Original Article
Running Title: Stage-related expression levels of eIF4E and 4E-BP1
Received: May 29, 2024; Accepted: November 30, 2024

Urothelial Carcinoma Stage and Grade Association with the Expression Levels of Eukaryotic Initiation Factor 4E and 4E Binding Protein 1

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Abstract

Background: Bladder cancer (BCa) accounts for the fourth most common cancer among men. Eukaryote translation initiation factor 4E (eIF4E) and its regulatory binding protein (4E-BP1) abnormal expression have been identified in numerous cancers. Therefore, we hypothesized and examined the existence of a connection between eIF4E and 4E-BP1 dysregulation and urothelial cancer (UC) in human subjects by employing more sensitive protein and gene expression experiments in both superficial and muscle-invasive UC.

Method: In this case-control study, the eIF4E and 4E-BP1 mRNA and protein levels were assessed in snap-frozen tissue samples of normal (n = 23), superficial (n = 38) and muscle invasive (n = 29) UC by immunohistochemical staining, quantitative real-time polymerase chain reaction and western blot. A comparison of different groups was carried out by Mann–Whitney U and Kruskal–Wallis tests using Graph Pad prism version 9. $P < 0.05$ were considered as significant.

Results: In addition to elevated expression for both eIF4E and 4E-BP1 in the UC group, we also considered upregulated patterns in both superficial and invasive UC as compared with the control group. An upregulated pattern was predominant in superficial group. Moreover, we observed diminished expression of eIF4E and 4E-BP1 in high-grade tissues of both superficial and invasive subjects compared with low-grade ones ($P < 0.05$).

Conclusion: The present data indicates a possible link between decreased 4E-BP1 expression and high-grade UC that might be associated with expression of cancer promoting genes. Whilst eIF4E may play a role in low grade UC.
Keywords: Carcinoma, Transitional cell, Eukaryotic initiation factor-4E, 4E-BP1, Urothelial cancer

Introduction

Bladder cancer (BCa) has proven to be the most diagnosed urinary system-related cancer in both sexes (168,560) and accounts for the fourth most common cancer among men with 6% (62,420) estimated incidence rate, and it is increasing 1.3% annually in men and women.[1] Smoking tobacco is considered the main urothelial cancer (UC) risk factor. Furthermore, occupational exposure to carcinogenic chemicals, chronic inflammation of the urinary bladder, and radioactive radiation are the next etiologies.[2] The most prevalent symptom of UC is painless gross hematuria, confirmed by cystourethrsocopy, transabdominal ultrasound, computed tomography and finally endoscopic transurethral resection of tumor are mostly recommended.[3, 4] The most common pathologic type of UC is transitional cell bladder cancer. Moreover, BCa can be categorized according to bladder wall diffusion severity, into superficial (non-invasive) and muscle-invasive which the first contains 80%-90% of cases and the remaining 10%-20% belongs to the second category.[5, 6]

Translation and translational initiation are considered prime processes for cancer cell adaption and response to the tumor environment and various conditions including distant-recurrence, immune system surveillance and cytotoxic drug resistance. The eukaryotic translation initiation factors (eIFs) play key roles in protein biogenesis; thus, their aberrant activity has been identified in numerous cancers, aging process and neurodevelopmental disorders like autism.[7] Translation initiation by eIFs is a multilevel mechanism in which at first eIF2 forms a ternary complex (TC) and then the other eIFs (eIF3, eIF5) with the 40S subunit compose 43S preinitiation complex. Next, eukaryotic initiation factor 4E (eIF4E) bounds to eIF4A and eIF4G forming cap binding eIF4F complex, which allow 43S preinitiation complex to recognize the mRNA. The latter step starts by moving the complex from the 5´ untranslated region to the initiation codon, forming 48S initiation complex and subsequently 60S subunit binds to the 48S complex with the help of eIF5B and eIF6 to make a complete 80S complex, which commences the translation initiation and proceed to the translation elongation.[8, 9]

A growing number of investigations have focused on the role of eIF4E in both tumor formation and metastasis by focusing on the translation of malignancy-related transcripts like cyclin D1, c-myc, vascular endothelial growth factor, FGF-2, and MMP9.[10, 11] The eIF4E-binding proteins (4E-BP1, 4E-BP2 and 4E-BP3) are heat-stable proteins that prevent eIF4G binding to eIF4E at the same binding site. Therefore, 4E-BPs prohibit assembly of eIF4F complex, and subsequently eIF4E-mediated translation initiation halts. Phosphatidylinositol 3-kinase (PI3K)-Akt- mammalian target of rapamycin (mTOR), Ras, and hypoxia-inducible factor-1 (HIF-1) signaling pathways play pivotal roles in regulating eIF4E and its binding proteins activity.[11, 12] The PI3K-Akt-mTOR related kinase and Ras/Raf/MAP kinase pathway phosphorylate 4E-BPs; thus, their affinity to eIF4E decreases, allowing cap-dependent translation activation and initiation of carcinogenesis-related function.[13, 14] Tumor hypoxic condition has been more considered for anti-cancer drug

development. HIF-1α is a heterodimeric transcription factor whose expression is stimulated by hypoxia and is crucial for tumor cell adaption and survival with low oxygen tension (Figure 1).[15] Activated eIF4E through PI3K-Akt-mTOR pathway, induce HIF-1α synthesis.[12] Interestingly, mTOR pathway may become down-regulated within high-stage patients because of HIF-1α activation.[16] This study aimed to investigate eIF4E and 4E-BP1 expression levels in human subjects by employing more sensitive protein and gene expression experiments than previous studies, in both superficial and muscle-invasive UC. Moreover, we evaluated both high and low-grade subgroups belonging to the above-mentioned stages of UC using fresh tumor tissue samples.

