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Prognostic Value of Copine 1 in Patients With Renal Cell Carcinoma

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Abstract. *Background/Aim: Renal cell carcinoma (RCC) is among the most common renal malignancies and requires reliable biomarkers for optimum diagnosis and prognosis. Copines are a family of calcium-dependent phospholipid-binding proteins that were reported to be associated with various cancers. We aimed to investigate the prognostic value of Copines 1 and 3 in RCC patients. Materials and Methods: Copines 1 and 3 bioinformatics analysis and immunohistochemical (IHC) staining were performed on patients with RCC. Results: The findings revealed significant association between Copine 1 expression and the patients' age, nuclear grade, and tumor stage. Bioinformatics analysis showed a similar trend for the mRNA expression of CPNE1, the gene that encodes Copine 1. Interestingly, results revealed a positive association between Copine 1 and both EphA and Ki-67 expression levels. Noteworthy, there was no significant association between Copine 3 expression and any parameters. Conclusion: Copine 1 may be used as an independent biomarker or in combination with both EphA2 and Ki-67 to predict disease outcome.*

Kidney cancers account for 2% of all human malignancies and are known to be one of the major causes of urological malignancies mortality (1). One-fourth of the patients eventually undergo disease recurrence or metastasis, despite radical surgical resection (2). Among all types of renal cell carcinoma (RCC), 70-80% of the cases are diagnosed as clear cell RCC (ccRCC), rendering it the main histologic subtype of RCC (3). Prognostic biomarkers are now useful tools that can be used to determine different responses to treatment regimens and direct individualized therapies. Hardly any marker is used in present clinical practice, notwithstanding the potential of the prognostic biomarker. Therefore, a successful evaluation of the prognostic value of biomarkers is desperately essential.

Copines are a family of calcium-dependent phospholipid-binding proteins that were found to be evolutionally conserved in several eukaryotic organisms in addition to protists. These proteins are encoded by the *CPNE* genes. At present, nine family members have been identified (4). It has been reported that these *CPNE* genes are differentially expressed in various tissues (5). Copine 1 is a member of the Copine family with two N-terminal type II C2 domains in addition to an integrin A domain located at the C-terminus, with no predicted transmembrane domains or predicted signal sequence. Copine 1 expression has been previously reported to be upregulated in triple-negative breast cancer, prostate cancer, lung cancer, and osteosarcoma (6-9). It was found to be associated with prostate cancer and lung cancer patients' survival, and its potential role in regulating tumorigenesis and chemoresistance was also reported (6-9). Copine 3 is another member of the Copine family exhibiting similar calcium-dependent membrane-binding properties. Copine 3 was found to play a different role in the cells' biological functions, specifically at the cell membranes' signalling interface and the cytoplasm. It is elevated in many tumors such as prostate, breast, and ovarian tumors (6, 10-12). In breast cancer, Copine 3 is located at the focal adhesions and was found to play an essential role in cell migration. Furthermore, Copine 3 was found to hinder metastasis and tumor invasion in non-small cell lung cancer (NSCLC) (13). Accordingly, it can be a promising potential therapeutic target for NSCLC secondaries (12). However, no studies have so far investigated the potential role of Copines 1 or 3 as diagnostic and predictive biomarkers in RCC. This study aimed at investigating the mRNA and protein

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expression levels of Copines 1 and 3 in patients diagnosed with RCC in correlation with the clinicopathological parameters and the patients' outcome. Additionally, the association of the previously identified biomarkers, Ki-67 and EphA2 (14), and Copines 1 and 3 was investigated in the same cohort.

Materials and Methods

The present study entailed 50 formalin-fixed paraffin-embedded (FFPE) blocks primarily diagnosed as RCC. The patients were surgically treated by radical or partial nephrectomy at Alexandria University Main Hospital, Egypt, from 2013 to 2018. Both stage IV RCC and locally recurring RCC cases were excluded. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the Research Ethics Committee of Alexandria Faculty of Medicine.

Clinicopathological data. Clinicopathological and 24-month follow-up data for all patients were retrieved from their files in both Pathology and Urology Departments, Alexandria Faculty of Medicine, Egypt. Accordingly, the patients were dichotomized into two groups: First group (G1), including patients free of the disease at the end of the follow-up period, and second group (G2), including patients who experienced either recurrence or distant metastasis at the end of the same period.

