Human papillomaviruses (HPVs) are the causative factor for >90% of cervical cancers and 25% of head and neck cancers. The incidence of HPV positive (+) head and neck squamous cell carcinomas has greatly increased in the last 30 years. E6 and E7 are the two key viral oncoproteins that induce and propagate cellular transformation. An immune response generated during cisplatin/radiation therapy improves tumor clearance of HPV(+) cancers. Augmenting this induced response during therapy with an adenoviral HPV16 E6/E7 vaccine improves long-term survival in pre-clinical models. Here, we describe the generation of an HPV16 E6/E7 construct, which contains mutations that render E6/E7 non-oncogenic, while preserving antigenicity. These mutations do not allow E6/E7 to degrade p53, pRB, PTPN13, or activate telomerase. Non-oncogenic E6/E7 (E6'/E7') expressed as a stable integrant, or in the [E1-, E2b-] adenovirus, lacks the ability to transform human cells while retaining the ability to induce an HPV-specific immune response. Moreover, E6'/E7' plus chemotherapy/radiation statistically enhances clearance of established HPV(+) cancer in vivo.

INTRODUCTION

The majority of individuals diagnosed with oropharyngeal (base of tongue, tonsil) head and neck squamous cell carcinoma (HNSCCs) are infected with high risk human papillomaviruses (HPVs). Greater than 90% of these individuals with HPV(+) cancers are infected with HPV type 16. Although the incidence of HPV negative (-) HNSCC is declining, the incidence of HPV(+) HNSCC is on the rise and has tripled in recent years. HPV(+) patients often do not have a significant tobacco history and present with a more advanced disease stage compared with their HPV(-) counterparts, suggesting HPV(+) HNSCC is a distinct virally mediated disease. The development of new treatments, which specifically target HPV(+) disease, and advancements in existing therapies that improve survival are imperative for this cancer type. Current treatment of HNSCC with non-specific chemotherapy and radiation, which results in significant morbidity, could be improved with addition of immunotherapy. Known viral oncogenes are attractive candidates as targets for immunotherapy. The HPV viral oncoproteins, E6 and E7, are predominantly responsible for transformation in HPV(+) HNSCCs. Like many viral oncoproteins, E6/E7 are expressed early in the course of infection and have multifunctional roles. E6 promotes degradation of p53, indirectly activates telomerase, and disrupts the function of the cellular phosphatase tumor suppressor PTPN13. E7 inactivates pRB and activates Mi2β. Together, these oncogenic alterations drive rapid cellular proliferation, suppress or downregulate key tumor suppressor proteins, and lead to cellular immortality. In addition, E6/E7 expression is vital for malignancy and is required to maintain a malignant transformed phenotype.

Currently, no HPV vaccine is effective at treating established disease, despite their success with preventing infection. Vaccine prevention of high risk HPV infection will likely fail as HPV infection of the tonsil epithelia occurs early in life, before when immunization is currently administered. Current HPV16 vaccines utilize viral coat proteins or virus-like particles with HPV16 late gene products; however, many HPV(+) HNSCC express early (E6/E7) rather than late viral genes such as the viral coat proteins. Thus, vaccination with viral coat proteins might be ineffective for treating established cancer and an immune-based therapeutic vaccine to the viral oncogenes, E6/E7, may prove more effective. Our pre-clinical model of HPV(+) HNSCC supports the effectiveness of an E6/E7 immunotherapeutic vaccine. The immune response that develops in animals bearing HPV(+) tumors is required to achieve tumor clearance in conjunction with cisplatin and radiation treatment, a regimen most often clinically used. Boosting the immune response with recombinant adenovirus expressing HPV E6/E7 improves long-term cures by as much as 30% over standard cisplatin/radiation alone. Though encouraging, two obstacles remain before bringing this therapy to trial in humans.

