EIF3D silencing suppresses renal cell carcinoma tumorigenesis via inducing G2/M arrest through downregulation of Cyclin B1/CDK1 signaling

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Abstract. There are no effective therapies for advanced renal cell carcinoma (RCC), except for VEGFR inhibitors with only ~50% response rate. To identify novel targets and biomarkers for RCC is of great importance in treating RCC. In this study, we observed that eukaryotic initiation factor 3d (EIF3D) expression was significantly increased in RCC compared with paracarcinoma tissue using immunohistochemistry staining and western blot analysis. Furthermore, bioinformatics meta-analysis using ONCOMINE microarray datasets showed that EIF3D mRNA expressions in CCRCC tissue specimens were significantly higher than that in normal tissue specimens. In addition, RCC tissue microarray demonstrated that elevated EIF3D expression was positively correlated with TNM stage and tumor size. EIF3D silencing in human 786-O and ACHN CCRCC cell lines by RNA interference demonstrated that EIF3D knockdown obviously inhibited cell proliferation and colony formation, caused G2/M arrest through downregulation of Cyclin B1 and Cdk1 and upregulation of p21, and induced apoptosis shown by sub-G1 accumulation and RARP cleavage. Moreover, correlation analysis using ONCOMINE microarray datasets indicated that increased EIF3D mRNA expression was positively correlated to PCNA, Cyclin B1 and CDK1 mRNA expression in RCC. Collectively, these results provide reasonable evidences that EIF3D may function as a potential proto-oncogene that participates in the occurrence and progression of RCC.

Introduction

Renal cell carcinoma (RCC) has been identified as the most commonly diagnosed subtype of kidney cancer, accounting for 2-3% of all malignancies (1). According to specific histopathological and genetic characteristics, three common pathologic types of RCC, including clear cell RCC (CCRCC; 80-90%), papillary RCC (PRCC; 10-15%) and chromophobe RCC (CRCC; 4-5%) account for 90% of all RCCs (2). At present, radical nephrectomy is an effective treatment for early-stage and localized RCC (3). However, there are still a certain part of patients diagnosed with advanced or metastatic RCC (mRCC) at the first visit (4). Some patients, although they have a small and localized RCC at the beginning, may still develop metastatic RCC after radical nephrectomy, where the underlying mechanisms are poorly understood. Therefore, it is urgent to develop novel biomarkers for early diagnosis and prognostic assessment. Although with the progress of molecular biology, neoadjuvant therapy targeting VEGFR (5) has brought a new hope for advanced mRCC, original drug resistance was seen in ≥50% of the patients, and acquired drug resistances were developed monthly. Therefore, an increasing understanding of the complex molecular basis of RCC is needed for the clinical treatment of this aggressive malignancy.

Translation of mRNA is a key process for protein synthesis in eukaryotes, including initiation, elongation and termination. Regulation of protein synthesis at translation initiation level is essential for the control of cell proliferation under normal physiological conditions. Eukaryotic translation initiation factor 3 (eIF3) is the largest known multi-protein complex containing 13 subunits (EIF3A-EIF3M), and is required for
eukaryotic protein synthesis by binding mRNA to 40S small ribosomal subunits and preventing premature binding of large ribosomal subunits (6). Interestingly, misregulation of EIF3 expression is closely related to cancer development and progression (6). It was recently reported (7) that over-expression of eIF3a could promote the growth of ovarian cancer cells and decrease the sensitivity of ovarian cancer cells to cisplatin. In addition, increased eIF3e expression was reported to be associated with colon tumor development and poor prognosis, whereas knockdown of eIF3e inhibited the proliferation of colon cancer cells (8). EIF3D, one of the core subunits of eIF3, was reported to play an oncogenic role in prostate cancer, glioma, melanoma, colon cancer and non-small cell lung cancer (NSCLC) (9-13). However, the clinical significance and biological function of eIF3 in human RCC remains unclear.

Therefore, the objective of this study was to explore the role of EIF3D in the pathogenesis of RCC, through IHC staining of RCC specimens, oncomine dataset analysis, and shRNA-mediated gene knockdown. The possible molecular mechanism involved in EIF3D-mediated function was also investigated.

