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Human Papillomavirus Type 16 Mutant E7 Protein Induces Oncogenic Transformation via Up-regulation of Cyclin A and cdc25A*

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Abstract: A new mutant human papillomavirus type 16 E7 gene, termed HPV16 HBE7, was isolated from cervical carcinoma biopsy samples from patients in an area with high incidence of cervical cancer (Hubei province, China). A previous study showed that the HPV16 HBE7 protein was primarily cytoplasmic while wild-type HPV16 E7 protein, termed HPV16 WE7, was concentrated in the nucleus. With the aim of studying the biological functions of HPV16 HBE7, the transforming potential of HPV16 HBE7 in NIH/3T3 cells was detected through observation of cell morphology, cell proliferation assay and anchorage-independent growth assay. The effect of HPV16 HBE7 on cell cycle was examined by flow cytometry. Dual-luciferase reporter assay and RT-PCR were used to investigate the influence of HPV16 HBE7 protein on the expression of regulation factors associated with G1/S checkpoint. The results showed that HPV16 HBE7 protein, as well as HPV16 WE7 protein, held transformation activity. NIH/3T3 cells expressing HPV16 HBE7 could easily transition from G1 phase into S phase and expressed high level of cyclin A and cdc25A. These results indicated HPV16 mutant E7 protein, located in the cytoplasm, induces oncogenic transformation of NIH/3T3 cells via up-regulation of cyclin A and cdc25A.

Key words: HPV16; Mutant E7; Transformation; Cell cycle

Cervical carcinoma is the second most common cancer in women worldwide, and it is the principal cancer of women in most developing countries where 80 percent of cases occur (11). There are about 500 000 patients with cervical carcinoma in the world and almost 250 000 patients die of this disease annually. In China, 131 500 cases occur and almost 20 000 patients die of this disease each year (3). Almost eighty percent of cervical carcinoma is related with four high-risk HPVs (HPV16, HPV18, HPV31 and HPV45) of which HPV16 is most closely linked to cervical carcinoma and comprises 40%-60% of HPV infections (29, 35). The HPV16 E7 gene is the major transforming gene, which can be continuously expressed in

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cervical cancer cells, and is vital to maintain the malignant phenotype of these cells (10). Moreover, HPV16 E7 can transform a variety of cell lines, including NIH/3T3 mouse fibroblasts (27). The most well-characterized biochemical property of HPV16 E7 is its ability to bind to the unphosphorylated retinoblastoma tumor suppressor protein (Rb) and disrupt Rb-E2F complexes, resulting in a loss of the G1/S checkpoint (1, 7, 35, 38). The HPV16 E7 protein is a 98-amino-acid nuclear phosphoprotein and can be divided into three main domains -CR1, aa 1-15; CR2, aa 16-37; and CR3, aa 38-98. All 3 domains are essential for the manifestation of the biological properties of E7 (33). CR1 and CR2 mediate binding of the viral oncoproteins to an overlapping set of cellular proteins, including the product of the retinoblastoma gene (pRB), cyclin A and cyclin E (18, 24, 36). The CR3 domain contains two "CXXC" sequences participating in Zn binding (9, 23). It was reported that zinc finger structure at the C-terminal was essential for HPV16 E7 to stabilize its structure and biological function (30).

Previous studies showed variants of HPV16 E7 gene were found in widely separated locations (5, 26, 31). A mutant E7 (HPV16 HBE7) protein derived from a clinical HPV16 was isolated from Hubei province in China (16). It was found that there were two mutations in HPV16 HBE7 (GenBank accession no. AF393782). What is more, a non sense mutation was found in codon 43 which made HPV16 HBE7 into a truncated protein, corresponding to the first 43 amino acids of HPV16 WE7. Generally speaking, the great variance of gene structure in the virus will lead to changes of its immunological characteristics and its biological functions, for example, mutations leading to changes in the structure of coronaviruses produces changes in host range and virulence (27). Previous research indicated that the HBE7 protein could induce mice to produce humoral immunity identical with the WE7 protein but with weaker specific cellular immunity (16). The integrity of the C-terminal of the truncated HBE7 protein affects its intracellular localization (17) and may affect its transformation activity. This research shows that HPV16 HBE7 (the HPV16 E7 fragment), which is predominantly cytoplasmic, is also competent for transformation of NIH/3T3 cells and can efficiently activate expression of cyclin A and cdc25A.

