

사람 유두종 바이러스 E6 단백질에 의한 death receptor 4의 발현 및 세포사멸의 선택적 유도

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Selective expression of death receptor 4 and induction of apoptosis by HPV E6

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Abstract : Human papillomaviruses, especially type 16 and 18, have been known to be closely associated with human cervix cancer. The E6 and E7 proteins encoded by these viruses disrupt normal cell cycle regulation. The E7 protein interacts with pRb and abrogates its tumor-suppressive activity, while the E6 protein cooperates with E6AP, a ubiquitin E3 ligase, to target p53 tumor suppressor protein for ubiquitin-dependent degradation. Telomerase activity is detected in more than 90% of immortalized and cancer cells but absent in most normal somatic cells and E6 oncoprotein could activate telomerase. In this experiment, we hypothesized that the hTERT promoter can be used for tumor-specific expression of transgenes to induce selective cancer cell death in HPV E6 positive cells. First, we tested whether hTERT core promoter is active only in cancer cells and whether the presence of E6 protein could potentiate the activation of the promoter by using luciferase construct and we found that hTERT promoter is active only in cancer cells, and the presence of E6 protein could augment hTERT promoter activity. Then, we constructed mammalian expression vector to express TRAIL-death Receptor (DR) 4 driven by hTERT promoter to test whether DR4 expression is induced by the promoter in the presence of E6 oncoprotein and we found that DR4 expression was induced. Finally, we tested whether the induction of DR4 could contribute to cancer cell death by observing caspase 3 activation and we found that in the presence of E6, caspase 3 activation is more evident than in the absence of E6. Taken together, our results reveal that the proper use of hTERT promoter could be useful and powerful for inducing selective cancer cell (especially for E6 positive cancer cells) death with effective death inducing transgene.

Abbreviations: HPV, human papillomavirus; DR4, Death receptor 4, TRAIL, TNF-related apoptosis inducing ligand

Key Words: HPV, E6, DR4, hTERT, apoptosis

Introduction

The human papillomaviruses (HPVs) designated as "high risk" types, such as HPV type 16 (HPV-16) and HPV-18, are associated with anogenital tract lesions that can progress to malignancy^{1, 2}. The E6 and E7 viral genes appear to be responsible for both the in vivo and in vitro transforming activity of these high-risk viruses^{3, 4}. The transforming activities of the E6 and E7 viral gene products reside in their ability to interact specifically with cellular regulatory proteins and interfere with their normal functioning. The E7 protein interacts with pRb and abrogates its tumor-suppressive activity^{5, 6}, while the E6 protein cooperates with E6AP, a ubiquitin E3 ligase, to target p53 tumor suppressor protein for ubiquitin-dependent degradation⁷⁻¹⁰. E6 oncoprotein could activate telomerase^{11, 12}, which is a ribonucleoprotein enzyme important for the maintenance of telomeric structures at the ends of chromosomes^{13, 14}. Telomerase activity is detected in more than 90% of immortalized and cancer cells but absent in most normal somatic cells^{15, 16}, suggesting that telomerase activation is an important event during the process of immortalization and malignant transformation. Telomerase activity is closely associated with the expression of the telomerase catalytic subunit, hTERT. The expression of hTERT RNA is detected at high levels in tumor tissues and tumor-derived cell lines but not in normal adjacent tissues or primary cells^{17, 18}. Ectopic expression of hTERT in telomerase-negative cells restores telomerase activity in these cells as well as extending their life span^{19, 20}. Recently, it has been shown that telomerase activity can be induced in primary human keratinocytes and mammary epithelial cells by oncogenic E6 viral protein expression¹¹. Taken together, we hypothesized that the hTERT promoter can be used for tumor-specific

(especially for E6 positive cancers) expression of transgenes.

In the present study, we have assessed the capability of plasmid-mediated transgene expression induced by the hTERT promoter in the absence or presence of E6 oncoprotein and the feasibility of targeting the pharmaceutical effects of the DR4 death receptor gene, a pro-apoptotic TNF-related apoptosis inducing ligand (TRAIL) binding cell surface receptor²¹ gene, to tumors by this promoter in vitro.