Materials and methods

Cancer specimens and tissue microarray. In total 20 CCRCC tissue specimens and corresponding paired paracarcinoma tissue specimens were provided by the Department of Urinary Surgery of Shanghai Changzheng Hospital from January 2015 to June 2015. All specimens were fixed in formalin and embedded with paraffin. RCC tissue specimens and corresponding paired paracarcinoma tissue specimens were snap frozen in liquid nitrogen and stored at -80°C before use. None of the patients had other renal diseases, other tumors or systemic diseases, and did not undergo any surgery before the radical nephrectomy. RCC tissue microarray containing 102 RCC specimens were obtained from the Department of Urinary Surgery of Shanghai Changzheng Hospital. Of the 102 RCC specimens, 92 were CCRCC, 6 were PRCC and the remaining 4 specimens were CRCC. This study was approved by the institutional review board of the Second Military Medical University (Shanghai, China). All the patients were required to provide written informed consent before enrollment.

Cell lines. Human CCRCC cell lines 786-O, ACHN, A498, 769-P, Caki-1 and Caki-2 were obtained from the cell bank of the Chinese Academy of Sciences (Shanghai, China). 786-O and 769-P cell lines were maintained in RPMI-1640 (HyClone) with 10% fetal bovine serum (FBS, Gibco). ACHN cell line was maintained in DMEM (HyClone) with 10% FBS (Gibco). Caki-1, A498 and Caki-2 cell lines were maintained in MEM (HyClone) with 10% FBS (Gibco). All of these cells were cultured in a humidified incubator containing 5% CO₂ at 37°C.

Immunohistochemistry. Paraffin blocks were sliced into 5-μm sections, and sequentially dewaxed in xylene, 100, 90, 80 and 70% ethanol and ddH₂O. The slides were first incubated with rabbit anti-EIF3D antibody (1:200, ab155419, Abcam) at 4°C overnight, labeled with biotinylated goat anti-rabbit serum and streptavidin-peroxidase conjugate for 15 min, and developed with diaminobenzidine (DAB). The positivity and intensity were assessed under a light microscope by two independent pathologists in a blinded manner. The distribution area (0, 0-20, 20-60 and 60-100%) of EIF3D-positive staining cells was given 0, 1, 2 and 3 scores, respectively. The final score was a combination of the two scores, classified into four grades: negative (0), weak (1), medium (2-4), and strong (5-6), respectively.

Lentivirus construction and transfection. ShRNA sequence targeting human EIF3D (NM_003753.3) (5'-GGCTCAATTGA CATCTGCATGACTCGAGTCATGCAGATGTCAATGACG CATTTTTT-3') and the control shRNA sequence (5'-CTAGCC CGGTTCCTCCGAACGTGTCACGTATCTCGAGATACGTG ACACGTTCGAGAATTTTTTAAAT-3') were designed and inserted into the plasmid pFH vector (Hollybio, Shanghai, China) containing green fluorescent protein (GFP) as a reporter gene. To generate recombinant lentivirus, the reconstructed EIF3D silencing plasmid and two packaging plasmids were cotransfected into HEK293T cells using calcium phosphate precipitation method. Approximately 72 h after transfection, recombinant lentivirus was harvested and purified by ultracentrifugation. For lentiviral transduction, 786-O cells (3x10⁴ cell/well) and ACHN cells (6x10⁴ cell/well) were seeded in 6-well plates overnight, and transduced with lentivirus containing shEIF3D or shCon at MOIs of 25 and 20, respectively. Transduction efficiency was monitored by relative GFP expression under fluorescent microscopy at 72 h post-transduction. At 120 h after transduction, cells were harvested to verify EIF3D silencing efficiency using RT-PCR and western blotting.

Quantitative real-time PCR. Total RNA was isolated from cells and samples using TRIzol reagent (Gibco) according to the manufacturer's instructions. Total RNA (2 μg) was reverse transcribed into cDNA using oligo(dT) primer and the M-MLV reverse transcriptase (Promega). Total 10 μl 2X SYBR premix ex-taq, 0.5 μl primer, 5 μl cDNA and 4.5 μl ddH₂O were mixed for quantitative real-time PCR (qRT-PCR) using CFX Connect™ Real-Time PCR Detection System (Takara). The primer sequences used were: 5'-CTGGAGGAGGGCACA TACCT-3' (forward) and 5'-CTCGTGGAGAACACAACTCT-3' (reverse) for EIF3D; 5'-GTGGGACATCCGGGAAA GAC-3' (forward) and 5'-AAAGGCTTGAACCGAACA CTA-3' (reverse) for β-actin. The 2^(-ΔΔCt) method was used to calculate relative expression level of EIF3D, being normalized to β-actin. The experiment was performed in triplicate and repeated three times.