MATERIALS AND METHODS

Cell culture

NIH/3T3 cells were purchased from the Cancer Research Institute of Zhongnan University (Hunan, China) and cultured in DMEM supplemented with 10% BCS at 37 ℃ in 5% CO_2 atmosphere.

Stable transfection methods

Recombinants of pcDNA3.1-HPV16HBE7 and pcDNA3.1-HPV16WE7 expressing HBE7 or WE7 respectively were generated previously (37). To evaluate the transformation ability of HPV16 HBE7 and its potential to regulate the G1/S phase of cell cycle, NIH/3T3 cells (1.5×10^5/well) were transfected with 0.8 μg plasmid DNA using lipofectamine^{TM}2000. Stable clones were selected using G418 (Wuhan TianYuan BioTechnologies co, China) at a concentration of 600μg/mL. Empty vector pcDNA3.1 (-) was also stably transfected into NIH/3T3 cells as a control.

Detection of expression of HPV16 E7 in stable clones

Expression of HPV16 E7 protein in stable clones was confirmed by reverse transcription-polymerase chain reaction (RT-PCR). Total RNAs of NIH/3T3 cells were extracted by Trizol reagent (MBI Fer-

mentas). 5μg of total RNA was reverse-transcribed to cDNA with a first-strand cDNA synthesis kit (MBI Fermentas). The PCR was performed in a 50μL volume under the following conditions: initial denaturation step of 94 ℃ for 5 min, cycling step of denaturation at 94 ℃ for 60 s, annealing for 60s and extension at 72 ℃ for 60s. The mRNA levels of β-actin served as an internal control. The products of PCR were identified by agarose gel analysis.

Morphological identification of cultured NIH/3T3 cells

Changes of cell morphology in stable NIH/3T3 cell transfectants with pcDNA3.1-HPV16HBE7 were observed by transmission electron microscope (Olympus, Japan). In the process of culturing, the samples of 5 $\times 10^6$ cells were collected and fixed with 2.0% glutaraldehyde, dehydrated, embedded, sectioned and finally visualized via transmission electron microscope. The cell morphology of parental NIH/3T3 cells was set as a control.

Cell proliferation assay

The growth rate of NIH/3T3 cells stably expressing HBE7 *in vitro* was measured using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method (25). Briefly, cells were seeded into 96-well plates (2×10^3 cells/well). On the day of harvest, 100μL of spent medium was replaced with an equal volume of fresh medium containing 10% MTT 5 mg/mL stock. Plates were incubated at 37 ℃ in a 5% CO_2 incubator for 4 h, then 100μL of dimethyl sulphoxide (DMSO) was added to each well and plates were shaken at room temperature for 10 min. The absorbance was measured at 570 nm on a Bio-Rad Model 550 microplate reader (BD Bioscience, San Jose, USA). NIH/3T3 cells transfected with empty vector and parental NIH/3T3 cells were set as controls.

Soft agarose colony formation assay

Soft agarose assay was essentially performed according to previous methods (22). The NIH/3T3 cells transfected with pcDNA3.1-HPV16HBE7 and parental NIH/3T3 cells were respectively suspended in 0.35% agarose with complete DMEM medium, plated at a density of 1×10^4 cells per well, previously coated with 0.7% agarose and maintained at 37˚C in 5% CO_2 atmosphere. The cultures were maintained for 3 weeks and colonies>150μm in diameter were counted under an inverted microscope at the magnification of 10 times.

Cell cycle analysis

The distribution of cells in the cell cycle phases was determined by FACS analysis of the DNA content. NIH/3T3 cells stably transfected with pcDNA3.1-HPV16HBE7 or vector pcDNA3.1(-) and parental NIH/3T3 cells were respectively fixed in ice-cold 70% ethanol and stored at -20 ℃. Prior to analysis, cells were washed and resuspended at 1.0×10^6 cells/mL in PBS and incubated with 0.1 mg/mL RNase A and 40 μg/mL propidium iodide (PI) at 37 ℃ for 30 min. Samples were analyzed by a FACS scanner (Becton Dickinson, San Jose, CA).

