ORIGINAL RESEARCH

Expression of 4E-BP1 and phospho-4E-BP1 correlates with the prognosis of patients with clear cell renal carcinoma

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Background: Eukaryotic translation initiation factor 4E (eIF4E) is a key regulator of protein synthesis. Changes in eIF4E activity disproportionally affect the translation of a subset of oncogenic mRNAs in some cancers.

Materials and methods: We have assessed the expression levels of vascular endothelial growth factor C (VEGFC), eIF4E, eIF4E-binding proteins (4E-BPs) and phospho-4E-BP1 in clear cell renal carcinoma (ccRCC; n=101) using immunohistochemistry and analyzed the relevant mRNA levels and survival using online databases.

Results: The protein levels of VEGFC, an eIF4E-regulated gene, were upregulated in ccRCC tissues compared with adjacent normal renal tissues, indicating an enhanced eIF4E activity in ccRCC. The expression of eIF4E had no significant changes in ccRCC tissues. However, 4E-BP1 and phospho-4E-BP1 were found to be overexpressed in ccRCC tissues ($P<0.05$), and the high mRNA and protein levels of 4E-BP1 and phospho-4E-BP1 correlated with an unfavorable clinical outcome in ccRCC patients. Meanwhile, the mRNA expression of PIK3CD and PIK3CG were enhanced in ccRCC.

Conclusion: From these results, we could infer that the increase in eIF4E activity may be caused by the increased phospho-4E-BP1 level, which was probably due to the activation of phosphoinositide 3-kinase (PI3K) pathway.

Keywords: eIF4E, 4E-BP1, phospho-4E-BP1, VEGFC, PI3K, ccRCC

Introduction

Kidney cancer is one of the most common tumors in the urinary system. In the past 2 decades, the amount of kidney cancer has been increasing. Kidney cancer consists of multiple types, including transitional cell carcinoma of kidney, renal cell carcinoma (RCC), inverted papilloma and kidney lymphoma. Among the four types of kidney cancer, RCC accounts for the largest proportion. RCC contains multiple pathological categories, including chromophobe RCC, clear cell renal carcinoma (ccRCC) and papillary RCC. Among them, ccRCC accounts for the highest proportion with ~75% of cases.[1]

The translations of mRNAs need to be tightly controlled in cells as the imbalance of translation leads to cancer occurrence and development.[2–4] At the transcription elongation step, nuclear-transcribed mRNAs have 5′-cap added. Translation in eukaryotic cells begins with binding between 5′-cap and the eukaryotic translation initiation factor 4F (eIF4F).[5,6] eIF4F complex includes eIF4A, eukaryotic translation initiation factor 4E (eIF4E) and eIF4G. eIF4E is able to recognize and bind to 5′-cap of mRNA, allowing the translation initiation.[7,8] Changes in eIF4E activity only have

a tiny influence on the mRNA translation of house-keeping genes such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH).[9] Most house-keeping mRNAs have short and low complexity 5′-untranslated regions (UTRs). Unlike them, some 5′-cap-dependent mRNAs own long 5′-UTRs with a high complexity, whose protein translation is tightly controlled by eIF4E including the mRNAs of cyclin D3, phosphoribosyl pyrophosphate synthetase 2 (PRPS2) and vascular endothelial growth factor (VEGF).[3,10–13]

Besides the amplification of eIF4E, eIF4E activity is also controlled by eIF4E-binding proteins (4E-BPs) such as 4E-BP1, 4E-BP2 and 4E-BP3.[14] 4E-BP1 is the most common isoform of 4E-BPs. 4E-BPs and eIF4G compete with eIF4E for the same binding site.[15–17] The phosphoinositide 3-kinase (PI3K) pathway can phosphorylate 4E-BPs, causing the separation of 4E-BPs and eIF4E.[6] Therefore, the phosphorylated 4E-BP1 is able to promote protein translation. PI3K can phosphorylate phosphatidylinositol 3,4 (PIP2) and activate the AKT.[18,19] The activity of AKT can be inhibited by phosphatase and tensin homolog (PTEN).[6,20,21] PI3Ks belong to three distinct categories called Class I, II and III.[22] PI3K class I consists of multiple genes including *PIK3CA*, *PIK3CB*, *PIK3CD* and *PIK3CG*.

The expression level of eIF4E or 4E-BP1 is deregulated in many cancers such as bladder, ovary and prostate.[23–33] In the present study, we focused on the expression and active levels of 4E-BP1 and eIF4E to confirm the prognostic values of these factors in ccRCC.

Materials and methods
Expression and clinical data obtained from Gene Expression Omnibus (GEO), The Cancer Genome Atlas (TCGA) and The Cancer Proteome Atlas (TCPA) databases

In the research, ccRCC datasets were extracted from the GEO database (http://www.ncbi.nlm.nih.gov/geo/). Four datasets with >400 specimens, GSE40435,[34] GSE46699,[35] GSE15641[36] and GSE66272,[37] were obtained. The probe ID was converted into a gene symbol first. When a gene was mapped to different probes, the genic expression value was calculated by the average expression value. Next, the data were translated into $\log_2$ logarithms, and the median normalization was performed using the robust multichip averaging method.[38] We compared the normal control vs cancer datasets using the Student's *t*-test. A *P* value of <0.05 was considered as statistically significant. The clinical data and mRNA expression data of eIF4E and 4E-BP1 in ccRCC

were extracted from the TCGA database.[39,40] The survival analysis of eIF4E and 4E-BP1 protein expression was directly obtained from the TCPA database (http://www.tcpaportal.org/tcpa/index.html).[41–44] According to the median value of gene or protein expression, samples were divided into low- and high-expression groups.

