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Oncolytic Adenoviruses Targeted to Human Papilloma Virus-Positive Head and Neck Squamous Cell Carcinomas

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Abstract

Objectives—In recent years, the incidence of Human Papilloma Virus (HPV)-positive head and neck squamous cell carcinomas (HNSCC) has markedly increased. Our aim was to design a novel therapeutic agent through the use of conditionally replicative adenoviruses (CRAds) that are targeted to the HPV E6 and E7 oncoproteins.

Methods—Each adenovirus included small deletion(s) in the E1a region of the genome (24 or CB016) intended to allow for selective replication in HPV-positive cells. In vitro assays were performed to analyze the transduction efficiency of the vectors and the cell viability following viral infection. Then, the UPCI SCC 090 cell line (HPV-positive) was used to establish subcutaneous tumors in the flanks of nude mice. The tumors were then treated with either one dose of the virus or four doses (injected every fourth day).

Results—The transduction analysis with luciferase-expressing viruses demonstrated that the 5/3 fiber modification maximized virus infectivity. In vitro, both viruses (5/3 24 and 5/3CB016) demonstrated profound oncolytic effects. The 5/3CB016 virus was selective for only HPV-positive HNSCC cells, whereas the 5/3 24 virus killed HNSCC cells regardless of HPV status. In vivo, single injections of both viruses demonstrated anti-tumor effects until only 6–8 days following viral inoculation. However, after four viral injections, there was statistically significant reduction in tumor growth when compared to the control group ($p < 0.05$).

Conclusion—CRAds targeted to HPV-positive HNSCCs demonstrated excellent in vitro and in vivo therapeutic effects, and they have the potential to be clinically translated as a novel treatment modality for this emerging disease.

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Conflicts of Interests

The authors have none to disclose.

Keywords

Human Papillomavirus; Head and Neck Cancer; Squamous Cell Carcinoma; Oncolytic Adenovirus

Introduction

In the United States, approximately 55,000 new cases of head and neck cancers were diagnosed in 2014 [1]. These cancers encompass a heterogeneous group of malignancies including oral, oropharyngeal, pharyngeal, and laryngeal tumors. Importantly, the vast majority of these malignancies are squamous cell carcinomas [2]. In recent years, the incidence of Human Papilloma Virus (HPV)-positive head and neck squamous cell carcinomas (HNSCC) has markedly increased [3]. Specifically, the population level incidence of HPV-positive oropharyngeal squamous cell carcinomas increased by 225% from 1998 to 2004, while there was a concomitant 50% decline in the incidence of their HPV-negative counterparts during the same time period [4]. Additionally, the annual number of HPV-positive oropharyngeal cancers is projected to surpass that of cervical cancers by 2020 [4].

HPV-positive HNSCCs are considered distinct biologic entities when compared to those without the viral association. When compared to HPV-negative HNSCCs, HPV-positive tumors tend to occur in younger individuals without exposures to the traditional risk factors such as smoking and alcohol use [5, 6]. While HPV-positive HNSCCs do have an improved survival when compared to HPV-negative tumors [7], it is key to note that there is still some difficulty with the management of distant recurrences. Distant metastases associated with HPV-positive tumors have been shown to occur in multiple organs, unusual sites, and after longer intervals when compared to those distant metastases in HPV-negative tumors [8]. These distant lesions are known to be a major cause of death in patients with oropharyngeal squamous cell carcinomas [9].

Much of the aforementioned differences in behavior can be attributed to the fact that HPV-positive tumors have distinct mechanisms of oncogenesis. Among the various HPV subtypes, HPV 16 and 18 (high risk) are most often associated with malignant transformation, and 95% of HPV-positive tumors within the oral cavity and oropharynx contained HPV 16 DNA [10, 11]. Unlike their low-risk counterparts, DNA from HPV 16 and 18 are known to contribute to the immortalization of human keratinocytes [12]. More specifically, it is the E6 and E7 oncoproteins encoded by only the high risk subtypes have been shown to transform and immortalize primary human keratinocytes [13, 14]. The E6 protein suppresses the function of p53 by promoting its degradation through the ubiquitin-dependent protease system [15, 16] and downregulates p53 by binding to the co-activator p300/CBP [17]. E7 binds to the retinoblastoma (Rb) protein to disrupt interactions with the E2F transcription factor, which results in the release of free E2F in its active form to potentiate tumorigenesis [18, 19].