Materials and Methods

Patients

The Ethics Committee at Urology and Nephrology Research Center approved the sampling procedure from human subjects (sbmu.unrc.890708.05). In this case-control study, UC tissue samples were collected during transurethral resection of bladder tumor and radical cystectomy procedures and classified into superficial (n = 38) and invasive UC (n = 29) based on TNM system (Table 1). All UC patients without any prior treatment for UC, Intravesical immuno or chemotherapy, systemic chemotherapy, pelvic radiation therapy, or other malignancies were included in the study. Informed written consent were received from participants. Bladder normal tissue was sampled from bladder of subjects during open prostate procedure (n = 23). Normal bladder samples gained from full-thickness open biopsy, measuring 5/5 mm, from the healthy bladder wall of those patients who underwent open surgical enucleation of prostate adenoma. Tissues were transferred to the laboratory at 4 ºC and after removal of blood remnants and necrotic parts, were snap-frozen in liquid nitrogen and stored at -80 ºC.

Immunohistochemical analysis of eIF4E and 4E-BP1 expression

UC and normal bladder tissue were fixed in 4% formaldehyde directly after collection for 24 h. After routine histologic preparation, tissues were embedded in paraffin and 5 µm sections were obtained. The tissue sections were stained for the expression of eIF4E and 4E-BP1 using their primary antibodies (mouse monoclonal, Santa Cruz Biotechnology, CA, USA). Then, to probe primary antibody binding sites, biotinylated universal secondary antibody of Vectastain Universal Elite ABC Kit (Vector Laboratories, Inc., Burlingame, CA) was applied. Finally, 3, 3′-diaminobenzidine substrate (Vector Laboratories, Inc., Burlingame, CA) was used as chromogen and sections were counterstained with Hematoxylin. In negative control tissue sections, primary antibodies were replaced by isotype control. Multiple slides and fields for each specimen were examined to determine the intensity and distribution of staining. The intensity was recorded on a scale of 0 to 4 (0, negative; 0.5, trace; 1, light; 2, moderate; and 3, intense; 4, very intense). The distribution of stain was categorized with a numerical score (0, negative areas; 0.1, 1-25% stained; 0.4, 26-50% stained; 0.6, 51-75%; and 0.9, 76-100%). Histoscores for each slide were calculated by multiplying mean intensity by mean stain distribution.

Western blotting

TTC and normal bladder tissues were lysed and homogenized in RIPA buffer containing protease and phosphatase inhibitors (50 mM Beta-glycerophosphate, 1 mM PMSF, 5 µg/ml Leupeptin, 10 µg/ml Pepstatin A, 1 mM EDTA, 5 mM EGTA, 10 mM NaF, 1 mM Sodium orthovanadate) by a Dounce homogenizer. The concentration of protein extracts was measured by Bradford method, and 40 µg of each sample was

electrophoresed by 12% sodium dodecylsulfate polyacrylamide gels the resolved proteins were blotted on polyvinylidene difluoride membranes by wet western blot transfer. The membranes were cut and casein solution was used for blocking the membranes at 4 °C overnight. Next, the membranes were incubated with primary antibodies against eIF4E (1:200, mouse anti human, Santa Cruz Biotechnology, CA, USA), 4E-BP1 (1:150, mouse anti human, Santa Cruz Biotechnology, CA, USA), and beta actin (1:500, mouse anti human, Santa Cruz Biotechnology, CA, USA). Then, horse radish peroxidase-conjugated anti mouse IgG secondary antibody (1;2000, Santa Cruz Biotechnology, CA, USA) was applied for probing primary antibodies binding sites. Finally, the membranes were subjected to ECL (beta actin) and ECL plus (eIF4E and 4E-BP1) reagents (Thermo Scientific, Waltham, MA, USA) and then exposed to western blotting films (Thermo Scientific, Waltham, MA, USA). Band intensities were calculated by ImageJ sofware (NIH, Bethesda, MD, USA) and normalized against beta actin.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA of tissue samples was isolated by RNX-plus reagent (Sinaclon, Tehran, Iran) and quantified by WPA spectrophotometer (Biochrom). DNA contamination was omitted using DNase I (RNase-free) (Sinaclon, Tehran, Iran) and cDNA was synthesized by cDNA synthesis kit (Sinaclon, Tehran, Iran). Primers were designed by AlleleID 6 software (Table 2) and synthesized by Macrogen (Macrogen, South Korea). PCR reactions for quantification of mRNAs were as follows: 10 µl 2X RealQ plus MasterMix Green (Ampliqon, Denmark), 0.8 µl of each primer, 2 µl cDNA template (1:5 in distilled water) and 6.4 µl distilled water. PCR reactions were performed by Rotor-Gene Q instrument (Qiagen) with parameters of 15 min at 95 °C for enzyme activation, and 35 cycles of 95 °C for 20 seconds followed by 60 °C for 60 seconds. GAPDH expression was used as a housekeeping gene, and the relative expression of mRNAs was normalized using $2^{-\Delta Ct}$.

Statistical analysis

Distribution of data was assessed by Kolmogorov-Smirnov test. A comparison of different groups was carried out using Mann–Whitney U and Kruskal–Wallis tests. All data were presented as mean ± standard error of mean and *P*-values < 0.05 were considered as significant. GraphPad prism version 9 was used for statistical analysis.

