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Clinicopathological Significances of EZH2 & Twist-1 Combined Expressions in Renal Cell Carcinoma (RCC)

ABLA S. MAHMOUD, M.D. and DINA A. KHAIRY, M.D.

The Department of Pathology, Faculty of Medicine, Beni-Suef University, Beni-Suef, Egypt

Abstract

Background: Renal cell carcinoma (RCC) is considered the commonest and most fatal malignant neoplasm of the kidney. Distant metastases are found in 30% of RCC patients at diagnosis, which is considered an important sign of prediction of patients' prognosis. So trying to clarify molecular mechanisms of metastases in RCC could help to discover a recent therapeutic target to decrease and control distant metastases and subsequently might improve patients prognosis.

Aim of the Study: Was to assess the EZH2 & Twist-1 combined expressions in RCC tissues with different histopathological subtypes and to correlate their expression with clinicopathological parameters.

Material and Methods: We have assessed expression of EZH2 & Twist-1 in tissue by using immunohistochemistry in 50 paraffin blocks of RCC different histopathological subtypes, analyzed correlations between the levels of combined expressions and clinicopthological data.

Results: Increased EZH2 expression was positively associated with advanced AJCC stage, Lymph Node (L.N) metastases (p=0.006), higher grade (p<0.001), and presence of distant metastases (p=0.045). Increased Twist-1 expression was positively associated with higher grade, L.N (p<0.001) & distant metastases (p=0.04) and AJCC stage (p=0.01). Both EZH& Twist-1 expressions were positively correlated to each other (p<0.001).

Conclusion: EZH2 & Twist-1 are considered markers of poor prognosis in RCC and so they could be used as novel therapeutic targets in RCC patients.

Key Words: *Renal cell carcinoma (RCC)– EZH2 – Twist-1 – Immunohistochemistry – Distant metastases.*

Introduction

RENAL cell carcinoma (RCC) is considered the commonest and most fatal malignant neoplasm of the kidney; globally [1]. Although many RCC patients are diagnosed at early stages, but 30% of patients will have subsequently metastatic and incurable disease [2]. Discovering recent biomarkers is needed to decrease and control distant metastases and subsequently might improve patients prognosis. Usually cancer size, grade and stage could predict patients' outcome [3], but it was stated that patients with RCC having the same pathological stage might have variable outcomes that need further clarification. Enhancer, of, zeste, homolog, 2 (EZH-2) is a histone methyl transferase that can silence plethora of variable tumor suppressor genes and was found to be mapped on chromosome 7q-35, is a member of the poly, comb, group genes family that function to allow the transmission of genes expression patterns to the daughter cells with a stability. EZH2 have vital roles in RCC-carcinogenesis [4]. Although it's exact role of action is still unproved.

The process in which cells could change their phenotype from the epithelial to the mesenchymal states is called the epithelial-mesenchymal transition (EMT). EMT leads to many changes in the differentiated characteristics of the cells e.g. Adhesion and apical basal polarity between cells, additionally, leads to change in the absence of mobility and acquire instead mesenchymal characters e.g. invasiveness motility, and resistance to apoptosis and by such process malignant cells could acquire invasive and metastatic capability [5]. EMT is regulated by plethora of transcription factors like Twist-1 that is a basic helix-loop-helix (bHLH) transcription factor which is characterized by a DNA binding site which could that target the consensus E-box sequence 59-CANNTG-39 and another helix-loop-helix site [6].

The aim was to assess the EZH2 & Twist-1 combined expressions in RCC tissues with different

Correspondence to: Dr. Abla S. Mahmoud,
The Department of Pathology, Faculty of Medicine,
Beni-Suef University, Beni-Suef, Egypt

histopathological subtypes and to correlate their expression with clinicopathological parameters.

Material and Methods

This is a retrospective cohort study where we have included 50 archival formalin-fixed, paraffin blocks of RCC with different histopathological subtypes that were processed and diagnosed in Pathology Department, Faculty of Medicine, Beni-Suef University and Cairo University Hospital, in the period from 2014 up to 2016. We recorded the detailed clinic-pathologic data for all cases. We used the T-N-M classification for pathologic-RCC-staging and The WHO 2016 classification of malignancies of the Urinary System for grading [7,8]. Our study followed Local-Ethics-Committee-guidelines.

