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Prognostic Implications of Serum Carbonic Anhydrase IX in Clear Cell Renal Cell Carcinoma

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Purpose: The present study aimed at determining the expression of carbonic anhydrase IX (CAIX) in tissue and serum of patients with clear cell renal cell carcinoma (ccRCC) and its relationship with clinical-pathological parameters; assessing CAIX as a marker of progression and survival; and studying the relationship of CAIX concentration in serum before and after surgical intervention.

Materials and Methods: Immunohistochemistry was used to assess the expression of CAIX in tumor and adjacent healthy renal tissues. The concentration of CAIX in serum was determined using commercial enzyme-linked immunosorbent assay before and 24 hours after radical nephrectomy in 60 patients diagnosed with ccRCC. SPSS ver. 28.0.1.0 was used for descriptive and inferential statistical analysis and graphics. A significance level of 0.05 was considered for all statistical tests performed.

Results: CAIX expression was positive in 59 of the 60 samples of tumor renal tissue, and negative in the 60 samples of healthy renal tissues. Median serum CAIX concentration was higher before nephrectomy (178.25 pg/mL) than after it (59.30 pg/mL), with a high correlation between pre-and postsurgical measurements (r=0.891). Significant differences were found in CAIX concentration according to the following variables: TNM, tumor stage, Fuhrman nuclear grade, progression, and death. Serum CAIX concentration before nephrectomy correlated with disease progression and overall survival, with a hazard ratio of 4.849 for CAIX values greater than 169.95 pg/mL.

Conclusion: CAIX expression in tumor renal tissue was specific, but not clinically useful as a prognostic marker. Measurements of CAIX in serum obtained pre- and postsurgical interventions showed good prognostic potential, correlating with clinical-pathological parameters and estimating the risk of progression. Presurgical intervention serum CAIX concentrations higher than 169.95 pg/mL indicated an almost 5-fold increased risk of death.

Key Words: Clear cell renal cell carcinoma, Carbonic anhydrase IX, Biomarker, Prognosis, Survival

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- **Conflicts of Interest:** The authors have nothing to disclose.

INTRODUCTION

1. Renal Cell Carcinoma

Renal cell carcinoma (RCC) accounts for 80 to 85% of all malignant renal tumors, and typically originates in the renal cortex [1]. The term RCC encompasses a broad spectrum of histopathological variants, with 3 main types of RCC being the most common, namely: clear cell RCC (ccRCC); papillary RCC; and chromophobe RCC. Among them, ccRCC is the most frequent (70%–85%) [1,2].

RCC is twice more common in men than in women and the average age at diagnosis is 64 years. In Spain, the number of deaths from RCC in 2022 was 2,243 individuals (1,512 men and 731 women) [3]. On the other hand, the estimated incidence of the disease for 2024 was 9,208 new cases (6,160 men and 3,048 women) [3,4].

RCC is characterised by remaining asymptomatic until a late stage of the disease [2]. Around 60% of the cases are usually detected by imaging tests due to suspicion of another pathology [1]. In the specific case of ccRCC, it occurs as a localised disease in 70% of patients, and, of them, 30% to 40% develop metastases after surgery [5]. Most recurrences occur in the first 3 to 5 years after radical nephrectomy [5,6].

2. Carbonic Anhydrase IX

Carbonic anhydrase IX (CAIX) is a membrane metalloenzyme that participates in the regulation of cellular pH. Its expression is regulated by the hypoxia-inducible factor-1α and is known to intervene in hypoxia-related processes [7-9]. It has been detected diffusely in healthy tissue, mainly in the larger bile ducts and gastric mucosa, but not in healthy renal tissue. In addition, it is overexpressed in some types of cancer, including bladder, breast, female genital tract, lung, gastrointestinal tract, head, neck, prostate, and kidney [6,8,9].

The role of CAIX as a tissue and serum marker in RCC has been a source of study in recent years. However, there is debate about its diagnostic and prognostic application in this disease. Its expression in tumor tissue has been related to better prognoses, unlike the expression of serum CAIX, given that higher expression is related to worse prognoses of the disease [6,7]. This is mainly true in the clear cell variant, but not in all RCC subtypes. It has been observed that patients with ccRCC had significantly higher serum CAIX levels than those with other RCC variants, and, in turn, they exhibited significantly higher CAIX levels than healthy individuals [6,8,9]. Therefore, serum CAIX levels are useful to differentiate ccRCC from other histopathological variants [7,9].