Results

Immunohistochemical results of eIF4E and 4E-BP1 expression

By comparing the histoscores of tissues after IHC (Figures 2A1 and 2A2), we observed a modest increased expression of eIF4E in TTC compared with the control group, which was not statistically significant (Figure 2B). However, significantly elevated levels of eIF4E were noticed in superficial UC in comparison with invasive UC ($P = 0.01$, Figure 2C). In addition, increased expression level of eIF4E was detected in low grade UC versus control and high-grade UC ($P = 0.046$, $P = 0.035$; respectively, Figure 2D). In this regard, when superficial UC group was categorized into low grade and high-grade superficial UC, a higher level of eIF4E was observed in the low grade superficial UC compared with the control group ($P = 0.023$, Figure 2E).

Regarding 4E-BP1 expression (Figures 3A1 and 2A2), relatively similar pattern to the expression of eIF4E was observed. Non-significant raise of 4E-BP1 was observed in UC, superficial UC, and invasive UC tissues compared with the control group (Figures 3B and C). Expression of 4E-BP1 was significantly higher in low grade UC versus

high grade UC and the control group (P = 0.047, P = 0.038; respectively, Figure 3D). Elevated level of 4E-BP1 was detected in low grade superficial UC when compared with high grade superficial UC (P = 0.015, Figure 3E). As almost all samples in the invasive UC group were high grade, no comparison between low grade and high- grade invasive UC was imaginable.

Evaluating the expression of eIF4E and 4E-BP1 proteins in tissue extracts

Western blot analysis of eIF4E revealed significantly increased levels in UC patients compared with control subjects (P = 0.0003, Figures 4A and 4I). Accordingly, significant raise of eIF4E was detected in superficial and invasive UC compared with the control group (P = 0.002, P = 0.023; respectively, Figure 4B). However, no difference between superficial and invasive UC was observed (Figure 4B). In addition, protein level of eIF4E was considerably higher in low grade UC versus control group (P = 0.0005, Figure 4C). No significant difference between levels of eIF4E in high grade UC in comparison with low grade UC was observed (Figure 4C). Superficial low-grade UC showed increased level of eIF4E in comparison with the control group (P = 0.0009, Figure 4D); while the difference between superficial low-grade and high-grade UC was not significant. Protein levels of 4E-BP1 were detected in considerably higher levels in UC patients compared with the control group (P = 0.0005, Figures 4E and 4I). The 4E-BP1 level was also significantly increased in superficial UC in comparison with the control group (P = 0.0005, Figure 4F), but the difference between superficial and invasive UC was not statistically significant. A comparison of 4E-BP1 levels between low-grade and high-grade UC did not reveal any significant alteration; however, low-grade UC levels of 4E-BP1 were significantly higher than those in the control group (P = 0.0007, Figure 4G). After classification of superficial UC into low- and high-grade, no significant difference was noticed; nevertheless, 4E-BP1 levels in low-grade UC were significantly elevated in comparison with the control group (P = 0.0007, Figure 4H).

Gene expression of eIF4E and 4E-BP1

In the analysis of the eIF4E expression level, we noticed significant up-regulation in UC patients compared with the control group (P = 0.0018, Figure 5A). The eIF4E level showed a dramatic increase in superficial UC group (P = 0.0022) compared with control. Furthermore, no statistically significant differences were seen between superficial and invasive groups (Figure 5B). The eIF4E expression level exhibited significantly higher levels in low-grade UC compared with control subjects (P = 0.001, Figure 5C). In addition, eIF4E expression showed elevated levels in superficial low-grade UC versus control group (P = 0.0014, Figure 5D). Although the levels of eIF4E in low-grade UC and superficial low-grade UC were increased compared with high-grade UC and superficial high-grade UC, these differences were not statistically significant.

Regarding 4E-BP1, the expression patterns were the same as the eIF4E but with some changes in statistical differences that are summarized in Figures 5E-H. Relative expression levels of 4E-BP1 were significantly higher in UC group as compared with the control group (P = 0.013, Figure 5E). In addition, increased levels of 4E-BP1 in superficial UC, low-grade UC, and superficial low-grade UC compared with other groups were observed, which did not reach the statistical threshold (Figures 5F-H).

Discussion

Our findings using protein and gene expression experiments, revealed elevated expression for both eIF4E and 4E-BP1 in the UC group. Moreover, our data exhibited up-regulated patterns in both superficial and invasive UC compared with the control

group. This upregulated pattern was predominant in the superficial group. Also, we observed diminished expression of eIF4E and 4E-BP1 in high-grade tissues of both superficial and invasive subjects compared with low-grade ones.

eIF4E plays a key role in cap-dependent translation initiation and its aberrant expression may lead to tumorigenesis.[2] Moreover, eIF4E is identified as an absolute proto-oncogene, the raised expression level of which increases the progression susceptibility of different kinds of tumors.[7, 8, 17] Chen et al.[18] noticed elevated expression of 4E-BP1/eIF4E using an HPV-immortalized cervical epithelial (H8) cell line; however, eIF4E activation was halted by 4E-BP1. Lu et al. interestingly observed that the p-eIF4E expression was significantly increased in lung adenocarcinoma, positively correlated with clinical stages.[19] Furthermore, different experiments introduced 4E-BP1 overexpression as a crucial element in different types of tumors including lung, prostate, breast and leukemia.[7, 20] In one study exploring eIF4E on BCa, increased p-eIF4E levels in both murine and human UC were reported.[21] However, in another study, that explored eIF4E expression by IHC staining UC tissues, no significant differences were reported between tumor and normal tissue.[2] Park et al. found the increased expression of p-4E-BP1 in UC patients versus the benign cohort.[16] Here, in our experiment using fresh snap-frozen tissue samples, protein, and gene expression levels of eIF4E and 4E-BP1 indicated increased pattern in the UC group compared with the control group, being statistically significant except for IHC results.