Immunohistochemical staining:

Streptavidine-biotin technique was used for assessment of the immunohistochemical staining [9], we have incubated sections from all paraffin blocks with primary monoclonal mouse anti-EZH2 antibody (MA, Danvers, USA Cell Signaling Technology) (dilution at 1:100) and primary polyclonal rabbit anti-Twist antibody (Abcam, Cambridge, UK ab50581) (dilution at 1:50). We have used carcinomas of the breast and thyroid sections as positive controls for EZH2 and Twist-1 respectively and we have omitted the primary antibodies for negative controls then we had replaced them with phosphate-buffered saline.

Assessment of EZH-2- & Twist-1 immunohistochemical-expressions:

We have evaluated nuclear stain intensity for both markers and graded it as 0; negative, 1; weak, 2; moderate& 3 as strong intensity, while we have assessed the nuclear stain extent for both markers as 1 calculated from 0 to 25%, 2 calculated from 26-50%, 3 calculated from 51-75%, and 4 calculated from 76-100%. Finally we have multiplied both extent and intensity of each marker expression to reach final staining scores from 0-12 [10], we have used 4 as a final cut-off score and we considered scores fewer than 4 as decreased expressions and a scores equal to or greater than 4 as increased expression.

Statistical-analysis:

We have used the SPSS 22.0 program for windows (SPSS Inc., USA Chicago) and MedCalc (MedCalc, Software 13, Belgium) in statistical assessments. We evaluated continuous variables by the mean ± SD & median (range), & categorical variables were evaluated by number (percentage), we used Mann-Whitney-U test to compare between non-normally distributed variables, also we have used Kraskall-Wallis-H test for comparison between more than 2 non-normally distributed groups of variables, finally we have used Fisher's-exact test or Pearson's Chi-square-test for comparison between categorical variables. p-value <0.05 was considered significant.

Results

Demographic data:

The demographic data are summarized in Table (1).

We have included 50 RCC patients that are diagnosed as 40 (80%) cases with clear cell renal cell carcinoma (cc-RCC), 6 (12%) papillary renal cell carcinoma (p-RCC) and 4 (8%) chromophope renal cell carcinoma (cp-RCC) with their ages ranged from (40-77) years. 40 (80%) patients were males and 10 (20%) were females.

Immunohistochemical results:

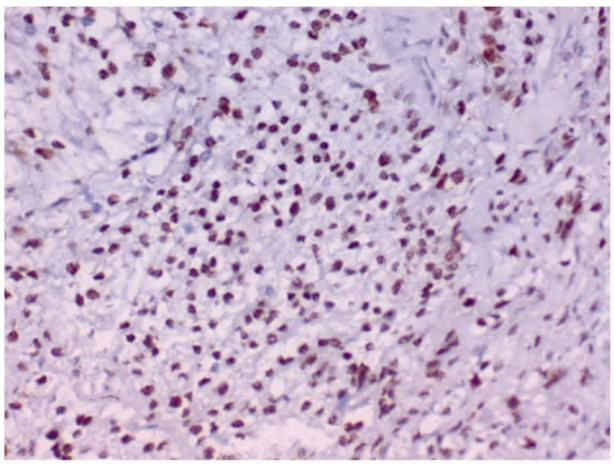
EZH2 expression:

EZH2 increased expression was detected in 28 out of 50 (56%) cases of RCC and was significantly positively correlated with higher grade of the tumor (p<0.001), higher T stage of the tumor (p=0.009), presence of distant metastases (p=0.045), presence of L.N metastases and advanced AJCC stage (p=0.006).

There were no significant correlations were found with age or sex of the patient, histopathological subtypes or size of the tumor (Figs. 1,2 & Tables 2,4).

Twist-1 expression:

- Twist-1 increased expression was detected in 30 out of 50 (60%) cases of RCC and was and was significantly positively correlated with higher grade of the tumor, higher T stage of the tumor (p<0.001), presence of distant metastases (p=0 .04), presence of L.N metastases and advanced AJCC stage (p=0.008),
- There were no significant correlations were found with age or sex of the patient, histopathological subtypes or size of the tumor (Fig. 3 & Tables 3,4).
- We found a significant positive association between both markers expression (p<0.001).
- Combination of EZH-2 & Twist overexpression was significantly positively correlated with grade, stage of the tumor, presence of L.Ns and D.M (p<0.001 for all) (Table 4).



