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The prognostic value of ephrin type-A2 receptor and Ki-67 in renal cell carcinoma patients

An Immunohistochemical and Bioinformatical Approach; A STROBE - compliant article

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Abstract

Patients with renal cell carcinoma (RCC), the most common malignant renal epithelial tumor, usually present with advanced disease and unpredicted clinical behavior. The receptor tyrosine kinase, ephrin type-A receptor 2 (EphA2) was found to be overexpressed in several malignancies and its expression was found to be associated with poor prognostic features.

Our study is an observational study with the aim of investigating the prognostic value of EphA2 in RCC patients and its association with clinicopathological parameters as well as Ki-67 expression, which is a well-known proliferative and prognostic marker in RCC.

EphA2 and Ki-67 immunohistochemical staining was performed on whole sections representative of 50 patients diagnosed with primary RCC from 2013 to 2018. In addition, the association between EphA2 mRNA expression and clinicopathological parameters as well as the patients' outcome was also evaluated using two large publicly available databases.

Our results showed a significant association between EphA2 immunohistochemical expression and tumor size, nuclear grade, tumor stage, patients' outcome and Ki-67 expression ($P < .05$ for all). The same trend was also observed with EphA2 mRNA expression using larger patients' cohorts in 2 publicly available databases. Notably, EphA2 protein expression showed higher levels of co-expression with the proliferative marker Ki-67.

Our results suggested that higher expression of EphA2 and Ki-67 in tumor tissues predicts a locally aggressive behaviour and poor outcome of patients with RCC. Moreover, our results give a rationale for the potential benefits of using novel therapeutic strategies with the aim of targeting EphA2 receptor in RCC patients that might help in improving their outcome.

Abbreviations: Eph = ephrin, EphA2 = ephrin type-A receptor 2, IHC = immunohistochemical, OS = overall survival, RCC = renal cell carcinoma.

Keywords: bioinformatics, ephrin A2 receptor, Ki-67, prognosis, renal cell carcinoma

1. Introduction

Worldwide, renal cell carcinoma (RCC) represents 2% to 3% of the adult malignancies with an increasing incidence.[1] One of the main challenges in RCC management is the fact that this disease is usually diagnosed in advanced stage.[2] Approximately 1 third of RCC patients present initially with metastasis or develop metastasis later in the course of the disease.[1] RCC is considered as 1 of the tumours that have unpredicted clinical behaviour; 30% of the patients who underwent radical nephrectomy usually end with recurrence. This resulted in a very low median 5-year survival for patients with metastatic RCC ranging from 5% to 30%. A variety of prognostic factors such as stage, grade and performance status of the patient, have been proposed as prognostically useful parameters, however many of them showed only limited clinical value.[3] All those together, highlight the need of more precise and accurate prognostic markers that help not only in predicting the patient's outcome, but also might help in the development of novel therapeutic modalities to treat this aggressive tumour.

The ephrin (Eph) subfamily represents the largest group of receptor protein tyrosine kinases identified to date and found to play a role in the development of tumours; regulation of cell

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All data generated or analyzed during this study are included in this published article [and its supplementary information files].

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growth, survival, migration and angiogenesis.[4–6] Ligands for Eph receptors include Eph -A4 (LERK-4) which binds EphA3 and EphB1. In addition, Eph -A2 (ELF-1) has been described as the ligand for EphA4, Eph -A3 (Ehk1-L) as the ligand for EphA5 and Eph -B2 (Htk-L) as the ligand for EphB4 (Htk).[7,8] High expression level of EphA2 is of a particular interest because it is often found in many types of cancers, including prostate,[9] breast[10] and non-small cell lung cancer,[11] indicating that EphA2 plays a role in tumour development. Moreover, recent reports showed that targeting EphA2 impairs cell cycle progression and growth of triple-negative breast cancers.[12]

All these together highlight the possible use of EphA2 as a potential prognostic marker in RCC patients that might have therapeutic application. Nevertheless, the expression of EphA2 in RCC has not been well investigated.[13]

Ki-67 is a nuclear antigen presented in almost all human malignancies. Ki-67 protein is expressed throughout the active phases of the cell cycle, and its expression is related to the proliferative activity in the cell nuclei.[14] It accumulates during the cell cycle from G1 to mitosis, having its lowest level after mitosis.[15] Ki-67 has a short half-life, as such, the detection of this antigen is more reliable than that of a long half-life proliferating cell nuclear antigen.

Ki-67 is regarded as 1 of the classical markers associated with tumour proliferation and several studies on RCC,[16] lymphomas,[17] gastric cancer,[18] colorectal cancer,[19] bladder cancer,[20] and breast cancer[20] have shown that overexpression of Ki-67 antigen is correlated with the biological behaviour and prognosis of these malignancies. Moreover, its expression in RCC was found to be positively associated with advanced tumour stage and grade.[21]

The aim of the present study was to investigate the prognostic value of EphA2 protein and mRNA expression in RCC and evaluate their association with other clinicopathological parameters as well as with patients' outcome. Moreover, we also investigated the prognostic value of Ki-67 and the significance of co-expression between Ki-67 and EphA2 in RCC.

2. Materials and methods

The present work encompassed 50 formalin-fixed paraffin embedded primary tumour blocks of RCC cases surgically managed by radical or partial nephrectomy in urology department, Alexandria University Main Hospital, starting from 2013 till the end of 2018. Exclusion criteria included both stage IV RCC and locally recurrent RCC cases. The study was approved by the Ethics Research Committee of the Faculty of Medicine, Alexandria University (Alexandria, Egypt).

2.1. Clinical data

The clinical data were collected by reviewing the pathology requests submitted to the pathology department, Alexandria Faculty of Medicine. The clinical data included patients' age, sex and clinical presentation as well as the type of operation (radical or partial nephrectomy). Follow up data were obtained from urology department and accordingly, the patients were dichotomized into 2 study groups; the first group (G1) included patients who were disease-free and the second group (G2) had either recurrence or distant metastasis at the end of 24 months duration.