Pre-existing adenoviral immunity severely limits the usefulness of an adenovirus-based vaccine strategy. To negate this problem, we utilized an E1-, E2b-deleted adenovirus (Ad5 [E1-, E2b-]) that induces effective immune stimulation despite the generation of vector-induced Ad5 immunity. The second obstacle is the expression of active viral oncogenes presents potential risks despite delivery via a non-replicating recombinant adenovirus. Potential transgene integration of the HPV oncogene(s) into the host genome may initiate cellular proliferation, transformation and immortalization. To address this risk, we inactivated the E6/E7 coding regions responsible for oncogenic transformation. E6 and E7 have been well studied and their oncogenic coding sites
Second, the C-terminal PDZ-binding domain of E6 (glutamic acid, generation of a full-length E6/E7 vaccine in which the known TAGGTGTATCTCCAGGCATG-3

St Louis, MO) added to a final concentration of 8

Eco

E6/E7 were then cut and ligated into the retroviral vector pLXSN using ratio of VP to PFU was 36.7/1 VP/PFU. The

E6/E7 (Ad5 [E1-, E2b-]-wt-E6/E7) were constructed and produced as based approach. The replication-deficient virus was propagated in the E.C7 packaging cell line, CsCl2 purified and titered as previously described.17

Viral construction

Ad5 [E1-, E2b-] containing E6/E7 (Ad5 [E1-, E2b-]-wt-E6/E7) were produced and constructed as previously described.17 Briefly, the transgenes were sub-cloned into the E1 region of the Ad5 [E1-, E2b-] vector using a homologous recombination-based approach. The replication-deficient virus was propagated in the E.C7 cell monolayer. The viral particle (VP) concentration was determined by SDS disruption and spectrophotometry at 260 and 280 nm.17,20

Viral infectious titer was determined as PFU (plaque forming units) on an E.C7 cell monolayer. The viral particle (VP) concentration was determined by SDS disruption and spectrophotometry at 260 and 280 nm.17,20

The ratio of VP to PFU was 36.7/1 VP/PFU. The E6/E717 inset as well as the wt-E6/E7 were then cut and ligated into the retroviral vector pLXSN using EcoRI and BamHI restriction sites. Retrovirus particles were generated in the Phoenix A cell line according to manufacturer’s recommendations (American Type Culture Collection, Manassas, VA) with polybrene (Sigma, St Louis, MO) added to a final concentration of 8 µg ml⁻¹.

Cells

A549 (human adenocarcinoma alveolar basal epithelium cell line) cells were grown in Dulbecco’s modified Eagle medium (Thermo Fisher, Logan, UT) supplemented with 10% fetal bovine serum (Thermo Fisher). Primary human tonsil epithelial (HTE) cells were isolated from surgical tonsillectomy of consented patients under institutional IRB approval as previously described.21 HTE cells were maintained in Keratinocyte SFM media (KSFM; Invitrogen, Carlsbad, CA). Mouse tonsil epithelial cells expressing HPV16 E6, E7, Ras and luciferase (mEELR) have been described previously and were maintained in DMEM supplemented with 22.5% Hams F-12 medium, 10% heat inactivated FCS, 100 U ml⁻¹ penicillin, 100 µg ml⁻¹ streptomycin, 0.5 µg ml⁻¹ hydrocortisone, 0.0084 µg ml⁻¹ cholaer toxin, 5 µg ml⁻¹ transferrin, 5 µg ml⁻¹ insulin, 0.00136 µg ml⁻¹ tri-iodo-thyronine and 5 µg ml⁻¹ EGF (Invitrogen).

Retroviral infection of cell lines

HTE and A549 cells were infected with retroviral supernatant containing wt-E6/E7, E6/E714, or empty vector retrovirus and incubated at 37 °C in 5% CO₂ overnight. Media was aspirated 24 h post-infection and fresh media supplemented with neomycin (RPI, Mount Prospect, IL) was added for selection. Individual colonies were ring cloned and put under further selection using 800 µg ml⁻¹ neomycin. Clones shown in figures are representative of multiple clones tested. Owing to their density dependence for cell growth, HTE cell lines were not placed under antibiotic selection but maintained in KSFM until cell death or immortalization.

Standard PCR was performed to analyze mRNA in stable cell lines expressing LXSXN, LXSXN wt-E6/E7 or LXSXN E6/E717 to validate that the changes made in E6/E7 did not affect its transcription rate. E6/E7 forward primer 5'-CAAAACCGTTGGTGTGTGTTAATA-3' and reverse primer 5'-GCTTTTGGTCAGATGTTGTC-3'; GAPDH forward primer 5'-GG-GGAAGGTT AAACTGGAG-3' and reverse primer 5'-TGGAAAGATTGATGAGGTTC-3' were used. All primer concentrations were at 450 µm. Preincubation was 94 °C for 10 min. Cycling conditions were 94 °C for 40 s, 55 °C for 40 s and 72 °C for 1 min for a total of 30 cycles using the Mx3000P thermocycler (Stratagene, La Jolla, CA).