Histopathological features. Two independent pathologists reviewed the Hematoxylin and Eosin-stained slides to confirm the primary diagnosis and the tumor typing according to the 2016 World Health Organization (WHO) classification of renal neoplasms. The RCC cases were graded and staged according to the International Society of Urological Pathology (ISUP)/WHO grading system and the American Joint Committee on Cancer (AJCC) 2018 TNM classification, respectively (15-17).

Immunohistochemical staining and interpretation. Immunohistochemical (IHC) staining was performed using the following primary antibodies in compliance with the manufacturer's instructions: 1. Rabbit polyclonal Copine 1 antibody (NBP1-32194, Novus Biologicals, Littleton, CO, USA) and 2. Rabbit polyclonal Copine 3 antibody (NBP1-85939, Novus Biologicals). Positive and negative controls were included in all runs.

Interpretation of the IHC staining was performed based on staining intensity and percentage as previously described (9). The percentage of positive cells was scored in each section as follows: 0, staining in 0% of the cells; 1, staining in £ 10% of the cells; 2, staining in >10 but £ 50% of the cells; 3, staining in >50 and £ 75% of the cells; and finally, 4 if staining in >75% of the cells. The scores for the intensity were: 0, negative; 1, mild; 2, moderate; and 3, strong. The final immunostaining score was calculated by multiplying both scores. Cases with negative and mild immunostaining were considered to have a low expression level, while those with moderate and strong staining were interpreted as of high protein expression level.

Table I. *Demographic and clinicopathological characteristics*.

	N	%
Age in years [mean, (sd)]	55.8 (9.97)	
Age group		
≤55	26	52.0
>55	24	48.0
Gender		
Male	32	64.0
Female	18	36.0
Diagnosis		
Non-clear cell	13	26.0
Clear cell	37	74.0
Tumor size		
≤7	24	48.0
>7	26	52.0
Nuclear grade		
1/2	18	36.0
3/4	27	54.0
Capsular invasion		
Positive	16	32.0
Negative	34	68.0
Vascular invasion		
Positive	4	8.0
Negative	46	92.0
Renal sinus invasion		
Positive	12	24.0
Negative	38	76.0
EPhA2 expression		
0/1	15	30.0
2/3	35	70.0
Ki-67 expression		
Positive	33	66.0
Negative	17	34.0
TNM Stage		
1/2	28	56.0
3/4	22	44.0
Extent of invasion		
Localized	37	74.0
Metastatic	13	26.0
Copine 1		
Low	8	16.0
High	42	84.0
Copine 3		
Negative	25	50.0
Positive	25	50.0
Survival status		
Alive	39	78.0
Dead	11	22.0

In silico validation

CPNE1 and CPNE3 mRNA expression in RCC in publicly available transcriptomic database. To validate the findings, the GEO Omnibus profiles (18) were explored using RCC+CPNE1 and RCC+CPNE3 in the search option. One dataset showed the presence of both probes (GSE781) and was investigated for the normalized gene expression of both genes between RCC (n=12) and normal kidney tissue (n=5).

Correlation of CPNE1 and CPNE3 mRNA expression with the overall survival of patients with RCC. To investigate the correlation of *CPNE1* and *CPNE3* mRNA expression with the overall survival (OS) of RCC patients, 530 ccRCC samples from the Pan-cancer RNA-seq dataset of the KM plotter database were explored (19).

