Antisense targeting human papillomavirus type 16 E6 and E7 genes contributes to apoptosis and senescence in SiHa cervical carcinoma cells

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Abstract

Objective. Human papillomavirus type 16 (HPV-16) is a high-risk DNA tumor virus involved in the development of cervical carcinomas. Substantial studies have demonstrated that E6 and E7 oncoproteins of HPV-16 could induce cell proliferation and immortalization. Repression of E6 and/or E7 oncogenes may induce cervical cancer cells to undergo apoptosis or senescence. The purpose of this study was to determine whether activation of the p53 and retinoblastoma (Rb) pathway by HPV-16 E6 and E7 repression was responsible for apoptosis and senescence of cervical cancer cells and to explore the potential of an antisense RNA (AS) transcript for gene therapy of cervical cancer.

Method. The antisense RNA directed against HPV-16 E6 and E7 (16AS) was constructed, and its effects on cell apoptosis and senescence of SiHa cervical carcinoma cells harboring HPV-16 were analyzed. The efficiency of 16AS was evaluated with RT-PCR, Western blotting, flow cytometry analysis, Hoechst 33258 staining, senescent cell morphology observation and senescence-associated β-galactosidase staining.

Results. The sufficient repression of HPV-16 E6 and E7 oncogenes were achieved in 16AS-transfected SiHa cells, which led to obvious apoptosis and replicative senescence of tumor cells. Furthermore, the downregulation of HPV-16 E6 and E7 by 16AS transfection resulted in remarkable increase of both p53 expression and hypophosphorylated p105Rb level in SiHa cells.

Conclusion. These results demonstrate that reduction of E6 and E7 expression is sufficient to induce SiHa cells to undergo apoptosis and senescence and suggest that transfection of cervical cancer cells with HPV-16 E6 and E7 antisense RNA is a potential approach to treat HPV-16-positive cervical cancers.

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Introduction

Cervical cancer is a major cause of death and the second most common malignancy in women worldwide with about 500,000 new cases every year [1]. Epidemiologic and molecular studies have convincingly demonstrated that infection with certain types of human papillomavirus (HPV) is the most important risk factor for cervical cancer [2]. Approximately 200 different HPVs have now been characterized, and new types are regularly added to the list [3]. These viruses can be designated as high risk or low risk according to the propensity for malignant progression of the lesions that they cause. The vast majority of human cervical cancers are associated with high-risk HPV infections [3], in which HPV-16 and HPV-18 are by far the most prevalent high-risk types [4] and are responsible for approximately 70% of cervical cancers worldwide [5,6].

Several experimental evidences indicate that the E6 and E7 oncogenes of high-risk HPV play major roles in the development as well as in the maintenance of the malignant phenotype of cervical cancer. The E6 protein binds to the cellular p53 tumor
suppressor protein and promotes its ubiquitin-dependent degrada-
tion [7,8], while the E7 protein associates with the retino-
blastoma (Rb) and interferes with its binding to E2F, resulting in
the loss of Rb/E2F complexes that represses transcription [9,10].

Therefore, the E6 and E7 oncogenes are ideal targets for gene
therapy in cervical cancer. In current studies, therapeutic strategies
including application of antisense RNA and oliognucleotides
have been used specifically to block translation of several genes
[11]. It was reported that antisense nucleic acid (AS) of HPV-16
E6 inhibited tumor cell growth and induced apoptosis in CaSki
cells by downregulation of E6 gene expression [12]. Introduction of
the bovine papillomavirus (BPV) E2 gene into HeLa cervical
carcinoma cells showed inhibitory effect on the expression of the
HPV-18 E6 and E7 genes and then caused HeLa cells to cease
proliferation and undergo senescence [13,14]. Senescence is the
first barrier that prevents the indefinite proliferation of cultured
cells, and it appears to be an important tumor suppressive
mechanism in animals [15].

