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HPV18 E7 induces the over-transcription of eIF4E gene in cervical cancer

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ABSTRACT

Objective(s): Eukaryotic translation initiation factor 4E (eIF4E) is overexpressed in cervical cancer (CC). However, the molecular mechanisms are unclear. This study aimed to investigate the molecular mechanism of eIF4E gene overexpression in CC.

Materials and Methods: The *human papillomavirus* (*HPV*) type 18 E7 and eIF4E mRNAs were measured following knock down or overexpression of E7 gene by RT-PCR and real-time PCR. Cell counting kit-8 assay was used to determine the cell proliferation. Flow cytometry was used to analyze the cell cycle and apoptosis. Transwell system was employed to determine the cell migration.

Results: Overexpression of E7 gene increased eIF4E mRNA level by 24.3% (P<0.01) in HPV negative *C33A* cells. Knock down of E7 decreased markedly eIF4E mRNA by 73% (P<0.01) in HPV18 positive *HeLa* cells. Under the state of high expression of E7, 1) up-regulation of eIF4E drastically promoted the cell proliferation, cell cycle and cell migration, and inhibited the cell apoptosis. 2) down-regulation of eIF4E significantly inhibited the cell proliferation, cell cycle and the ability of cell migration, and also promoted the apoptosis of cervical cancer cells.

Conclusion: HPV E7 induced eIF4E gene over transcription which might be a new marker for CC. The finding broadens the understanding of the CC carcinogenesis.

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Introduction

Cervical cancer (CC) is one of the most common female cancers (1, 2), which is closely related to *human papillomavirus (HPV)* infection. Among the molecules encoded by the high risk HPV DNAs, E7 plays a key role in carcinogenesis. E7 binds the retinoblastoma (Rb) protein family members (pRb, P107, P130) and degrades Rb proteins through the ubiquity 26S protease pathway (3, 4). The Rb degradation leads to the release of elongation 2 factors (E2F) and the activation of proteins which are associated with DNA synthesis and promotes DNA replication, cell division and transformation (5).

Eukaryotic translation initiation factor (eIF4E) is a rate-limiting molecule in the cap-dependent translation initiation (6). Human eIF4E gene is located at chromosome 4q21-q25 and encodes a 24KD protein. EIF4E appears as a cap-binding protein (CBP) that recognizes explicitly the mRNA cap and regulates the mRNA translation. Studies revealed that eIF4E works as a key node of the signal pathway of carcinogenesis and tumor development (7-9). EIF4E was overexpressed in various tumors, including breast cancer, head and neck squamous cell carcinoma, and bile duct cancer (10-12). eIF4E also promotes tumor occurrence, invasion and metastasis by strengthening the translational expression of oncogenes and growth factors such as cellular homolog of the retroviral v-myconcogene (c-Myc), vascular endothelial growth factor (VEGF) and matrix metalloproteinase-9 (MMP9) (10-12). However, the molecular mechanism for eIF4E overexpression remains poorly studied in cervical cancer.

Recent studies reported that eIF4E protein overexpresses in CC (13-15). However, little is known on eIF4E regulation. Van Tranppen *et al* (13) observed eIF4E over expression in CC tissues by reverse transcription-polymerase chain reaction (RT-PCR) and speculated that eIF4E might take a great part

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in tumor development. Lee *et al* (14) further showed through immunohistochemistry (IHC) that eIF4E expression was markedly enhanced following the progression of cervical malignant lesions. So far, studies on eIF4E in CC are still rare, leading to poor understanding of the role and regulation of eIF4E in CC. In our previous study, we found the overexpression of eIF4E was correlated to CC development (15). However, whether HPV E7 could induce eIF4E expression is not known yet, which is an important missing point to clarify the full role of HPV and eIF4E in CC. This paper aimed to explore the mechanism of eIF4E gene overexpression in CC. Here, we provided evidence that E7 can induce eIF4E gene transcription in CC.

Materials and Methods

Cell lines and transfection

Two CC cell lines, HPV positive *HeLa* and HPV negative *C33A* were utilized. The cells were cultured in Roswell Park Memorial Institute (RPMI) cell culture medium 1640, which contains 10% Fetal Bovine Serum (FBS), 2 mmol/l l-glutamine, 50 U penicillin and 50 microgram/ml streptomycin) at 37 °C in 5% CO_2 in air.

