Journal Homepage: - www.journalijar.com
INTERNATIONAL JOURNAL OF ADVANCED RESEARCH (IJAR)
Article DOI: 10.21474/IJAR01/3949
DOI URL: http://dx.doi.org/10.21474/IJAR01/3949

RESEARCH ARTICLE

ENHANCER OF ZESTE HOMOLOG 2 AND VASCULAR ENDOTHELIAL GROWTH FACTOR IMMUNOHISTOCHEMICAL EXPRESSION ARE ASSOCIATED WITH ADVERSE CLINICAL OUTCOME IN NON-METASTATIC CLEAR CELL RENAL CELL CARCINOMA.

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Manuscript Info

Manuscript History

Received: 28 March 2017
Final Accepted: 16 April 2017
Published: 30 April 2017

Key words:-
Clear cell renal cell carcinoma, Immunohistochemistry, EZH2, VEGF, Ki-67.

Abstract

Background: Clear cell renal cell carcinoma (CCRCC) represents the most common malignant adult renal tumor. Its poor prognosis is due to poor response to current treatment options. Identification of new therapeutic targets is a priority. Enhancer of zeste homolog 2 (EZH2) expression is associated with aggressive behavior in different cancers. Vascular endothelial growth factor (VEGF) is strong angiogenic agent that promotes angiogenesis during the growth of tumor. **Aim:** To assess the significance of immunohistochemical expression of EZ2H and VEGF on tumor behavior, prognosis and patient survival in clear cell renal cell carcinoma. **Methods:** Immunohistochemical expressions of EZH2, VEGF,Ki-67were evaluated in 25 cases of CCRCC, correlated with each other , with clinicopathological parameters and patients survival. **Results:** Expression of EZH2 was significantly correlated with tumor grade and stage ($p< 0.001$), metastasis to lymph node ($p=0.016$), Ki67 expression ($p= 0.014$) and with local recurrence of the tumor ($p= 0.001$) in CCRCC cases. Vascular Endothelial Growth Factor was significantly positively correlated with tumor grade and stage ($p< 0.001$), Ki67 expression ($p= 0.008$) and local recurrence of the tumor ($p= 0.001$) while no correlation was found with lymph node metastasis ($p=0.096$). Direct correlation was detected between EZH2 and VEGF expression ($p< 0.001$), and a significant positive correlation was detected between ki-67 and both EZH2 ($p= 0.003$), and VEGF ($p<0.001$).High EZH2 and VEGF expression was significantly positively correlated with worse 2-year overall survival (OS) ($p < 0.001$ and $p= 0.009$, respectively).**Conclusion:** EZH2 and VEGF can be considered as independent prognostic markers in CRCC and may be used as a target for therapy in these cases.

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Introduction:-

Renal cell carcinoma (RCC) represents 85 % of primary malignant tumors that originates from the renal parenchyma in adults **(Znaor et al., 2015).** In Egypt, RCC represents 68.2% of primary kidney tumors **(Helal et al., 2015).**The most frequently used prognostic factors for this tumor are grade and stage, but these factors are widely considered less than reliable as several researches detected metastatic RCC cases with low grade and stage **(Cheng et al., 2014).** Enhancer of zeste homolog 2 (EZH2) is a polycomb group protein necessary for the transmission of gene expression patterns to progeny cells during normal development and it is responsible for histone and DNA methylation **(Vire et al., 2006).** EZH2 overexpression has oncogenic effect by giving cellular growth advantage **(Wagener et al., 2010).** Von-Hippel Lindau gene inactivation is common in both hereditary and sporadic renal cell carcinomas resulting in hyperactivity of the hypoxia-inducible factor leading to production of factors that stimulates blood vessel formation as vascular endothelial growth factor. VEGF is a potent angiogenic factor that induces angiogenesis during tumorigenesis **(Lainakis and Bamias**, **2008).** Ki-67 is a protein that present in all the phases of cell cycle (G1, S, G2 and mitosis) while it is absent in non-dividing cells (G0) **(Gerdes et al., 1984).**That is why it is considered as excellent indicator for the proliferating fraction of tumor cells. Instead of relying on multiple parameters, Ki-67 needs only evaluation of nuclear staining for interpretation. Proliferation determined by Ki-67 is known to be of prognostic importance in CCRCC and is known to correlate with tumor grade **(Mehdi et al., 2016).** The aim of the study is to assess the significance of immunohistochemical expression of EZ2H and VEGF on tumor behavior, prognosis and patient survival in clear renal cell carcinoma.

Patients And Methods:-

This study was conducted in Pathology, Clinical oncology and General surgery departments, Zagazig University, in the period from January 2015 to February 2017. The study was carried out on 25 patients of clear cell renal cell carcinoma (CCRCC). Sections from the involved cases were stained, evaluated by routine H&E stain and were graded according to Fuhrman grading system **(Fuhrman et al., 1982),**and the stage is evaluated conferring to the American Joint Committee on Cancer staging system**(Edge and Compton, 2010).**Clinical, radiological and pathological data were abstracted from files of the corresponding departments**.** Patients with a negative postoperative PET/CT scan were included in this study while those with evidence of metastasis or deficient data were omitted. None of the patients had received chemo or radiotherapy preceding surgery. Clinical follow-up was done every three months to all cases and information concerning follow up was abstracted from hospital records or patient contact.

Immunohistochemistry:-

The immunohistochemical staining procedure was done using streptavidin–biotin immunoperoxidase technique (Dako-Cytomation, Glostrup, Denmark). Sections of 3–5 μm from the formalin-fixed-paraffin-embedded blocks were cut and mounted on positively charged slides then deparaffinized by xylene, and rehydrated in graded alcohol. Thereafter, sections were boiled in buffered citrate (pH 6.0) for about 20 minutes then washed in PBS (pH 7.3). Then, endogenous peroxidase activity was blocked with 6% H2O2 in methanol. The slides were incubated overnight with a mouse monoclonal EZH2 (ab14389; Abcam, Cambridge, UK, 1:100); rabbit polyclonal VEGF (Lab Vision, Fremont, CA, USA, 1:200) and mouse monoclonal Ki-67 (Lab Vision, Fremont, CA, USA, 1:50) antibodies. After rinsing in PBS, the slides were immersed with a biotin-conjugated secondary antibody (Lab Vision Corporation, Fermont, USA). DAB was used as a chromogen and Mayer's Hematoxylin was used as a counter stain, and then the slides were washed with distilled water and PBS. Positive and negative controls were stained with the same setting of the studied cases. Sections from normal testis were used as positive controls for EZH2 and prostate adenocarcinoma tissues as positive controls for VEGF and Ki-67. The negative controls were done using the same tissue with the omission of the primary antibody.