In this study, an EGFP transcription vector expressing the
antisense sequences directed against HPV-16 E6 and E7 genes
was constructed and then transfected into a human cervical
cancer cell line SiHa cells to determine the effect of antisense
technology on HPV-16 E6 and E7 expression and to investigate
whether the reduction of E6 and E7 genes expression efficiently
triggered apoptosis and/or senescence in the SiHa cervical
carcinoma cells.

Materials and methods

Cell lines and culture conditions

SiHa epithelial cell line is derived from human cervical carcinoma and
contains integrated HPV-16 genome about one or two copies. C-33A cells which
have no HPV genome were used as control cells. The two cells were both
obtained from the American Type Culture Collection (ATCC, Manassas, USA)
and cultured in Dulbecco’s modified Eagle’s medium (DMEM, GibCO, USA)
supplemented with 15 mM (pH 7.3) HEPES and 10% heat-inactivated fetal
bovine serum (FBS) at 37 °C in a humidified atmosphere with 5% CO2.

Construction of recombinant plasmid

The most coding sequence of HPV-16 E6 and E7 genes was amplified by
PCR (95 °C for 30 s, 50 °C for 30 s and 72 °C for 1 min, for 30 cycles in standard
buffer) from the plasmids containing cloned full-length genomic DNA for HPV-16
(kindly provided by Pro. Ethel-Michele de Villiers) using oligonucleotide
primers P1 (5′-CGG GAT CCC GCG ACC CAG AAA GTT ACC ACA G-3′) and
P2 (5′-CGG AAT TCC GAC AAC CGA AGC GTA GAG TCA C-3′). These
primers were designed to place EcoRI and BamHI restriction sites at the
5′ and 3′ ends of the E6 and E7 coding sequence respectively. Then the 649-bp
PCR product was inversely inserted into a eukaryotic expression vector pEGFP
(Clontech, USA) between the EcoRI and BamHI sites. The resulting pEGFP-
HPV-16 E6 and E7 AS recombinant vector (16AS) expressing antisense E6 and
E7 and the green fluorescent protein was sequenced accurately by Invitrogen
(Shanghai, China).

Plasmid transfections

Twenty-four hours before transfection, 1.5×105 SiHa cells were plated
into each well of 6-well sterile plates (10 cm2). When reached about 80–90% conflu-
cy, cells were transfected with either a recombinant vector 16AS
encoding human papillomavirus E6 and E7 in the antisense orientation fused
with EGFP or a control vector pEGFP encoding only EGFP by use of Lipo-
fectamine 2000 (Invitrogen, USA) in accordance with the manufacturer’s
protocol. For each dish, 3–5 μg purified plasmid mixed with 8–10 μl Lipo-
fectamine in 2 ml serum-free DMEM was used. Six hours later, the medium was
replaced by fresh growth medium. The cells were further incubated in 5% CO2 at
37 °C and harvested for analysis at different time points.

RT-PCR analysis

Changes in gene expression were verified by RT-PCR using β-actin as an
internal normalization standard. At 48 h after transfection with 16AS or pEGFP,
the total RNA was isolated from the cells using TRIZol reagent (Sigma, USA).
Then cDNA was synthesized from 5 μg of RNA using a one-step RT-PCR kit
(Promega, USA) according to the manufacturer’s instructions. The parameters
for RT-PCR were 42 °C, 60 min; 95 °C, 5 min for synthesizing first strand cDNA
and RNA/cDNA/primer denaturation; 95 °C, 30 s; 50 °C, 30 s; 72 °C, 1 min; 30 cycles
for second strand cDNA synthesis and PCR amplification; final extension
for 10 min at 72 °C. The primers used were as follows: for HPV-16 E6, 5′-TTA
CCA CAG TTA TG C AC A GA 3′ and 5′-ACA GTG GCT TTT GAC AGT TA-
3′; for HPV-16 E7, 5′-AGA AAC CCA GCA G TA ATC AT-3′ and 5′-TTA TGG
TTT CTG AG A GA GA 3′; for β-actin, 5′-AGC CAT GTA CG TGC TAT CC-
3′ and 5′-TTG GCG TAC AGG TCT TTG C-3′. The PCR products were
electrophoresed in a 1.5% agarose gel and visualized under ultraviolet light by
ethidium bromide staining.