Liposome method was employed for the plasmid transfection. Cells were divided into three groups: Mock group (untreated group, no plasmid was used for the transfection), NC group (negative control group, the plasmid containing a negative DNA fragment was used for the transfection) and treated group (the plasmid containing a tested DNA fragment was used for the transfection). 2.5 μl Lipofectamine™ 2000 (Invitrogen, Guangzhou, China) was used per microgram DNA (15).

eIF4E immunocytochemistry (ICC)

ICC was performed using mAbs specific for eIF4E (Santa Cruz, California, USA). The primary antibody eIF4E in 1:25 dilutions was added to the cell smears and incubated at 4 ˚C overnight. PBS instead of the eIF4E antibody was used as the negative control staining. Plasmids *shE7* RNA interference plasmids were constructed by Genechem Company, Shanghai. Three pairs of *shE7* sequences were: ShE7-1 (stem:GCATGGACCTAAGGCAACA, Loop:AGTGAAGCCACAGATGTA, stem: TGTTGCCTTAGGTCCATGC), ShE7-2 (stem: GGCAACATTGCAAGACATT, Loop:AGTGAAGCCACAGATGTA, stem: AATGTCTTGCAATGTTGCC), ShE7-3(stem:GCAAGACATTGTATTGCAT, Loop:AGTGAAGCCACAGATGTA, Stem:ATGCAATACAATGTCTTGC). E7 and E7 mutant expression plasmids were constructed with *pEGFP-C5* vector as described previously (16). E7 contains the wild type E7 of HPV16. E7 mutant contains mutations (TTCGGTTG to TACGTAGG) from nt 191 to nt 198 of E7. The *sieIF4E* was received as desalted and unprotected oligonucleotides. The sequences of sieIF4E were: 5'-GGAUAUUAUAAAUAGAUUATT-3' and 5'-UAAUCUAUUUAUAAUAUCCTT-3'. Normal control (NC) sequences were: 5'-UUCUCCGAACGUGUCACGUTT-3' and 5'-ACGUGACACGUUCGGAGAATT-3'.

Detection of E7 and eIF4E mRNA by RT-PCR and real time PCR

E7, eIF4E and GAPDH primer sequences were used for RT-PCR and real-time PCR: 5'-GCGTTAGAGCCCCAAAATGA-3', 5'-CGTCGGGCTGGTAAATGTTGA-3' for E7. 5'-CTGCGGCTGATCTCCAAG-3', 5'-CTGCGGCTGATCTCCAAG-3' for EIF4E. 5'-GAAGGTCGGAGTCAACGGATTT-3', 5'-CCTGGAAGATGGTGATGGGATT-3' for glyceraldehyde-3-phosphate dehydrogenase (GAPDH). RT-PCR was performed using the Access RT-PCR kit (Promega, USA) according to the manufacturer's protocols. Data was analyzed by sequence detection software from Applied Biosystems. Real-time PCR was performed by Fast Start Universal SYBR Green Master (ROX) assay on the Gene Amp PCR System 9700 (ABI company, USA).

CCK-8 cell proliferation assay

Cell proliferation was determined with Cell counting Kit-8 (CCK-8) (Beyotime Institute of Biotechnology, Shanghai, China) Assay. Cell proliferation ability was represented by the mean absorbance value (AV). The proliferation rate and inhibition rate were calculated:
proliferation rate (%) = (AV treated group/AV mock -1)×100%. Inhibition rate (%)= (1-AV treated group/AV mock) ×100%.

Cell cycle assays and apoptosis assay

Cell cycle and apoptosis were evaluated by flow cytometry. 3×10^5 cells in 300 μl PBS were stained with 3 μl PI at 4 ˚C for 30 min in cell cycle assay, and with 3 μl Annexin V-FITC at room temperature for 10 min. The samples were analyzed with MultiCycle software using the flow cytometer BD FACS CantoTM (Becton Dickinson, CA, USA).

Transwell migration assay

Cell migration was determined in a transwell system. 6×10^4 cells were vaccinated into the upper surface of the transwell membrane and cultured at 37°C in 5% CO_2 in air for 24 hr, 48 hr and 72 hr. The number of migrated cells was counted under a microscope (200X).