The telomere repeat amplification protocol assay to test telomerase activity in the A549 parental, A549 wt-E6/E7 and A549 E6/E717 cell lines were measured as described previously.22,23 Cells were grown to 80% confluency, harvested by trypsinization, counted and re-suspended to 400 000 cells per ml. Cells were lysed for 30 min on ice in CHAPS lysis buffer (0.5% Chaps; 10 µm Tris, pH 7.5; 1 µm MgCl₂; 1 µm EGTA, 5 µm 2-ME; 10% glycerol; 0.1 µm 4-(2-amino-ethyl)benzene-sulfon fluoride hydrochloride (AEBSF). Membranes were pelleted by centrifugation (10 000 r.p.m. at 4 °C) for 20 min and soluble proteins collected. The primer pair used was H3 primer 5'-CGGGGTTAATGTTGACCGGTG-3' and ACX primer 5'-GCCGGGCTTTACACCTCC-3'; H3ACC-3 each at a final concentration of 100 ng. The PCR reaction contained SYBERGreen PCR Master Mix (Applied Biosystems, Carlsbad, CA), 1 µm EGTA pH 7.5 and 1 µl lysate (2000 cells) in a 50 µl reaction volume and completed in triplicate. The PCR reaction was incubated at room temperature for 30 min. Cycling conditions were 95 °C for 10 min with 40 cycles at 95 °C for 15 s and 60 °C for 60 s using the Stratagene Mx3000P thermocycler. The human foreskin keratinocyte HTERT cell line was used as a reference gene.

Adenoviral infection of cell lines

HTL cells grown to 80% confluency were infected with Ad5 [E1-, E2b-]; null (empty vector), Ad5 [E1-, E2b-]-wt-E6/E7, Ad5 [E1-, E2b-]-E6/E717, or Ad GFP at an MOI of 100 for 24 h. DNA was collected at passages 1, 2, 3, 4 and 5 post-infection. Cells were trypsinized, rinsed and re-suspended in 1 x phosphate buffered saline. DNA extraction was performed using a standard animal tissue spin-column protocol from DNeasy DNA Blood and Tissue Kit (Qiagen, Valencia, CA).

Quantitative real-time PCR was performed to assay for HPV16 copy number. Briefly, HPV 16 primer set 520 5'-TTGCAGATCTACAAAGACGG TAGA-3' and 671 5'-CTTGTCAGCTGGACCATCTAC-3' along with an 185 base pair set (Applied Biosystems) were used. The amplification reaction contained SyberGreen PCR Master Mix (Applied Biosystems) 250 nM HPV 16 primers or 100 nM 185 primers and 25 ng template. Cycling conditions were 95 °C for 10 min with 40 cycles at 95 °C for 15 s and 60 °C for 60 s using the Stratagene Mx3000P thermocycler.

Western blot analysis

The following stable cell lines A549 wt-E6/E7, A549 E6/E717, and parental A549 cells were grown to 80–90% confluency, rinsed with PBS and harvested with lysis solution (50 mM Tris HCl pH 7.5; 150 mM NaCl; 5 mM EDTA; 2 mM NaVO₃; 100 mM NaF; 10 mM NaPi; 10% glycerol; 1% Triton; 1 x halte protease inhibitors; 17.4 µg ml⁻¹ PMSF). Membranes were pelleted by centrifugation (10 000 r.p.m. at 4 °C) and soluble proteins collected. Total protein was quantified using the BCA protein assay kit as per the manufacturer’s directions (Pierce, Logan, UT) and equal total protein was analyzed by western blot. Briefly, proteins were separated by SDS–PAGE, transferred to PVDF-membranes (Immobilon-P), blocked with either 5% bovine serum albumin (MP Biomedicals, Solon, OH) or non-fat dry milk and visualized by chemiluminescence on film or via UVP bioimaging system (Upland, CA). Membranes were incubated with the following antibodies: FAP-1 (1:500, Santa Cruz, Santa Cruz, CA), p53 (1:500, Calbiochem, Dartmouth, Germany), pBf (1:250, BD Biosciences, Sparks, MD) and GAPDH (1:5 000, Ammon, Carlsbad, CA).