Patients and specimens

The research consisted of 202 samples from 101 patients with ccRCC. All the patients had a renal resection at the Fujian Provincial Hospital between January 2016 and June 2017. The standard requirements for patients included in the study were 1) histologically confirmed ccRCC; 2) no history of other malignancy, and 3) no prior neoadjuvant chemotherapy. The study was performed with the approval of the ethics committee of Fujian Provincial Hospital. Written informed consent was given by the patients for their information, and specimens were stored in the hospital database and used for research.

Immunohistochemical staining

Paraffin blocks that contained sufficient formalin-fixed tumor specimens were serial sectioned at 3 μm and mounted on silane-coated slides for immunohistochemical staining analysis. Dimethylbenzene rehydrated through 100% ethanol, 100% ethanol, 95% ethanol, 85% ethanol and 75% ethanol were applied to deparaffinize. In all, 0.01 mol/L of sodium citrate buffer (pH 6.0) was used to the progress of antigen retrieval treatment (autoclaved at 121°C, 2 min). Then, 3% H_2O_2 was applied to block endogenous peroxidase at room temperature for 10 min. The sections were washed in PBS solution subsequently and blocked with 10% goat serum (ZhongShan Biotechnology, Beijing, China) for 30 min and incubated with anti-eIF4E (ab33766, 1:100 dilution, monoclonal; Abcam, Cambridge, MA, USA) or anti-eIF4E-BP1 (ab32024, 1:100 dilution, monoclonal; Abcam) antibody or anti-vascular endothelial growth factor C (VEGFC; ab83905, 1:100 dilution, polyclonal; Abcam) or anti-phospho-4E-BP1 (Thr37/46) (236B4) (2855, 1:200 dilution, monoclonal; Cell Signaling Technology, Boston, MA, USA) at 4°C for 12 h. The sections were washed in PBS solution three times and incubated with HRP-conjugated secondary antibody for 30 min at room temperature. All slides were counterstained with diaminobenzidine (DAB) solution and 20% hematoxylin and dehydrated. The primary antibody diluent was regarded as negative control.

Evaluation of immunostaining intensity

Immunohistochemical staining tissue sections were reviewed and scored by two independent pathologists. The score was

calculated according to the proportion of stained tumor cells and intensity of cellular staining. The intensity of cellular staining was scored between 0 and 3: 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining. The proportion of stained tumor cells was scored between 1 and 4: 1, 0–25%; 2, 26–50%; 3, 51–75% and 4, 75–100%. The multiplication of these two variables was calculated as the final score. The staining was divided into five grades according to the final score as follows: 0 score, 0; 1 score, 1–2; 2 score, 3–4; 3 score, 6–8 and 4 score, 9–12.

Statistical analyses

In the research, the student's *t*-test was used to calculate the mRNA expression level in ccRCC tissues and adjacent normal renal tissues by using GraphPad Prism 5 (GraphPad Software, Inc., La Jolla, CA, USA). Log-rank test was used to calculate the survival analysis by using IBM SPSS version 19.0 (IBM Corporation, Armonk, NY, USA). Multivariate survival analysis was performed by using stepwise Cox proportional hazards regression model. *P* value of <0.05 was considered as statistically significant.

Results
Expression levels of eIF4E were not changed in ccRCC

In order to explore the expression of eIF4E in ccRCC patients, a total of four related publications were used. Based on these

datasets (GSE40435, GSE46699, GSE15641, GSE66272; Table S1), no change in eIF4E expression was observed in the tissues of ccRCC compared with adjacent normal renal tissues (Figure 1A–D). These results suggested that the mRNA of eIF4E expression did not change in ccRCC. Moreover, the expression of eIF4E protein in 101 tissues of ccRCC and their adjacent normal renal tissues was detected by immunohistochemical staining. Representative immunohistochemical-stained tissue sections and the frequency distributions of the immunohistochemical scores are presented (Figure 1E–G). The mean scores of eIF4E proteins in ccRCC and adjacent normal renal tissues were 2.11 and 2.29, respectively (Figure 1H), consistent with the conclusion from mRNA analysis that the level of eIF4E did not change in ccRCC.

Protein expression level of VEGFC was upregulated in ccRCC

VEGFC, an important downstream target of eIF4E, is regulated by eIF4E activity. In order to further investigate the activity of eIF4E in ccRCC, we set out to evaluate the expression of VEGFC protein in ccRCC. The expression of VEGFC protein in 101 tissues of ccRCC and their adjacent normal renal tissues was detected by immunohistochemical staining. Representative immunohistochemical-stained tissue sections and the frequency distributions of the immunohistochemical scores are presented (Figure 2A–C).The mean scores of
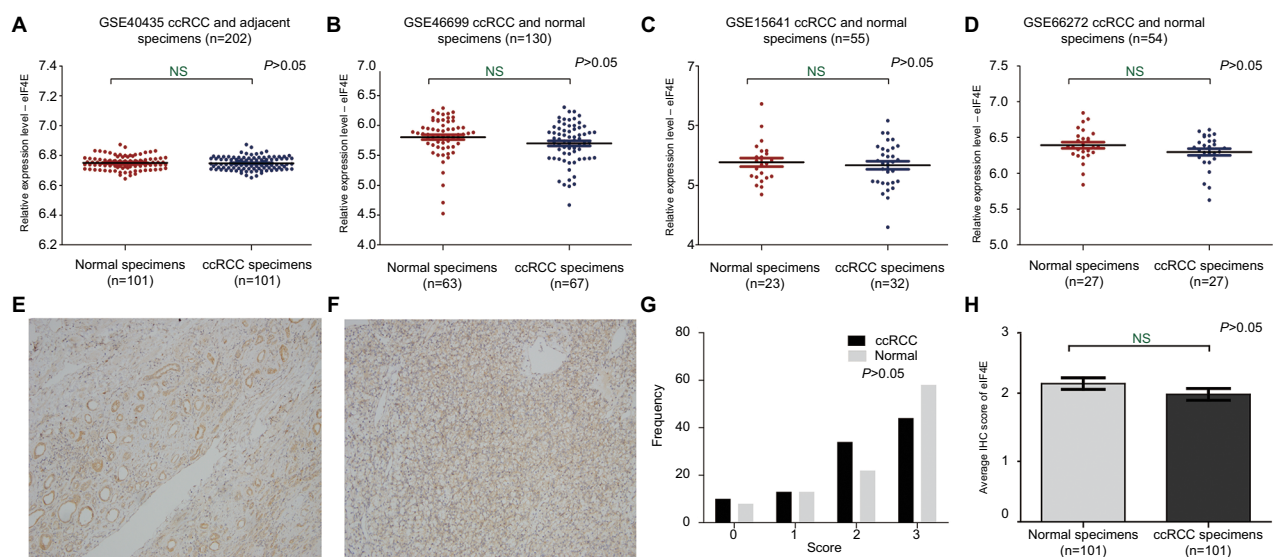