3. Objectives of the Study

The primary objectives of the present study were to determine the expression of CAIX in tissues and serums of patients diagnosed with ccRCC and assess the relationship of CAIX expression with a series of clinical-pathological parameters, in addition to assessing the potential of CAIX as a serum marker of progression and survival. The secondary objective was to study the relationship between the concentration of CAIX in serum before and after 24 hours of radical nephrectomy.

MATERIALS AND METHODS

1. Study Design

The present study was designed as observational and analytical with prospective follow-up.

2. Patients Included in the Study

Patients were aged ≥18 years, diagnosed with ccRCC and underwent radical nephrectomy at the Lozano Blesa University Hospital in Zaragoza, Aragón, Spain. They were admitted exhibiting any tumor stage, including new diagnoses and relapses, and signed an informed consent form approved by the Clinical Research Ethics Committee of Aragón.

3. Sample Collection

Sixty samples that met the inclusion criteria of the study were collected between 11th May 2011 and 2nd September 2014. The age range at the time of nephrectomy was 38 to

84 years, with a mean of 61 years. The patients included were 39 men and 21 women. They were staged based on the 8th edition of TNM classification published in 2017 by the American Joint Committee on Cancer [10]. The clinical-pathological parameters studied are included in Table 1.

4. Laboratory Tests

Positive or negative expressions of CAIX in tumors and adjacent healthy renal tissues were assessed using immunohistochemistry (IHC). Determination of CAIX in presurgical intervention (pre-SI) and postsurgical intervention (post-SI) serum samples was performed using commercial enzyme-linked immunosorbent assay (ELISA). The statistical analysis of the data was subsequently performed.

Table 1. Clinical-pathological parameters and descriptive statistics.

Characteristic	Value
Tumor cm (N=60), median (IQR)	6.10 (4.5–9.0)
TNM classification	
Primary tumor (T) (N=60)	
T1	30 (50.0)
T2	13 (21.7)
T3	17 (28.3)
T4	0 (0)
Regional lymph nodes (N) (N=60)	
N0	42 (70.0)
N1	8 (13.3)
Nx	10 (16.7)
Metastasis (M) (N=60)	
M0	51 (85.0)
M1	9 (15.0)
Tumor stage (N=60)	
Stage I	27 (45.0)
Stage II	13 (21.7)
Stage III	11 (18.3)
Stage IV	9 (15.0)
Fuhrman nuclear grade (N=49)	
Grade I	11 (22.5)
Grade II	19 (38.8)
Grade III	14 (28.6)
Grade IV	5 (10.2)
Progression (N=59)	
Yes	22 (37.3)
No	37 (62.7)
Death (N=59)	
Yes	19 (32.2)
No	40 (67.8)

Values are presented as median (interquartile range) or number (%).
TNM, tumor size - cancer spread to the regional lymph nodes - metastasis.

1) Immunohistochemistry

Tissue samples were deparaffined and hydrated in graduated alcohol series from 100% to 70% and running water for 5 minutes. Antigen was recovered by PT-Link (Dako, Glostrup, Denmark) through tissue samples heating for 20 minutes in buffer at 92℃ with acid pH (Target Retrieval Solution, Low pH, Dako). Then, it was washed in buffer (Dako) and IHC study was performed by Dako EnVision FLEX+ Mouse Kit. Next, endogen peroxidase (Peroxidase-Blocking Reagent, EnVision, Dako) was briefly added followed by CAIX (Human Carbonic Anhydrase IX/CA9 Antibody#: MAB2188. R&D Systems, Minneapolis, MN, USA). IHC study was performed such in tumor as healthy tissue samples. Then, sections obtained from each patient were incubated with EnVision FLEX + Mouse linker followed by horseradish EnVision/HRP peroxidase marked polymer. Colour reaction was developed by DAB + chromogen in substrate buffer (Dako) resulting brown reaction product. Tissue sections were treated with Mayer haematoxylin, dehydrated in alcohol gradient series, cleared with xylene and assembled for microscopic observation. Presence or absence of CAIX staining was evaluated, as positive or negative staining.