The increasing incidence of HPV-positive HNSCCs and their unique biology have necessitated the development of novel treatment methods. Adenovirus-based vectors have

emerged as a powerful tool for the treatment of many types of cancers, and could have a great deal of applicability for the treatment of HPV-positive HNSCCs. Specifically, conditionally replicative adenoviruses (CRAd) are attractive as therapeutic agents as they have the potential to selectively replicate within the target cells of interest. Importantly, adenoviruses and the HPV E6/E7 oncoproteins interact with similar regulatory proteins to modulate the cell cycle. The adenovirus E1A protein is the first unit transcribed and its products are key components for successful adenoviral replication. The CR1 region of the adenovirus E1A protein has been shown to bind to p300, which then interacts with and suppresses the function of p53 [20]. Additionally, the CR2 region of the E1A protein is able to disrupt the interaction between the pRb-E2F complex, rendering the retinoblastoma protein unable to perform its normal cell cycle checkpoint functions [19, 21].

Adenoviral replication can be controlled by the inclusion of a tumor-specific promoter (e.g. Cox2, CXCR4) within the adenoviral genome. Alternatively, vectors can be designed with genomic deletions to confer replication selectivity. Due to the similarities in the way the HPV and adenovirus interact with cell cycle regulators, deletion in the adenovirus E1 region can be used to target HPV positive tumors. For example, a 24 base pair deletion (Δ24) in the E1A CR2 region (the area normally responsible for binding to the retinoblastoma protein) has been used to selectively target HPV-positive tumors [22]. Furthermore, another CRAd (CB016) that contains an additional deletion in the adenovirus E1A CR1 region (along with the Δ24 deletion) has been developed [23]. The deletions in the adenoviral genome within the Δ24 and CB016 viruses do not allow for the encoding of key viral protein products that would normally allow for viral replication. However, within HPV positive cells that express the E6 and E7 oncoproteins, adenoviral replication proceeds due to functional transcomplementation from the HPV proteins [24, 25].

In this study, we employed the Δ24 and CB016 conditionally replicating adenoviruses in the treatment of HPV-positive HNSCCs. The vectors ability to selectively replicate within and kill HPV-positive HNSCCs was demonstrated in vitro. Thereafter, the oncolytic potential of the adenoviruses was studied in a xenograft model using nude mice. The results of these experiments provide insight into the clinical utility of adenovirus-based therapy for the treatment of HPV-positive HNSCCs.

Materials and Methods

Cell Lines and Culture Conditions

Two human HPV-negative (SCC-4, SCC-15 [provided by Dr. David Wong, University of California Los Angeles]) and three HPV-positive HNSCC cell lines (93VU147T [provided by Dr. Hans Joenje, VU University Medical Center, The Netherlands], UPCI SCC 090 [gift from Dr. Suzanne Gollin, University of Pittsburgh], and UM047 [obtained from Dr. Thomas Carey, University of Michigan]) were cultured in Dulbecco's modified Eagle medium (DMEM) (Mediatech, Herndon, VA). All cell lines were supplemented with 10% (V/V) fetal bovine serum and a 1% penicillin-streptomycin mixture (100 IU/mL and 100 µg/mL, respectively). They were maintained as adherent monolayers at 37°C in a humidified incubator with 5% CO₂ in air.