Figure 1 The mRNA and protein level of eIF4E in ccRCC.
Notes: The mRNA expression level of eIF4E in ccRCC was measured. Four mRNA datasets were used including GSE40435 (**A**), GSE46699 (**B**), GSE15641 (**C**) and GSE66272 (**D**). The protein expression level of eIF4E in 101 ccRCCs was measured using immunohistochemical staining. Representative adjacent normal renal tissues' staining (**E**), ccRCC tissues' staining (**F**), frequency distributions of protein expression across the cohort (**G**) and the average score of immunohistochemical staining for eIF4E (**H**) are shown. (**E** and **F**) Magnification ×100.
Abbreviations: ccRCC, clear cell renal carcinoma; eIF4E, eukaryotic translation initiation factor 4E; IHC, immunohistochemistry; NS, not significant.
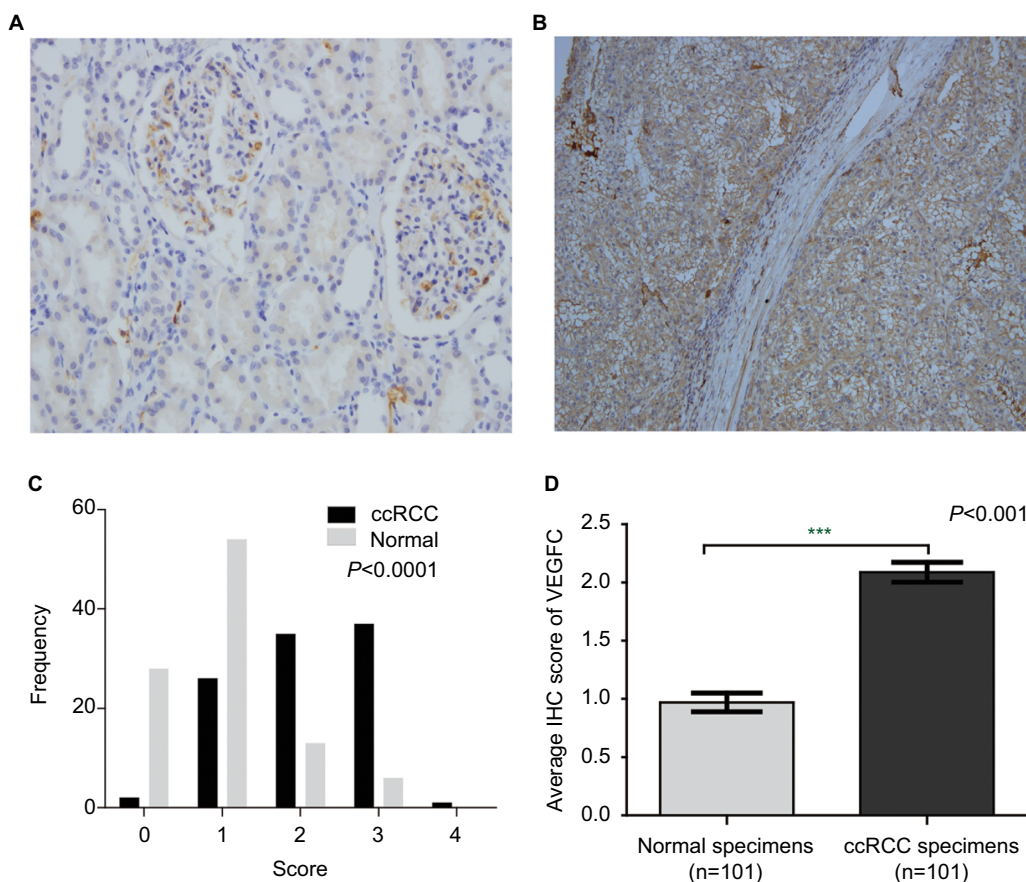
Figure 2 The protein expression of VEGFC in ccRCC.

Notes: The protein expression level of VEGFC in ccRCC was measured using immunohistochemical staining. Representative adjacent normal renal tissues' staining (**A**), ccRCC tissues' staining (**B**), frequency distributions of protein expression across the cohort (**C**) and the average score of immunohistochemical staining (**D**) are shown. (**A** and **B**) Magnification ×100. ***$P<0.001$.