2) Enzyme-linked immunosorbent assay

The R&D DCA900 Quantikine Human Carbonic Anhydrase IX ELISA was used for the quantitative measurement of CAIX. EDTA plasma samples were stored at -20℃. Calibrators and samples were incubated in microplate wells precoated with monoclonal anti-CAIX antibody. After 2 hours incubation and washing, monoclonal anti-f Human Carbonic Anhydrase IX Conjugate to each well was added to the wells and was incubated for 120 minutes. Following another washing step, the remaining horseradish peroxidase conjugate was allowed to react with the substrate solution (tetra methyl benzidine). The reaction was stopped by addition of acidic solution and absorbance of the resulting yellow product was measured. A calibration curve was constructed by plotting absorbance values against concentrations of calibrators, and concentrations of unknown samples were determined using this calibration curve.

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5. Statistical Analysis

A descriptive and inferential statistical analysis was performed using appropriate tests for each set of variables, taking into account their distribution, type of variable, and possibility of data pairing. Shapiro-Wilk test indicated that the data of CAIX in serum did not exhibit a normal distribution; therefore, medians and interquartile ranges (IQRs) were calculated in the descriptive statistics.

In addition, the following nonparametric tests were also performed: Mann-Whitney U-test and Kruskal-Wallis test to compare the concentration of CAIX between 2 and more than 2 groups respectively for the different parameters; Spearman rank correlation coefficient to measure the correlation between 2 variables; and the receiver operating characteristics (ROC) curve to find a value that could serve as an estimator of disease progression. Furthermore, the Cox proportional hazards regression model and the Kaplan-Meier method were used to calculate the relative risk of serum CAIX in overall survival (OS). The IBM SPSS Statistics ver. 28.0 (IBM Co., Armonk, NY, USA) was used for the statistical analysis and graphics, and a p-value <0.05 was considered statistically significant.

RESULTS

1. CAIX in Tissue

CAIX expression was measured using IHC in tumor renal tissues and adjacent healthy renal tissues from patients with ccRCC who underwent radical nephrectomy. Among the 60 tumor renal tissue samples analysed, 59 were positive, whereas none of the healthy renal tissue samples from the same patients exhibited positive CAIX expression.

2. CAIX in Serum

CAIX concentration was measured in serum samples collected before nephrectomy (178.25 pg/mL; IQR, 112.30–324.20) and 24 hours after (59.30 pg/mL; IQR, 30.78–141.90) using commercial ELISA. A direct correlation was found between the expression of CAIX in pre- and post-SI serum (r=0.891, p=0.001), with the concentration of CAIX being approximately 3 times higher before nephrectomy.

1) Tumor size

The mean tumor size was 6.91 cm (range, 2.20–16.50 cm). A direct correlation was found between the size in centimetres and the concentration of CAIX in pre-SI serum (r=0.516, p<0.001). Likewise, statistically significant differences were observed between the concentration of CAIX and tumor sizes greater or smaller than 4 cm (p=0.021), 7 cm (p<0.001), and 10 cm (p=0.012).

2) Clinical-pathological parameters

Statistically significant differences were observed for the following variables: TNM, tumor stage, Fuhrman nuclear grade, progression, and death (Table 2). Regarding the classification based on size (T), the concentration in T3 was significantly higher than in T1. On the other hand, when the cancer spread to the regional lymph nodes (N1), the expression of CAIX was significantly higher in comparison to when the nodes had not been affected (N0). The same fact was observed in patients who exhibited distant metastasis (M1) and those who exhibited localised tumors (M0).

When the expression of CAIX in pre-SI serum was compared based on stage grouping in accordance with the TNM classification, statistically significant differences were found between stages I–II, I–III, I–IV, and II–IV in patients with stage IV exhibiting much higher concentrations of CAIX in serum (Fig. 1). In addition, statistically significant differences were observed between grades I–III and II–III in the Fuhrman nuclear grade classification.

