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Original Article

Pathology Section

Expression of Carbonic Anhydrase-IX and Vascular Endothelial Growth Factor in Renal Cell Carcinoma and their Prognostic Significance

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ABSTRACT

Introduction: Renal Cell Carcinoma (RCC) is the most common adult renal malignancy. Histopathologically, clear cell RCC accounts for 65-70% of all RCCs. Carbonic Anhydrase-IX (CA-IX) is a transmembrane protein and takes a role in cancer development and progression. A 75-100% clear cell RCCs show CA-IX expression. But this varies with grade and stage of tumour. Vascular Endothelial Growth Factor (VEGF) is responsible for tumour angiogenesis and expressed variedly in RCCs. Both VEGF and CA-IX expression is mediated by Hypoxia Inducible Factor-1α (HIF-1α).

Aim: The present study aimed to evaluate the expression of CA-IX and VEGF in respect to different grades and stages of RCC and assessing their prognostic significance.

Materials and Methods: This was a cross-sectional, observational study done on 45 histopathogically diagnosed cases of RCC. It was performed in the Department of Pathology, Nil Ratan Sircar Medical College, Kolkata, West Banglore, India over a period of two years (February 2018 to January 2020). Expression of VEGF and CA-IX were studied by immunohistochemistry. Results were analysed in Statistical Package for Social Sciences (SPSS) software (version 16.0) using Pearson Chi-square test. A p-value of <0.05 was regarded as significant.

Results: Out of 45 cases of RCC, 34 tumours (32 clear cell carcinoma and two multilocular cystic renal neoplasm of low malignant potential) were evaluated for CA-IX immuno expression. About 25 cases showed CA-IX positivity which inversely associated with grade and stage of RCC (p-value <0.05). The CA-IX had a diagnostic value in detecting clear cell RCC with sensitivity 73.53%, specificity 100% and accuracy 80%. With 28 (62.2%) cases of RCC showed VEGF positivity among which nine were VEGF 1+ and 19 cases VEGF 2+. The VEGF expression showed a positive association with the grade and Tumour-Node-Metastasis (TNM) stage of tumour (p-value <0.05). Finally, authors found a statistically significant inverse association between CA-IX and VEGF expression in RCCs with clear cell morphology including clear cell RCC and multilocular cystic renal neoplasm of low malignant potential (p-value=0.001).

Conclusion: High grade RCCs show low expression of CA-IX and strong positivity with VEGF. Both these markers have a prognostic significance. From the therapeutic point of view, VEGF positive tumours, especially inoperable and metastatic cases, may be benefited by anti-VEGF therapy whereas CA-IX positive tumours respond well by treatment with Interleukin-2.

Keywords: Angiogenesis, Cancer, Immunohistochemistry, Kidney, Targeted therapy

INTRODUCTION

According to the World Health Organisation (WHO) data (2012), kidney cancer was the 9th most common cancer in men and 14th most common in women with a male:female ratio of 2:1; it was the 16th most common cause of death from cancer worldwide [1]. Approximately, one-third of all patients present with metastasis and 50% develop recurrence even after complete surgical excision [2]. Due to resistance of RCC to chemotherapy, radiotherapy and hormonal therapy, it carries a poor prognosis among all urologic malignancies. Common histopathological subtypes of RCC in adults are clear cell RCC, papillary RCC, chromophobe RCC, collecting duct carcinoma, renal medullary carcinoma, multilocular cystic renal neoplasm of low malignant potential, etc.

Clear cell RCC accounts for 65-70% of all renal cancers [3]. They are associated with inactivation of the Von Hippel-Lindau (VHL) tumour suppressor gene and upregulation of HIF-1α. This induces expression of hypoxia inducible genes like CA-IX and VEGF [3,4]. The CA-IX is a transmembrane protein having a role in carbon dioxide transport and intracellular pH regulation. This was first identified in the cervical carcinoma cell line *HeLa* in 1992 [5]. It is expressed in diffuse membranous pattern in 75-100% of clear cell RCCs and has a critical role in cancer development and progression [3,6]. The CA-IX expression has been identified as possible immunohistochemical predictor of RCC patient outcome although reduced expression is seen in high grade cases and basolateral positivity in clear cell papillary RCC [7,8].

