Pretreatment p53 Protein Expression Correlates with Decreased Survival in Patients with End-Stage Head and Neck Cancer

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ABSTRACT
Relatively little is known about p53 changes in far-advanced head and neck cancer for several reasons: (a) most patients respond well to initial treatment; (b) most institutions do not encounter large numbers of these patients; (c) recurrent or metastatic disease is often within body cavities inaccessible for analysis; and (d) the variety of treatment regimens and disease sites makes meaningful conclusions difficult to draw in such a heterogeneous group. The purpose of this study was to evaluate the clinical significance of p53 mutations and overexpression in a homogeneous group of patients with end-stage squamous cell carcinoma of the head and neck.

Pretreatment tumor specimens from a homogeneous group of 16 patients with end-stage squamous cell carcinoma of the head and neck were obtained. All patients had recurred after surgery and radiation ± induction chemotherapy, and all met the criteria for enrollment in a Phase II chemotherapy trial consisting of 5-fluorouracil, N-phosphoacetyl-L-aspartate, and recombinant IFN-α. Each was analyzed for mutations in exons 5–8 of the p53 gene and protein expression using the p53 polyclonal antibody CM-1.

No relationship was found between p53 immunostain or p53 mutations and age, gender, site of primary tumor, or site of disease recurrence. p53 alterations also did not correlate with response to Phase II chemotherapy. p53 immunostain (but not p53 mutations) correlated with a shorter survival (P = 0.0124) after diagnosis with end-stage disease. This suggests that mechanisms other than p53 mutations which alter the half-life of p53 protein may contribute to the outcome of these patients.

INTRODUCTION
While it is clear that p53 alterations occur early (1, 2) in the development of SCCHN, there is controversy in the literature regarding the prognostic role of p53 in these tumors. Most studies have concluded that there is no correlation between p53 expression and disease progression (3) or survival (4) in patients with SCCHN, although at least one study did find a correlation between p53 mutations and disease progression (2). Exceptions to the general lack of correlation with survival are two studies which evaluated patients with advanced tongue base tumors (5) and patients with end-stage disease (6), with conflicting results. Over half of adult cancers, including lung, breast, colon, esophagus, and skin cancers contain p53 mutations (7), and increased p53 expression is one of the most common genetic features in a variety of human malignancies. The p53 gene product functions as a transcription factor (4), and has a central role in cell cycle regulation (8) and apoptosis (9).

p53 alterations may influence treatment response since wt p53 is required for G1 arrest following ionizing radiation (10), wt p53 protein has been shown to repress the activity of the human MDR1 gene promoter in vitro, and mutant p53 protein has a stimulatory effect on MDR1 activity (11). Overexpression of the MDR1 gene product, a Mr 170,000 transmembrane glycoprotein (P-glycoprotein), has been associated with drug resistance in a variety of malignancies (12). The purpose of this study was to evaluate the influence of p53 mutations and p53 protein expression on response to treatment and on survival in patients with incurable SCCHN who had already received standard therapy and were enrolled in a Phase II trial using combination chemotherapy and biological modifiers.

PATIENTS AND METHODS
Patient Characteristics. Formalin-fixed, paraffin-embedded tumor tissue was analyzed from 16 patients (9 males and 7 females) with distant metastases or uncontrolled local or regional tumor. These patients were enrolled between 1991 and 1994 in a Phase II trial of combination chemotherapy consisting of 5-FU (2600 mg/m²/week by 24-h continuous infusion), PALA (250 mg/m²/week by rapid i.v. infusion), and rIFN-α (10 million units × 3/week by s.c. injection) administered weekly until evidence of disease progression or intolerable toxicity ensued.

Information was available regarding gender, tumor stage, site of initial disease, and site of first recurrence (Table 1). All patients underwent surgery and radiation prior to enrollment in the trial. Pretreatment tissue from the primary disease site was available for analysis in all patients. Comparison tumor from a local or regional recurrence was available in five patients before and in five patients (four different, one overlap) after treatment with 5-FU/PALA/rIFN-α chemotherapy. To be considered...
evaluate for response to and toxicity of 5-FU/PALA/IFN-α chemotherapy, a patient had to receive at least 6 weeks of therapy and satisfy the response criteria as outlined: complete remission, disappearance of all clinical evidence of tumor by scan and physical examination for a minimum of 4 weeks; partial remission, 50% or greater decrease in tumor size lasting a minimum of 6 weeks, with no new tumors appearing; stable disease, <50% decrease or <25% increase in tumor size for at least 8 weeks, with no new tumors; and progressive disease, increase in tumor size of at least 25%.