3) Progression and survival

The concentration of CAIX in pre-SI serum was analysed based on disease progression and an area under the curve of 0.793 was obtained with a 95% CI (0.673–0.913), indicating that this was an effective model for predicting progression (p<0.05). The cutoff point for CAIX was 169.95 pg/mL. It was selected in the coordinates of the ROC curve due to its good sensitivity (0.81) and specificity (0.68).

The median OS time of the study population was 62.40 months after nephrectomy. Significant differences were found in the pre-SI CAIX concentration when comparing those who died during follow-up. The assessment of OS was

Table 2. Clinical-pathological parameters with medians and interquartile ranges of CAIX values in pre- and post-SI serum

Characteristic	CAIX pre-SI (pg/mL)	p-value		CAIX post-SI (pg/mL)	p-value	
CAIX						
ccRCC (60)	178.25 (112.30–324.20)	-		59.30 (30.78–141.90)	-	
TNM classification						
Primary tumor (T)			<0.001			<0.001
T1 (30)	112.30 (68.4–143.53)	T1–T2	0.054	32.70 (22.78–60.15)	T1–T2	0.186
T2 (13)	198.30 (169.95–213.55)	T1–T3	<0.001	65.30 (46.45–78.60)	T1–T3	<0.001
T3 (17)	356.90 (292.70–1191.35)	T2–T3	0.087	245.10 (69.65–502.80)	T2–T3	0.090
Regional lymph nodes (N)						
N0 (42)	139.35 (108.50–211.53)	N0–N1	<0.001	46.45 (28.08–69.38)	N0–N1	0.002
N1 (8)	694.10 (287.00–1230.55)			284.65 (87.95–611.20)		
Nx (10)	263.20 (85.35–1291.08)			111.55 (22.78–596.53)		
Metastasis (M)						
M0 (51)	147.20 (99.80–241.20)	M0–M1	<0.001	47.20 (25.60–78.30)	M0–M1	<0.001
M1 (9)	1258.30 (1136.30–1441.70)			725.30 (502.80–945.05)		
Tumor stage (N=60)			<0.001			<0.001
Stage I	111.40 (65.70–125.40)	I–II	0.015	28.90 (21.80–47.20)	I–II	0.068
Stage II	198.30 (169.95–213.55)	I–III	<0.001	65.30 (46.45–78.60)	I–III	0.001
Stage III	321.50 (258.70–356.90)	I–IV	<0.001	96.20 (69.20–245.10)	I–IV	<0.001
Stage IV	1258.30 (1136.30–1441.70)	II–III	1.000	725.30 (502.80–945.05)	II–III	1.000
		II–IV	0.017		II–IV	0.009
		III–IV	0.579		III–IV	0.356
Fuhrman nuclear grade (N=49)			0.001			0.006
Grade I	98.70 (65.30–136.40)	I–II	1.000	28.90 (23.10–65.20)	I–II	1.000
Grade II	174.20 (96.80–215.20)	I–III	0.004	58.70 (25.40–78.90)	I–III	0.009
Grade III	299.65 (233.65–1241.23)	I–IV	0.060	170.65 (62.53–630.20)	I–IV	0.317
Grade IV	356.90 (179.30–1341.80)	II–III	0.047	74.10 (46.80–678.90)	II–III	0.054
		II–IV	0.348		II–IV	1.000
		III–IV	1.000		III–IV	1.000
Progression (N=59)						
Yes	318.15 (180.28–1169.33)		<0.001	148.70 (73.35–568.60)		<0.001
No	125.40 (93.25–211.20)			42.10 (23.90–68.50)		
Death (N=59)			0.005			<0.001
Yes	325.10 (174.20–1235.40)			245.10 (59.90–632.50)		
No	139.35 (101.88–214.38)			40.65 (25.10–78.75)		

CAIX, carbonic anhydrase IX; ccRCC, clear cell renal cell carcinoma; pre-SI, pre-surgical intervention; post-SI, post-surgical intervention; TNM, tumor size - cancer spread to the regional lymph nodes - metastasis.
The p-value of the applied statistical tests is indicated.

