p16 and p53 Protein Expression as Prognostic Indicators of Survival and Disease Recurrence from Head and Neck Cancer

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ABSTRACT

Purpose: Markers of somatic mutation such as p16 and p53 remain controversial prognostic indicators for patients with squamous cell carcinoma of the head and neck (SCCHN). The relationship between p53 protein expression and radiation therapy is also unclear.

Experimental Design: We made a prospective cohort study (n = 171) of incident cases receiving standardized therapy for SCCHN.

Results: Patients whose tumors showed increased p53 protein expression had over twice the risk of all-cause mortality after 550 days [hazard ratio (HR), 2.7; 95% confidence interval (CI), 1.07–6.66] and three times the risk of dying from cancer-specific causes after 550 days [HR, 3.09; 95% CI, 1.15–8.30] after adjustment for age, therapy, and stage. Tumors demonstrating alteration of both p16 and p53 did not confer any additional diagnostic information over p53 alone. Patients whose tumors expressed increased levels of p53 protein and received radiation were almost three times more likely to die as compared with those who received radiation but whose tumors did not express increased p53 protein after adjustment for age and stage (HR, 2.6; 95% CI, 1.03–6.50).

Conclusions: p53 protein expression was found to violate the proportional hazards assumption for our cohort, which may explain the controversial prognostic ability of this protein in the literature. p53 protein expression, but not p16 protein expression, was related to poor survival in general for men and women. In addition, an interaction between p53 expression and radiation therapy was demonstrated. Additional studies are needed to confirm and extend our results.

INTRODUCTION

Twenty-five years of incidence and mortality data from the SEER registry system suggest that despite decreasing incidence of oral cavity, pharynx, and larynx cancer in the United States, mortality from head and neck cancer remains substantial (1). Five-year relative survival of 58% for whites and 34% for blacks diagnosed with oral cavity or pharynx cancer, and 66% for whites and 53% for blacks diagnosed with larynx cancer have been reported for 1992–1997 (1). Although these percentages reflect a slight improvement in survival for patients with head and neck cancer as compared with those diagnosed in 1973, the decrease in mortality is not as great as might be expected given the advances in medicine and surgery over the past quarter of a century.

Mortality trends are proportional to the number of newly diagnosed cases each year (incidence) and survival. Treatment is a component of survival, and treatment modalities such as radiation are not without morbidity. Identifying patients likely to be poor responders to treatment such as radiation might increase quality of life and shed light on the molecular biology of the cancer. Considerable interest lies, therefore, in classifying patients in relation to prognosis to guide treatment decisions. A genetic progression model has been proposed for SCCHN and researchers have used this model to focus their efforts on the evaluation of somatic tumor mutations of SCCHN in relation to prognosis.

One of the earliest known events in head and neck squamous cell carcinogenesis may occur at chromosome 9p21 (2–4). This chromosome contains the locus for two proteins, ARF and p16 (5). The p16 protein exerts a tumor suppressor function by binding to the cyclin D1 CDK4/CDK6 complex preventing phosphorylation of the retinoblastoma protein, resulting in G1 arrest (4). Inactivation of p16 may occur through several different mechanisms including homozygous deletion, methylation of the gene promoter with subsequent transcriptional silencing, and single bp mutation (6–8). The most frequent method of inactivation is homozygous deletion (7).

Only one study has evaluated p16 protein expression and survival among 148 patients with carcinoma of the anterior tongue (9). Using IHC to measure p16 protein expression, investigators found that decreased expression of p16 was pre-

The abbreviations used are: SEER, Surveillance, Epidemiology, and End Results; CI, confidence interval; DAB, 3,3′-diaminobenzidine; IHC, immunohistochemistry; SSCP, single-strand conformational polymorphism; HR, hazard ratio.
dictive of disease-free survival and overall survival after adjustment for stage, primary treatment, and cyclin D1 expression. HRs of 3.2 (95% CI, 1.65–7.50) for disease-free survival and 2.8 (95% CI, 1.27–6.06) for overall survival were estimated (9). No study has evaluated the interaction of p16 protein expression and treatment in relation to prognosis.

Loss of heterozygosity (LOH) at chromosome 17p13, the location of the locus for p53, has been suggested as a later event than LOH at 9p21 for SCCHN (2). Studies of genetic progression have suggested that p53 alteration occurs at greater frequency in invasive carcinomas than in noninvasive lesions (10). Several studies have demonstrated that ~40–70% of SCCHN contain mutations in exons 5–9 at the p53 locus (10–13). Wild-type p53 protein has numerous functions including gene transcription, DNA synthesis and repair, and apoptosis (14).

Despite its important role in carcinogenesis, p53 remains a controversial prognostic indicator for SCCHN. Using IHC methods, 16 studies of SCCHN have found p53 protein expression to be prognostic of survival (11, 15–29), whereas eleven have found no correlation with survival or disease recurrence (30–40). The relationship of p53 protein accumulation and response to radiation has been evaluated by only a few studies. Exposure of cells to ionizing radiation leads to rapid accumulation of wild-type p53 protein, and two studies have suggested that inactivation of the p53 locus leads to radioresistance in SCCHN (41, 42).

The purpose of this study was to examine the relationship of p16, p53, and tumors with abnormal expression of p16 and p53 in relationship to all-cause mortality, disease-specific mortality, and locoregional recurrence for a well-defined cohort of incident cases receiving standardized therapy. In addition, prognosis based on p16 and p53 protein expression as modified by radiation therapy was also examined.