Abbreviations: VEGFC, vascular endothelial growth factor C; ccRCC; clear cell renal carcinoma; IHC, immunohistochemistry.

VEGFC proteins in ccRCC and adjacent normal renal tissues were 2.09 and 0.97, respectively(Figure 2D), suggesting that although eIF4E expression levels were unchanged, the activity of eIF4E in ccRCC tissues was enhanced.

Expression levels of phospho-4E-BP1 and 4E-BP1 were enhanced in ccRCC

In order to investigate how the activity of eIF4E was enhanced in ccRCC, we next investigated the expression and activity of 4E-BP1. To our surprise, the mRNA and protein expression of 4E-BP1 were overexpressed in ccRCC tissues based on four datasets (GSE40435, GSE46699, GSE15641, GSE66272; Figure 3A–D and Table S1) and immunohistochemical staining of 4E-BP1 in 101 ccRCC tissues and their adjacent normal renal tissues. Representative immunohistochemical-stained tissue sections of 4E-BP1 and the frequency distributions of these scores are presented (Figure 3E–G). The mean scores of 4E-BP1 proteins in ccRCC and adjacent normal renal tissues were 3.10 and 0.19, respectively (Figure 3H).

Then, we detected the protein expression of phospho-4E-BP1 in the 101 sample cohort. Representative immunohisto-chemical-stained tissue sections of phospho-4E-BP1 and the frequency distributions of these scores are presented (Figure 4A–C). The mean scores of phospho-4E-BP1 in ccRCC and adjacent normal renal tissues were 2.52 and 0.18, respectively (Figure 4D), suggesting that both 4E-BP1 and phospho-4E-BP1 levels were much higher in ccRCC tissues.

Expression levels of *PIK3CD* and *PIK3CG* were upregulated in ccRCC

4EBPs could be phosphorylated due to the activation of PI3K pathway. Phosphorylated 4EBPs enable the assembly of eIF4F by dislodging 4EBPs from eIF4E. In order to further investigate the mechanisms underlying eIF4E activation in ccRCC, the expression levels of *PIK3CA*, *PIK3CB*, *PIK3CD*, *PIK3CG* and *PTEN* were examined (Table S2). The mRNAs of *PIK3CD* and *PIK3CG* were upregulated in ccRCC tissues based on the TCGA database (Figure 5).
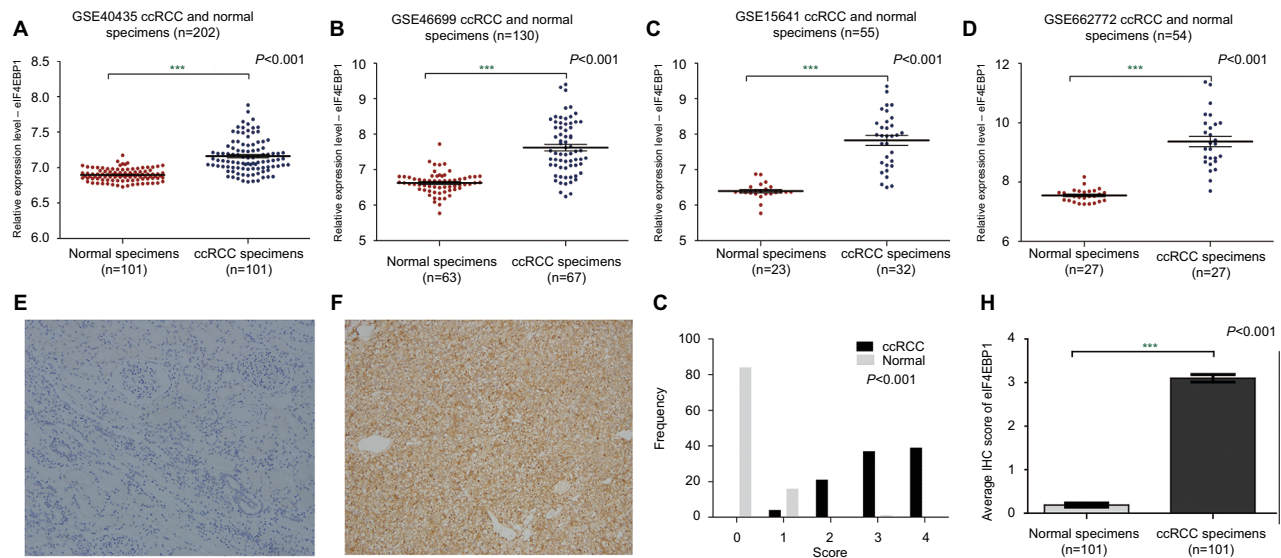
Figure 3 The mRNA and protein expression of 4E-BP1 in ccRCC.
Notes: The mRNA expression level of 4E-BP1 in ccRCC was measured. Four mRNA datasets were used including GSE40435 (**A**), GSE46699 (**B**), GSE15641 (**C**) and GSE66272 (**D**). The protein expression level of 4E-BP1 in ccRCC was measured using immunohistochemical staining. Representative adjacent normal renal tissues' staining (**E**), ccRCC tissues' staining (**F**), frequency distributions of protein expression across the cohort (**G**) and the average score of immunohistochemical staining (**H**) are shown. (**E** and **F**) Magnification ×100. ***P<0.001.
Abbreviations: ccRCC; clear cell renal carcinoma; eIF4E, eukaryotic translation initiation factor 4E; BP, binding protein.
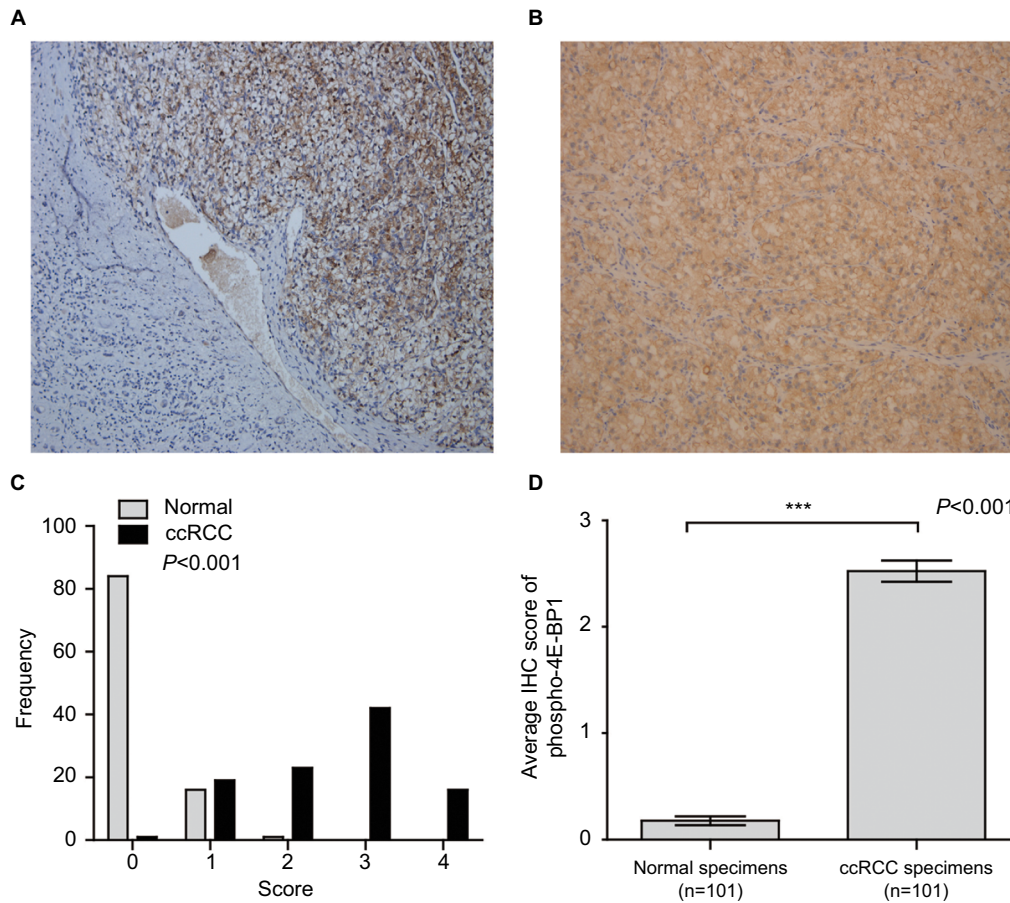