Immunohistochemistry assessment:-

EZH2 immunostaining: Frequency of nuclear staining was evaluated using a semiquantitative score: 0 = no expression; 1 = positivity in 1 to 5% = low expression; 2 = positivity in >5 to 25% = intermediate expression; 3 = positivity in >25 to 50% =high expression; and 4 = positivity in more than 50% = very high expression **(Wagener et al., 2010).**

VEGF immunostaining:-

Cells with dark brown cytoplasmic staining were regarded as having positive protein expression. The percentages of VEGF positive tumor cells were scored as 0 (no staining), 1 (1–25% positive cells), 2 (26–50% positive cells) and 3 (>50% positive cells). The VEGF staining intensity was scored as 0 (negative), 1 (weak), 2 (intermediate), and 3

(strong). The sum of the percentage and intensity scores was evaluated. Cases were divided into, group 1 (score 0–3) considered low expression and group 2 considered high expression (score 4–6) **(Ebru et al., 2016).**
Ki-67 immunostaining: Ki-67 antigen staining was detected in the nucleus and scored as following**:** 0- no nuclear staining is observed in tumor cells; 1 - nuclear staining is detected in 1-10% of the tumor cells; 2-11-20%; 3->20% **(Kocarslan et al., 2014).**

Statistical Analysis:-
Continuous variables were expressed as the mean ± SD & median (range), and the categorical variables were expressed as a number (percentage). Continuous variables were checked for normality by using Shapiro-Wilk test. Independent samples Student's t-test was used to compare between two groups of normally distributed variables. Kruskal Wallis H test was used to compare between more than two groups of non-normally distributed variables. Percent of categorical variables were compared using Pearson's Chi-square test or Fisher's exact test when was appropriate. Strength of relationship between immunohistochemical staining for EZH2, VEGF and Ki-67 were examined using computing Kendall's tau-c correleation coefficient, (+) sign was indicator for direct relationship & (-) sign was indicator for inverse relationship, also values near to 1 was indicator for strong relationship & values near 0 was indicator for weak relationship.

Disease free survival (DFS) was calculated as the time from surgery to reappearance of the disease (local or regional or distant metastasis) or the most recent follow-up in which relapse free. Overall Survival (OS) was calculated as the time from diagnosis to death or the most recent follow-up contact (censored). Stratification of DFS and OS was done according to clinicopathological data and markers score. These time-to-event distributions were estimated using the method of Kaplan-Meier plot, and compared using two-sided exact log-rank test. A p-value <0.05 was considered significant. All statistics were performed using SPSS 22.0 for windows (SPSS Inc., Chicago, IL, USA) and MedCalc windows (MedCalc Software bvba 13, Ostend, Belgium).

Results:-

Patients and their clinicopathological parameters:-
Our study included 25 patients; 64% of them were males, with an age range from 22 to 81 years (mean: 57.08±14.78). The histologic grades of tumor were as follows: 15 (60%) grade I, 4 (16%) grade II, 5 (20%) grade III and 1 case (4%) grade IV. T1 was the most frequent tumor size (60%) and 16% of cases showed nodal metastasis. Different stages of tumor were detected, stage I (n =13), stage II (n =3), and stage III (n =9) (Table 1).

Immunohistochemical results:-
EZH2 immunohistochemical results:-
EZH2 was expressed in 88% of our studied cases. There was a statistically significant correlation between its expression and tumor grade ,where most of low grade tumors (GI,II) showed low scores (1and 2),and none of them showed high scores, in contrast to high grade tumors (GII,VI) that showed only high scores (3 and 4)($p< 0.001$).
As regards the relationship between EZH2 and tumor size, there was a gradual increase in its expression with increasing tumor size ,as 6.7% and 25% of T1 and T2 tumors showed score 4 compared to 80% and 100% of T3 and T4 respectively, with a significant statistical difference ($p< 0.001$).EZH2 was also correlated with nodal metastasis ($p< 0.0016$).

The relationship between EZH2 score and tumor stage was statistically significant as none of stage I or II tumors showed score 4 ,but 77.8% of stage III tumors showed this score($p< 0.001$).
However, no significant difference was detected between EZH2 and age or sex of the patient ($p= 0.771$ & 0.417) respectively (Table 2, Figure 1).

VEGF immunohistochemical results:-
VEGF showed low expression in 48% and high expression in 52% of the studied cases. A significant association was observed between VEGF expression and tumor grade, as all grade 1 cases showed low expression ,while all grade III and VI cases showed high expression($p< 0.001$). VEGF expression was also correlated with tumor size ($p=0.001$) and tumor stage,99.3% of stage I showed low expression, while all cases of stage II and III showed high expression($p< 0.001$). However, no significant correlation was observed between VEGF and nodal metastasis ($p=0.09$), age ($p= 0.56$) or sex ($p= 1.00$)of the patients (Table 3,Figure2).

Correlation between EZH2, VEGF and Ki-67 immunohistochemical expressions: Using Kendall's tau-b correleation coefficient, a significant correlation was detected between EZH2 and VEGF expression (τ tau correleation coefficient = +0.941 ,p< 0.001), and a significant positive correlation was detected between ki-67 and both EZH2 (τ tau correleation coefficient =+0.414,p= 0.003), and VEGF (τ tau correleation coefficient =+0.602 ,p<0.001) (Table 4,Figure 1,2,3)

Survival analysis results:-

During 24 months of follow up, fourteen patients relapsed. Disease Free Survival (DFS) was considered from the time of surgery to confirmation of locoregional recurrence (LRR) or distant metastasis (DM), either radiological or clinical, or death by any cause and patients were censored at the last time known to be disease free and alive. Overall Survival (OS) was calculated from the time of surgery to death from any cause and patients were censored at the date last known to be alive.

Cases with high EZH2 expression were significantly associated with disease progression (both loco-regional recurrence and distant metastasis), disease free survival and a poor overall survival (p< 0.001).

The expression of VEGF was significantly associated with the progression of disease (both loco-regional recurrence and distant metastasis), disease free survival and a poor overall survival (p< 0.001, p< 0.001and p= 0.009 respectively)(Table 5&6 ,Figure 4).