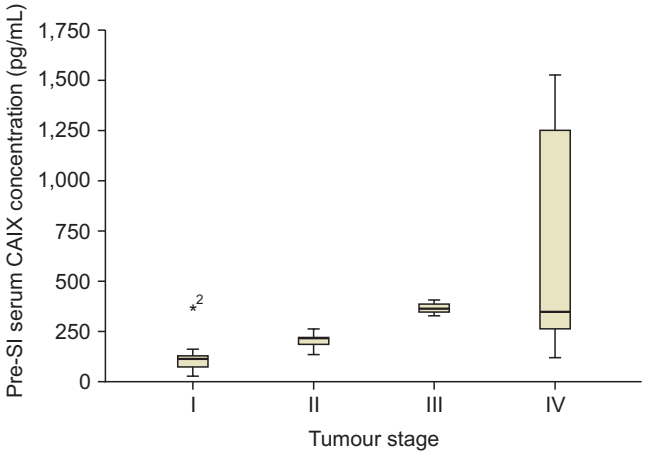

Fig. 1. Pre-SI serum CAIX concentration (pg/mL) as a function of tumor stage. Pre-SI, presurgical intervention; CAIX, carbonic anhydrase IX.

performed and the hazard ratio (HR) for death was found to be 1.001 for the CAIX variable (p<0.001), 95% CI (1.001–1.002).

OS was assessed using the CAIX concentration of 169.95 pg/mL in pre-SI serum as a cutoff point. Statistically significant differences were found when comparing OS between groups based on the CAIX concentration in pre-SI serum (p=0.02). In the group of patients with CAIX concentrations less than 169.95 pg/mL, 17.24% (5 of 29) died with a median OS of 64.27 months, whereas in the group of patients with concentrations greater than 169.95 pg/mL, 50% (15 of 30) died with a median OS of 47.49 months (Fig. 2). On the other hand, the HR value of 4.849 (p<0.05), 95% CI

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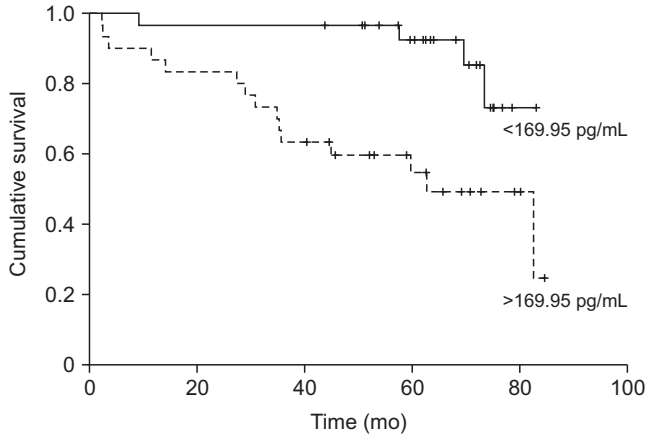

Fig. 2. Overall survival graph as a function of pre-SI serum CAIX concentration. The Kaplan-Meier survival curve predicts lower survival for individuals belonging to the group with the highest concentration (log-rank test, p=0.02). Pre-SI, presurgical intervention; CAIX, carbonic anhydrase IX.

(1.596–14.729) was obtained for the group with the highest concentration.

DISCUSSION

The effectiveness of CAIX in tumor tissue and serum as a prognostic factor for ccRCC has been widely studied. However, the results published to date are contradictory. This way, we proposed to study the expression of CAIX in tumor renal tissue and adjacent healthy renal tissue, as well as in the serum of 60 patients diagnosed with ccRCC, before and 24 hours after undergoing radical nephrectomy.

1. CAIX in Tissue

Among the 60 samples of tumor renal tissues assessed by IHC, 59 (98.33%) exhibited positive expression of CAIX. In contrast, none of the samples from adjacent healthy renal tissues belonging to the same patients exhibited it. This result confirms what has been previously described in the literature, i.e., CAIX is expressed in tumor renal tissue but not in healthy renal tissue [11,12].

For routine clinical practice, CAIX in tissue should not be considered as a useful prognostic marker by measuring only positive or negative expression. However, as other authors have proposed, it would be interesting to determine the percentage of expression in tissue and its relationship with the progression of the disease after surgery [11,13-20], as well as its association with other markers in tissue [17,21].