2.2. Pathological examination

2.2.1. Macroscopic examination. The macroscopic features of the specimens were retrieved from the pathology reports archived in the pathology department; the tumour site, size, consistency, the appearance of the cut section of the tumour, the degree of haemorrhage and necrosis and the macroscopic extent of the tumour including infiltration of surrounding structures.

2.2.2. Histopathological examination. The formalin-fixed paraffin embedded tissue blocks were cut into 5 micron-thick sections, stained with haematoxylin and eosin stain[22] and examined under light microscope to determine tumour typing according to the 2016 WHO classification of renal neoplasms,[23] tumour staging using TNM classification and stage grouping of RCC (AJCC 2018)[24] and nuclear grading using ISUP/WHO grading system.[25]

2.3. Immunohistochemical (IHC) staining

IHC staining was done using the following primary antibodies:

(1) Mouse monoclonal EphA2 antibody (C-3), sc-398832, Santa Cruz Biotechnology, TX.
(2) Mouse monoclonal, anti-human Ki-67 antigen, clone MIB-1, code M7240, Denmark A/S.

Positive and negative controls were included in all runs. External positive control cases were selected as recommended by the manufacturer's protocols[26–28] as follow:

(1) Breast cancer for EphA2 as external positive control and normal kidney tubules included in some slides as internal control.
(2) Tonsils for Ki-67 as external positive control.

2.4. Interpretation of IHC staining

The stained slides were examined by two independent pathologists who were totally blinded to the clinical data. For EphA2 antibody, positivity was considered with cytoplasmic staining. The proportion of stained cells was scored as: 0 (<5%), 1 (5% to 25%), 2 (26% to 50%), and 3 (>50%). Staining intensity was graded as 0 (negative), 1+ (weak intensity), 2+ (moderate intensity), and 3+ (strong intensity); followed by the summation of two scores. Final scores of < 3 were classified as "EphA2-low" and those ≥ 3 as "EphA2-high".[29]

For Ki-67 antibody, cells labelled by the antibody displayed a nuclear staining pattern. The score was assigned according to the average of the extent of immuno-expression (0%–100% percentage of cells staining) and the RCC sections were considered positive for Ki-67 when >15% of tissue showed positive nuclear staining.[30]

2.5. Data mining

Two large, publicly available databases were used in this study. The ONCOMINE database, that includes more than 700 different datasets for different types of cancers. In this database we used Jones (92 cases), Higgins (44 cases) and Bittner (256 cases) to investigate the mRNA expression of EphA2 in normal versus malignant samples as well as its expression in different tumour stages.

In addition, we also used a cohort of 530 kidney clear cell carcinoma samples from the Pan-cancer RNA-seq dataset of the

KM plotter database. This is user friendly large publicly available database that allow investigators to evaluate the association between different genes and patients' outcome presented as overall survival (OS).

2.6. Statistical analysis

Statistical analysis was carried out using SPSS statistics software version 23. Quantitative data were tested for normality using Kolmogorov-Smirnov test. The age variable was normally distributed and described by mean, standard deviation and 95% confidence interval while tumour largest dimension variable was not normally distributed and described by median, range, and interquartile range.

Non-parametric statistical tests of significance were applied; Kruskal Wallis test was used to compare 2 independent groups. Qualitative data was expressed by numbers and percent. Monte-Carlo Exact test was used to test the association between qualitative variables. Logistic Regression (Stepwise Approach) was used to predict the variables contributing in the prognosis. In all applied statistical tests of significance, P value ($<.05$) was considered significant.

3. Results

3.1. EphA2 mRNA expression is upregulated in RCC samples compared to normal tissue

Due to the absence of normal samples in our cohort, we initially investigated the EphA2 mRNA expression in normal kidney samples compared to RCC cases using publicly available ONCOMINE database (Jones dataset, 92 cases) (Fig. 1). Interestingly, our results showed that EphA2 mRNA expression to be significantly up-regulated ($P=.002$) in RCC tissue samples (papillary subtype) compared to normal tissue (Fig. 1A). The same trend was also observed with clear cell RCC; however, it does not reach to statistical significance ($P=.136$) (Fig. 1B). These results were further confirmed using Higgins dataset, which includes 44 RCC patients' samples. It also showed EphA2 mRNA expression to be significantly up-regulated in RCC samples from papillary ($P=.025$) (Fig. 1C) and clear cell carcinoma subtypes ($P=.017$) (Fig. 1D).

3.2. Clinicopathological features of our patient's cohort

To explore the prognostic value of EphA2 in RCC, we initially investigated the association between EphA2 protein expression and different clinicopathological parameters by immunohistochemistry.

Our cohort included 50 cases of RCC (Fig. 2A). Their ages ranged from 29 to 75 years with a mean age of 55.8 ± 9.9 years and a peak age incidence at the 6th decade of life (50% of the patients). Thirty-two patients (64%) were males and eighteen (36%) were females with male to female ratio of 1.7:1. The size of the examined RCCs ranged from 2 to 23 cm with a median 7.65 ± 5.6. RCC of ≥ 7 cm was detected in 24 cases (48%) and >7 cm in 26 cases (52%). In the present study, 5 histologic types of RCC were recognized (according The WHO 2016 classification) (Fig. 2B). This includes 35 cases of clear cell RCC, 7 cases of papillary RCC, 5 cases of chromophobe RCC, two cases of Clear cell papillary RCC and 1 case of collecting duct carcinoma (Table 1). Due to the low number in some histological subtypes, all non-clear cell carcinoma cases were clustered into 1 group. Nuclear grading was performed using ISUP/WHO grading system. Three of the cases were grade I, 15 were grade II, 19 grade III and 8 grade IV. Moreover, 20 cases classified as stage I, 16 as stage II and 14 as stage III. Cases were graded according to ISUP/WHO 2016 grading system (Fig. 2C). In addition, the cases were staged according to the TNM staging system 2018, depending on the clinical and radiological findings and then finalized by the histopathological examination as shown in Figure 2d.