Dual-luciferase reporter assay

The cyclinA promoter activity was determined by dual-luciferase reporter assay. NIH/3T3 cells were grown in 24 well plates to about 70%-80% confluence and were cotransfected with recombinants of pcDNA3.1-HPV16HBE7 or empty vector pcDNA3.1-, pGL-cyclinA or pRL-CMV using lipofectamine^{TM}2000 (Wuhan Tianyuan Biotechnologies co, China) according to manufacturer's instructions. pGL-cyclinA gifted from Dr. Xue-Feng Liu (Georgetown University

Table 1. Primers for amplification of each gene

Gene	Upper primer	Lower primer
E7	5'-GG*GAATTC*ATGCATGGAGATAC-3'	5'-GG*GATCCT*TATGGTTTCTGAGAAC-3'
cyclin A	5'-CTGTAGTTCTTCCCCTGCA-3'	5'-GCAAACAGCAAGTTGTTTATT-3'
cdc25 A	5'-GTCGCAGAGCAGTAAGCAAA-3'	5'-GCCAGGGTCTACCAATAAGG-3'
cyclin E	5'-ACGTTCTACTTGGCACAGGA-3'	5'-CACAATGGTCAGAGGGCTTA-3'
cdk2	5'-AAACAAGTTGACGGGAGAAGT-3'	5'-GAAGGACACGGTGAGAATGG-3'
β-actin	5'-CCATGTACGTGGCCATCCAGGCTGT-3'	5'-ATCTGCTGGAAGGTGGACAGCGAG-3'

Medical Center) contains the cyclinA promoter. Cells were cultured at 37 ℃ in 5% CO_2 atmosphere for 36 h, and then the transfected cells were lysed with Passive Lysis Solution (Promega, Madison, WI). The luciferase activity was measured with the Dual-Luciferase® reporter assay system (Promega, Madison, WI) on a TD-20/20 luminometer. Renilla luciferase activity was used to normalize the transfection efficiency.

Semi-quantitative RT-PCR

RT-PCR was performed in a semiquantitative manner with cyclinA, cdc25A, cyclin E and cdk2. Primers for amplification of each gene are listed in Table 1. Total RNAs of NIH/3T3 cells were extracted by Trizol reagent (MBI Fermentas). 4μg of total RNA was reverse-transcribed to cDNA with a first-strand cDNA synthesis kit (MBI Fermentas). The PCR was performed in a 50μL volume under the following conditions: initial denaturation step of 94 ℃ for 5 min, cycling step of denaturation at 94 ℃ for 60 s, annealing for 60s and extension at 72 ℃ for 60s. The mRNA levels of β-actin served as an internal control. The products of PCR were identified by agarose gel analysis.

Statistical analysis

Data were presented as mean ± SD and analyzed with SPSS 11.0 software. $P < 0.05$ was considered statistically significant.

RESULTS

Expression of HPV16 E7 in stable clones

A specific band could be seen in both NIH/3T3 cells stably transfected with pcDNA3.1-HPV16HBE7 and NIH/3T3 cells stably transfected with pcDNA3.1-HPV16WE7 (Fig.1). It showed that NIH/3T3 cells stably expressing HBE7 (NIH/3T3-HBE7) and NIH/3T3 cells stably expressing WE7 (NIH/3T3-WE7) were successfully obtained.

Morphology of the cultured NIH/3T3 cells

Transmission electron microscopy revealed that NIH/3T3 cells stably expressing HBE7 (NIH/3T3-HBE7) grew quite well. There were several bigger and abnormally shaped nucleoli in NIH/3T3-HBE7 cells and more pathologic nuclear phase and immature cell organs were found in NIH/3T3-HBE7 cells than in parental NIH/3T3 cells (Fig. 2).

