Adenovirus-mediated Wild-Type p53 Gene Transfer as a Surgical Adjuvant in Advanced Head and Neck Cancers

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ABSTRACT
A high incidence of locoregional failure contributes to the poor overall survival rate of around 50% for patients with squamous cell carcinoma of the head and neck (SCCHN). In vitro and in vivo preclinical work with adenovirus-mediated wild-type p53 gene transfer using the recombinant p53 adenovirus (Ad-p53) has shown its promise as a novel intervention strategy for SCCHN. These data have translated into Phase I and Phase II studies of Ad-p53 gene transfer in patients with advanced, locoregionally recurrent SCCHN. The safety and overall patient tolerance of Ad-p53 has been demonstrated. Of 15 resectable but historically noncurable patients in the surgical arm of a Phase I study, 4 patients (27%) remain free of disease, with a median follow-up time of 18.25 months. Surgical and gene transfer-related morbidities were minimal. These results provide preliminary support for the use of Ad-p53 gene transfer as a surgical adjuvant in patients with advanced SCCHN. The implications of our findings for the management of SCCHN in general are discussed.

INTRODUCTION
The treatment of advanced primary human SCCHN in the upper aerodigestive tract remains a major therapeutic challenge, despite advances in surgical and radiotherapeutic techniques. Locoregionally recurrent disease, which has a particularly dismal prognosis and few meaningful treatment options, remains the principal cause of death among patients with advanced SCCHN. In addition, it has been shown that detection of clonal specific p53 mutations at tumor margins in SCCHN is a predictor of local recurrence. These molecular pathological advances suggest that despite adjuvant radiotherapy, residual disease (microscopic as well as histologically normal but genotypically abnormal) is a major problem in the treatment of patients with SCCHN. Our interest in developing new treatment strategies for SCCHN is generated by the humbling overall survival rate of approximately 50%, which has not changed over the last several decades.

Mutation of the p53 tumor suppressor gene is one of the most common genetic alterations in human malignancy. Approximately 60% of human tumors are thought to possess mutation at the p53 locus. Transient overexpression of the wild-type p53 gene in various malignancies has been considered a potential molecular intervention strategy. This strategy is based on the role that wild-type p53 plays as a tumor suppressor gene and an inducer of cell cycle arrest and apoptosis. Our laboratory has focused on the potential of wild-type p53 gene transfer as a strategy for the selective induction of apoptosis in SCCHN. The recombinant adenovirus Ad-p53 has been used as the gene delivery tool in all of our preclinical studies. The tropism of the adenovirus for tissues of the upper aerodigestive tract, the ability to produce the adenovirus in high titers, and the efficiency of adenovirus-mediated gene transfer have made this vector an attractive tool for transient gene delivery.

In our preclinical studies with Ad-p53, transduction of wild-type p53 into several different SCCHN cell lines induced apoptosis without adversely affecting normal cells. We have also shown that Ad-p53 reduces the growth of established tumors in xenograft models of SCCHN. Additionally, we have demonstrated that in a nude mouse xenograft model of microscopic residual disease, Ad-p53 can prevent the establishment of tumors from subcutaneously deposited SCCHN cell lines in a dose-dependent fashion.

In our recently completed Phase I clinical trial of Ad-p53 gene transfer in patients with advanced locoregionally recurrent SCCHN who were unsuccessfully treated with conventional therapy including radiotherapy, two treatment arms were established. Our previous report demonstrated the feasibility and tolerance of Ad-p53 administered to patients with nonresectable disease and to patients who could be surgically treated but were historically deemed incurable; tissue vector biodistribution was evaluated in this publication as well. In this current focused analysis with longer patient follow-up, we report the potential antitumor activity and complications of Ad-p53 in a surgical adjuvant setting, based on our Phase I experience.

MATERIALS AND METHODS

Study Subjects. Of the 33 total patients entered into the Phase I study, 15 patients with advanced locoregionally recur-
rent or refractory SCCHN were placed into the surgical treatment arm. These 15 patients are the subjects of this report. For this report, we also examined biopsy samples of tumor margin and untreated adjacent normal tissues from a representative nonsurgical patient for evidence of apoptosis as well as expression of the wild-type p53 and p21\textsuperscript{wild} gene products.

Patients typically had multiple treatments for either refractory or locoregionally recurrent disease before study entry (Table 1).