Adenoviral Vectors

The 5/3 Δ 24 E3-ADP-Luc and 5/3 CB016 E3-ADP-Luc vectors (hereafter referred to as 5/3 Δ 24 and 5/3 CB016 respectively) were based on adenovirus type 5 (Ad5) (Figure 1). The adenoviral vector plasmids encoding Δ 24 and CB016 mutations (pAd Δ 24 and pCB016 respectively) [22, 23] (provided by Drs. Ramon Alemany and Cristina Balgué) were recombined with the pAd- E3-ADP-Luc adenoviral backbone as previously described [26]. Briefly, in the pAd- E3-ADP-Luc structure, most of the non-essential adenovirus E3 genes were deleted (with the exception of the adenovirus death protein (ADP) which is designed to facilitate viral spread and oncolysis) and replaced with the luciferase reporter gene [26, 27].

For infectivity enhancement analysis, CMV promoter-driven luciferase expression vectors with RGD-modified Ad5 fiber (RGD-CMV-Luc), Ad5/Ad3-chimeric fiber (5/3-CMV-Luc), or wild type Ad5 fiber (Ad5-CMV-Luc) were used. All of these replication-incompetent Ad vectors are identical, except for their genetically modified fibers. The Ad 5/3 chimeric fiber replaces the knob region of Ad5 with that of Ad3, while the RGD fiber adds an arginine-glycine-aspartate-containing peptide into the HI loop of the fiber knob domain [28]. The wild type Ad5 (Ad5Wt) and the 5/3 E3-ADP-Luc viruses were utilized as non-selective replicative control vectors [26].

All viruses were propagated in the 293 cell line, purified by double cesium chloride density gradient ultracentrifugation, and dialyzed against phosphate-buffered saline (PBS) with 10% glycerol. The vectors were titrated using a plaque-forming assay, and the viral particle (vp) number was measured spectrophotometrically.

Analysis of Fiber Structure for Optimized Infectivity

The human HNSCC cell lines (SCC-4, SCC-15, 93VU147T, UPCI SCC 090, and UM047) were cultured as above in 24-well plates (5×10^4 cells/well). On the following day, they were infected with replication-deficient (CMV promoter-driven), luciferase expressing vectors with the Ad 5 Wt, RGD, or chimeric Ad 5/3 fiber at a titer of 100 viral particles (vp)/cell. Two days after infection, the cells were lysed with 100 μ l of cell culture lysis buffer, and the luciferase activity was determined with the Luciferase Assay System (Promega, Madison, WI). Results were standardized with cellular protein concentration as quantitated using the DC protein assay (Bio-Rad, Hercules, CA).

Analysis of Selective Viral Replication

The 5/3 Δ 24 and 5/3 CB016 vectors were used to infect the same five human HNSCC cell lines (24-well plates; 5×10^4 cells/well) at a titer of 100 vp/cell. A replication deficient vector (5/3-CMV-Luc) and wild type replication vector (5/3 E3-ADP-Luc) were used as non-selective controls. Two days after infection, the cells were lysed with 100 μ l of cell culture lysis buffer, and the luciferase activity was determined with the Luciferase Assay System (Promega, Madison, WI). Results were standardized with cellular protein concentration quantitated using the DC protein assay (Bio-Rad, Hercules, CA).

Qualitative Cytocidal Effect

Four human HNSCC cell lines (SCC-15, 93VU147T, UPCI SCC 090, and UM047) were cultured in 24-well plates, and a viral infection was performed the next day using a titer of 100 vp/cell. Vectors included a wild type control (Ad5 Wt), a non-replicating control (5/3 CMV Luc), and two CRAds (5/3 24 and 5/3 CB016). After 3, 6, and 9 days of cultivation, the cells were fixed with 10% buffered formalin for 10 minutes, stained with 1% crystal violet in 70% ethanol for 20 minutes, washed 3 times with tap water and allowed to air dry.

