**p16<sup>INK4A</sup> Adenovirus-mediated Gene Therapy for Human Head and Neck Squamous Cell Cancer**

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**ABSTRACT**

Inactivation of the tumor suppressor gene p16<sup>INK4A</sup> is the most common genetic alteration in human head and neck squamous cell cancer (HNSCC), making it an ideal target for gene replacement. We constructed a replication-defective, recombinant adenovirus capable of targeting p16<sup>INK4A</sup> protein expression (Ad5-p16) to investigate its benefit in treating HNSCC. Initial in vitro experiments in four human HNSCC cell lines demonstrated that Ad5-p16 treatment significantly inhibits cell growth with up to 96% efficiency. Flow cytometric analysis showed that Ad5-p16 induced a maximum G<sub>1</sub>-S cell cycle arrest of 90%. Subsequent in vivo studies in a nude mouse model demonstrated that Ad5-p16 treatment significantly reduced (cell line 011) or stabilized (cell line 012) established tumors when compared with control treatments (P < 0.008). These results demonstrate for the first time a significant antitumor effect of Ad5-p16 against human HNSCC in vivo and support the potential application of Ad5-p16 to treat locally advanced, unresectable, or metastatic head and neck cancer, as well as microscopic residual disease after surgical resection.

**INTRODUCTION**

Worldwide, there are approximately 500,000 new cases of HNSCC each year, with over 50,000 of these cases occurring here in the United States (1). Standard therapies for advanced HNSCC include radical surgical procedures with adjuvant radiotherapy and the possible use of chemotherapy in selected cases. Surgical resection frequently results in significant cosmetic deformity, along with functional deficits in speech, swallowing, and upper extremity strength. Radiation and chemotherapy are not innocuous and also bring substantial morbidity including: mandible and laryngeal cartilage radionecrosis, tissue fibrosis, mucosal atrophy, and xerostomia, as well as gastrointestinal, bone marrow, renal, and ototoxicity (2). Unfortunately, despite the widespread use of these aggressive therapeutic modalities, the overall rate of survival for patients with HNSCC has failed to significantly improve over the last 30 years. Most studies still report overall survival rates of ~45%, with 2-year survival rates of 30% or less for patients with advanced stage III or IV disease (3).

As a consequence of these poor outcomes in advanced HNSCC, new treatment strategies based on the therapeutic application of genes are being investigated. Because most treatment failures in HNSCC patients tend to be local-regional, with less than 10% of patients succumbing to distant metastasis alone (4), these patients may be ideal candidates for direct gene transfer to persistent or recurrent disease. Promising gene therapy strategies to date have been founded on the use of highly efficient adenovirus vectors to deliver therapeutic genes to advanced HNSCC. The herpes virus thymidine kinase gene (suicide gene therapy) and the tumor suppressor gene p53 are the two most widely studied therapies in preclinical studies (5-9).

The tumor suppressor gene p16<sup>INK4A</sup> is also a strong candidate HNSCC gene therapy for several reasons: (a) inactivation of p16<sup>INK4A</sup> is the most frequent genetic alteration in human HNSCC, with over 80% of primary HNSCCs inactivating p16<sup>INK4A</sup> through deletion, mutation, or promoter methylation (10); (b) the transfection of plasmid vectors containing p16<sup>INK4A</sup> into numerous tumor cell lines (11), including HNSCC cell lines (12), has led to both growth suppression and G<sub>1</sub>-S cell cycle arrest; (c) recombinant p16<sup>INK4A</sup> adenoviruses have been shown to inhibit in vitro and in vivo tumor cell proliferation in cell lines derived from human lung (13) and prostate carcinomas (14), as well as suppressing glioma (15) invasion in vitro.

The p16<sup>INK4A</sup> gene encodes for a 156-amino acid protein that was initially identified as a specific inhibitor of the CDKs, CDK4 and CDK6 (16). CDK4 and CDK6 are the major catalytic partners of cyclins D1, D2, and D3 and regulate progression through the G<sub>1</sub>-S transition of the cell cycle by assisting in the cyclin D-dependent phosphorylation of the retinoblastoma susceptibility gene product, pRb (17). Phosphorylation of pRb is equivalent to functional inactivation and results in the release of transcription factors essential for S-phase progression (18, 19). Thus, by binding to and inhibiting CDK4 and CDK6, p16<sup>INK4A</sup> prevents both pRb phosphorylation and subsequent progression into the S phase of the cell cycle (20).