Jana et al. showed that p-eIF4E expression level was increased with the progression of normal urothelium to aggressive carcinoma in vivo.[21] Targeting p-4E-BP1 and p- eIF4E by mTOR inhibitors suppressed bladder tumor invasion which pointed the vital role of these two proteins in bladder tumor progression.[22] Kwon et al., by manipulating the expression of eIF4E in urothelial carcinoma cell lines (T24 and 5637), demonstrated that eIF4E silencing was associated with reduced cell migration and invasion.[23] According to an investigation by Crew et al., eIF4E expression level was greater in muscle invasive bladder tumors than superficial one. In addition, they found that individuals with T1G3 tumor were more susceptible than subjects carrying T1G1 or T1G2 bladder tumors for disease progression.[24] Based on our findings, both superficial and invasive UC indicated increased levels of eIF4E and 4E-BP1 compared with the control group. In addition, the superficial group showed higher levels of eIF4E and 4E-BP1 than the invasive one, which was only statistically significant in IHC results. The conflicting results observed in this study compared to Crew et al. could be attributed to the use of significantly different techniques, as they used an unreliable semi-quantitative method. In addition, the larger sample size of the present study makes our findings more robust. In this regard, Kwon et al.'s findings on the contribution of eIF4E in cancer cells migration and invasion were totally derived from an in-vitro study, which can be remarkably different from our work dealt with human samples.

An elevated level of p-eIF4E is strongly related to the high-risk prostate cancer and poor survival.[25] Mice with eIF4E phosphorylation resistance, are resistant to lung metastasis and its induction by transforming growth factor-beta can be resulted in epithelial-to-mesenchymal transition.[26] Moreover, another study on colorectal adenocarcinoma showed that eIF4E expression is highly correlated with lymphovascular and perineural invasion, tumor and nodal stage, metastatic status and disease stage.[27] An elevated level of eIF4E in breast cancer subjects also is associated with

the aggressiveness degree and poor survival of disease.[28] Although elevated eIF4E expression was stated to be related to exacerbation of tumor state in different kinds of tumors, there were not sufficient experiments exploring this gene in high-grade BCa. One of the advantages of present study is sub grouping superficial and invasive UC into high-grade and low-grade levels before assessing eIF4E and 4E-BP1. As our gene and protein expression analyses showed, we observed diminished expression of eIF4E at protein and gene levels in high-grade subjects. Inconsistency of our results with other kinds of malignancies may originate from different molecular basis of BCa development; in addition, larger-scale experiments on different grades and levels of BCa and phosphorylated form of eIF4E and 4E-BP1will be recommended to acknowledge our findings.

Diab-Assef et al. indicated the bi-phasic manner of 4E-BP1 during carcinogenesis as a tumor suppressor protein which means that its expression decreased by the progression of the disease.[29] The fact that 4E-BP1 can repress eIF4E overexpression reveals the inhibitory role of 4E-BP1.[30] Another cohort experiment on BCa revealed that p-4E-BP1level was not related to progression or recurrence in superficial BCa.[16] According to Fahmy et al., 4E-BP1 was highly expressed in different stages of BCa, but there were not any significant differences among them.[31] Accordingly, Kim et al. also added that despite the high expression of 4E-BP1 in low-grade BCa, 4E-BP1 cannot be a reliable recurrence predictive marker for high-grade subjects as there were no correlation between this protein and clinicopathological variables.[32] Conversely, raised expression of 4E-BP1 in invasive bladder urothelial carcinoma and its relevance to pathological stage was reported.[33] Therefore, there were diverse findings about 4E-BP1 association with pathological stages and exacerbation of BCa.

Based on our findings, 4E-BP1 can be down-regulated through high-grade stages of both superficial and invasive BCa. Our Western blot findings also support this hypothesis. Although our findings have raised more questions about the role of eIF4E and 4E-BP1in UC than the answered ones, addressing the limitations of this work by investigating the phosphorylated form of these proteins and their upstream molecules in the signaling pathway will provide a more detailed insight about the function of eIF4E and 4E-BP1 in UC.

Conclusion

Translation initiation and its regulatory mechanisms can play significant roles in UC molecular pathology. Our findings using fresh UC tissue indicated that there could be a relationship between decreased 4E-BP1 expression and high-grade UC. This may be associated with the specific gene expression profile promoting cancer invasion. While extended experiments about eIF4E role in different grades of UC levels are needed, a plausible connection between its expression and superficial UC can be imagined.

Acknowledgments

We would like to thank Hootan Yazdani for his technical assistance for western blotting and IHC staining.

Funding

This study was financially supported by the Urology and Nephrology Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.

Authors' Contribution

Study designing: M.N., F.S., S. A. A. Data gathering and drafting: M.Y., S. M., H. S., and M. P. Drafting the paper and data analysis: M.Y., M. N. Reviewing the

manuscript: F. S. All authors read and approved the final manuscript version and agree with all parts of the work in ensuring that any queries about the accuracy or integrity of any component of the work are appropriately investigated and handled.

Conflict of Interest

None declared.