Quantitative Analysis of Cell Viability

Three human HNSCC cell lines (SCC-15, 93VU147T, UPCI SCC 090) were cultured in 96-well plates (3,000 cells/well), and subsequently infected with adenoviral vectors 24 hours thereafter at strengths of 1, 10, or 100 vp/cell. The number of surviving cells was estimated using a colorimetric method (Cell Titer Aqueous One Solution Cell Proliferation Assay; Promega; Madison, WI) according to the manufacturer's instructions. Absorbance attributable to living cells was measured at a wavelength of 490 nm in a FLUOstar Omega spectrophotometer (BMG Labtech, Ortenberg, Germany). The number of infected, living cells at each time point was normalized to the number of uninfected living cells.

In Vivo Tumor Growth Analyses

Using the UPCI SCC 090 HPV-positive HNSCC cell line, 2×10^6 cells (in 100 μ L of PBS) were inoculated subcutaneously into the flanks of female athymic nude mice to generate tumors. When the tumor nodules achieved a diameter of approximately 6–8 mm, virus was injected intratumorally. For the single injection experiment, a dose 3.5×10^{10} vp/tumor was used. For the multiple injection experiment, a viral dose of 3.5×10^{11} vp/tumor was used, and a total of four intratumoral injections were delivered. The tumor diameter was measured twice per week with calipers. The tumor volume was calculated using the following formula: Tumor Volume = (Width² $\times$ Length)/2. All procedures were carried out according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Minnesota.

Statistical Methods

Statistical analyses of in vitro and in vivo viral effects were performed using Excel (Microsoft, Redmond, WA). Differences in viral infectivity and replication, cell viability, and the relative tumor volumes of in vivo treatment groups were analyzed using the Student's t-test. The results were considered statistically significant when a two-tailed *P* value was less than 0.05. Data are expressed as a mean $\pm$ standard deviation.

Results

Augmented Viral Infectivity due to Fiber Modification

Replication-deficient vectors (Ad5-CMV-Luc, RGD-CMV-Luc, and 5/3-CMV-Luc) expressing the luciferase transgene and equipped with one of three different fibers (Ad 5 Wt, RGD, or Ad 5/3) were used to analyze viral infectivity in five HNSCC cell lines (Figure 2). In all cell lines tested (three HPV-positive and two HPV-negative), luciferase expression

reflecting the extent of viral infectivity was significantly greater with the Ad 5/3 chimeric fiber than the Ad 5 Wt or RGD fibers ($p<0.01$). Since the Ad 5/3 chimeric fiber modification demonstrated the greatest infectivity enhancement in both HPV-positive and HPV-negative cell lines, it was used exclusively in all additional studies.

Replication Selectivity of E1-Mutant Viruses

The replication of the 5/3 CB016 and 5/3 24 viruses was tested in the HPV-negative (SCC-4 and SCC-15) and HPV-positive cell lines (93VU147T, UM047, and UPCI SCC 090) (Figure 3). The 5/3 E3-ADP-Luc virus was used as a positive control vector as it demonstrated ubiquitous replication that was independent of HPV status (all data was then expressed as a percentage relative to this control virus), and the 5/3-CMV-Luc virus served as a replication-deficient (negative) control. The 5/3 24 virus demonstrated a high level of viral replication in all cell lines, which was similar to the replicating control vector. Notably, the 5/3 CB016 vector replicated selectively in the HPV-positive cell lines [UPCI (49%) and UM047 (36%)], and demonstrated only 5 to 10% replication in the HPV-negative cell lines (SCC-4 and SCC-15). These results indicated that the replication of 5/3 CB016 virus was quite selective, while 5/3 24 virus showed stronger but HPV independent replication.

In Vitro Cytocidal Effects of HPV-targeted CRAds

To demonstrate the qualitative cytocidal effects of the 5/3 CB016 and 5/3 24 viruses, a crystal violet stain was performed at multiple time points following viral infection (Figure 4). The 5/3-CMV-Luc (replication deficient) and Ad5 Wt vectors were used as controls. In the HPV-positive cell lines, the 5/3 CB016 virus killed cells effectively in two of the three cell lines at early time points and by day 9 there was total cell death in all HPV-positive cells. Additionally, the 5/3 CB016 virus had minimal effect on the HPV-negative cell line (SCC-15). In contrast, the 5/3 24 virus killed all cell lines efficiently, regardless of HPV status.

