Reversal of the Malignant Phenotype of Cervical Cancer CaSki Cells through Adeno-Associated Virus–Mediated Delivery of HPV16 E7 Antisense RNA

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Abstract  Human papillomavirus (HPV) infection is the most important risk factor for the development of cervical cancer. The oncogene E7 from high-risk HPV strains has the ability to immortalize epithelial cells and increase cellular transformation in culture. In this study, we explored the possibility of preventing cervical cancer growth by inhibiting HPV16 E7 expression through gene transfer of an antisense construct. A recombinant adeno-associated virus (rAAV) vector was chosen for the transfer, based on its transfection efficiency, in vivo stability, and lack of detectable pathology. In vitro transfer of an rAAV vector expressing antisense HPV16 E7 (AAV-HPV16E7AS) inhibited cell proliferation, induced apoptosis, reduced cell migration, and restrained in vivo proliferation of HPV16/HPV18–positive cervical cancer CaSki cells. These results indicate that down-regulation of HPV16 E7 with antisense RNA is beneficial in reducing the tumorigenicity of CaSki cells, and rAAV vectors ought to be a new efficient approach for delivering the expression of therapeutic genes.

Most acquired and inherited diseases are rooted at the genetic level, and the ability to correct genetic defects that cause disease remains the ultimate goal of gene therapy. For some diseases, gene therapy presents the only hope for patients, but truly effective gene therapy continues to be elusive. Although a great variety of genes show potential for the treatment of human disease, delivering genetic material with therapeutic potential to specific target sites remains a challenge (1, 2).

Gene delivery systems can be categorized as viral or nonviral systems. The more commonly used viral gene delivery systems are retrovirus (3), adenovirus (4), and adeno-associated virus (AAV; ref. 5) vectors. Commonly used nonviral delivery systems include cationic liposome (6), HVJ-liposome (7), and mechanical approaches, such as “gene gun” (8), DNA infusion, and DNA injection (9). Many gene delivery systems have shown some degree of success in vitro, but all have fallen short in in vivo trials. The major problems encountered have been low efficiency of gene delivery and/or inability to achieve targeted gene placement.

Antisense technology has been used mainly to knock out or down-regulate the expression of certain genes associated with disease. Antisense constructs are small and generally do not code for any known biological activities in the host, and in early trials, they have proven to be well tolerated (10, 11). However, the effectiveness of antisense therapy has generally been short lived, and problems with the efficiency and specificity of gene delivery have also limited its use. The side effects that result from nonspecific gene delivery can be circumvented if the chosen target for antisense attack is an acquired genetic material, such as a viral sequence, and not a somatic gene. Targeting acquired genes has been proposed for diseases associated with infectious agents, including some cancers, such as cervical cancer associated with human papillomavirus 16 (HPV16)–infected CaSki cells (12).

Cervical cancer is one of the leading neoplastic causes of death in women worldwide (13, 14). The single most important etiologic factor for cervical cancer is HPV infection (15, 16). More than 95% of all cervical cancers have been found to be positive for HPV (17, 18). Mounting evidence suggests that high-risk HPV strains are oncogenic, and their activity depends on the viral E6 and E7 oncoproteins that are selectively retained in HPV-associated cervical cancers (15, 16, 19). E6 and E7 have the ability to immortalize epithelial cells in culture alone, and in concert with other oncogenes, they increase cellular transformation (20–22). The E6 oncoprotein binds to p53 and promotes its rapid degradation via the ubiquitin-dependent pathway (23, 24), whereas the E7 oncoprotein binds to pRB, rendering it ineffective in normal cell growth regulation (3, 25–27). The E6 and E7 oncoproteins of high-risk HPV strains, such as HPV16 and HPV18, have higher affinity for p53 and pRB, respectively, compared with those of...
low-risk HPV strains, and thus may have a greater ability to interfere with the normal cellular function of p53 and pRB (22, 28, 29). Although our understanding of the pathogenic role of HPV in cervical cancer is not complete, inhibition of the virus life cycle has led to beneficial clinical outcomes in HPV-positive cancers (30, 31).

In HPV16, the E6 coding sequence is always expressed on an mRNA with the entire E7 coding sequence (32), a single antisense blockage of RNA targeting the E7 coding sequence could be used to knock down the expression of both E6 and E7 because they are expressed bicistronically (33). Thus in this study, we used antisense RNA to knock out the E7 oncogene carried by HPV16 E7 in CaSkI cells and tested the hypothesis that the tumorigenesis potential of HPV16 oncogenes could be reversed by means of recombinant AAV (rAAV)–mediated HPV16 E7 antisense RNA gene transfer.

