EXPRESSION OF VEGF-A, HIF-1 A, CD34 AND KI67 IN CLEAR CELL RENAL CELL CARCINOMAS AND THEIR RELATIONSHIP WITH CONVENTIONAL PROGNOSTIC MARKERS

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RESUMEN

El carcinoma renal de células claras es la variante más frecuente de carcinoma renal. En los últimos años, la atención se ha enfocado en la expresión de factores angiogénicos por estos tumores, lo que justificaría en parte su capacidad de crecer, invadir y diseminarse, determinando una peor evolución de aquellos pacientes con un perfil angiogénico desfavorable. Se estudiaron 83 piezas de nefrectomía con diagnóstico de carcinoma renal de células claras. Se recolectaron datos clínicos y patológicos. Los tumores fueron estudiados para evaluar la expresión inmunohistoquímica de los siguientes marcadores: VEGF-A, HIF-1α, CD34 y Ki67. Los resultados indicaron una relación lineal directa entre la expresión de estos cuatro marcadores. Además, la expresión de HIF-1α se encontraba directamente relacionada con el grado de Furhman, la invasión de la vena renal y el estadio tumoral. Asimismo, el índice de proliferación tumoral, evaluado con Ki67, se hallaba directamente relacionado con la presencia de necrosis, la invasión capsular y el estadio tumoral avanzado. Con respecto a la expresión de CD34, mientras mayor es la densidad vascular, menor es la necrosis tumoral y menor la sobrevida global. Los hallazgos resultan controvertidos en comparación con la literatura disponible. Se abriría, entonces, un escenario de investigación donde se destaca la importancia de generar estudios prospectivos y más estandarizados para determinar el rol que cumplen estos factores angiogénicos en la evolución tumoral y la posibilidad de estandarizar resultados que permitan un mejor estudio diagnóstico y pronóstico de estos tumores.

Palabras clave: carcinoma renal, factor de crecimiento endotelial vascular, factor inducido por hipoxia, densidad microvascular, índice de proliferación, sobrevida global

ABSTRACT

Clear cell renal carcinoma is the most frequent type of renal carcinoma. Recently, attention has been focused in the expression of angiogenic factors by these tumors, which would justify in part their capacity to grow, invade and disseminate, stating a worse evolution of those patients with an unfavorable angiogenic profile. 83 samples of nephrectomy with a diagnosis of clear cell renal cell carcinoma were studied. Clinical and pathological data were collected. Tumors were studied to assess immunohistochemical expression of the following markers: VEGF-A, HIF-1α, CD34 and Ki67.

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Results indicated a direct linear relationship between expressions of these four markers. Besides, the expression of HIF-1α was directly related to Furhman grade, invasion of the renal vein and tumor stage. Likewise, tumor proliferation index, assessed with Ki67, was directly related to the presence of necrosis, capsular invasion and advanced tumor stage. Regarding the expression of CD34, vascular density was inversely related to tumor necrosis and overall survival. These findings are controversial compared with the available literature. Then, a research scenery would be open, where the importance of generating prospective and more standardized studies are highlighted to determine the role of these angiogenic factors in tumor evolution and prognostic evaluation of these tumors.
Key words: renal carcinoma, vascular endothelial growth factor, hypoxia inducible factor, microvessel density, proliferation index, overall survival

Introduction

In recent years attention has been focused on the expression of different angiogenic factors in renal cell carcinomas, especially in clear cell renal cell carcinomas (CCRCC) because it is the most frequent variant of renal carcinoma (RCC), accounting for 80% of the total. To date, tumor stage and nuclear grade were considered the most important prognostic variables for patients with CCRCC.

It is known that angiogenesis is an important factor that enhances tumor growth, favoring invasion and dissemination. Its development would respond to the biallelic loss of tumor suppressor gene von Hippel Lindau (VHL) present in 50 to 70% of sporadic CCRCC. This gene encodes a protein component of the unit cell that breaks down various angiogenesis-inducing factors such as hypoxia inducible factor 1α (HIF-1α). This factor acts regulating the cellular stress in an hypoxic microenvironment, determining the expression of growth factors, including the vascular endothelial growth factor A (VEGF-A). By altering the degradation of HIF-1α, its persistence leads to increased expression of VEGF-A, allowing increased tumor vascular development [1,2,3]. High HIF-1α and VEGF-A levels have been correlated with high vascular density, higher proliferation rate, higher nuclear grade and advanced tumor stage, determining a poor clinical outcome. Nevertheless, in RCC conflicting results have been presented about protein levels of HIF-1α and VEGF-A, vascular density and different clinicopathological factors.