To quantitate the cytocidal effect of the vectors, cells were infected with increasing doses of the 5/3 CB016 and 5/3 24 viruses, and the percentages of living cells over time were analyzed (Figure 5). At day 3, the 5/3 24 virus showed strong cytocidal effects at low viral titers. By day 7 after viral infection, there were no viable cells after infection with the lowest titer (1 vp/cell) in all cell lines. In contrast, at day 3, the CB016 virus at the highest titer (100 vp/cell) tested caused only modest cell death in the 93VU147T cell line (HPV-positive). At the later time point, the same titer of the 5/3 CB016 virus killed over 80% of the 93VU147T cell line. Moreover, the 5/3 CB016 virus had a minimal effect on the HPV-negative (SCC-15) cell line at both time points.

Limited Antitumor Effect of a Single Intratumoral Injection

The in vivo antitumor effects of the vectors were assessed with a nude mouse model using subcutaneous xenografts established with the UPCI SCC 090 (HPV-positive) cell line (Figure 6). The resulting tumors were then directly injected with a single dose of 3.5×10^{10} viral particles. Up until day 4, the 5/3 CB016 vector demonstrated an equivalent tumor growth suppression to the wild type vector (when compared to untreated controls) after which time the 5/3 CB016 group demonstrated tumor regrowth. Additionally, the antitumor

effect of 5/3 24 virus persisted until approximately day 12 (similar to wild type vector) when compared to the untreated controls, after which it too demonstrated regrowth when compared to the wild type vector. It was clear that both the 5/3 24 and 5/3 CB016 vectors demonstrated tumor growth suppression after a single intratumoral injection, but the effect was short-lived.

Multiple Intratumoral Injections Yields Improved Antitumor Effect

As the single intratumoral injections failed to sustain antitumor effects, subcutaneous xenografts were established in the same fashion as the previous experiment, and four intratumoral injections (4 day intervals) were performed using a dose of 3.5×10^{11} vp/tumor (Figure 7). The growth curves of the treatment groups and control group diverged soon after the initial injection of virus, and continued to separate throughout the experiment. The 5/3 24 and 5/3 CB016 viruses significantly suppressed tumor growth ($p < 0.05$) for up to 30 days when compared to the untreated controls. Additionally, the 5/3 24 group demonstrated tumor regression with a relative volume of less than 1.0 from day 9 until day 26 (Figure 7 inset).

Discussion

As a way to develop therapeutics targeting HPV viral protein expression, our research group has employed conditionally replicating adenoviruses (CRAd) for the treatment of HPV-positive HNSCCs. Unlike some other virus-associated cancers, HPV-induced cancers continue to express key oncoproteins even after carcinogenesis [29]. Our viral vectors are designed to target and utilize this continued oncoprotein expression for therapeutic purposes. In these studies, we have demonstrated the improved infectivity afforded with a 5/3 fiber modification, as well as the selective replication of the 5/3 CB016 virus only within the HPV-positive cell lines. Furthermore, we also show the impressive in vivo oncolytic effect of multiple intratumoral injections in a subcutaneous xenograft model.