Table 1:-Clinicopathological features, immunohistochemical staining and outcome of in 25 patients with CCRCC

	No.	**Percent**		**No.**	**Percent**
Age (years)			**EZH2**		
Mean ± SD	57.08	±14.78	0	3	12%
Median (Range)	58	(22-81)	1	6	24%
< 60 years	14	56%	2	6	24%
≥ 60 years	11	44%	3	3	12%
Sex			**VEGF**		
Male	16	64%	Low	12	48%
Female	9	36%	High	13	52%
Grade			**Ki-67**		
Grade I	15	60%	0	2	8%
Grade II	4	16%	1	5	20%
Grade III	5	20%	2	11	44%
Grade IV	1	4%	3	7	28%
Tumor size			**Relapse**		
T1	15	60%	Absent	11	44%
T2	4	16%	Present	14	56%
T3	5	20%	**LRR**	5	20%
T4	1	4%	**DM**	3	12%
			LRR+DM	6	24%
Lymph node			**Survival**		
N0	21	84%	Alive	14	56%
N1	4	16%	Died	11	44%
Stage			**Follow-up (months)**		
Stage I	13	52%	Mean ± SD	19.48 ±5.47	
Stage II	3	12%	Median (Range)	24 (8-24)	
Stage III	9	36%			

LRR: Locoregional recurrence; DM: Distant metastasis.

Categorical variables were expressed as number (percentage).

Continuous variables were expressed as mean ± SD & median (range).

Table 2:- Correlation between clinicopathological features and immunohistochemical staining for EZH2 in 25 patients with CCRCC.

Characteristics	**All (N=25)**		**EZH2**								**p-value**
			0 (N=3)		1 (N=6)		2 (N=6)		3 (N=3)		4 (N=7)		
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
Age (years)													
Mean ± SD	57.08±14.78		61	±7.21	57.50	±14.34	55	±11.93	64.66	±21.73	53.57	±18.82	0.839•
Median (Range)	58 (22-81)		63	(53-67)	50.50	(46-80)	58.50	(33-68)	73	(40-81)	55	(22-75)	
< 60 years	14	(56%)	1	(7.1%)	4	(28.6%)	4	(28.6%)	1	(7.1%)	4	(28.6%)	0.771‡
≥60 years	11	(44%)	2	(18.2%)	2	(18.2%)	2	(18.2%)	2	(18.2%)	3	(27.3%)	
Sex													
Male	16	(64%)	1	(6.3%)	4	(25%)	4	(25%)	1	(6.3%)	6	(37.5%)	0.417‡
Female	9	(36%)	2	(22.2%)	2	(22.2%)	2	(22.2%)	2	(22.2%)	1	(11.1%)	
Grade													
Grade I	15	(60%)	3	(33.3%)	5	(55.6%)	1	(11.1%)	0	(0%)	0	(0%)	<0.001§
Grade II	4	(16%)	0	(0%)	1	(16.7%)	5	(83.3%)	0	(0%)	0	(0%)	
Grade III	5	(20%)	0	(0%)	0	(0%)	0	(0%)	3	(75%)	1	(25%)	
Grade IV	1	(4%)	0	(0%)	0	(0%)	0	(0%)	0	(0%)	6	(100%)	
Tumor size													
T1	15	(60%)	3	(20%)	6	(40%)	4	(26.7%)	1	(6.7%)	1	(6.7%)	<0.001§
T2	4	(16%)	0	(0%)	0	(0%)	2	(50%)	1	(25%)	1	(25%)	
T3	5	(20%)	0	(0%)	0	(0%)	0	(0%)	1	(20%)	4	(80%)	
T4	1	(4%)	0	(0%)	0	(0%)	0	(0%)	0	(0%)	1	(100%)	
Lymph node													
N0	21	(84%)	3	(14.3%)	6	(28.6%)	6	(28.6%)	3	(14.3%)	3	(14.3%)	0.016‡
N1	4	(16%)	0	(0%)	0	(0%)	0	(0%)	0	(0%)	4	(100%)	
Stage													
Stage I	13	(52%)	3	(23.1%)	6	(46.2%)	4	(30.8%)	0	(0%)	0	(0%)	<0.001§
Stage II	3	(12%)	0	(0%)	0	(0%)	2	(66.7%)	1	(33.3%)	0	(0%)	
Stage III	9	(36%)	0	(0%)	0	(0%)	0	(0%)	2	(22.2%)	7	(77.8%)	
VEGF													
Low	12	(48%)	3	(25%)	6	(50%)	3	(25%)	0	(0%)	0	(0%)	0.001‡
High	13	(52%)	0	(0%)	0	(0%)	3	(23.1%)	3	(23.1%)	7	(53.8%)	
Ki-67													
0	2	(8%)	1	(50%)	1	(50%)	0	(0%)	0	(0%)	0	(0%)	0.014§
1	5	(20%)	1	(20%)	1	(20%)	1	(20%)	1	(20%)	1	(20%)	
2	11	(44%)	1	(9.1%)	4	(36.4%)	3	(27.3%)	1	(9.1%)	2	(18.2%)	
3	7	(28%)	0	(0%)	0	(0%)	2	(28.6%)	1	(14.3%)	4	(57.1%)	

•Kraskall Wallis H test; ‡ Chi-square test; § Chi-square test

Table 3:- Correlation between clinicopathological features and immunohistochemical staining for VEGF in 25 patients with CCRCC.

Characteristics	**All (N=25)**			**VEGF**					**p-value**
				Low (N=12)			**High (N=13)**		
	No.	(%)		No.	(%)		No.	(%)	
Age (years)									
Mean ± SD	57.08	±14.78		59.58	±10.60		54.76	±17.94	0.428*
Median (Range)	58	(22-81)		59.50	(46-80)		55	(22-81)	
< 60 years	14	(56%)		6	(42.9%)		8	(57.1%)	0.561‡
≥ 60 years	11	(44%)		6	(54.5%)		5	(45.5%)	
Sex									
Male	16	(64%)		8	(50%)		8	(50%)	1.000‡
Female	9	(36%)		4	(44.4%)		5	(55.6%)	
Grade									
Grade I	15	(60%)		9	(100%)		0	(0%)	<0.001§
Grade II	4	(16%)		3	(50%)		3	(50%)	
Grade III	5	(20%)		0	(0%)		4	(100%)	
Grade IV	1	(4%)		0	(0%)		6	(100%)	
Tumor size									
T1	15	(60%)		12	(80%)		3	(20%)	0.001§
T2	4	(16%)		0	(0%)		4	(100%)	
T3	5	(20%)		0	(0%)		5	(100%)	
T4	1	(4%)		0	(0%)		1	(100%)	
Lymph node									
N0	21	(84%)		12	(57.1%)		9	(42.9%)	0.096‡
N1	4	(16%)		0	(0%)		4	(100%)	
Stage									
Stage I	13	(52%)		12	(92.3%)		1	(7.7%)	<0.001§
Stage II	3	(12%)		0	(0%)		3	(100%)	
Stage III	9	(36%)		0	(0%)		9	(100%)	
EZH2									
0	3	(12%)		3	(100%)		0	(0%)	<0.001§
1	6	(24%)		6	(100%)		0	(0%)	
2	6	(24%)		3	(50%)		3	((50%)	
3	3	(12%)		0	(0%)		3	(100%)	
4	7	(28%)		0	(0%)		7	(100%)	
Ki-67									
0	2	(8%)		2	(100%)		0	(0%)	0.008§
1	5	(20%)		3	(60%)		2	(40%)	
2	11	(44%)		7	(63.6%)		4	(36.4%)	
3	7	(28%)		0	(0%)		7	(100%)	

*Independent samples Student's test.
‡ Chi-square test.

