

TCEAL2 as a Tumor Suppressor in Renal Cell Carcinoma is Associated with the Good Prognosis of Patients

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Background: Renal cell carcinoma (RCC) is one of the most common tumors in urinary tract tumors. However, the mechanism that supports renal cell carcinoma is unclear. The function of transcription elongation factor A (SII)-like 2 (TCEAL2) and its association with human cancer have not been reported.

Materials and Methods: To explore the role of TCEAL2 in carcinogenesis of clear cell renal cell carcinoma (ccRCC), we performed bioinformatics analysis to determine the expression levels of TCEAL2 in ccRCC specimens and normal kidney tissue and then verified findings with our samples by qPCR, Western blot and immunohistochemistry staining. Furthermore, the lentiviral vectors were used to increase the expression of TCEAL2 in ccRCC cell lines. The immunofluorescence assay was taken to observe the subcellular location of TCEAL2 in ccRCC cells, and CCK-8 and flow cytometry were introduced for assessing cell proliferation and cell cycle of ccRCC cells, respectively.

Results: Compared with adjacent normal kidney tissue and human proximal tubular epithelial cells, the expression of TCEAL2 in ccRCC tissues and cell lines was down-regulated. Patients who had low expression of TCEAL2 had a statistically significant late tumor stage. Restore of TCEAL2 in ccRCC cells inhibited cell proliferation and induced cell cycle arrest in S phase of ccRCC cells.

Conclusion: To our knowledge, this is the first report of TCEAL2 expression changes in ccRCC. We found that the decrease of TCEAL2 expression may be related to the occurrence of ccRCC. Further research is needed to clarify the molecular mechanism of TCEAL2 in progress of ccRCC.

Keywords: TCEAL2, renal cell carcinoma, tumorigenesis, cell cycle

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Introduction

Renal cell carcinoma (RCC), also known as renal adenocarcinoma, is one of the common tumors in the urinary system and the second leading cause of death in the world. About 200,000 people were diagnosed with renal cell carcinoma every year around worldwide. The national age-standardized incidence rate is 12/100,000, accounting for 2% to 3% of adult malignancies, and accounting for approximately 90% of kidney tumors, and its incidence is increasing in Europe.¹ Renal cell carcinoma originates from renal tubular epithelial cells, clear cell carcinoma of the kidney is most common.² Its treatment plan is still based on radical surgery. Postoperative adjuvant treatment options are diverse, but effective adjuvant treatment options are lacking.³ However, due to asymptomatic and non-specific markers

in the early stages of renal cancer, most patients come to the hospital when tumor immersion and painless hematuria appear in the renal pelvis.⁴ Therefore, an exploration of potential molecular mechanisms and the identification of effective biomarkers involved in the pathogenesis of clear cell renal cell carcinoma (ccRCC) is urgently needed to improve the effectiveness of therapeutic strategies.

The transcription elongation factor A-like 2 (TCEAL2) gene (also known as WEX1, my048 and MY0876G05) was first cloned and located on the Xq22.1 chromosome in 2005.^{5,6} It spans 2025 bp and consists of three exons. Human TCEAL2 mRNA is 1100 bp long and encodes a protein of 227 amino acids with a relative molecular mass of approximately 26 kDa.⁷ TCEAL2 belongs to the transcription elongation factor A (SII)-like (TCEAL) gene family containing TFA domains.⁸ It has been reported as a nuclear phosphoprotein that modulates transcription in a promoter context-dependent manner and has been recognized as an important nuclear target for intracellular signal transduction.⁸ It was reported that up-regulation of TCEAL2 might be associated with a poor prognosis patients with ovarian cancer.⁹ Recent studies have shown that TCEAL2 expression was found to be reduced in ccRCC, TCGC and TCC whole-genome sequencing.¹⁰ However, the expression and physiological effects of TCEAL2 in ccRCC have not been studied so far.

Here, we confirmed that TCEAL2 is generally reduced in the mRNA and protein levels of ccRCC tissues and cell lines, and the decreased TCEAL2 expression is related to the clinicopathological parameters of renal cell carcinoma. Therefore, the change of TCEAL2 protein level might be used for early detection of RCC, monitoring tumor progression and predicting clinical markers of patient prognosis.

