Actas Urológicas Españolas. 2011;**35(2)**:80–86

Actas Urológicas Españolas

www.elsevier.es/actasuro

ORIGINAL ARTICLE

Immunohistochemical expression of microvascular density and carbonic anhydrase IX in renal carcinoma. Relation to histological type and tumoral progression

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Received August 13, 2010; accepted October 10, 2010

KEYWORDS
Carbonic anhydrase IX;
Immunohistochemistry;
Microvascular density;
Prediction;
Prognosis;
Renal cell carcinoma

Abstract

Purpose: To correlate the immunohistochemical expression of microvascular density (MVD) and carbonic anhydrase IX (CAIX) with the different histological subtypes of renal cell carcinoma and its progression.

Material and methods: We studied 93 patients with renal cell carcinoma operated between 1990 and 2008. Antibodies employed for immunohistochemistry (IHC); CD31 (1: 40, Dako) and CD 34 (1: 50, Dako) for MVD and CAIX (1: 100, Santa Cruz). CAIX was validated semi-quantitatively as: strongly positive (>85%); weakly positive (10%-85%); and negative (< 10%), independently of the intensity of the stain. MVD was validated with both anti-CD31 and anti-CD34 by means of a whole section to select the microscopic field (x100) with highest density of stained vessels, counting the number of vessels in a photographic field of 0.53 mm^2. Results are expressed as the maximal number of vessels by mm^2 of tumour tissue.

Results: The median follow-up was 40 months (1-160). We found no differences of expression with any of the 3 IHC markers between tumours that progressed (49) and tumours that did not progress (44). The IHC expression of CAIX was strongly related to MVD, measured for both CD31 and CD34 ($p< 0.0001$). MVD with both antibodies was inversely related to tumour size and the Fuhrman grade and was also stronger in clear cell carcinomas compared to the rest of histological subtypes, measured by CD31 ($p=0.001$) and CD34 ($p=0.003$).

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Conclusions: Neither MVD nor CAIX expressions were related to tumour progression, but were related to histological subtypes. This fact, added to their co-expression, could prompt the use of the CAIX expression, which is far more reproducible as a quick and easy approximation to MVD. More research should be done to use it as a marker for targeted therapy.

PALABRAS CLAVE
Anhidrasa carbónica IX;
Carcinoma renal;
Inmunohistoquímica;
Densidad microvascular;
Predicción;
Pronóstico

Expresión inmunohistoquímica de la densidad microvascular y de la anhidrasa carbónica IX en carcinoma renal. Relación con el tipo histológico y con la progresión tumoral

Resumen

Objetivos: Relacionar la expresión inmunohistoquímica (IHQ) de la densidad microvascular (DMV) y de la anhidrasa carbónica IX (ACIX) con los tipos histológicos de carcinoma renal (CR) y con su progresión.
Material y métodos: Se estudiaron 93 pacientes operados por CR entre 1990-2008. Anticuerpos: CD31 (1: 40, Dako) y CD 34 (1: 50, Dako) para DMV y ACIX (1: 100, Santa Cruz). ACIX se valoró semicuantitativamente; intensamente positivos (> 85%), débilmente positivos (10%-85%) y negativos (< 10%), independientemente de la intensidad de la tinción. La DMV se valoró independientemente con anti-CD31 y anti-CD34. Campo de bajo aumento (x100) con mayor densidad de vasos teñidos; se contabilizó el número de vasos en un campo fotográfico de 0,53 mm^2. Resultados expresados como número máximo de vasos/ mm^2 de tejido tumoral.
Resultados: Mediana seguimiento; 40 meses (1-160). No encontramos diferencias IHQ para ninguno de los 3 marcadores entre tumores que progresan (49) y no progresan (44). La expresión de ACIX estaba relacionada con la DMV ($p < 0,0001$). La DMV se relacionó inversamente con el tamaño tumoral y con el grado de Fuhrman de forma significativa. Así mismo, fue significativamente mayor en los CR de células claras tanto medida con CD31 ($p = 0,001$) como con CD34 ($p = 0,003$) frente al resto de subtipos histológicos.
Conclusiones: La DMV y la expresión de ACIX no se relacionan con la progresión, pero sí con el tipo tumoral. Ello y su coexpresividad permitiría usar la expresión de ACIX como medida orientativa rápida y fácil para medir DMV y su posible relación con la respuesta a antiangiogénicos.