Table 4:- Correleation between EZH2, VEGF and Ki-67 immunohistochemical staining in 25 patients with CCRCC.

	EZH2			**VEGF**			**Ki-67**	
	τ	p-value		τ	p-value		τ	p-value
EZH2	---	---		+0.941	<0.001		+0.414	0.003
VEGF	+0.941	<0.001		---	---		+0.602	<0.001
Ki-67	+0.414	0.003		+0.602	<0.001		---	---

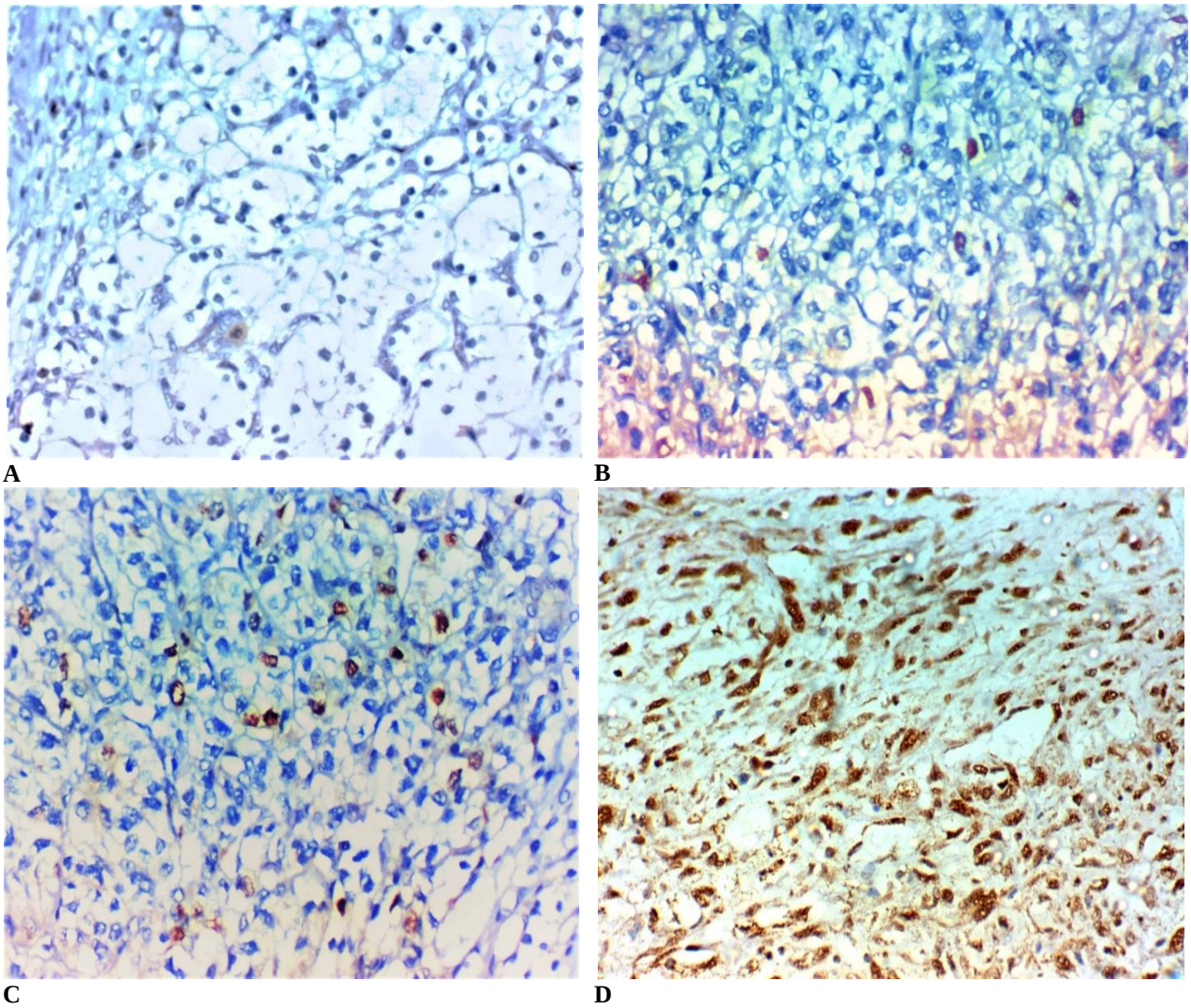
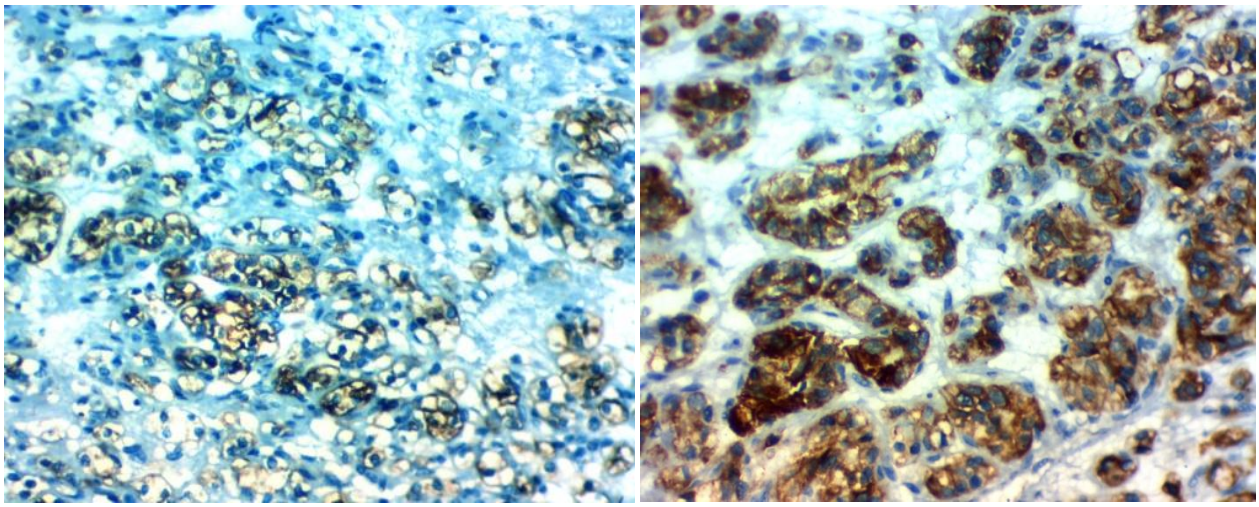
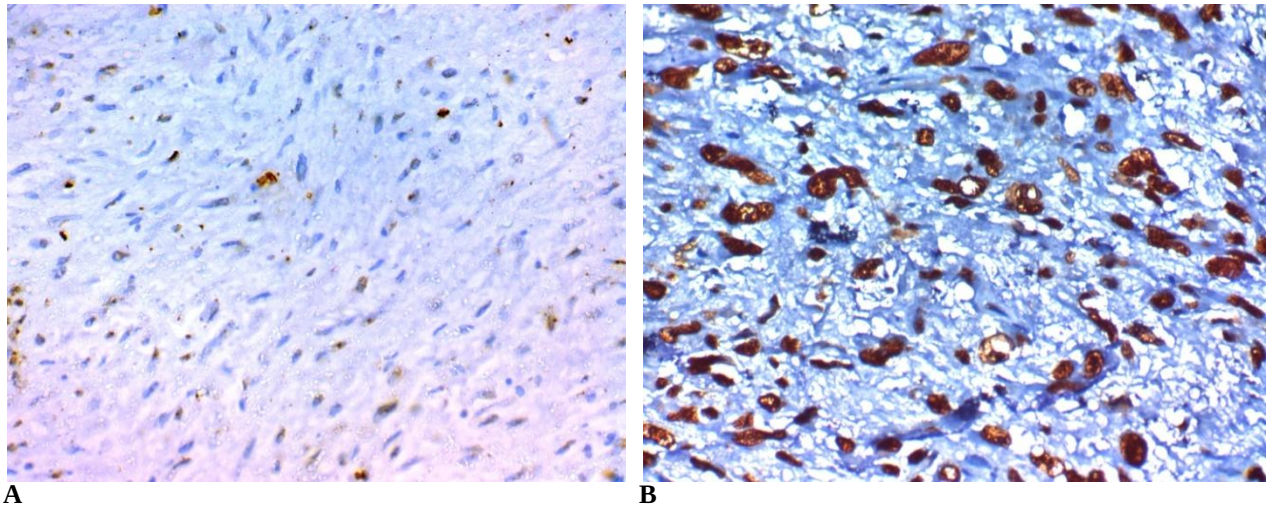