Materials and Methods

Cell Culture and ccRCC Tissue Specimens

The clear cell renal cell carcinoma cells (786-O, 769-P, ACHN, Caki-1 and Caki-2) and human proximal tubular epithelial cell line (HK-2) were obtained from the American Type Culture Collection. All cell lines were maintained in culture mediums as described previously.¹¹ All tissue samples for RT-qPCR were obtained from ccRCC patients without undergoing radiotherapy and chemotherapy between 2013 and

2015 at the Department of Urology, Peking University Shenzhen Hospital, China. All experiments followed the “Helsinki Declaration” and were approved by the Ethics Committee of Peking University Shenzhen Hospital (NO.20090017). All patients were informed of their specimen content, potential risks, purpose and signed written informed consent.

Database Mining

The TCGA data and GEO dataset GSE53757 were downloaded to investigate the expression of TCEAL2 mRNA levels in ccRCC and normal kidney tissues, and the correlation of TCEAL2 and clinicopathological characteristics of patients with ccRCC on April 18, 2019.

RT-qPCR

Total RNA was isolated from freshly frozen clear cell renal cell carcinoma tissues or cell lines using RNAiso Plus lysis reagent (Takara, Japan). One microgram of total RNA was reversely transcribed to cDNA using the PrimeScript RT reagent Kit (Takara, Japan). The TCEAL2 gene primer amplification sequence: forward: 5'-AACATGGCTAGGGTGGAGGA-3'; reverse: 5'-TCCACGAATCATGACGTCTCT-3'. The human GAPDH gene was used as an endogenous control for RNA standardization and amplified with the following primer pairs: forward: 5'-GCTCTCCAGAACATCATCCCTGCC-3'; reverse: 5'-CGTTGTCATACCAGGAAATGAGCTT-3'. The qPCR was performed using SYBR Premix Ex TaqTMII Kit (Takara, Japan).

Western Blotting

Western blotting was performed as described previously.¹² Primary antibodies used were against TCEAL2 (1:1000, Santa Cruz Biotechnology, Inc. USA) and β -tubulin (1:5000, Abcam, UK). The secondary antibody was anti-rabbit (1:2000, Cell Signaling Technology, USA). Luminescence of protein bands was observed by using a chemiluminescence imaging system (Tanon-5200Multi, Tanon, China).

Immunohistochemistry Staining

Immunohistochemistry staining was performed on Single spot tissue micro arrays (TMA) slides (90 points of RCC, 90 points of adjacent normal tissue, HKid-CRC180Sur-01, Shaanxi Avila Biotechnology Co., Ltd, Shaanxi, China) using the TCEAL2 rabbit antibody (1:100, Santa Cruz Biotechnology, Inc. USA) and the rabbit streptavidin-biotin detection system (Beijing

Zhongshan Golden Bridge Biotechnology Co., Ltd. Peking, China).

The TMA was manually scored by two pathologists (X. H. and G.Y.) according to a 4-layer scoring system. We defined tumor cells with $\geq 30\%$ strong staining or $\geq 70\%$ moderate staining as a score of 3+. Tumor cells with $>70\%$ weak staining or >30 and $\leq 70\%$ medium staining or $\leq 30\%$ strong tumor cell staining were defined as a score of 2+. When $\leq 70\%$ of tumor cells were weakly positive or $\leq 30\%$ were moderately stained, the score was

1+. Less staining was defined as negative (score 0). Inconsistent results are resolved through consensus review.

Immunofluorescence Staining

For immunofluorescence, 1×10^4 ccRCC cells with or without TCEAL2 overexpression were seeded on cell slides. Cells in logarithmic phase were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, blocked with blocking buffer, and incubated with