The VEGF is a dimeric glycoprotein and plays an important role in tumour angiogenesis. Increased expression of VEGF is associated with high nuclear grade and stage of tumour [3,9]. Additionally, over expression of VEGF is associated with HIF-1α. In this background, this study was undertaken with the objectives of assessing expression of CA-IX and VEGF in RCC, and thereafter finding their association with prognostic parameters, viz., tumour grade, Lymphovascular Space Invasion (LVSI) and tumour stage.

MATERIALS AND METHODS

This was a cross-sectional, observational study done in the Department of Pathology, Nil Ratan Sircar Medical College, Kolkata over a period of two years from February 2018 to January 2020, in collaboration with Department of Urology. Necessary approval from the Institutional Ethics Committee was obtained for this purpose (NO/NMC/1521).

Inclusion criteria: Total 45 cases of partial or radical nephrectomy specimens received from Department of Urology with confirmed histopathological diagnosis of renal cell carcinoma were included in the study.

Exclusion criteria: Histopathological diagnosis of malignancies other than RCC, benign renal tumours and non neoplastic lesions were excluded from the study.

Histopathology and Immunohistochemical Interpretation

Gross examination of the specimens and histopathological reporting was done as per the College of American Pathology protocol 2017 (CAP protocol 2017) [10]. After diagnosis of RCC on Haematoxylin and Eosin (H&E) stained sections, histological type, nuclear grade, presence of LVSI, regional lymph node status and pathological stage were assessed. The nuclear grading was done as per World Health Organisation/International Society of Urologic Pathologists (WHO/ISUP) criteria of 2013 [10].

Immunohistochemistry was performed on all cases of RCC using sections from formalin-fixed paraffin-embedded blocks of tumours. For VEGF, monoclonal rabbit antibody (RTU clone: RBT-VEGF Bio-SB) was used and sections of lobular capillary haemangioma were taken as positive control. The VEGF expression was interpreted in the form of three-tiered scoring as described by Yildiz E et al. [11] in which,

Score 0 = No staining of tumour cells.

Score 1+ = Membranous stain with no cytoplasmic stain or light cytoplasmic stain in some tumour cells (< 50%).

Score 2+ = Diffuse and strong membranous and cytoplasmic stain in most of the tumour cells (>50%).

While assessing CA-IX immunostaining, monoclonal rabbit antibody (CA-IX: EP 151 clone) was utilised. Normal kidney tissue adjacent to the tumour was taken as internal control while sections of cervical squamous cell carcinoma were used as positive control. The interpretation was done on the basis of degree of cell membrane positivity as described by Genega EM et al., where intensity of staining was scored on the scale of 0 to 3 [12]. But high score was regarded as >85% positivity (2+) and low score as <85% positivity (1+) irrespective of intensity of stain.

STATISTICAL ANALYSIS

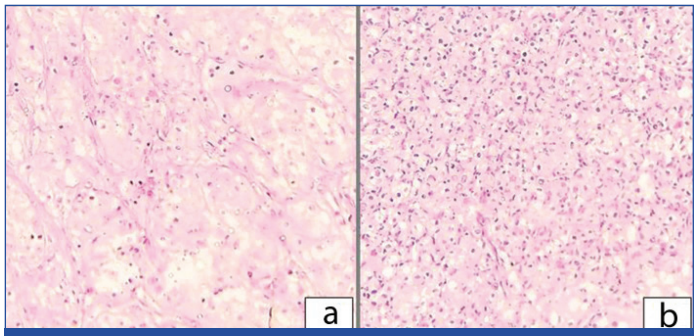
Statistical analysis was done using SPSS software (version 16.0). At first frequency distribution tables were prepared. Pearson Chi-square test was done to assess the association between different variables of this study. A p-value <0.05 was regarded as statistically significant.

RESULTS

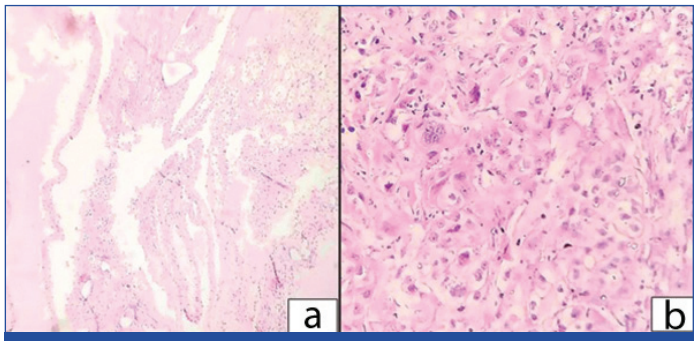
In this study, total 45 cases of RCC were studied among which 32 cases (71.1%) were clear cell carcinoma, nine cases (20%) papillary carcinoma, two cases each (4.4%) of collecting duct carcinoma and multilocular cystic renal neoplasm of low malignant potential [Table/Fig-1 a,b, 2 a,b, 3 a,b]. The study population had male:female ratio of 2:1. A 55.6% cases were left-sided and 68.9% cases showed presence of LVSI.