Materials and Methods

Cell culture. CaSkI cell, a HPV16/HPV18–positive cervical carcinoma cell line, and hypotriploid human cell line 293 cell, an AAV packaging cell line, were purchased from the American Type Culture Collection (Rockville, MD). CaSkI cells were grown in RPMI basal medium; the viral packaging cells were grown in high glucose DMEM basal medium. All complete media were supplemented with fetal bovine serum (5%), penicillin G (100 units/mL), and streptomycin (100 µg/mL).

Construction and generation of rAAV encoding antisense HPV16 E7 (rAAV-HPV16E7AS). The coding sequence of HPV16E7 was isolated after total RNA isolation of CaSkI cells, reverse transcription-PCR, and subsequent PCR, using the following primer pair: 5′-GCCGCCGCCGAAACCAGCTCTAACATC-3′ (sense) and 5′-GCCGCCGCTTATCCCTTCTCGAGAACAGA-3′ (antisense). The isolated PCR product was cloned into pGEM-Teasy (Promega, Madison, WI); the correct sequence was confirmed by sequencing. Then it was subcloned in antisense orientation into plasmid pLFI (a gift from Xiao Xiao) by NotI digestion to generate a vector named pLFI-HPV16E7AS, the correct orientation of which was verified by AccI and KpnI digestion. rAAV-HPV16E7AS was generated by cotransfection of pLFI-HPV16E7AS, PXX2, and PXX6 into 293 cells. rAAV-Laz was produced using the same protocol. Preparation of rAAV-HPV16E7AS was done by cotransfection according to published protocols with modifications. Cells from 80 dishes were harvested 48 to 72 hours after transfection, then cell mixtures were homogenated, and CsCl was added to a final density of 1.37 g/mL. The virus particles were then purified by CsCl density gradient centrifugation as previously published (34). Titers of AAV-HPV16E7AS were determined by dot blot hybridization as recommended (35) to calculate the relative concentration of viral particles per mL.

Cell proliferation assays. Cells were plated in 96-well plates. Various concentrations of trichostatin A were added into wells, and cell growth was monitored. Each treatment group had triple replicates. Cells were fixed with trichloroacetic acid for 6 hours after plating the cells. The absorbance obtained with this plate is taken as 0% growth. The absorbance obtained with wells containing untreated cells was taken as 100% growth. An increase and decrease at 570 nm values in the experimental wells relative to the initial value indicate cell growth and death, respectively. When plotted as a percentage of untreated control growth and death, values settle on the positive and negative scales of the Y axis, respectively (36).

Flow cytometry. Confluent cultures of CaSkI cells were trypsinized and recovered with mild centrifugation. The cell pellets were washed twice in PBS and resuspended in 200 µL of PBS followed by the addition of 2 mL of 70% ice-cold ethanol for 30 minutes at 4°C. The cells were centrifuged again, and the pellets were resuspended in 200 µL PBS. The cell suspension was kept at 4°C overnight. One hundred microliters of RNase (1 mg/mL) and 100 µL of propidium iodide (100 mg/mL) were added to the cell suspensions and incubated at 37°C for 30 minutes. The flow cytometer was used with the Workstation, version 1.5, for apoptosis evaluation (Beckman Coulter, Fullerton, CA).

Northern blot. To determine whether the rAAV-E7AS can reduce HPV16 E7 mRNA, Northern blot analysis was done as reference (37).

Total RNA was isolated using an Atlas Pure RNA isolation kit (Clontech, Palo Alto, CA). For Northern blots, RNA samples were electrophoresed through 1.2% agarose-formaldehyde gels and transferred to Hybond-N⁺ membranes from Pharmacia (Uppsala, Sweden) by capillary diffusion for 12 to 14 hours. Immobilized RNA was membrane cross-linked to exposure to UV light using a Stratalinker (Stratagene, San Diego, CA) and hybridized with horseradish peroxidase–labeled HPV16E7- or glyceraldehyde-3-phosphate dehydrogenase–specific cDNA probes prepared by random primer labeling (NorthSouth Direct HRP System; Pierce, Rockford, IL). HPV16E7 hybridization signals were quantitated using a Molecular Dynamics Phosphorimage (Sunnyvale, CA), and values were normalized to glyceraldehyde-3-phosphate dehydrogenase mRNA levels.