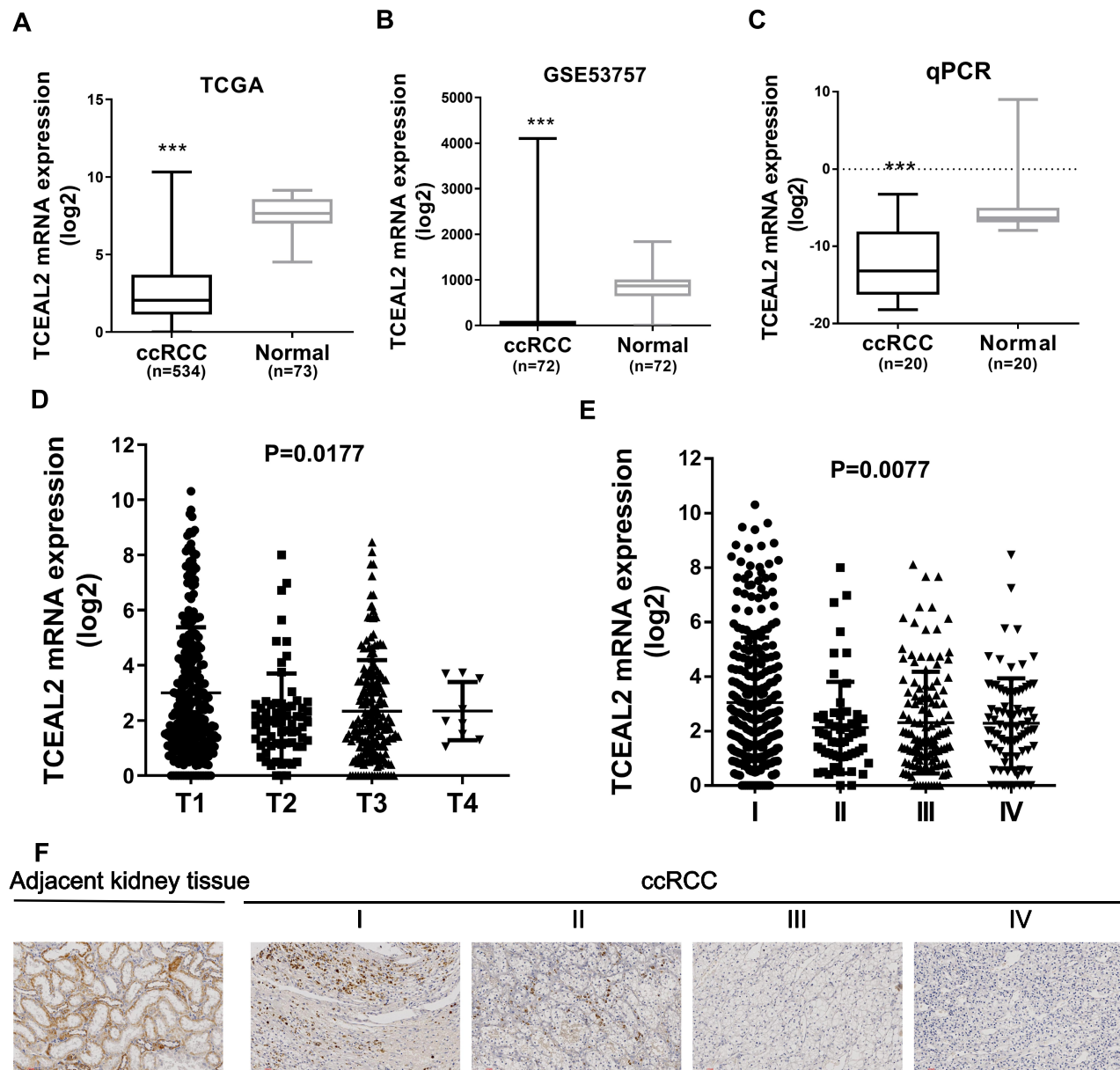


Figure 1 TCEAL2 was down-regulated in ccRCC tissues. (**A** and **B**) The expression of TCEAL2 in ccRCC tissues was significantly lower than that in normal kidney tissues by TCGA and GSE53757. (**C**) RT-qPCR result revealed that TCEAL2 mRNA in 20 cases of ccRCC tissues compared to the adjacent normal kidney tissues. (**D** and **E**) The expression of TCEAL2 mRNA was related to the tumor grade and stage in renal cancer patients. (**F**) The expression of TCEAL2 protein in kidney cancer tissues was lower than that in adjacent normal kidney tissues, and was related to tumor stage by immunohistochemistry staining. Scale bar = 100 μ m. t-test, Mean $\pm$ SD, ***p < 0.001.

primary antibody against TCEAL2 overnight at 4°C. Samples were then labeled with fluorochrome-conjugated secondary antibodies (Life Technology, USA). Finally, DAPI staining for DNA was performed. Cells covered with fluorescence mounting medium (DAKO, CA, USA) were examined using a fluorescence system mounted on a BX53 upright microscope (Olympus, Japan).

