



Role of CAIX Expression in Conventional Renal Cell Carcinomas as a Diagnostic Marker and its Prognostic Importance

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Abstract

Significant advances in understanding of the biology of renal cell carcinoma (RCC) have been achieved recently, which led to novel targeted therapies, revolutionising the management of patients with advanced disease. To date, there are no molecular markers which can reliably predict RCC outcome. We investigated whether a novel kidney cancer marker, carbonic anhydrase IX (CAIX), is associated with progression and survival. A retrospective study was done on patients diagnosed with renal cell carcinoma over a period of 5 years. Immunohistochemical analysis using a CAIX monoclonal antibody was performed on paraffin-embedded blocks from patients treated with nephrectomy for clear cell RCC. Patients were segregated into two categories based on CA IX expression as CA IX \leq 85% and CA IX $>$ 85%. A comparison was made based on the survival (from date of diagnosis) with CA IX expression. Correlation of CA IX expression and TNM staging, nuclear grading, tumour volume and age was statistically studied using Student's *t* test. The association between survival and CA IX was done using Mann-Whitney test. The association of CA IX with rest of the prognostic variables were analysed using Fisher's exact test. In our study, CA IX expression $>$ 85% had longer survival compared with those with lower expression \leq 85%. A significant statistical association was seen with CAIX and lymphovascular emboli, major vessel, perinephric fat, renal sinus fat involvement and distant metastasis. CAIX reflects significant changes in tumour biology that predicts clinical outcome and identify high-risk patients for adjuvant immunotherapy and CAIX targeted therapies.

Keywords Clear cell RCC · CAIX · Hypoxia · Metastasis · Prognosis · Survival

Introduction

Carbonic anhydrase IX is a transmembrane enzyme with an extracellular portion composed of an N-terminal proteo-glycan-like region and a large central carbonic anhydrase domain (CA) linked via a single transmembrane anchor to a short C-terminal intracytoplasmic tail [1]. CA IX gene is located on chromosome 9 p12–13 loci, and is positively induced by HIF-1 α , which is upgraded by the mutation, hypermethylation or deletion of the

VHL gene in clear cell RCC [2]. CA IX is expressed in 94–97% of CCRCC and in half the papillary RCC.

Function of CAIX

Adequate tissue perfusion is essential for cellular haemostasis. The delicate balance between oxygen supply and demand is disrupted in highly metabolic neoplastic cells. In RCC, CAIX allows survival in hypoxic and acidotic conditions by facilitating transmembrane proton exchange to buffer intracellular PH [3]. In normal kidney tissue, hypoxia inducible factor-1 α (HIF-1 α) is hydroxylated by prolyl hydroxylase domain (PHD) proteins and bound by Von Hippel-Lindau protein (pVHL). Subsequently, the complex is ubiquitinated, which causes degradation of HIF-1 α . In clear cell renal cell carcinoma (ccRCC), pVHL is mutated and binding with HIF-1 α is prohibited. Subsequently, HIF-1 α forms a heterodimeric complex with HIF-1 β , translocates to the nucleus, where it activates hypoxia inducible genes, such as vascular endothelial growth factor and CA IX,

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which is expressed on the tumour cell membrane [4].

CA IX Immunohistochemical Staining and Evaluation For CAIX staining intensity (Fig. 1c–f),

SCORE 3: strong circumferential membranous staining,

SCORE 2: weak circumferential membranous,

SCORE 1: weak partial membranous staining,

SCORE 0: negative staining

The percentage of cells corresponding with each staining intensity was also recorded.

The CA IX high-score was calculated by multiplying the highest CA IX intensity (0–3) by the percentage of cells exhibiting the highest CA IX intensity. The CA IX total score was calculated by adding together the products of each of the CA IX intensities (0–3) and the % of cells exhibiting that respective staining intensity.

CA IX and its Role in Predicting Early Metastasis

The expression level of CA IX alone or the combination of stage or grade with the expression level of CA IX showed a significant difference in the metastasis-free survival rates. The most significant was seen when the stage, grade and expression level of CA IX were combined [5]. Level of CA IX expression could be useful to predict the early metastasis after surgery. The addition of the CA IX marker to the stage and grade could enable the identification of a subpopulation characterized for a high risk of early metastasis. Therefore, the quantitative RT-PCR of CA IX may be a useful tool for guiding post-operative follow-up and treatment [5].