Figure 4 The protein expression of phospho-4E-BP1 in ccRCC.
Notes: The protein expression level of phospho-4E-BP1 in ccRCC was measured using immunohistochemical staining. Representative adjacent normal renal tissues' staining (**A**), ccRCC tissues' staining (**B**), frequency distributions of protein expression across the cohort (**C**) and the average score of immunohistochemical staining (**D**) are shown. (**A** and **B**) Magnification ×100. ***P<0.001.
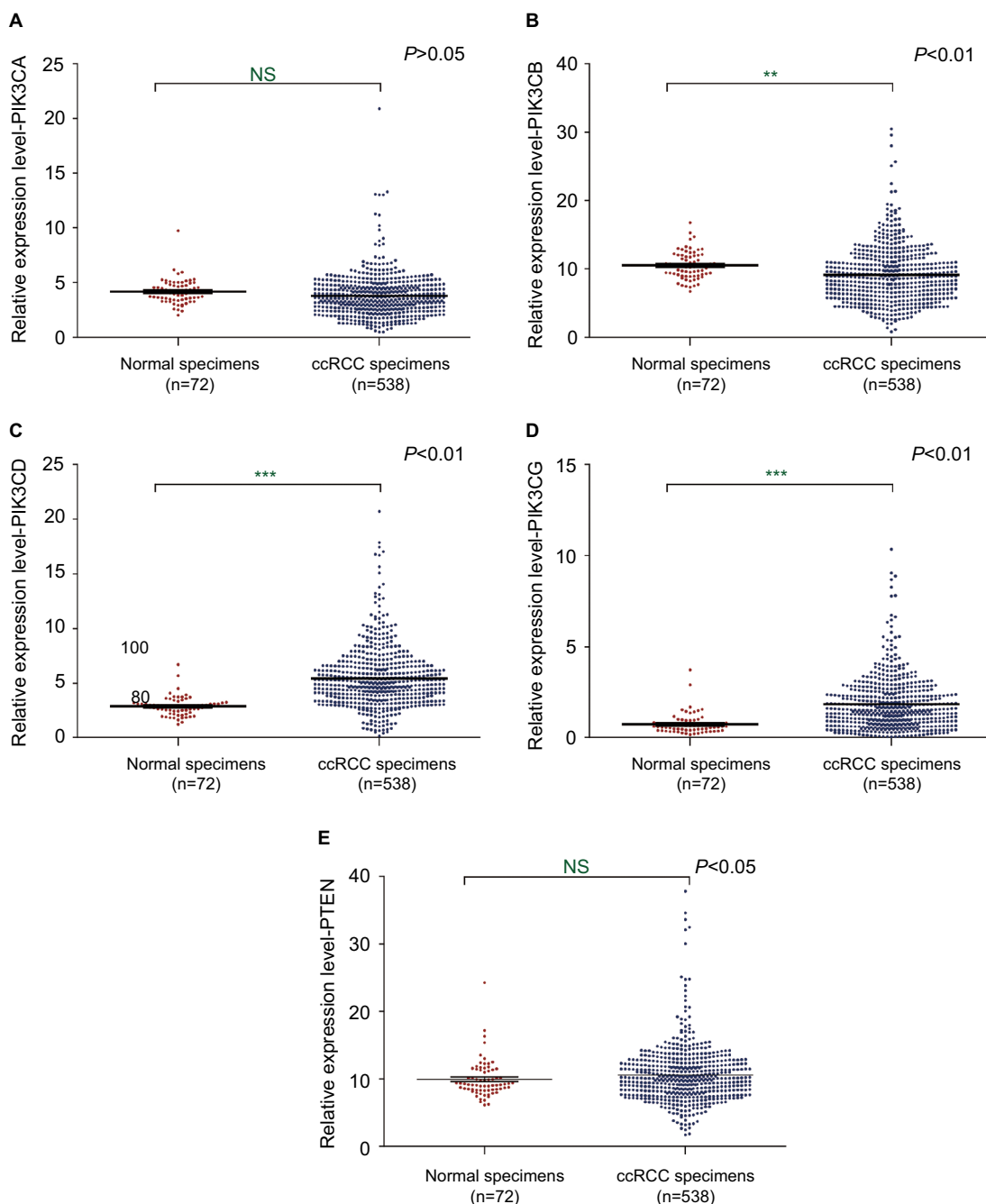**Abbreviations:** BP, binding protein; ccRCC; clear cell renal carcinoma; IHC, immunohistochemistry.