DNA Extraction from Fixed Tissue. DNA was extracted from formalin-fixed tissue blocks as previously described (13), with slight modifications. After confirmation of tumor location through hematoxylin and eosin tissue staining, tumor tissue was microdissected from surrounding stroma with a scalpel and placed in xylene followed by rehydration in graded alcohols to water. The tissue was then boiled for 10 min, and a suspension of Chelex 100 ion exchange resin was added (Bio-Rad Laboratories, Melville, NY) followed by proteinase K (0.4 μg/μl), boiling for 8 min, and centrifugation.

PCR-SSCP Analysis. Nested primers (14) were used to perform two sets of PCR reactions for exons 5 to 8 of the p53 gene to obtain more efficient DNA amplification. In the first set, 4 μl extracted DNA, 2.8 μl deoxynucleotide triphosphates (1.25 mM of each deoxynucleotide triphosphate), 1 μl primer mix (each primer 20 μM), 0.25 μl (5 units/μl) AmpliTaq DNA polymerase (Perkin Elmer/Cetus, Norwalk, CT), 1 μl 10X PCR buffer, and 10.9 μl water were used to make a 20-μl first reaction mixture. Samples were overlaid with 20 μl mineral oil. Eight PCR cycles of melting at 94°C for 1.6 min, annealing at 37°C for 1.6 min, and extension at 72°C for 1.6 min were then performed. For the second set of cycles, 2 μl template DNA from the first set of cycles, 2.5 μl primer mix (each primer 4 μM), 0.20 μl AmpliTaq DNA polymerase, 0.25 μl (10 μCi/μl) [α-32P]dCTP (DuPont New England Nuclear Products, Wilmington, DE), 1 μl 10X PCR buffer, and 2.55 μl water were used to make a 10-liter reaction mixture. Thirty-five cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min were then performed. The resulting DNA solution was diluted 1:12 in 0.1% SDS-10 mM EDTA and then mixed 1:1 with a solution containing 20 mM EDTA, 95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol. These samples were heated at 95°C for 5 min, chilled on ice, and 3 μl immediately loaded onto gels. For exons 5 to 7, 0.089 mM Tris-0.089 mM borate-0.002 mM EDTA-10% glycerol-6% polyacrylamide gel was used and run at 3 W overnight. Autoradiography was performed at 24 h at −70°C with an intensifying screen (Fig. 1). Tumor DNA known to have a p53 mutation in the evaluated exon and a water blank substituting for DNA were used as positive and negative controls.

The proportion of p53-positive samples was compared between groups using Fisher’s exact test and survival curves generated using log rank analysis. Phase II chemotherapy did not significantly alter patient survival. Thus, although a minority of patients did not fit the protocol criteria for evaluation of chemotherapeutic response and toxicity, all 16 were considered evaluable for survival.

RESULTS

Eleven of 16 patients completed the chemotherapeutic regimen. Five patients received less than full treatment either due to toxicity or early death. There were no complete responses and two partial responses to treatment. Neither the ability to evaluate response nor evidence of response significantly altered patient survival. There were no treatment-related deaths. All patient mortality was due to progressive head and neck cancer or its sequelae.
In pretreatment tissue, 7 (44%) of 16 specimens had increased p53 protein expression, and p53 mutations were identified in 8 (50%) of 16 samples (Table I). Mutations were identified in each of the four exons analyzed. No correlation was found between either p53 expression or p53 mutations and gender, site of primary tumor, tumor stage, site of first disease recurrence (Table 1), or age (data not shown). There was concordance between p53 mutations and protein expression in 11 (69%) of 16 cases. In five patients with tissue available before and after standard radiation/chemotherapy but prior to Phase II chemotherapy, protein/mutation analysis was the same in three patients and different in two patients. In one patient, a p53 mutation and increased p53 overexpression were identified after but not before treatment; in the second patient, the reverse occurred, with decreased expression/absence of a mutation post-treatment.