The aim of the present study was to evaluate the relationship between immunohistochemical expression of HIF-1α VEGF-A, CD34 and Ki67 and clinicopathological parameters in 83 samples of CCRCC.

Material and Methods

Case selection, clinical data and population features

This study included tumor specimens of CCRCC obtained from patients undergoing partial or total nephrectomy at Hospital Privado, Córdoba, Argentina. 83 cases were selected from filed formalin fixed and paraffin embedded tumor tissues collected consecutively from January 2006 to December 2010. All cases were reviewed by two pathologists using WHO (2009) tumor classification criteria. Clinicopathologic data obtained from patient medical records and from files kept at Department of Pathology, included sex, age, tumor size, pathological stage, presence of necrosis, nuclear grade as assessed using Fuhrman nuclear grading system, vascular invasion, capsular involvement, sinus tissue invasion, urinary tract involvement and overall survival (OS).

Selection of tumor samples

Tumor samples were chosen using original hematoxilin/eosine slides. All the slides from each case were carefully evaluated to determine which the better block was, considering the presence of enough viable tumor cells to perform the evaluation of the four different immunohistochemistry markers. Each sample corresponded to a representative block of each case, avoiding those ones with important areas of necrosis or fibrosis. The chosen block was studied in full. In patients with metachronic carcinomas, the larger tumor was studied.

Immunohistochemistry

The selected samples for immunohistochemistry were deparafinated and rehydrated.

The process of antigenic recovery were carried out by using a citrate buffer ph6 (DAKO) for HIF-1α and Ki67, EDTA buffer ph9 (DAKO) for VEGF-A and pepsin for 20 minutes at 37ºC to determine CD34.

The blockage of endogenous peroxidase was performed with H2O2 at 0,3% in methanol for 30 minutes.

Primary antibodies used were HIF-1α, EP1215Y clone, Millipore, in a dilution 1/200 for 45 minutes;, Ki67, Mib-1 clone, DAKO, in a dilution of 1/100 for 30 minutes; VEGF-A, VG1 clone, DAKO, in a dilution of 1/50 for 60 minutes; and CD34, QEnd10 clone, DAKO, in a dilution 1/50 for 30 minutes. All antibodies were incubated at room temperature.

To reveal the technique, LSAB + kit HRP of DAKO were used according to the manufacturer protocol (15 minutes of the secondary antibody and 15 minutes of the tertiary reagent). Diaminobencidine (DAB-DAKO) as chromogen was used for 10 minutes.

All samples were processed in an Autostainer Plus of DAKO and they were counterstained with hematoxylin before their visualization.

Evaluation of immunostaining

Immunohistochemical staining results were evaluated independently by two pathologists, without knowledge of clinicopathologic data on each individual case. Appropriate positive and negative controls were included for each antibody.

HIF-1α immunoreactivity was evaluated as percentage of nuclear positivity. At least 10 high-power fields including tumor were evaluated. Weak cytoplasmic staining was detected in normal renal tubules and mesangial cells.

The immunostaining of VEGF-A was evaluated as percentage of cytoplasmic staining pattern in tumor cells. At least 10 high-power fields including tumor were evaluated. Moderate cytoplasmic staining was seen in normal renal tubules.

CD34 density was assessed by counting vessels of small caliber in three high-power fields and calculating the mean value.

Ki67 index was also quantified assigning a percentage value that was calculated by scoring 500 tumor cell nuclei.

Illustrative pictures are shown in figure 1.

Statistical analyses

All statistical analyses were done using the software IBM SPSS Statistics version 15.0. Different statistical tests were performed to assess the influence of angiogenic factors (HIF-1α and VEFG-A), vascular density (CD34) and proliferation factor (Ki67) among each other and among other clinicopathological variables such as tumor size, Furhman grade, capsular invasion, sinus tissue invasion, urinary tract involvement, presence of necrosis and tumor stage according TNM - UICC 2009. The linear association among variables of continuous nature was assessed with Pearson´s correlation. By means of studies of binary logistic regression, variables associated to an increase or decrease of the probability of events of interest were stated. Kruskal Wallis test was

applied as a non-parametric alternative to the analysis of variance (ANOVA).
Overall survival was calculated from date of diagnosis to date of death or last follow-up. Distribution of OS was estimated using the method of Kaplan-Meier and differences in OS was assessed by the stratified log-rank test.