Materials and methods

• Generation of HPV type 16 E6 ORF expressing adenovirus (Ad-E6)

To make an adenoviruses expressing HPV type 16 E6 ORF, we used pAdTRACK/CMV and pAdEasy system as previously described²². Briefly, E6 ORF of HPV type 16 was amplified by PCR. The amplified fragment was cloned into pAdTRACK/CMV and then, recombinant plasmid was generated in BJ5183 *E. Coli* by co-transforming PmeI digested E6 cloned pAdTRACK/CMV and pAdEasy. The recombinant plasmid was digested with PacI and was used for transfection into 293 cells using Superfect (QIAGEN, Valencia, CA). Approximately five days after transfection, when comet-like patches of the green cells appeared, the cells were harvested, and the viruses were amplified and purified as previously described²³. The infectivity of the viruses was checked by the observation of green fluorescence on the fluorescence microscope. Adenoviruses expressing only GFP were also generated and used it as control virus.

• Assessment of biological activity of E6 protein

The expression and the biological activity of E6 protein were evaluated indirectly as follows. Wild

type p53 containing H460, lung cancer cell lines, was treated with 0.5 μ M adriamycin for 16 hrs. Then, adriamycin treated cells were infected with either Ad-GFP or Ad-E6 followed by 24 hrs incubation. The cell lysates were prepared and 12% SDS-PAGE was performed. Western blot immunostaining was done with anti p53 antibody (Pab 1801, 1:500; Calbiochem) to check stabilization in the absence of E6 protein and degradation of p53 in the presence of E6 protein.

• Construction of phTERT promoter

To check the selective activation of hTERT promoter only in cancer cells and an augmentation of the activation of the promoter in the presence of E6 ORF protein, phTERT-luciferase construct (phTERT-Luc) was generated using pGL3 basic plasmid (Promega Corp., Madison, WI). To amplify hTERT core promoter (from -240 to + 50, based on transcriptional start site)²⁴, PCR was performed using two primers. The sequences of upstream primer were 5'-GAAGATCTGGAGCAGC TGCCTGT-3' and the sequences of downstream primer were 5'-GAAGATCTAAGCTTGCCAGGG CTT-3'. For directional cloning, each primer has a BglII recognition site and BglII/HindIII sites, respectively. Two hundreds microgram of human genomic DNAs were used as a template. To clone the promoter region into pGL3 basic plasmid, amplified PCR products and pGL3 plasmid were digested with BglII and HindIII followed by ligation and transformation. The resulting cloned plasmid was named as phTERT-luc. The sequences of amplified hTERT core promoter were confirmed by sequencing.

• Luciferase assay for checking the selective activation of the promoter in cancer cells and for checking an augmentation of the activation by E6

protein

For the transient transfection assay, cells were plated at a density of 2×10^5 cells/6 well dish. On the next day, transfection was performed with 1.8 μ g of the indicated luciferase constructs, 0.2 μ g of the pCMV β -galactosidase plasmid (CLONTECH Laboratories, Inc., Paolo Alto, CA). As a positive or a negative control plasmid, pGL3 basic and pGL3 control plasmids were used (Promega Corp., Madison, WI). Transfection was performed using LipofectAMINE Plus transfection reagent (Life Technologies, Inc.) for 3hrs according to the manufacturer's instructions. After 24 hrs of transfection, the cells were infected either with Ad-GFP or Ad-E6. Twenty-four hrs after infection, the cells were washed with PBS and lysed in 200 μ l of reporter lysis buffer (Promega Corp.). Luciferase activities were measured using the Luciferase Assay System (Promega Corp.) and normalized by the β -galactosidase activities to correct for the transfection efficiency. NIH3T3 cells were used as a normal cell lines, and 293, transformed human embryonic kidney epithelial cells were used as a cancer cell lines and it has been known that 293 has telomerase activity.