Western blotting. Cell samples and fresh RCC tissues were lysed in 2X SDS lysis buffer at 4°C and boiled for 5 min. The supernatant was separated by centrifugation at 12,000 x g for 10 min at 4°C. Protein quantification was carried out using BCA protein assay kit. Proteins after quantification were separated in 10% SDS-PAGE gel electrophoresis and transferred onto PVDF membranes. Next, the transferred PVDF membranes were blocked in 5% non-fat milk dissolved
were identified. One dataset (Cutcliffe Renal, Clin Cancer Analysis’ ‘Kidney Cancer’ and ‘mRNA’. Then, six datasets were used: ‘EIF3D’, ‘Cancer vs. Normal tissue vs. normal kidney tissue, we conducted a meta-analysis of Oncomine.org) including previously published and publicly available 715 datasets and 86,733 samples. The following online microarray data in the Oncomine database (www.oncomine.org) were performed three times in triplicate.

MTT assay. Cell viability after EIF3D knockdown was measured using 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-

tetrazolium bromide (MTT) assay. After 72-h infection, 2580-2590, 2016

876-O and ACHN cells from different groups (Con, Lv-shCon, Lv-shEIF3D) were trypsinized, suspended, counted and plated in a 96-well plate at a density of 3x10³ cells/well for five points in time (days 1, 2, 3, 4 and 5). At each time-point, cells were incubated with MTT solution for 4 h and then incubated with acidified isopropanol overnight. The absorbance at 595 nm was measured using microplate reader. The experiment was performed three times and each group sample had five repetitions.

Plate colony formation assay. After 72-h infection, 786-O and ACHN cells from different groups (Con, Lv-shCon, Lv-shEIF3D) were trypsinized, suspended, counted and plated in a 6-well plate at a density of 400 cells/well. Subsequently, culture medium was replaced every 3 days. After incubation for 2 weeks, cells were fixed in 4% paraformaldehyde for 30 min, and stained with Giemsa solution for 20 min. The number of colonies (>50 cells per colony) in each well was counted under a light microscope and photographed using a digital camera. The experiment was performed three times and each group had three repetitions.

Cell cycle analysis. After 120-h infection, 786-O and ACHN cells from different groups (Con, Lv-shCon, Lv-shEIF3D) were trypsinized, centrifuged, washed with ice-cold PBS, fixed in 75% ethanol overnight, washed again with PBS, centrifuged, and incubated in 500 μl PI staining solution (PI and RNase A) at 37°C for 1 h. Cell cycle distribution was analyzed by flow cytometer (BD Biosciences, USA). The experiment was performed three times in triplicate.

Bioinformatics meta-analysis using ONCOMINE microarray datasets. To investigate the expression of EIF3D in RCC tissue vs. normal kidney tissue, we conducted a meta-analysis on online microarray data in the Oncomine database (www.oncomine.org) including previously published and publicly available 715 datasets and 86,733 samples. The following search terms were used: ‘EIF3D’, ‘Cancer vs. Normal Analysis’, ‘Kidney Cancer’ and ‘mRNA’. Then, six datasets were identified. One dataset (Cutcliffe Renal, Clin Cancer Res, 2005) that did not include a differential analysis of RCC vs. normal kidney was excluded from our meta-analysis. All data are reported Log2 Median-Centered intensity in the Oncomine database. Correlation analysis of two gene expressions was performed on the same dataset (Jones Renal, Clin Cancer Res, 2005).

Table I. IHC staining of EIF3D expression in prostate cancer and paired paracarcinoma tissues.

<table>
<thead>
<tr>
<th>Positive level</th>
<th>No. of specimen</th>
</tr>
</thead>
<tbody>
<tr>
<td>+/-</td>
<td>0</td>
</tr>
<tr>
<td>+</td>
<td>15</td>
</tr>
<tr>
<td>++</td>
<td>2</td>
</tr>
<tr>
<td>P-value</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

| Paracancerous | 2 | 8 | 10 | 2 |

*Wilcoxon signed rank text.