Introduction

Renal cell carcinoma (RCC) is characterized molecularly by a high rate of inactivation of the *von Hippel-Lindau* tumour-suppressor gene, which entails an accumulation of the hypoxia-inducible factor and the resulting expression of different hypoxia-responsive genes that will activate the angiogenesis process, including the carbonic anhydrase IX gene (CAIX) and the vascular endothelial growth factor gene. Targeted therapies have been a revolution in its treatment in the last decade. Today, there are currently five drugs with different mechanisms of action approved for its first or second-line indication. There are significant gaps as concerns the treatment of each histological type, sequential or concomitant treatment, neoadjuvancy and adjuvancy with these drugs, etc. Several groups have searched for serous, pathological and molecular markers that allow optimizing each treatment for each patient.[1,2]

Angiogenesis can be quantified in solid tumours by means of immunohistochemistry (IHC) in terms of microvascular density (MVD), defined as the number of small vessels in a specific tumoural area. In RCC, the association of MVD with prognosis is controversial,[3] probably due to problems in the homogenization of the results of the different works that follow the traditional manual count method[4] among others, as well as to the small sample sizes used, different IHC markers and to the limitations themselves of the IHC.[5]

CAIX is an enzyme that belongs to a family of metalloenzymes that catalyze the rapid conversion of carbon dioxide and water to bicarbonate and protons and that regulates intracellular and extracellular pH during hypoxic stress. Its active part is extracellular and participates in the acidification of the extracellular medium. The acidification of the extracellular matrix induces the expression of pro-angiogenic factors; it enhances cellular immunity and boosts tumour aggressiveness.

CAIX is a very interesting marker in RCC for several reasons, firstly, as a diagnostic tool, given that it is expressed in 86%-96% of the tumours, whereas its practically nil expression in healthy kidney tissue. Furthermore, it has a different expression depending on the histological type, is prevalent in that of clear cells and is not expressed in oncocytomas.[6] Secondly, it has been used as a prognostic tool, given that it has been included in predictive nomograms for progression,[7]

although the results have been controversial.[8-10] Thirdly, it has been proposed as a predictive tool for response to treatment, established for interleukin-2[11-13] and currently in investigation with targeted therapies.[14-16] Lastly, it has potential use as a staging tool. In fact, given the similarity of the anti-CAIX antibody to the G250 monoclonal antibody (mAbG250), it has been possible to diagnose RCC in small renal masses and metastatic foci with high specificity and sensitivity.[17] Consequently, its possible therapeutic value is currently under investigation in radioimmunotherapy protocols.[18,19]

Our aim is to assess the MVD, CAIX and the relation between the two in a group of renal carcinomas, as well as to also associate them with progression and the different histological types of the disease.

Materials and methods

Patients

Subsequent to approval by the Ethics Committee of the Oncological Institute of Valencia, we retrospectively checked 100 patients operated for renal cell carcinoma between 1990 and 2008 by means of open or laparoscopic radical nephrectomy, with sufficient paraffin-embedded tissue in good state to be studied anatomopathologically. We selected 50 patients that had progressed with local, nodal and/or distant recurrence, and 50 patients that had not at the time the study began.

Immunohistochemical methods

We used formalin-fixed paraffin-embedded tissue in all the cases. We examined the hematoxylin-eosin-stained sections to confirm the diagnosis and to select a representative block of the peripheral area of the tumour, where its proliferative activity and vascularization are greater. For the IHC techniques, the antibodies we used were CD31 (1: 40, Dako), CD 34 (1: 50, Dako) and CA IX (1: 100, Santa Cruz).

In all the cases, we obtained 4-micron sections on slides treated with poly-lysine-coated slides using blocks of formalin-fixed paraffin-embedded tissue. After deparaffinizing the tissue, we subjected the sections to a process of antigen retrieval with heat and subsequent staining using the automated PTLink Dako® and AutostainerPlus Dako® systems respectively.