Figure 5 The mRNA expression of PIK3s and PTEN in ccRCC.
Notes: The mRNA expression levels of *PIK3CA* (**A**), *PIK3CB* (**B**), *PIK3CD* (**C**), *PIK3CG* (**D**) and *PTEN* (**E**) in ccRCC were analyzed using the TCGA database. **$P<0.01$; ***$P<0.001$.
Abbreviations: PTEN, phosphatase and tensin homolog; ccRCC, clear cell renal carcinoma; TCGA, The Cancer Genome Atlas; NS, not significant.

Expression levels of 4E-BP1 and phospho-4E-BP1 were strongly associated with ccRCC survival

In the TCGA ccRCC cohort, we observed that patients with advanced stage and grade were at significantly increased risk of death. Patients with age >60 years, laterality=left, pharmaceutical therapy and radiation therapy also have a high risk of death in ccRCC (Table 1).

The survival analyses of 4E-BP1and eIF4E mRNA are shown in Figure 6A and B. We found that the mRNA expression of 4E-BP1 (Figure 6A), but not eIF4E (Figure 6B), was associated with the clinical outcomes of ccRCC patients (Table S3), after adjusting for tumor location, grade, stage and patients' age, gender and race (Table 2). The survival analyses of 4E-BP1, eIF4E and phospho-4E-BP1 protein were obtained from the TCPA database. With the cutoff value

Table 1 Univariate analysis of the correlation between clinicopathological parameters and survival of ccRCC patients in the TCGA cohort

Variables	Patients (n)	MST (days)	Log-rank test	P-value
Age (years)				
≤60	261	NA		
>60	261	2415	13.256	0.000*
Gender				
Female	182	2564		
Male	340	2830	0.04	0.841
Location				
Left	246	2343		
Right	275	3695	4.657	0.031*
Race				
White	463	2752		
Other	59	1913	0.284	0.594
Tumor stage				
I/II	314	NA		
III/IV	205	1133	76.153	0.000*
Histologic grade				
G1/G2	242	NA		
G3/G4	280	1913	34.145	0.000*
Pharmaceutical therapy				
Yes	73	1378		
No	85	2830	11.489	0.001*
Radiation therapy				
Yes	45	927		
No	116	2764	17.13	0.000*
Surgery locoregional procedure				
Yes	16	1724		
No	146	1964	0.103	0.748
Surgery metastatic procedure				
Yes	31	1567		
No	132	1964	0.996	0.318

Note: *$P<0.05$, statistical significance.
Abbreviations: ccRCC, clear cell renal carcinoma; TCGA, The Cancer Genome Atlas; MST, median survival time, NA, not available.

set at the median, we found that the protein expression of 4E-BP1 (Figure 6C) and phospho-4E-BP1 (Figure 6E), but not eIF4E (Figure 6D), was also associated with the clinical outcomes of 445 ccRCC patients.

Discussion

In this study, the expression and prognostic relevance of eIF4E, 4E-BP1 and phospho-4E-BP1 in ccRCC were examined. Our analysis included four datasets with >400 specimens from the GEO database, one web tool for interactively exploring survival correlations performed by the TCPA database and one ccRCC cohort (n=101) with immunohistochemistry (IHC) analyses.

eIF4E is highly elevated and deregulated in many cancers such as lung and breast.[45,46] Surprisingly, no association was found between eIF4E expression and survival (Figure 6B and D), whereas the protein level of VEGFC (Figure 2) was upregulated in ccRCC tissues compared with adjacent normal renal tissues. The protein translation of VEGFC mRNA was regulated by eIF4E. Our results indicated that eIF4E activity is upregulated in ccRCC.

