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Prognostic Significance of Renin and Erythropoietin (Proteins Related to Renin Angiotensin system Pathway) Expression in Clear Cell Renal Cell Carcinoma (Immunohistochemical Study)

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ABSTRACT

Background: Clear cell renal cell carcinoma (ccRCC) is considered the most common histopathological subtype and constituting nearly 70% of all primary renal cell carcinomas. Dysregulation of proteins related to Renin-Angiotensin System (RAS) could offer a new prognostic indicator in this malignant neoplasm.

Objective: This study aimed to demonstrate renin (REN) & erythropoietin (EPO) immunohistochemical expression in ccRCC cases & their expression was correlated with the clinicopathological data & survival of the studied cases to explore their potential prognostic role.

Material and methods: This retrospective research carried upon 30 cases ccRCC immunohistochemically stained with REN and EPO & the results to be correlated with different clinicopathological variables and patient's survival.

Results: REN & EPO were expressed in 70% & 80% of ccRCC cases, respectively. Positive REN expression was significantly related to younger patients' age, lower nuclear grade, absence of (renal vein thrombosis, necrosis, perirenal fat invasion, renal sinus invasion, lymph/vascular invasion, lymph node metastasis & distant metastasis) and lower American Joint Committee on Cancer (AJCC) stage. While EPO positive expression was significantly related to younger patients' age, greater grade, greater T stage & higher AJCC stage. Negative REN & positive EPO expression were correlated to poor 5-year overall survival (*P*= 0.008 & 0.045 respectively) & disease-free survival (*P*= 0.006 & 0.038 respectively).

Conclusion: Renin and Erythropoietin expression may be prognostic indicators of ccRCC and could predict patient's survival**.** Further researches are recommended to declare prognostic significance of other proteins of RAS pathway providing new insights and treatment modalities of ccRCC.

Keywords: Renin, Erythropoietin, ccRCC.

INTRODUCTION

Renal cell carcinoma (RCC) is considered one of the most diagnosed tumors globally with an estimated 434,419 new cases diagnosed in 2022, accounting for 2.2% of all cancer diagnoses worldwide [(1)]. Clear cell renal cell carcinoma (ccRCC) is considered the most frequent subtype of primary RCCs and constitutes nearly 70% of all renal cell carcinomas [(2)]. In Egypt, renal cancer ranks the 14th, and accounts for 1.5% of all cancer diagnoses at a rate of 3.0/100,000 in men and 2/100,000 in women according to GLOBOCAN 2022 [(1)]. Egypt is one of countries that had the highest mortality rates in Africa with mortality risk 1% in this cancer [(3)].

However the incidence of RCC has been gradually rising and there is a slight increase in the last 10 years, the mortality rates have decreased. An increase in the number of unintentional abdominal imaging diagnosis and the enhancements in methods of treatment may explain the declining in mortality rate[(4)]. Recently, several studies have searched for new prognostic markers in RCC specially clear cell type, which is the most prevalent subtype intending to discover a valuable predictive value in this lethal disease. The renin angiotensin system (RAS) is known as a poly peptide-based system that has hormonal axes and has an eminent role in regulation of blood pressure and maintenance of fluid balance. Dysregulation of the RAS system has been demonstrated by several studies attributed to angiogenesis, inflammation and apoptosis [(5)]. Renin (REN) is a hormone that is produced mainly within the juxtaglomerular cells (JGCs), which are located at afferent glomerular arterioles; its main function is modulation and activation of the RAS pathway as it is considered a main precursor in this pathway. It has been found that RCC could secrete REN and also shares REN its cortical origin. So, examination of REN expression in renal cell carcinoma may provide a challenge in treatment modalities of this malignant neoplasm [(6)].

Erythropoietin (EPO) is a glycoprotein, which is formed in the renal cortex mainly in the proximal tubules, the same site from which the majority of the RCC originates and is considered a protein related to RAS, which has an effect upon it. Erythropoietin plays a crucial role in hematopoiesis and some studies discovered its role in various nonhaematopoietic tissues such as uterus, liver, endothelial cells, central nervous system and different solid malignant neoplasms including RCC [(7)]. The role of EPO in hematopoiesis is well known, but its role in nonhaematopoietic neoplasms is not well clarified. Few research aimed to detect prognostic value of these proteins that the kidneys secrete.

The aim of this study was to evaluate the immunohistochemical expression of REN and EPO in ccRCC and to clarify their roles, that may provide valuable prognostic immunohistochemical markers and

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can also provide new insights in prognosis and treatment modalities in this malignant neoplasm.

MATERIAL AND METHODS

1- Study design & data collection: This retrospective study was conducted at the Pathology, Internal Medicine-Oncology Unit & Urology Departments at Faculty of Medicine-Benha University. The study included 30 patients who had partial or complete nephrectomy for a renal mass and were diagnosed as ccRCC during the period from March 2015 to March 2019 with follow up the patients' survival for 5-years. The study was performed on formalin-fixed paraffin-embedded blocks that included representative tumor samples. Collection of patients' clinical & pathological data that included tumor size, nuclear grade, stage, sex, age at diagnosis and follow-up data was performed by revising patients' files & pathological reports.