A single pathologist specialized in uropathology (CA) evaluated CAIX semi-quantitatively. He did not possess the clinical data at the time of the evaluation and collected the percentage of membrane-stained tumour cells. Following the criteria previously described in literature and used by many researchers in a reproducible manner, the cases were classified as intensely positive (>85%), weakly positive (10%-85%) and negative (<10%), regardless of stain intensity.[8,20]

Density was assessed independently in the sections stained with anti-CD31 and anti-CD34. In each case we studied the complete section to choose the low power field (x 100) with greater density of stained vessels. We photographed and counted the number of vessels in a photographic field of 0.53 mm^2. We considered all those stained cells or groups of cells without a muscular layer around them to be vessels that could be separated from other vessels or adjacent structures, although they did not have an identifiable vascular illumination. In tumours in which vascularization formed a dense network, we interpreted each different branch to be an independent vessel. We counted the large anastomotic sinusoids as a single vessel. We expressed the results as the maximum number of vessels per mm^2 of tumour tissue.[21-23]

Statistical methods

We compared the groups using the chi-squared method for categorical variables (Fisher test for 2x2 tables) and the Kruskal-Wallis or Mann-Whitney tests to compare categorical variables with continuous variables. In all the studies, we considered a value of $p<0.05$ (two-tailed test) to be statistically significant. The statistical analyses were carried out using SPSS software (version 15.0.1, SPSS Inc., Chicago, Illinois, USA).

Results

Follow-up

During the performance of the series, we did not consider the IHC technique to be informative due to different technical problems in seven cases. We therefore considered a total of 93 patients to be valid for the study, 49 of whom had progressed (53%) and 44 had not (47%). The mean follow-up was 40 months, with a range of 1-160 months.

Results of the immunohistochemical techniques

We evaluated the MVD independently with both CD31 and CD34 antibodies. The exact location of the stained membrane and the cytoplasm of the endothelial cells was not precise (fig. 1). On the contrary, staining with CAIX is localized in the membrane and is clearly delimitable (fig. 2). We did not observe positive staining in the non-tumoural renal tissue.

Table 1 List of the positive or negative expression of carbonic anhydrase IX with vascular microdensity (VMD) measured with both antibodies (CD31, CD34) and expressed as a maximum number of vessels per mm^2 of tumour tissue

CAIX	VMD CD31/mm^2 (Dt)	p Value	VMD CD34/mm^2 (Dt)	p Value
Negative	247.6 (169.9)	<0.0001	197.4 (135.9)	<0.0001
Positive	521.6 (227.8)		433.3 (226.1)	