Establishment of ccRCC Cells with TCEAL2 Stable Expression

The lentiviral vector GV358-TCEAL2 and control vector were obtained from Genechem technology company (Shanghai, China). To establish ccRCC cells stably expressing TCEAL2, 2×10^4 ccRCC cells per well were seeded in 6-well plate and infected with lentiviral vector GV358-TCEAL2. One-milliliter fresh culture mediums were added in cells at 24 h after infection. The cells were observed under fluorescent microscope at 72 h after infection.

Cell Proliferation

3×10^3 /well ccRCC cells with lentiviral vector GV358-TCEAL2 or control vector were inoculated into 96-well plates, and the proliferation was analyzed at 0, 24, 48 and 72 hours by using cell counting kit 8 (CCK-8) (Medium: Cell counting kit 8=10:1, Dojindo Laboratories, Kumamoto, Japan).

Flow Cytometry for Cell Cycle

ccRCC cells with or without TCEAL2 stable expression cultured for 48h were collected and washed twice with PBS, and then fixed with 70% ice-cold ethanol overnight at 4°C. The fixed cells were resuspended in PBS containing PI/RNase staining buffer (BD Biosciences, San Diego, CA), and incubated for 15 minutes at room temperature. Cells were subjected to flow cytometric analysis of DNA content using a flow cytometer (BD Accuri™ C6 Plus, BD Bioscience, CA). The percentages of cell cycle distribution were calculated by Flowjo VX software.

Statistical Analysis

All data from three repeated experimental results are expressed as mean $\pm$ SD. The correlation of TCEAL2 and each clinical pathological variable was comparatively analyzed by χ^2 test, the Fisher exact test and Chi-square test. A p value of less than 0.05 indicated the presence of statistically significant difference between groups. All statistical analyses were carried out with SPASS 19.0 and GraphPad Prism 6 software.

Results

TCEAL2 Was Down-Regulated in ccRCC Tissues and Cell Lines

To identify our preliminary findings, the TCGA visual database GEPIA and cBioPortal and the GEO Profile GSE53757 were adopted. The expression of TCEAL2 in patients with ccRCC was significantly lower than that in patients without ccRCC (Figure 1A and B). In addition, immunohistochemical staining showed that 82 cases (91.1%) of RCC cases had negative

Table 1 Expression of TCEAL2 in ccRCC and Adjacent Normal Kidney Tissues

TCEAL2	No. of Cases	Type of Tissues		χ^2	P
		Adjacent Tissue	ccRCC		
Negative	92	10	82	115.3	<0.0001***
Positive	88	80	8		

Notes: Pearson's χ^2 test; ***p values < 0.001.

Table 2 Correlation Between TCEAL2 Expression and the Clinicopathological Characters of Patients with Clear Cell Renal Cell Carcinoma (IHC)

Clinico-Pathologic Variables	No. of Cases	TCEAL2 Expression		χ^2	P
		Negative	Positive		
All cases	90	82	8		
Gender				1.202	0.2730
Male	51(56.7%)	45(88.2%)	6(11.8%)		
Female	39(43.3%)	37(94.9%)	2(5.1%)		
Age				0.2297	0.6317
<60	49(54.4%)	44(89.8%)	5(10.2%)		
≥60	41(45.6%)	38(92.7%)	3(7.3%)		
Tumor stage				1.817	0.6112
I	65(72.2%)	60(66.7%)	5(5.5%)		
II	19(21.1%)	16(17.8%)	3(3.3%)		
III	4(4.4%)	4(4.4%)	0(0%)		
IV	2(2.2%)	2(2.2%)	0(0%)		
T grade				0.5979	0.7416
T1	67(74.4%)	61(67.8%)	6(6.6%)		
T2	18(20%)	16(17.8%)	2(2.2%)		
T3	5(5.6%)	5(5.6%)	0(0%)		
T4	0	0(0%)	0(0%)		
Years of survival				0.0363	0.8489
<5	31(34.4%)	28(90.3%)	3(9.7%)		
≥5	59(65.6%)	54(91.5%)	5(8.5%)		

Note: Pearson's χ^2 test.