In a previous study, we transfected plasmids containing full-length p16<sup>INK4A</sup> cDNA under the control of a CMV promoter into three well-characterized HNSCC cell lines and dem-
Adenovirus-mediated Gene Therapy for Human HNSCC

As a therapeutic agent against HNSCC, we needed to overcome the low transfection efficiencies inherent in plasmid-based transfection techniques. We subsequently constructed a replication-defective recombinant adenovirus, Ad5-p16, to take advantage of the high transduction efficiency of the adenovirus system. Major advantages of the recombinant adenovirus system are: its well-documented ability to infect multiple cell types; its capability to infect nonproliferating cells; and the ease in which a recombinant adenovirus can be produced at the high titers needed for in vivo use. To directly examine the therapeutic potential of p16INK4A against human HNSCC, we treated four cell lines. Ad5-p16 was able to significantly arrest tumor cell growth both in vitro and in vivo, regardless of the status of the endogenous p16INK4A gene, demonstrating that effective in vivo treatment of human HNSCC in nude mice can be achieved using adenovirus-mediated transfer of the human p16INK4A gene.

MATERIALS AND METHODS

Cell Lines. Primary tumor explants were derived from squamous cell carcinomas of the head and neck from patients at the Johns Hopkins Hospital Department of Otolaryngology (cell lines 011, 012, 022, and 029) and have been well characterized by both double-stranded DNA sequencing and Western blot analysis (Table 1). All four human HNSCC cell lines were maintained in RPMI 1640 with 10% fetal bovine serum and 1% penicillin/streptomycin. Cell line 293 (American Type Culture Collection) was maintained in DMEM (Life Technologies, Inc.). All four human HNSCC cell lines were treated with Ad5-p16 stocks were determined by plaque assays on 293 cells using standard methods (22). The recombinant adenovirus Ad5-lacZ (gift from Dr. Frank Graham, McMaster University, Hamilton, Ontario, Canada) carries the lacZ gene from Escherichia coli under the control of the strong CMV immediate-early gene promoter and is similar to Ad5-p16.

Western Blotting. Confirmation of p16INK4A protein expression after Ad5-p16 infection was performed in all four HNSCC cell lines. Duplicate samples of log phase cells were seeded at a density of 5 × 10^5 in 35-mm culture dishes 24 h before treatment. Cells were either mock-treated with PBS or treated with the viruses Ad5-lacZ or Ad5-p16 as indicated. Cell monolayers were incubated with 0.5 ml of media containing virus at a concentration of 5 PFU/cell for 1 h at 37°C before adding an additional 2.5 ml of media and continuing the incubation for an additional 23 h. The virus-containing medium was removed by aspiration, and cells were incubated in 2.0 ml of fresh media and maintained at 37°C. Forty-eight h after treatment, the medium was removed, and the cell monolayers were washed with PBS at room temperature. Cell monolayers were then lysed by adding 0.2 ml of ice-cold RIPA buffer containing 100 μg/ml of phenylmethylsulfonyl fluoride, followed by sonication for 3 s to reduce viscosity. The resulting cell lysate was than incubated on ice for 1 h before centrifuging at 15,000 × g to remove cell debris. The cell lysate was then cleared through centrifugation at 14,000 rpm for 15 min. Cell lysates were then transferred to a fresh tube, and the protein concentration was determined by the Bio-Rad protein assay. A 0.1 ml of sample was loaded onto each lane and subjected to electrophoresis on a 4%–20% gradient gel according to the manufacturer’s protocol (Bio-Rad). Following electrophoresis, the proteins on the gel were transferred to nitrocellulose according to the manufacturer’s protocol (Bio-Rad). The membranes were incubated with primary antibodies against p16INK4A and GAPDH (Amersham, Piscataway, NJ). The membranes were then washed and incubated with secondary antibodies conjugated to horseradish peroxidase. The proteins were visualized using the ECL detection system (Amersham). Protein bands were quantified using the Gel-Pro Analyzer software (Media Cybernetics, Silver Spring, MD).

Table 1. Characterization of HNSCC cell lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tissue origin</th>
<th>p16</th>
<th>p53</th>
<th>Rb</th>
</tr>
</thead>
<tbody>
<tr>
<td>011</td>
<td>Larynx</td>
<td>WT*</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>012</td>
<td>Oral cavity</td>
<td>Meth*</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>022</td>
<td>Larynx</td>
<td>WT*</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>029</td>
<td>Larynx</td>
<td>HD</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