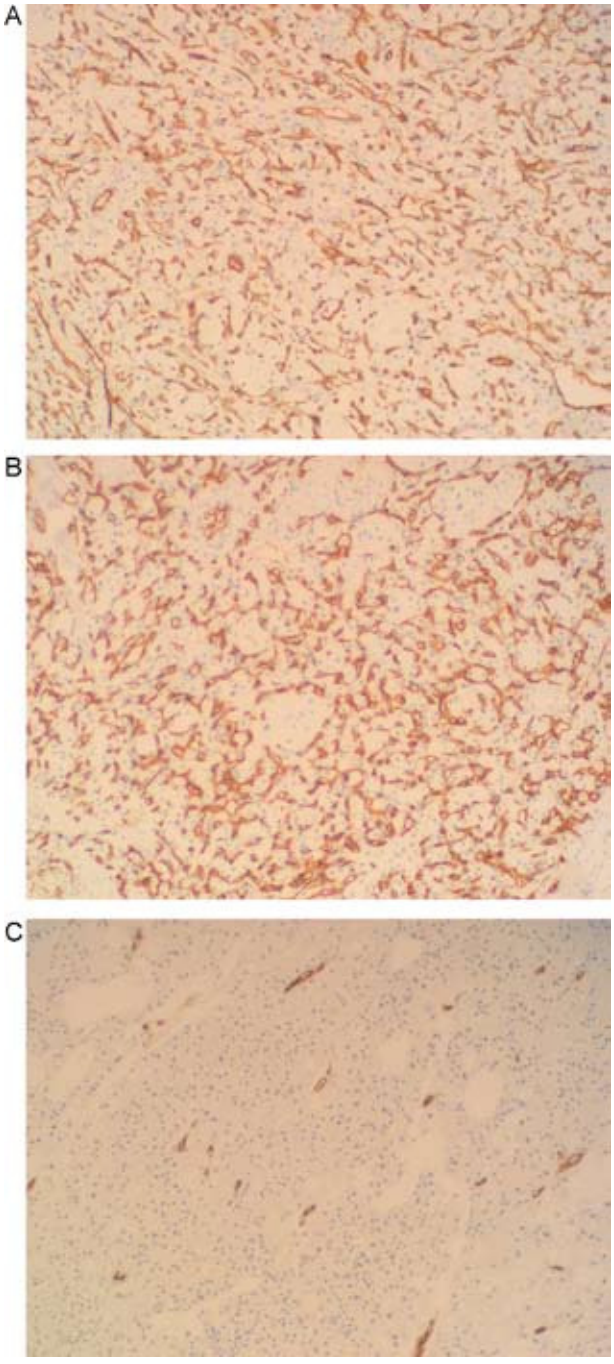

Figure 1 (A) Clear cell RCC, Fuhrman grade 2, CD31 (x100), VMD 977/mm^2, (B) Clear cell RCC, Fuhrman grade 2, CD34 (x100), VMD 883/mm^2, (C): Chromophobe RCC, CD31 (x100), VMD 49/mm^2.

Pathological results

We analysed a total of 79 (85%) clear cell renal cell carcinomas, 8 (9%) chromophobe, 5 (5%) papillary and 1 (1%) sarcomatoid. The MVD was significantly greater in the clear cell RCC, measured both with CD31 (p=0.001) and with CD34 (p=0.003) in comparison to the rest of the histological subtypes (fig. 3). It was inversely related with the tumour grade both with CD31 (p=0.0005) and with

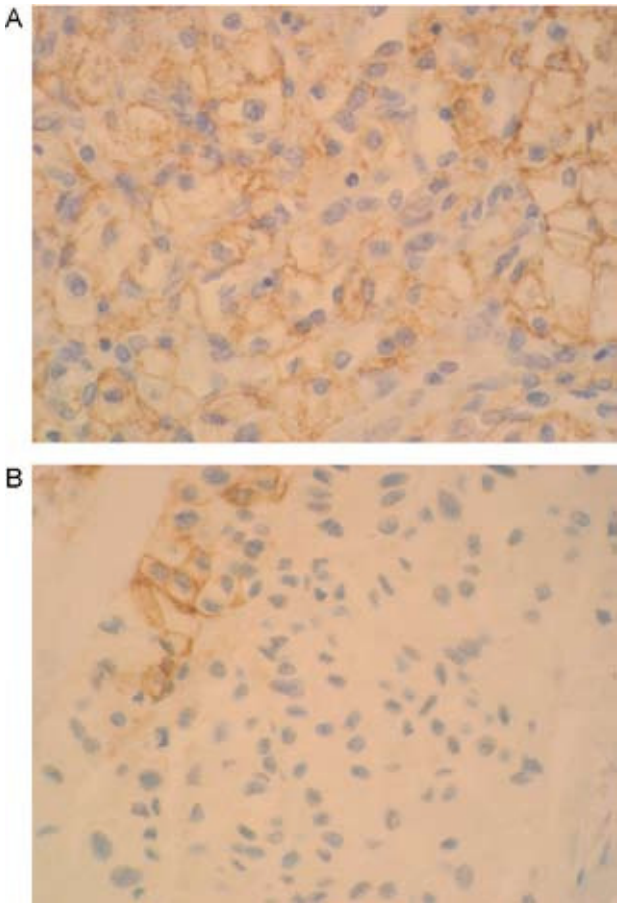

Figure 2 (A) Clear cell RCC, Fuhrman grade 2, CA9 (x100) intensely positive (stain 100% of the cells), (B): Chromophobe RCC, (CA9 (x100) negative (stain 5% of the cells).