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Table 1. Pathological characteristics of urothelial cancer human subjects

Clinical stage, n (%)	
pTa	7 (10/45%)
pT1	31 (46/27%)
pT2	21 (31/34%)
pT3	5 (7/46%)
pT4	3 (4/48%)
Controls	23 (100%)
Lymph node stage, n (%)	
NX	51 (76/12%)
N0	5 (7/46%)
N1	0
N2	1 (1/49%)
Missing data	10 (14/93%)
Metastasis stage, n (%)	
MX	57 (85/07%)
M0	0
Missing data	10 (14/93%)
Clinical grade, n (%)	
High-grade	47 (55/22%)
Low-grade	20 (29/85%)

n: Number; pT: Pathologic stage; pTa: Non-invasive papillary carcinoma; pT1: Tumor invades sub epithelial connective tissue layer; pT2: Tumor invades muscle; pT3: Tumor invades perivesical tissue; pT4: Tumor invades surrounded organs; NX: Regional lymph nodes cannot be assessed; N0: No regional lymph node metastasis; N1: Metastasis in a single lymph node in the true pelvis; N2: Metastasis in multiple regional lymph nodes in the true pelvis; MX: Metastasis cannot be assessed; M0: No distant metastasis

Table 2. The sequences of oligonucleotides used for quantification of mRNAs by real-time PCR

Oligonucleotide	Sequence (5′-3′)
eIF4E-Forward	TAGCAATATGGACTACTGAATGTG
eIF4E-Reverse	CTGCGTGGGACTGATAACC
4E-BP1-Forward	CGGGCGGGCGGTGAAGAG
4E-BP1-Reverse	CCTGGCTGGTGGGACTCCTC
GAPDH-Forward	GCCACATCGCTCAGACAC
GAPDH-Reverse	GCAACAATATCCACTTTACCAGAG

eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1; PCR: Polymerase chain reaction

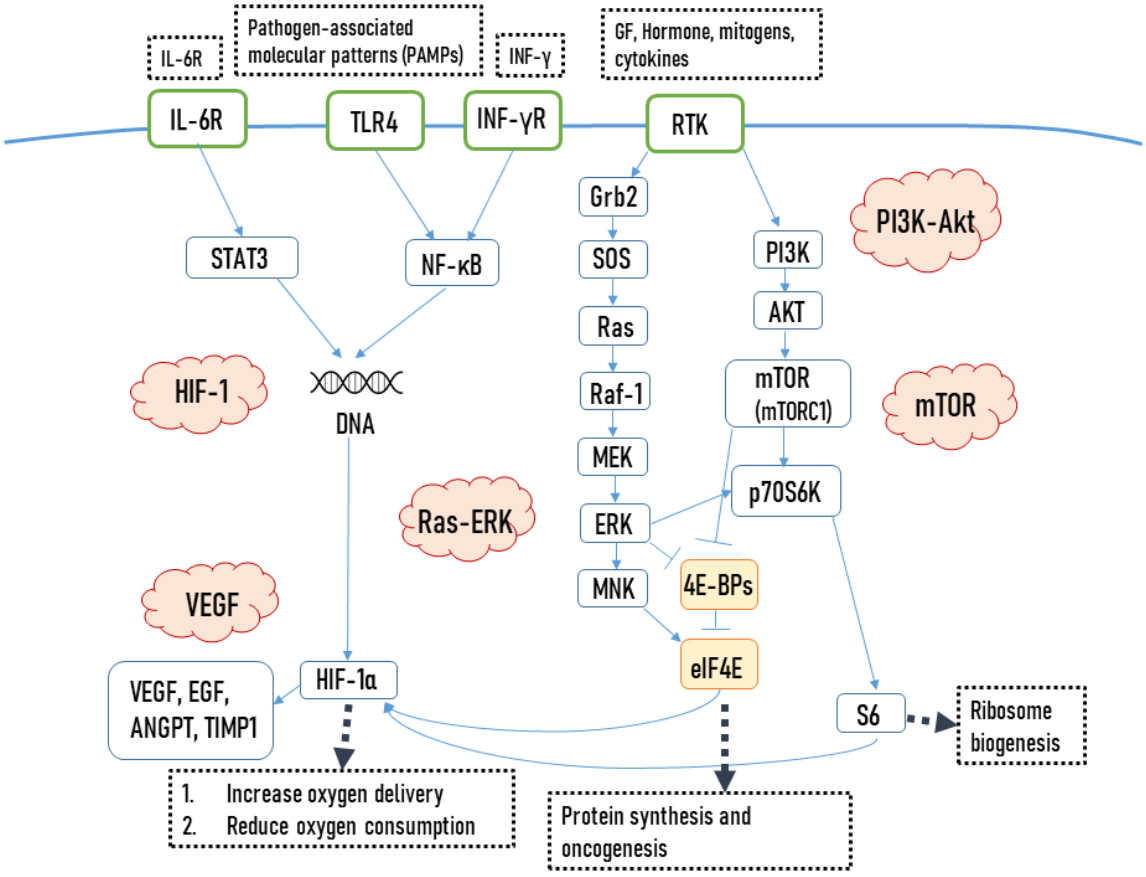

Figure 1. Schematic diagram of signaling pathways that can liberate eIF4E from its binding proteins like 4E-BP1 is depicted in this figure. As the result of eIF4E activation by altered function of PI3K/AKT/mTOR pathway, ribosome biogenesis, protein synthesis and its aberrant expression promote oncogenesis in different kind of cancers.[34] Also, HIF-1 pathway activation, through VEGF signaling pathway activation, will enable cancer cells more compatible with different oxygen level existed in their intercellular matrix. This pathway can be substituted by mTOR pathway in more aggressive level of bladder cancer.[15, 16]

ERK: Extracellular signal-regulated kinases; TLR4: Toll-like receptor 4; RTK: Receptor tyrosine kinase; HIF-1: Hypoxia-inducible factor-1; mTOR: Mammalian target of rapamycin; PI3K: Phosphatidylinositol 3-Kinase; VEGF: Vascular endothelial growth factor; eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1; IL: Interleukin; INFγ: Interferon gamma; STAT3: Signal transducers and activators of transcription 3; NFκB: Nuclear factor kappa B ; ANGPT: Angiopoietins; TIMP1:Tissue inhibitor of metalloproteinase 1; AKT: Alpha serine/threonine- protein kinase; SOS: Son-of-sevenless protein; MEK: Mitogen-activated protein kinase; Ras: Renin-angiotensin system; Raf-1: Rapidly accelerated fibrosarcoma-1; p70S6K: Ribosomal protein S6 kinase beta-1