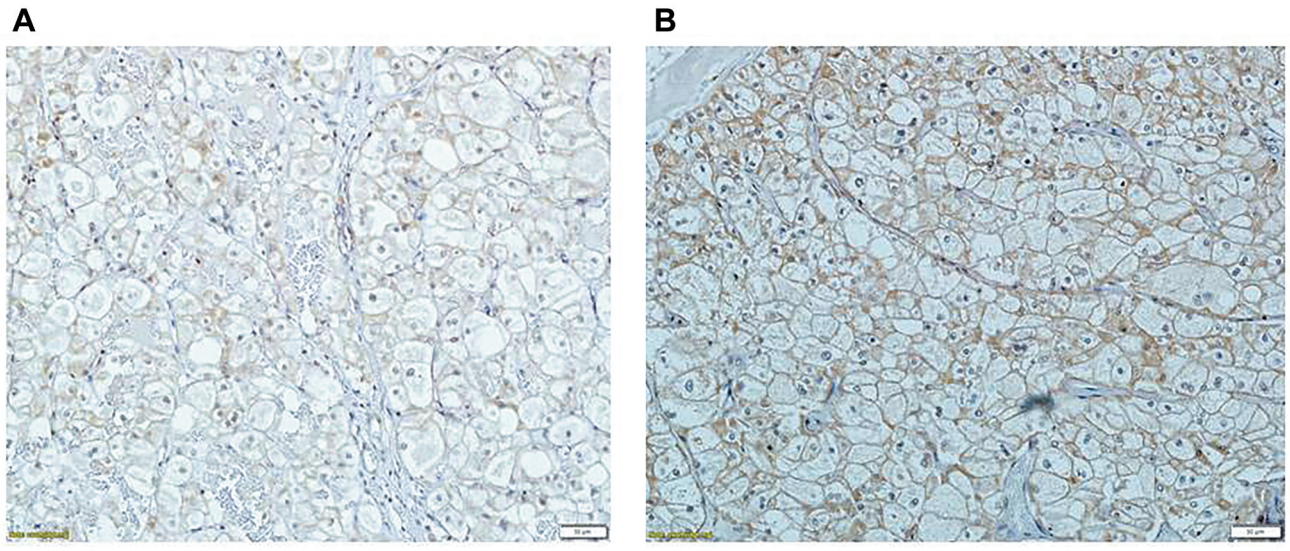


Figure 1. *Immunohistochemical staining of Copine 1 in renal cell carcinoma tissues. (A) Representative image of low expression. (B) Representative image of high expression (Scale bar: 50 μm).*

Different clinical attributes in patients with clear cell carcinoma with high versus low CPNE1 mRNA expression. The differences in clinical attributes in patients with high (above median) *CPNE1* mRNA expression and those with low (below median) expression were investigated. ccRCC (TCGA, Firehose Legacy) dataset (n=537 patients) was extracted using the public domain (http://gdac.broadinstitute.org/runs/stddata__2016_01_28/data/KIRC/20160128/). The patients were divided and compared according to their CPNE1 mRNA expression and their clinical attributes using cBioportal online tool.

Statistical analysis. Data were analyzed using Statistical Package for Social Sciences (SPSS) version 26. Categorical data were summarized and presented as frequencies and proportions, while continuous normally distributed data were summarized as means and standard deviations. The normality of data was checked visually using the Q-Q plots and statistically using the Kolmogorov-Smirnov test. Associations between categorical variables were studied using the Chi-square test, where the level of significance was set at 5%. The strength of association was measured and reported using the odds ratio (OR) and its 95% confidence interval (CI).

Results

The clinicopathological and demographic data of the studied patients' cohort (n=50), along with the expression levels of Copines 1 and 3, EphA2, as well as the proliferation marker Ki-67, are presented in Table I.

The IHC results showed that Copine 1 was highly expressed in 84% (n=42) of the cases, whereas Copine 3 was expressed in only 50% (n=25) of the cases. Figure 1 shows representative sections of low Copine 1 expression (Figure 1A) *versus* high expression levels of the same protein (Figure 1B).

Table II. *Association between Copine 1 levels and demographic factors.*

Variable	High level n (%)	Low level n (%)	Chi-square	*p*-Value
Age group				
≤55	25 (96.2%)	1 (3.8%)	5.953	0.021
>55	17 (70.8%)	7 (29.2%)		
Gender				
Male	27 (84.4%)	5 (15.6%)	0.009	1.000
Female	15 (83.3%)	3 (16.7%)		

Based on the demographic data, Copine 1 expression was inversely associated with age (p=0.021) as 96.2% of patients under 55 years old exhibited high levels of Copine 1 expression. Furthermore, Copine 1 expression was not associated with sex, as shown in Table II. On the other hand, Copine 3 expression showed no association with the demographic factors.

The clinicopathological data shown in Table III revealed a positive association between Copine 1 expression and nuclear grade, EphA2 and Ki-67 expression, and pathological TNM stage of the tumor (p=0.004, 0.006, 0.013, 0.006, respectively).

Although the correlation between Copine 1 expression and the OS of patients did not reach statistical significance, the present data showed that 26.2% of the cohort with high Copine 1 expression deceased within five years. Interestingly, no patients with low Copine 1 expression deceased within the same time interval (Table IV).