### Table 1: Demographics of surgical treatment arm study participants

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (yr)</th>
<th>Sex</th>
<th>p53 genotype</th>
<th>Index tumor site</th>
<th>Prior treatments related to index tumor</th>
<th>Recurrent tumor site</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>31</td>
<td>F</td>
<td>Mutant</td>
<td>Tongue, floor of mouth</td>
<td>1. laser exc. L. tongue\textsuperscript{a}</td>
<td>Left face and neck</td>
</tr>
<tr>
<td>2</td>
<td>58</td>
<td>M</td>
<td>Mutant</td>
<td>Supraglottic larynx</td>
<td>1. supraglottic laryngectomy</td>
<td>Submental and submaxillary area</td>
</tr>
<tr>
<td>3</td>
<td>72</td>
<td>M</td>
<td>WT</td>
<td>Unknown primary</td>
<td>1. L. MRND</td>
<td>Left neck</td>
</tr>
<tr>
<td>4</td>
<td>46</td>
<td>M</td>
<td>Mutant</td>
<td>Tongue base</td>
<td>1. XRT to L. and R. necks and tongue base (6600 cGy)</td>
<td>Tongue base</td>
</tr>
<tr>
<td>5</td>
<td>58</td>
<td>M</td>
<td>Mutant</td>
<td>Larynx</td>
<td>1. wide field laryngectomy</td>
<td>Hypopharynx</td>
</tr>
<tr>
<td>6</td>
<td>48</td>
<td>M</td>
<td>Mutant</td>
<td>Tongue base</td>
<td>1. XRT to tongue base, chemotherapy</td>
<td>Right superior larynx</td>
</tr>
<tr>
<td>7</td>
<td>76</td>
<td>F</td>
<td>Mutant</td>
<td>Supraglottic larynx</td>
<td>1. XRT to larynx, retropharyngeal and subdigastric nodes</td>
<td>Left supraclavicular area</td>
</tr>
<tr>
<td>8</td>
<td>53</td>
<td>M</td>
<td>WT</td>
<td>Unknown primary</td>
<td>1. R. RND</td>
<td>Right submentum</td>
</tr>
<tr>
<td>9</td>
<td>56</td>
<td>M</td>
<td>Mutant</td>
<td>Larynx</td>
<td>1. verticle hemilaryngectomy</td>
<td>Neopharynx and peristomal region</td>
</tr>
<tr>
<td>10</td>
<td>49</td>
<td>F</td>
<td>NE</td>
<td>Left oral tongue</td>
<td>1. wide local exc. L. tongue</td>
<td>Tongue</td>
</tr>
<tr>
<td>11</td>
<td>68</td>
<td>M</td>
<td>Mutant</td>
<td>Left tonsil</td>
<td>1. XRT to L. tonsil, L. upper neck, L. supraclavicular fossa (7000 cGy)</td>
<td>Left tonsil</td>
</tr>
<tr>
<td>12</td>
<td>37</td>
<td>M</td>
<td>Mutant</td>
<td>Left oral tongue</td>
<td>1. hemiglossecotomy, 2. re-excison L. tongue, 3. XRT to tongue (6400 cGy)</td>
<td>Left tongue base</td>
</tr>
<tr>
<td>13</td>
<td>34</td>
<td>F</td>
<td>NE</td>
<td>Floor of mouth and submentum</td>
<td>1. wide local exc. tongue and floor of mouth, L. MRND</td>
<td>Left tongue and floor of mouth</td>
</tr>
<tr>
<td>14</td>
<td>56</td>
<td>M</td>
<td>Mutant</td>
<td>Right hypopharynx</td>
<td>1. 2 cycles of 5FU and cisplatin</td>
<td>Right hypopharynx</td>
</tr>
<tr>
<td>15</td>
<td>73</td>
<td>F</td>
<td>Mutant</td>
<td>Left buccal mucosa</td>
<td>1. exc. L. buccal lesion</td>
<td>Left buccal mucosa</td>
</tr>
</tbody>
</table>