Predicting Response to Immunotherapy

Interleukin 2 (IL-2) remains the only form of therapy that can lead to a durable response [3]. Bui et al. first suggested that CAIX expression might be associated with a response to IL 2. In an analysis of 86 patients with metastatic RCC receiving IL-2, 84% of patients had high expression of CAIX. The overall response rate to IL-2 for patients with high and low CAIX expression was 27% and 14%, respectively [3]. Atkins et al. [3] confirmed the association of high CAIX expression with IL-2 response.

Aims and Objectives

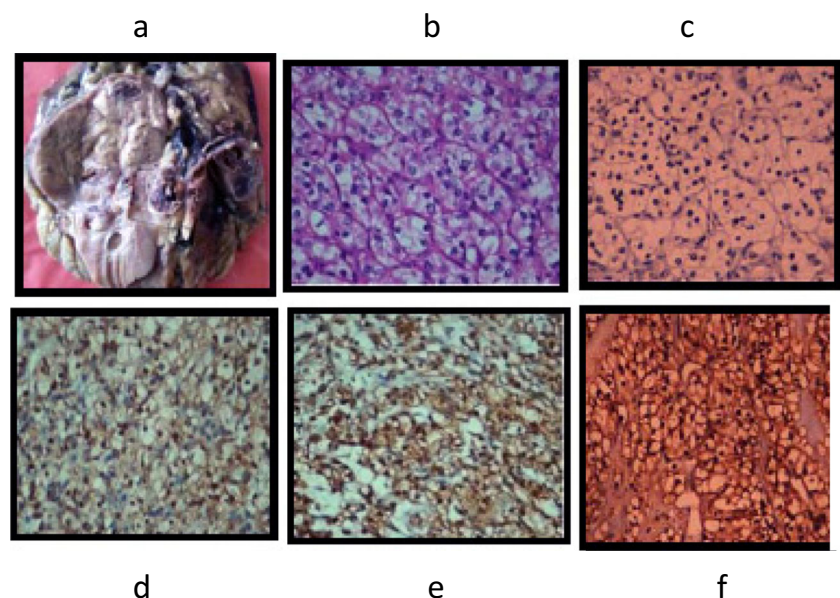
The primary objective was to estimate the percentage of clear cell subtype of renal cell carcinoma among the various types of RCC and to assess the association between CA IX and different variables if any. The secondary objective was to assess the role of CA IX in predicting early metastasis in clear cell RCC.

Material and Methods

Study Design

A retrospective study was done on patients diagnosed with renal cell carcinoma over a period of 5 years from (January 1, 2008, to December 31, 2012) based on clinical assessment and pathologic/microscopic analysis. Patient details and respective cell blocks were obtained from the hospital database. Each cell block was subjected to an immunohistochemical analysis for CA IX expression. Patients were segregated into

Fig. 1 **a** Gross image renal cell carcinoma. **b** CcRCC, H&E \times 100. **c** Immunohistochemistry: CA IX score 0. **d** CA IX score 1. **e** CA IX score 2. **f** CA IX score 3



two categories based on CA IX expression as CA IX \leq 85% and CA IX $>$ 85%.

Correlation of CA IX expression and TNM staging, nuclear grading, tumour volume and age was statistically studied using Student's *t* test and the association between survival was done using Mann-Whitney test. The association of CA IX with rest of the variables was statistically analysed using Fisher's exact test.

Inclusion Criteria

All patients who underwent nephrectomy for renal cell carcinoma and all metastatic renal cell carcinoma patients were included in the study.

Exclusion Criteria

Other histological variants of RCC like mucinous tubular, spindle cell carcinoma, collecting duct of Bellini carcinoma, unclassified carcinoma, renal medullary carcinoma and renal oncocytomas were excluded.

Statistical Analysis

Percentage of clear cell RCC out of the total RCC cases was computed. To test the statistical significance of the association between CA IX and early metastasis in clear cell RCC, chi square test/Fisher's exact test was done.