The coxsackie and adenovirus receptor (CAR) is the primary receptor for adenovirus type 5; however, it is expressed at low and often variable levels in HNSCCs [30, 31]. Furthermore, the levels of CAR expression have also been shown to decrease with the malignant progression of HNSCCs [32]. Low CAR levels on target tumor cells have been a limiting factor with certain gene therapy approaches for some time, but modification of the adenovirus capsid proteins has been shown to improve infectivity in both HNSCCs as well as other cancers [33, 34]. Previously, our group has used an Ad5/Ad3 fiber in the setting of adenoviral therapy for pancreatic cancer [27]. Based on this experience, we have applied the same fiber modification to overcome the CAR deficiency for HNSCCs. This particular fiber replaces the Ad5 knob with that of Ad3, the receptor of which has been recently identified to be human CD46 and desmoglein 2 [35–37]. Our data indicate that the Ad 5/3 fiber significantly improves infectivity when compared to either a RGD or wild type fiber, and the Ad 5/3 fiber was therefore used in these studies.

The 5/3 CB016 and 5/3 24 vectors were compared to a replication competent control virus to analyze their replication profile. The 5/3 CB016 virus selectively replicated in HPV-positive HNSCC cells, while the 5/3 24 virus demonstrated a high degree of non-selective

replication. The cytocidal effect of the viruses analyzed by both qualitative and quantitative assays exhibited a HPV-targeted cell-killing effect of the 5/3 CB016 virus and a strong (albeit non-selective) effect of the 5/3 24 virus, which corresponded well to the aforementioned replication profile. It is likely that the 5/3 24 virus' single deletion in the adenoviral E1A CR2 region was not enough to confer selectivity in this setting, thereby suggesting that the additional deletion in the CR1 region is quite necessary to achieve selective replication. These results are not unexpected as an adenoviral infection in raft cultures of keratinocytes expressing HPV 18 E6 and E7, the CB016 virus replicated selectively, while the 24 virus did not [23].

In our in vivo studies, only a short-lived antitumor effect resulted from a single adenovirus injection. While this was encouraging, we wanted to further optimize the therapeutic utility of the vectors. This observation led us to design a multiple injection experiment where four intratumoral doses of virus were delivered within two weeks. The multiple injection regimen conferred a profound improvement in oncolytic effect, and this statistically significant difference was present from a very early time point and persisted throughout the duration of the experiment. In fact, clinical trials employing multiple intratumoral injections of replication-selective adenoviruses have been conducted for the treatment of head and neck cancers [38]. Therefore, a multiple injection regimen is a reasonable protocol for these types of tumors, considering that HNSCCs usually have relatively accessible anatomic location in patients.

There have also been clinical trials specifically using derivatives of the 24 virus for the treatment of solid tumors in patients. For example, the 24 virus has been used in the treatment of gynecologic malignancies, and it is notable that there was no significant toxicities directly attributed to the administration of the virus during these trials [39, 40]. While we do not address the bio-toxicities or safety profile of the vectors in this study, it is crucial to note that similar vectors have already been tested in humans with minimal adverse effects. We would anticipate that our 5/3 CB016 vector, given its selective replication, would also show a favorable toxicity profile.

HPV-associated tumors are becoming more widespread, and this is especially true for head and neck cancers. Distant metastases continue to present treatment challenges for clinicians, and these difficulties translate into increased morbidity and mortality for patients. Our oncolytic adenoviruses (5/3 24 and 5/3 CB016) represent a novel application of viral therapy to address a burgeoning problem in clinical medicine. This work is an important step towards clinical translation and maximizing the vectors' potential for the treatment of HPV-positive HNSCCs.

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Abbreviations

HPV	Human Papilloma Virus
HNSCC	Head and neck squamous cell carcinoma
CRAd	Conditionally replicating adenovirus
ADP	Adenovirus death protein
Luc	Luciferase

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Figure 1. Schematic of oncolytic adenoviruses targeted to HPV-positive HNSCCs

Key deletions of CR1 and CR2 within the E1 region of the adenoviral genome account for the conditional replication in HPV-positive HNSCC cells. $\Delta 24$ has one deletion in CR2, while CB016 has that same deletion and an additional deletion in CR1. Additionally, an expression cassette containing the adenoviral death protein (ADP) and the luciferase reporter gene (Luc) were placed into the adenovirus E3 region. Wild type, RGD, and 5/3 fibers were tested, although the 5/3 fiber has the greatest increase in infectivity.