expression of TCEAL2 in cancer tissues, while only 8 cases (8.9%) of adjacent normal tissues had low expression of TCEAL2 ($P < 0.001$) (Table 1). By analyzing the clinicopathological data of 90 patients with tissue microarray, the expression of TCEAL2 was not related to the patient's tumor stage, and the difference was not statistically significant ($P > 0.05$) (Table 2). Furthermore, in accordance with the immunohistochemical staining, RT-qPCR data also showed a significant decrease of TCEAL2 mRNA in 20 cases of ccRCC tissues compared to the adjacent normal kidney tissues ($P < 0.001$) (Figure 1C and F). And in the TCGA visual database, the expression of TCEAL2 was related to the tumor stage and T grade in renal cancer patients ($P < 0.001$) (Figure 1D and E). Then, TCEAL2 expression levels were detected in ccRCC cell lines (786-O, 769-P, ACHN and Caki-1) and normal renal tubular epithelial cell lines (HK-2). As shown in Figure 2A and B, TCEAL2 expression was significantly reduced in the

786-O, 769-P, ACHN and Caki-1 cell lines at both transcription and translation levels, compared to the HK-2 cell line ($p < 0.001$, respectively).

TCEAL2 Under-Expression is Associated with the Tumor Stage of Patients with ccRCC

We analyzed the possible correlation between the TCEAL2 mRNA level in 534 ccRCC samples and the clinical characteristics of ccRCC through TCGA data. As summarized in Table 3, patients were divided into two subgroups based on TCEAL2 expression level: low-expression group ($n = 510$) and high-expression group ($n = 24$). Compared with the high-expression group, the TCEAL2 low-expression group more appeared in advanced tumor stage ($P = 0.0364$), T grade ($P = 0.0466$) and male ($P = 0.0073$). There was no significant correlation between the expression level of TCEAL2 and age and 5-year