In five patients with specimens available both before standard radiation/chemotherapy and after receiving 5-FU/PALA/rIFN-α chemotherapy, p53 protein/mutation analysis was the same in two patients and different in three patients: in two of these patients, a mutation found before treatment was not identified in the postchemotherapy specimen, while in the third patient a p53 mutation and increased p53 expression were identified after Phase II chemotherapy but not before treatment.

There was no correlation between either pretreatment p53 mutations or increased protein expression and survival calculated from the time of initial diagnosis (Table 2). On the other hand, when survival was calculated from the time of diagnosis with end-stage disease (Table 2 and Fig. 3), increased p53 protein expression (but not p53 mutations) correlated with a poor outcome ($P = 0.0124$).

**DISCUSSION**

Patients with end-stage head and neck cancer have few options. They have failed standard therapy, and further treatment using radiation, surgery, or standard chemotherapy is either inadvisable or inevitably futile. Such patients may be offered investigational chemotherapy. To date, no studies have demonstrated a survival benefit with this approach. All of the patients enrolled in this study meet these criteria.

Studying the tumors of patients with far-advanced head and neck cancer is more difficult than investigating those with early or newly diagnosed disease for a variety of reasons. By the time most patients with far-advanced disease are evaluated, their primary tumors have been treated and residual or recurrent tumor is either intertwined with scar or otherwise not readily accessible to biopsy. Tissue is generally obtained only if confirmation of disease recurrence or persistence is essential, and is
was believed to have diagnosis, there was a significant correlation between increased patients with end-stage SCCHN who met the criteria for enrollment into a chemotherapy trial. To analyze a relatively homogeneous population, we chose patients with end-stage disease who were heterogeneous in their disease stage at presentation; (b) the regimen of treatment administered; (c) the response to therapy; and (d) the time course to end-stage SCCHN. Thus, the most consistent element in the cancer of these individuals is that they all developed end-stage SCCHN. That p53 immunostain, but not p53 mutations, correlated with a poor prognosis suggests either that mutations occurred outside the exons analyzed and therefore were not detected, or that factors other than gene mutations are important to the behavior of recurrent SCCHN. The former proposition is unlikely, since 98% of p53 mutations are found in tumors expressing a mutant ras protein (SV40 T antigen, Elb protein of adenovirus, HPV E6) and host proteins (mdm 2). Accumulation of wt p53 in cells has been reported in tumors expressing a mutant ras oncogene (15). Unlike wt p53, some mutant p53 proteins are able to form usually accomplished by the least invasive means, yielding small samples for analysis. Thus, it is difficult to obtain specimens for research. In addition, these patients have been treated with radiation and or chemotherapy, either of which may alter the target gene to be analyzed, obscuring spontaneous genetic events that might otherwise have led to tumor progression.

As a result, most p53 analysis in SCCHN has been performed at the time of initial diagnosis. To avoid the problems outlined above, we chose to focus on tumor tissue obtained at diagnosis of the primary tumor, before treatment with radiation or chemotherapy commenced. To analyze a relatively homogeneous population, we chose patients with end-stage disease who were eligible for a Phase II chemotherapy trial.

Field et al. (6) studied the role of p53 abnormalities in 24 patients with end-stage SCCHN who met the criteria for enrollment into a chemotherapy trial. Their evaluation using immunohistochemistry alone demonstrated that, while p53 immunostain did not correlate with survival from the time of initial diagnosis, there was a significant correlation between increased p53 protein expression and progression to death after the patient was believed to have "end-stage disease." They found no correlation between p53 staining and age, sex, site of primary tumor, or site of recurrence.