Table III. *Prevalence of clinical indicators according to Copine 1 levels.*

Variable	High level n (%)	Low level n (%)	Chi-square	*p*-Value
Diagnosis				
Clear cell	31 (73.8%)	6 (75.0%)	0.005	1.000
Non-clear cell	11 (26.2%)	2 (25.0%)		
Tumor size				
≤7	18 (42.9%)	6 (75.0%)	2.782	0.132
>7	24 (57.1%)	2 (25.0%)		
Nuclear grade				
1/2	11 (29.7%)	7 (87.5%)	9.147	0.004*
3/4	26 (70.3%)	1 (12.5%)		
Capsular invasion				
Positive	15 (35.7%)	1 (12.5%)	1.664	0.409
Negative	27 (64.3%)	7 (87.5%)		
Renal sinus invasion				
Positive	12 (28.6%)	0 (0.0%)	3.008	0.173
Negative	30 (71.4%)	8 (100.0%)		
EPhA2 expression				
0/1	9 (21.4%)	6 (75.0%)	9.184	0.006*
2/3	33 (78.6%)	2 (25.0%)		
Ki-67 expression				
Positive	31 (73.8%)	2 (25.0%)	7.134	0.013*
Negative	11 (26.2%)	6 (75.0%)		
TNM stage				
1/2	20 (47.6%)	8 (100.0%)	7.483	0.006*
3/4	22 (52.4%)	0 (0.0%)		
Extent of invasion				
Localised	30 (71.4%)	7 (87.5%)	0.902	0.662
Metastatic	12 (28.6%)	1 (12.5%)		

*$p<0.05$.

Table IV. *Patients' deaths according to demographic and clinicopathological parameters.*

Variable	Dead n (%)	OR [95%CI]	Chi-square	*p*-Value
Age group				
≤55	9 (34.6%)	5.824	5.024	0.025*
>55	2 (8.3%)	[1.110-30.559]		
Gender				
Male	9 (28.1%)	3.130	1.943	0.287
Female	2 (11.1%)	[0.595-16.459]		
Diagnosis				
Clear cell	9 (24.3%)	1.768	0.448	0.704
Non-clear cell	2 (15.4%)	[0.328-9.518]		
Tumor size				
≤7	6 (25.0%)	1.400	0.242	0.623
>7	5 (19.2%)	[0.365-5.365]		
Nuclear grade				
1/2	2 (11.1%)	0.250	2.888	0.156
3/4	9 (33.3%)	[0.047-1.333]		
Capsular invasion				
Positive	5 (31.3%)	2.121	1.173	0.297
Negative	6 (17.6%)	[0.535-8.403]		
Renal sinus invasion				
Positive	4 (33.3%)	2.214	1.182	0.424
Negative	7 (18.4%)	[0.517-9.475]		
EPhA2 expression				
0/1	2 (13.3%)	0.444	0.938	0.468
2/3	9 (25.7%)	[0.084-2.362]		
Ki-67 expression				
Positive	10 (30.3%)	6.944	3.899	0.073
Negative	1 (5.9%)	[0.808-58.823]		
TNM Stage				
1/2	3 (10.7%)	0.210	4.723	0.042*
3/4	8 (36.4%)	[0.048-0.922]		
Extent of invasion				
Localised	4 (10.8%)	0.104	10.383	0.003*
Metastatic	7 (53.8%)	[0.023-0.468]		
Copine 1				
Low	0 (0.0%)	0.795	2.686	0.174
High	11 (26.2%)	[0.677-0.932]		
Copine 3				
Negative	5 (20.0%)	1.262	0.117	0.733
Positive	6 (24.0%)	[0.329-4.831]		

*$p<0.05$. OR: Odds ratio; CI: confidence interval.

On the contrary, no significant correlation was observed between Copine 3 expression and the clinicopathological parameters, indicating that it cannot be considered as a prognostic or predictive marker in RCC (Table IV).

The obtained results were validated using the GEO Omnibus profiles (GEO Profiles Search database). Figure 2 shows that *CPNE1*, but not *CPNE3* mRNA expression, was higher in RCC compared to healthy controls ($p<0.05$).

The correlation between increased mRNA expression of *CPNE1* and *CPNE3* and the OS of patients with RCC was investigated. A cohort of 530 ccRCC samples from the Pan-cancer RNA-seq dataset of the KM plotter database was explored. Figure 3 shows that RCC patients with high (above median) *CPNE1* expression showed poor prognosis compared to those with low (below median) expression (log-rank p=2.4e-10). Interestingly, *CPNE3* showed the opposite trend, where higher *CPNE3* expression was associated with a good prognosis (log-rank p=4.2e-8).