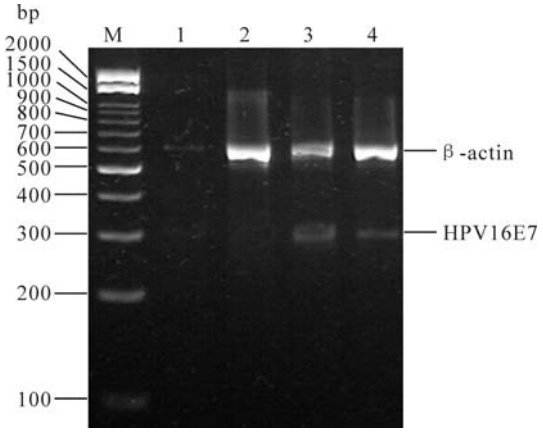

Fig. 1. Detection of HPV16E7 mRNA in NIH/3T3 cells by RT-PCR. M, molecular weight markers; 1, NIH/3T3 cells stably transfected with pcDNA3.1(-); 2, control NIH/3T3 cells; 3, NIH/3T3 cells stably transfected with pcDNA3.1-HPV16-HBE7; 4, NIH/3T3 cells stably transfected with pcDNA3.1-HPV16WE7.

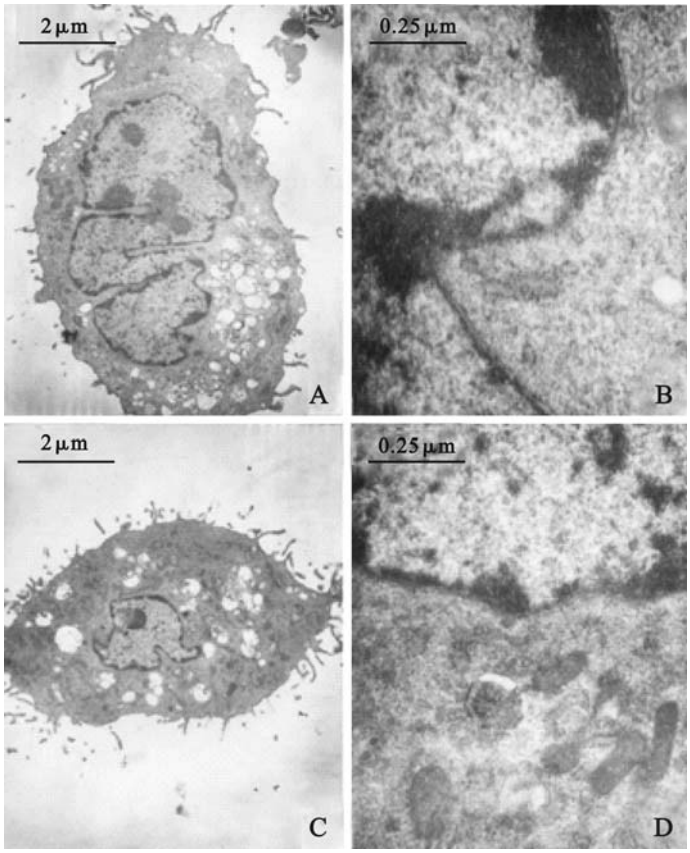

Fig. 2. Morphology of NIH/3T3 cells under transmission electron microscopy. A and B, morphology of NIH/3T3 cells stably expressing HBE7 (NIH/3T3-HBE7); C and D, morphology of parental NIH/3T3 cells. Transmission electron microscope revealed different shapes of cells. Under transmission electron microscope, obvious nuclei, few mitochondriae, immature endoplasmic reticulum and Golgi bodies, inadequate ribosomes (glycogens) and underdeveloped non-sarcomered myofibrils scattered around nuclei were observed in NIH/3T3-HBE7 cells. And parental NIH/3T3 cells exhibited a small-sized nucleus in the center of the cell body and a relatively large-sized cytoplasm.

Effects of HBE7 on cell progression

Four groups of NIH/3T3 cells were used, NIH/3T3 cell clones stably expressing exogenous HPV16 HBE7 (NIH/3T3-HBE7), NIH/3T3 cells stably expressing exogenous HPV16 WE7 (NIH/3T3-WE7), NIH/3T3 cells obtained by transfecting the empty vector pc DNA3.1(-) (NIH/3T3-vector) alone, and parental NIH/3T3 cells (NIH/3T3) as controls. Through the MTT assay, it was found that NIH/3T3-HBE7 cells had a significantly increased growth rate compared with other control cells (P<0.05). There was no significant difference between the growth rate of NIH/3T3-vector and parental NIH/3T3 cells（P>0.05）(Fig. 3).