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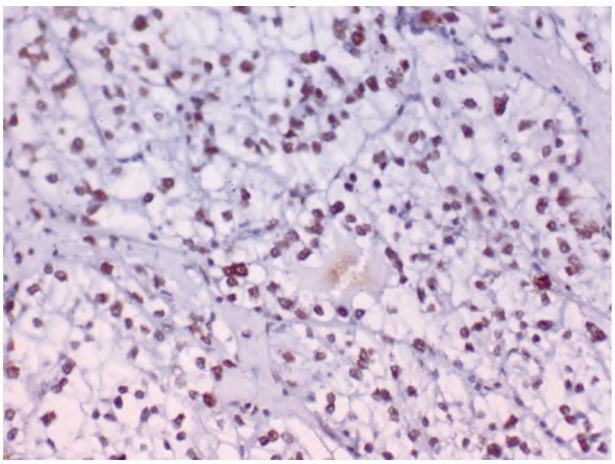

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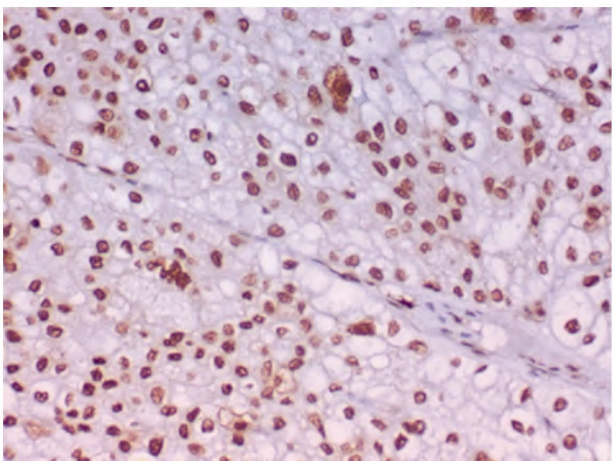
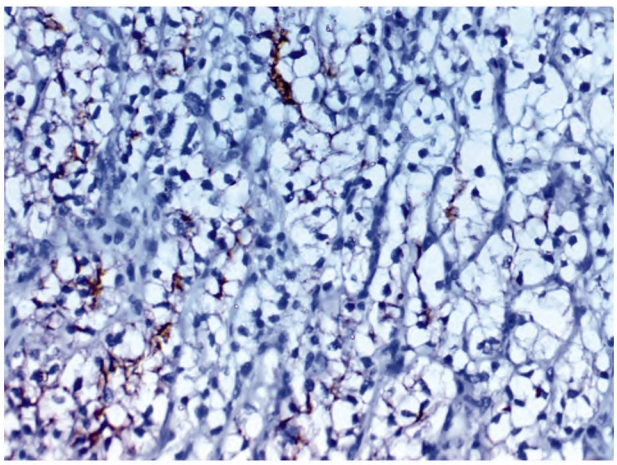


(C)

Fig. (1): Immunohistochemical expression of EZH2 in renal cell carcinoma (RCC): (A) High expression in nucleus of clear cell RCC grade III stage IVx400 (B) High expression in nucleus of clear cell RCC grade II stage IIIx400 (C) High expression in nucleus of chromophope RCC grade II stage III x400. Note: High EZH2 immunohistochemical expression in high grade & stage RCC.



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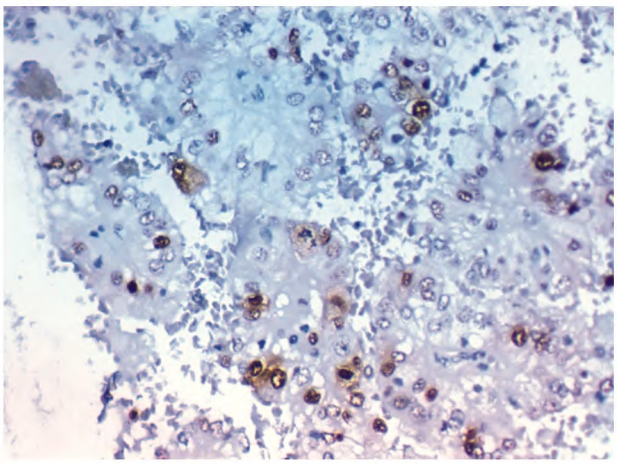