Results

Clinical and pathological features

The study included 83 patients who had a diagnosis of CCRCC. 73.5% (61) were males and 26.5% (22) were females. The mean age was 57 years (range 27 to 78 years); median age was 59 years.
26.5% (22) had a partial nephrectomy and 73.5% (61) underwent total nephrectomy.
Regarding tumor size, the mean value was 49 mm (range 18 to 130 mm); median value was 50 mm.
Most cases presented with localized tumors (80%) (66). 64% (53) were at pathological stage I, 16% (13), at stage II and 20% (17), at stage III.
According to Fuhrman nuclear grading system, 12% (10) tumors were grade IV, 53% (44), grade III, 34% (28), grade II and 1% (1), grade I.
31% (26) showed necrosis above 10%.
Renal vein invasion was informed in 17% (14), capsular invasion in 18% (15), sinus tissue invasion in 10% (8) and urinary tract involvement in 7% (6) (table 1).
OS time was 24 months (range 6 to 68 months). Twelve patients developed metastases in their follow-up, affecting lung, bone and adrenal gland. Four of them died of the disease. Three patients died immediately after surgery due to hemorrhagic complications. At the end of this study, 84% of the patients were alive.
Five patients had tumors with sarcomatoide differentiation and three patients had metachronic bilateral carcinomas.

Statistical analisis

Pearson´s correlation stated that CD34 expression was linearly associated with HIF-1α expression (p=0,029). Likewise, HIF-1α expression was directly associa-

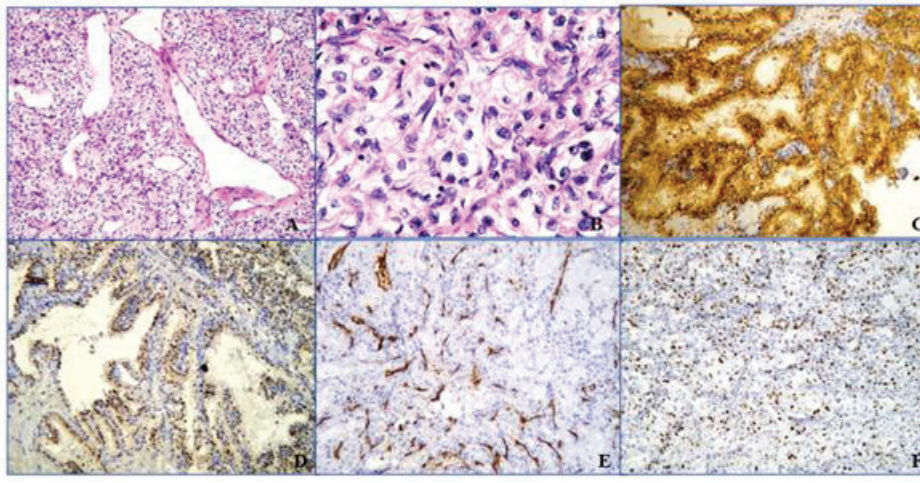

Figure 1
A: clear cell renal cell carcinoma (H/E – 10x)
B: clear cell renal cell carcinoma (H/E – 40x)
C: VEFG-A staining in clear cell renal cell carcinoma (10x)
D: HIF-1α staining in clear cell renal cell carcinoma (10x)
E: CD34 staining in clear cell renal cell carcinoma (10x)
F: Ki67 staining in clear cell renal cell carcinoma (10x)

ted with VEGF-A expression (p<0,000) and VEGF-A expression was related to the proliferation index (Ki67) (p=0,010) (direct linear association). Finally, OS was found in inverse linear relationship with tumor size (p=0,006) and CD34 expression (p=0,048).

Kruskal Wallis test found that CD34 expression was lower in presence of necrosis (p=0,029), while Ki67 expression showed statistically significant differences according to tumor stage (p=0,006). Proliferation index was higher in T3 stage regarding T1 and T2 stages (localized tumors).

In the studies of logistic regression, the following results were found. Expression of HIF-1α was directly related to Furhman grade (I-II vs. III-IV) (p=0.029), invasion of the renal vein (p=0.016) and tumor stage (T1-T2 vs. T3) (p=0.040). Likewise, tumor proliferation index, assessed with Ki67, was directly related to the presence of necrosis (p=0.008), capsular invasion (p=0.036) and advanced tumor stage (p=0.007). Regarding expression of CD34, a higher vascular density was inversely related to tumor necrosis (p=0.031).