The most common WHO/ISUP grade amongst all cases were grade 2 (40% cases) and pT-stage was pT3 (48.9% cases). A 26.7% cases showed lymph node metastasis (pN1 stage) while 15.6% cases had distant metastasis (M1 stage).

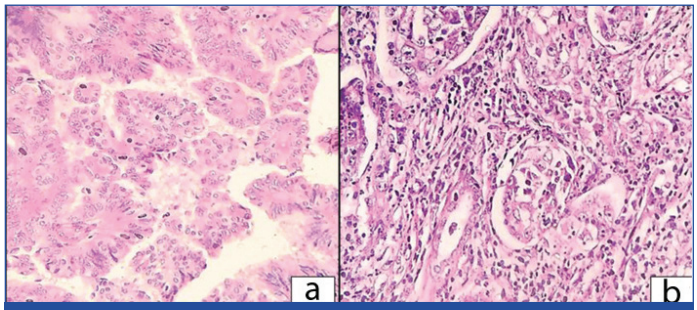
The VEGF immunostaining was done on all 45 cases of RCC. Majority of the tumours were VEGF 2+ positive (19 cases, 42.2%). A statistically significant positive association was observed between VEGF expression (staining intensity) and WHO/ISUP grade (p-value <0.05) as well as pT stage (p-value=0.002). There was also a statistically significant association with presence of LVSI (p-value <0.001), lymph node metastasis (pN stage) (p-value=0.002) and distant metastasis (M stage) (p-value=0.003) [Table/Fig-4a-c,5].



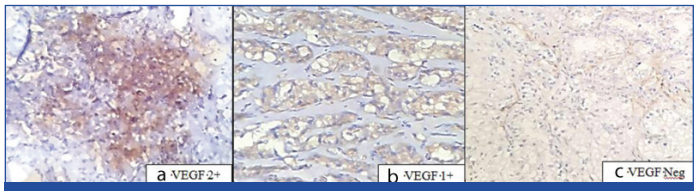
[Table/Fig-1]: a) Microphotograph showing clear cell RCC grade 2 (WHO/ISUP) (H&E x400); b) Microphotograph showing clear cell RCC grade 3 (WHO/ISUP) (H&E x400).



[Table/Fig-2]: a) Microphotograph showing multilocular cystic renal neoplasm of low malignant potential grade 2 (WHO/ISUP) (H&E x100); b) Microphotograph showing clear cell RCC grade 4 (WHO/ISUP) (H&E x400).



[Table/Fig-3]: a) Microphotograph showing papillary RCC grade 3 (WHO/ISUP) (H&E x400); b) Microphotograph showing collecting duct carcinoma (H&E x400).



[Table/Fig-4]: a) Microphotograph showing clear cell RCC grade 4 with VEGF 2+ score (x 400); b) Microphotograph showing clear cell RCC grade 3 with VEGF 1+ score (x400); c) Microphotograph showing clear cell RCC grade 2 VEGF negative (x400).

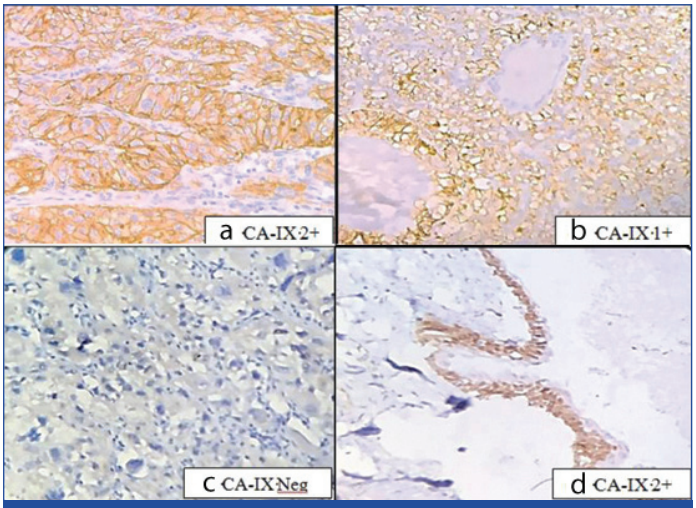
The CA-IX immunostaining was done in all 45 cases of RCC. All nine cases of papillary RCC and two cases of collecting duct carcinoma were CA-IX negative irrespective of grade and stage. Among remaining 34 cases, including clear cell RCC (32 cases) and multilocular cystic renal neoplasm of low malignant potential (two cases) which showed clear cell morphology, 25 cases were CA-IX positive. So, during statistical evaluation between grade, pathological Tumour -Node-Metastasis (pTNM) stage, LVSI and CA-IX expression in tumour cells 11 cases (with histological diagnosis of papillary RCC and collecting duct carcinoma) were excluded. Instead of the evaluation of prognostic parameters was restricted to 34 cases of RCC with CA-IX expression.