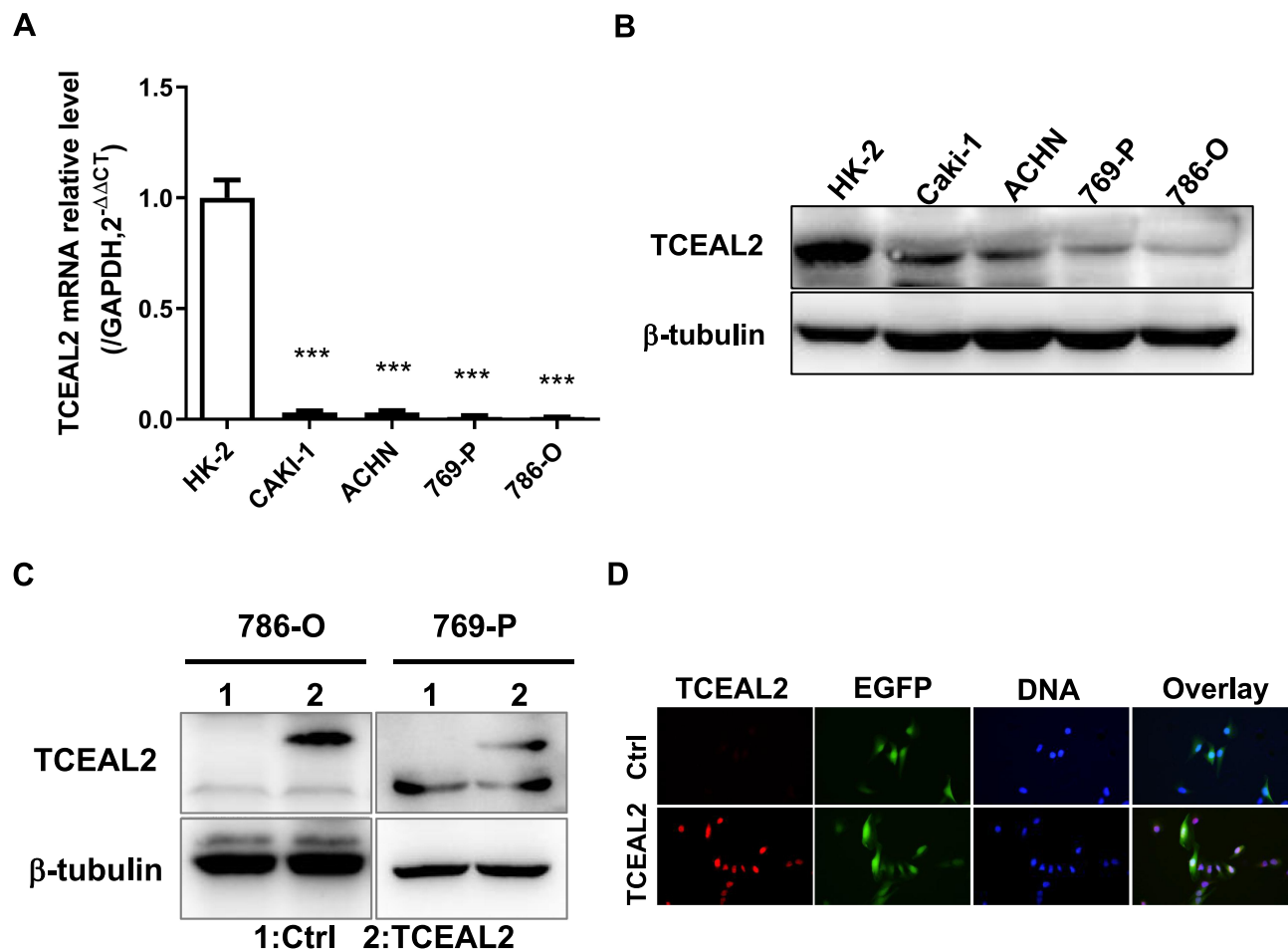


Figure 2 TCEAL2 was down-regulated and located in the nucleus of ccRCC cell lines. (A and B) TCEAL2 was remarkably decreased in ccRCC cell lines 786-O, 769-P, ACHN and Caki-1 compared to human proximal tubular epithelial cell line HK-2 at both transcription and translation levels. (C) The protein level of TCEAL2 in ccRCC cell lines 786-O and 769-P with infection of TCEAL2 lentivirus was noteworthy higher than that in the control group. (D) TCEAL2 was primarily located in the nucleus by Immunofluorescence assay. Red: TCEAL2, Green: EGFP, Blue: DNA. T-test, Mean $\pm$ SD, *** $p < 0.001$.

Table 3 Correlation Between TCEAL2 Expression and the Clinicopathological Characters of Patients with Clear Cell Renal Cell Carcinoma (TCGA)

Clinico-Pathologic Variables	No. of Cases	TCEAL2 Expression		χ^2	P
		Low	High		
All cases	534	510	24		
Gender					
Male	346	332	14	7.191	0.0073**
Female	188	178	10		
Age					
<60	265	251	14	0.2073	0.6489
≥60	269	259	10		
Tumor stage					
I	268	249	19	8.518	0.0364*
II	57	56	1		
III	123	120	3		
IV	84	83	1		
T grade					
T1	274	255	19	7.913	0.0466*
T2	69	68	1		
T3	180	176	4		
T4	11	11	0		
Years of survival					
<5	510	364	17	0.0033	0.9545
≥5	24	146	7		

Notes: Pearson's χ^2 test; *p values < 0.05; **p values < 0.01.

survival rate (Table 3). According to the above-mentioned TCEAL2 mRNA level and clinicopathological characteristics of ccRCC patients, the multivariate cox-regression analysis showed that the patient's age and tumor stage were related with survival time, and the risk coefficients are 1.428 and 1.988, respectively (Table 4). However, the expression of TCEAL2 in ccRCC has not been a variable that affects their survival time, which may require more samples for evaluation.

TCEAL2 Can Inhibit the Proliferation of ccRCC Cells

To further identify the biological significance of TCEAL2 in ccRCC cells, we first performed the lentivirus to over-express TCEAL2. As shown in results (Figure 2C), after TCEAL2 lentivirus infection, the protein level of TCEAL2 in ccRCC cell lines 786-O and 769-P was significantly higher than that in the control group by Western blot. It indicated that we successfully overexpressed TCEAL2 using lentiviral vector. Immunofluorescence data showed

Table 4 Multivariate Cox-Regression Analysis for Patients After Surgery (TCGA)

Factor	P value	Hazard Ratio	95% Confidence Interval	
			Lower	Upper
Gender				
Male vs female	0.523	1.107	0.81	1.514
Age				
≤60 yrs vs >60 yrs	0.031*	1.428	1.033	1.974
Tumor stage				
I vs II vs III vs IV	0.000**	1.988	1.593	2.481
T grade				
T1 vs T2 vs T3 vs T4	0.393	0.882	0.662	1.176
TCEAL2				
Neg vs pos	0.847	0.906	0.331	2.475

Notes: Cox regression analysis; *p values < 0.05; **p values < 0.01.

that TCEAL2 was located in the nucleus (Figure 2D). CCK-8 assay showed that ccRCC cell lines 786-O and 769-P with TCEAL2 overexpression had notable decreased proliferation ability compared to the control group (Figure 3A-B). This indicated that TCEAL2 might be associated with tumorigenesis of ccRCC.