Statistical analysis

Data were analyzed using SPSS 17.0 software and were reported as means±standard deviation (SD). Independent samples t-test was used for analyzing the two groups and one-way analysis of variance (ANOVA) was used for examining multiple

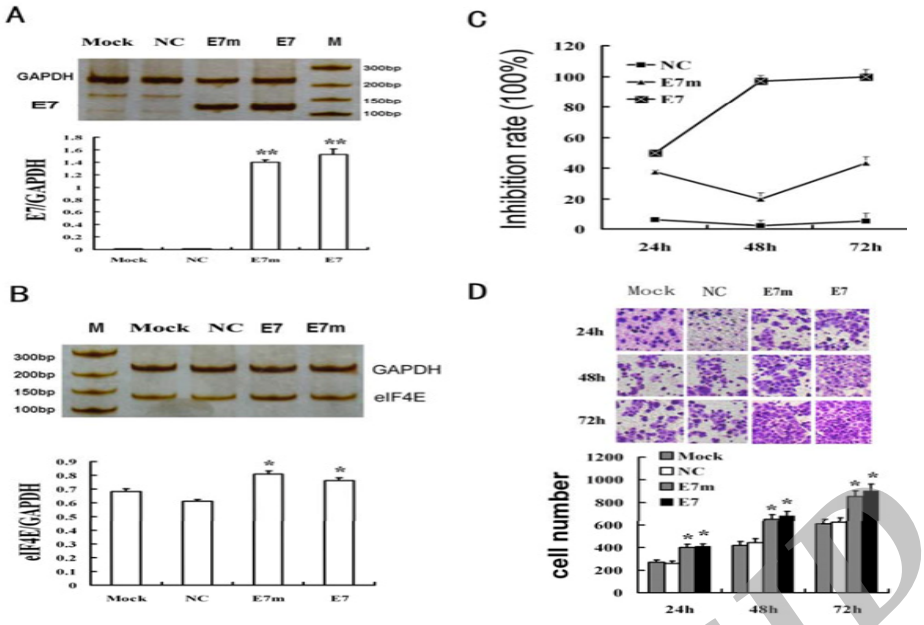

Figure 1. *Human papillomavirus* e7 gene induced eukaryotic translation initiation factor 4e expression and promoted the proliferation and migration of *C33A* cells. Cells were divided into 4 groups: untreated *C33A* cell group (MOCK), p-EGFP blank plasmid group (NC); E7m, E7 mutant group; E7, E7 expression vector group. (A) ecto-E7 gene expression of *C33A* cells at 20 hr, detected by RT-PCR. (B) eIF4E gene expression of *C33A* cells at 20 hr, detected by RT-PCR. (C) Transfection of E7 gene promoted the proliferation of *C33A* cells, detected by CCK-8 assay. (D) Transfection of E7 gene promoted the migration of *C33A* cells, detected by the transwell migration assay. *: vs Mock, P <0.05; **: vs Mock, P<0.01

groups. The level of statistical significance was set at P <0.05 or P <0.01.

Results

HPV E7 induced eIF4E expression and promoted the proliferation and migration of HPV negative C33A cells

The E7 expression vector was transfected into *C33A* cells. For RT-PCR results, two bands with expected sizes for E7 (271bp) (Figure 1A), E7 mutant (271bp) and GAPDH (224bp) were seen. The eIF4E mRNA detection showed two bands for eIF4E (132bp) and GAPDH (224bp) (Figure 1B). By Image J analysis, the relative eIF4E mRNA levels (eIF4E/GAPDH) were 0.682 (mock), 0.613 (NC), 0.808 (E7m group) and 0.762 (E7 group). The eIF4E bands were stronger in E7 and E7m groups than the mock and NC groups. The changes of the eIF4E mRNA were consistent with the changes of E7 mRNA. The lighter changes of the eIF4E mRNA in E7m group suggested that the E7m had a part of function in inducing eIF4E transcription. The results of real-time PCR were similar to that of RT-PCR.

The cell proliferation in the mock and NC groups was similar. Compared with the NC group, the proliferation rates increased by 44.3% (24 hr), 97.4% (48 hr) and 96.0% (72 hr) in E7 group, and 32.4% (24 hr), 19.9% (48 hr) and 40.5% (72 hr) in E7m group, respectively (Figure 1C). Here, E7m also influenced the cell proliferation, suggesting E7m maintained partial function of E7.

At any time point, the number of migrating cells in the mock and NC groups was similar. Compared with the NC group, the number of the migrating cells significantly increased by 412.01±21.523 (24 hr), 680.25±40.032 (48 hr) and 900.11±63.22(72 hr), respectively, in the E7 group, and significantly increased by 400.14±29.218 (24 hr), 651.23± 40.036(48 hr) and 850.99±51.001 (72 hr), respectively, in the E7m group (Figure 1D).

