC, showing cytoplasmic pattern. Original magnification × 400. **b** Semiquantitative evaluation of Bcl-x_L immunoreactivity. (0) No, (+) weak, and (++) strong immunohistochemical expression in ccRCC samples

B

Bcl-x_L expression

	0		+		++		Total
	n	%	n	%	n	%	
ccRCC	10	41.7	5	20.8	9	37.5	24
Non-ccRCC	12	50.0	11	45.8	1	4.2	24

ccRCC (Clear cell renal cell carcinoma)

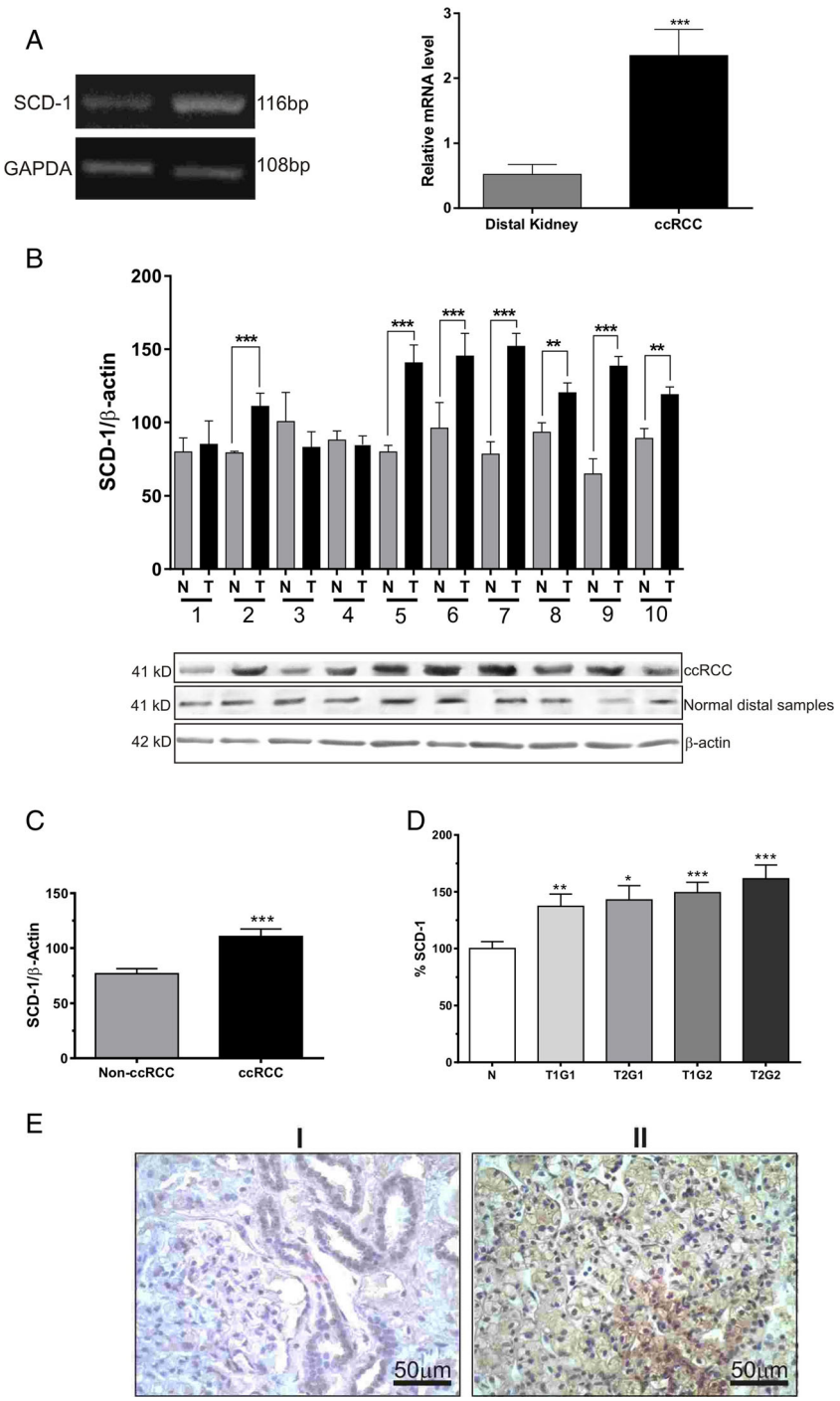


Fig. 8 Expression of SCD-1 in ccRCC. **a** SCD-1 mRNA levels determination by RT-PCR of a representative sample. *Bars* indicate band quantifications of all samples ($n = 24$). **b** Immunoblottings of SCD-1. Illustrative blots of ten samples comparing SCD-1 expression in ccRCC versus normal distal renal tissue of the same patient are shown. SCD-1 has a constitutive basal expression in controls. SCD-1 was overexpressed in more than 75 % of ccRCC whole homogenates. Immunoblottings were performed by triplicate from a single sample of three independent experiences. Data were normalized to beta-actin used as loading control. **c** SCD-1/β-actin ($n = 24$). **d** Percentage of increment of SCD-1 expression related to the pathological stage and the nuclear grade of the tumors' samples compared with the matched adjacent non-tumor tissues, respectively. **e** Immunohistochemistry of SCD-1. Representative photomicrographs corresponding to normal distal renal tissue (*I*) and ccRCC (*II*) are shown. Original magnification ×400. Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ indicate significant differences

VEGF and VEGFR-2, reinforcing the evidence of the ccRCC proangiogenic phenotype associated with non-metastatic stages of ccRCC in accordance with Song et al. [27] findings.

EPO is also under the direct control of hypoxia, since HIF-1α binds to the hypoxia-responsive element of the EPO gene [39]. The expression of EPO and EPO-R in cancer cells, an apparent consequence of cellular dedifferentiation, may serve analogous pathways of growth and survival that could ultimately enhance tumor aggressiveness. However, the contribution of the EPO/EPO-R axis to cancer progression is not completely understood because its influence on different carcinomas appears to be quite variable [24, 40–42].