Inclusion criteria: Cases with available clinicopathological data concerning age, grade, stage, lymph node status. Follow-up data and survival outcomes of the cases for 5-years. Paraffin blocks with good quality.

Exclusion Criteria: Cases of other histologic types but clear cell types. Patients whose clinical data were unavailable. Cases without available follow-up data. Paraffin blocks with bad quality or insufficient tissue for immunostaining.

Revision of the diagnosis & other histopathologic parameters were performed by two pathologists blinded to the original diagnosis. Tumors were classified according to standard histopathologic criteria following 5th edition WHO Urinary and Male Genital Tumors Classification [8] and graded using standard WHO/ISUP criteria [9]. The tumor, node, and metastasis (TNM) staging for RCC were made depending on the timing of surgical resection based on American Joint Committee on Cancer's (AJCC) (8th Edition) staging system [10].

2- Immunohistochemical staining: Immunohistochemical staining of 4-μm tissue sections from whole tissue paraffin blocks was executed using a primary rabbit polyclonal antibody against human renin ***(Cat# 14291-1-AP 100 μL, diluted 1/200; Proteintech, Rosemont, IL, USA)*** and a primary rabbit polyclonal antibody against human erythropoietin ***(Cat# 17908-1-AP 100 μL, diluted 1/200; Proteintech, Rosemont, IL, USA)***. Anti- REN and Anti-EPO antibodies were applied for each slide utilizing Avidin-Biotin complex technique in compliance with manufacturer's guidelines. Antigen retrieval was executed utilizing 10 mmol/1 citrate buffer (ph. 6.0) for both. Tissue sections were incubated overnight at room temperature with the primary antibodies. Immunoreaction was visualized by adding DAB as a chromogen. The slides of positive and negative controls were comprised in each run. Representative sections of normal kidney tissue were utilized as positive controls for both REN [11] & EPO [12]. Replacement of the primary antibody with nonimmune IgG was done to get negative controls.

3- Interpretation of immunohistochemical staining

Interpretation of REN positivity: Strong cytoplasmic expression in more than 95% of tumor cells of cases was considered positive REN expression [13].

Interpretation of EPO positivity: Positive moderate or strong cytoplasmic expression in more than 5% in tumor cells was considered positive for EPO. Negative expression was considered for cases with no immunohistochemical staining within the tumor cells of cases with weak staining in <5% of tumor cells [14].

Ethical approval: This research received approval from Benha Faculty of Medicine's Ethical Committee for Research. Code of Ethics (RC 30-10-2024). After receiving all of the information, each participant signed a permission. The Helsinki Declaration was followed throughout the course of the investigation.

Statistical analysis

Statistical package for the social sciences (SPSS) software package version 22 was utilized for data categorization and analysis, Quantitative data were described utilizing numbers and percentages. Chi-Square (χ^2) test and Fisher Exact test (FET) were employed for categorical variables, to contrast between different variables. Survival curves were plotted using Kaplan–Meier method and contrasted by Log-rank test. Disease-free survival (DFS) was the time from primary treatment to the time of recurrence/metastasis. Overall survival (OS) was characterized as the time from the beginning of the primary treatment to death from any cause. Univariate & multivariate survival analyses were executed by applying Cox proportional hazards model. A statistical significance was regarded for P value ≤ 0.05 in all tests.

RESULTS

1- Clinicopathological characteristics:

This study included 30 cases diagnosed as ccRCC. The median age of the studied cases was 50 (ranged from 40- to 70) years and 19 (63.3%) cases were ≤ 50 years. Eighty cases (80%) were males, and 6 (20%) cases were females. Other clinicopathologic features are illustrated in table (1).

Table (1): Clinicopathologic features of studied ccRCC cases (N=30)

Parameters	**Number (%)**
Age (years)	
Median (range)	50 (40-70)
≤50	19 (63.3%)
>50	11 (36.7%)
Sex Male	24 (80%)
Female	6 (20%)
Nuclear grade (G)	
G1	13 (43.3%)
G2	13 (43.3%)
G3	4 (13.4%)
Tumor necrosis No	17 (56.7%)
Yes	13 (43.3%)
Perirenal fat invasion: Absent	25 (83.3%)
Present	5 (16.7%)
Renal sinus Invasion: Absent	26 (86.7%)
Present	4 (13.3%)
Renal vein thrombosis: Absent	26 (86.7%)
Present	4 (13.3%)
Lymph/vascular Invasion: Absent	22 (73.3%)
Present	8 (26.7%)
Primary tumor (T) stage	
T1	14 (46.7%)
T2	10 (33.3%)
T3	6 (20%)
Lymph node (LN) stage	22 (73.3%)
N0	8 (26.7%)
N1	
Distant Metastasis (M) stage	28 (93.3%)
M0	2 (6.7%)
M1	
AJCC stage	
I	14 (46.7%)
II	6 (20%)
III	8 (26.7%)
IV	2 (6.6%)
Survival	
Alive	15 (50%)
Died	15 (50%)

N, Number; *AJCC*, American Joint Committee on Cancer.