3.3. EphA2 protein expression is marker of more aggressive phenotype and more advance disease

EphA2 immunostaining for the 50 cases was analyzed (Fig. 3) and our results revealed that most of the RCC cases showed moderate staining (21 cases, 42%) and strong staining (14 cases, 28%) with only 9 cases (18%) showed weak or negative staining (6 cases, 12%).

Initially, we investigated the relationship between Eph A2 expression and different RCC histologic types (Table 1). In clear cell RCC, out of the 35 cases investigated, Eph A2 staining was positive (57.24%). This includes 14 cases with moderate staining (score 2) (40.1%) and 6 cases with strong staining (score 3) (17.1%). Eph A2 staining intensity was negative (score 0) in 9 cases (25.7%) and weak (score 1) in 6 other cases (17.1%) (Table 1).

Interestingly, all the non-clear cell RCC cases showed moderate- strong EphA2 expression with moderate stain (46.67%) and 8 (53.33%) strong stain.

While EphA2 expression showed no statistically difference with patients' age or sex (0.054 and 0.158 respectively), our univariate statistical analysis revealed a significant positive correlation between EphA2 protein expression and RCC histologic type, grade and stage ($P=.002$, .021 and .008 respectively, Monte Carlo Exact test) (Table 1). Indeed, all grade 4 tumors showed high EphA2 immunostaining (62.5% moderate and 37.5% strong), in contrast, none of the grade 1 samples showed high EphA2 immunostaining. Similarly, while 92.85% of stage III tumors showed high EphA2 immunostaining (57.1% moderate & 35.71% strong), only 40% of stage I tumors showed high EphA2 immunostaining (15% moderate & 25% strong). Moreover, the protein expression of EphA2 was also found to increase with increasing the size of RCC and this relation was statistically significant ($x^2=14.840$, $P=.002$, Kruskal Wallis test) (Table 1). Regarding sinus, capsular and vascular invasion of RCC, EphA2 expression was statistically insignificant with all of them ($P=.287$, 0.161 and .620 respectively, Monte Carlo Exact test) (Table 1). In summary our results showed EphA2 protein expression to be a marker of more aggressive phenotype and more advance disease in our RCC patient cohort.

3.4. EphA2 mRNA expression is a marker of more advanced disease stage

For better understanding of the association between EphA2 and different clinicopathological parameters in RCC, we investigated the association between EphA2 mRNA expression and tumor stage, which is 1 of the major pathological variables that determine patient prognosis using Bittner dataset of the ONCOMINE database. This dataset involves 265 RCC patients' samples. Similar to our immunohistochemistry findings, our results here also showed a positive association between EphA2 mRNA higher levels and advanced tumor stage, with more