Figure 1:- Enhancer of zeste homolog 2(EZH2) immunohistochemical expression in clear cell renal cell carcinoma (CCRCC):
A;:Grade I CCRCC showing positivity in < 5% (Score1)
B:Grade II CCRCC showing positivity in >5 but < 25% (Score2)
C:Grade III CCRCC showing positivity in >25 to 50% (Score 3)
D:Grade IV CCRCC showing positivity in more than 50% (Score 4)
(EZH2 immunohistochemistry, original magnification x400).



A **B**

Figure 2:- Vascular Endothelial Growth Factor (VEGF) immunohistochemical expression in CCRCC:
A:Grade II CCRCC showing low VEGF expression
B: Grade III CCRCC showing high VEGF expression
(VEGF immunohistochemistry, original magnification x400).



A **B**

Figure3:- Ki-67 immunohistochemical expression in CCRCC:
A:Ggrade II CCRCC showing score 1 Ki-67 nuclear expression
B: Grade III CCRCC showing score3 Ki-67 nuclear expression
(Ki-67 immunohistochemistry, original magnification x400).

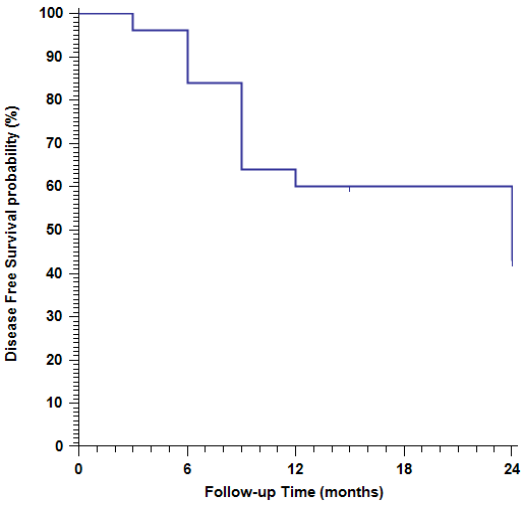
Table 5:- Correlation between clinicopathological features/immunohistochemical staining and events in 25 patients with CCRCC.