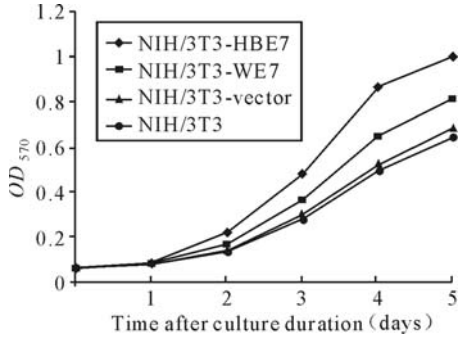

Fig. 3. Effects of HBE7 on the growth of NIH/3T3 cells *in vitro*. Growth of NIH/3T3 cells stably expressing HBE7 was detected by MTT assay. NIH/3T3-HBE7 cells had a significantly increased growth rate compared with the control cells (P<0.05). Optical density representing numbers of viable cells is indicated on the y-axis, and time points are indicated on the x-axis. Values represent means _ SD.

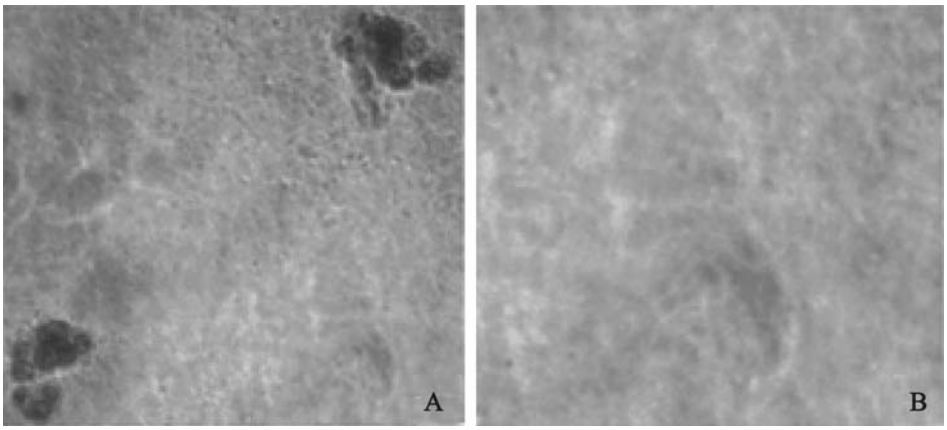

Fig. 4. Anchorage independent growth of NIH/3T3 cells in soft agarose. Anchorage independent growth is considered as an *in vitro* test for tumorigenesis. Therefore, the two NIH/3T3 cell groups, NIH/3T3-HBE7 cells (A) and parental NIH/3T3 cells (B) were also examined for anchorage independent growth in a soft agarose medium. On day 21, the colonies formed in soft agarose of NIH /3T3-HBE7 cells and parental NIH/3T3 cells group were 41±2.94 and 0±0.00, respectively. These data suggested an effective expression of HBE7 protein in NIH/3T3 cells and its potential tumorigenesis.

Transforming potentials of HBE7 protein

The tumorigenic potential of transformed cells was determined from their ability to grow as anchorage-independent colonies in soft agarose. Small colony formation could be seen in NIH/3T3-HBE7 cells two weeks later. At 15 to 21 days, obvious cell colonies were formed. On day 21, NIH/3T3-HBE7 cells could form some colonies in the soft agarose, while parental NIH/3T3 cells could not form colonies (Fig. 4). These data suggested there was tumorigenesis in the HBE7 protein.

Cell cycle assay

The effect of the HBE7 gene on cell cycle distribution was determined to gain insights into the mechanism of its proliferative activity. As can be seen in Table 2, the presence of the HBE7 gene resulted in significant accumulation of cells in S phase, accompanied with a significant decrease in the percentage of cells in the G2-M phase, compared with control cells. The percentage of NIH/3T3-HBE7 cells in S phase was increased by about twofold compared with that of control NIH/3T3 cells. The results show that HBE7 can influence the cell cycle by increasing the ratio of cells in S phase.