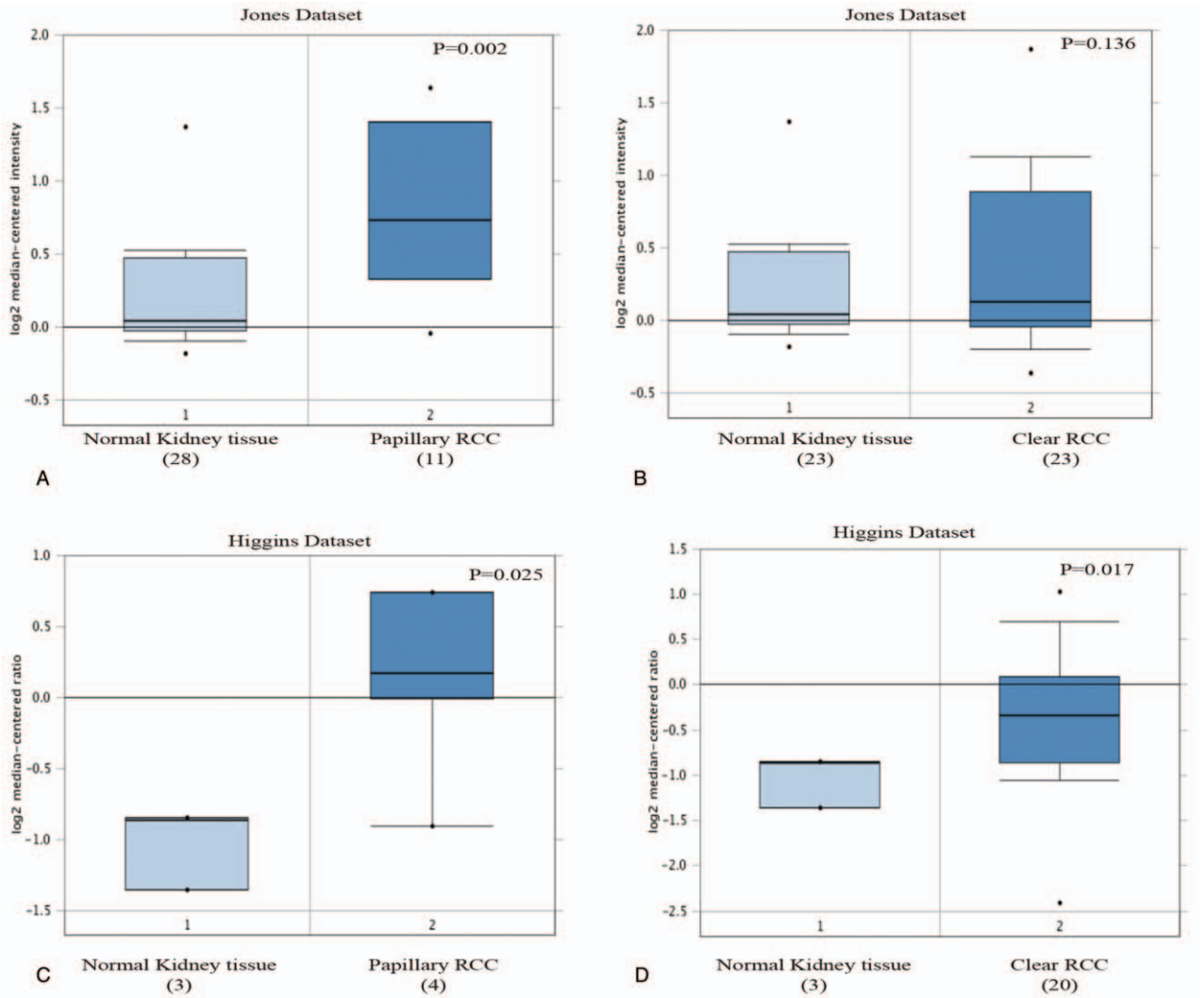


Figure 1. The mRNA expression of Ephrin A2 in normal tissue compared to renal cell carcinoma samples. The EphA2 mRNA expression in normal kidney tissue versus papillary RCC patient sample using Jones Dataset of ONCOMINE (ONCOMINE is a Cancer Microarray Database and Integrated Data-Mining Platform) database. The EphA2 mRNA expression in normal kidney tissue versus clear RCC patient sample using Jones Dataset of ONCOMINE database. The EphA2 mRNA expression in normal kidney tissue versus papillary RCC patient sample using Higgins Dataset of ONCOMINE database. The EphA2 mRNA expression in normal kidney tissue versus clear RCC patient sample using Higgins Dataset of ONCOMINE database. EphA2 = ephrin type-A receptor 2, mRNA = messenger ribose nucleic acid.

expression in the more advanced stage III and IV compared to stage I and II (Fig. 4A). Moreover, we also found that EphA2 mRNA to be more expressed in samples from patients with distance metastasis (M1) compared to patients with no evidence of distance metastasis (M0) (Fig. 4B).

3.5. EphA2 protein and mRNA expression is a marker of poor patient's outcome in RCC patients

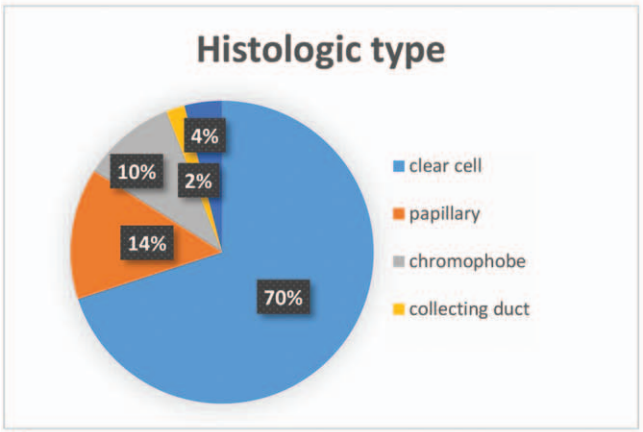
Finally, and for better understanding of the prognostic value of EphA2 in RCC, we next investigated the association between EphA2 protein expression and patient outcome in our 50 RCC cases. Indeed, our results revealed that patients with higher levels of EphA2 immunostaining to be associated with worse patient outcome presented as shortened 2 years recurrence or metastatic free disease (88.89%) compared with (59.37%) in the EphA2 low cases.

The association between high EphA2 and poor patient outcome was statistically significant ($P=.034$, Monte-Carlo Exact Test).

Due to the limited number of the cases in our cohort and the fact that many of the previous reports that investigated the association between EphA2 and patient's outcome were limited to small sample size, we next investigated the association between EphA2 mRNA expression and patient's outcome using large RCC cohort (530 cases) from the KM Plotter database. Interestingly, while EphA2 mRNA expression was found to be associated with favorable outcome in the early stage tumors (Fig. 4C), its expression in the more advanced stage III tumors was found to be significantly associated with poor patient outcome ($P=.0053$) represented by shortened OS (Fig. 4D). This further confirms the association between EphA2 expression and poor patient's outcome that we observed in the protein level.

A

Renal mass		Number of cases (%)
Size	7cm≤	24 (48%)
	> 7cm	26 (52%)
Capsule	Well circumscribed polar mass with pseudocapsule	34 (68%)
	Poorly circumscribed central mass	6 (12%)
	Diffuse infiltrative mass	9 (18%)
	Multicentric nodules	1 (2%)
Cut section	Variegated with haemorrhage and necrosis	21 (42%)
	Predominantly cystic	8 (16%)
	Lobulated, tan coloured with minimal necrosis	11 (22%)
	Greyish white	10 (20%)
Extension	Localized to the kidney	38 (76%)
	Perinephric fat invasion	12 (24%)
	Gerota's fascia	3 (6%)
	Renal sinus invasion	12 (24%)
	Adrenal gland	0 (0%)
Venous invasion	Renal vein thrombus	4 (8%)



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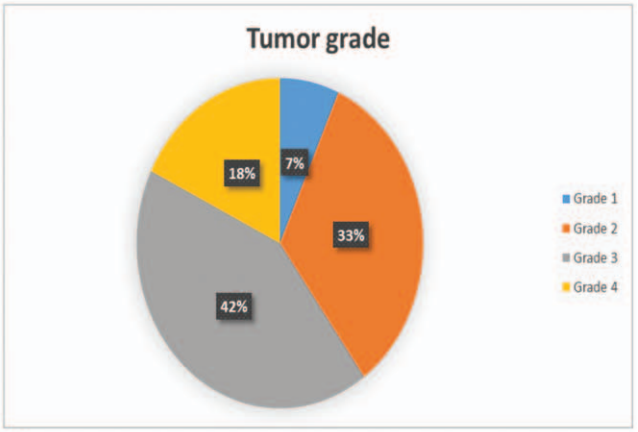

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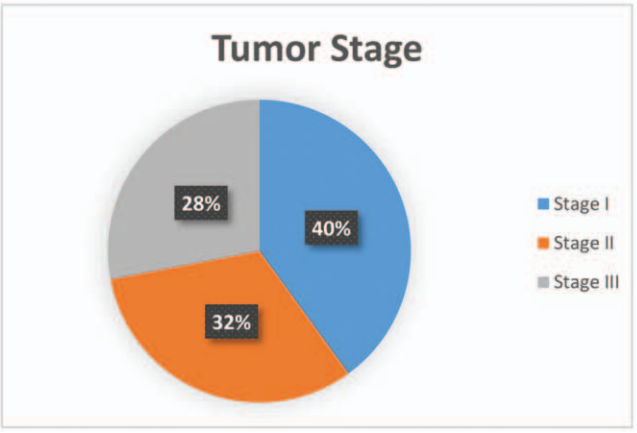

D

Figure 2. The clinicopathological characteristics of the patient's cohort. The distribution of patient cohort according to clinicopathological parameters. The distribution of patient cohort according to histologic types. The distribution of patient cohort according to according to ISUP/ WHO 2016 grading system. The distribution of patient cohort according to Tumor, Node, Metastasis (TNM) staging system-2018.