Results

In our study, a total of 140 cases were studied over a period of 5 years from 2008 January to 2012 December. Out of the 140 cases of renal cell carcinoma, 90 cases were conventional clear cell carcinoma which constituted around 64.28%, 6.4%

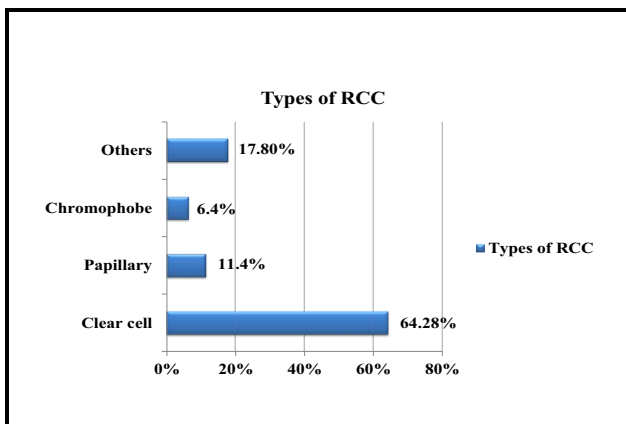


Fig. 2 Types of RCC

of chromophobe, 11.4% of papillary and 17.8% constituted the rest of the renal tumours, which is comparable with the study conducted by Namita et al. (2012, $n = 65$) [6] and Atif Ali Hashmi et al. (2014, $n = 64$) [7]. (Fig. 2).

Out of the 65 cases of conventional clear cell renal cell carcinoma, 4 cases had radical nephrectomy along with aortocaval, paraaortic and IVC exploration; 5 cases had partial nephrectomy and the rest had radical nephrectomy. The most common age group in our study among the 65 cases of clear cell renal cell carcinoma was 50–70 years, and the mean age was 61. This data is comparable with that of a study conducted by Mathew et al. [5] (2003), Bedke et al. [8] (2008), Zhou et al. [9] (2010) and Sakai et al. [10] (2009). Tumour size varied from 1.2 to 13.5 cm in GD. Mean was 7.2 cm. According to WHO, the average size was 7 cm. According to an article by Zhou et al. (2010) [9], the mean tumour size was 3.8 cm (range 1.6–8.4). According to a study by Mathew et al. [5] (2003), the mean tumour size was 7 cm. So, our study is comparable with that of the study conducted by Atif Ali Hashmi [7] (2014). A male preponderance among the total renal cell carcinomas was noted in our study which was 78.50% ($n = 110$) compared with 21.4% ($n = 30$) in females.

Two cases were known cases of Von Hippel-Lindau disease. Two cases each had sarcomatoid and rhabdoid differentiation. A study by Bedke et al. [8] (2008) reported 7 cases of conventional clear cell renal cell carcinoma which had sarcomatoid differentiation. Grossly, only 1 case had multiple tumours (3 in number) and the rest of them were single. Twenty-four cases presented incidentally, and in the rest of the 41 cases, 1 case presented as mass alone; 7 cases presented as pain, mass and haematuria and the rest of the 33 cases had either of the following presentation: haematuria, pain, mass or any of the two combinations. Sakai et al. (2009) [10] studied cases which presented incidentally and symptomatically and found that 109 cases (71.2%) were detected incidentally and 44 cases (28.8%) were symptomatic. We grouped the T stage into high group which comprised T3 and T4 and the low group which comprised T1 and T2. Majority of the cases belonged to the lower group comprised around 64.6% compared with 35.4% in the highest group. N stage was segregated into 2 groups. Nx, N0 comprised the group which had no lymph node involvement and in which lymph node assessment could not be made and the group with lymph node involvement that is N1, N2. Majority (96.9%) belonged to the group which had no lymph node involvement. Fuhrman grade was divided into two groups, grade 1, 2 in one group which was the low-grade group and the high-grade group which was grade 3, 4. Seventy three percent were in the low-grade group compared with the high-grade group. None of the conventional clear cell carcinomas had perineural invasion. Major vessel involvement was seen in only 18.5% ($n = 12$) of the cases; the most common was the renal vein. A total of 13.8% ($n = 9$) of the cases had perinephric fat involvement. A total of 27.70%

($n = 18$) had renal sinus involvement. Adrenal gland involvement was seen in only 1 case. No evidence of recurrences was seen. One case had metastasis at the time of presentation and survived for 3 months following the date of diagnosis. Distant metastasis was observed in 5 patients, and the metastatic sites were liver, lung, skull and pubic ramus.