* Sequence is wild-type (WT) with low but detectable endogenous protein by Western blotting.
* Hypermethylated promoter region that fails to make detectable endogenous p16INK4A protein by Western blotting.
* HD, homozygous deleted.
for 20 min at 4°C. The resulting supernatant (total cell lysate) was stored at −80°C. Cell lysates representing 5 × 10^5 cells were separated on 16.5% tricine gels as described (24). Protein was electrophoretically transferred to Immobilon-P membranes (Millipore, Danvers, MA) by semidry blotting. Nonspecific binding sites were blocked by incubating the membrane in Tris-buffered saline with 0.1% Tween (TBS-T) and 5% nonfat dried milk for 1 h at room temperature. Primary antibody incubations were carried out in TBS-T using a murine antibody against human p16INK4A (p16INK4A Ab-1; Neomarkers, Fremont, CA) diluted 1:1000 for 1 h at room temperature. After washing with TBS-T, secondary antibody incubations were carried out using a horseradish peroxidase-conjugated goat antibody against murine IgG diluted to a concentration of 1:1000 for 1 h at room temperature. Subsequent protein detection was performed using the enhanced chemiluminescence system (Amersham Corp., Arlington, IL).

**Growth Curves.** The effect of p16INK4A expression on cell growth was examined in all four HNSCC cell lines. Triplicate samples of log phase cells were seeded at a density of 1 × 10^5 in 35-mm^2 culture dishes 24 h before infection. Cells were either mock-treated with PBS or treated with the viruses Ad5-lacZ or Ad5-p16 as indicated. Treatment consisted of incubating the cell monolayers with 0.5 ml of media containing purified virus at a concentration of 5 PFU/cell for 1 h at 37°C before adding an additional 2.5 ml of media and continuing the incubation for an additional 23 h. The virus-containing media was than aspirated, and cells were incubated in 2.0 ml of fresh media and maintained at 37°C for an additional 24 h before harvesting. Cells were harvested by centrifugation at 800 rpm for 10 min, followed by two washes in ice-cold PBS. Cells were then fixed in 2.0 ml of 70% ethanol and stored at 4°C for a minimum of 1 h. Prior to FACS analysis, cells were washed twice with ice-cold PBS, and the cell pellet was resuspended in 10 μg/ml of propidium iodide (Sigma Chemical Co.) and 100 μg/ml of RNase (Sigma Chemical Co.) and incubated at 37°C for 30 min. FACS analysis was done on a Becton Dickinson FACScan using an excitation laser of 480 nm and detection light at 575 nm. Data analysis was done by ModFit LT software.

**In Vivo Tumor Treatment.** The therapeutic potential of Ad5-p16 was studied in athymic, nude mice (BALB/c nu/nu). All animal experiments were performed using sterile technique under a laminar flow hood. Nude mice, ages 6–10 weeks, were anesthetized by i.p. injection of 0.7 ml of avertin at a concentration of 20 mg/ml. Using a 100-μl syringe and 26-gauge needle, a 75-μl solution containing 1 × 10^7 of 011 or 012 HNSCC cells in PBS was slowly injected s.c. into the dorsal flanks of nude mice.

For adenovirus injection, the nude mice were anesthetized as before, and the tumors were exposed by surgical elevation of a s.c. flap. After measuring the tumors in three dimensions with calipers, each tumor was carefully injected with 5 × 10^8 PFU of Ad5-lacZ or Ad5-p16 virus in 50 μl of PBS or 50 μl of PBS alone using a 25-gauge needle and microliter syringe. The actual delivery of adenovirus or PBS was performed with four separate needle passes, two parallel to the long axis of the tumor and two perpendicular to this axis. Skin incisions were closed with 4-0 silk (Ethicon). Additional control and Ad5-p16 injections of 5 × 10^8 PFU were performed on treatment days 3 and 5 in a similar manner. Total treatment was 1.5 × 10^9 PFU/tumor.

The mice were sacrificed 21 days after tumor implantation, and the lesions were carefully excised. Tumor masses were measured immediately after excision using calipers as described previously. The PBS and Ad5-lacZ treatments served as controls for both technical and nonspecific adenovirus affects. The change in tumor volume was calculated by subtracting pretreatment tumor volumes from posttreatment values. Statistical significance was assessed by Mann-Whitney statistical analysis (Statmost for Windows).
RESULTS

Gene Transfer Efficiency and p16INK4A Expression in Vitro. All four HNSCC cell lines growing in monolayers were stained with X-Gal after Ad5-lacZ treatment for 48 h to determine adenoviral gene transfer efficiency. X-Gal staining showed that 90–95% of the cells in all four HNSCC cell lines stained positive when a multiplicity of infection of 5 PFU/cell was used (data not shown). Use of higher PFU resulted in only minimal gains in gene transfer efficiency.