The OS showed the same trend, which confirms the previous findings that *CPNE1* mRNA expression is a poor prognostic marker. RCC patients with high *CPNE1* mRNA expression showed a higher percentage of death, more metastases, and worst stage and grade than the low expression group (Figure 4) ($p<0.05$).

Discussion

RCC is a heterogeneous disease with a broad spectrum of prognosis. Selection of the treatment and follow-up protocols depends on reliable disease outcome prediction based primarily on clinical and pathological prognostic factors.

The pathological TNM stage and histological grade are currently the best prognostic markers. Recently, several attempts

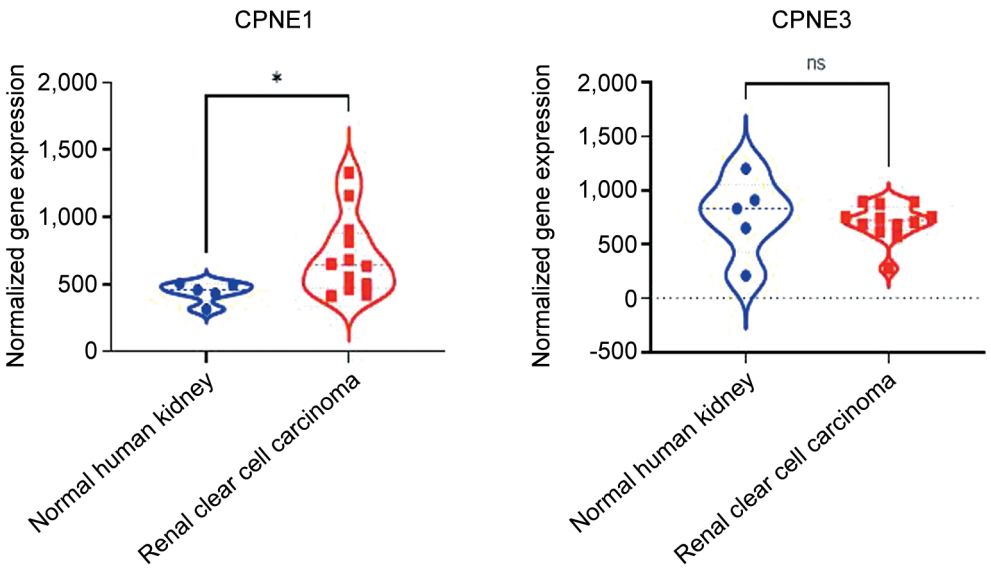


Figure 2. *Normalized mRNA expression of CPNE1 and CPNE3 between renal cell carcinoma (n=12) and normal kidney tissue (n=5) using (GSE781), *p<0.05.*