It has been communicated that ccRCC consistently coexpresses EPO and EPO-R [43] and that the cytoplasmic expression of EPO in this carcinoma has been shown to be adversely associated with patient survival [44]. Papworth and colleagues reported the co-occurrence of EPO and EPO-R in this tumor tissue without implications in worse prognosis [13].

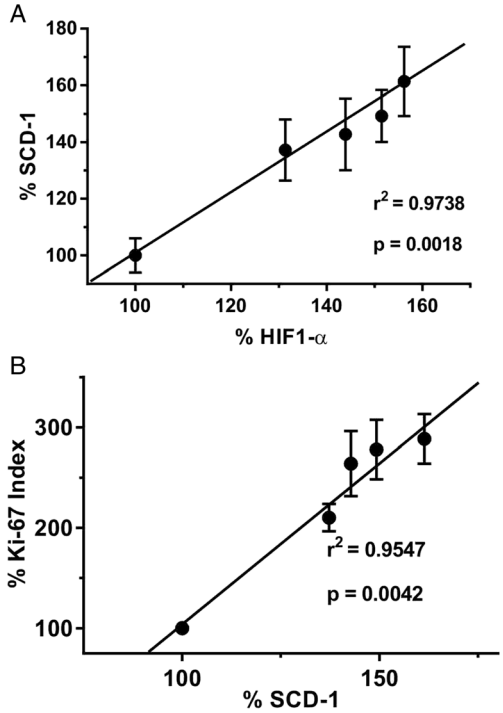


Fig. 9 Correlation analyses related to SCD-1 expression, hypoxia, and proliferation in ccRCC samples. **a** Correlation between the percentage of increment of SCD-1 and the percentage of increment of HIF-1α in each experimental group. **b** Correlation between the percentage of increment of Ki-67 index (Ki-67 % increment) and the percentage of increment of SCD-1 in each experimental group

Thus, the clinical significance of all these findings is, at the present time, controversial.

The present results showed a strong overexpression of HIF-1α, EPO, and EPO-R in ccRCC in agreement with Papworth et al. [13]. However, when EPO expression was related to TMN stage and nuclear grades in ccRCC samples, a strong significance was noted in the initial stage group (T1G1). These results are different from those reported by the mentioned authors, who found majority EPO expression in the higher stages of tumors (III–IV). Hence, we hypothesize that this EPO overexpression in the early tumor stages could be essential for triggering the cell proliferation. As urinary excretion of EPO may be correlated with its tissue levels, current studies performed in our laboratory are evaluating the urinary excretion of EPO as a potential early biomarker of ccRCC. Moreover, as it can be noticed, the EPO and EPO-R expression profiles are not similar, especially in T1G1 group. As in any other biologic pair ligand/receptor, a stoichiometric or evenly, a close relationship between ligands and receptor levels for triggering the subsequent biological effects is not always required [45].