• Construction of hTERT promoter containing DR4 expression plasmid

To generate hTERT promoter containing DR4 expression plasmid, we used pIRES plasmid (CLONTECH Laboratories, Inc., Paolo Alto, CA). To make the expression of DR4 driven by hTERT promoter, we replace the CMV promoter region that is normally present in pIRES with cloned hTERT core promoter and then, we named the plasmid as pIRES-hTERT. Then, we cloned PCR amplified DR4 fragment into pIRES-hTERT. For amplification of DR4, following primers were used. Upstream primer was 5'-CCGCTCGAGCCGCCATGGCGCCACCAC-3' and downstream primer was 5'-GGAATTC

TCACTCCAAGGACACGGC-3'²¹. Each primer has a XhoI recognition site and an EcoRI recognition site for directional cloning, respectively. After ligation and transformation, we obtained a clone and we named it as pIRES-hTERT-DR4.

• Detection of DR4 expression and caspase 3 activation

293 cells were transfected with pIRES, pIRES-DR4, pIRES-hTERT, and pIRES-hTERT-DR4, respectively. After 24 hrs of transfection, the cells were infected with either Ad-GFP or Ad-E6. Cell lysates were prepared by directly adding 1xSDS loading buffer after 24 hrs of infection. SDS-PAGE (12%) was performed followed by western blot immunostaining. Blotted membrane was immunostained with anti-DR4 (1:500; Pharmingen, San Diego, CA), anti-caspase 3 (E-8, 1:200; Santa Cruz, Santa Cruz, CA), or anti-tubulin (Ab-1, 1:1000; Pharmingen).

Results

• Biological activity of HPV type 16 E6 ORF

The expression and the biological activity of E6 were confirmed indirectly by western blot immunostaining with anti-p53 Ab. Wild type p53 expressing H460, the human lung cancer cell lines, were treated with adriamycin (0.5 μ M) for 16 hrs followed by infection either with Ad-GFP or Ad-E6. Infectivity of the viruses were 100% when observed on fluorescence microscope (data not shown). After adriamycin treatment, p53 tumor suppressor protein was stabilized as observed in control (Fig.1, lane 2) or Ad-GFP infected cells (Fig.1, lane 4). When infected with Ad-E6, p53 protein was not observed even in the presence of adriamycin (Fig.1, lane 6) suggesting that the E6 protein derived from

Ad-E6 infection degraded stabilized p53 protein. Thus, we use this Ad-E6 virus for next experiments to simulate the cells as if they were infected with HPV viruses.

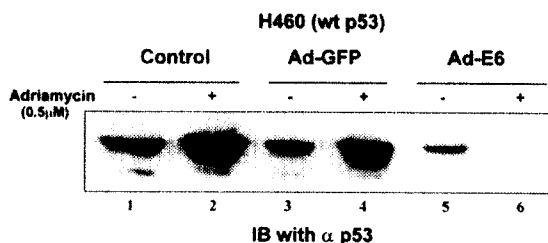


Fig 1. E6 expressing adenoviruses: Its expression, and biological activity. Wt p53 containing human lung cancer cell line, H460, was used. To stabilize p53, the cells were treated with adriamycin (0.5 μ M) for 16 hrs (lanes 2, 4, and 6). Then, treated cells were infected either with Ad-GFP (lanes 3 and 5) or Ad-E6 (lanes 4 and 6) for 24 hrs. Lysates were prepared from the cells by directly adding 1x SDS loading buffer. SDS-PAGE (12%) was performed followed by western blot immunostaining with anti p53 Ab (PAb 1801).