2. CAIX in Serum

In spite of the high diagnostic precision of imaging tests, and taking in account that the role of biopsy of a renal mass has a limited value, in many cases the final diagnosis of certainty is not reached until the anatomopathological result of the nephrectomy specimen is obtained. In some series nephrectomies due to benign tumors may be performed in more than 30% of cases. Therefore, the identification of a marker in peripheral blood that can refine this diagnosis would be of great interest.

All patients with ccRCC assessed exhibited CAIX in serum pre-and post-SI. A ratio of 3:1 and a direct correlation were observed between the variables. An individual study had revealed a decrease in serum CAIX of 2.5 to 5.5 times in 70% of the patients examined between the 8th and 9th day after radical nephrectomy [22]. This finding is associated with the fact that the main tumor is the primary source of CAIX production in ccRCC [23]. It would be convenient to measure the concentration of serum CAIX over time to determine whether, once the tumor is removed, this concentration is reduced to values found in healthy individuals or, on the contrary, increases indicating tumor recurrence.

Regarding the results obtained with serum CAIX and tumor size, it was observed that the larger the tumor size, the higher the serum CAIX concentration. The association with size is of great interest for the follow-up of patients with ccRCC. Possibly the subgroup of patients that would benefit most from the application of serum CAIX for follow-up is that whose patients exhibit larger tumors, since they have a high risk of tumor recurrence. An onset of elevated concentration could be indicative of these recurrences. As with tumor size, statistically significant differences were obtained according to lymph node involvement and distant metastasis; therefore, the greater the tumor load, the higher the serum CAIX concentration.

Given the results obtained in the present study, it seems that patients with higher serum CAIX concentrations have more aggressive ccRCC in terms of stage, size, lymph node involvement, distant metastasis, Fuhrman nuclear grade, and progression. However, there is no consensus with similar

studies regarding the association of tumor aggressiveness and the different clinical-pathological parameters mentioned [12,22,24-27].

Determining a cutoff point that allows estimating the risk of recurrence can be of great help in the clinical management of the disease. Several authors have assessed survival in association with serum CAIX concentrations in patients with ccRCC. Some obtained statistically significant differences between the groups defined based on the cutoff points, considering CAIX as an independent prognostic factor for survival in these patients [24,27]. At the same time, other authors did not find differences [26].

In the present study, a cutoff point of 169.95 pg/mL of CAIX in pre-SI serum was obtained. This cutoff point is based on the best sensitivity and specificity for our sample and used to estimate the risk of progression in patients with ccRCC, unlike the previously mentioned studies that used the median serum concentration of CAIX obtained in their samples as cutoff points.

With respect to the high percentage of patients who develop metastasis after nephrectomy, the defined cutoff point was used to compare OS between the groups with the lowest and highest pre-SI serum CAIX concentrations, observing a lower survival for the group with the highest concentration. This result allows to predict that those patients with a pre-SI serum CAIX concentration higher than the cutoff point could have approximately 5 times (HR, 4.849) more chances of death than those with concentrations below the cutoff point. The existence of a cutoff point could serve as support for clinical decision-making, whether in planning treatment strategies or in closer patient follow-up, complementing imaging tests with serum CAIX determinations. Nevertheless, the broad confidence interval (95% CI, 1.596–14.729) suggests considerable variability, so it is necessary to adjust this effect with other established prognostic factors such as TNM staging. The present study, like other similar studies published, points to the need to conduct subsequent multicenter studies, with larger numbers of patients, prospective monitoring of the serum concentrations of CAIX, and assessing and associating different serum markers in the progression of the disease.

CONCLUSIONS

The expression of CAIX in renal tumor tissues confirmed its specificity, with 98.33% (59 of 60) of tumor samples being positive and all adjacent healthy tissue samples being negative. However, its clinical utility requires surgical intervention and it is not useful as a prognostic marker for ccRCC. On the other hand, the measurement of CAIX in serum pre-SI and post-SI has good prognostic potential, directly relating to different clinical-pathological parameters characteristic of ccRCC (Table 2). The cutoff point of 169.95 pg/mL for CAIX in pre-SI serum allows estimating a higher risk of progression, in addition to predicting a five-fold higher probability of death in patients with concentrations higher than this cutoff point.

NOTES

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