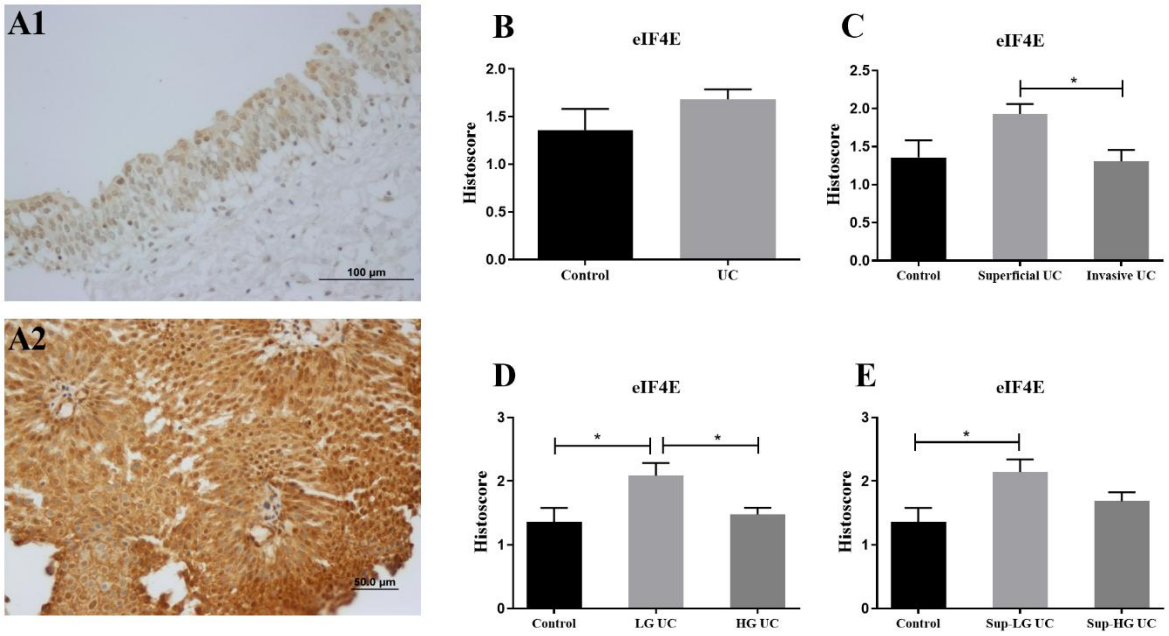

Figure 2. Immunohistochemistry staining of eIF4E expression in UC and control tissues was shown. (A1) eIF4E protein was expressed in transitional cells of normal bladder (bar: 100 µm), and (A2) UC tissue (bar: 50 µm). eIF4E histoscore differences between control and UC group (B); control group versus superficial and invasive UC groups (P = 0.01) (C); control group versus LG and HG UC groups (P = 0.046, P = 0.035; respectively) (D); control group compared with Sup-LG UC and Sup-HG UC groups (P = 0.023) (E).

UC: Urothelial carcinoma; LG UC: Low grade urothelial carcinoma; HG UC: High urothelial carcinoma; Sup-LG UC: Superficial low grade urothelial carcinoma; Sup-HG UC: Superficial high grade urothelial carcinoma; eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1. Error bars represent standard error ($^{*}P < 0.05$)