2- Immunohistochemical expression results

Renin expression in the studied ccRCC cases & its relation to clinicopathological variables: Renin expression was positively expressed in the cytoplasm of 21 (70%) ccRCC cases, while 9 (30%) showed negative expression with no staining of the tumor cells (Figure 1). There was a significant statistical relation between REN positive expression and younger patients' age (*P* = 0.026), lower nuclear grade (*P* = 0.002) and lack of necrosis (*P* = 0.001), absence of perirenal fat invasion (*P* = 0.008), absence of renal sinus invasion (*P* = 0.035), absence of renal vein thrombosis (*P* = 0.035) absence of lymph/vascular invasion (*P* = 0.001), negative lymph node metastasis (*P* = 0.001), absence of metastasis (*P* = 0.025), and lower AJCC stage (*P* = 0.005). These relations are demonstrated in table (2).

Erythropoietin expression in the studied ccRCC cases & its relation to clinicopathological variables: Erythropoietin (EPO) expression was positively expressed in the cytoplasm of 24 (80%) ccRCC cases, while 6 (20%) showed negative expression with no staining of the tumor cells (Figure 2). A significant statistical relation was existed between EPO positive expression and younger patients' age (*P* = 0.008), higher grade (P = 0.007), greater T stage (*P* = 0.014) and higher AJCC stage (*P* = 0.036) with no significant relation found with the other parameters (Table 2).

Table (2): Relation between renin and erythropoietin expression and the clinicopathologic variables of the studied ccRCC cases (N=30).

Parameters (n)	**REN Expression**		***P* value**	***EPO Expression***		***P* value**
	Negative (n=9)	**Positive (n=21)**		**Negative (n=6)**	**Positive (n=24)**	
	n (%)	**n (%)**		**n (%)**	**n (%)**	
Age (years)						
≤50 (19)	3 (15.8%)	16 (84.2%)	**0.026***	1 (5.3%)	18(94.7%)	**0.008****
>50 (11)	6 (54.5%)	5 (45.5%)		5 (45.5%)	6 (54.5%)	
Sex						
Male (24)	7 (29.2%)	17 (70.8%)	0.842	4 (16.7%)	20 (83.3%)	0.361
Female (6)	2 (33.3%)	4 (66.7%)		2 (33.3%)	4 (66.7%)	
Nuclear Grade (G)						
G1 (13)	1 (7.7%)	12 (92.3%)		6 (46.2%)	6 (46.2%)	
G2 (13)	4 (30.8%)	9 (69.2%)	**0.002****	0	0	**0.007****
G3 (4)	4 (100%)	0		0	0	
Tumor necrosis						
No (17)	1 (5.9%)	16 (94.1%)	**0.001****	5 (29.4%)	12 (70.6%)	0.141
Yes (13)	8 (61.5%)	5 (38.5%)		1 (7.7%)	12 (92.3%)	
Perirenal fat invasion						
Absent (25)						
Present (5)	5 (20%)	20 (80%)	**0.008****	6 (24%)	19 (76%)	0.221
	4 (80%)	1 (20%)		0	5 (100%)	
Renal sinus Invasion						
Absent (26)						
Present (4)	6 (23.1%)	20 (76.9%)	**0.035***	6 (23.1%)	20 (76.9%)	0.283
	3 (75%)	1 (25%)		0	4(100%)	
Renal vein thrombosis						
Absent (26)						0.283
Present (4)	6 (23.1%)	20 (76.9%)	**0.035***	6 (23.1%)	20 (76.9%)	
	3 (75%)	1 (25%)		0	4(100%)	
Lymph/vascular Invasion						
Absent (22)						
Present (8)	3 (13.6%)	19 (86.4%)	**0.001****	6 (27.3%)	16 (72.7%)	0.099
	6 (75%)	2 (25%)		0	8 (100%)	
Primary tumor (T) stage						
T1 (14)						
T2 (10)	3 (21.4%)	11 (78.6%)	0.090	6 (42.9%)	8 (57.1%)	**0.014***
T3 (6)	2 (20%)	8 (80%)		0	10 (100%)	
	4 (66.7%)	2 (33.3%)		0	6 (100%)	
Lymph node (N) stage						
N0 (22)						
N1 (8)	3 (13.6%)	19 (86.4%)	**0.001****	6 (27.3%)	16 (72.7%)	0.099
	6 (75%)	2 (25%)		0	8 (100%)	
Distant Metastasis (M) stage						
M0 (28)	7 (25%)	21 (75%)	**0.025***	6 (21.4%)	16 (72.7%)	0.464
M1 (2)	2 (100%)	0		0	8 (100%)	
AJCC stage						
I (14)	2 (14.3%)	12 (85.7%)	**0.005****	6 (42.6%)	8 (57.1%)	**0.036***
II (6)	0	6 (100%)		0	6 (100%)	
III (8)	5 (62.5%)	3 (37.5%)		0	8 (100%)	
IV (2)	2 (100%)	0		0	2 (100%)	

N, Number; REN, Renin; EPO, Erythropoietin AJCC, American Joint Committee on Cancer