Western blotting analysis

Cells were harvested 48 h after transfection with 16AS or pEGFP and
resuspended in lysis buffer. The cell lysates were incubated at 4 °C for 30 min
and spun at 12,000 rpm for 20 min at 4 °C, then the supernatants were collected.
Fifty micrograms of total protein from each sample was subjected to a 10% SDS–PAGE
gel electrophoresis and then electrotransferred to nitrocellulose membranes.
Blocked with TBS/Tween 20 containing 5% skimmed milk for 1 h, the
membranes were incubated overnight at 4 °C with primary antibodies
specific for the proteins as follows: HPV-16 E6 (sc-460), p53 (SC-126) and
β-actin (sc-1616-R) from Santa Cruz Biotechnology (Calif, USA); HPV-16 E7
(MS-768-P0) from NeoMarkers (Fremont, USA); pRb (554136) form BD
PharMingen (Calif, USA). After four washes with TBST for 15 min each at
room temperature, the membranes were incubated with horseradish peroxidase-
labeled secondary antibody for 1 h at 37 °C. Then they were washed again in
TBST four times for 15 min each at room temperature and incubated with ECL
reagents (Pierce, USA), and the signals were detected by Kodak film (Eastman
Kodak Co., Rochester, USA).

Flow cytometry analysis

Cell apoptosis was evaluated by flow cytometry (FCM) according to the
methods described by Ishibashi and Lippard [16]. At 48 h post-transfection with
16AS or pEGFP, cells were harvested with 0.2% trypsin, washed in cold PBS,
fixed in 70% ice-cold ethanol and then stained with propidium iodide (PI)
onight at −20 °C. On the following day, the stained cell were run on a
FACSort flow cytometer (Becton-Dickinson, Franklin Lake, USA) and
evaluated using the CELLQuest software system (BD Biosciences, USA) to
calculate the percentage of sub-G1 (hypodiploid) apoptotic cells.

Nuclear staining with Hoechst 33258

To observe the apoptosis on the cell morphology, SiHa cells were transfected
with 16AS or control vector (pEGFP). Forty-eight hours after the transfection,
the cells were stained with 10 μg/ml of bisbenzimide (Hoechst 33258; Sigma,
USA) for 30 min at 37 °C. Then the stained cells were observed under the
fluorescence microscope (Nikon, Japan) to show apoptotic nuclear degradation.

Senescent cell morphology observation with phase-contrast
microscopy

For cell morphology analysis, SiHa cells were seeded on 24-well sterile
plates and transfected with 16AS or pEGFP the next day. Ten days after the
transfection, the cells were microphotographed under the phase-contrast microscopy (Nikon, Japan) with a magnification of 200-fold.

**In situ staining of senescence-associated β-galactosidase (SAβ-gal) activity**

SiHa cells were seeded into 24-well plates and then transfected with 16AS or pEGFP respectively on the following day. Ten days after the transfection, the cells were stained with β-gal (5-bromo-4-chloro-3-indolyl-D-galactopyranoside, pH 6.0) which was characteristic of the senescent cells as described previously [17] and examined under bright-field illumination.

**Statistical analysis**

All experiments were repeated at least thrice. The data were analyzed with the software package SPSS 12.0. The χ² test was performed to compare frequencies among the groups. P value less than 0.05 were considered significant statistically.

**Results**

**Antisense targeting HPV-16 E6 and E7 (16AS) inhibits the expression of E6 and E7 mRNAs in SiHa cells**

To observe whether the antisense HPV-16 E6 and E7 RNA decreased the expression of both E6 and E7 genes, the empty vector (pEGFP) and recombinant vectors containing the sequence directed against HPV-16 E6 and E7 (16AS) were used to transfect SiHa cells, a cell line transformed with HPV-16. At 48 h after the transfection, a substantial reduction of E6 and E7 mRNA expression in 16AS-transfected SiHa cells was demonstrated by RT-PCR compared with that in pEGFP-transfected or non-transfected SiHa cells. The RT-PCR product levels were normalized to β-actin expression (Fig. 1). These results demonstrated that the reduction of E6 and E7 expression through an antisense mechanism was effective and pragmatic.