Western blot. For Western blot analysis, the cell lysate was prepared by adding 20% SDS containing 1 mmol/L phenylmethylsulfonyl fluoride. The lysate was sonicated for 30 seconds on ice followed by centrifugation for 30 minutes at 4°C. Twenty micrograms of total protein from each sample were subjected to a 10% SDS-polyacrylamide gel and electrophoresed to a nitrocellulose membrane. After blocking with PBS/Tween 20 containing 5% powdered milk, the membrane was incubated with antibodies against HPV 16 E7 (Ab-1, Bio-Rad, Richmond, CA) overnight at 4°C. Primary antibody binding was detected with horseradish peroxidase–labeled secondary antibody (Bio-Rad) and enhancer chemiluminescence reagent (Pierce). β-Actin expression was detected in the same way as an internal control.

Migration and invasion assays. For chemotaxis assays, modified Boyden chambers (Millicell, 8-µm pore size, 12-mm diameter; Millipore, Bedford, MA) were coated for 24 hours at 4°C with 5 µg/mL rat tail collagen (Boehringer Mannheim, Indianapolás, IN), washed with PBS, air-dried, and placed into 24-well chambers containing 0.4 mL of migration medium (DMEM with 0.5% bovine serum albumin) with or without epidermal growth factor at the indicated concentrations. For invasion assays, growth factor–reduced Matrigel (Collaborative Biomedical Products, Bedford, MA) was diluted in 4°C water, and the indicated microgram amount was added to the membrane topside in 100-µL total volumes. The Matrigel was allowed to polymerize for 1 hour at 37°C; the chambers were air-dried for 16 hours; the Matrigel barrier (1 mm) was reconstituted with 100 µL DMEM for 2 hours at 37°C; and chambers were placed into 24-well dishes containing 0.4 mL of migration media with or without 10% FCS. Serum-starved cells (0.1% FCS for CaSkI and 0.5% FCS for fibroblasts) were added to the upper compartment (1 × 10⁵ cells in 0.3 mL of migration media) and after 6 hours (chemotaxis migration) or 48 hours (invasion) at 37°C. Cells on the upper membrane surface were removed by a cotton tip applicator; chambers were washed with PBS; and migratory cells on the lower membrane surface were fixed by treatment with 2% formaldehyde/0.5% glutaraldehyde. Cells were stained with 0.1% crystal violet, 0.1 mol/L borate (pH 9.0), and 0.2% ethanol, and migration values were determined either by dye elution and absorbance measurements at 600 nm or by counting five high-power (×40) fields per chamber. Mean values were obtained from three individual chambers for each experimental point per assay. Pharmacologic inhibitors or DMSO were preincubated (30 minutes) with suspended
cells and were also included in the migration assay at the indicated concentrations (38).

**Tumorigenicity rate in s.c. tumor mouse model.** The relative antitumoral efficacy of AAV-HPV16E7AS in vivo versus AAV-Laz was tested in the following tumor models, which have been approved by the Animal Committee of Huazhong University of Science and Technology. Female nu/BALB-c mice were obtained from Shanghai Laboratory Animal Center of Chinese Academy of Science. Mice were used when 4 to 6 weeks old and were maintained in a laminar flow cabinet under specific pathogen-free conditions, in accordance with institutional guidelines. For in vivo tumorigenicity experiments, $2 \times 10^7$ AAV-Laz–transfected CaSki cells or AAV-HPV16E7AS–transfected CaSki cells were collected at 72 hours after transfection and washed twice with PBS. The cells pellets were suspended in 100 μL of PBS and injected into the buttocks of nu/BALB-c mice subcutaneously (38). Animal models (n = 5 for each group) were kept under observations, and the new growth masses were measured every 3 days until animals were euthanized (tumor size in diameter >5 cm or 90 days after treatment).

**Statistical analysis.** All experiments were repeated at least thrice. One-way ANOVA analysis was used to evaluate the differences among groups. The results of the two-sided test were considered significant when $P < 0.05$.

**Results**

**Construction and characterization of AAV vectors.** The efficient production of AAV virions coding for HPV16E7AS and Laz was achieved. Two vectors based on the AAV serotype 2 were constructed, expressing HPV16E7AS or Laz under the control of the human cytomegalovirus promoter. The molecular size and restriction enzyme map of HPV16E7AS was confirmed by restriction enzyme digestion and agarose gel electrophoresis (Fig. 1A). The vectors AAV-Laz and rAAV-HPV16E7AS were packaged and purified, resulting in high titers up to $1 \times 10^{10}$ particles/mL (Fig. 1B and C).