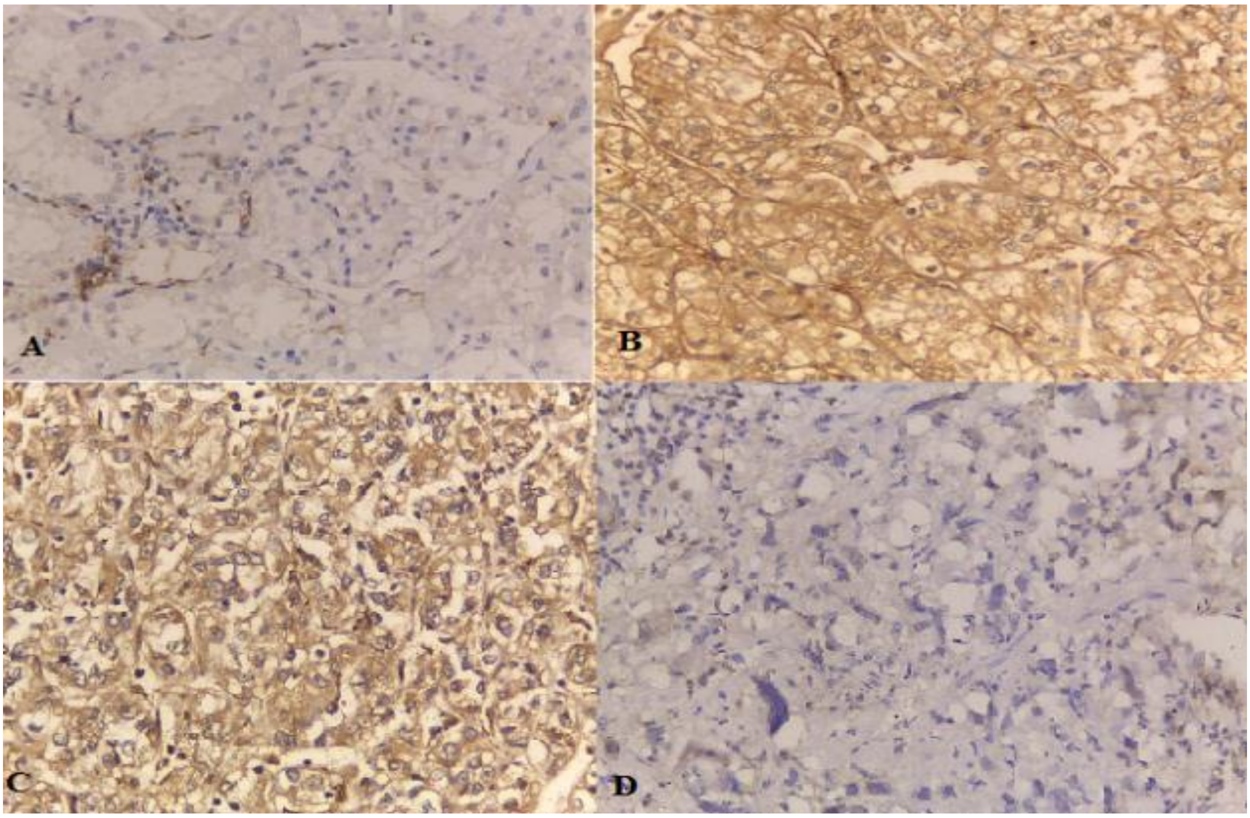

Figure (1): A photomicroghraph shows renin (REN) immunohistochemical expression; (A) Positive REN cytoplasmic expression in juxtaglomerular cells of normal renal parenchyma (IHC, X400); (B) Positive REN cytoplasmic expression in ISUP/WHO grade 1 clear cell renal cell carcinoma (IHC, X200); (C) Positive REN cytoplasmic expression in ISUP/WHO grade 2 clear cell renal cell carcinoma(IHC, X400); (D) Negative REN expression in ISUP/WHO grade 3 clear cell renal cell carcinoma (IHC, X400).