Statistical analysis. All results were drafted in diagrams by GraphPad Prism 5 software. Wilcoxon signed rank test was carried out to evaluate differences in EIF3D expression between RCC tissue and paired paracarcinoma tissue. Chi-square test was used to analyze EIF3D expression and patient characteristics of RCC tissue microarray. Statistical comparisons on the continuous variables of two groups (Lv-shCon vs. Lv-shEIF3D) were performed by the Student's t-test. All statistical calculations were carried out using SPSS version 19 (IBM Corp., USA), and P-value <0.05 was considered statistically significant.

Results

EIF3D mRNA and protein are upregulated in RCC tissue. To compare the expression levels of EIF3D in clinical RCC tissue and paired paracarcinoma tissue, three pairs of fresh RCC tissues and paired paracarcinoma tissues were analyzed by western blotting. It was found that the EIF3D expression level in RCC tissue was significantly higher than that in the paracarcinoma tissues (P=0.005) (Fig. 1A and B). Representative images of EIF3D staining are shown in Fig. 1C. Moreover, EIF3D expressions in RCC tissues was also significantly higher than that in the paracarcinoma normal kidney tissues (Fig. 1D), and the difference was considered to be statistically significant (Table I, P<0.001).

Correlations between EIF3D expression and clinicopathologic features in RCC patients. To further confirm the clinical significance of EIF3D in RCC, an RCC tissue microarray containing 102 cases of RCC sections were used to assess the relationships between EIF3D proteins and corresponding patient clinicopathological features by Chi-square test. As shown in Table II, EIF3D expression in RCC tissues was significantly correlated with tumor size (P=0.004) and TNM stage (P=0.03), while no significant associations were observed between EIF3D expression and sex, age, Fuhrman grade, histologic type or metastasis. Notably, the percentage of EIF3D staining intensity was positively correlated with the TNM stage (Fig. 1E and F).

Knockdown of EIF3D in RCC cells through lentivirus-mediated expression of shRNA. Considering that EIF3D was upregulated in RCC and correlated to tumor stage, we further explored the cellular functions of this protein in...
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RCC cell lines. Consistent with the findings in RCC tissues, the expression of EIF3D protein was overexpressed in RCC cell lines, including A498, 769-P, 786-O, ACHN, Caki-1 and Caki-2 (Fig. 2A). Two RCC cell lines, 786-O and ACHN cell lines, with relatively higher EIF3D expression were selected for loss-of-function study. Lentivirus-introduced shRNA was used to silence EIF3D expression in 786-O and ACHN cells. As shown in Fig. 2B, most of the cells presented GFP-positive signals of >90% under fluorescence microscopy, indicating successful infection efficiency. To further determine EIF3D
silencing efficiency, the level of EIF3D protein and mRNA was analyzed through RT-PCR and western blotting in the 786-O and ACHN cells after EIF3D knockdown. As shown in Fig. 2C and D, EIF3D protein expression in Lv-shEIF3D group of the two cell lines was significantly suppressed as compared with those in Lv-shCon group. In addition, the mRNA levels of EIF3D were significantly reduced by 95.5% in 786-O cells and 91.1% in ACHN cells after EIF3D knockdown (Fig. 2E and F, P<0.001), suggesting that shRNA targeting EIF3D successfully inhibited gene expression.