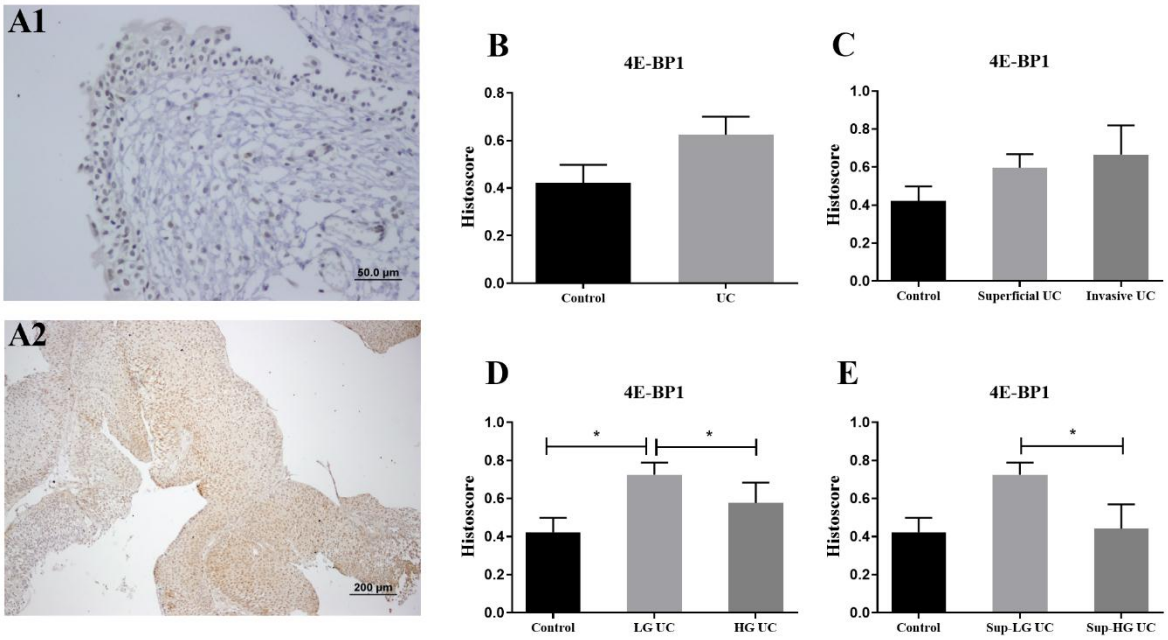

Figure 3. Immunohistochemistry staining of 4E-BP1 expression in UC and control tissues. (A1) Cross-section of bladder in control and (A2) UC bladder tissue showed the expression of 4E-BP1 (bar: 200 µm). Histoscore differences of 4E-BP1 levels between control and UC group ($P = 0.01$) (B); control group versus superficial and invasive UC groups ($P = 0.01$) (C); control group versus LG and HG UC groups ($P = 0.047$, $P = 0.038$; respectively) (D); Sup-LG UC and Sup-HG UC groups compared to normal group ($P = 0.015$) (E).

eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1; UC: Urothelial carcinoma; LG UC: Low grade urothelial carcinoma; HG UC: High grade urothelial carcinoma; Sup-LG UC: Superficial low grade urothelial carcinoma; Sup-HG UC: Superficial high grade urothelial carcinoma. Error bars represent standard error ($^{*}P < 0.05$)

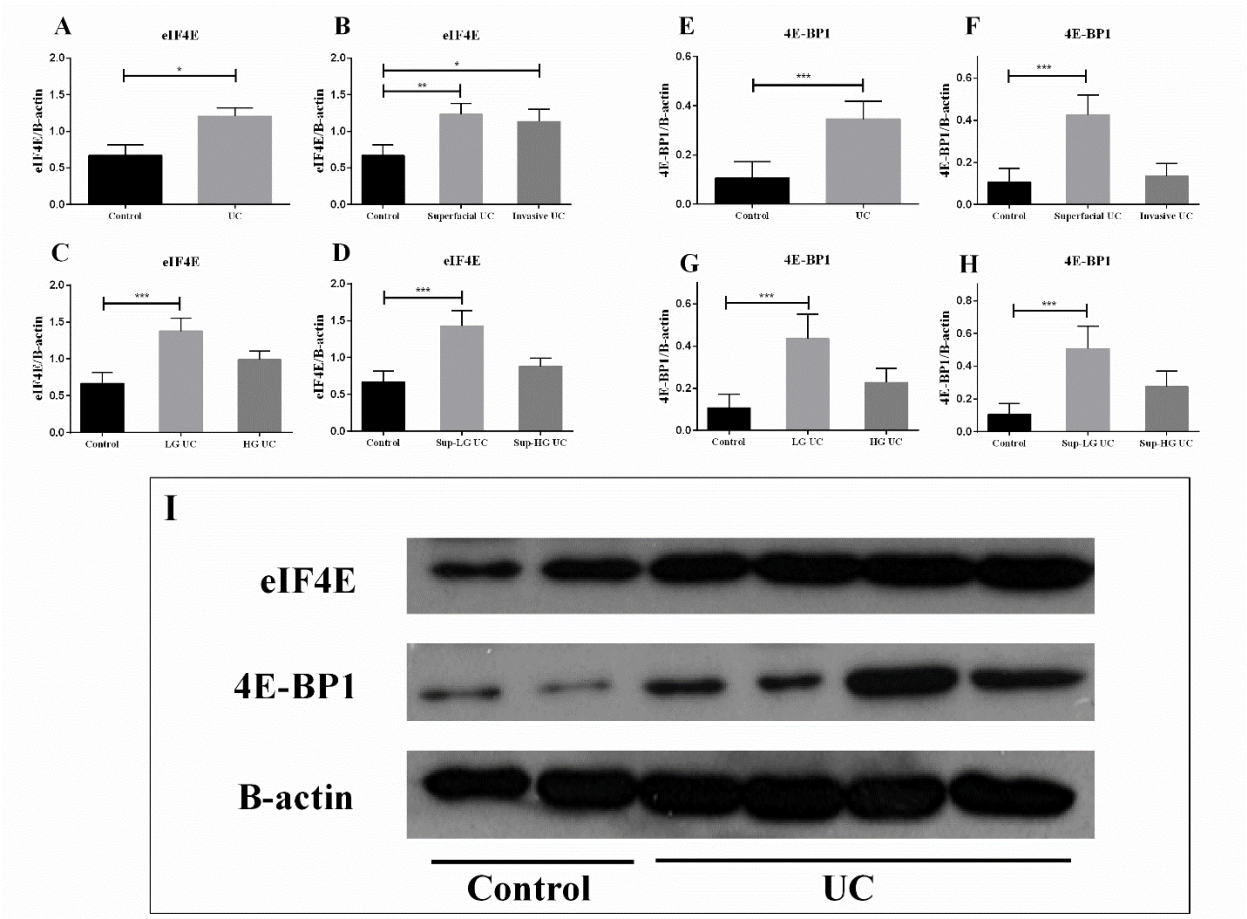

Figure 4. Western blot results of the eIF4E and 4E-BP1in UC and normal tissue. (A-D) Band intensities of eIF4E, between control and UC group (A), control group versus superficial and invasive UC groups (B), control group versus LG and HG UC groups (C), Sup-LG UC and Sup-HG UC groups compared with the control group (D) were calculated. (E-H) Band intensities of 4E-BP1 with the same order of eIF4E charts were depicted. (I) Immunoblot analysis was performed on 40 µg of total protein (loaded in each lane) from control and UC samples (β-Actin served as loading control).