Tumor size turned out to be a factor which was constantly related to features of locoregional aggressiveness such as capsular invasion (p=0.018), invasion of the renal vein (p=0.000), sinus tissue invasion (p=0.037), urinary tract involvement (p=0.006), presence of necrosis (p=0.000) and advanced tumor stage (p=0.000). Besides, it was found in inverse relationship with OS.

OS regarding CD34 expression was lower in those patients with vascular density equal or higher than 50 (Long-Rank of Mantel-Cox, p=0.017). With respect to proliferation index, OS did not turn out statistically different in patients with an expression lower than 10% and higher or equal than 10% (Long-Rank of Mantel-Cox, p=0.354). A similar result was obtained for the survival analysis regarding VEGF-A factor, comparing patients with an expression lower than 30% and higher or equal than 30% (Long-Rank of Mantel-Cox, p=0.189). There were no differences found in OS for patients with HIF-1α expression under 10% compared to patients with HIF-1α expression, higher or equal to 10% (Long-Rank of Mantel-Cox, p=0.562). These results may be appreciated from the observation of Kaplan-Meier curves on figure 2.

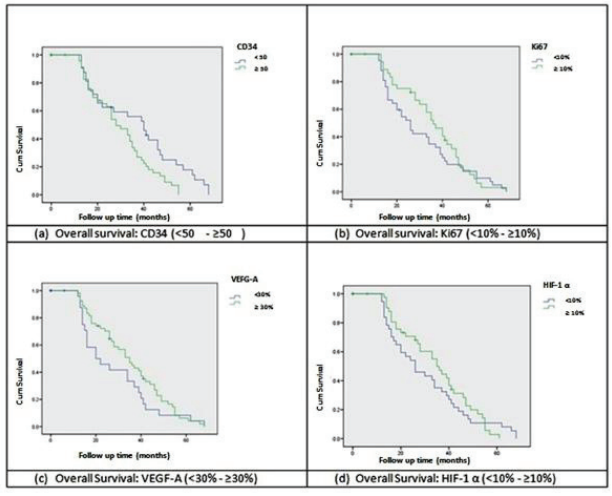

Figure 2
Kaplan-Meier survival analysis curves comparing groups according immunohistochemical expression of the four different markers

Discussion

In the last years, attention has been focused in the expression of several angiogenic factors in RCC, especially in CCRCC since they are the most frequent variety of renal carcinoma and they would have more vascularization regarding the remaining subtypes. It is known that angiogenesis is a favoring event of tumor growth, although the mechanisms involved in its development have not been elucidated clearly yet. Besides, it plays an important role in tumor invasion and spread.

In this study, 83 cases of CCRCC processed in the Pathology Department of Hospital Privado de Córdoba were reviewed so as to interpret the expression of angiogenic factors, vascular density and proliferation index in order to correlate findings with regularly assessed clinicopathological characteristics in this type of tumors.

According the different angiogenic factors studied in the available literature, VEFG-A and HIF-1α have been the most important ones, the results being controversial and inconsistent according to different studies. Attention has also been focused in tumor microvascular density, a feature directly associated to the expression of angiogenic factors. Proliferation index was also assessed with different results.

Concerning expression of VEGF-A, in 1997, Nicol et al showed that it was increased in RCC, using Western Blot techniques and immunohistochemistry (4). In 1994, Takahashi et al, evaluated the expression of mARN of VEGF-A in RCC and detected an increase compared to normal renal parenchyma [5]. Afterwards, two studies, (Tomisawa et al; Zhang et al) showed that expression of VEGF-A was directly related to tumor stage [6,7]. Besides, Zhang et al proposed that a higher expression of VEGF-A was directly associated to a higher vascular density [7]. Paradis et al showed that expression of VEGF-A increased with higher tumor size and was correlated to vascular density [8]. Jacobsen et al found a direct relationship between VEGF-A expression and tumor stage [9]. Yilmazer et al found that a higher expression of VEGF-A was found in direct relationship with tumor size, stage, vascular density and capsular invasion [10]. In 2009 Djorevic et al reported that VEGF-A expression increased related to a higher nuclear grade, higher stage and higher size. VEFG-A expression was not related to proliferation index [11]. Even though, in 2007, the same group had reported that a higher expression of VEGF-A was related to a higher nuclear grade and a higher proliferation index [12]. On the other hand, Minardi et al did not find any association between VEGF-A expression and tumor stage, nuclear grade, capsular invasion and necrosis. They did report a direct relationship with HIF-1α expression and vascular density [13]. In this study, the expression of VEGF-A was only directly related to proliferation index. A statistically significant relationship between the VEGF-A expression and the remaining analyzed variables was not found.