Our findings in a similar patient population expand on their results. As is characteristic of patients with far-advanced head and neck tumors, the individuals whose tumors we evaluated were heterogeneous in (a) their disease stage at presentation; (b) the regimen of treatment administered; (c) the response to therapy; and (d) the time course to end-stage SCCHN. Thus, the most consistent element in the cancer of these individuals is that they all developed end-stage SCCHN. The p53 immunostain, but not p53 mutations, correlated with a poor prognosis suggests either that mutations occurred outside the exons analyzed and therefore were not detected, or that factors other than gene mutations are important to the behavior of recurrent SCCHN. The former proposition is unlikely, since 98% of p53 mutations are found in exons 5–8 (7). In addition to inactivation by mutant p53 protein, wt p53 can be inactivated (3) by binding to viral proteins (SV40 T antigen, Elb protein of adenovirus, HPV E6) and host proteins (mdm 2). Accumulation of wt p53 in cells has been reported in tumors expressing a mutant ras oncogene (15). Unlike wt p53, some mutant p53 proteins are able to form

Table 1 Clinical and tumor characteristics in 16 patients with end-stage head and neck cancer

<table>
<thead>
<tr>
<th>Patient</th>
<th>Sex</th>
<th>Primary</th>
<th>Initial stage</th>
<th>Recurrence</th>
<th>Prior treatment</th>
<th>Response</th>
<th>Survival (mo)</th>
<th>IHC</th>
<th>SSCP</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>Larynx</td>
<td>III</td>
<td>Local only</td>
<td>S + XRT</td>
<td>NE</td>
<td>1, DOD</td>
<td></td>
<td>Exon 6</td>
</tr>
<tr>
<td>2</td>
<td>F</td>
<td>Larynx</td>
<td>III</td>
<td>Locoregional</td>
<td>S + XRT</td>
<td>SD</td>
<td>10, DOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>Pharynx</td>
<td>III</td>
<td>dis</td>
<td>S + XRT + CMT</td>
<td>NE</td>
<td>9, DOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>M</td>
<td>Larynx</td>
<td>III</td>
<td>Regional + dis</td>
<td>S + XRT + CMT</td>
<td>NE</td>
<td>1, DOD</td>
<td></td>
<td>Exon 7</td>
</tr>
<tr>
<td>5</td>
<td>F</td>
<td>Pharynx</td>
<td>III</td>
<td>Locoregional</td>
<td>S + XRT</td>
<td>PD</td>
<td>11, DOD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>F</td>
<td>Larynx</td>
<td>III</td>
<td>Local + dis</td>
<td>S + XRT</td>
<td>PD</td>
<td>7, DOD</td>
<td></td>
<td>Exon 7</td>
</tr>
<tr>
<td>7</td>
<td>M</td>
<td>Larynx</td>
<td>IV</td>
<td>Local only</td>
<td>S + XRT + CMT</td>
<td>NE</td>
<td>1, DOD</td>
<td></td>
<td>Exon 8</td>
</tr>
<tr>
<td>8</td>
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<td>III</td>
<td>Local only</td>
<td>S + XRT</td>
<td>SD</td>
<td>7, DOD</td>
<td></td>
<td>Exon 8</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>Oral</td>
<td>IV</td>
<td>Local only</td>
<td>S + XRT</td>
<td>PD</td>
<td>7, DOD</td>
<td></td>
<td>Exon 8</td>
</tr>
<tr>
<td>10</td>
<td>M</td>
<td>Larynx</td>
<td>IV</td>
<td>Locoregional</td>
<td>S + XRT + CMT</td>
<td>PR</td>
<td>22, AWD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>F</td>
<td>Oral</td>
<td>I</td>
<td>Local only</td>
<td>S + XRT + CMT</td>
<td>PD</td>
<td>21, DOD</td>
<td></td>
<td></td>
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<tr>
<td>12</td>
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<td>IV</td>
<td>Locoregional</td>
<td>S + XRT</td>
<td>PD</td>
<td>5, DOD</td>
<td></td>
<td>Exon 7</td>
</tr>
<tr>
<td>13</td>
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<td>III</td>
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<td>PD</td>
<td>2, DOD</td>
<td></td>
<td>Exon 5</td>
</tr>
<tr>
<td>14</td>
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<td>Larynx</td>
<td>IV</td>
<td>Regional</td>
<td>S + XRT + CMT</td>
<td>NE</td>
<td>11, AWD</td>
<td></td>
<td>Exon 8</td>
</tr>
<tr>
<td>15</td>
<td>F</td>
<td>Oral</td>
<td>II</td>
<td>Regional + dis</td>
<td>S + XRT</td>
<td>PR</td>
<td>14, AWD</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>M</td>
<td>Oral</td>
<td>III</td>
<td>Locoregional</td>
<td>S + XRT + CMT</td>
<td>SD</td>
<td>6, DOD</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*a* Surgery (S), radiation (XRT), and/or neoadjuvant chemotherapy (CMT) prior to phase II chemotherapy.