Characteristics	All (N=25)		Relapse				p-value	Survival				p-value
			Absent (N=11)		Present (N=14)			Alive (N=14)		Died (N=11)		
	No.	(%)	No.	(%)	No.	(%)		No.	(%)	No.	(%)	
Age (years)												
Mean ± SD	57.08	±14.78	58.81	±10.76	55.71	±17.60	0.613*	53.57	±10.35	61.54	±18.60	0.222*
Median (Range)	58	(22-81)	59	(46-80)	56.50	(22-81)		52.50	(33-71)	68	(22-81)	
< 60 years	14	(56%)	6	(42.9%)	8	(57.1%)	1.000‡	10	(71.4%)	4	(28.6%)	0.116‡
≥ 60 years	11	(44%)	5	(45.5%)	6	(54.5%)		4	(36.4%)	7	(63.6%)	
Sex												
Male	16	(64%)	7	(43.8%)	9	(56.3%)	1.000‡	7	(43.8%)	9	(56.3%)	0.208‡
Female	9	(36%)	4	(44.4%)	5	(55.6%)		7	(77.8%)	2	(22.2%)	
Grade												
Grade I	15	(60%)	9	(100%)	0	(0%)	<0.001§	8	(88.9%)	1	(11.1%)	<0.001§
Grade II	4	(16%)	2	(33.3%)	4	(66.7%)		5	(83.3%)	1	(16.7%)	
Grade III	5	(20%)	0	(0%)	4	(100%)		1	(25%)	3	(75%)	
Grade IV	1	(4%)	0	(0%)	6	(100%)		0	(0%)	6	(100%)	
Tumor size												
T1	15	(60%)	11	(73.3%)	4	(26.7%)	0.001§	11	(73.3%)	4	(26.7%)	0.019§
T2	4	(16%)	0	(0%)	4	(100%)		2	(50%)	2	(50%)	
T3	5	(20%)	0	(0%)	5	(100%)		1	(20%)	4	(80%)	
T4	1	(4%)	0	(0%)	1	(100%)		0	(0%)	1	(100%)	
Lymph node												
N0	21	(84%)	11	(52.4%)	10	(47.6%)	0.105‡	14	(66.7%)	7	(33.3%)	0.026‡
N1	4	(16%)	0	(0%)	4	(100%)		0	(0%)	4	(100%)	
Stage												
Stage I	13	(52%)	11	(84.6%)	2	(15.4%)	<0.001§	11	(84.6%)	2	(15.4%)	0.001§
Stage II	3	(12%)	0	(0%)	3	(100%)		2	(66.7%)	1	(33.3%)	
Stage III	9	(36%)	0	(0%)	9	(100%)		1	(11.1%)	8	(88.9%)	
EZH2												
0	3	(12%)	3	(100%)	0	(0%)	<0.001§	3	(100%)	0	(0%)	<0.001§
1	6	(24%)	6	(100%)	0	(0%)		5	(83.3%)	1	(16.7%)	
2	2	(8%)	2	(33.3%)	4	(66.7%)		5	(83.3%)	1	(16.7%)	
3	3	(12%)	0	(0%)	3	(100%)		1	(33.3%)	2	(66.7%)	
4	7	(28%)	0	(0%)	7	(100%)		0	(0%)	7	(100%)	
VEGF												
Low	12	(48%)	11	(91.7%)	1	(8.3%)	<0.001‡	10	(83.3%)	2	(16.7%)	0.008‡
High	13	(52%)	0	(0%)	13	(100%)		4	(30.8%)	9	(69.2%)	
Ki-67												
0	2	(8%)	2	(100%)	0	(0%)	0.007§	2	(100%)	0	(0%)	0.405§
1	5	(20%)	3	(60%)	2	(40%)		2	(40%)	3	(60%)	
2	11	(44%)	6	(54.5%)	5	(45.5%)		7	(63.6%)	4	(36.4%)	
3	7	(28%)	0	(0%)	7	(100%)		3	(42.9%)	4	(57.1%)	
Relapse												
Absent	11	(44%)						10	(90.9%)	1	(9.1%)	0.004‡
Present	14	(56%)						4	(28.6%)	10	(71.4%)	

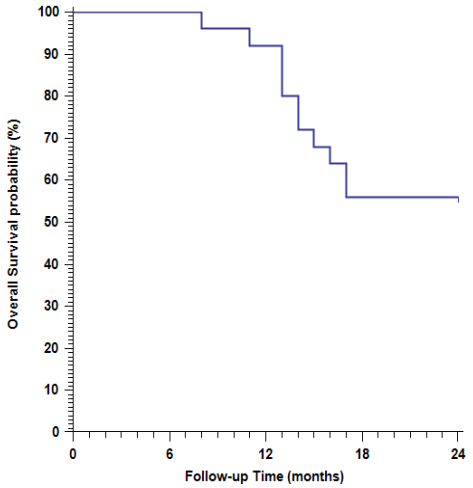
Table 6:- Correlation between clinicopathological features/immunohistochemical staining survival in 25 patients with CCRCC.

Characteristics	All (N=25)			Disease Free Survival (DFS)				p-value†	Overall Survival (OS)				p-value†
	No.	(%)		Mean DFS (months)	6 month DFS (%)	12 month DFS (%)	24 month DFS (%)		Mean OS (months)	6 month OS (%)	12 month OS (%)	24 month OS (%)	
All patients	25	(100%)		17.5 month	84%	60%	42.9%	-----	19.5 month	100%	92%	56%	-----
Age (years)													
< 60 years	14	(56%)		19.1 month	85.7%	71.4%	42.9%	0.752	20.9 month	100%	92.9%	71.4%	0.095
≥60 years	11	(44%)		15.6 month	81.8%	45.5%	45.5%		17.7 month	100%	90.9%	36.4%	
Sex													
Male	16	(64%)		15.8 month	75%	50%	42.9%	0.529	18.3 month	100%	87.5%	43.8%	0.132
Female	9	(36%)		20.7 month	77.8%	77.8%	44.4%		21.7 month	100%	100%	77.8%	
Grade													
Grade I	15	(60%)		24 month	100%	100%	100%	<0.001	23 month	100%	100%	88.9%	<0.001
Grade II	4	(16%)		22 month	100%	83.3%	33.3%		22.8 month	100%	100%	83.3%	
Grade III	5	(20%)		11.3 month	50%	25%	0%		15.3 month	100%	75%	25%	
Grade IV	1	(4%)		7.5 month	66.7%	-----	-----		13.7 month	100%	83.3%	----	
Tumor size													
T1	15	(60%)		21.2 month	100%	80%	72.7%	0.001	21.5 month	100%	93.3%	73.3%	0.115
T2	4	(16%)		14.3 month	50%	50%	0%		16.8 month	100%	50%	50%	
T3	5	(20%)		10.8 month	60%	60%	0%		16 month	100%	100%	20%	
T4	1	(4%)		9 month	100%	-----	-----		17 month	100%	100%	----	
Lymph node													
N0	21	(84%)		19.6 month	90.5%	71.4%	51%	<0.001	20.7 month	100%	95.2%	66.7%	0.002
N1	4	(16%)		6.8 month	50%	-----	-----		13 month	100%	75%	----	
Stage													
Stage I	13	(52%)		23.1 month	100%	92.3%	83.9%	<0.001	22.8 month	100%	100%	84.6%	<0.001
Stage II	3	(12%)		18 month	66.7%	66.7%	0%		19.7 month	100%	66.7%	66.7%	
Stage III	9	(36%)		9.3 month	66.7%	11.1%	0%		14.7 month	100%	88.9%	11.1%	
EZH2													
0	3	(12%)		24 month	100%	100%	100%	<0.001	24 month	100%	100%	100%	<0.001
1	6	(24%)		24 month	100%	100%	100%		22.5 month	100%	100%	83.3%	
2	2	(8%)		22 month	100%	83.3%	33.3%		22.8 month	100%	100%	83.3%	
3	3	(12%)		13 month	66.6%	33.3%	0%		16 month	100%	66.7%	33.3%	
4	7	(28%)		7.3 month	57.1%	-----	-----		13.6 month	100%	85.7%	-----	
VEGF													
Low	12	(48%)		23 month	100%	91.7%	91.7%	<0.001	22.7 month	100%	100%	83.3%	0.009
High	13	(52%)		12.5 month	69.2%	30.8%	0%		16.5 month	100%	84.6%	30.8%	
Ki-67													
0	2	(8%)		24 month	100%	100%	100%	0.087	24 month	100%	100%	100%	0.438
1	5	(20%)		16.8 month	60%	60%	60%		17.4 month	100%	80%	40%	
2	11	(44%)		18.6 month	90.9%	63.6%	54.6%		20.5 month	100%	100%	63.6%	
3	7	(28%)		14.6 month	85.7%	42.9%	0%		18.1 month	100%	85.7%	42.9%	