CD34 (p=0.001), grouping Fuhrman classification I-II vs. III-IV. Similarly, we objectified an inverse relation between tumour size (≤7cm vs. >7cm) and the MVD measured with both antibodies (fig. 4).

The expression of CAIX is strongly associated with the expression of the MVD, both if measured with CD31 and with CD34 (p< 0.0001) (table 1). None of the expressions of the three antibodies used were significantly associated with tumour progression, or CAIX (p=0.771) or the MVD measured with CD31 (p=0.250) or with CD34 (p=0.842).

Discussion

The lack of effective serous, pathological or molecular markers in renal cell carcinoma does not help to resolve the controversies of the new therapeutic panel for this neoplasia. In this respect, it would be logical to think that as targeted therapies aimed at combating tumour angiogenesis, the measurement of MVD should be an important parameter in the management of these drugs in RCC. However, this is not the case. Probably one of the reasons why this does not occur lies in its form of evaluation. The majority of authors, including our work, follow the method described by Weidner et al.[4] based on a manual count, and although it has allowed developing this

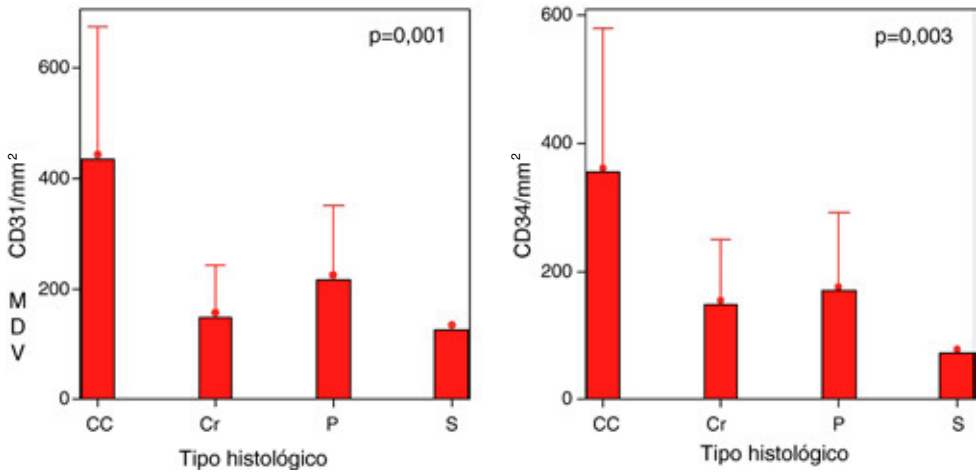

Figure 3 Expression of VMD measured with the CD31 antibody (left diagram) and with the CD34 antibody (right diagram) in RCC according to histological type.

concept in solid tumours, it is subjected to significant inter-observer variability and is considered to be cumbersome and tedious by pathologists. We ourselves ratified that it is difficult to reproduce in the routine activity of a healthcare laboratory. These methodological problems have been handled by applying an automated quantitative analysis system capable of automatically measuring variations in the protein expression in tumours in tissue microarrays or standard preparations, adding objectiveness as opposed to the traditional evaluation method. This system has allowed introducing a new concept, the area of microvessels, which was directly associated with a better prognosis in two independent studies of 284 cases.[3]

Our results show greater MVD in clear cell renal cell carcinomas. This has also been described by other authors,[21] as well as its inverse association with tumour diameter[23,24] and tumour grade.[23,24] We did not observe any relation between MVD and tumour progression, which coincides with the publications of some authors,[21,22,25] although not in other series.[26,27]

The contradiction that RCC with low MVD has a worse prognosis as opposed to what occurs in other tumours has resulted in different hypotheses. Of them, worth mention is the inability of microvascularization to adapt to aggressive growth, giving way to an increase in vascular permeability[28] Another hypothesis focuses on the fact of considering that MVD manifests good differentiation, given that the neoplasia originated in the renal cortex that is considerably vascularised.[29] It has also been postulated that the formation of macrovessels by rapidly growing tumours conditions a decrease in MVD.[26]