Table 2. Analysis of cell cycle

Group	G_0/G_1 (%)	G2/M (%)	S (%)
NIH/3T3-HBE7	35.9	18.5	45.6
NIH/3T3-vector	50.7	32.1	17.2
NIH/3T3	49.7	34.0	16.3

Transcriptional activation of the cyclin A by HBE7

For these experiments NIH/3T3 cells transfected with the reporter vectors, which contained luciferase genes, were chosen. To examine whether HBE7 gene increased the accumulation of cyclin A mRNA by stimulation of transcription, the effects of HBE7 on the cyclin A promoter in NIH/3T3 cells were studied. The recombinant pGL3-CyclinA, pRL-CMV and pcDNA3.1-HBE7 were co-transfected into NIH/3T3 cells and luciferase activity was examined 36 h later. The results showed that compared with pcDNA3.1(-) transfecting the cells, the activity of cyclin A promoter increased by 2 times in HBE7 transfecting cells (Fig. 5). These results demonstrate that HPV16HBE7 protein

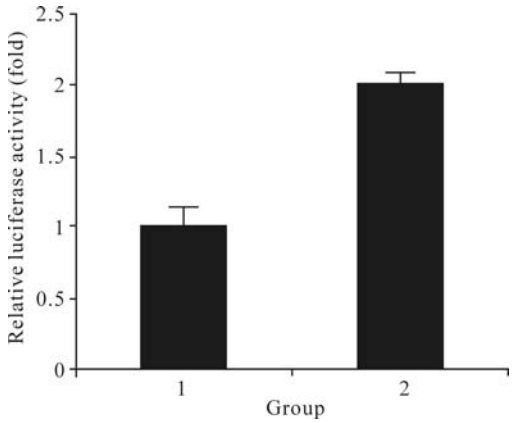

Fig. 5. Relative activation of the cyclin A promoter. The recombinants pGL3-cyclin A, pRL-CMV and empty vector pcDNA3.1(-) or pcDNA3.1-HPV16HBE7 were co-transfected into NIH/3T3 cells. Group1, NIH/3T3 cells group transfected with pGL3-cyclin A, pRL-CMV and empty vector pcDNA3.1 (-). Group2, NIH/3T3 cells group transfected with pGL3-cyclin A, pRL-CMV and pcDNA3.1-HPV16HBE7.

promotes the proliferation of NIH/3T3 cells and the HBE7 gene is a transforming gene.

Activation the G1/S phase of the cell cycle by HBE7

To further investigate the potential of HBE7 to regulate the G1/S phase of cell cycle, the expression level of regulation factors associated with G1/S checkpoint was examined. Semi-quantitative RT-PCR results are presented in Fig.6. The relative mRNA expression levels of each gene are listed in Table 3. As shown, the mRNA expression level of cyclin A or cdc25A in NIH/3T3-HBE7 cells was significant up-regulated compared with NIH/3T3- vector cells (P<0.05) and the expression level of cyclin A or NIH/3T3-HBE7 cells and NIH/3T3-vector cells (P>0.05). The results showed that NIH/3T3 cells expressing HBE7 could express higher level of cyclin A and cdc25A, which is consistent with the results of the dual-luciferase reporter assay.