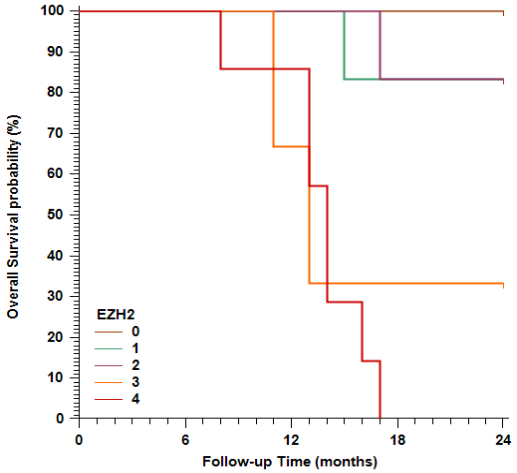
† Log rank test


Disease Free Survival probability (%)
Follow-up Time (months)

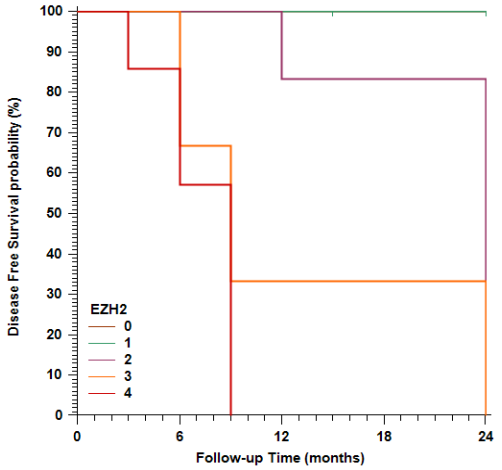
A


Overall Survival probability (%)
Follow-up Time (months)

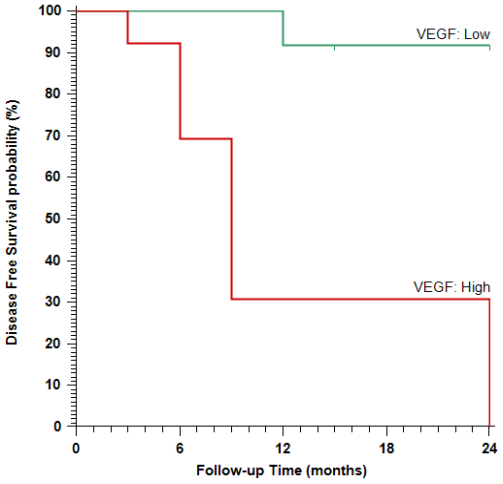
E


Overall Survival probability (%)
EZH2
0
1
2
3
4
Follow-up Time (months)

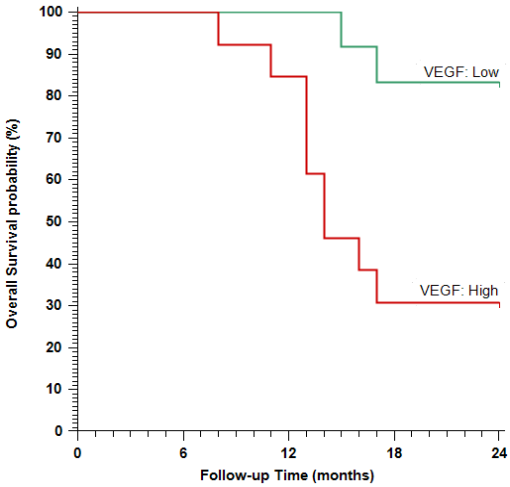
B


Disease Free Survival probability (%)
EZH2
0
1
2
3
4
Follow-up Time (months)

F


Disease Free Survival probability (%)
VEGF: Low
VEGF: High
Follow-up Time (months)

C


Overall Survival probability (%)
VEGF: Low
VEGF: High
Follow-up Time (months)

G

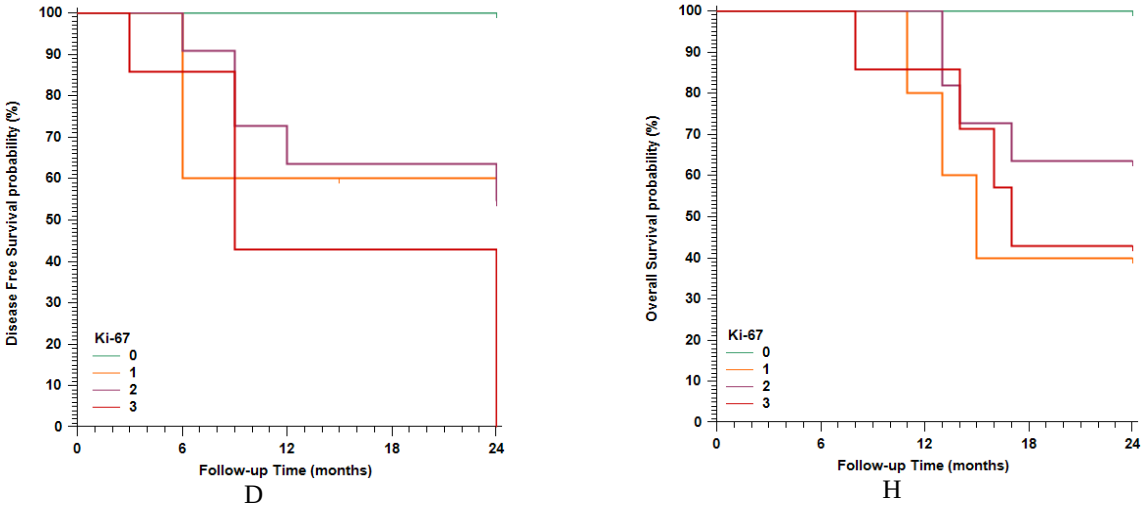


Figure (4):- Kaplan Meier plots, Left panel: Disease Free Survival, Right panel: Overall Survival: (A) & (E) All studied RCC patients (N=25); (B) & (F) Stratified by EZH2; (C) & (G) Stratified by VEGF;(D) & (H) Stratified by Ki-67.

Discussion:-

Renal cell carcinoma (RCC) is the third commonest malignancy in the urinary system representing 5% of all cancer diagnoses. Clear cell renal cell cancers (CCRCC) represent about 70% of all renal cancers, and several clinical and histopathologic factors are implicated in the prognosis of renal cancers (**Jemal et al.,2011)**.