The ability of Ad5-p16 to direct a high level of p16INK4A expression was confirmed in all four cell lines by Western blot analysis. As shown in Fig. 1, high expression of p16INK4A was detected in all cells treated with Ad5-p16 (Lane 3 for all cell lines) but not in the PBS (Lane 1) or Ad5-lacZ (Lane 2)-treated cells. Cell lines 011 and 022, which are wild-type for J61NK4A, only demonstrated endogenous p16INK4A expression when the total cell lysate loaded was significantly increased to >75 μg/lane (data not shown), and most likely reflects the low abundance of p16INK4A protein in cell lines that are wild-type for pRb (12, 25, 26).

Ad5-p16 Inhibition of Human HNSCC in Vitro. The effect of p16INK4A expression on cell growth was examined in the same four HNSCC cell lines. Triplicate sets of mock (PBS) and treated cells (Ad5-lacZ or Ad5-p16 at 5 PFU/cell) were counted for 5 consecutive days after treatment, and the mean cell number for each day was calculated. As shown in Fig. 2, in vitro growth rates of cell lines 011, 012, 022, and 029 after Ad5-p16 treatment were significantly inhibited by 77.4, 89.3, 96.7, and 89.2%, respectively, when compared with the growth rate of Ad5-lacZ-treated cells. At the virus concentration used (5 PFU/cell), minimal toxicity due to expressed viral proteins or lacZ gene expression was noted in any of the cell lines. The status of the endogenous p16INK4A gene had no relation to the effect of Ad5-p16 treatment on cell growth, as demonstrated by the similar results seen in cell lines containing wild-type p16INK4A (011 and 022) and those containing inactivated p16INK4A (012 and 029).

Ad5-p16 Induces a G1-S Cell Cycle Arrest. The underlying mechanism of in vitro growth inhibition seen in the Ad5-p16-treated cells was investigated by cell cycle analysis using flow cytometry. Cell lines 011 and 012 in log phase growth were treated with PBS, Ad5-lacZ, or Ad5-p16 as indicated. As shown in Fig. 3, treatment with Ad5-p16 resulted in a significant increase in the percentage of cells in G0-G1 in both cell lines (>88%), consistent with a cell cycle arrest at the G1-S transition. This data confirmed the results of our earlier study, which demonstrated that increased expression of p16INK4A can block tumor cell entry into the S phase (12). The high percentage of cells seen at the G1-S transition reflects the high efficiency of gene transfer seen with the use of an adenovirus system. Of note, treatment with Ad5-lacZ did not result in a significant change in any cell cycle parameters when compared
Fig. 3  Treatment of two of our HNSCC cell lines with Ad5-p16 induces almost total G1-S cell cycle arrest. The effect of p16<sup>INK4a</sup> expression on cell cycle dynamics was examined in both the 011 and 012 HNSCC cell lines by flow cytometric analysis of Ad5-p16-treated cells. Triplicate samples of log phase cells were seeded at a density of 5 × 10<sup>5</sup> in 35-mm<sup>2</sup> culture dishes 24 h before treatment. Cells were either mock-treated with PBS or treated with the viruses Ad5-lacZ or Ad5-p16 at a concentration of 5 PFU/cell for 48 h as indicated. A, representative flow cytometric data 48 h after treatment in the human HNSCC cell line 012. B, results of triplicate samples for both the 011 and 012 cell lines. Note that the increase in the percentage of cells in G<sub>0</sub>-G<sub>1</sub>, in AD5-p16-treated cells is coincident with a decrease in the percentage of cells in S and G<sub>2</sub>-M. The height of each data bar represents the mean percentage of cells in that phase of the cell cycle; bars, SD.
with PBS-treated cells, effectively controlling for nonspecific viral effects (Fig. 3A, middle panel). Three representative flow cytometry analysis output curves of cell line 012 treated with PBS, Ad5-lacZ, or Ad5-p16 are shown in Fig. 3A. The tabulated results for both cell lines are presented in Fig. 3B.