In a simple model to calculate the regulatory effect toward eIF4E activity, one phospho-4E-BP1 protein could eliminate the inhibitory effect of two 4E-BP1 proteins on eIF4E activity,[47,48] suggesting that phospho-4E-BP1 plays a greater role than unphosphorylated 4E-BP1 in the regulation of eIF4E activity. In our study, high mRNA and protein expression level of 4E-BP1 were observed in ccRCC tissues compared with adjacent normal renal tissues (3.10 vs 0.19, tumor vs normal; Figure 3). At the same time, a strong phosphorylation status of the 4E-BP1 in ccRCC was also observed (2.52 vs 0.18, tumor vs normal; Figure 4). Therefore, the increased eIF4E activity observed in our study may be due to an enhanced proportion of phospho-4E-BP1 in ccRCC compared with adjacent normal renal tissues (Figure 7). As we know, eIF4E activity can be controlled by the phospho-4E-BP1 and the expression levels of eIF4E, and the increased eIF4E activity drives cancer progression. Therefore, phosphorylated 4E-BP1 and eIF4E overexpression synchronously drive disease progression in ccRCC.[49]

The simultaneous increase in 4E-BP1 protein expression and phosphorylation may be due to the malfunction of the feedback loop between 4E-BP1 and eIF4E. When eIF4E is overactivated, the enhanced 4E-BP1 is supposed to suppress its activity. However, due to the upregulation of the PI3K pathway, i.e., overexpression of *PIK3CD* and *PIK3CG* mRNA (Figure 5), most of newly synthesized 4E-BP1 proteins were phosphorylated and lost the ability to suppress eIF4E activity. Thus, the protein expression of 4E-BP1 cannot be restored and remained high in ccRCC; albeit most of the 4E-BP1 protein were phosphorylated and inactive.

Collectively, our data displayed enhanced activity of eIF4E in ccRCC, which is probably due to the increased ratio of phospho-4E-BP1 against 4E-BP1 (Figure 7). In addition, there was a significantly unfavorable influence of 4E-BP1 and phospho-4E-BP1 expression on the survival (Figure 6). These results suggested that 4E-BP1, eIF4E and phospho-4E-BP1 are important determinants of diagnosis and disease progression in ccRCC.
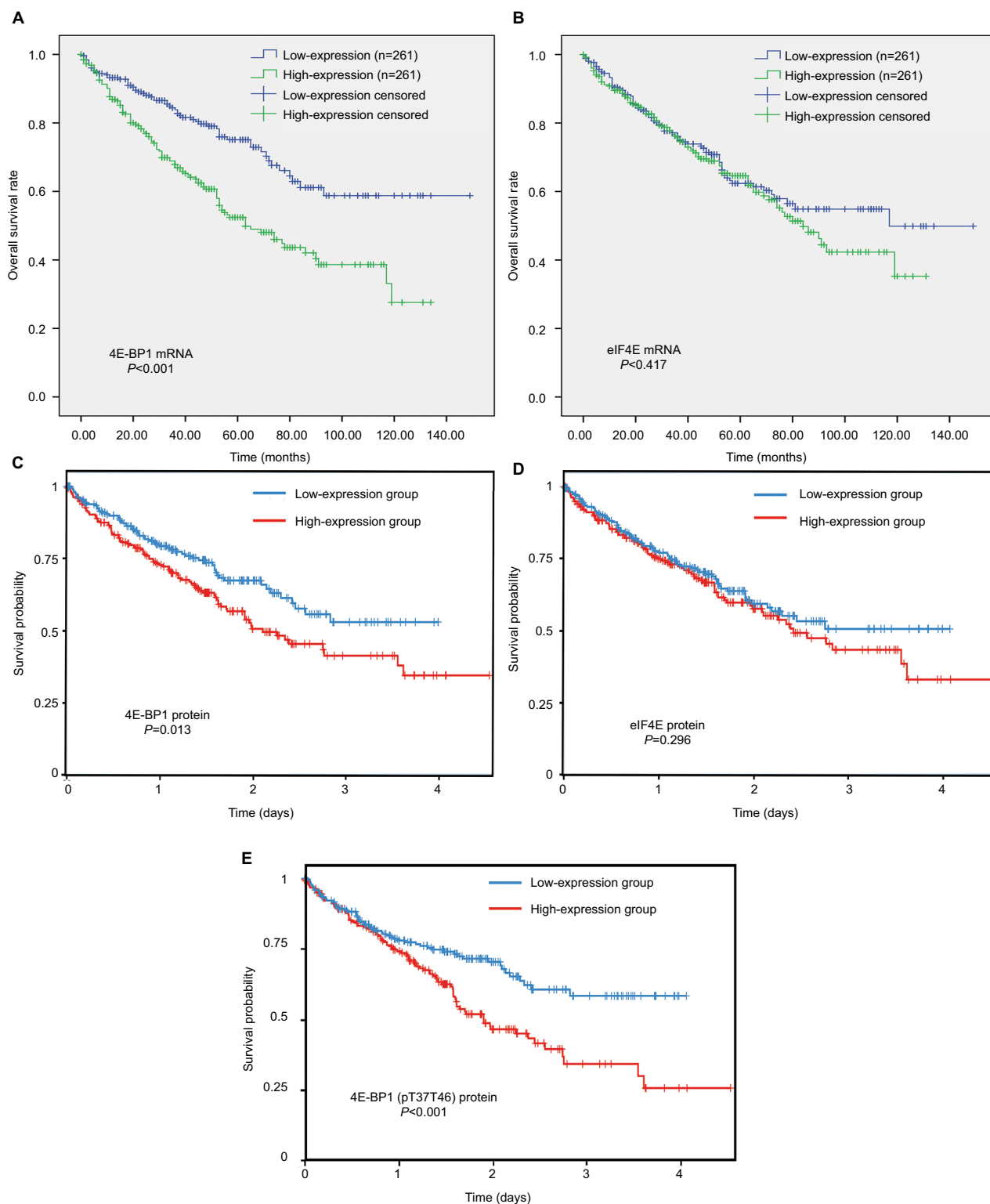
Figure 6 The prognostic value of 4E-BP1, eIF4E and phospho-4E-BP1 in ccRCC.
Notes: The Kaplan–Meier survival analyses of 4E-BP1 (**A**) and eIF4E (**B**) mRNA expression of the overall survival time of ccRCC patients using the OncoLnc database. The Kaplan–Meier survival analyses of 4E-BP1 (**C**), eIF4E (**D**) and phospho-4E-BP1 (**E**) protein expression of the overall survival time of ccRCC patients using the TCPA database.
Abbreviations: BP, binding protein; eIF4E, eukaryotic translation initiation factor 4E; ccRCC; clear cell renal carcinoma; TCPA, The Cancer Proteome Atlas.