**Effect of AAV-HPV16E7AS transfection on CaSki cell proliferation.** To investigate whether E7 antisense is able to inhibit the proliferation of HPV16/HPV18–positive CaSki cells, the cells were treated with AAV-HPV16E7AS for 24, 48, and 72 hours, and the growth rates were calculated. Two groups, nontreated CaSki cells and AAV-Laz–transfected CaSki cells, were set up as controls. In the AAV-HPV16E7AS group, the cell proliferation rate decreased gradually and reached its lowest level 72 hours after treatment. No significant changes in cell proliferation rate were observed for either the nontreated cells or the AAV-Laz–transfected cells through 72 hours (Fig. 2).

**Effect of rAAV-HPV16E7AS transfection on CaSki cell apoptosis.** The E7 protein of HPV16 has been shown to inhibit apoptosis of cervical cancer cells (25). It is thus conceivable that HPV16 E7 antisense transfection might reverse the inhibition and induce cancer cell apoptosis. To test this hypothesis, we transfected AAV-HPV16E7AS into CaSki cells and determined the time course for the induction of apoptosis by measuring the DNA content (apoptotic nuclei) with FACSscan analyses and propidium iodide staining. In AAV-HPV16E7AS–transfected CaSki cells, the morphologic alterations characteristic of apoptosis, including nuclear condensation and fragmentation, were detected (data not shown). In the quantitative DNA analysis, the number of apoptotic cells increased with time, accounting for 11.9% of the total cell population by 72 hours, significantly higher than with the nontreated cell or the AAV-Laz–transfected CaSki cells control ($P < 0.01$; Fig. 3A-C).

**Effect of AAV-HPV16E7AS transfection on HPV16 E7 expression.** In the proliferation assay and apoptosis experiment,
AAV-HPV16E7AS achieved its greatest effect 72 hours after transfection; thus, we chose 72 hours as the time point to ascertain whether rAAV-HPV16E7AS treatment might inhibit the expression of HPV16 E7 in CaSki cells. Northern blot and Western blot analyses were done to determine E7 mRNA and protein expression. As show in both Fig. 4A and B, a significant reduction of HPV16 E7 expression in both mRNA and protein level were observed in AAV-HPV16E7AS–transfected cells when compared with the nontreated or AAV-Laz–transfected group. We also observed a notable reduction in the levels of E6 mRNA in cells transfected with AAV-HPV16E7AS compared with those in controls (data not shown). These results are consistent with the hypothesis that the reduction of HPV16 oncogenes expression led to the proliferation inhibition and apoptosis reduction in cervical cancer CaSki cells.

Effect of AAV-HPV16E7AS transfection on migration and invasion. Cell migration or invasion is a characteristic feature of carcinoma cells. To investigate the role of HPV16 E7 in tumor cell invasion, we measured the ability of CaSki cells to migrate through Matrigel-coated filters in a modified Boyden chamber assay. Nontreated CaSki cells and AAV-Laz–transfected CaSki cells showed HPV16 E7 overexpression and had high invasive activity in vitro, whereas AAV-HPV16E7AS–transfected cells displayed low levels of HPV16 E7 expression and poor ability to traverse the Matrigel barrier. Almost 50% invasive activity was induced in AAV-HPV16E7AS–transfected cells when compared with nontreated cells or AAV-Laz–transfected cells (Fig. 4C; \( P < 0.01 \)). These results suggest HPV16 E7 may play an important role in invasion of cervical cancer cells. AAV-HPV16E7AS transfection might reverse this malignant feature.

Effect of AAV-HPV16E7AS transfection on tumorigenicity. CaSki cells produce a tumor mass when incubated into nude mice. Although we have showed that AAV-HPV16E7AS transfection may inhibit the proliferation of CaSki cells, an intriguing unresolved question is whether AAV-HPV16E7AS treatment can inhibit tumorigenicity of CaSki as well. To address this question, CaSki cells transfected either with AAV-Laz or AAV-HPV16E7AS were injected into the buttoks of nu/BALB-c mice subepidermally, and the tumorigenicity rate was determined by successive observation. In the AAV-HPV16E7AS–transfected group, only one mouse of the five injected developed a new-growth tumor (0.8 cm in diameter) until 90 days after treatment. In comparison, all five mice in the AAV-Laz–transfected group developed large tumor masses, tumor sizes in diameter of which were 5.0, 4.2, 2.5, 2.8, 4.5 cm, respectively, in the injection area just 4 weeks after treatment (Fig. 5).
Inhibition of HPV16E7 oncogene in CaSki cells retarded tumor formation. Equal numbers of cells (2 x 10^7) were injected into the buttocks of nu/BALB-c mice subepidermally. There were five mice in each group. A, mice were inoculated with CaSki cells transfected with AAV-HPV16E7AS for 72 hours. Only one of the five mice injected developed a new-growth tumor (0.8 cm in diameter) until 90 days after treatment. B, mice were inoculated with CaSki cells transfected with AAV-Laz for 72 hours. All five mice in this group developed large tumors masses, tumor sizes in diameter of which were 5.0, 4.2, 2.5, 2.8, and 4.5 cm, respectively, in the injection area just 4 weeks after treatment.