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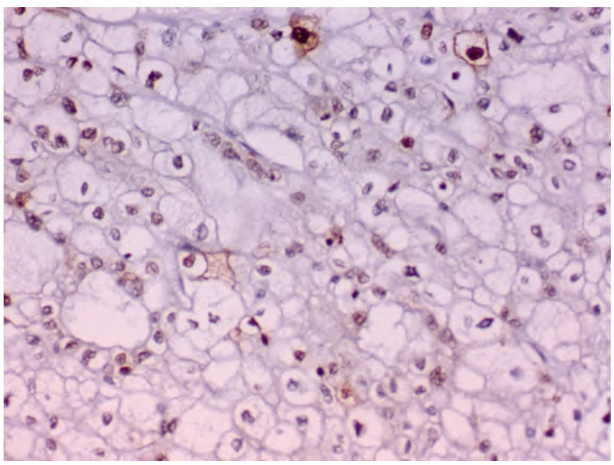
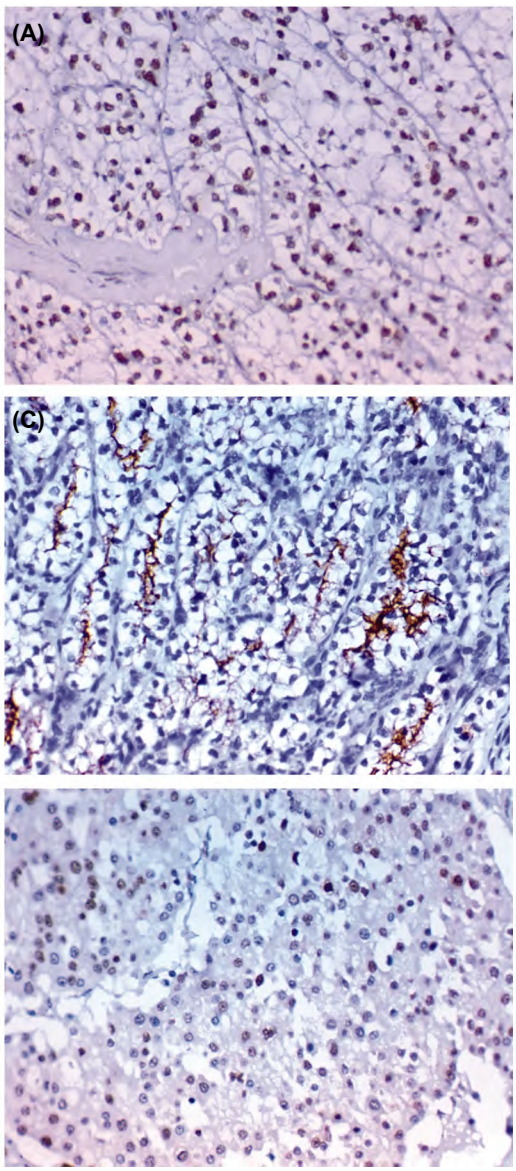


(C)

Fig. (2): Immunohistochemical expression of EZH2 in renal cell carcinoma (RCC): (A) Low expression in nucleus of clear cell RCC grade I stage Ix400 (B) Low expression in nucleus of papillary RCC grade I stage IIx400. (C) Low expression in nucleus of chromophope RCC grade I stage Ix400. Note: Low EZH2 immunohistochemical expression in Low grade & stage RCC.



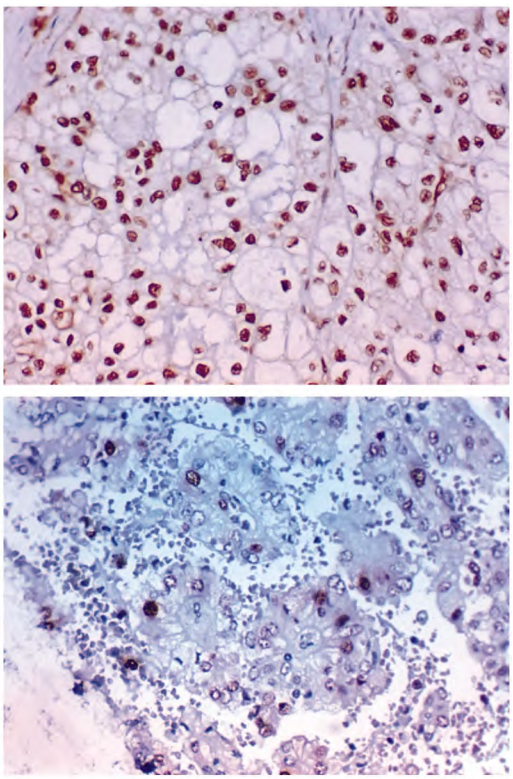


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Table (1): Clinicopathological data, EZH2 & Twist-1 expressions in our cases.