Out of 34 cases of RCC, seven cases had nuclear grade 4 of which six tumours showed CA-IX negativity and one was CA-IX 1+. Whereas among 14 grade 2 RCC, 11 (78.6%) showed CA-IX 2+, two (14.3%) with CA-IX 1+ and one (7.1%) case CA-IX negative

	VEGF negative	VEGF 1+	VEGF 2+	Total	p-value
Grade (WHO/ISUP)					
G2	15 (83.3%)	2 (11.1%)	1 (5.6%)	18	p<0.001
G3	2 (11.8%)	6 (35.3%)	9 (52.9%)	17	
G4	0 (0%)	1 (10%)	9 (90%)	10	
pTNM stage					
pT1	7 (100%)	0 (0%)	0 (0%)	7	p=0.002
pT2	4 (57.1%)	3 (42.9%)	0 (0%)	7	
pT3	6 (27.3%)	4 (18.2%)	12 (54.5%)	22	
pT4	0 (0%)	2 (22.2%)	7 (77.8%)	9	
pN0	17 (51.5%)	7 (21.2%)	9 (27.3%)	33	p=0.002
pN1	0 (0%)	2 (16.7%)	10 (83.3%)	12	
M0	17 (44.7%)	9 (23.7%)	12 (31.6%)	38	p=0.003
M1	0 (0%)	0 (0%)	7 (100%)	7	
LVSI					
LVSI (-)	17 (100%)	0 (0%)	0 (0%)	17	p<0.001
LVSI (+)	0 (0%)	9 (32.2%)	19 (67.8%)	28	

[Table/Fig-5]: Association of VEGF expression with grade, LVSI and pTNM stage of Renal Cell Carcinoma (RCC) (n=45).
LVSI (-): Lymphovascular space invasion absent; LVSI (+): Lymphovascular space invasion present; Pearson Chi-square test was used for statistical analysis; p-value <0.05 was regarded as statistically significant

negative [Table/Fig-6a-d]. A significant inverse association was seen between CA-IX expression and grade of RCC (p-value <0.001) [Table/Fig-7]. LVSI was seen in 25 out of 34 cases; among these nine cases were CA-IX negative, nine cases CA-IX 1+ and only seven cases CA-IX 2+. Out of nine cases of RCC without LVSI, eight cases expressed CA-IX 2+. So, the authors observed loss of CA-IX expression with presence of LVSI and it was statistically significant (p-value=0.006).



[Table/Fig-6]: a) Microphotograph of clear cell RCC grade 2 (ISUP/WHO) with CA-IX 2+ score (x400); b) Microphotograph of clear cell RCC grade 3 (ISUP/WHO) with CA-IX 1+ score (x400); c) Microphotograph of clear cell RCC grade 4 (ISUP/WHO) with CA-IX negative (x400); d) Microphotograph of multilocular cystic renal neoplasm of low malignant potential showing CA-IX 2+ score (x400).