3.6. Ki-67 protein expression is another marker of more aggressive phenotype and more advance disease in RCC samples

Another marker which we also investigated in our cohort is the Ki-67. Indeed, Ki-67 is 1 of the classical proliferation markers widely used in the IHC analysis due to its expression in all cell cycle phases except G0. Many reports demonstrated an important association between Ki-67 expression and poor prognosis as well as advanced clinicopathological features, however, the association between Ki-67 and EphA2 in RCC samples was not thoroughly investigated.

Out results showed high Ki-67 expression in 33/50 RCC cases (66%) and only 17 cases with negative staining (34%). Similar to

Table 1

Association between EphA2 immunohistochemistry and different clinicopathological parameters.

	EphA2 expression					
	EphA2-low		EphA2-high			
Sex	**Negative**	**Weak**	**Moderate**	**Strong**	**Total**	***P* value**
Male	5 (15.62%)	6 (18.75%)	11 (34.37%)	10 (31.25)	32	*P*=.158
Female	4 (22.22%)	0 (0%)	10 (55.55%)	4 (22.22%)	18	
Histological type						
Clear cell RCC	9 (25.71%)	6 (17.14)	14 (40%)	6 (17.14)	35	*P*=.002
Non-clear cell RCC	0 (0%)	0 (0%)	7 (46.67%)	8 (53.33%)	15	
Nuclear grade						
Not applicable	0 (0%)	0 (0%)	1 (20%)	4 (80%)	5	*P*=.021*
1	2 (66.67%)	1 (33.33%)	0 (0%)	0 (0%)	3	
2	5 (33.33%)	2 (13.33%)	4 (26.67%)	4 (26.67%)	15	
3	2 (10.52%0	3 (15.78%)	11 (57.89%)	3 (15.78%)	19	
4	0 (0%)	0 (0%)	5 (62.5%	3 (37.5%)	8	
Tumor stage						
Stage I	7 (35%)	5 (25%)	3 (15%)	5 (25%)	20	*P*=.008**
Stage II	1 (6.25%)	1 (6.25%)	10 (62.5%)	4 (25%)	16	
Stage III	1 (7.14%)	0 (0%)	8 (57.14%)	5 (35.71%)	14	
Size						
Tumor largest dimension (mean)	16.83	10.08	32.31	27.46		*P*=.002**
Capsular Invasion						
Negative	7 (20.58%)	6 (17.64%)	14 (41.17%)	7 (20.58%)	34	*P*=.161
Positive	2 (12.5%)	0 (0%)	7 (43.75)	7 (43.75)	16	
Vascular Invasion						
Negative	9 (19.56%)	6 (13.04%)	19 (41.30%)	12 (26.08%)	46	*P*=.620
Positive	0 (0%)	0 (0%)	2 (50%)	2 (50%)	4	
Renal Sinus Invasion						
Negative	8 (21.05%)	6 (15.78%)	15 (39.47%)	9 (23.68%)	38	*P*=.287
Positive	1 (8.33%)	0 (0%)	6 (50%)	5 (41.66%)	12	
Ki-67 expression						
Negative	9 (52.94%)	5 (29.41%)	3 (17.64%)	0 (0%)	17	*P*=.000***
Positive	0 (0%)	1 (3.03%)	18 (54.54%)	14 (42.42%)	33	
Prognosis						
No recurrence or metastasis at the end of 24 mo	9 (28.12%)	4 (12.5%)	11 (34.37%)	8 (25%)	32	*P*=.034*
Recurrence or metastasis at the end of 24 mo	0 (0%)	2 (11.11%)	10 (55.56%)	6 (33.33%)	18	

For tumor size, Kruskal Wallis test was used, for all the other variables the Monte-Carlo exact test was used.
EphA2 = ephrin type-A receptor 2, RCC = renal cell carcinoma.

the EphA2 protein expression, our univariate statistical analysis revealed a significant positive association between Ki-67 expression and RCC histological type, grade, stage and tumor size. (*P*=.04, .017, .048 and .038 respectively). Similarly, the relationship between Ki-67 expression and outcome of the patient was significant (Table 2). It also increases with worse outcome. (*P*=.01, Monte Carlo Exact test). Regarding sinus, capsular and vascular invasion of RCC, Ki-67 expression was statistically insignificant with all of them. (*P*=.450, .118, and .134 respectively, Monte Carlo Exact test) (Table 2). No statistically significant difference was detected between Ki-67 expression and histologic types of RCC, patient's age or sex. (*P*=.352, .630 and .587 respectively). Moreover, in our 50 cases cohort, patients with negative Ki-67 expression showed better patients' outcome presented as 2-year metastasis/recurrence-free survival (46.87%) compared to only 11.11% in the Ki-67 positive group (*P*=.01) (Table 2).

3.7. RCC samples showed high levels of EphA2 and Ki-67 protein co-expression

Next, we investigated the association between Ki-67 and EphA2 in RCC samples. As can be seen in Figure 4 E, all of the 9 cases (100%) with negative EphA2 staining were also negative for Ki-67 expression and 5 out of 6 cases (83.3%) that showed weak EphA2 expression (score 1), were also negative for the Ki-67 expression. In addition, 18 out of 21 cases (85.7%) with moderate EphA2 expression (score 2) were also positive for Ki-67 staining, while only 3 cases (14.3%) were negative. Moreover, all of the 14 cases (100%) that showed strong EphA2 expression (score 3), showed concomitant positive Ki-67 expression. The association between EphA2 and Ki-67 co-expression staining intensity was statistically significant. (*P* is less than .001, Monte-Carlo Exact Test) (Table 1).