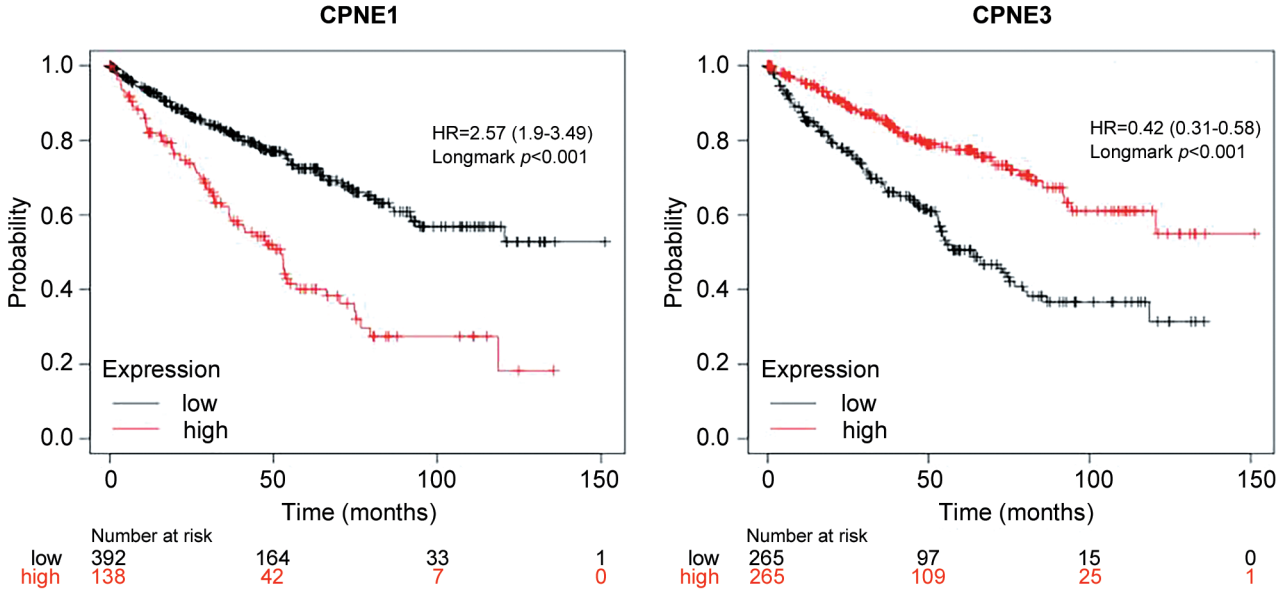
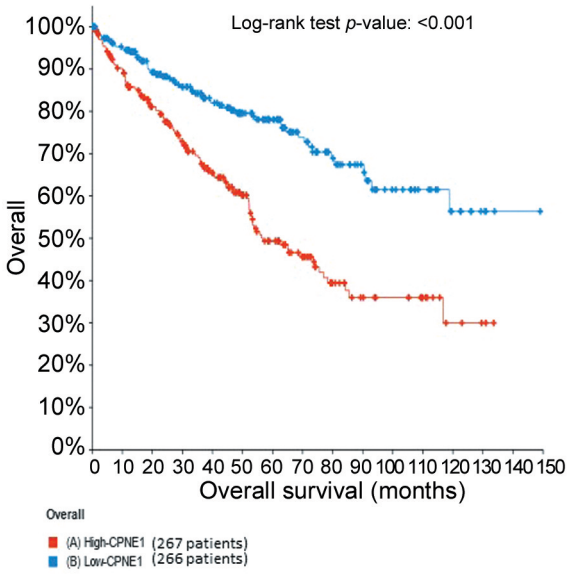


Figure 3. *Overall survival plot of the renal cell carcinoma patients with high and low mRNA expression of CPNE1 and CPNE3 in a cohort of 530 ccRCC samples from the Pan-cancer RNA-seq dataset of the KM plotter database were explored.*

have been made to incorporate multiple prognostic biomarkers, both pathological and clinical, into comprehensive frameworks designed to enhance outcome prediction for RCC patients and to help achieve stronger prognostic tools (20).

Copines are a family of calcium-binding proteins encoded by *CPNE* genes found to be differently expressed in various tissues (4, 21). In particular, Copines 1 and 3 are reported to be associated with different types of cancers (6-9, 22-25). This study aimed to investigate the prognostic role of Copines 1 and 3 in RCC, and their association with disease outcome using a combined approach of immunohistochemistry and bioinformatics analysis.

A



B

Clinical Attribute	Attribute Type	Statistical Test	p-Value	q-Value
Person Neoplasm Status	Patient	Chi-squared Test	9.16E-08	5.40E-06
American Joint Committee on Cancer Metastasis Stage Code	Patient	Chi-squared Test	9.30E-06	1.37E-04
Neoplasm Disease Stage American Joint Committee on Cancer Code	Patient	Chi-squared Test	2.45E-04	2.90E-03
Neoplasm Histologic Grade	Patient	Chi-squared Test	1.15E-03	9.73E-03
Tissue Source Site	Patient	Chi-squared Test	2.84E-03	0.021