CA-IX staining was done in 65 cases and was scored according to Cleveland scoring system, and the total score ranged from 5 to 300. The total number of cases in the CA-IX > 85% group was 52, and the total number of cases in the CA-IX group \leq 85% was 13. Thus, the variable extent and intensity of immunostaining for CAIX in ccRCC seems to be associated with the proportion of the granular cell component, and it may be dependent on the numbers of mitochondria in the cytoplasm of neoplastic cells [11]. The granular cell component occurs commonly in ccRCC usually manifesting a higher nuclear grade than the clear-cell part of the tumour, and therefore, it could be interpreted as a sign of tumour dedifferentiation, with a possible impact on the patient's prognosis [11].

In the selected 65 cases, association of CA-IX with stage of disease, gender, lymphovascular emboli, renal sinus involvement, perinephric fat involvement, capsular invasion, metastasis, adrenal gland involvement, major vessel involvement, number of tumour, cytoplasmic changes, Furhman's grade, perineural invasion and the overall survival were assessed.

Absence of lymphovascular emboli was 84.20% in the high group (CA-IX > 85%) compared with the 50% which had lymphovascular emboli. This observation has statistical significance. (p value = 0.044). No comparable studies were noted in the literature.

Absence of major vessel involvement was seen in 84.90% in the higher group (CA-IX > 85%), compared with 58.30% which had major vessel involvement. This observation has got statistical significance with a p value of 0.053. However, no similar studies were observed.

In group CA-IX > 85%, 83.90% did not reveal perinephric fat invasion compared with the 55.60% which had perinephric fat involvement; with a p value of 0.07, this observation has statistical significance. But no similar studies were noted in the literature.

Significant association between CA-IX > 85% and absence of renal sinus fat involvement was noted. A total of 87.20% had no renal sinus involvement compared with the 61.10% which had renal sinus involvement in group CA-IX > 85%. With a p value of 0.034%, this observation has got statistical significance. No similar studies were noted in the literature.

Significant statistical association between CA-IX expression and metastasis was noted. No metastasis was noted in 85% of cases with CA-IX expression > 85% compared with the 20% which had metastasis, suggesting that high CA-IX expression was related with relatively good prognosis. The

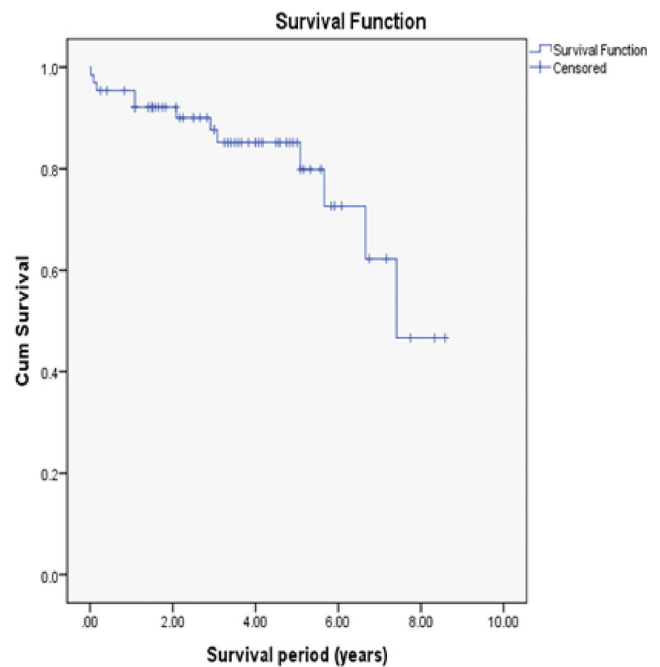


Fig. 3 Kaplan-Meier survival analysis. The mean in CA-IX > 85% group is 6.930 with 95% confidence interval of (lower bound 6.242 + upper bound 7.618), and the mean in CA-IX \leq 85% was 5.542 with 95% confidence interval of (lower bound 3.800 + upper bound 7.284). The median value was 7.410. These observations were found to be statistically significant with a p value of 0.049. This observation highlights that in the CA-IX > 85% group, the overall survival period was greater than the CA-IX \leq 85%

below observation is statistically significant (p value = 0.005). Our study was comparable with a similar study done by Guorong Li et al.