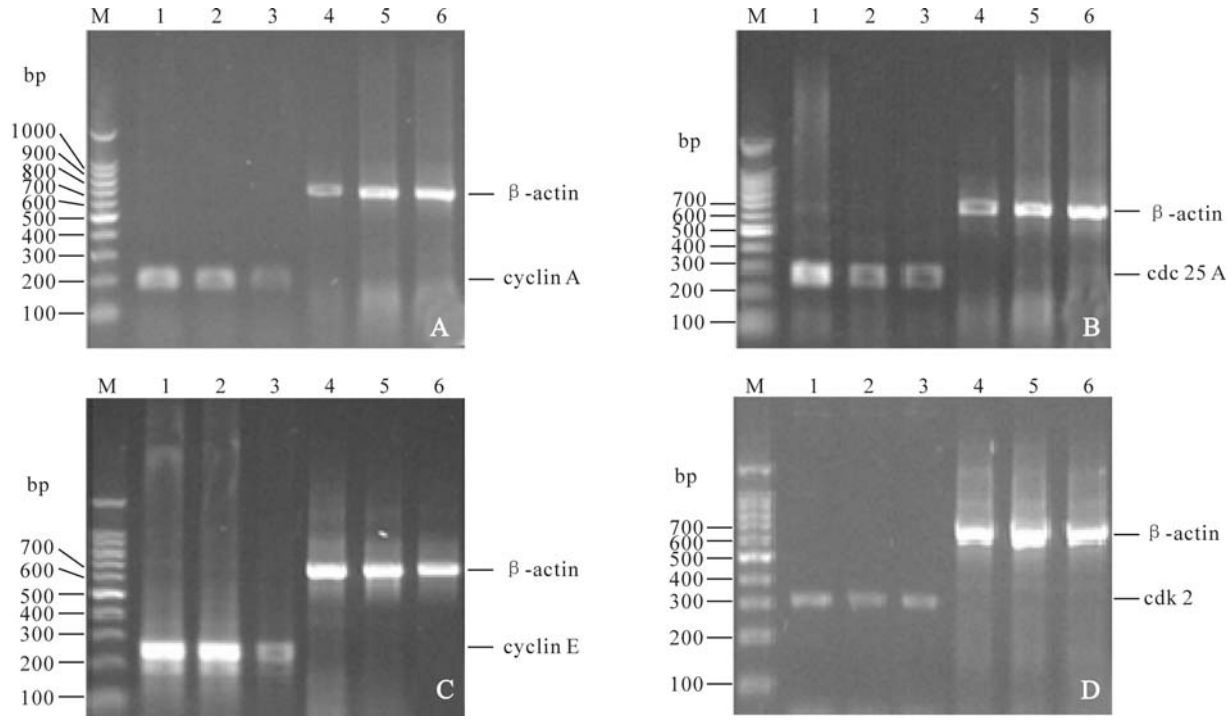

Fig. 6. Expression level of regulation factors in NIH/3T3 cells by semiquantitative RT-PCR. The mRNA expression levels of cyclin A (A), cdc25A (B), cyclin E(C) and cdk2 (D) in NIH/3T3-HBE7 cells were investigated. The mRNA levels of β-actin served as an internal control. M, DNA Marker; Lanes 1,2 and 3, the mRNA expression levels of regulation factors in NIH/3T3-HBE7 cells, NIH/3T3-WE7 cells and NIH/3T3-vector cells，respectively; Lanes 4,5 and 6, the mRNA expression levels of β-actin in NIH/3T3-HBE7 cells, NIH/3T3-WE7 cells and NIH/3T3-vector cells, respectively. The HPV16 HBE7 protein greatly up-regulated the mRNA expression of cyclin A and cdc25A.

Table 3. The relative mRNA expression levels of each gene

Group	cyclin A/ β-actin	cdc25 A / β-actin	cyclin E / β-actin	cdk2/ β-actin
NIH/3T3-HBE7	1.043±0.1401	1.072±0.1463	1.869±0.1025	0.939±0.0540
NIH/3T3-WE7	0.708±0.0145	0.578±0.0240	1.983±0.0413	0.878±0.0320
NIH/3T3-vector	0.429±0.0275	0.402±0.0300	0.896±0.0290	0.870±0.0480

DISCUSSION

Precise and timely intracellular localization of proteins is essential to study their biological functions. HPV16 E7 interacts with tumor suppressor protein pRb to promote cell cycle progression (34). Thus, interactions with cellular tumor suppressor proteins and perturbation of normal cell cycle control by HPV16 E7 are believed to be the most important influences for malignant conversion (19). Deletion of the C-terminal region of HPV16 WE7 (amino acids 44 to 98) abolishes its nuclear import, suggesting that this region functions as a nuclear localization signal (NLS) and a NLS-mediated import machinery mediates the nuclear import of HPV16 WE7. Two point mutations in the structure of HPV16 HBE7 resulted in cytoplasmic retention of the fusion protein, which may be correlated with its loss of NLS after mutation.