Table 2 Multivariate analysis of the correlation between clinicopathological parameters and survival of ccRCC patients in the TCGA cohort

Covariates	Coefficient	Standard error	HR	95% CI for HR	P-value
Gender (female vs male)	0.067	0.161	1.070	0.780–1.467	0.676
Age (≤60 vs >60 years)	0.425	0.159	0.654	0.479–0.893	0.008*
Race (white vs other)	0.100	0.301	1.105	0.613–1.993	0.739
Location (left vs right)	0.372	0.152	1.450	1.073–1.959	0.015*
Tumor stage (I/II vs III/IV)	1.118	0.301	0.532	0.372–0.762	0.001*
Histologic grade (G1/G2 vs G3/G4)	0.631	0.183	1.105	0.613–1.993	0.000*
eIF4E expression (low vs high)	0.212	0.154	0.809	0.598–1.093	0.168
4E-BP1 expression (low vs high)	0.463	0.167	1.589	1.146–2.204	0.006*

Note: *$P<0.05$, statistical significance.
Abbreviations: ccRCC, clear cell renal carcinoma; HR, hazard ratio; TCGA, The Cancer Genome Atlas; eIF4E, eukaryotic translation initiation factor 4E; BP, binding protein.
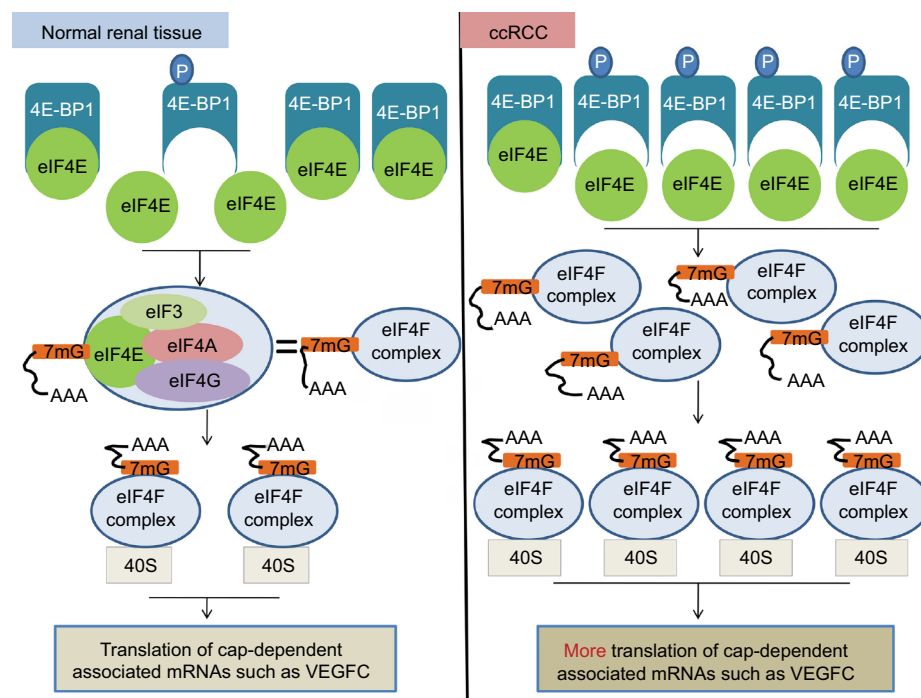

Figure 7 A diagram illustrating the potential molecular mechanisms underlying the regulation of eIF4E activity by 4E-BP1 and phospho-4E-BP1 in ccRCC.
Notes: In normal renal tissue, most eIF4E activity is inhibited due to the combination of eIF4E and 4E-BP1. This ensures that the translations of cap-dependent associated mRNA are tightly controlled. In ccRCC, the mRNA level of eIF4E does not change. However, the PI3K pathway was activated and 4E-BP1 was phosphorylated, leading to the release of 4E-BP1 from eIF4E and the increased activity of eIF4E. The increased activity of eIF4E actively promoted cap-dependent translation. eIF4F complex, eIF4E+eIF3+eIF4A+eIF4G.
Abbreviations: eIF4E, eukaryotic translation initiation factor 4E; BP, binding protein; ccRCC; clear cell renal carcinoma; PI3K, phosphoinositide 3-kinase; VEGFC, vascular endothelial growth factor C.

Author contributions

Feng Li and Qingshui Wang performed the experiments, wrote the paper and prepared figures and/or tables.

Xiaoxue Xiong and Chenyi Wang performed the experiments and analyzed the data.

Xiaohua Liu, Ziqiang Liao, and Bifeng Xie contributed to reagents/analysis tools.

Yao Lin conceived and designed the experiments, wrote the paper and prepared figures and/or tables.

All authors contributed toward data analysis, drafting and revising the paper and agree to be accountable for all aspects of the work.

Disclosure

The authors report no conflicts of interest in this work.

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