• Selective activation of hTERT promoter in cancer cells and augmentation of the promoter activation in the presence of E6 protein

It has been known that E6 protein could transcriptionally transactivate the hTERT promoter with yet unknown mechanism. Thus, we chose hTERT core promoter region to regulate the expression of effector gene such as death receptor 4. First of all, we tested whether hTERT promoter can be activated only in cancer cells on our hands. Lack of materials made us use mouse NIH3T3 cell lines as a normal control although it may not fully represent human normal epithelial cells. When transfected with pGL3 basic plasmid along with Ad-GFP that does not have any promoter region, basal luciferase activity was observed. Contrary to pGL3 basic-luc result, pGL3 control-luc that contains CMV promoter showed the highest luciferase activity. When transfected with pHTERT-luc, the luciferase activity was slightly elevated than that of pGL3 basic but a lot lesser than that of pGL3-control (Fig. 2A).

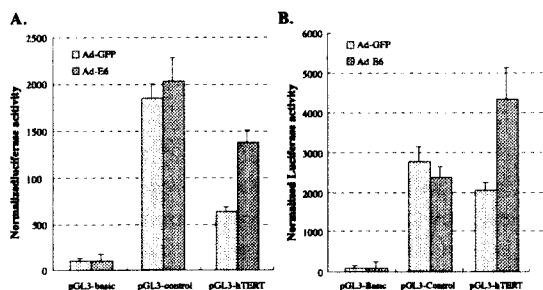


Fig 2. Activation of hTERT promoter only in cancer cells and augmentation of the activation by E6 protein. Transfection and luciferase activity assay were performed as described in the text. A: Results from NIH3T3 cells; B: Results from 293 cells. Dotted bar represents the results from Ad-GFP infection and black bar represents the results from Ad-E6. Experiments were done triplicate (mean value \pm SD).

When infected with Ad-E6, pGL3 basic showed almost the same as Ad-GFP. When transfected with pGL3-hTERT, the luciferase activity was slightly augmented with Ad-E6 infection. At present, we do not exactly understand why the luciferase activity was dropped in the presence of E6 protein in pGL3 control-luc. Transformed human embryonic kidney epithelial cells were used as cancer cells and it has been known that telomerase is active in 293 cells. PGL3 basic showed basal luciferase activity and there was no difference between Ad-GFP and Ad-E6. When transfected with pGL3 control, luciferase activity was elevated and Ad-E6 did not show any augmentative effect on the activity. In case of phTERT-luc, luciferase activity was elevated up to almost the same level as pGL3 control and the presence of E6 protein could augment the activation of hTERT promoter almost two folds (Fig. 2B). These results suggest that hTERT promoter is active only in cancer cells, and the presence of E6 protein could augment hTERT promoter activity.

• DR4 expression and induction of apoptosis

Western blot immunostaining with anti-DR4 Ab after transfection with DR4 expressing constructs

showed that DR4 was induced in the presence of E6 protein as seen in Figure 3 (DR4, lane 8) compared to the result shown in lane 7. Along with this result, immunostaining with anti-caspase 3 revealed that the activation of caspase 3 was augmented with the presence of E6 (Fig. 3, procaspase 3, lane 8). These results suggest that hTERT promoter can be used as a promoter for driving gene expression that could induce selective cancer cell death and in the presence of E6 protein, its activation could be potentiated.

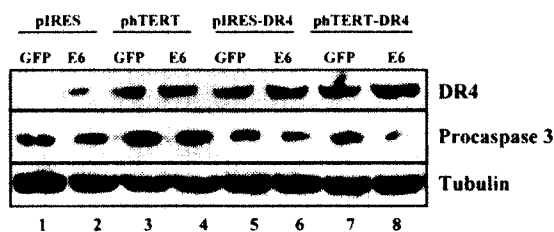


Fig 3. Induction of DR4 expression and caspase 3 activation. 293 cells were transfected with indicated constructs. Twenty hours after transfection, the cells were infected with either Ad-GFP (lanes 1, 3, 5, and 7) or Ad-E6 (lanes 2, 4, 6, and 8) for 24 hrs. Lysates were prepared by adding 1xSDS loading buffer directly, and subjected to 10 or 12% SDS-PAGE, then immunoblotted and stained with indicated Abs. Tubulin was used for internal loading control.