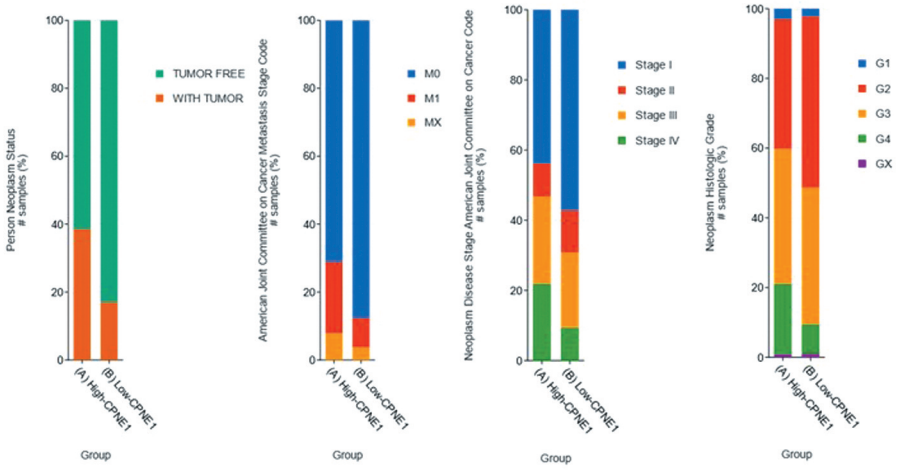


Figure 4. *Clinical attributes of the patients with clear cell renal cell carcinoma (ccRCC) with high (above median) CPNE1 mRNA gene expression and those with low (below median) expression using ccRCC (TCGA, Firehose Legacy) dataset (n=537 patients): Patients were divided according to their CPNE1 mRNA expression and their clinical attributes. The groups were compared using cBioportal online tool. (A) Overall survival plot. (B) Most significant clinical characteristics.*

Consistent with previous studies demonstrating increased Copine 1 expression in lung cancer, prostate cancer, triple-negative breast cancer (TNBC), and osteosarcoma, we also found increased Copine 1 expression in RCC patients' samples. Copine 1 expression was found to be markedly associated with distant metastasis, TNM stage, and OS, but not with age and tumor size in patients with NSCLC (9, 26), whereas it was correlated with tumor stage and recurrence-

free survival, but not with age in patients with prostate cancer (7). Copine 1 expression was also found to be associated with tumor size, distant metastasis, and OS, but not with age and TNM stage in patients with TNBC (6). Also, a significant correlation was observed between Copine 1 over-expression and the occurrence of osteosarcoma (8). However, the current study results revealed that Copine 1 expression is inversely associated with age but not with sex and positively associated with nuclear grade, EphA2, Ki-67 expression, and pathological TNM stage.

High expression levels of EphA2 and Ki-67 were previously reported by our team to be significantly associated with RCC on the same cohort sample (14). Interestingly, our results also showed a significant association between the expression of EphA2, Ki-67, and Copine 1. Within the same studied cohort, the expression EphA2 and Ki-67 was reported to be highly expressed in an aggressive phenotype with high grade and advanced stage. This observed correlation between Copine 1, EphA2, and Ki-67 in RCC would offer a powerful multi-biomarker panel signature in RCC to predict the disease prognosis and potential future outcomes.

On the other hand, high expression of Copine 3 was inversely associated with OS and event-free survival in acute myeloid leukemia (27). Also, Copine 3 was reported to be a poor prognostic marker in NSCLC as it is significantly associated with advanced TNM stages (10, 24). On the contrary, our findings did not support the presence of any association between Copine 3 expression and the demographic or clinicopathological parameters of our patients. The potential role of Copine 3 in the pathogenesis of other cancers is well documented in the literature (10, 11, 25, 27). However, this role could be selective to certain types of cancer, and Copine 3 may not influence the pathogenesis of RCC. Further studies on the role of Copine 3 in RCC pathogenesis are recommended.

The bioinformatics analysis using publicly available databases was used to validate our findings. *CPNE1* but not *CPNE3* mRNA expression was higher in RCC compared to healthy controls, and Copine 1 expression showed poor prognostic value in patients with RCC. Those results are in accordance with the findings obtained by immunohistochemical analysis.

Conclusion

The identification of reliable and validated biomarker(s) in the context of specificity and sensitivity is essential for better disease diagnosis and prognosis. Our results revealed that high Copine 1 expression is associated positively with RCC aggressiveness and advanced stages. Interestingly, Copine 1 can be used as an independent prognostic biomarker in RCC as well as in combination with EphA2 and Ki-67. This study gives a rationale to develop a biomarker signature panel with potential benefits of using personalized therapeutic strategies to improve the disease outcome.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

Conceptualization, W.E.-H., I.T.; Methodology, W.E.-H., E.A.-G.; Statistical Analysis, A.H., E.A.-G.; Investigation, W.E.-H., E.A.-G., I.S., M.H.; Writing—Original Draft Preparation, W.E.-H., I.T., E.A.-G.; Data curation, W.E.-H., I.T., E.A.-G., M.E.; Writing—Review & Editing, W.E.-H., I.T., E.A.-G.; Supervision, W.E.-H., I.T.; Project Administration, W.E.-H., I.T.; Funding Acquisition, W.E.-H. All Authors have read and agreed to the published version of the manuscript.

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