This tumor has a poor response to existing treatment options with relapse in 20-30% of cases after complete primary tumor resection. New targeted treatments, as tyrosine kinase inhibitors, showed limited benefit **(Wagener et al., 2010).** Prognosis and response to treatment are still not sufficiently predictable **(Eichelberg et al., 2009).** That is why searching for new predictive and prognostic factor for RCC and targets for therapy is a high priority.

Enhancer of zeste homolog 2 (EZH2) protein is part of is polycomb repressive complex (PRC2).It acts as a histone methyltransferase by the addition of 3 methyl groups to lysine 27 of histone 3 (H3K27).The trimethylation of H3K27 leads to chromatin condensation and mediates epigenetic silencing of a large number of genes involved in tumor proliferation, invasion, and angiogenesis **(Au et al.,2011).**

In this study, significant direct correlation between EZH2 expression and Fuhrman grade ($p< 0.001$) that acts as independent prognostic factor for CCRCC was detected. Low EZH2 expression was noticed in lower grades renal cancers, while high expression was noticed in higher grades renal cancers. These results were in line with that of **Wagener et al. (2010)** and **Ebru et al. (2016)** who found significant correlation between EZH2 expression and grade ($p < 0.0001$and $p < 0.00001$) respectively.

A significant correlation between EZH2 expression and lymph node metastasis was detected ($p= 0.016$), this was in agreement with the finding of **Xu et al. (2015).**One possible explanation for this is suppression of cadherin by EZH2 (**Lee and Choe, 2012).**

Our results revealed direct significant correlation between EZH2 expression and TNM stage ($p< 0.001$), suggesting its role in the development of renal cancer. This may be explained by finding of **Tian et al. (2016)** who found that EZH2 modulates epithelial mesenchymal transition (EMT) signaling and promotes cancer cell migration and invasion in CCRCC cells. These results were in line with that of **Ebru et al. (2016)** who found significant correlation between EZH2 expression and tumor stage ($p < 0.0001$).

Our findings suggest that high EZH2 expression in CCRCC can predict aggressiveness of the tumor. This is in agreement with the putative role of EZH2 in other tumors as breast cancer **(Takawaet al., 2011;Testoni et al., 2011).** A cell line study of CCRCC by **Wagener et al.(2008),** showed that EZH2 contributes to proliferation and apoptotic resistance that supports our results.
Different mechanisms of EZH2 overexpression was detected in many malignant tumors. The RB gene pathway regulates EZH2 expression by transcriptional activation leading to tumor proliferation **(Bracken et al., 2003).**The loss of miRNAs such as miR-26a, miR-101 and miR-214 lead also to EZH2 accumulation (**Danget al.,2012).**Treatment with EZH2 inhibitor drugs as 3-deazaneplanocin A33, or blocking the effect of EZH2 may provide a major advance in the treatment of CCRCC **(Crea et al., 2012).**

Ki-67 has an independent prognostic importance in renal cell carcinoma, and also correlates with Fuhrman nuclear grade but is more objective and reproducible and can be used in conjunction with it to determine prognosis in renal cell carcinoma **(Mehdi et al., 2016).**
Significant correlation between Ki-67 and EZH2 expression (p= 0.003) was found. As far as we know, no previous studies tested the relation between EZH2 and Ki67 expression in CCRCC.
According to current work, EZH2 is a negative prognostic marker in patients of CCRCC; that is why the assessment of its expression may improve selection of patient for systemic therapies. EZH2 status integration into current prognostic models may improve survival prediction.

Vascular endothelial growth factor (VEGF) is considered as the most potent endothelial cell-specific angiogenesis factor. It increases vascular permeability leading to endothelial cell proliferation with subsequent tube formation. In addition, CCRCC, a clinically angiogenic activity, has a direct relation with the expression of VEGF. This led to VEGF inhibition-based treatment methods used today against CCRCC (**Ebru et al., 2016).**

According to our study, VEGF expression was significantly associated with Fuhrman grade (p< 0.001), **Ebru et al. (2016)** and **Xu et al. (2015)** reported same results, it was also associated with TNM stage (p< 0.001), this was in line with results of **Xu et al. (2015)** (p=0.001).Direct correlation between VEGF and both grade and stage prove the relationship between its expression and progression and development of CCRCC.

No significant association was detected between VEGF expression and lymph node metastasis (p= 0.096).This may be explained by results of **Baldewijns et al. (2009)** that concluded that there is only limited lymphangiogenesis and predominance of haemangiogenesis s in CCRCC and this explain why CRCC prefer haematogenous dissemination to lymphatic spread. VEGF-targeted therapies have therefore been implicated in the management of advanced CCRCC where they demonstrated an improvement in survival of these patients (**Lampinem et al., 2016).**

Direct correlation (p= 0.001) between EZH2 and VEGF was found. These results were in line with **Xu et al., (2015)**, who supported the role of EZH2 on angiogenesis in CCRCC and **Tian et al.(2016)** who found that EZH2 promotes tumor development by increasing VEGF expression in CCRCC.

EZH2 dowenregulation inhibits vascularization in different tumors. These results suggest a possible therapeutic potential for EZH2 inhibition in tumors with aberrant vascularization **(Kim and Roberts, 2016).**

A statistically significant relation was noticed between VEGF and Ki-67(p< 0.001),this was in agreement with results of **Burgesseret al. (2014)** who found that VEGF expression is directly related to the proliferation index.

According to our study high expression of EZH2 correlated with advanced stages of CCRCC and poor survival outcomes which were consistent with the results of the studies carried out by **Liu et al, (2016)** and **Wang et al.(2015)** who demonstrated that EZH2 represents independent prognostic factor in multivariate analysis for DFS (p = 0.004) and OS (p = 0.017).The results of this study was in concordance with meta-analysis carried out by **Tian et al.(2016)** who suggested that EZH2 overexpression is associated with a higher risk of RCC and it is also associated with worsened survival of CCRCC patients.

In this work, VEGF expression in CCRCC was significantly related to more progressive disease in correlation with lower survival rates and this in line with results of **Osman and Youssef (2015).**

Conclusion:-

Compared with early staged tumors, advanced CCRCC had significant detectable EZH2 and VEGF expressions. The expression level of EZH2 correlated positively with that of VEGF in CCRCC. The present study suggests that EZH2 overexpression has a role in the progression, development and angiogenesis in renal cell carcinoma and consequently can be used as an indicator for predicting the prognosis of CCRCC patients. These results suggest that EZH2 targeting might be an attractive therapeutic approach in the treatment of renal cancer.

Conflict of interest: There are no conflicts of interest.

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