*b* Response (PR, partial response; SD, stable disease; PD, progressive disease; NE, not evaluable) to Phase II chemotherapy.

*C* DOD, dead of disease; AWD, alive with disease.

*d* IHC, immunohistochemistry; presence (+) or absence (−) of p53 overexpression in pretreatment tumor tissue.

*e* SSCP analysis of exons 5–8 of the p53 gene in pretreatment tumor tissue.

*f* M, male; F, female; dis, distant.

Table 2 Overall survival (months) in patients with and without p53 alterations in pretreatment tumor

<table>
<thead>
<tr>
<th>IHC</th>
<th>No. of patients</th>
<th>Mean</th>
<th>Med</th>
<th>Range</th>
<th>Mean</th>
<th>Med</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal p53</td>
<td>9</td>
<td>35.6</td>
<td>32</td>
<td>10-100</td>
<td>14.6</td>
<td>11</td>
<td>1-22</td>
</tr>
<tr>
<td>Altered p53</td>
<td>7</td>
<td>33</td>
<td>22</td>
<td>8-89</td>
<td>5.3</td>
<td>6</td>
<td>1-9</td>
</tr>
<tr>
<td>SSCP</td>
<td>Normal p53</td>
<td>9</td>
<td>35.9</td>
<td>35</td>
<td>10-100</td>
<td>10.7</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>Altered p53</td>
<td>7</td>
<td>32.1</td>
<td>22</td>
<td>8-89</td>
<td>6.1</td>
<td>7</td>
</tr>
</tbody>
</table>

*Med, median; IHC, immunohistochemistry.*
complexes (7) with a Mr 70,000 HSP70. Mutants that form a complex with HSP70 have a longer half-life than normal p53. Although mutant p53 proteins that do not complex with HSP70 may still have an extended half-life, they are more weakly transforming in vitro than those proteins that associate with HSP70 (16).

Disparity between p53 mutations and immunostain in 31% of tumors is consistent with the findings of others (15, 17). p53 immunostain is influenced both by p53 mutations and posttranslational events. A p53 mutation may result in a mutant protein with a prolonged half-life, a single base change which does not alter the protein, or the absence of detectable p53 protein due to a truncated protein or a p53 deletion. Posttranslational effects, including the binding of viral, host, or HSPs, can influence the half-life of both wt and mutant p53 proteins.

A lesser incidence of p53 immunostain but not p53 mutations in women was found which approached statistical significance ($P = 0.06$). Although the explanation for this is not clear, it is interesting that all three of the subjects with p53 mutations but negative p53 immunostain were women.

The fact that 5 of the 10 samples retested after either standard treatment or Phase II chemotherapy had different molecular and immunohistochemical results is noteworthy. Although most metastatic and recurrent tumors contain the same wt or altered codon in the p53 gene as is found in the primary tumor, p53 gene differences between the primary and the metastatic or recurrent tumor from the same patient have been seen both in tobacco-related (18) and other (19) tumors. Thus, at least two plausible reasons for the differences in our p53 results before and after treatment can be proposed. One possibility is that during tumor progression, an aggressive clone, differing in p53 sequence or protein expression from the predominant primary tumor clone, survived treatment and became the predominant clone in the tumor recurrence. An alternative possibility is that the perceived tumor recurrence was instead a second primary tumor.

In pretreatment tumor specimens, p53 protein expression but not p53 mutations correlated with a worse prognosis in patients with end-stage SCCHN after enrollment in a Phase II chemotherapy trial. This suggests that some posttranslational event altering the half-life of p53 is important in tumor behavior. This small series is the second to correlate increased p53 immunostain in end-stage SCCHN with poor survival and should prompt confirmation in a larger patient population, preferably in a prospective Phase II or Phase III trial.

REFERENCES
p53 in End-Stage SCCHN

Pretreatment p53 protein expression correlates with decreased survival in patients with end-stage head and neck cancer.


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