\textsuperscript{a} L., left; R., right; exc., excision; XRT, radiation therapy; CDDP, cis-diamminedichloroplatinum; 5FU, 5-fluorouracil; MRND, modified radical neck dissection; RND, radical neck dissection; WT, wild-type; NE, could not be evaluated. Recurrent tumor site refers to the recurrent lesion that was treated in this Phase I trial.
ble 1). All patients had previously received radiotherapy at some point during their treatment. Entry into the surgical treatment arm required only that the tumor could be resected to microscopic residual disease (without resection of the internal carotid artery), but resection offered little or no opportunity for cure as determined by the Multidisciplinary Head and Neck Oncology Treatment Planning Committee at The University of Texas M. D. Anderson Cancer Center. There were 10 males and 5 females, with a mean patient age of 54.3 years. Tumor p53 genotype was analyzed by direct sequencing for each patient, although a mutant genotype was not a prerequisite for study entry. Patients were required to practice contraception while in the study, and women of child-bearing age had to have negative pregnancy tests. A detailed description of the 15 subjects can be found in Table 1. The study was reviewed and approved by the Institutional Surveillance Committee of The University of Texas M. D. Anderson Cancer Center, the NIH Recombinant DNA Advisory Committee, and the Food and Drug Administration. Informed consent was obtained from all patients before study entry, with emphasis placed upon the investigational nature of the study and the absence of therapeutic intent.

Recombinant Adenovirus. The recombinant adenovirus Ad-p53 was used to directly introduce the wild-type p53 gene into all subjects. Preparation of the recombinant adenovirus was described previously (18). Ad-p53, also designated as INGN201, is a replication-defective adenovirus serotype 5 vector with a cytomegalovirus-promoted p53 cDNA insert replacing the E1 region of the vector. Ad-p53 is a BL-2 agent and was handled with the appropriate level of biological containment. Ad-p53 was produced by Magenta, Inc. (now MA Biosciences, Rockville, MD) and Introgen Therapeutics (Houston, TX) and stored at −80°C at concentrations of 2 × 10^10 to 3 × 10^10 pfu/ml in PBS supplemented with 10% glycerol. Ad-p53 was thawed and diluted in PBS at 4°C within 2 h of use.

Administration of Ad-p53. All Ad-p53 was administered on an inpatient basis under strict aseptic conditions. Ad-p53 was delivered to sites of disease recurrence only. There were three Ad-p53 intervention approaches/patient: (a) preoperative; (b) intraoperative; and (c) postoperative.

Ad-p53 was given in escalating doses to determine a maximum tolerated dose for this treatment strategy. The Ad-p53 dose did not vary throughout each patient’s treatment (Table 2). Doses started at 1 × 10^6 pfu and were increased in log increments until 1 × 10^9 pfu was reached and then increased in one-half log increments until 1 × 10^11 pfu was reached.

The preoperative Ad-p53 administration consisted of direct tumor injections given three times weekly for 2 consecutive weeks (six treatments overall). The preoperative injection volumes were based on the estimated volume of the injected mass and the number of injection sites. Ad-p53 was administered using 27-gauge needles and 3–10-ml syringes, depending on the volume injected. Ad-p53 was injected directly into tumors by inserting the needle to the tumor depth and injecting upon withdrawal. Ad-p53 was diluted in a volume of PBS concordant with the number of tumor injections to be performed. Generally, we injected about 0.5 ml of vector solution at 1-cm (surface area) tumor increments. Thus, a very large tumor required the appropriate amount of vector to be diluted in a larger volume of PBS. Tumor maps were generated depicting the injection sites so that these areas could be reinjected. A typical tumor map for a recurrent oral tongue lesion is shown in Fig. 1.

Seventy-two h after the last Ad-p53 intratumoral injection, patients had their surgery. At the time of surgery, after total gross tumor removal and just before closure, another dose of Ad-p53 (diluted to 10 ml in PBS) was delivered by injection to the surgical sites where microscopic residual disease was presumed to be present, including mucosal margins of the resected neoplasms (Fig. 2). A small amount of this dose was saved and administered liberally (a vector wash) to the tumor bed via a syringe and left in contact for 60 min before wound closure.

Seventy-two h after surgery, the patients received the final Ad-p53 administration (again diluted to 10 ml in PBS) via retrograde instillation through wound catheters that had been placed intraoperatively in the areas of presumed microscopic residual disease. Clamps were used to prevent efflux of the Ad-p53 for 1 h. The drains were removed 24–48 h after the postoperative instillation.

Statistical Analysis of Patient Outcome. Kaplan-Meier disease-specific survival and disease-free intervals were analyzed for all 15 patients entered into the surgical arm of the study. The time of study entry was the day of the first preoperative Ad-p53 administration. All patients were macroscopically free of disease after surgical resection.