Regarding the expression of HIF-1α, Lidgren et al in 2006 did not find any association between HIF-1α expression with tumor stage, nuclear grade, tumor size or invasion of the renal vein [14]. Klatte et al did not find any significant differences between the expression of this factor concerning stage or nuclear grade as well. They report that the lower expression of HIF-1α was associated to smaller tumors [15]. On the other hand, Djorevic et al reported that the expression of HIF-1α increased in tumors with higher nuclear grade, higher stage and higher size [11]. In our study, the expression of HIF-1α was directly related to nuclear grade, tumor stage and invasion of the renal vein.

If we focus in the vascular density, Sharma et al found that tumor vascular density was higher in tumors of advanced stage

[16]. Kavantzas et al showed that a higher vascular density was related to a higher nuclear grade [17]. On the other hand, Nativ el al showed that vascular density was lower regarding a higher nuclear grade [18]. Herbst et al found an inverse relationship between vascular density in regards to nuclear grade and proliferation index [19]. MacLennan et al did not find a significant association between vascular density and stage or nuclear grade [20]. Yagasaki et al reported that vascular density was lower as the tumor size increases [21]. In this study, vascular density was inversely related to the presence of necrosis, and in a direct relationship with HIF-1α expression.

About to proliferation rate, Onda et al found that the expression of Ki67 was in direct relationship with tumor stage [22]. Zhang et al showed a direct relationship with stage and nuclear grade [23]. In this study, this index showed an association with presence of necrosis, capsular invasion and advanced tumor stage.

Concerning OS, Jacobsen et al reported that those renal tumors with an expression of VEGF-A which was higher than 30% had less survival [9]. Minardi et al found similar results [13]. Yildiz et al found that a highest expression of VEGF-A and a high proliferation index were correlated to a lower survival [24]. In another study, Lidgren et al reported that the highest expression of HIF-1α was a favorable prognostic factor [25]. On the other hand, Klatte el al reported that patients whose tumors showed high expression of this marker have less survival [15]. Regarding vascular density, MacLennan et al did not find a relationship between tumor vascular density and OS [20]. On the other hand, Joo et al found that a higher vascular density was related to a worse survival [26]. Rioux-Leclercq el at showed that a higher proliferation rate was associated to less survival [27]. In our study, OS was significantly lower in those patients whose tumors showed a higher vascular density, assessed by CD34 expression.

If we analyze the reasons which may explain the existence of very controversial results regarding the expression of these factors related to angiogenesis in RCC, the differences may be due to the use of different antibody clones for immunohistochemistry technique, to the intra and inter-observer variability to evaluate immunohistochemical expression of the different markers and to the absence of standardized values to interpret these immunohistochemistry studies. What we must highlight in our study, given certain technical limitations, is the measurement of the vascular density which was performed through staining with CD34 and estimating the average of small vessel counts in fields of great magnification in a conventional optical microscope [16,17,28].

Up to date, there are no other studies where the interrelationship between the expression of VEGF-A, HIF-1α, CD34 and Ki67 was assessed among each other and with other clinicopathological variables.

Finally, when assessing the interrelationship between the expression of the four markers evaluated in this study, we found a linear relationship regarding the tumor expression of HIF-1α and VEGF-A. Besides we observed that they were related to a higher vascular density and a higher proliferation index, which suggests a close relationship between the expression of angiogenic factors by the tumor, which would induce a higher formation of vascular channels and a higher proliferation rate. In addition to, the results showed that VEGF-A predicted proliferation, a reduction in CD34 expression predicted necrosis, Ki67 expression correlated with a higher stage of cancer as well as a reduction in OS, and HIF-1α expression predicted a higher grade and a higher stage of cancer but did not correlate with OS.

As a conclusion, although the findings are

controversial, the expression of angiogenic factors such as the ones studied, VEGF-A and HIF-1α, in CCRCC is a acknowledged fact and opens a research scenery where the importance of generating prospective and more standardized studies is highlighted to specify the role of these angiogenic factors in tumor evolution and to evaluate the ability to standardize results that allow better diagnostic and prognostic studies of these tumors.

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