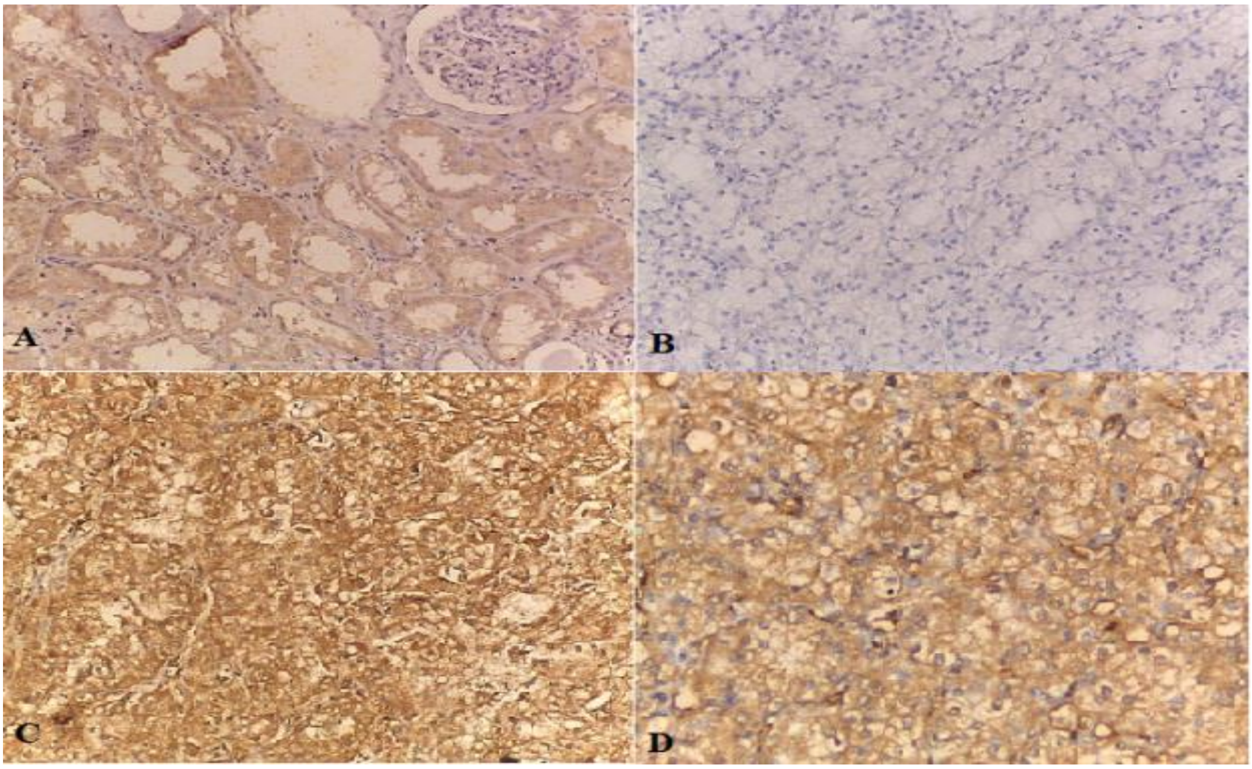

Figure (2): A photomicroghraph shows erythropoietin (EPO) immunohistochemical expression; (A) Positive EPO cytoplasmic expression in the cortex of normal renal parenchyma (IHC, X200); (B) Negative EPO expression in ISUP/WHO grade 1 clear cell renal cell carcinoma (IHC, X200); (C) Positive EPO cytoplasmic expression in ISUP/WHO grade 2 clear cell renal cell carcinoma (IHC, X200); (D) Positive EPO cytoplasmic expression in ISUP/WHO grade 3 clear cell renal cell carcinoma (IHC, X400).

3-Survival Analysis:

With the completion of the follow-up period (5-years), overall survival & disease-free survival rates for the studied cases were 50%, each with a median follow-up time 49 months. Kaplan-Meier analysis exhibited that cases with positive **REN** expression were correlated to better both OS and DFS with the median OS for cases with positive & negative **REN** expression was 50.74 vs. 23.33 months respectively (Log rank=6.938, *P*=0.008), and the median DFS for cases with positive & negative *REN* expression was 45.92 vs. 26.72 months, respectively (Log rank=7.587, *P*=0.006) (Figure 3). While, positive **EPO** expression was correlated to poorer both OS and DFS. The median OS for cases with positive & negative **EPO** expression was 38.13 vs. 59.66 months respectively (Log rank=4.032, *P*=0.045), and the median DFS for cases with positive & negative **EPO** expression was 34.93 vs. 59.66 months, respectively (Log rank=4.288, *P*=0.038) (Figure 3).

In the univariate analysis for OS: REN expression was an indicator for OS (HR 0.277; 95% CI 0.099-0.78; *P*=0.015), while **EPO** expression showed no significance. The clinical and pathological variables that were indicators for OS in univariate analysis were comprised in a multivariate cox regression model analysis and none of them was an independent indicator for OS (Table 3).

In the univariate analysis for DFS: REN expression was a prognostic indicator for DFS (HR 0.26; 95% CI 0.09-0.73; *P*=0.011), while **EPO** expression showed no significance. The clinical and pathological variables that were indicators for DFS were exhibited in Table (3). The multivariate cox regression model demonstrated that tumor T stage was an independent predictor for DFS (HR 46.4; 95% CI 2.59-830.4; *P*=0.009) (Table 3).