**Knockdown of EIF3D impaired RCC cell proliferation and colony formation ability.** To investigate the function of EIF3D in RCC cells, we examined whether reduction of EIF3D expression has an effect on cell proliferation and colony formation ability. MTT assay was used to measure cell viability in 786-O and ACHN cells after knockdown of EIF3D. As shown in Fig. 3A and B, the growth curves of 786-O and ACHN cells remarkably declined upon EIF3D knockdown. The number of viable cells was greatly reduced in Lv-shEIF3D groups than that in Lv-shCon groups in 786-O and ACHN cells (P<0.001). Moreover, cell colony formation assay was performed to gain additional insight into the effect of EIF3D on malignant proliferation of RCC cells. There was a significant difference in the colony sizes between Lv-shEIF3D group and Lv-shCon group (Fig. 3C), especially in 786-O cell line. The number of colonies of the two RCC cell lines was decreased significantly in Lv-shEIF3D group as compared with that in Lv-shCon group (786-O cells: 0.0±0.0 vs. 83.0±2.0, P=0.001; ACHN cells: 19.0±2.0 vs. 85.0±2.6, P<0.001) (Fig. 3C and D). These results indicated that EIF3D plays a vital role in the malignant growth of RCC cells.
Silencing of EIF3D induces G2/M phase arrest in RCC cells. To explore the potential mechanism underlying the suppressive effect of EIF3D silencing on tumor cell proliferation, cell cycle distribution was evaluated using flow cytometry. As shown in Fig. 4A and C and E, knockdown of EIF3D in 786-O and ACHN cells significantly increased the percentage of cells in G2/M phase (786-O cells: 39.33±1.27 vs. 15.74±1.28%, P<0.001; ACHN cells: 56.04±0.92 vs. 19.70±0.43%, P<0.001) and decreased the percentage of cells in G0/G1 phase (786-O cells: 32.85±0.34 vs. 54.00±0.61%, P<0.001; ACHN cells: 31.27±0.48 vs. 49.31±0.64%, P<0.001). Moreover, the number of S-phase ACHN cells treated with Lv-shEIF3D was also markedly decreased (12.69±0.48 vs. 30.99±0.35%, P<0.001). Besides, more cells were accumulated in sub-G1 phase in 786-O and ACHN cells after EIF3D knockdown as compared with the control (786-O cells: 6.09±0.36 vs. 4.37±0.36%, P=0.01; ACHN cells: 1.76±0.25 vs. 2.46±0.30%, P<0.01) (Fig. 4D and F), suggesting that knockdown of EIF3D might induce cell apoptosis.

To further understand the mechanism underlying G2/M phase arrest, the expression alterations of cell cycle regulators were detected in 786-O and ACHN cells after EIF3D knockdown using western blot analysis. As shown in Fig. 4B, the expression levels of CDK1 and Cyclin B1, associated with G2-M transition, were decreased in Lv-shEIF3D groups, whereas the expression levels of p21 and RARP cleavage were increased in 786-O and ACHN cells following Lv-shEIF3D transduction.

### Table III. EIF3D expressions in ONCOMINE microarray datasets.

<table>
<thead>
<tr>
<th>Dataset</th>
<th>Subtype</th>
<th>N (cancer/normal)</th>
<th>P-valuea (cancer/normal)</th>
<th>Fold change (cancer/normal)</th>
<th>% Gene ranking</th>
<th>Database references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Jones</td>
<td>CCRCC</td>
<td>32/23</td>
<td>5.14E-12</td>
<td>1.824</td>
<td>572</td>
<td>Clin Cancer Res 2005/08/15</td>
</tr>
<tr>
<td></td>
<td>PRCC</td>
<td>11/23</td>
<td>7.32E-06</td>
<td>1.637</td>
<td>1,552</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CRCC</td>
<td>6/23</td>
<td>0.326</td>
<td>1.076</td>
<td>7,495</td>
<td></td>
</tr>
<tr>
<td>Beroukhim</td>
<td>Hereditary CCRCC</td>
<td>32/11</td>
<td>3.08E-07</td>
<td>1.393</td>
<td>1,059</td>
<td>Cancer Res 2009/06/01</td>
</tr>
<tr>
<td></td>
<td>Non-hereditary CCRCC</td>
<td>27/11</td>
<td>1.32E-04</td>
<td>1.286</td>
<td>1,731</td>
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<tr>
<td>Lenburg</td>
<td>CCRCC</td>
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<td>0.007</td>
<td>1.323</td>
<td>2,052</td>
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<tr>
<td>Cumz</td>
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<td>0.004</td>
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<td>Clin Cancer Res 2007/08/15</td>
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<td>Yusenko</td>
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<td>26/5</td>
<td>0.105</td>
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<td>7,645</td>
<td>BMC Cancer 2009/05/18</td>
</tr>
<tr>
<td></td>
<td>PRCC</td>
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<td>1.236</td>
<td>8,673</td>
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</tr>
<tr>
<td></td>
<td>CRCC</td>
<td>4/5</td>
<td>0.127</td>
<td>1.542</td>
<td>8,039</td>
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</tr>
</tbody>
</table>