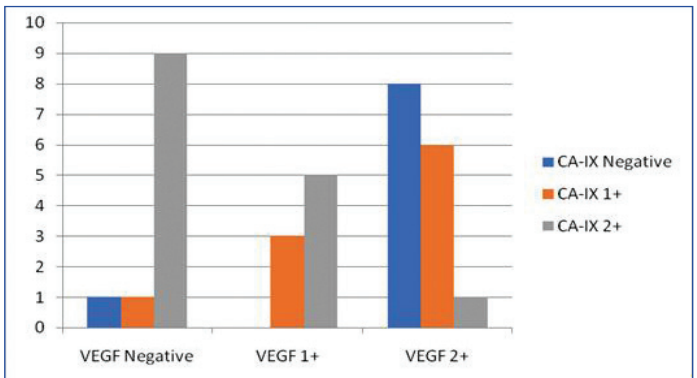
Upon associating pTNM stage of 34 RCC cases with CA-IX expression, all seven cases in pT1 stage showed CA-IX 2+ positivity. On the other hand, among nine cases of pT4 stage, five were CA-IX negative, three CA-IX 1+ and 1 case CA-IX 2+. One case out of seven cases of pT2 stage and three cases out of 11 cases with pT3 stage did not show CA-IX expression. Here, the authors deduced that pT-stage and CA-IX positivity had an inverse association which was statistically significant (p-value=0.038). Among these 34 RCC cases, six cases had lymph node metastasis (pN1 stage). Of these six N1 stage cases, five did not express CA-IX; whereas among 28 cases of pN0 stage, only four cases were CA-IX negative and rest were CA-IX positive. Here also, statistically significant inverse association between pN-stage and CA-IX expression (p-value=0.002) was found. Among six cases with distant metastasis (M1 stage), five cases (83.3%) were CA-IX negative. But only four (14.3%) out of 28 cases of M0 stage showed CA-IX negativity. Again, distant metastatic potential of RCC and CA-IX expression demonstrated inverse association which was statistically significant (p-value=0.002) [Table/Fig-7].

	CA-IX negative	CA-IX 1+	CA-IX 2+	Total	p-value
Grade (WHO/ISUP)					
G2	1 (7.1%)	2 (14.3%)	11(78.6%)	14	p<0.001
G3	2 (15.4%)	7 (53.8%)	4 (30.8%)	13	
G4	6 (85.7%)	1 (14.3%)	0 (0%)	7	
pTNM stage					
pT1	0 (0%)	0 (0%)	7 (100%)	7	p=0.038
pT2	1 (14.3%)	2 (28.6%)	4 (57.1%)	7	
pT3	3 (27.3%)	5 (45.4%)	3 (27.3%)	11	
pT4	5 (55.6%)	3 (33.3%)	1 (11.1%)	9	
pN0	4 (14.3%)	9 (32.1%)	15 (53.6%)	28	p=0.002
pN1	5 (83.3%)	1 (16.7%)	0 (0%)	6	
M0	4 (14.3%)	9 (32.1%)	15 (53.6%)	28	p=0.002
M1	5 (83.3%)	1 (16.7%)	0 (0%)	6	
LVSI					
LVSI (-)	0 (0%)	1 (11.1%)	8 (88.9%)	9	p=0.006
LVSI (+)	9 (36%)	9 (36%)	7 (28%)	25	

[Table/Fig-7]: Association of grade, pTNM stage and LVSI of Renal Cell Carcinoma (RCC) with CA-IX expression (n=34).
LVSI (-): Lymphovascular space invasion absent; LVSI (+): Lymphovascular space invasion present; Pearson Chi-square test was used for statistical analysis; p-value <0.05 was regarded as statistically significant

Overall, CA-IX has a sensitivity 73.53%, specificity 100% and accuracy 80% in immunohistochemical diagnosis of clear cell RCC.

Finally, the authors noted that with gradual increase in degree of expression of VEGF there was a simultaneous gradual decrease in degree of CA-IX expression [Table/Fig-8]. This inverse association between VEGF and CA-IX had a strong statistical significance (p-value=0.001).



[Table/Fig-8]: Bar diagram showing relation between VEGF and CA-IX expression in Renal Cell Carcinoma (RCC).
The values along y-axis represent number of cases

DISCUSSION

The RCC is notorious for showing unpredictable biological behaviour and clinical outcome [1,13]. Computed-tomography scan and magnetic resonance imaging are good diagnostic modalities in suspected adult renal masses. However, core needle biopsy in expert hands has been shown to provide adequate diagnostic material in 80% of cases additionally providing material for immunohistochemistry [14,15]. In low stage tumours some centres opt for partial nephrectomy without biopsy for preserving the rest of the kidney [16].