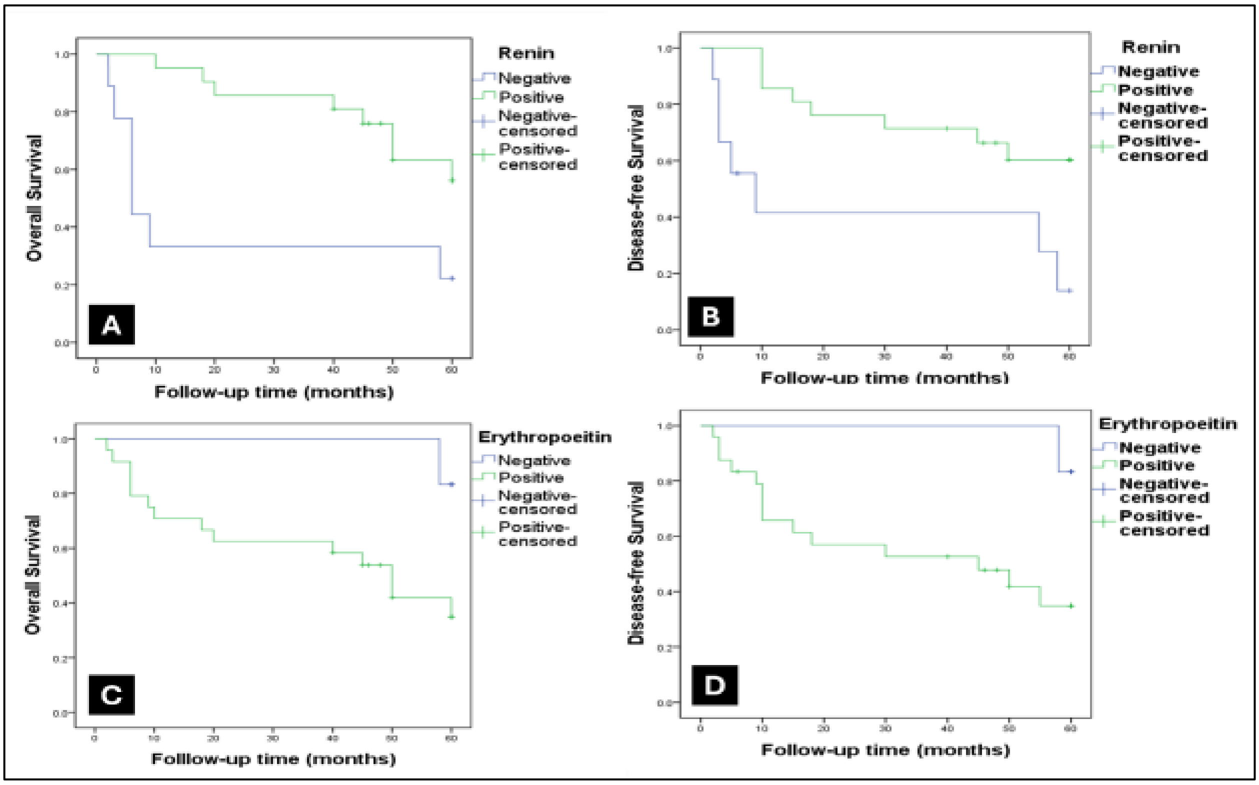

Figure (3): Kaplan-Meier survival analysis for 5-year OS & 5-year DFS according to renin (A & B) and erythropoietin expression (C & D). **OS**: overall survival; **DFS:** Disease-free survival.

Table (3): Univariate and Multivariate Cox regression analysis for overall survival and disease-free survival

Parameters	Overall survival				Disease-free survival			
	Univariate		Multivariate		Univariate		Multivariate	
	HR (95% CI)	**P value**	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**	**HR (95% CI)**	**P value**
Age	1.21 (0.43-3.44)	0.715			1.22 (0.43-3.45)	0.712		
Sex	0.527 (0.12-2.36)	0.402			0.25 (0.03-1.89)	0.178		
Grade	3.67 (1.46-9.24)	**0.006****	2.25 (0.60-8.48)	0.229	4.32 (1.60-11.61)	**0.004****	3.97 (0.73-21.64)	0.110
Tumor necrosis	2.23 (0.8-6.22)	0.125			3.94 (1.33-11.69)	**0.013***	0.16 (0.018-1.41)	0.099
Perirenal fat invasion	6.65 (1.87-23.65)	**0.003****	3.51 (0.03-395.7)	0.602	11.03 (3.22-37.85)	**0.000****	0.24 (0.003-17.90	0.515
Renal sinus Invasion	4.55 (1.17-17.62)	**0.028***	1.32 (0.09-17.98)	0.835	8.07 (2.3-28.38)	**0.001****	0.07 (0.00-12.35)	0.314
Renal vein thrombosis	5.56 (1.44-21.50)	**0.013***	0.07 (0.00-13.73)	0.331	9.23 (2.61-32.64)	**0.001****	6.66 (0.28-156.66)	0.239
Lymph/vascular Invasion	3.95 (1.35-11.56)	**0.012***			6.37 (2.15-18.83)	**0.001****		
Primary tumor (T) stage	3.82 (1.78-8.20)	**0.001****	4.39 (0.40-47.91)	0.225	7.88 (3.16-19.63)	**0.000****	46.4 (2.59-830.4)	**0.009****
Lymph node (N) stage	3.95 (1.35-11.56)	**0.012***	1.29 (0.02-82.80)	0.904	6.37 (2.15-18.83)	**0.001****	4.75 (0.06-379.5)	0.486
Distant metastasis	1.29 (0.169-9.91)	0.805			3.18 (0.71-14.35)	0.131		
AJCC stage	2.06 (1.34-3.18)	**0.001****	0.79 (0.10-6.25)	0.826	2.66 (1.69-4.20)	**0.000****	0.31 (0.03-2.98)	0.309
Renin	0.277 (0.099-0.78)	**0.015***	0.49 (0.08-2.97)	0.439	0.26 (0.09-0.73)	**0.011***	0.103 (0.08-1.29)	0.078
Erythropoietin	6.18 (0.80-47.74)	0.81			6.54 (0.84-50.72)	0.072		

CI, Confidence Interval; *AJCC*, American Joint Committee on Cancer.