The great variation of structure and localization in HPV16 HBE7 will lead to changes of its biological functions. Transformation of rodent fibroblasts was frequently used as an experimental approach to study the biological functions of viral oncogenes. In the present study, HPV16 HBE7 was expressed in established rodent fibroblasts (NIH/3T3 cells), which were used previously to analyze the transforming potential of single candidate genes, including HPV16 E7 (12). It was found that the HBE7 gene increased the growth of NIH/3T3 cells and eventually transformed NIH/3T3 cells into malignant cells. NIH/3T3 cells expressing HBE7 grew significantly fast and could form some colonies. These results demonstrated that the HBE7 gene as well as the WE7 gene held transformation activity; the C-terminal deletion did not delete the transforming activity of HPV16 HBE7. The conserved domain CR2 of HPV16 E7 plays a critical role in its oncogenic function by interaction with the Rb family of proteins via its LxCxE motif (residues 22-26). This truncated HBE7 corresponds to the first 43 amino acid residues of HPV16 E7, so it also contains the LXCXE sequence. Whether this sequence is also important for transformation by the truncated HBE7 requires further investigation.

It is generally presumed that the main function of HPV16 E7 is to disrupt the cell cycle by promoting G1/S progression, thus establishing an environment that is more conducive for viral replication and subsequently promoting tumorigenesis (6, 13). The G1/S point involves the initiation of cellular DNA synthesis. Since DNA tumor viruses rely on the host's replication machinery to duplicate their own genome, infected cells must be shifted into S phase. The results in this study showed that the percentage of NIH/3T3-HBE7 cells in S phase was significant higher than that of NIH/3T3-vector or parental NIH/3T3 cells in S phase. It indicated that NIH/3T3 cells expressing HBE7 gene could more easily transit from G1 phase into S phase. HPV16 HBE7 held strong ability to promote the G1/S progression. It was consistent with this study that HPV16 HBE7 significantly increased the growth of NIH/3T3 cells.

The promoter of the human cyclin A gene is silent during G1 phase and strongly induced as cells enter S

phase (15). In this study, the activity of cyclin A promoter increased obviously in NIH/3T3-HBE7 cells compared with NIH/3T3-vector cells. It demonstrated expression of HPV16 E7 in NIH/3T3 cells also induced transcription of the cyclin A promoter and that promoter activation was involved in the observed change in the level of cyclin A. The results above showed that cyclin A may be a critical target of HPV16 HBE7 in the disruption of the G1/S phase transition.

As HPV16 HBE7 demonstrated a strong ability to promote the G1/S progression, we then investigated whether the levels of regulation factors associated with G1/S checkpoint were altered in NIH/3T3-HBE7 cells. HPV16 E7 has been reported to be associated with cyclin A and cyclin E. Cyclin A was believed to participate in DNA replication and may have a role in transcription control during S phase (14). Cyclin E was expressed in mid-G1 and rate-limiting for passage through G1 (32). Cyclin E combines with cdk2 later in G1, and cyclin A associates with cdk2 at the beginning of S phase (8, 28). cdc25A tyrosine phosphatase was involved in the regulation of the G1/S phase transition by activating cyclin E-cdk2 and cyclin A-cdk2 complexes (2, 20). The results showed that the expression level of cyclin A and cdc25A in NIH/3T3-HBE7 cells was significantly higher than for NIH/3T3-WE7 cells. But there was no significant difference in the expression level of cyclin E and cdk2. These results indicated HPV16 HBE7 could up-regulate expression level of cyclin A and cdc25A more effectively. As such, cyclin A and cdc25A were likely to be important mediators of HPV16 HBE7-enforced cell cycle progression. However, the molecular mechanisms of HBE7 protein interacting with its intracellular ligands are not fully understood.

In summary, this study showed that HPV16 HBE7 was primarily distributed in the cytoplasm and HPV16 HBE7 also held strong transformation activity. It could more easily transition from G1 phase into S phase and could up-regulate expression level of cyclin A and cdc25A more remarkably. These results indicated that activation of cyclin A and cdc25A was especially important for HPV16 HBE7-dependent transformation of NIH/3T3 cells. The effect and mechanisms of the HPV16 HBE7 gene in tumorigenicity are in further study. It could be important to the design of therapeutics and vaccines in Hubei province in China.

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