Recent identification of some molecular markers in diagnosis, prognosis and treatment of RCC are expected to play an important

role both in surgically resectable and non resectable cases. In this study immunohistochemistry for VEGF was done in all RCC cases to assess association between its expression and different prognostic parameters in an attempt to propose anti-VEGF therapy to VEGF positive cases. The CA-IX is characteristically expressed in diffuse cytoplasmic membranous distribution in 75-100% of clear cell RCCs, although high grade tumours have less expression [3]. It may be noted that this study has assessed the expression of CA-IX only in RCC with clear cell morphology and associated it with grade, stage and other prognostic parameters.

The present study found CA-IX positivity in 73.6% of all clear cell RCC cases among which 44.2% showed high expression and 29.4% showed low expression. 26.4% of clear cell RCCs were CA-IX negative and most of these had high WHO/ISUP grade. No case of RCC other than clear cell carcinoma and multilocular cystic renal neoplasm of low malignant potential were CA-IX positive. Genega EM et al., and Liao SY et al., have also stated similar findings [12,17]. Leibovich BC et al., and Ebru T et al., have detected high CA-IX expression in >70% of clear cell RCC cases [18,19]. This study also observed a negative association between CA-IX expression and WHO/ISUP grade of clear cell RCC, i.e., expression of CA-IX is decreased in high grade tumours (p-value <0.001). These results are consistent with those of Genega EM et al., and Leibovich BC et al., [12,18]. Regarding the CA-IX expression and pTNM stage of the clear cell RCC, a statistically significant negative association was found separately with pT-stage (p-value=0.038), pN-stage (p-value=0.002) and M-stage (p-value=0.002). Although the studies of Genega EM et al., and Leibovich BC et al., did not show any significant association in these two parameters, Bui MH et al., showed a statistically significant association between CA-IX expression and stage of tumour [12, 18, 20]. In this study, out of 34 cases of clear cell RCC, 25 had LVSI and only seven cases among those showed high expression of CA-IX. Simultaneously, an inverse association between presence of LVSI and CA-IX expression (p-value=0.006) was also noted. To the authors' knowledge, similar finding has not been appreciated in any study. So, it may be derived that CA-IX is not only a highly specific immunohistochemical marker for clear cell RCC, but also its low or negative expression is a predictor of worse outcome.

In the present study, VEGF positivity was seen in 62.2% cases of RCC while 37.8% were VEGF negative; 42.2 % RCC showed strong VEGF expression (2+). These findings are corroborating with those of Ebru T et al., and Yang S et al., [19, 21]. The authors noted a statistically significant positive association between WHO/ISUP grade of RCC and VEGF expression (p-value <0.001). Expression of VEGF was seen to increase significantly with increasing TNM stage of tumours, separately with pT-stage (p-value=0.002), pN-stage (p-value=0.002) and M stage (p-value=0.003). These observations were concordant with those of Ebru T et al., [19]. So, it can be predicted that high VEGF expression is associated with poor prognosis. This study also revealed CA-IX expression to be inversely associated with VEGF expression, a finding that is statistically significant (p-value=0.001). Phuoc NB et al., had published similar observations [22].

As RCC is seldom responsive to radiotherapy or chemotherapy, targeted molecular therapy has a great role to play especially in surgically non resectable tumours and metastatic diseases. Anti-VEGF antibody may be useful in VEGF expressive RCC, viz., Bevacizumab, Sorafenib and Sunitinib. Another mode of molecular therapy is with high dose Interleukin-2 (IL-2). Unfortunately, long lasting responses are low and high dose IL-2 has significant side effects. So, selection of patients for this therapy is very important [23]. Bui MH et al., stated that response rate of IL-2 therapy was higher (27%) in patients with CA-IX high expressing tumours than in CA-IX low expressing tumours (14%) [20].

Limitation(s)

The authors acknowledge the inherent limitations of this study owing to small sample size and limited duration of observation, cases confined to one geographic region and that too at single institute. Therefore, they propose further multi-institutional studies with larger number of cases over longer duration with scope of follow-up to provide better information about the subject matter.

CONCLUSION(S)

The CA-IX is a specific immunohistochemical marker for diagnosing clear cell RCC particularly in small biopsies. Its expression is inversely associated with grade and stage of tumours. On the other hand VEGF expression is positively associated with grade and stage of all RCCs. So, we can say that CA-IX negative and VEGF strongly positive tumours definitely carry poor prognosis. Both the markers have therapeutic importance also. All VEGF positive RCCs which are surgically non resectable or presented with metastasis may be benefited by anti-VEGF therapy. Such cases of clear cell RCC which are VEGF negative but CA-IX positive can be treated with IL-2 to prolong the survival.

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