Characteristics		No.	%
Age years:	Mean ± SD	59.65± 10.12	
	Median (Range)	64 (40–77)	
	≤55 years	20	40
	>55 years	30	60
Sex:	Male	40	80
	Female	10	20
Pathological type:	Clear cell	40	80
	Papillary	6	12
	Chromphobe	4	8
Grade:	Grade I	8	16
	Grade II	18	36
	Grade III	12	24
	Grade IV	12	24
Size:	<7cm	10	20
	>7cm	40	80
T:	T1	10	20
	T2	20	40
	T3	10	20
	T4	10	20

Table (1): Cont.

Characteristics		No.	%
T:N:	N0	30	60
	N1	20	40
M:	M0	40	80
	M1	10	20
AJCC Stage group:	Stage I	10	20
	Stage II	20	40
	Stage III	10	20
	Stage IV	10	20
EZH2:	Low	22	50
	High	28	50
Twist-1:	Low	20	40
	High	30	60
EZH2/Twist-1	Low/Low	20	40
	Low/High	5	10
	High/Low	5	10
	High/High	20	40

Table (2): Correlations between clinicopathological features and EZH2 expressions in our patients.

Characteristics	EZH2						*p*-value
	All (N=50)		Low (N=22)		High (N=28)		
	No.	%	No.	%	No.	%	
Age (years):							
Mean ± SD	59.65± 10.12		557.57± 10.01		61.73±9.97		0.037•
Median (Range)	64 (40–77)		62.50 (40–77)		65.50 (40-75)		
55 years	20	40	12	(58.3)	8	(41.7)	0.292‡
> 55 years	30	60	10	(44.4)	20	(55.6)	
Sex:							
Male	40	80	20	(50)	20	(50)	1.000‡
Female	10	20	2	(50)	8	(50)	
Pathological type:							
Clear cell	40	80	18	(46.6)	22	(53.4)	0.368‡
Papillary	6	12	2	(55.6)	4	(44.4)	
Chromphobe	4	8	2	(20)	2	(80)	
Grade:							
Grade I	8	16	7	(93.3)	1	(6.7)	<0.001§
Grade II	18	36	9	(50)	9	(50)	
Grade III	12	24	3	(23.5)	9	(76.5)	
Grade вг	12	24	3	(16.7)	9	(83.3)	
Size:							
<7cm	10	20	4	(58.8)	6	(41.2)	0.390‡
>7cm	40	80	18	(46.5)	22	(53.5)	
T:							
T1	10	20	7	(66.7)	3	(33.3)	0.009§
T2	20	40	12	(66.7)	8	(33.3)	
T3	10	20	3	(35.3)	7	(64.7)	
T4	10	20	0	(0)	10	(100)	
N:							
N0	30	60	20	(67.6)	10	(32.4)	0.006‡
N1	20	40	8	(26.9)	12	(73.1)	
M:							
M0	40	80	19	(55.1)	21	(44.9)	0.045‡
M1	10	20	3	(27.3)	7	(72.7)	
AJCC Stage group:							
Stage I	10	20	7	(66.7)	3	(33.3)	0.006§
Stage II	20	40	12	(68.4)	8	(31.6)	
Stage III	10	20	2	(33.3)	8	(66.7)	
Stage IV	10	20	1	(21.4)	9	(78.6)	
EZH2:							
Negative	22	40					
Positive	28	60					
Twist-1:							
Low	20	80	18	(89.7)	2	(10.3)	<0.001‡
High	30	20	4	(12.9)	26	(87.1)	

* Mann Whitney U test. ‡ Chi-square test. § Chi-square test for trend.

Table (3): Correlations between clinicopathological features and Twist-1 expressions in our patients.