**Ad5-p16 Treatment of Established Tumors.** The therapeutic potential of Ad5-p16 to treat tumors in vivo was studied in BALB/c nu/nu mice. Tumors were created by injecting $1 \times 10^7$ of 011 or 012 HNSCC cells in 50 µl of PBS into the s.c. dorsal flanks of nude mice as described. The range of mean pretreatment tumor volumes for the three treatment groups were 25.5–42.0 mm$^3$ for cell line 011 and 51.3–78.7 mm$^3$ for cell line 012. Tumors from both cell lines were treated 10 days after implantation by direct intratumoral injection of PBS (four tumors), Ad5-lacZ (six tumors), or Ad5-p16 (six tumors). Each tumor was injected on three alternate days with $5 \times 10^8$ PFU for a total of $1.5 \times 10^9$ PFU/tumor. The PBS and Ad5-lacZ treatments served as controls for both technical and nonspecific adeno virus affects. As shown in the box-whisker plots of Fig. 4, the PBS and Ad5-lacZ-treated tumors from both cell lines experienced significant growth after treatment. This was in direct contrast to the results seen with Ad5-p16 treatment, where in the 011 cell line, established tumors decreased to 57.6% of pretreatment size, whereas tumors from cell line 012 exhibited a 57.3% reduction in growth rate. Mann-Whitney statistical analysis demonstrated the therapeutic effect of Ad5-p16 treatment to be significant in both the 011 ($P = 0.008$) and 012 ($P = 0.014$) cell lines when compared with PBS and Ad5-lacZ controls. The tumor volume differences between PBS and Ad5-lacZ-treated cells were not significant ($P > 0.05$) for both cell lines.

**DISCUSSION**

The hypothesis that the inactivation or loss of certain genes, specifically tumor suppressor genes, leads to both tumor growth and progression is now well established and provides a unique opportunity in cancer gene therapy. The decision to use p16$^{INK4A}$ as a therapeutic agent against human HNSCC was based on our earlier in vitro work where we had transfected a p16$^{INK4A}$ cDNA plasmid into three well-characterized human HNSCC cell lines and noted a marked in vitro growth inhibition through a G1-S cell cycle arrest (12). Because of these promising initial results, we constructed, purified, and characterized a replication-defective adenovirus that expressed high levels of the tumor suppressor protein p16$^{INK4A}$. We also successfully introduced and applied a number of effective quality control measures to ensure the production of a high-titer, wild-type, contaminant-free (23) recombinant adenovirus for both in vitro use and possible later application in human clinical trials.

Initial in vitro experiments demonstrated that the reintroduction of p16$^{INK4A}$ with Ad5-p16 significantly inhibited cell growth by inducing a G1-S cell cycle arrest in the four HNSCC cell lines examined. Subsequent in vivo studies demonstrated that Ad5-p16 treatment of established HNSCC tumors in nude mice resulted in either a reduction in tumor volumes, suggesting cell death (cell line 011), or stabilization of tumor volumes, suggesting growth arrest (cell line 012). A number of studies have demonstrated that xenografts in nude mice accurately predict clinical outcome (27). However, the relationship between growth arrest and cell death is becoming increasingly complex (28), and Ad5-p16-treated tumors that have decreased in size may or may not have undergone the extended G1-S growth arrest seen in vitro. Future studies using assays that distinguish between long-term growth arrest versus cell death...
may allow the different in vivo therapeutic responses to Ad5-p16 treatment to be characterized (28).

All four of our HNSCC cell lines responded to Ad5-p16 treatment. The consistency of this response was a direct consequence of the wild-type Rb status of the four cell lines studied (Table 1). Only 12% of primary HNSCC tumors have been shown to display a loss of Rb expression (29), whereas greater than 80% exhibit a loss of p16INK4A expression (10). Furthermore, these two events tend to be mutually exclusive and are most likely a consequence of a lack of selection to inactivate multiple components along the Rb pathway during tumor evolution (30). Consequently, greater than 80% of human HNSCCs may be susceptible to Ad5-p16 treatment.

The construction of a recombinant adenovirus capable of directing a high level of p16INK4A protein expression, will allow the therapeutic efficacy of Ad5-p16 as a potential gene therapy agent against human HNSCC to be examined. Our recombinant adenovirus was easily purified to the high viral titers (>1012/ml) needed for in vivo and clinical use (31). Future investigations to enhance the overall treatment efficacy of Ad5-p16 will focus on enhancing adenovirus delivery within the tumor, the use of new adenoviral vectors, and possible combination therapy with cooperating tumor suppressor genes. Specifically, recent studies have demonstrated bystander killing of nontransduced cells by transduced cells (5-7) and significant tumor regression with adenoviral vectors, and possible combination therapy with cooperating tumor suppressor genes. Our data support the use of adenovirus-mediated p16INK4A treatment for HNSCC and provide an important foundation from which to build future preclinical studies and initiate future human clinical trials. If Ad5-p16 proves to be effective in the clinical setting, it will offer a much needed addition to present therapeutic regimens. Ad5-p16 may potentially develop into an important adjuvant therapy, useful in treating unresectable HNSCC or microscopic residual disease after surgical resection (34).

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