Immunization of mice with Ads [E1-, E2b-]-E6/E717 for immune assays

Male C57BL/6 mice (N = 4) to 8 weeks old were immunized three times intrataneously at 7-day intervals. Control mice (N = 4) were given with buffer
solution or with 10^10 VP Ad5 [E1-, E2b-] intranasally using the same injection schedule as that for mice immunized with Ad5 [E1, E2b-]E6^1/E7^1. Two weeks after the last injection/imunization, all mice were killed and spleens harvested. Splenocytes from each mouse were isolated for enzyme-linked immunospot (ELISpot) testing as described below. Serum from each mouse was collected and stored at -20 °C until testing.

Preparation of mEERL cell lysates for ELISpot
HPV (+) (mEERL) cells were grown in a T125 flask until confluent, after which cells were aseptically scraped off the plastic surface, washed three times with sterile PBS, and re-suspended in 1 ml of sterile PBS. Cells were lysed by freeze-thawing three times and cellular debris removed by centrifugation. Soluble protein was brought to a final volume of 2 ml with sterile PBS. The presence of HPV-E7 in the lysate was confirmed by western blot analysis performed as described.24

ELISpot assays
HPV16 E6 and E7-specific IFN-γ and IL-2 production from splenocytes isolated from individual mice following immunizations was detected by ELISpot as previously described.10 Briefly, cells were stimulated with HPV16 E6 and E7 peptides (15-mer peptide complete sets for each; JPT Peptide Technologies, Berlin, Germany). Peripheral blood mononuclear cells were used at a concentration of 2 x 10^6 cells per well and reported as the number of spot forming cells (SFCs) per 10^6 cells per well. All E6 peptides were combined and tested as a single pool. Each peptide pool was tested in duplicate. To test for specificity, splenocytes were also exposed to an HIV-gag peptide pool and a cytomegalovirus (CMV) peptide pool. Peptides were utilized at 0.1 μg of each peptide per well. To test for reactivity to mEERL cell lysate, 25 μl of lysate was added to test wells in duplicate. In all, 5 x 10^5 splenocytes were added to wells of ELISpot plate and placed at 37 °C with 5% CO₂ for 2 h.

Figure 1b shows an amino acid alignment of the E6 protein. The specific changes that disrupt E6 and E7 function are outlined in the scheme (a) and specific protein changes are shown in bold in the protein alignment (b).

In vivo tumor growth and treatment
Male C57Bl/6 mice were obtained from the Jackson Labs and maintained at Sanford Research LARF in accordance with USDA guidelines. All experiments were approved by Sanford Research IACUC and performed within institutional guidelines. Briefly, 1 x 10^6 mEERL cells were implanted s.c. in the right hind flank of mice using a 23-gage needle. After palpable tumors were present, on days 7, 14 and 21 mice were given 10^10 VP Ad5[E1-, E2b-] null or Ad5 [E1-, E2b-]E6^1/E7^1 intranasally. Cisplatin (Calbiochem) 20 mg m^-2 was administered i.p. and concurrent radiation (8 Gy Xray (Rad Source RS2000 irradiator, Brentwood, TN)) was administered on days 13, 20, 27 days post-tumor implantation. Tumor growth was monitored weekly using caliper measurements and tumor volume calculated using the following formula, volume = (width^2)(depth). Mice were killed when tumors reached 1.5 cm in any dimension, the animal became emaciated, or demonstrated functional leg impairment. Long-term survival was followed for > 70 days.

Statistical analysis
Statistically significant differences in the mean immune responses between groups of animals were determined by Student’s t-test using GraphPad Prism (GraphPad Software, La Jolla, CA). Statistically significant differences for mouse in vivo survival analysis were determined by Kaplan-Meier log-rank survival using SigmaPlot 11 (Systat Software, San Jose, CA).

RESULTS
E6/E7 mutations
To decrease the risk associated with adenoviral delivery of HPV E6/ E7 we mutated six known oncogenic regions within E6 and E7^10,25 (Figure 1a). The introduced mutations destroy the ability of E6 to mediate degradation of p53, activate telomerase and bind at the PDZ motif.27 The E6 PDZ binding motif participates in transformation by inactivating the tumor suppressor protein PTPN13 and other proteins with PDZ domain interactions.26,27 Mutations introduced in E7 prevent its ability to bind/inactivate p53 and other pocket proteins, and to associate with Mi2β that enhances cellular growth.26 Figure 1b shows an amino acid
alignment of wild-type E6/E7 (wt-E6/E7) and non-oncogenic E6/E7 (E6/E7
d1) proteins.