3.8. Multivariate logistic regression analysis

Finally, in a trial to investigate the actual role of EphA2 as new predictive factor in RCC and whether it is beneficial to be used alone or in combination with other factors, the prognostic significance of EphA2 and Ki-67 was studied by using multivariate logistic regression analysis in order to correlate the results of these factors and outcome data of the patients. For statistical purpose, cases with negative and weak EphA2 staining intensity were grouped together and referred to as (negative) (15 cases, 30%), while cases with moderate and strong EphA2

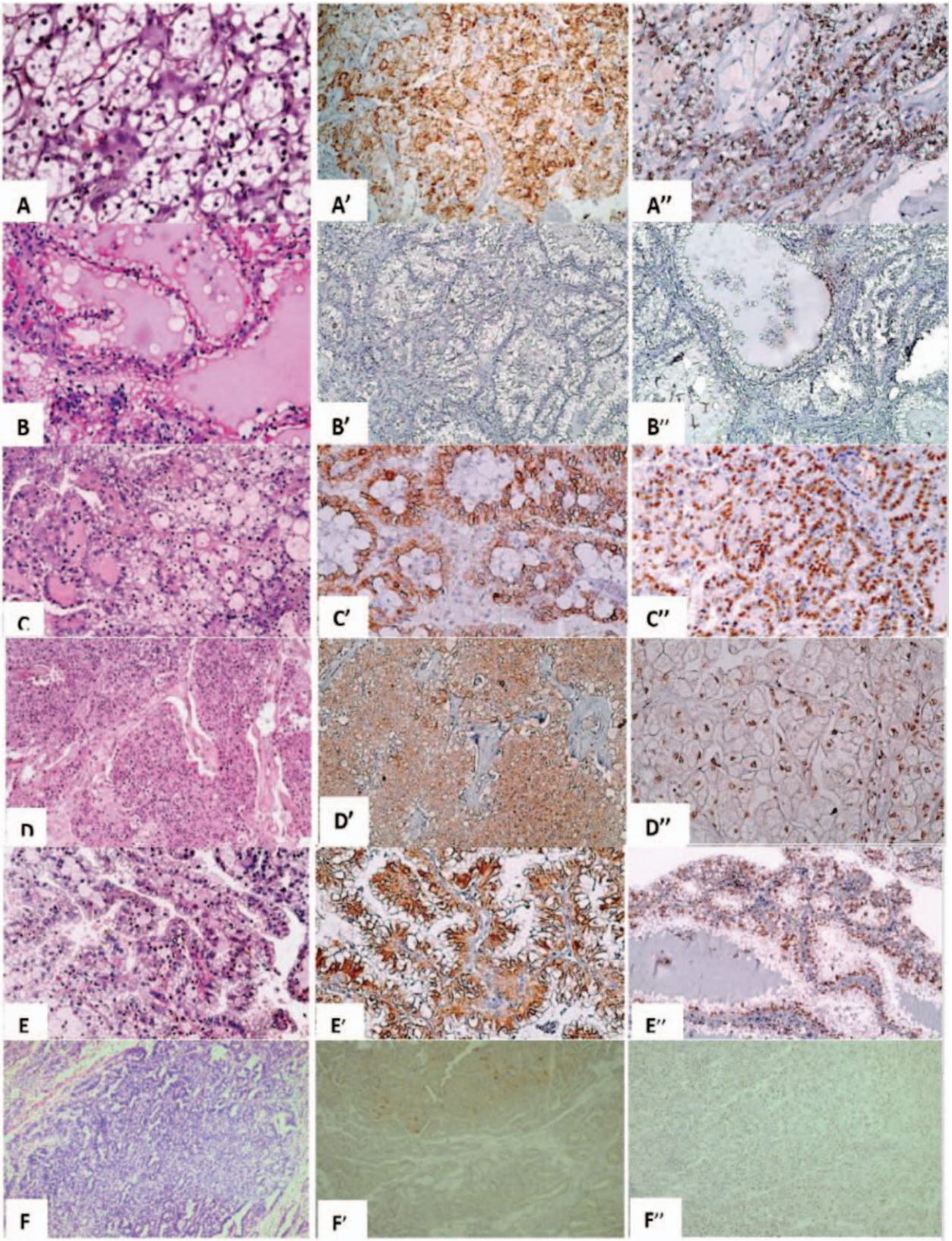

Figure 3. Example of the different histological subtypes of RCC included in the study (H and E in the first column) and both EphA2 expression (second column marked by ′) and Ki-67 expression (the third column marked by ″) in them (A: CCRCC, B: Multilocular cystic renal neoplasm of low malignant potential, C: PRCC, D: chromophobe RCC, E: clear cell papillary RCC, F: collecting duct carcinoma). EphA2 = ephrin type-A receptor 2, H and E = haematoxylin and eosin, RCC = renal cell carcinoma.