DISCUSSION

Clear cell renal cell carcinoma (ccRCC) is considered the most prevalent histopathological subtype and constituting nearly 70% of all primary RCC [(1)].

The renin-angiotensin system (RAS) is known as a complex peptide-based hormonal pathway that is important for regulation of the blood pressure, fluid balance, and other related cardiovascular physiological and pathophysiological processes. RAS has two axes to modulate its action: the first one, angiotensin-converting enzyme (ACE)/angiotensin (Ang) II/angiotensin type 1 receptor (AT1R) axis and the second one, ACE2/Ang-(1-7)/Mas receptor (MasR) axis [(2)]. Although the role of proteins related to RAS in ccRCC is debatable, the pertinent literature reported that stimulation of RAS may lead to metastasis and progression of ccRCC [(15)]. So, It is hypothesized that RAS has a crucial function in development and progression of ccRCC. Few researches have focused on this enzyme system introducing the values of these enzymes as prognostic factors in RCC and drugs acting on them to provide new and effective treatment modalities to the favor of the patient [(6)].

Our study aimed to detect immunohistochemical expression of renin and erythropoietin in ccRCC and also to detect their relations with different clinicopathological variables and survival.

The current research demonstrated that renin (REN) expression was positively expressed in the cytoplasm of 70% of ccRCC cases, while 30% showed negative expression with no staining of the tumor cells. This finding was in line with **de Almeida *et al.*** [(6)], **Siljee *et al.*** [(11)] and **Mourao *et al.*** [(14)] studies, which reported that renin expression was positive in 72%, 70.6% and 73% respectively of ccRCC cases of their studies.

There was a significant statistical relation between REN positive expression and younger patients' age (P = 0.026), lower nuclear grade (P = 0.002), lack of necrosis ($P = 0.001$), absence of perirenal fat invasion ($P = 0.008$), absence of renal sinus invasion ($P = 0.035$), absence of renal vein thrombosis ($P = 0.035$) , absence of lymph/vascular invasion ($P = 0.001$), negative lymph node metastasis ($P = 0.001$), lack of metastasis ($P = 0.025$), and lower tumor stage ($P = 0.005$). These findings come in parallel with results of **de Almeida *et al.*** [(6)] who found that lack of renin expression was significantly correlated with high-grade ccRCC cases (by Fuhrman classification and ISUP, both P < 0.001), more existence of microvascular invasion (P = 0.046), and existence of renal vein invasion (P = 0.026). Also, come in agreement with **Mourao *et al.*** [(14)] study, which revealed that REN positive expression was significantly correlated to younger patients' age ($P = 0.028$), low grade ccRCC cases (P < 0.001), lower tumor size (P < 0.001), lack of necrosis ($P = 0.007$), absence of perirenal fat infilteration (P = 0.001), lack of renal sinus venous (P < 0.001), absence of microvascular invasion (P = 0.003), negative lymph node metastasis ($P = 0.001$), and lower tumor stage ($P = 0.001$).

Based on our current results, positive REN expression was correlated to favorable prognostic factors. This result could be explained by the fact that REN is produced from the juxtaglomerular cells that present within the renal parenchyma converting angiotensinogen to angiotensin I, which is further converted to angiotensin II that has a potent vasoconstrictor effect and REN is a part of RAS, which has two axes, the first one ACE/Ang II/AT1R axis, which has pro-tumorigenic effects, as its activation rises the blood pressure and triggers tumor progression while the second one ACE2/Ang-(1-7)/MasR neutralizes these effects. So, downregulation of this second axis may increase the bad behavior of the tumor. In fact , It is pivotal for presence of balance between two axes and both the Ang-(1-7)/MasR and Ang II/AT1R signaling pathways might lead to the progression of ccRCC [(15)]. So positive REN expression may cause dysregulation of granular cells producing REN, which may cause upregulation of the second axis [ACE2/Ang-(1-7)/MasR], which takes the upper hand and prevents tumor progression through vascular protection by its anti-inflammatory effect, antioxidant effect and increased bioavailability of nitric oxide, which in turn causes vasodilatation and antagonizes the pro-tumorgenic effect of the first axis.

The present work revealed that Erythropoietin (EPO) expression was positively expressed in the cytoplasm of (80%) ccRCC cases, while (20%) showed negative expression with no staining of the tumor cells. There was a significant statistical relation between EPO positive expression and younger patients' age (P = 0.008), higher grade (P = 0.007) and greater T stage (P = 0.014) and higher AJCC stage (P = 0.036) with no significant relation was found with the other parameters.

In agreement with our result**, Ferreira *et al.*** [(12)] study reported that EPO expression was positive in 73.3% of cases of his study and positive EPO expression was statistically related to reduced patients' age and greater tumor size ($P = 0.003$, $P = 0.015$ respectively). This finding is partially in agreement with **Mourao *et al.*** [(14)] study that revealed positive EPO expression in 86.6% of ccRCC cases of his study, and a significant statistical relation was discovered between EPO positive expression and younger patients' age (P = 0.015), but not associated with a significant statistical relation with either the stage or the grade. This discrepancy may be due to the difference in number of the studied cases between both studies and the greater proportion of ccRCC cases of his study was in clinical stage I (67%). This finding also is in accordance with **Mjønes *et al.*** [(16)] study, which found that positive immunohistochemical EPO expression was present in up to 97% of ccRCC cases . And partly, in line with our result, **Stoyanoff *et al.*** [(17)] study revealed that EPO was positively expressed in most samples of ccRCC cases but earlier tumors (T1G1) showed higher EPO expression than tumors of the control group (≥T2). Inversely, **Michael *et al.*** [(18)] study demonstrated that positive EPO expression was only in 33% of RCC cases of the study. This different finding may be due to different histopathological subtypes of RCC that were included in his study beside ccRCC.