UC: Urothelial carcinoma; eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1; LG UC: Low grade urothelial carcinoma; HG UC: High grade urothelial carcinoma; Sup-LG UC: Superficial low grade urothelial carcinoma; Sup-HG UC: Superficial high grade urothelial carcinoma. Error bars represent standard error. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$

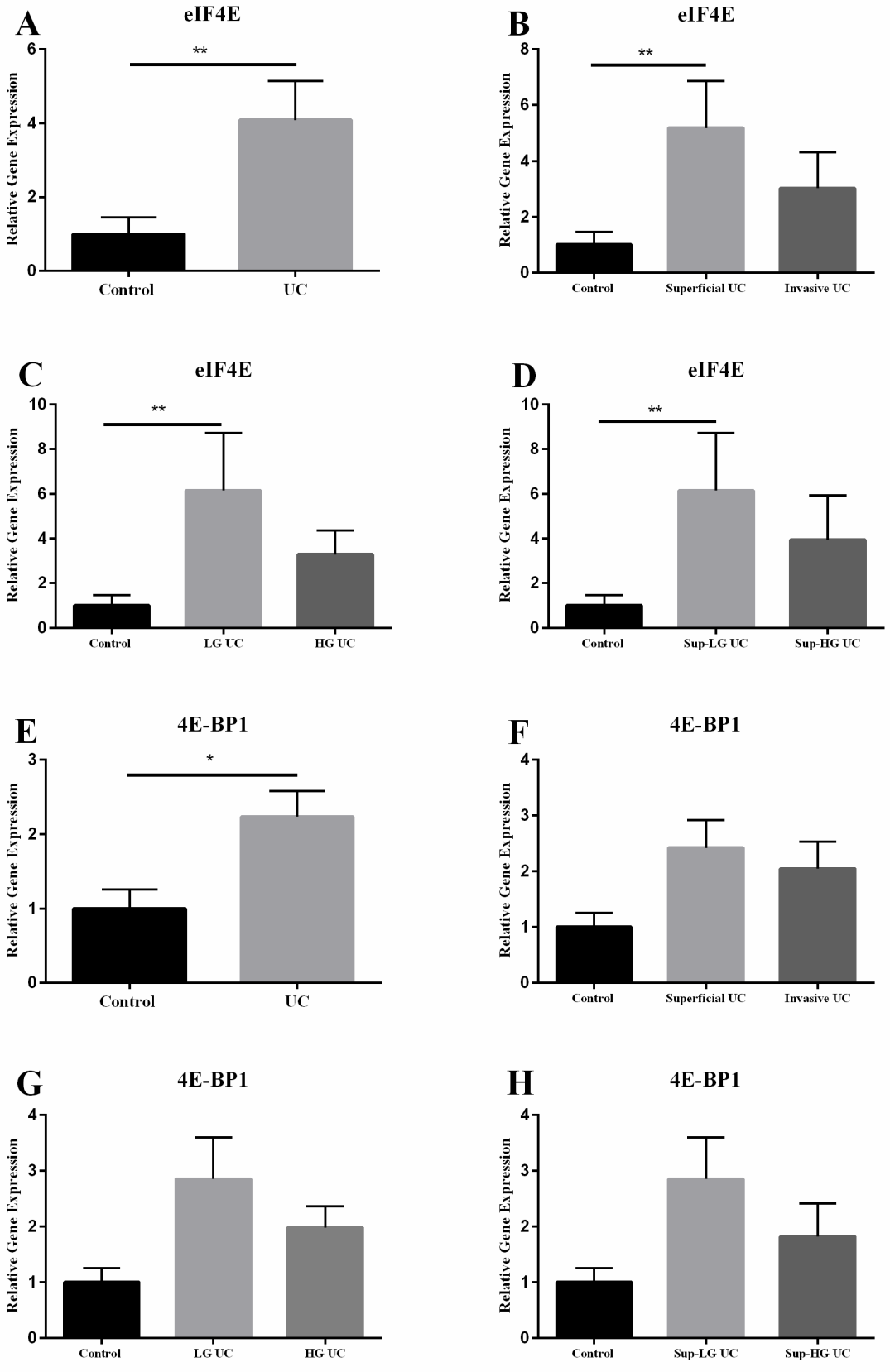
A
eIF4E
Relative Gene Expression
**
Control
UC
B
eIF4E
Relative Gene Expression
**
Control
Superficial UC
Invasive UC
C
eIF4E
Relative Gene Expression
**
Control
LG UC
HG UC
D
eIF4E
Relative Gene Expression
**
Control
Sup-LG UC
Sup-HG UC
E
4E-BP1
Relative Gene Expression
*
Control
UC
F
4E-BP1
Relative Gene Expression
Control
Superficial UC
Invasive UC
G
4E-BP1
Relative Gene Expression
Control
LG UC
HG UC
H
4E-BP1
Relative Gene Expression
Control
Sup-LG UC
Sup-HG UC

Figure 5. The expression levels of eIF4E (A-D) and 4E-BP1 (E-H) mRNA in UC and control groups. All classes of UC (A), superficial and invasive UC (B), LG and HG UC (C), sup-LG and sup-HG UC (D) were compared with the control group for the expression of eIF4E mRNA. The expression of 4E-BP1 is reported with the same order of eIF4E charts (E-H).

eIF4E: Eukaryotic initiation factor 4E; 4E-BP1: 4E binding protein 1; UC: Urothelial carcinoma; LG UC: Low grade urothelial carcinoma; HG UC: High grade urothelial carcinoma; Sup-LG UC: Superficial low grade urothelial carcinoma; Sup-HG UC: Superficial high grade urothelial carcinoma. Error bars represent standard error ($^{*}P < 0.05$, $^{**}P < 0.01$)