Discussion

Gene delivery may provide new therapeutic approaches for cervical cancer. Thus far, this goal has been preliminarily achieved with adenoviral vectors (39). With these vectors, transduction efficiency is generally high; yet, their clinical use might be limited by vector-related toxicity as well as short-term expression of the transgene, due to nonintegration of the therapeutic gene in the tumor cell DNA or to the clearance of adenoviral vector—carrying cells by the immune response.

rAAV is a gene delivery system that has great potential for cancer gene therapy. AAV vectors do not induce strong cellular immune responses to the transduced cells, allowing the persistence of gene expression. For example, previous studies have shown that a single peroral administration of the rAAV vector resulted in persistent expression of transgene and long-lasting phenotypic correction in rat model for >6 months (40, 41). The gene expression in skeletal muscle transduced by AAV has been shown to persist for >1.5 years, and many researches have shown that rAAV can be used as an efficient, safe, and practical in vivo gene therapy vector (5, 34, 42). In the present study, we used a novel AAV packaging system, pXX2 and pXX6. These two new plasmids can increase rAAV vector yields 40-fold (34, 35, 43). Here, for the first time, we constructed high titer and pure viral preparations of AAV-HPV16E7AS, which could perceptibly inhibit the HPV-16 E7 expression of cervical cancer CaSki cells and partly reverse their malignant features.

In addition, the viral genome of rAAV can stably integrate into a specific location within the host genome, facilitating long-term gene transfer. We thus conceivable that the transfection with rAAV vector endowed a long-term expression of E7 antisense in CaSki cells and achieved prolong inhibiting effect in vitro or even in animal experiment in our study.

Papillomaviruses have proven to be the most complex group of human pathogenic viruses. Eighty-five genotypes have been fully characterized. High-risk HPV strains encode two oncoproteins, E6 and E7, which subvert crucial cellular regulatory nodes to reactivate and maintain DNA synthesis in the host cell (44). Recent studies have shown that the viral oncogenes E6 and E7 are required for the initiation and maintenance of the malignant phenotype in HPV-positive cancers. HPV E6 mediates the accelerated proteosomal degradation of the p53 tumor suppressor protein, whereas the oncogenic effect of E7 derives from its ability to interact with a number of cell cycle regulators and perturb their functions. Among the major targets of E7 are the ‘‘pocket protein’’ p105Rb, p107, and p130. The interaction between p105Rb and E7 prevents binding of Rb to the E2F transcription factor, leading to activation of E2F target genes. Furthermore, E7 can induce abnormal centrosome synthesis before the development of extensive nuclear abnormalities. As such, the E7 protein has been shown to be necessary to efficiently immortalize their natural host cells, primary human squamous epithelial cells (42, 45, 46).

CaSki cells contain 200 to 400 copies of HPV-16 DNA per cell and have a high E7 mRNA expression level (47). Continuous high-level expression of E6 and E7 is required for primary human keratinocytes to become immortalized and for the progression and maintenance of cancer cells (48). In this study, we used AAV-HPV16E7AS to reduce HPV 16 oncogenes expression in CaSki cells and observed a reduction of malignant biological behaviors. In addition, our data showed inhibited E6 and E7 expression, retarded cell proliferation, and reduced apoptosis in AAV-HPV16E7AS–transfected CaSki cells when compared with controls. Accordingly, AAV-HPV16E7AS transfection restrained the CaSki cell migration and invasion capability noticeably. In animal experiment, we found that AAV-mediated delivery of antisense HPV16E7 RNA was able to maintain a long-term inhibiting effectiveness on tumorigenicity of CaSki cells in nude mice. Our results showed E7 regions of the viral genome seems to be necessary for the maintenance of the malignant phenotype of CaSki cells, and these malignant biological behaviors can be reversed by blocking HPV oncogenes expression.

In cancer gene therapy, the goal is to eliminate the cancer cells or enable the cancer cells to be subjected to normal growth regulation and differentiation. In this experiment, the down-regulation of HPV16 oncogenes in CaSki cells was not sufficient to kill the cells absolutely, but it did allow the cells to undergo normal differentiation or apoptosis. AAV-mediated delivery of antisense HPV16E7 RNA potentially provides a therapeutic tool for the prevention and treatment of cervical cancer.

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