**Suppression of E6 and E7 expression by 16AS and its effect on expression and activities of p53 and Rb proteins**

RT-PCR analysis above showed a substantial reduction of E6 and E7 mRNA in 16AS-transfected SiHa cells. The expression levels of HPV-16 E6, HPV-16 E7, tumor suppressor gene p53, and Rb proteins were then determined by Western blot. As expected, 2 days after 16AS transfection, expression of the HPV-16 E6 and E7 proteins was markedly repressed in the 16AS-transfected SiHa cells (Fig. 2, panels 1, 2). Down-regulation of HPV-16 E6 and E7 by 16AS transfection resulted in remarkably elevation of p53 expression in SiHa cells (Fig. 2, panel 3). Furthermore, the introduction of 16AS led to an obvious increase in the levels of hypophosphorylated p105Rb (Fig. 2, panel 4), which was due to the repression of the HPV-16 E6 and E7 genes and subsequent loss of the E7-mediated destabilization of p105Rb. These results demonstrated that specific repression of the HPV-16 E6 and E7 proteins could activate the p53/Rb pathway in SiHa/16AS cells.

**16AS transfection induces the apoptosis of the SiHa cervical carcinoma cells**

To determine whether repression of E6 and E7 induced SiHa cells undergoing apoptosis, the cells were transfected with 16AS or pEGFP empty vector. At 48 h after the transfection, the cells were stained with PI and subjected to flow cytometry analysis. As shown in Fig. 3C, up to 56.22% of SiHa cells exhibited sub-G1 DNA content. Fig. 2. The repression of HPV-16 E6 and E7 and its effect on the p53 and Rb pathways by Western blot analysis. Protein was extracted from the SiHa cells 2 days after transfection with the 16AS or empty vector (EGFP). A 50-μg amount of each sample was resolved by gel electrophoresis, transferred to a membrane and probed with antibodies specific to HPV-16 E6, HPV-16 E7, p53 or pRb, as indicated. The major hyperphosphorylated and hypophosphorylated species of pRb were indicated by P and O, respectively. The β-actin was used as an internal control.

**Fig. 3. Effect of pEGFP-HPV-16 E6 and E7 AS on apoptosis of SiHa cells by Flow cytometric analysis. Flow cytometry was used to analyze the changes of the propidium iodide-stained SiHa cells (A), mock (EGFP)-transfected SiHa cells (B) and 16AS-transfected SiHa cells (C). The number of apoptotic cells was counted with a FACSort flow cytometer according to the methods described in Materials and methods. At 48 h post-transfection, the percentages of apoptotic cells in 16AS-transduced cells were 56.22%, compared to only 12.70% of EGFP-transfected (P=0.0075) or 1.97% of non-transfected SiHa cells (P=0.0032) exhibited sub-G1 DNA content.**
transfected with 16AS displayed sub-G1 DNA content, which was an indication of DNA fragmentation and often associated with apoptotic cell death. In contrast, only 12.70% of mock-transfected (transfected with pEGFP empty vector) or 1.97% of untreated cells exhibited sub-G1 DNA content \((P < 0.05)\). These results were consistent with the hypothesis that the reduction of HPV-16 oncogenes expression led to the proliferation inhibition and apoptosis induction in SiHa cervical carcinoma cells.

Since the flow cytometric analysis of sub-G1 might not adequately determine the apoptotic fraction, we also observed the morphology of the cells stained with Hoechst 33258. As shown in Fig. 4A, an increased number of cells with chromosome condensation and fragmentation were found in 16AS-transfected SiHa cells as compared with mock-transfected SiHa cell. The results clearly demonstrated that transfection of 16AS induced apoptosis in SiHa cells.