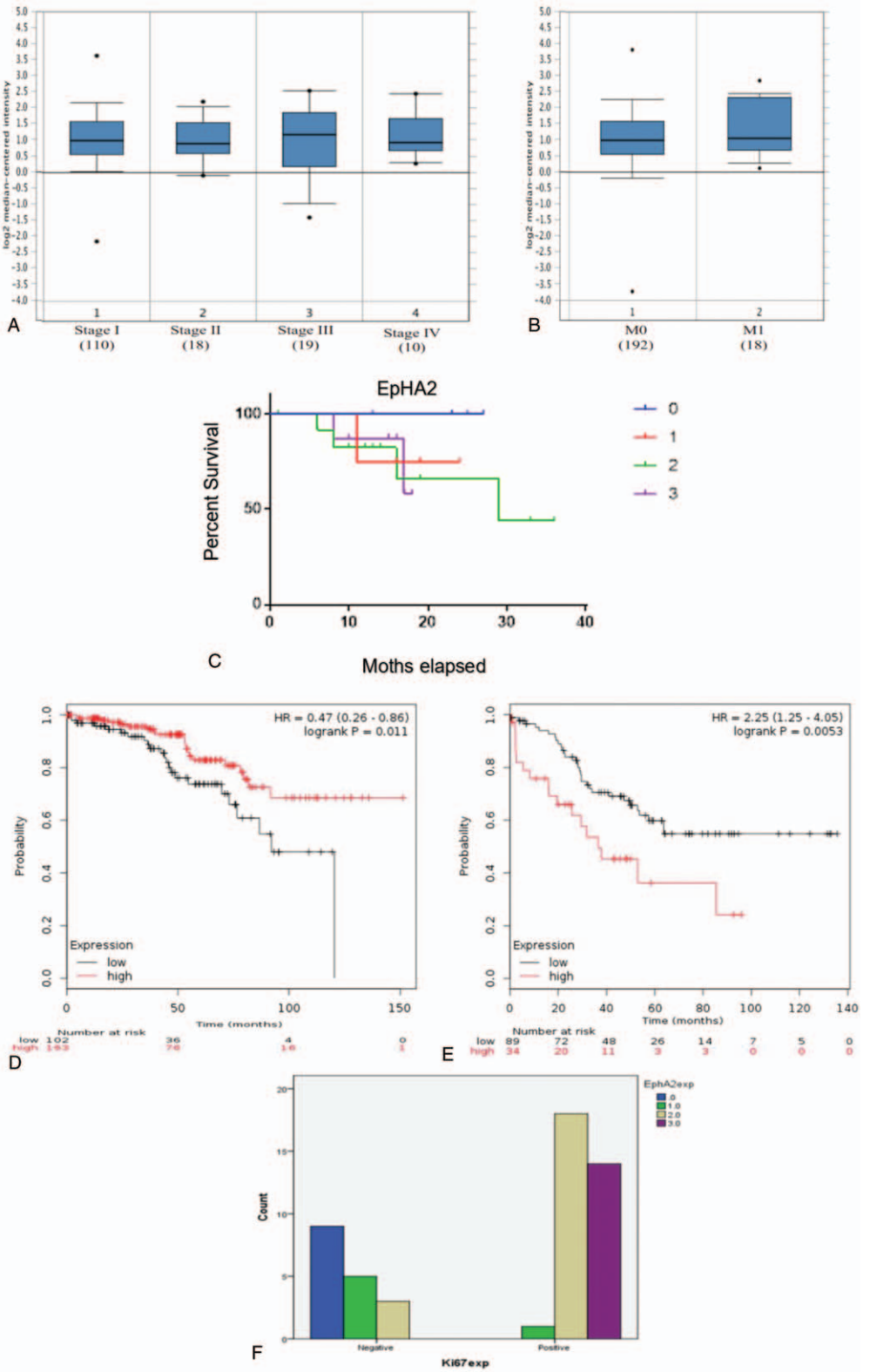

Figure 4. The EphA2 mRNA expression and its association with tumor stage using Bittner database of ONCOMINE database. The EphA2 mRNA expression and its association with presence or absence of distance metastasis using Bittner database of ONCOMINE database. The EphA2 protein expression and its association with patients' outcome in our patients' cohort. The EphA2 mRNA expression and its association with patient outcome presented as Overall Survival (OS) using KM plotter database in stage I renal cell carcinoma patients. The EphA2 mRNA expression and its association with patient outcome presented as OS using KM plotter database in stage III renal cell carcinoma patients. The relationship between EphA2 expression and Ki-67 expression. EphA2 = ephrin type-A receptor 2, mRNA = messenger ribose nucleic acid.

Table 2

Association between Ki-67 immunohistochemistry and different clinicopathological parameters.

	Ki-67 Expression			
	Negative	Positive	Total	*P* value
Sex				
Male	10 (31.25%)	22 (68.75%)	32	*P*=.158
Female	7 (38.89%)	11 (61.11%)	18	
Histological type				
Clear cell RCC	15 (42.85%)	20 (57.14%)	35	*P*=.04*
Non-clear cell RCC	2 (13.33%)	13 (86.67%)	15	
Nuclear grade				
Not applicable	1 (20%)	4 (80%)	5	*P*=.017*
1	3 (100%)	0 (0%)	3	
2	7 (46.66%)	8 (53.33%)	15	
3	6 (31.57%)	13 (68.42%)	19	
4	0 (0%)	8 (100%)	8	
Tumor stage				
Stage I	11 (55%)	9 (45%)	20	*P*=.048*
Stage II	3 (18.75%)	13 (81.25%)	16	
Stage III	3 (21.42%)	11 (78.57%)	14	
Capsular Invasion				
Negative	14 (41.17%)	20 (58.82%)	34	*P*=.118
Positive	3 (18.75%)	13 (81.25%)	16	
Vascular Invasion				
Negative	17 (36.95%)	29 (63.04%)	46	*P*=.134
Positive	0 (0%)	4 (100%)	4	
Renal Sinus Invasion				
Negative	14 (36.84%)	24 (63.15%)	38	*P*=.450
Positive	3 (25%)	9 (75%)	12	
Prognosis				
No recurrence or metastasis at the end of 24 months	15 (46.87%)	17 (53.12%)	32	*P*=.01**
Recurrence or metastasis at the end of 24 mo	2 (11.11%)	16 (88.89%)	18	

For all variables the Monte-Carlo Exact Test was used and $P<.05$ was considered significant.
RCC = renal cell carcinoma.

staining intensity were grouped together and referred to as (positive) (35 cases, 70%). On the other hand, grade 1 and grade 2 RCCs were grouped together and referred to as (low grade) (18 cases, 36%), while cases of grade 3 and grade 4 RCCs were grouped together and referred to as (high grade) (32 cases, 64%).

Logistic regression model is made including the patient outcome as the dependent factor and tumor largest dimension, nuclear grade, TNM Staging, EphA2 expression and Ki-67 as independent factors.

It was found that EphA2 staining intensity, RCC grade, stage, and Ki-67 expression could be used as independent predictors of RCC patient's outcome ($P=.001$, .001, .030 and .007 respectively) but not the tumor largest dimension ($P=.94$) (Table 3).