E7 mRNA expression was knocked down effectively by the shE7s in HeLa cells

The *shE7* vectors carrying GFP were successfully constructed. The E7 mRNA level in the NC group was similar with that in the mock group, determined by both real-time PCR and RT-PCR test. By the real-time PCR detection, the E7 mRNA in *shE7*-1, 2, 3 treated groups were significantly lower than that of the NC group (Figure 2A). Among *shE7*-1, 2, 3, *shE7-2* showed the most effective inhibition of E7 mRNA, with an inhibition rate of approximately 81%. By the RT-PCR and agarose gel detection, two distinct bands with expected sizes were seen for E7 gene (119bp) and GAPDH gene (224 bp) in all groups (Figure 2B). By Image J band analysis, the E7 mRNA in *shE7*-1 and *shE7-2* but not *shE7*-3 groups was considerably knocked down, compared with the NC group. The E7 mRNA level in *shE7-2* group was lower than that in *shE7*-1 group (Figure 2B). The changes of the E7 mRNA were comparable in both the real-time PCR and RT-PCR detection (Figure 2).

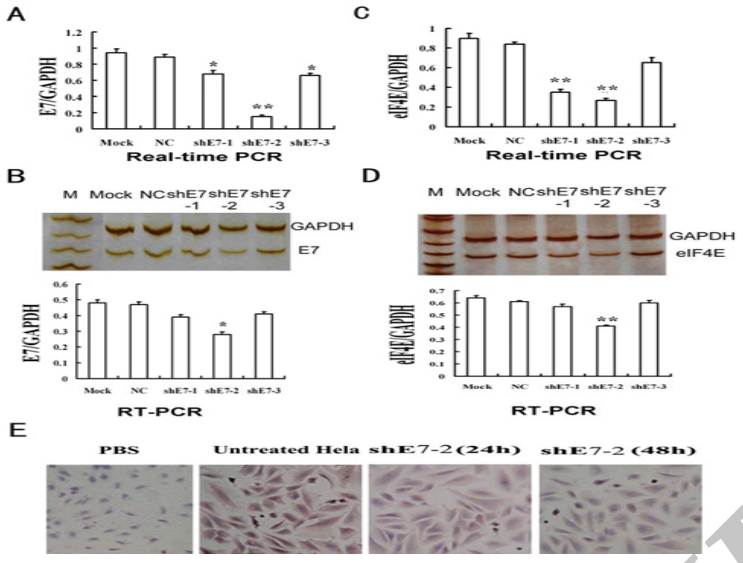

Figure 2. Knockdown of e7 in *HeLa* cells down regulated eukaryotic translation initiation factor 4e gene expression. (A) E7 mRNA expression was decreased by *shE7*s at 48 hr detected by real-time PCR. (B) Detection of E7 mRNA expression by RT-PCR; (C) eIF4E mRNA expression decreased after E7 knockdown at 48 hr detected by real-time PCR. (D) Detection of eIF4E mRNA expression by RT-PCR; (E) eIF4E protein expression decreased in *HeLa* cells at 24 hr and 48 hr after the transfection of *shE7*. Mock, *HeLa* cells; NC, blank vector group; *: vs Mock, P <0.05; **: vs Mock, P<0.01

E7 knockdown down regulated eIF4E expression in HPV⁺ HeLa cells

The eIF4E mRNA level in the NC group was analogous to that in the mock group, by both real-time PCR and RT-PCR detection. EIF4E mRNA levels in *shE7*-1, 2, 3 treated groups were significantly decreased, compared with the NC group. Among the treated groups, the eIF4E mRNA level in *shE7-2* group was reduced most, with an inhibition rate of approximately 73% (Figure 2C). By RT-PCR and agarose gel detection, two discrete bands with expected sizes were seen for eIF4E gene (132 bp) and GAPDH gene (224 bp) (Figure 2D). By Image J band analysis, the eIF4E mRNA levels of *shE7*-1 and *shE7-2* but not *shE7*-3 groups were substantially decreased, compared with the NC group. The eIF4E mRNA in *shE7-2* group was decreased more than that in *shE7*-1 group. The changes of eIF4E mRNA were comparable in both the real-time PCR and RT-PCR detection. The changes of eIF4E mRNA followed tightly the changes of E7 mRNA in *shE7*-1, 2, 3 groups.