**Effects of 16AS on cellular morphology in SiHa cervical carcinoma cells**

Based on previous work, it was confirmed that the morphology of E7 siRNA-treated cells revealed changes character-istic of senescent keratinocytes [14]. In our research, subtle morphological changes were first apparent in SiHa/16AS cells at 6 to 7 days after the transfection. By 10 to 12 days post-transfection, there were striking morphological differences. As shown in Fig. 4B, the appearance of the cells was assessed by phase-contrast microscopy 10 days after mock transfection or transfection with 16AS. The depression of E6 and E7 caused the SiHa/16AS cells to cease proliferation and adopt a flattened and enlarged appearance, suggesting that the cells efficiently underwent senescence. In contrast, most SiHa/EGFP cells failed to cease proliferation and a significant number of proliferating colonies developed. The results implied that the downregulation of E6 and E7 had apparent effects on the morphology or growth of SiHa cells.

**16AS induces senescence in SiHa cervical carcinoma cells**

As a more specific marker of senescence, SAβ-gal expression was measured on 10 days after the transfection. As shown in Fig. 4C, the colonies arising from the mock-transfected showed faint background staining, whereas almost all of the cells transfected with 16AS displayed intense blue staining indicative of SAβ-gal activity and senescence. We also found that 16AS induced neither morphological changes nor SAβ-gal activity in the HPV-negative C-33A cells (date not shown). Taken together, these results indicated that the immortal cell lines such as SiHa cells were likely induced to a senescent state through the extinction of E6 and E7 expression.

**Discussion**

Cervical carcinoma, a leading cause of cancer mortality in women worldwide, is initiated by infection with high-risk HPVs such as HPV-16 and whose E6 and E7 genes have been identified as the major HPV oncogenes [18]. It is believed that continued expression of the high-risk HPV E6 and E7 proteins is required to initiate and maintain the proliferative state of cervical cancer cells [19].

The high-risk HPV E6 protein binds to p53 tumor suppressor protein and targets it for accelerated ubiquitin-mediated degradation [20]. The E6 protein also stimulates the activity of telomerase, which maintains the ends of chromosomes [21]. The high-risk HPV E7 protein binds to Rb family members and disrupts Rb/ E2F complexes, resulting in increased expression of E2F-responsive genes [22]. In addition, the E7 protein induces rapid degradation of hypophosphorylated Rb family members [23]. Since the main tumorigenic effects of the high-risk HPVs have been attributed to E6 and E7 genes, much of the efforts to develop gene therapy for these infections have been made to block the expression of E6 and E7 genes. Antisense RNA-mediated repression of HPV gene expression in cervical carcinoma cell lines is an innovative method to induce the specific decrease of the viral target oncoprotein and result in a several-fold inhibition of proliferation [24]. Recently, some studies showed that inhibition of human HPV E6 and E7 transcription in HeLa cells by means of the E2 protein of bovine papillomavirus 1 (BPV1) induced the cells to undergo growth arrest and senescence [13, 25].
Adenovirus-mediated transfer of human papillomavirus 16 E6/E7 antisense RNA suppressed expression of E6/E7 and induced apoptosis of human cervical cancer SiHa cells [26]. Transfection of cervical cancer CaSki cells with HPV-16 E7 antisense RNA expressed by a recombinant adeno-associated virus vector inhibited cell proliferation, induced apoptosis, reduced cell migration and restrained in vivo proliferation of CaSki cells [27]. These papers paid most attention to the role of reduced cell migration and restrained in vivo proliferation of antisense RNA expressed by a recombinant adeno-associated virus vector and HPV-16 E7 antisense RNA and cervical cancer cells senescence. In this paper, to demonstrate whether the E6 and E7 repression in SiHa cells by antisense RNA caused growth arrest and induced apoptosis and senescence, the antisense transcript of E6 and E7 genes of HPV-16 were introduced into cervical SiHa cells harboring HPV-16 via an enhanced green fluorescent protein (EGFP) vector, pEGFP-HPV-16 E6/E7 AS (16AS) and analyzed the effects of these genes’ downregulation on SiHa cells.