In the case of CAIX, the cut value of 85% of the stained tumour cells was established in a series of 321 carcinomas through the analysis of the survival tree[8] and has been ratified by other authors that consider this IHC to be easily reproducible and comparable. The co-expressiveness of MVD and CAIX that we found in our work (table 1), knowing that CAIX is one of the pro-angiogenic genes overexpressed after activation of HIF-1, allows evaluating this molecule as a rapid, reliable and reproducible method for evaluating MVD. In fact, in a study on 122 renal neoplasias, the co-expression CAIX-VEGF was objectified as an independent prognostic factor in the multivariate analysis for cancer-specific survival (RR 2,353; IC 95% 1,473-3,375, p=0.0002). This co-expression was able to stratify cancer-specific survival into three risk groups: high, medium and low (p<0.001).[30]

Therefore, added to the possibilities indicated to consider CAIX as a molecule of interest in the management of RCC, is the fact that it shows MVD in a direct manner. In our results on non-metastatic renal neoplasias, we did not

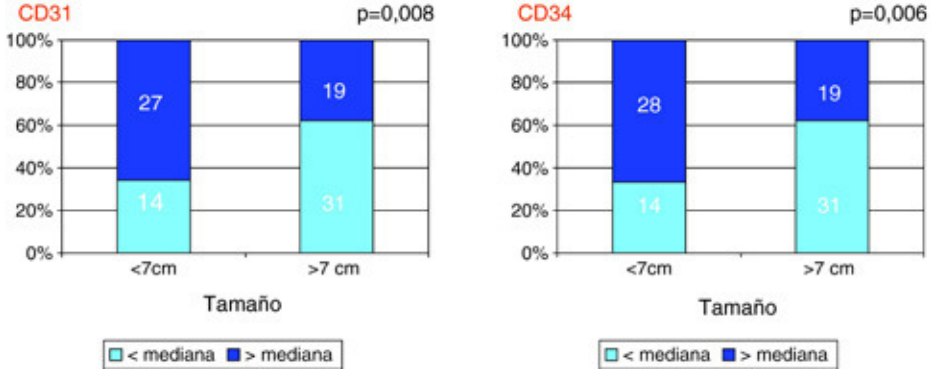

Figure 4 List of VMD measured with CD31 (left diagram) and with CD34 (right diagram) depending on its expression above or below its mean with the tumour size, with cut value at 7 cm.

objectify that it has any prognostic value, and the same has been observed by other authors.[8,9] However, it does appear to have a prognostic value in metastatic cancers.[8,10]

Regarding the controversy on its prognostic value, it can be inferred that CAIX may have more predictive value of response to the treatment than a prognostic value in itself. This is clearly validated in metastatic tumours treated with IL-2.[11-13] In relation to targeted therapies, the expression of CAIX has been validated as a predictive factor of response to Sorafenib[16] and has not been associated with the response to Temsirolimus, where a high expression of phospho-Akt or phospo-S6. However, upstream and downstream molecules of the cell signalling path governed by mTOR have logically been related with the response to this drug.[14] A trial in athymic rats with RCC implants demonstrated that after simultaneous treatment with mAbG250 and different tyrosine kinase inhibitors, the capture of mAbG250 in the tumour implants, a manifestation of the expression of CAIX, decreased in parallel with different MVD markers. After stopping treatment, the expression of CAIX increased once again, especially in the peripheral area of the tumours where neovascularization was more visible.[15]

It is possible that in the near future CAIX will find a place in the differential diagnosis of renal cell carcinoma, as well as regarding the prognostic value and the predictive value of response to treatment, both with respect to the use of cytokines and tyrosine kinase inhibitors, as it is a molecule that is involved in angiogenesis. It could also be used as a reflection of the MVD index.

Funding

Study financed by Fundación para la Investigación en Urología.

Conflict of interest

The authors declare not to have any conflict of interest.

Thanks

Additional thanks to Vanesa Pérez (Data Manager of Servicio de Urología del IVO) and to María García Flores and Tania Mazcuñán (Lab Technicians).

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