Figure 2. Infectivity maximized with the Ad 5/3 fiber

The adenoviral vector infectivity with three different fibers (Ad 5Wt, Ad RGD, and Ad 5/3) was compared in three HPV-positive cell lines (93VU147T, UPCI SCC 090, UM047) and two HPV-negative (SCC15, SCC4) HNSCC cell lines. Non-replicating vectors employing a luciferase transgene and these fibers were used at a titer of 100 vp/cell. The luciferase expression was analyzed two days after infection. Results are shown in relative light units (RLU) per mg of protein and are relative to the Ad 5 Wt fiber. The virus with the Ad 5/3 fiber showed significantly higher infectivity in all cell lines. Error bars indicate standard deviation. N=4 for each experimental condition.

Figure 3. Selective replication in HPV-positive HNSCC cells

Three HPV-positive cell lines (93VU147T, UPCI SCC 090, UM047) and two HPV-negative (SCC15, SCC4) HNSCC cell lines were infected with the 5/3 CB016 and 5/3 Δ24 viruses along with replicating (5/3 E3-ADP-Luc) and non-replicating (5/3 CMV Luc) control vectors. A viral titer of 100 vp/cell was used and the luciferase expression was quantified two days following infection. Results are shown as RLU per mg protein relative to the replicating control vectors. The 5/3 CB016 virus selectively replicated in HPV-positive cell lines, while the 5/3 Δ24 virus was potent but non-selective. Error bars indicate standard deviation. N=4 for each experimental condition.

Figure 4. In vitro qualitative cell viability

Crystal violet staining was used to analyze the cells remaining alive following viral infection with the 5/3 CB016, 5/3 Δ24, 5/3-CMV-Luc (non-replicating control), and Ad5 Wt (replicating control) vectors at a titer of 100 vp/cell. One HPV-negative (SCC15) and three HPV-positive (93VU147T, UPCI SCC 090, UM047) HNSCC cell lines were used. Staining was performed at days 3, 6, and 9 following viral infection. N=3 and a representative plate is shown in the figure.

Figure 5. Quantitative cell viability

The cytotoxic effect of the 5/3 CB016 and 5/3 Δ24 viruses were tested in two HPV-positive HNSCC cell lines (93VU147T, UPCI SCC 090) and one HPV-negative HNSCC cell line (SCC15). The Ad5 Wt virus was a replicating control vector. Gradually increasing doses of virus were used (1, 10, and 100 vp/cell), and the percentages of living cells were quantified using the MTS assay at days 3 and 7 after viral infection. Results are shown as a percentage of living cells. Error bars indicate standard deviation. N=3 for each experimental condition.

Figure 6. Anti-tumor effect of a single adenovirus injection

Subcutaneous tumors of UPCI SCC 090 (HPV-positive HNSCC cell line) in nude mice were treated with a single intratumoral injection of adenovirus (3.5×10^{10} vp/tumor). At early time points, the 5/3 CB016 and 5/3 Δ 24 groups demonstrated a moderate therapeutic effect but it was not sustained at later time points. Tumor size is shown as a relative volume compared to day 0. Error bars indicate standard deviation. N=6 tumors in each experimental group.

Figure 7. Improved In vivo therapeutic effect of multiple injections

Subcutaneous tumors (established with the UPCI SCC 090 HPV-positive HNSCC cell line) in female nude mice were treated with multiple intratumoral injection of adenovirus (3.5×10^{11} vp/tumor). Injections began on day 0 and were every 4 days for a total of four injections. Both the 5/3 CB016 and 5/3 Δ24 groups significantly outperformed the control animals treated with PBS (* $p < 0.05$). The figure inset highlights the tumor regression achieved with the 5/3 Δ24 virus. Tumor size is shown as a relative volume compared to day 0. Error bars indicate standard deviation. N=7 tumors in each experimental group.