Using immunocytochemistry, the eIF4E protein expression was detected in *HeLa* cells (Figure 2E). The eIF4E positive cells were stained yellow brown in cytoplasm and/or nucleus. In the NC group, the rate of eIF4E positive cells was up to 96.8%. When *shE7-2* transfection was done for 24 hr and 48 hr, the rates of eIF4E positive cells were decreased to 91.26% and 42.97% (P<0.001), respectively. The intensity of cell staining became considerably weaker in the *shE7-2* treated group than in the NC group.

shE7-2 inhibited proliferation, migration and promoted cell apoptosis

After *shE7-2* transfection, cell proliferation was significantly inhibited in *shE7-2* group. The inhibition rates of cell proliferation were 9.9% (24 hr), 17.3% (48 hr) and 11.3% (72 hr) (Figure 3A).

Under the condition of high E7 expression, sieIF4E significantly inhibited the proliferation of the *HeLa* cells, to a degree comparable with the effect of *shE7*s. Compared with the NC group, the cell proliferation in sieIF4E group was markedly inhibited. The changes of the inhibition rates of cell proliferation in sieIF4E group were 7.635±1.143%(24 hr), 27.505±1.679% (48 hr) and 32.143±3.031% (72 hr), close to that in *shE7* group at 24 hr, but significantly higher than that in *shE7* group at 48 hr and 72 hr (Figure 3B).

Compared with the NC group, the cell cycle in *shE7-2* group was noticeably changed (Table 1). The cell numbers were significantly increased by 14.4% (24 hr), 19.8% (48 hr) and 25.7% (72 hr) in G0/G1 phase, decreased by 15.1% (24 hr), 23.2% (48 hr) and 28.3% (72 hr) in S phase, and changed without significance in G2/M phase. The transfection effect suggested that *shE7-2* inhibits the proliferation of *HeLa* cells through arresting cells at G0/G1 phase.

Annexin V-FITC/PI double staining method was adopted for cell apoptosis detection after the *shE7-2* transfection (Table 2). The change of apoptosis between the mock and NC groups was similar. Compared with the NC group, the V-FITC+/PI- cells (early apoptosis cells) in *shE7-2* group were significantly increased by 30.1% (24 hr) and 39.2% (48 hr), respectively, while the V-FITC+/PI+ cells (late apoptosis cells) were increased without significance.

Table 1. Cell cycle change of *HeLa* cells after the interference plasmid of e7 transfection

Group	Percentage ($\bar{x}$±s, n=3)		
	G1%	S%	G2%
HeLa	32.124±1.425	50.263±1.651	14.598±1.254
Nc	36.987±1.251	49.541±1.625	14.028±1.669
shE7-2(24 hr)	47.915±1.805	36.255±1.155	18.835±1.951
shE7-2 (48 hr)	53.354±1.552*	28.101±1.202*	17.547±1.752
shE7-2 (72 hr)	59.206±1.401*	22.987±1.703*	17.804±1.300

*: VS NC, *P*<0.01

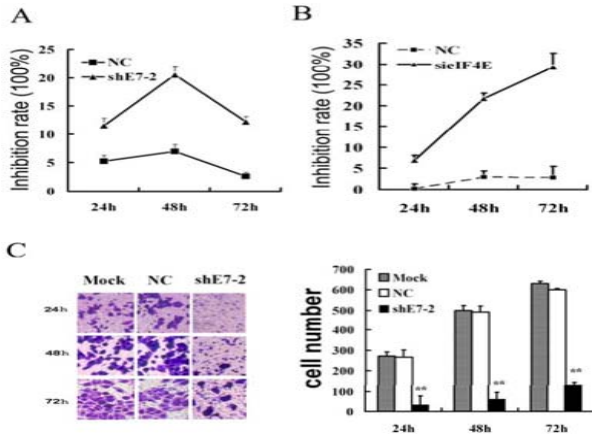

Figure 3. The interference plasmid of e7 or the siRNAs of eukaryotic translation initiation factor 4e transfection influenced *HeLa* cell biology. (A) *shE7-2* inhibited the proliferation of *HeLa* cells detected by CCK-8 assay; (B) sieIF4E inhibited the proliferation of *HeLa* cells detected by CCK-8 assay; (C) *shE7-2* inhibited the migration of *HeLa* cells detected by transwell assay. Mock: untreated *HeLa* cells. NC: blank vector group. *shE7-2*: *shE7-2* group. *: vs Mock, *P*<0.05;**: vs Mock, *P*<0.01

To analyze the migration of *HeLa* cells after the *shE7* transfection, Transwell Migration Assay was performed (Figure 3C). The cell numbers of migration were comparable between the mock and NC groups. Compared with the NC group, the cell numbers of migration in *shE7-2* group were decreased by 33.47±11.563 (24 hr), 61.25±6.629 (48 hr), and 131.68±11.051 (72 hr), respectively (*P*<0.01) (Figure 3).