Loss of oncogenic function in E6/E7
d1
To confirm the loss of oncogene function, A549 cells were infected with a retrovirus containing wt-E6/E7, E6/E7
d1, or control vector and subsequently ring cloned. Clones were analyzed by western blot. Figure 2a shows that expression of wt-E6/E7 decreases PTPN13, pRb, and p53 protein expression, while the E6/E7
d1 expression rescues this phenotype. PCR analysis of clones confirmed similar levels of viral oncogene expression (Figure 2b) suggesting that the changes evident by western blot were a consequence of altered oncogene function rather than expression and confirm the biochemical loss-of-function in the E6/E7
d1 construct. Telomerase activity was also examined in these clones. As predicted, the E6/E7
d1 vector control did not show telomerase activation, whereas the wt-E6/E7 enhanced it (Figure 2c). Previous studies have shown that HPV wt-E6/E7 induces morphological mesenchymal type changes. Therefore, the morphological characteristics of clones were also examined. Figure 2d shows that control and E6/E7
d1 grow in tight colonies, while wt-E6/E7 expression induces a mesenchymal-like change in morphology and cells grow in a non-adherent manner. Together, these data suggest that, unlike expression of wt-E6/E7, stable expression of E6/E7
d1 does not induce the biochemical or morphological changes associated with cellular transformation.

To further confirm that E6/E7
d1 cannot transform cells, HTEs were infected with retrovirus containing wt-E6/E7, E6/E7
d1, or empty vector. Expression of wt-E6/E7 results in cellular immortalization, consistent with previous findings. However, HTEs expressing E6/E7
d1 and uninfected (control) HTEs did not immortalize (Figure 3a). These findings along with the biochemical evidence above demonstrates that stable expression of the E6/E7
d1, even with an integrating retrovirus, does not result in cellular immortalization.

Neither WT-E6/E7 nor E6/E7
d1 genes in Ad5 [E1-, E2b-] transform primary HTE cells
To determine the function of wt-E6/E7 or E6/E7
d1 in a non-replicative adenoviral vector, transfection of HTE was examined and compared with a control expressing GFP. Previous studies have demonstrated that the Ad5 [E1-, E2b-] vector transgenes do not replicate in transfected cells. However, the effect(s) of HPV E6/E7 in the context of this vector have not been previously examined. Wt-E6/E7 was able to induce loss of p53, whereas E6/E7
d1 was able to rescue p53 levels compared with control (Figure 3b). However, neither wt-E6/E7 nor E6/E7
d1 were able to immortalize primary tonsil epithelial cells after infection.
positive responses to concanavalin A. Granzyme B assay was also
performed with CMV. Functionally active splenocytes in all groups was verified by
splenocytes were exposed to the irrelevant antigens HIV-gag or
buffer solution or Ad5 [E1-, E2b-]-null. The specificity of the CMI
cells (SFCs) in immunized mice but not control mice injected with
Ad5 [E1-, E2b-] was determined in mice immunized with
splenocytes harvested from individual mice. As shown in Figures
numbers of IFN-γ were determined in control and vaccinated mice by assessing the
response, ELISpot assays were performed to assess CMI responses
in mice immunized with Ad5 [E1-, E2b-] and vaccinated mice showed significant increase in cell-mediated tumor cell
toxicity compared with non vaccinated mice splenocytes (Figure 4c). These results indicate that the non-oncogenic
E6/E7 is immunogenic and induces an HPV-specific E6/E7 CMI response.