Lenburg and Cumz datasets, and slightly upregulated CCRCC samples in Yusenko datasets. Meta-analysis of the five datasets collectively revealed that increased EIF3D mRNA expression was associated with CCRCC as compared with the normal kidney tissue (gene median rank: 1891.5, P=0.004) (Fig. 5A). In the Beroukhim renal microarray dataset, EIF3D mRNA expression in CCRCC containing hereditary CCRCC and non-hereditary CCRCC exhibited a significant difference as compared with the normal tissue (P<0.001) (Fig. 5B). To study whether EIF3D is differentially expressed in distinct RCC subtypes, Jones renal dataset (Fig. 5C) was further analyzed. It was found that RCC tended to have higher EIF3D expression than renal oncocytoma, a benign renal tumor (P=0.187) (Fig. 5D). Among RCCs, CCRCC had significantly higher EIF3D expression than CRCC and PRCC (Fig. 5E, CCRCC vs. PRCC: P=0.01; CRCC vs. PRCC: P=0.05; CCRC vs. CRCC: P=0.157).

EIF3D is positively correlated with PCNA, Cyclin B1 and CDK1. We also investigated the correlation of EIF3D and potential downstream signaling molecules using ONCOMINE datasets. As depicted in Fig. 6, EIF3D was positively correlated with PCNA, a proliferative biomarker (r²=0.1687, P<0.0001), suggesting a correlation of EIF3D with the malignant growth of RCC. In addition,
EIF3D expression was also positively correlated with Cyclin B1 ($r^2=0.2354$, $P<0.0001$) and CDK1 ($r^2=0.1332$, $P=0.0003$) expressions in RCC, providing evidence at the *in vivo* level that EIF3D may regulate cell cycle progression in RCC.
Figure 5. EIF3D mRNA expression in CCRCC vs. normal kidney tissues was analyzed by using ONCOMINE microarray database. (A) Five microarray datasets regarding EIF3D mRNA expression in CCRCC vs. normal tissues were included in our meta-analysis: 1-2, Beroukhim Renal (hereditary CCRCC and non-hereditary); 3, Gumz Renal; 4, Jones Renal; 5, Lenburg Renal; 6, Yusenko Renal. Data are shown as the median rank of IF3d through each dataset analysis. P-value for EIF3D is presented using the median ranked analysis on CCRCC vs. normal tissues. (B) Beroukhim renal microarray dataset showed that EIF3D mRNA expression in CCRCC was significantly upregulated compared with the normal tissue (**P<0.001). Data are presented as log2 median-centered intensity. (C-E) Differential expression of EIF3D mRNA expression in several common tissue subtypes was analyzed in Jones independent renal microarray dataset (*P<0.05, **P<0.01; ***P<0.001).

Figure 6. Correlation analysis of EIF3D expression and relevant gene expression using Jones independent renal microarray dataset. (A-C) Increased EIF3D mRNA expression was positively correlated with PCNA, Cyclin B1 and CDK1 mRNA expression. Correlation coefficient of $r^2$ and P-value are shown in images. Data are presented as log2 median-centered intensity.
Discussion

Gene transcription and mRNA translation are two indispensable processes of eukaryotic gene expression. When eukaryotic mRNA translation is out of control, the process of gene expression will be deregulated, resulting in uncontrolled cell growth or even leading to tumor formation (14). Hence, targeting abnormal translation factors in human cancers may be a potential targeted treatment strategy. EIF3D, as a subunit of EIF3 complex, has been reported to participate in nearly all stages of translation and associated with various cancers. However, its clinical significance and cellular functions in RCC has not been reported yet. Here, we observed that EIF3D was overexpressed in RCC tissues, especially in CCRCC specimens, and that EIF3D expression was positively correlated with malignant features of RCC. Silencing EIF3D by shRNA inhibited malignant growth of 786-O and ACHN RCC cell lines, caused G2/M arrest, and induced apoptosis, through misregulation of cell cycle regulators. These results suggested that increased EIF3D expression was associated with malignant transformation of RCC, and may serve as a biomarker for RCC.