Discussion

This study discovered that E7 induced eIF4E expression in CC cells. Here, the ecto-E7 gene expression significantly induced eIF4E transcriptional gene expression in *C33A* cells (HPV$^-$, eIF4E$^+$). Furthermore, the decreased expression of eIF4E mRNA and protein directly followed the knockdown of E7 in HPV positive *HeLa* cells; the degree of eIF4E down regulation correspondingly matched the degree of E7 knockdown in the *shE7*-1, 2, 3 transfection groups of *HeLa* cells. To the best of our knowledge, this is the first study to suggest that E7 induces eIF4E transcription independently. This could be one of the mechanisms for eIF4E gene overexpression in CC.

However, whether E7 induces eIF4E transcription directly or indirectly is not yet known. Additional study will be done in our laboratory to confirm this issue. Evidence revealed that the conserved sequence of E7 could bind pRb, P107 and P130 (17-20). These Rb family members can form the pRb/E2F complex and inactivate the transcription and function of c-Myc, which negatively regulates the progression of G1/S and prohibits the cell cycle. In the process, E7 binds G1-specific pRb to obstruct the formation of the pRb/E2F complex and rescue the transcription and function of c-Myc, leading to the accelerated cell cycle. It was reported that the transcription of eIF4E is induced by c-Myc via a positive eIF4E/c-Myc feedback loop in lymphangiectasis (21). As a result, we concluded that E7 up regulates eIF4E through pRb/c-Myc pathway.

The results suggested that E7 enhanced the cell proliferation, migration and cell cycle progression, and also inhibited cell apoptosis through eIF4E. E7 regulates the transcription of a series of oncogenes by complicated molecular mechanisms (16, 22-24). Since eIF4E directly enhances the translation of many oncogenes whose gene transcription was regulated by E7, the E7/eIF4E pathway might be efficient for E7 to initiate and promote CC.

Table 2. Apoptosis change of *HeLa* cells after the interference plasmid of e7 transfection

Group	Percentage ($\bar{x}$±s, n=3)	
	V-fitc$^+$/pi$^-$	V-fitc$^+$/pi$^+$
Hela	0.783±0.251	0.187±0.165
Nc	11.054±1.254	5.817±1.816
shE7-2（24 hr）	41.164±3.755*	10.104±1.020
shE7-2（48 hr）	50.205±3.503*	7.604±1.402

*: VS NC, *P*<0.01

However, a few studies reported the effects of eIF4E on CC cell biology. Here, ecto-E7 gene expression was performed in *C33A* cells to investigate the function of E7 on eIF4E. We found that up-regulation of eIF4E gene expression by E7 accelerated the cell proliferation and inhibited the apoptosis (Figure 1). In HPV$^+$ *HeLa* cells, *shE7* down regulated eIF4E gene expression, inhibited the cell proliferation and speeded up the cell apoptosis (Figure 3). These results indicate that eIF4E engaged in the key process of HPV caused carcinogenesis.

In addition, HPV E6 and E7 are proven to produce a bicistronic transcript. Thus, the knockdown of E7 might also lead to the knockdown of E6 gene (25, 26). In this study, we knocked down E7 in HPV$^+$ *HeLa* cells. The results showed down regulation of eIF4E gene expression and the cell proliferation. This result may be caused by single E7 or E6/E7 because of the presence of the E6/E7 bicistronic transcript. Thus, HPV$^-$ *C33A* cells were chosen to demonstrate the ability of E7 gene affecting eIF4E. After the E7 expression vector was transfected into *C33A* cells, the expression of E7 up-regulated eIF4E markedly in the absence of E6. The result showed clearly that E7 could induce eIF4E gene transcription. Thus, the down-regulation of eIF4E was caused, at least, mainly by the knockdown of E7. We concluded that E7 could induce eIF4E in *HeLa* and *C33A* cells.

Conclusion

EIF4E gene overexpression in CC cell lines was further confirmed in this study. More importantly, we discovered that the transcription of eIF4E could be induced by HPV E7. In addition, the down regulation or up regulation of eIF4E on the condition of sustained high expression of E7 significantly influenced the cell proliferation, cell cycle progression, migration and apoptosis. Our finding suggests that eIF4E is an important target for the treatment and prevention of HPV associated cancers such as CC.

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