Patient Monitoring. Because the treatment of patients with Ad-p53 was within the context of a Phase I clinical trial, diligent patient monitoring for the detection of untoward and toxic effects was obligatory. Surgical complications as well as potential Ad-p53-related toxic effects were recorded. Vital signs were recorded, performance status was evaluated, and chest X-rays and hematologic and blood chemistry testing were performed daily. Patients were closely monitored for 2 h after each Ad-p53 administration.

Detection of Wild-Type p53 and p21 Waf1 Gene Product Expression and Apoptosis. Biopsy samples taken from the tumor margins of a representative nonsurgical patient were analyzed 48 h after Ad-p53 delivery (10^6 pfu) to the tumor. This immunohistochemical analysis examined the expression of the wild-type p53 gene product and the gene product of the downstream p53-transactivated gene p21 Waf1 (19) via an avidin-biotin-peroxidase complex method (20). The DO-1 anti-p53 mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and the anti-p21 Waf1 mouse monoclonal antibody (Oncogene, Uniondale, NY) were used for all p53 and p21 Waf1 immunohistochemical studies, respectively. Standard H&E staining as well as TdT end-labeling to detect apoptotic cells were performed on similarly prepared tumor margin biopsy samples 48 h after Ad-p53 delivery to the tumor. TdT end-labeling was performed with the ApoTag Plus kit (Oncor, Gaithersburg, MD) according to the manufacturer’s instructions. All of the these studies were matched with biopsy samples taken from adjacent un.injected grossly normal tissues of the same patient 48 h after Ad-p53 delivery to the tumor.

RESULTS

Patient Outcome. The Kaplan-Meier disease-specific survival curve for the patients enrolled in the surgical arm of the
Phase I Ad-p53 clinical trial is shown in Fig. 3. Median survival was 12.4 months. Currently, four patients are alive with no evidence of disease (at 29.1, 23.8, 11.5, and 12.7 months). One patient is alive with disease (at 13.1 months), and eight patients have died of disease. Two patients died of unrelated causes (at 13.4 and 4.8 months). The current disease status of each study participant is shown in Table 2.

The median disease-free interval was 3.9 months for the nine patients enrolled in the surgical treatment arm whose disease recurred. The four patients without evidence of disease (disease free at 29.1, 23.8, 11.5, and 12.7 months) and the two patients who were without evidence of disease at the time of death (at 13.4 and 4.8 months) were not included in this calculation.

Table 2  Ad-p53 and surgical treatment and related complications of surgical treatment arm study participants