Several proapoptotic and antiapoptotic proteins of the Bcl-2 family, as well as proliferative and apoptotic indexes, have previously been studied in different types of RCC [46, 47]. It is well known that cell proliferation and cell death determine tumor growth and progression. The present data focused in early stages of ccRCC show an enhancement in Bcl-x_L expression and proliferative indexes, as well as a non-significant increment of AI.

Globally, the EPO, EPO-R, and Bcl-x_L overexpression concomitant with a non-significative apoptosis suggest that EPO/EPO-R axis might be part of an autocrine/paracrine loop involved in tumor survival, in accordance with Szejanch et al. [48].

An enhancement of the lipogenic pathway activity is a universal metabolic feature of proliferating tumor cells, given their huge requirements for large amounts of lipids as building blocks for biological membranes. Many observations have shown that this demand is met by an increased de novo lipid biosynthesis. Therefore, the process of lipid synthesis itself may contribute to the tumorigenic phenotype [18]. Many enzymes (acetyl-CoA acetyltransferase; ATP citrate lyase; acyl-CoA synthetase long-chain, or stearoyl-CoA desaturase) within the fatty acid and cholesterol-biosynthesis pathways are regulated by SREBPs, and some of them show overexpression in different cancers. SCD-1, a target gene of SREBP, has been observed upregulated in oncogene-transformed cells [49] and in several human cancers [50, 51].

Von Roemeling and collaborators have shown that SCD-1 overexpression supports ccRCC viability. Therefore, they have proposed SCD-1 as a novel molecular target for therapy [22] in agreement with former studies [20, 52].

The present results showed an increased RNA messenger and protein expression of SCD-1 in this renal cancer cells. Notably, SCD-1 expression increased with the tumor size and with Fuhrman nuclear grade.

It has also been reported that HIF is crucial in modulating cellular hypoxic responses through altering cell energy metabolism, which includes the modification of glucose and lipid metabolism-associated gene expression, thus promoting tumor progression. Lee et al. [53] have recently demonstrated that hypoxia treatment stimulates the expression of SCD-1 in human mesenchymal stem cells concomitantly with their proliferation. Particularly, data analysis reveals statistical positive correlations between SCD-1 and HIF-1α, as well as between SCD-1 and Ki-67, what have not been previously reported, suggesting a putative involvement in tumor progression in early stages of ccRCC. These new data add encouraging information to support SCD-1 inhibition as a potential therapeutic strategy to stop cell proliferation. However, the molecular interplay between these molecules needs further research with the accomplishment of knockdowns assays of SCD-1 and HIF-1α in ccRCC cell lines.

Focusing on the relationship between the studied proteins and patient's survival, there are controversial reports about the role of EPO-EPO-R signaling in RCC tumor growth and progression [13, 43, 44]. Moreover, the combination of EPO-R expression and sEPO levels may effectively predict clinical outcome [54]. Similarly, Rioux-Lectercq et al. [26] have

shown that VEGF expression in ccRCC was significantly correlated with advanced tumor stage, as well as tumor necrosis, tumor aggressiveness, and progression. Besides, Holder et al. [55] found that high levels of SCD-1 expression are associated with significant shorter relapse-free survival (RFS) and overall survival (OS) in breast cancer patients. Additionally, the prognostic significance of HIF-1α expression has been evaluated in a number of solid tumors such as cervical, colon, breast, lung, and gastric cancers [56, 57]. It has been reported that the increased nuclear expression of HIF-1α and the cytoplasmic expression of HIF-2α indicate unfavorable prognosis in RCC patients [58].

To our knowledge, there are no reports regarding the correlation of SCD-1 and HIF-1α expression with survival rate of ccRCC. Thus, we will carry out a survival analysis in our patients in order to analyze if there exists any correlation between these key molecules and survival rate of ccRCC.

In summary, this is the first report concerning the relationship among proliferation, survival, apoptosis, with the expression of key molecules related to tumoral hypoxia (HIF-1α, EPO, VEGF), their receptors (EPO-R, VEGFR-2), and SCD-1 in early stages of ccRCC. Additionally, this study demonstrates for the first time that EPO shows a highest expression in the initial stage group of ccRCC (T1G1), allowing us to postulate this protein as a potential early biomarker of this pathology.

This study provides new information of tumoral biology of ccRCC regarding to hypoxic microenvironment, lipogenesis, and cell proliferation that might contribute to a better understanding of this complex carcinoma.

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