Although there were significant and insignificant factors, the analysis of the logistic regression model reveals that those contributing factors can affect the outcome of RCC patients by 78% (R2=78%) and the overall model was significantly affecting the outcome ($P=.02$) (Table 3). In other words, although the tumor largest dimension could not be used alone as a predictive factor for outcome, this model tells that it could be used significantly with those other factors to predict patient's outcome.

4. Discussion

RCC is a heterogeneous and complex disease with a widely varying prognosis. The treatment decision making, and selection of appropriate follow-up regimens depend on accurate prediction of disease outcome which is mainly based on the assessment of clinical and pathological prognostic factors.

Table 3

Logistic regression analysis of outcome of RCC patients.

					95% C.I. for (B)	
Factor	S.E.	Wald	df	Sig. (*P*)	Lower	Upper
Tumor largest dimension	0.113	0.303	1	0.940	.753	1.173
Nuclear grade	0.993	0.475	4	0.001***	0.000	22.890
TNM Staging	0.993	1.899	2	0.030*	0.018	3.004
EphA2 expression	0.876	0.405	3	0.001***	0.019	3.200
Ki-67 expression	1.591	1.250	1	0.007**	.007	3.817

Logistic regression (Outcome=Prognosis): $R^2=78\%$, $X^2=12.96$, $P=.02$. Variable (s) entered: tumor largest dimension, Nuclear Grade, TNM Staging, EphA2 expression, and Ki-67expression.
EphA2 = ephrin type-A receptor 2, RCC = renal cell carcinoma, TNM staging = Tumor, Node, Metastasis staging.

Currently, pathologic stage (TNM) and histologic grade represent the most powerful prognostic variables in literature. Recently, many attempts have been made to combine several prognostic factors, both pathological and clinical, into integrated systems designed to improve the prediction of outcomes for patients with RCC and help in achieving stronger prognostic tools.[31]

Recently, several reports found the receptor tyrosine kinase, EphA2 to be overexpressed in many types of carcinomas including breast, prostate lung, ovary and other tumors.[9–11] Moreover, its expression in different tumors was found to be associated with poor prognostic features such as high grade, more advanced stage and higher incidence of tumor recurrence.[32] Nowadays, many therapeutic strategies were proposed to treat cancers based on targeting EphA2 receptor in these tumors.[32] These together highlight the possible use of EphA2 as a possible novel prognostic marker in RCC patient with therapeutic applications. Despite this fact, the prognostic role of this receptor in RCC and its association with patient outcome was not thoroughly investigated. For that reason, here we used a combined immunohistochemistry and bioinformatical approach to investigate the prognostic value of EphA2 in RCC. In addition, we also evaluated the prognostic value and co-expression level of Ki-67, which is an important classical proliferative marker, long known to have independent prognostic importance in RCC.

Our results demonstrated that both EphA2 mRNA and protein levels to be highly expressed in RCC samples. Our results goes with Xu et al, 2014,[28] who also found EphA2 mRNA and protein levels to be significantly higher in RCC specimens compared to corresponding tissues. These results clearly demonstrate a possible role of EphA2 in the process of renal cancer development.

Moreover, our results also showed a significant association between EphA2 expression and more aggressive phenotype presented as high grade and more advanced disease presented as larger tumors and advanced stage. Interestingly, we were able to confirm the association between EphA2 expressions and advanced tumor stage using large cohort from publicly available database that includes 256 RCC patient samples. These findings are also similar to Xu et al, 2014 and L. Wang et al, 2015, who also found that EphA2 expression was positively related to advanced stage and grade.[28,29]This association between EphA2, high tumor grade and stage, was not restricted to RCC, but also was found in other tumors including ovarian and prostate carcinomas.[33,34] One of the hallmarks of this study is to investigate the ability of EphA2 to predict the outcome of RCC patient. In the present study, EphA2 expression was found to be significantly high in patients who suffered tumor recurrence and/or metastasis in the period of 24 months of follow up ($P = .034$). Nevertheless, 1 of the main limitations of our study and other studies as well is the limited number of patients that have complete record of follow up, for that reason we also investigated the association EphA2 mRNA expression and patient outcome in 530 RCC patients' samples using the KM plotter database. Indeed, our results showed that in the more advanced tumors, EphA2 mRNA expression to be significantly associated with poor patient's outcome presented as shortened OS. These findings go with other reports that also found EphA2 expression to be associated with shorter survival times in different tumors including RCC,[28] breast cancer[35] and colon cancer.[36]

This association between EphA2 expression, poor clinico-pathological parameters and poor patient's outcome, which was observed in our study can be contributed to its role in regulation of group of processes essential for tumor progression including malignant transformation, angiogenesis, and metastasis.[37–39] Moreover, the EphA2 receptor and its family was found to be associated with group of downstream signaling pathways that are found to be essential for cancer cells proliferation and survival including RAS/MAPK and PI3K/AKT/mTOR pathways.

Finally, our report is the first study to compare the expression of Ki-67 and EphA2, as 1 of tyrosine kinase inhibitor targets, in RCC. The idea was about proving the prognostic ability of EphA2 by comparing its expression with a well-known prognostic marker as Ki-67. As we mentioned above, our results showed a great similarity in the prognostic power of both receptors, in addition, our results also showed high levels of co-expression between both markers in RCC samples. This similarity in the expression pattern and co-expression levels between the 2 markers further confirm our finding regarding the potential use of EphA2 expression as a marker of poor prognosis and patient's outcome in RCC patients.

5. Conclusions

Our results showed high EphA2 expression to be a marker of more aggressive phenotype and more advanced disease in RCC patients. Besides, it also showed the possible use of EphA2 as an independent prognostic marker that can predict RCC patient's outcome. Our results also showed a high association between EphA2 expression and the classical proliferation marker Ki-67 further confirming its role in RCC cell progression and proliferation. Moreover, our results give a rationale for the potential benefits of using novel therapeutic strategies to target EphA2 receptor in RCC that might help in improving the outcome of the patients.

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