Characteristics	All (N=50)		Twist-1 Low (N=20)		High (N=30)		*p*-value
	No.	%	No.	%	No.	%	
Age (years):							
Mean ± SD	59.65±10.12		57.76±10.18		61.42±9.91		0.083*
Median (Range)	64 (40–77)		63 (40–77)		65 (40–75)		
≤ 55 years	20	40	10	(50)	10	(50)	0.460‡
> 55 years	30	60	10	(44.4)	20	(55.6)	
Sex:							
Male	40	80	16	(45)	24	(55)	0.605‡
Female	10	20	4	(41.7)	6	(58.3)	
Pathological type:							
Clear cell	40	80	16	(40)	24	(60)	0.397‡
Papillary	6	12	3	(50)	3	(50)	
Chromphobe	4	8	1	(20)	3	(80)	
Grade:							
Grade I	8	16	6	(80)	2	(20)	<0.001§
Grade II	18	36	10	(55)	8	(45)	
Grade III	12	24	4	(35.3)	8	(64.7)	
Grade IV	12	24	0	(0%)	12	(100)	
Size:							
<7cm	10	20	5	(50)	5	(50%)	0.307‡
>7cm	40	80	15	(44.2)	25	(55.8)	
T:							
T1	10	20	6	(66.7)	4	(33.3)	<0.001§
T2	20	40	14	(71.4)	6	(28.6)	
T3	10	20	1	(23.5)	9	(76.5)	
T4	10	20	0	(0)	10	(100)	
N:							
N0	30	60	18	(70.6)	12	(29.4)	0.008‡
N1	20	40	2	(19.2)	18	(80.8)	
M:							
M0	40	80	19	(57.1)	21	(42.9)	0.04‡
M1	10	20	1	(9.1)	9	(90.9)	
AJCC Stage group:							
Stage I	10	20	6	(66.7)	4	(33.3)	0.008§
Stage II	20	40	10	(50%)	10	(50%)	
Stage III	10	20	4	(33.3)	6	(66.7)	
Stage IV	10	20	0	(0%)	10	(100)	
EZH2:							
Negative	22	40	17	(86.7)	5	(13.3)	<0.001‡
Positive	28	60	3	(10)	25	(90)	
Twist-1:							
Low	20	80					
High	30	20					

* Mann Whitney U test. ‡ Chi-square test. § Chi-square test for trend.

Table (4): Correlations between clinicopathological features, expressions of both markers together in our patients.

Characteristics	All (N=50)		Low/Low (N=20)		Low/High (N=5)		High/Low (N=5)		High/High (N=20)		*p*-value
	No.	(%)	No.	(%)	No.	(%)	No.	(%)	No.	(%)	
Age (years):											
Mean ± SD	59.65±10.12		57±10.38		57±9.53		62.80±7.32		61.81±10.18		0.155*
Median (Range):	64 (40-77)		62 (40-77)		58 (47-66)		65 (50-68)		66 (40-75)		
≤55 years	20	(40)	8	(50)	2	(8.3)	1	(4.2)	9	(37.5)	0.409‡
>55 years	30	(60)	12	(36.1)	1	(2.8)	4	(11.1)	13	(50)	
Sex:											
Male	40	(80)	17	(45.8)	1	(2.1)	1	(6.3)	21	(45.8)	0.095‡
Female	10	(20)	3	(25)	1	(16.7)	1	(16.7)	5	(41.7)	
Pathological type:											
Clear cell	40	(80)	17	(41.3)	3	(6.5)	5	(10.9)	15	(41.3)	0.554‡
Papillary	6	(12)	2	(55.6)	0	(0)	0	(0)	4	(44.4)	
Chromphobe	4	(8)	1	(20)	0	(0)	0	(0)	3	(80)	
Grade:											
Grade I	8	(25)	5	(80)	1	(6.7)	1	(6.7)	1	(6.7)	<0.001§
Grade II	18	(36.7)	8	(45.5)	1	(4.5)	1	(4.5)	8	(45.5)	
Grade III	12	(28.3)	2	(17.6)	0	(0)	2	(17.6)	8	(64.7)	
Grade IV	12	(10)	0	(0)	1	(16.7)	0	(0)	12	(83.3)	
Size:											
<7 cm	10	(20)	5	(52.9)	0	(0)	1	(11.8)	4	(35.3)	0.433‡
>7 cm	40	(80)	17	(45.8)	1	(2.1)	1	(6.3)	21	(45.8)	
T:											
T1	10	(20)	6	(60)	0	(0)	1	(13.3)	3	(26.7)	0.001§
T2	20	(40)	10	(61.9)	2	(0)	2	(9.5)	6	(28.6)	
T3	10	(20)	2	(17.6)	2	(17.6)	1	(5.9)	5	(58.8)	
T4	10	(20)	0	(0)	0	(0)	0	(0)	10	(100)	
N:											
N0	30	(60)	16	(61.8)	0	(0)	4	(11.8)	10	(26.5)	<0.001‡
N1	20	(40)	4	(15.4)	4	(11.5)	2	(3.8)	10	(69.2)	
M:											
M0	40	(80)	15	(45.8)	1	(2.1)	1	(6.3)	13	(45.8)	<0.001‡
M1	10	(20)	1	(9.1)	2	(18.2)	0	(0)	7	(72.7)	
AJCC Stage group:											
Stage I	10	(20)	6	(60)	0	(0)	2	(13.3)	2	(26.7)	<0.001§
Stage II	20	(40)	12	(63.2)	0	(0)	3	(10.5)	5	(26.3)	
Stage III	10	(20)	2	(25)	1	(8.3)	0	(8.3)	7	(58.3)	
Stage IV	10	(20)	1	(7.1)	2	(14.3)	0	(0)	7	(78.6)	