TCEAL2 Induces Cell Cycle Arrest in S Phase of ccRCC Cells

To better understand why cells overexpressing TCEAL2 proliferated slowly, the cell population of ccRCC cells with or without TCEAL2 stable expression was analyzed by flow cytometry, and the role of TCEAL2 on cell cycle progression was evaluated. As shown in Figure 3, a significantly lower G2/M phase percentage (15.1% for 786-O and 10.1% for 769-P) was observed in TCEAL2 stably expressing ccRCC cells than that in control group cells (22.1% for 786-O and 20.8% for 769-P), while a significantly higher G0/G1 (51.0% for 786-O and 56.2% for 769-P) or S phase (16.2% for 786-O and 15.9% for 769-P) percentage was observed in ccRCC cells stably expressing TCEAL2 than that in control group cells. The above results indicated that TCEAL2 is correlated with the cell cycle progression in ccRCC.

Discussion

The elongation of transcription in eukaryotes is a very complicated process.¹³ When RNAPII slides along the DNA

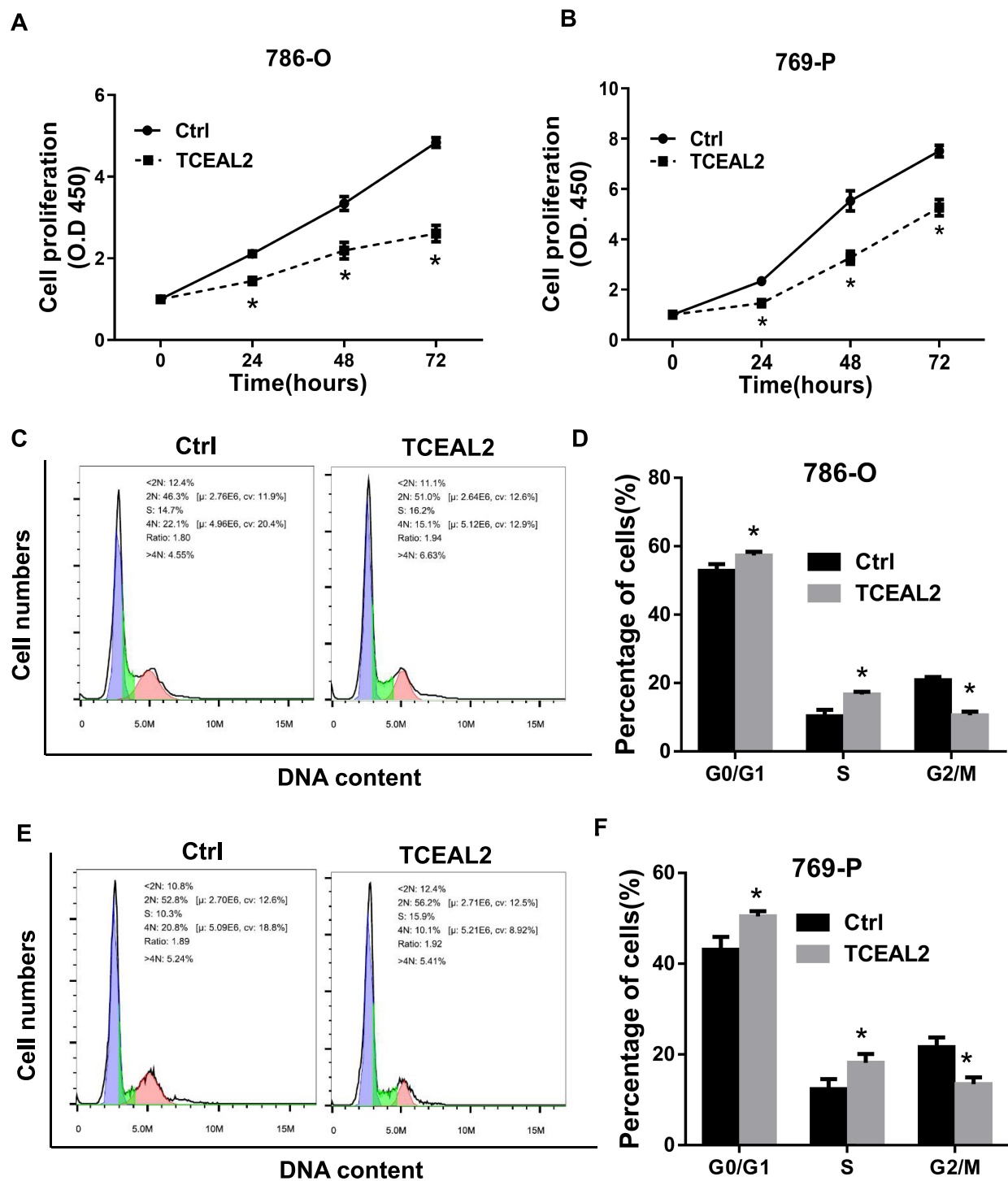


Figure 3 TCEAL2 inhibit the proliferation and induced cell cycle arrest in S phase. **(A and B)** After infection TCEAL2 lentivirus, the proliferation ability of 786-O and 769-P was conspicuous lower than that of control group. **(C–F)** In 786–O and 769 cell line, a significantly lower G2/M phase percentage was observed in TCEAL2 stably expressing ccRCC cells than control group cells. t-test, Mean $\pm$ SD, * $p < 0.05$.

template, it will encounter obstacles and produce instantaneous pauses and arrests. Some protein factors can directly or indirectly interact with RNAP II during this process to inhibit or affect its transient rest and stagnation and thus affect the

efficiency of transcription. A handful of studies showed that transcription elongation factors involved in human ccRCC tumorigenesis and metastasis. Two transcription elongation factors, Elongin B and C were shown to bind in vitro and

in vivo to a short, colinear region of the VHL protein (pVHL) that is frequently mutated in human ccRCC.^{14–16} In our previous study, we found that transcription elongation factor A-like protein TCEAL2 was down-regulated in ccRCC tissues.¹⁰ In this study, we firstly investigated the expression and function of TCEAL2 in clear cell renal cell carcinoma. Compared with normal kidney tissue, the expression of TCEAL2 in ccRCC was significantly reduced at mRNA and protein levels. In addition, the low expression of TCEAL2 was associated with a higher tumor stage and male.