E6/E7 induces HPV-specific cell-mediated immune (CMI)
responses
To determine whether the changes induced in the non-oncogenic E6/E7 after the ability to mount an HPV-specific immune response, ELISpot assays were performed to assess CMI responses in mice immunized with Ad5 [E1-, E2b-] and E6/E7. CMI responses were determined in control and vaccinated mice by assessing the numbers of IFN-γ, IL-2 and granzyme B secreting cells in splenocytes harvested from individual mice. As shown in Figures 4a and b, CMI responses were detected in mice immunized with Ad5 [E1-, E2b-]. This was demonstrated by the induction of significantly elevated levels of IFN-γ, IL-2 and granzyme B secreting cells (SFCs) in immunized mice but not control mice injected with buffer solution or Ad5 [E1-, E2b-]. The specificity of the CMI responses was demonstrated by a lack of reactivity when splenocytes were exposed to the irrelevant antigens HIV-gag or CMV. Functionally active splenocytes in all groups was verified by positive responses to concanavalin A. Granzyme B assay was also examined because it reflects immune related tumor cell toxicity
assays.32 Splenocytes from mice vaccinated with both E6/E7 vaccines showed significant increase in cell-mediated tumor cell
toxicity compared with non vaccinated mice splenocytes (Figure 4c). These results indicate that the non-oncogenic E6/E7 is immunogenic and induces an HPV-specific E6/E7 CMI response.

E6/E7 immunotherapy increases survival during concurrent
chemotherapy/radiation
We have previously shown that mice intranasally vaccinated with and Adenovirus expressing wild-type E6/E7 improved long-term survival in mice receiving standard cisplatin/radiation.16 Although the in vitro tests suggested that mice receiving the Ad5 [E1-, E2b-] vector had a similar anti-HPV immune response, we wanted to test if it would enhance clearance of HPV+ tumors similar to the wild-type oncogenes. To test the efficacy of the non-
ononcogenic E6/E7 we established HPV(+) tumors in wild-type mice identically as previously shown.16 Once tumors had established for 1 week Ad5 [E1-, E2b-] was delivered intranasally weekly for 3 weeks. Mice receiving only cisplatin/rxt + Ad5 [E1-, E2b-] had similar and long-term clearance as we had previously reported.16 However, mice receiving Ad5 [E1-, E2b-] had significantly improved long-term survival (Figure 5) mimicking the response we had seen in the past studies when we had delivered wild-
type E6/E7.16 These data show that in immune competent mice vaccination with Ad5 [E1-, E2b-] enhances immune related clearance in vivo during standard therapy for HPV-related cancer.

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Additionally, infectious disease models utilizing the CMV responses. Adenovirus can be mass-produced, is stable, and has been repeatedly used in humans to induce robust autoimmunity than with other tumor-associated antigens.

The improved HPV(+) therapeutic vaccine for HPV(+) tumors contained a combination of modifications that render these viral oncogenes non-oncogenic while retaining the antigenicity necessary to produce an immunotherapeutic vaccine against HPV(+) tumors. We demonstrated biochemically that these mutations lack the capacity to degrade p53, pRb and PTPN13. In addition, we found that whether E6/E7 expression as a vaccine in the new Ad5 [E1-, E2b-]-based vaccine could be used for homologous vaccination regimes to induce robust CMI responses in the presence of Ad5 vector immunity.

Several pre-clinical studies suggest a therapeutic immune-based vaccine strategy using E6 and/or E7 may be of value in the treatment of established disease.35–37 However, these published studies include vaccines utilizing specific regions of either E6 and/or E7.38–40 By expressing slightly varied non-oncogenic full-length forms of both E6 and E7, our strategy has the advantage of maintaining native antigenic processing and thus theoretically increasing the chance of an immune response in vivo. Given the heterogeneity of immune response in humans, which is much less predictable than inbred strains of mice, expression of full-length proteins allows for antigen processing and development of a CMI response that may produce a broad and effective anti-tumor response.

HPV16 is the most common cause of cervical, oropharynx and anal cancers and common therapies (cisplatin and radiation) are used for treatment in the advanced stages of these cancers. Even though we examined a pre-clinical model of HPV(+) oropharynx cancer, it is likely that similar principles of therapy and vaccination can be used for other HPV(+) associated cancers. Thus, use of the non-oncogenic Ad5 [E1-, E2b-]-E6/E7+ vaccine in conjunction...
with chemoradiation represents an important advancement in the therapy of HPV(+)–associated cancers. The pre-clinical and in vitro data show loss of transformation and the equivalent augmentation of immune-mediated clearance in mice suggest that further clinical trials in patients in a phase 1 setting would be safe and efficacious. We believe that this new Ad5 [E1-, E2b-]-E6/E7 vector vaccine should be investigated further as a new immune-based therapy for HPV16-associated cancers.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Cancer Gene Therapy website (http://www.nature.com/cgt)