* Mann Whitney U test. ‡ Chi-square test. § Chi-square test for trend.

Discussion

In more than 65% of RCC cases of different histopathological features the disease is localized and curable at time of diagnosis but there are still about 30% of cases present with advanced disease or with L.Ns and distant metastases at the time of diagnosis, that could worsen the prognosis and leads to fatal outcome **[11]**. So studying and understanding the molecular pathogenesis of invasion, progression and metastases of RCC might help in identification of recent therapeutic targets for treatment and improving the prognosis of patients with such type of cancer. As cancer is now considered a genetic disease, so it needs many genetic alteration and combinations of many genes for its initiation, promotion and progression. Recently the epigenetic alterations that include DNA-methylations, histone-modifications, and miro-RNA changes, were considered cancer promoting factors **[12]**. The epigenetic alterations differed from the genetic changes in that; they could be reversed by their inhibitors, but that could not happen in the genetic changes or in DNA mutations. We choose the EZH-2 for evaluation as it was found to play a role of such epigenetic change in cancer.

In our study, we found that high expression of EZH2 was significantly positively correlated with higher grade and stage of the tumor, presence of distant metastases and presence of L.N metastases. Our results were near to results of Xu et al., who proved that EZH2 expression levels were positively related to poor clinicopathological and prognostic parameters, RCC progression, higher grade of RCC and advanced stage which points to role of EZH2 as a novel PCC prognostic marker [13].

Liu et al., proved that that increased EZH2 expression could be a predictive of RCC patients' overall and progression free survival rates that were in line with our results [14]. Our results and results of previous studies will add to the prognostic role of TNM-stage in RCC as such value will improve when it is combined with EZH2 levels of expression. Also EZH2 expression could be considered a clinically easily applicable method of differentiation of RCC patients with different outcomes. That will lead to individualized management of RCC patients. In line with our results; Wagener et al.,: Have proved that EZH2 could be considered a recent marker of dismal prognosis in RCC-patients [15].

Many other previous researchers have proved results similar to us that increased EZH2 expression was related to worse clincopathological features and dismal outcomes of patients having malignancies of organs other than the kidney [16,17].

Different from us were results of Stefan Hinz and colleagues who showed that increased levels of expression of EZH2 were related to less aggressive parameters and were related to favorable outcomes of RCC patients' if they are assessed by real-time PCR [18].

The different method of evaluation of EZH-2 expression levels between us and the other study is responsible for such conflicting results.

Our results were explained by Z. Q. Xu et al., who clarified that increased expression of EZH2 enhanced RCC progression by increasing angiogenesis that subsequently increased RCC size, invasion, L.N and distant metastases [13].

Additionally silencing of EZH2 gene suppresses malignant cells growth, leads to cell cycle arrest and stimulate apoptosis of cancer cells, while its overexpression promotes malignant cells growth and inhibited apoptosis in them. So EZH2 knocking down might inhibit malignant cells proliferation and increased apoptosis [19,20]. All these results that are in line with us point to the benefit of using targets against EZH-2 in RCC therapy, as EZH2 inhibitors decreased malignant growth, invasion and metastases.

The process in which cells could change their phenotype from the epithelial to the mesenchymal states is called the epithelial-mesenchymal transition (EMT). EMT leads to many changes in the differentiated characteristics of the cells e.g. Adhesion and apical basal polarity between cells, additionally, leads to change in the absence of mobility and acquire instead mesenchymal characters e.g. invasiveness motility, and resistance to apoptosis and by such process malignant cells could acquire invasive and metastatic capability [5]. EMT is regulated by plethora of transcription factors like Twist-1.