The current study revealed that positive expression of EPO was statistically related to higher grade (P = 0.007) and greater T stage (P = 0.014) & higher AJCC stage (P = 0.036) as previously aforementioned. This outcome might be accounted for the fact that the von HippelLindau (VHL)-HIF-EPO pathway regulates EPO secretion via hypoxia-inducible factor (HIF). As von Hippel-Lindau (VHL) gene mutation is associated with development of sporadic and familial RCC, its mutation can stabilize and upregulate HIF and consequently activates other moelecules including EPO, which promotes proliferation and progression of solid tumours, angiogenesis and drug resistance [(20)]. In addition to the hypoxic cellular microenvironment that the tumor is exposed giving the chance to HIF to be accumulated, which in turn stimulates EPO expression triggering tumor progression in general and specially in RCC, it had a particular significance due to the relation between VHL, HIF and EPO [(13)].

On survival analysis of this research, Kaplan-Meier analysis showed that cases with positive **REN** expression was statistically related to better both OS and DFS, (P=0.008, P=0.006) respectively. REN expression was an indicator for OS & DFS (HR 0.277; 95% CI 0.099-0.78; P=0.015), (HR 0.26; 95% CI 0.09-0.73; P=0.011) respectively. Our result regarding REN expression came in line with **de Almeida *et al.*** [(6)] who discovered negative renin expression that was related to unfavorable prognosis for DFS (RR = 2.923, P < 0.001). Also, in agreement of **Mourao *et al.*** [14] study that showed REN expression lack was related to unfavorable prognosis of 10-year OS and cancer specific survival (CSS).

The worse prognosis that was associated with negative REN expression could be explained by that REN is a part of RAS in addition to its ability to promote tumor growth and proliferation through the first ACE/Ang II/AT1R axis of RAS, which has strong pro-tumorigenic effects. In case of ccRCC, dysregulation of these granular cells producing REN may occur and absence of REN expression may activate this axis upregulating the pro-angiogenic factors expression such as basic fibroblast growth factor (BFGF), vascular endothelial growth factor (VEGF), and angiopoietins. Therefore lack of renin can give the tumor bad prognostic behavior by increasing tumor angiogenesis and supporting tumor cell survival and proliferation [15].

While survival analysis regarding EPO revealed positive EPO expression that was associated with poorer both OS and DFS, (P=0.045) & (P=0.038) respectively. Few studies were published in the literature concerning its role and its effect on survival of ccRCC cases. In line with our result **Michael *et al.*** [18] study revealed that the risk of death in patients with positive EPO expression was roughly twice as high as that for patients with negative EPO expression (HR 2.34; 95% CI 1.27–4.3; p=0.006). So, positive EPO expression in ccRCC cases was associated with worse survival. Inversely, **Ferreira *et al.*** [12] study revealed that lack of EPO expression was a bad prognostic factor and affected the OS (p < 0.001) and DFS (p < 0.001) rates negatively. And patients with positive EPO expression had a better prognosis, and after 5 years 82.9% were alive. However, these opposite results concerning EPO, **Mourao *et al.*** [8] study revealed that expression of EPO didn't affect survival rates even with its concomitant assessment with REN. This conflict and opposite results may be due to different immunohistochemical techniques among these studies and may be also due to different ethnicities.

The current study could explain this finding, as ccRCC is commonly associated with hypoxic media, which stimulates hypoxia-inducible-factor alpha (HIF1a) accumulation within tumor cells in association with VHL protein deletion. All these reasons can in turn cause dysregulation of juxtaglomerular cells, decreasing renin production, causing a phenotypic transition and converting the renin producing granular cells into cells producing erythropoietin via the von HippelLindau (VHL)-HIF-EPO pathway. So, absence of REN expression is adversely connected to increased positive EPO expression, which is associated with aggressiveness of the tumor through its stimulation of neoangionesis and tumor cell proliferation and increase the chance for tumor metastasis [7].

Our study revealed that tumor T stage was an independent predictor for DFS (P=0.009) in ccRCC by using the multivariate cox regression model. This finding actually comes in agreement with **Mattila *et al.*** [19] study, which concluded that tumor stage was an independent prognostic indicator in ccRCC as advanced tumot stages are frequently associated with microvascular invasion (MVI), tumor necrosis and distant metastasis.

CONCLUSION

So, we can conclude that REN and EPO are concomitantly proteins related to Renin Angiotensin system (RAS) pathway that could predict prognosis in ccRCC and REN affect EPO production. Detection of their expression and molecular biology in ccRCC provides new insights in prognosis and treatment strategies of this malignant neoplasm.

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