In our experimental system, the reduction of E6 and E7 mRNA was verified by RT-PCR at 48 h after the transfection with 16AS using β-actin as an internal normalization standard, and the reduction of E6 and E7 proteins was reconfirmed by Western blotting analysis. The biochemical and physiological responses of SiHa cells to the E6 and E7 repression were also determined. As expected, 48 h after the transfection with 16AS, the expression of p53 markedly increased in the 16AS-transfected cells (Fig. 2, third panel). Similarly, there was an evident raise in the level of hypophosphorylated p105Rb following E6 and E7 repression in the SiHa/16AS cells but not in the control SiHa/EGFP cell lines (Fig. 2, fourth panel). The results suggested that introduction of antisense RNA directed against E6 and E7 genes of HPV-16 was efficient to inhibit E6 and E7 expression in SiHa cervical carcinoma cell lines.

Based on published literatures, E6 and E7 repression may have obvious effects on cell proliferation and apoptosis [13]. Thus, we attempted to perform flow cytometry analysis of PI-stained 16AS-transfected SiHa cells and found it to be an effective method. In our studies, the peak of sub-G1 (hypodiploid) fraction (apoptotic cells) was markedly higher in the SiHa cells transfected with 16AS (SiHa/16AS) than those transfected with pEGFP empty vector. Moreover, E6 and E7 repression caused a substantial fraction of SiHa/16AS cells to undergo apoptosis through Hoechst 33258 staining 48 h after the transfection (Fig. 4A). Taken together, the above results suggest that the transfection of SiHa cells with 16AS results in the induction of growth arrest and apoptosis of the cells. Therefore, targeted induction of apoptosis in SiHa cells can be a potential therapeutic strategy for those conditions.

Several previous studies showed that the observed reduction in replication was most likely to be due to either apoptosis or senescence [14]. This hypothesis was confirmed when observation of the morphology of BPV E2-infected cells revealed changes characteristic of senescent keratinocytes [13]. Subtle morphological changes were first apparent 6 to 7 days after the original transfection. By 10 to 12 days post-transfection, there were striking morphological differences between 16AS-transfected cells and mock-transfected cells (Fig. 4B). The 16AS-transfected cells were larger and flatter than the controls. The cells were frequently multinucleate and had long cytoplasmic projections, which were also more dispersed than the mock-transfected cells.

To further confirm that the above cells had become senescent, the cells were stained for senescence-associated β-galactosidase (SAβ-gal), a well-established marker of senescence in keratinocytes [17]. As shown in Fig. 4C, the majority of 16AS-transfected cells showed intense blue staining, reflecting high levels of SAβ-gal activity. In contrast, mock-transfected cells did not show significant staining. We also found that 16AS induced neither morphological changes nor SAβ-gal activity in the HPV-negative C-33A cells (data not shown). Based on these findings, it appears that reducing E6 and E7 levels by antisense cause SiHa cells to undergo dramatic changes that are characteristic of senescent cells such as enlargement and flattening of cells and the expression of SAβ-gal activity. These results imply that the reduction of E6 and E7 expression by the 16AS is sufficient to induce senescence in SiHa cervical carcinoma cell. As a result, repression of E6 and E7 expression may induce a great deal of SiHa cells undergoing apoptosis while others survive and are prone to senescence.

HPV E6 and E7 genes, which initiate carcinogenesis, are also required in the maintenance of malignant phenotype of cervical carcinoma cells. Our results demonstrate that the reduction of E6 and E7 genes activates apoptosis and senesence pathways by antisense technology, so therapies directed against the viral proteins may be effective and pragmatic. According to these findings, it appears that transfection of pEGFP vector expressing the antisense sequences directed against HPV-16 E6 and E7 genes may be a good strategy for gene therapy of cervical cancer.

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