In our study we have proved that increased Twist-1 expression was significantly positively correlated with higher grade and stage of the tumor, presence of distant metastases and presence of L.N metastases.

Results of our study were similar to results of Kojiro Ohba, et al., [21], that proved that Twist over expression was positively correlated with RCC higher grade, advanced pathological tumor stage stage, higher incidence of invasion and metastasis in RCC patients.

The meta-analysis that was done by Wushou et al., [22], proved similar to us that; immunohistochemical expression of Twist-1 is related to poor prognosis in cancer patients that point to the development of therapeutic targets against such transcription factor is a useful therapeutic method.

Additionally Wright et al., [23], found results similar to ours that demonstrated the role of Twist-1 in EMT induction in RCC cells.

Additionally, similar to our results Harada et al., [24], have found that increased Twist expression is associated with poor prognosis in RCC patients.

Previous researches regarding different types of cancer found nearly similar results to ours in RCC that Twist-1 overexpression is related to poor clinicopathological and prognostic parameters in various cancer types [25,26], increased Twist-1 expression is associated with poor patients prognosis, higher grade and advanced stage [22]. In squamous cell carcinoma of the oral cavity increased Twist-1 expression could predict L.Ns, lung and distant metastases occurrence and also related to poor patient survival. So near our results in RCC Twist-1 over-expression is considered a

poor prognostic sign and was related to poor survival rates of cancer patients having different types of cancer **[27-29]** . Our results are clarified by the Twist-1 played its pro-metastatic role by its ability to stimulate the EMT which is the process that transforms cells to more invasive and motile subtypes that is able to break through the basement membrane and invade the surrounding extracellular matrix **[30]**.

Results of our research regarding Twist-1 expression and its role in RCC progression may help to detect a new therapeutic target aiming for adequate management to decreases the invasiveness and metastases of RCC, that was in line with **[31]**.

When we correlated the expression of EZH-2 and Twist-1 expression together we demonstrate that there is a significant direct relation between both markers expressions in RCC and Combination of EZH-2 & Twist overexpression was significantly positively correlated with grade, stage of the tumor, presence of L.Ns and D.M (p<0.001 for all). Suggesting that a combination of EZH-2 & Twist expression could predict disease outcome and it will be beneficial to use therapeutic inhibitors of both markers.

Summary and Conclusions:

In summary, we tried to correlate the tissue protein expression of EZH-2 and Twist-1 aiming to detect novel therapeutic targets our results and found that overexpression of both EZH-2 & Twist-1 expression is a marker of poor prognosis in patients with RCC.

The positive association between both markers proved that the combination of molecular inhibitors against both of them could be used for inhibition of occurrence of invasion and metastases in RCC. We recommend performing other studies on EZH-2& Twist-1 expression in different types of cancer to prove their value on cancer molecular targeted therapy.

Conflicts of interest:

Authors declared no conflicts of interest.

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الأهمية الإكلينيكية والباثولوجية لإظهار بروتين HDAC & SPoCK1 فى حالات سرطان المثانة

يعتبر سرطان خلايا الكلية الورم الخبيث الأكثر شيوعاً و الأكثر خطورة فى الكلى. حيث وجد أن ٣٠ من المرضى المصابين به يكون أنتقل لأماكن أخرى أثناء التشخيص والذى يعتبر مؤشر مهم فى مسار المرض. لذا فإن محاولة توضيح الآليات الجزئية للانتشار فى سرطان خلايا الكلية يمكن أن تساعد فى أكتشاف هدف علاجى حديث لتقليل الأنتشارات البعيدة والسيطرة عليها، وبالتالى قد يؤدى إلى تحسين تشخيص المرضى.

الهدف: تقييم تعبيرات EZH2 وTwist-1 مجتمعة فى أنسجة سرطان خلايا الكلية مع أنواع فرعية مرضية مختلفة وربط تعبيرهم مع المعايير الإكلينيكية .

الأساليب: قمنا بتقييم التعبير عن EZH2 وTwist-1 فى الأنسجة باستخدام دراسة هستوكيميائية فى ٥٠ بلوك البارافين من أنواع فرعية مختلفة مرضية الأنسجة، ثم تحليل الإرتباطات بين مستويات ظهورهم والبيانات الإكلينيكية.