The survival analysis was done using Kaplan–Meir method (Fig. 3). The observations highlight that in the CA-IX > 85% group, the overall survival period was greater than the CAIX < 85% group. The relationship between CAIX expression and survival period in (years) was also analysed using Mann-Whitney analysis and was found to be statistically significant with a p value of 0.071. In a study conducted by Mathew et al. (2003), only the association of CA IX and metastasis had comparable p values. Our study did not observe significant association of CA IX with grade, lymph node status or T stage of the tumour. In our study, there was no statistical association of CAIX expression with the age and sex of the patient.

Although studies by Guorong et al. [5] (2007) obtained statistical significance of association of CA IX with T stage and Furhman grade with significant p values, these observations were not comparable with our studies. According to Genega et al' [12] (2010), the association between CAIX expression and tumour grade for primary clear cell and primary papillary RCC was studied. There was a significant association between CAIX expression levels and grade of primary clear cell RCC ($p < 0.01$) with high CAIX expression in the

lower grades. But in our study, no significant association was observed. This was comparable with study conducted by Grace X Zhou *et al.* [9] (2010).

Although a study by Chamie *et al.* (2015) obtained statistical significance of CA IX with nodal status, our study was not comparable with this study (p value = 0.363). There was no statistical association observed between CA-IX and tumour volume, number of tumour, adrenal involvement, capsular invasion and cytoplasmic changes. No similar studies stating the association of CA IX and the above variables were noted in literature.

Conclusion

In our study, a total of 140 cases were studied over a period of 5 years from 2008 January to 2012 December. Ninety cases were conventional clear cell carcinoma which constituted around 64.28%.

The most common age group in our study was 50–70 years and the mean age was 61. Tumour size varied from 1.2 to 13.5 cm in GD and the mean was 7.2 cm. A male preponderance among the total renal cell carcinomas was noted in our study which was 78.50% ($n = 110$) compared with 21.4% ($n = 30$) in females. Our study also noted a gender preponderance in conventional clear cell renal cell carcinoma in which males constituted around 90.8% ($n =$) and females 9.2% ($n = 6$). None of the conventional clear cell carcinomas had perineural invasion.

Eight cases had lymphovascular emboli (12.3%). Major vessel involvement was seen in only 18.5% ($n = 12$) of the cases; the most common was the renal vein. A total of 13.8% ($n = 9$) of cases had perinephric fat involvement. A total of 27.70% ($n = 18$) had renal sinus involvement. Adrenal gland involvement was seen in only 1 case.

Majority of the cases belonged to the lower group (T1, T2 stage) comprised around 64.6% compared with 35.4% in the higher group (T3, T4 stage). Majority (96.9%) had no lymph node involvement. A total of 73.8% of cases had low nuclear grade group (G1, G2) compared with the high-grade group (G3, G4). Sixty-five cases were scored according to Cleveland scoring system, and the total score ranged from 5 to 300. There was no statistical association observed between CA IX and age, tumour volume, number of tumour, adrenal involvement, capsular invasion, Furhman grade, T stage, N stage, cytoplasmic changes, age and gender of the patient. In our study, we have been able to point out a significant statistical association of CAIX with lymphovascular emboli, major vessel involvement, perinephric fat involvement, renal sinus fat involvement, distant metastasis and survival period.

Hence, by the end of the study, we have been able to point out that there is a role of CAIX expression in conventional renal cell carcinomas as a diagnostic as well as a prognostic marker

for predicting metastasis and long-term survival. In addition to TNM stage, several other clinical, molecular and pathological prognostic factors are relevant. Our understanding is increasing concerning the genetic basis and molecular pathways involved in RCC, and there has been identification of several novel potential prognostic and predictive markers in RCC, such as carbonic anhydrase (CA IX), VEGF, Ki67, p53 [13].

The ability to stratify patients into more sophisticated risk categories will ultimately permit the goal of moving from nonspecific treatments to designing and targeting therapies for targeted populations of patients [14]. Targeted therapies directed at CAIX are being developed to exploit the exclusivity of CAIX expression in RCC for the treatment of metastatic disease. A recent study showed that a carbonic anhydrase inhibitor, acetazolamide, was able to inhibit the invasive capacity of renal cancer cells *in vitro* [15]. Therefore, CAIX reflects significant changes in tumour biology, which should be used to predict clinical outcome and identify high-risk patients in need for adjuvant immunotherapy and CAIX targeted therapies [15].

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