Discussion

We have shown here that the hTERT promoter has high transcriptional activity in a cancer cell lines but not in normal cells and can be used to prevent the toxic effects of the DR4 gene *in vitro*. The fact that telomerase is active in various tumor types and in >85% of all primary tumors suggest that the hTERT promoter will find broad applications in cancer gene therapy. In addition to these, the fact that hTERT promoter activity can be induced in the presence of oncogenic E6 protein, hTERT promoter could be more potentially useful for targeting the pharmaceutical effects of a therapeutic gene to HPV E6 positive human cervix cancer cells. In this experiment, we just showed the results obtained from one cancer and one normal cell lines, and thus,

further study should be needed to confirm the safety of using this strategy *in vitro* before applying this to clinical trial by using many different cancer cell lines that are positive or negative for E6 and normal primary epithelial cells. One of the major concerns about the use of the hTERT promoter to drive expression of pro-apoptotic or cytotoxic genes is its potential toxicity to stem cells. However, evidence suggests that hTERT may not be active in quiescent or G₀ stem cells²⁵. As a therapeutic gene, we used DR4 gene that codes pro-apoptotic TRAIL death receptor and it has been known that overexpression of death receptor alone could induce cancer cell death²⁶. Now, we are developing a new strategy to express simultaneously both TRAIL-death receptor and extracellular portion of TRAIL driven by hTERT promoter to enhance the cancer cell killing effect in broad range of cancer cells.

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■ 국문 조록 ■

사람 유두종 바이러스 특히 16형과 18형은 사람 자궁 경부암과 밀접한 연관성이 있음이 알려져 있다. 유두종 바이러스 16형과 18형으로부터 발현되는 종양 단백질중의 하나인 E6 단백질은 ubiquitin E3 ligase 활성을 가진 E6AP 단백질과 함께 p53종양 억제 단백질을 파괴하고, E7 단백질은 pRb 단백질에 대하여 E2F 전사 인자와 경쟁적으로 결합함으로써 pRb로부터 E2F를 유리하여 E2F의 전사 활성을 증가시킴으로써 정상적인 세포 주기 조절을 파괴한다고 알려져 있다. Telomere의 길이를 유지시키는 데 중요한 역할을 하는 효소인 telomerase는 정상 체 세포에서는 그 활성이 없지만 불멸화 된 세포 또는 암세포의 90 % 이상에서 활성화되어 존재하며, 또한 E6 단백질에 의해 활성화 된다고 보고되어있다. 이에 본 연구에서는 사람 유두종 바이러스가 양성인 자궁 경부 암에서 telomerase의 전사를 조절하는 promoter를 이용하여 주입한 세포 사멸 유발 유전자를 암세포에서만 선택적으로 발현하도록하여 암세포를 죽일 수 있는 방법을 개발하고자 하였다. 결과로서 첫째, telomerase의 전사를 조절하는 promoter 부위가 암세포 내에서만 활성화됨을 확인하였고 E6 단백질의 존재 하에 그 활성화 효과가 증폭되는 것을 확인하였다. TRAIL (TNF-related apoptosis inducing ligand)의 수용체인 DR4의 발현이 telomerase promoter에 의해 조절되도록 포유동물 세포 발현 벡터를 제조한 후 DR4의 발현 여부를 확인한 결과 DR4의 발현이 암세포에서 telomerase promoter에 의해 발현 증가되는 것을 확인하였고 E6 단백질 존재 시에 그 발현이 더욱 증가되는 것도 확인하였다. 또한 이러한 DR4의 발현이 암세포의 사멸을 유발하는 지 세포 사멸의 마지막 단계에서 활성화되는 caspase 3의 활성 여부를 통해 확인한 결과 E6 단백질 존재 시에 세포 사멸이 더욱 활성화됨을 확인할 수 있었다. 이상의 결과들로 telomerase의 promoter를 적절히 사용하게 되면 E6 양성인 암세포에서만 선택적으로 세포 사멸을 유도할 수 있는 유용하고 강력한 암 치료법을 개발할 수 있을 것으로 사료된다.