TCEAL2 like other members of the transcription elongation factor A-like family contains a BEX protein conserved domain that regulates gene transcription. Tceal1/SIIR/p21, a nuclear phosphoprotein, can inhibit the transcriptional activity of the long end of Rous sarcoma virus.^{8,17} Down-regulation of Tceal7 encoding a cell death-regulating protein¹⁸ enhances the transcriptional activity of NF- κ B by increasing the expression levels of the cell-promoting genes cyclin D1 and c-Myc, and the angiogenic genes IL-6, IL-8, and VEGF.¹⁹ Up to date, there are few studies about the expression of TCEAL2 in cancers. Kim et al have found up-regulation of TCEAL2 might be associated with a poor prognosis of stage III serous ovarian cancer.⁹ Here, we reported that under-expression of TCEAL2 in ccRCC was associated with higher tumor stage, and TCEAL2 could inhibit cell proliferation and induced cell cycle arrest into S phase.

In addition to the BEX protein domain, TCEAL2 also contains a K⁺-dependent Na⁺/Ca⁺ exchanger (data not shown). It is reported that K⁺-dependent Na⁺/Ca⁺ exchanger can inhibit the proliferation of tumor cells and induce apoptosis by regulating the concentration of calcium ions inside and outside the cell.^{20–22} Since TCEAL2 contains the conserved domains BEX and K⁺-dependent Na⁺/Ca⁺ exchanger, we speculate that TCEAL2 may have similar functions with other members of the transcription elongation factor A-like family.

In short, this is the first study to describe the role of TCEAL2 expression in the development of renal clear cell carcinoma. The results suggest that loss of expression of TCEAL2 might play a role as a tumor suppressor in clear cell renal cell carcinoma. To clarify the mechanisms of TCEAL2 in renal cancer, further molecular biology experiments are required.

Abbreviations

RCC, renal cell carcinoma; TCEAL2, transcription elongation factor A (SII)-like 2; ccRCC, clear cell renal cell carcinoma.

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Disclosure

The authors have no conflicts of interest to declare for this work.

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