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Ad-p53 dose per treatment</th>
<th>Adenovirus complications</th>
<th>Surgical treatment of recurrence</th>
<th>Surgical complications</th>
<th>Current disease status</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>6</td>
<td>Headache with first preoperative injection</td>
<td>Left maxillectomy and mandibulectomy, bilateral NDs, total laryngectomy, partial pharyngectomy, latisimus free flap and bilateral pectoralis flap reconstruction</td>
<td>Postoperative fever with positive blood cultures for Staphylococcus</td>
<td>DOD</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>Erythema at preoperative injection site</td>
<td>Total glossectomy, total pharyngectomy, bilateral NDs, resection anterior neck skin, right pectoralis flap reconstruction</td>
<td>Intraoperative bradycardia and atrial flutter, electrolyte imbalance</td>
<td>DOD</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>None</td>
<td>Left extended RND, left pectoralis flap reconstruction</td>
<td>None</td>
<td>NED</td>
</tr>
<tr>
<td>4</td>
<td>7</td>
<td>Pain with preoperative injections</td>
<td>Total laryngectomy, total glossectomy, right MRND, marginal mandibulectomy, left verteicle rectus myocutaneous flap reconstruction</td>
<td>None</td>
<td>DOD</td>
</tr>
<tr>
<td>5</td>
<td>8</td>
<td>None</td>
<td>Total pharyngectomy, cervical esophagectomy, completion thyroidectomy, bilateral MRND, mediastinal nodal dissection, free jejunum reconstruction</td>
<td>Anemia, electrolyte imbalance, confusion, fever, mild respiratory insufficiency, acute renal insufficiency</td>
<td>NED</td>
</tr>
<tr>
<td>6</td>
<td>8</td>
<td>None</td>
<td>Total laryngectomy, right MRND, left RND, partial pharyngectomy, subtotal thyroidectomy, total glossectomy, free rectus flap and pectoralis flap reconstruction</td>
<td>Anemia, electrolyte imbalance, acute renal insufficiency</td>
<td>DOD</td>
</tr>
<tr>
<td>7</td>
<td>9</td>
<td>Tenderness at preoperative injection site</td>
<td>Left extended RND, pectoralis flap reconstruction</td>
<td>Anemia, electrolyte imbalance, aspiration pneumonia</td>
<td>DOC</td>
</tr>
<tr>
<td>8</td>
<td>9.5</td>
<td>Fever during early preoperative injections, pain with preoperative injections</td>
<td>Right ND</td>
<td>None</td>
<td>DOD</td>
</tr>
<tr>
<td>9</td>
<td>10.5</td>
<td>Sore throat, increased dysphagia, sinus congestion, and headache during preoperative injections</td>
<td>Total pharyngectomy, total thyroidectomy, bilateral MRND, free jejunum reconstruction</td>
<td>Anemia, electrolyte imbalance, ascites, hypothyroidism, pneumatosis intestinalis</td>
<td>DOD</td>
</tr>
<tr>
<td>10</td>
<td>10.5</td>
<td>Fever, sore throat, headache, and increased odynaphagia and dysphagia during preoperative injections</td>
<td>Partial glossectomy, hemimandibulectomy, left MRND, free fibula osseocutaneous flap reconstruction</td>
<td>Anemia, electrolyte imbalance, hypertension, pneumonia, delayed cervical wound healing</td>
<td>DOD</td>
</tr>
<tr>
<td>11</td>
<td>10.5</td>
<td>Fever during preoperative injections, and pain associated with injections</td>
<td>Left partial pharyngectomy, left partial mandibulectomy, left RND, right verteicle rectus free flap reconstruction</td>
<td>Anemia, electrolyte imbalance, pleural effusion, pneumonia and respiratory failure, hypertension, cellulitis left neck</td>
<td>DOC</td>
</tr>
<tr>
<td>12</td>
<td>11</td>
<td>Pain after preoperative injections, throat swelling after first preoperative injection</td>
<td>Total glossectomy, total laryngectomy, partial pharyngectomy, partial mandibulectomy, bilateral ND, free transverse rectus abdominis flap reconstruction</td>
<td>Anemia, electrolyte imbalance and hypovolemia, fever</td>
<td>DOD</td>
</tr>
</tbody>
</table>
The known role of p53 as a tumor suppressor gene and an inducer of cell cycle arrest and apoptosis in mammalian cells (6, 13–16), as well as our encouraging preclinical in vitro and in vivo animal findings with Ad-p53 in SCCHN (7–9), made this an attractive treatment strategy.

As indicated earlier, the Phase I study of patients with
advanced locoregionally recurrent SCCHN revealed that Ad-p53 gene transfer is safe and well tolerated (17). Furthermore, in the current analysis, apoptosis and expression of the wild-type p53 and p21\textsuperscript{Waf1} (a downstream p53-transactivated gene) gene products were demonstrated in tumor margin biopsy samples taken from a representative nonsurgical patient after Ad-p53 delivery. The findings with regard to median survival in the surgical arm of the study (Ad-p53 delivered as an adjuvant to surgical therapy) prompted the current report, although our sample size was small, and thus the results should not be overinterpreted. The median survival for these patients (12.4 months) was about 60% longer than that found in chemotherapy trials for similar patients (21). Furthermore, the median disease-free interval of 3.9 months among those patients whose disease recurred suggests that this trial was not preselecting a favorable patient population. The observations made with regard to potential antitumor activity among patients with resectable tumors is encouraging as we proceed with the international Phase II evaluation of Ad-p53 gene transfer in patients with SCCHN. Recurrence rates and mortality are higher in patients with molecular evidence of residual disease (as determined by PCR-based assay of p53 mutation) at tumor margins (1, 2). Thus, the use of Ad-p53 as an adjuvant modality in surgical wound beds may lower those rates.

There are several implications of our findings. Given the low toxicity of Ad-p53, this agent may be applied as an adjuvant therapy after primary definitive treatment of advanced lesions (or early lesions), as indicated above. Furthermore, Ad-p53 gene transfer may be efficacious in dysplastic lesions because p53 mutations have been found in head and neck premalignancies.
Finally, Ad-p53 gene therapy may be applied in combination with radiotherapy or chemotherapy because enhanced antitumor activity has been seen in such combination treatment models in preclinical studies (23, 24).

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REFERENCES
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