CCRCC is the major histologic subtype of RCC, also called conventional RCC, accounting for >80% RCC (15). There are no effective therapies for advanced mCCRCC, except for VEGFR inhibitors with about only 50% response rate. To identify novel targets and biomarkers for RCC is of great importance in treating RCC. Here, we demonstrated that EIF3D protein expression in RCC tissues was significantly higher than that in the paracarcinoma tissues among 20 clinical specimens. EIF3D mRNA was obviously elevated in 136 CCRCC tissues compared to 58 normal tissues from ONCOMINE database. EIF3D expression in 102 RCC tissue microarray was positively correlated with tumor size (P=0.004) and TNM stage (P=0.03). Moreover, EIF3D expression was significantly increased in CCRCC with a higher malignancy compared with CRCC and PRCC in Jones renal dataset. To further explore the biological function of EIF3D in CCRCC, shRNA was used to knockdown the expression of EIF3D in CCRCC cell lines, 786-O and ACHN cells. Functional analysis indicated that knockdown of EIF3D observably inhibited the capacity of cell proliferation and colony formation, which is in agreement with the effect of EIF3D-silencing observed in prostate cancer, glioma, melanoma, colon cancer and NSCLC cells (9-13). These results implied that EIF3D is a potential proto-oncogene, participating in malignant transformation of RCC.

We found that EIF3D knockdown induced G2/M phase arrest. Consistent with our results, G2/M phase arrest induced by EIF3D knockdown has also been observed in different types of malignant cells (10,11,13,14). However, the potential molecular mechanisms of EIF3D affecting the cell cycle distribution has not yet been reported. G2/M checkpoint in eukaryotic cells is an important cell cycle DNA damage checkpoint, which play an important role in regulating cell cycle progression (16). G2/M arrest is considered to prevent eukaryotic cells from entering the process of mitosis and could be an effective antitumor strategy (17,18). In eukaryotes, cell cycle is regulated by cyclins, cyclin-dependent kinases (CDKs) and cyclin-dependent kinase inhibitors (CDKIs) (19). Cell cycle G2 phase into M phase for mitosis was reported to be regulated by activation of Cyclin B1/Cdk1 complex (19,20). Cyclin-dependent kinase inhibitors (CDKIs) that bind to cyclin/Cdk complexes regulate cell cycle by suppressing the kinase activity of cyclin/Cdk complexes (21). p21, a downstream target of p53 and an important CDK1 that induces G2/M phase arrest, was reported to work with proliferating cell nuclear antigen (PCNA)/Cdk complexes to regulate DNA repair (22-24). Our results showed that EIF3D knockdown increased the protein expression of p21 and decreased the protein expression of Cyclin B1 and CDK1, indicating that EIF3D-silencing arrested cell cycle at G2/M phase in CCRCC cells. Furthermore, gene correlation analysis using ONCOMINE microarray database also demonstrated that EIF3D mRNA expression was positively association with the mRNA expression of Cyclin B1, CDK1 and PCNA. These results have been demonstrated by Wu et al, who showed that G2/M arrest was attributed to the downregulation of Cyclin B1 and CDK1 and upregulation of p21 (25).

Promoting cell apoptosis is another important mechanism underlying the inhibitory effect of antitumor therapy on the growth of tumor cells (26). Our study demonstrated that silencing of EIF3D markedly induced more cells accumulated in sub-G1 phase in CCRCC cells, suggesting that the percentage of apoptosis cells increased after knockdown of EIF3D. PARP is a vital protein that plays an important role in DNA repair and programmed cell death (27). Once the PARP has been cut by the cleavage of caspases, apoptosis will be induced (28). Western blotting in this study showed that cleaved PARP was upregulated in Lv-shEIF3D group as compared with Lv-shCON group. Recently, it was reported that 5-Nitro-5’-hydroxy-indirubin-3’-oxime (AGM130) induced cell apoptosis through upregulating the level of cleaved PARP to inhibit the growth of A549 NSCLC cells (29). In brief, silencing of EIF3D expression in CCRCC cells increased the production of cleaved PARP to suppress cell growth.

In conclusion, this study provides insights into the clinical significance of EIF3D in RCC, demonstrated that knockdown of EIF3D in RCC cells significantly induced cell growth suppression and cell cycle arrest through down regulation of Cyclin/CDK1 signaling. These results provide reasonable evidence that EIF3D may function as a potential oncogenic molecule that plays a pivotal role in the occurrence and progression of RCC.

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