PATIENTS AND METHODS

Subjects. The study cohort consisted of 190 consecutive incident cases of invasive SCCHN [International Classification of Diseases 9th edition (ICD-9) codes 141, 143–146.9, 148–149.9, and 161] diagnosed at University of North Carolina at Chapel Hill Memorial Hospital from April 1994 until June 1997. Nineteen (10%) patients had insufficient tumor tissue for IHC and were excluded from the analysis. Those excluded from the analysis were found to have no significant difference in prognosis compared with those included in the analysis. The primary tumor site of those included in the analysis was distributed as 80 oral cavity, 36 pharyngeal, and 55 larynx.

Enrollment of cohort members began in April 1994 and concluded in June 1997. All of the members were followed prospectively from the date of diagnosis until death or March 31, 1999, whichever came first. Locoregional recurrence was defined by the treating surgeon as the first recurrence of squamous carcinoma at the initial primary site. Follow-up data were collected on cohort members using a prospective longitudinal tracking system maintained by the UNC Hospitals and Lineberger Comprehensive Cancer Center. Vital status was verified using the National Death Index (NDI) Plus. Information on vital status, site, TNM stage [consistent with the American Joint Committee on Cancer (AJCC) 1992 system], lymph node status, disease recurrence, and treatment were collected and updated at least every 6 months from clinician’s notes, pathology reports, laboratory reports, and death certificates.

Primary therapy received was abstracted from medical records and defined as receiving surgery and radiation with intent to cure; surgery only with intent to cure; radiation only with intent to cure; and chemoradiation only with intent to cure.

IHC. IHC for p16 and p53 protein expression was assessed on 4-μm serial sections of formalin-fixed paraffin-embedded SCCHN. H&E slides from each tumor underwent pathological review, and blocks were then selected that best represented invasive squamous cell carcinoma for that tumor. All of the unstained tissue sections were stored at 4°C until used to minimize antigen deterioration.

Appropriate quality control and quality assurance procedures were implemented including positive and negative tissue controls run with each assay. SCCHN known to contain a mutation at the p53 loci as determined by sequencing, served as positive controls, and squamous epithelium of the tonsil served as a negative control for p53. Similarly, two SCCHN which had been sequenced and known to contain either a methylation or a deletion at the p16 loci served as positive controls, a tumor sequenced and known not to contain either a deletion or a methylation of the p16 promoter region served as a negative control.

All of the antibodies were used in an immunoperoxidase system incorporating the Vector Elite avidin-biotin horseradish peroxidase complex system. DAB was used as a chromogen, and the assays were performed using the DAKO Autostainer Universal staining system. This system provides for clean, high-quality resolution of immunostaining in a quality-controlled environment, while minimizing the quantity of antibody and other required reagents.

Steam antigen retrieval in citrate buffer (Citra; Biogenex) for 30 min was used for both p16 and p53 before primary antibody staining. Tissue sections were then quenched of endogenous peroxidase with methanol/hydrogen peroxide and blocked from nonspecific binding with normal horse serum.

For p53 expression, the DO7 clone (Oncogene Science; 1:1000 dilution) was added dropwise to tissue sections and allowed to incubate for 60 min at room temperature. For p16 expression, the p16INK4 mouse monoclonal antibody (Neomarkers; 1:60 dilution) was added dropwise and allowed to incubate overnight at 4°C in a humidified chamber. After washes with PBS, secondary antibody was added, followed by preformed avidin-biotin reagent. Localization of antigen was visualized using the chromogen DAB. Tissue sections were then counterstained in dilute hematoxylin, dehydrated, cleared in xylene, and mounted with permount.

IHC scoring was completed by three evaluators (S. A. G., K. V., W. K. F.), who underwent standardization training with a selection of tumor sections with a known sequencing status for p16 and p53. All of the scorers were blinded to the outcome and molecular status of each tumor specimen. At least 10 fields were examined under high power, and only nuclear staining was considered. For p16, low protein expression was considered positive if less than 1% of cells with nuclear staining were measured, and p53 protein overexpression was scored as positive if greater than or equal to 50% of cells with nuclear staining were measured.
Molecular Analysis for p16 and p53. Molecular analysis was carried out on DNA purified from 10-μm sections of fresh-frozen paraffin-embedded tumors on a random subset of tumors ($n = 15$) to validate IHC results. Tumor sections were subjected to pathological review in which tumor areas were identified and isolated, and DNA was extracted using a standardized protocol (43).

Tumors were screened for mutations in p53 exons 5–8 and p16 exon 2 (44). Gene amplification for p53 and p16 was carried out using the PCR according to standard methods. SSCP-PCR was performed on p53 exons 5, 6, 7, and 8 and p16 exon 2 using the first PCR products as a template. Negative control samples containing only wild-type p53 or p16 genes were included on each gel. In addition, all of the experiments were run using a blank control without template DNA, to rule out contamination.

Tumors showing altered SSCP bands were further analyzed as follows. The first PCR product or gel-purified abnormal SSCP bands were used as a template for asymmetric PCR to generate single forward and reverse DNA strands. Both forward and reverse asymmetric PCR products were purified and used as a template in sequencing reactions. Mutations were verified by repeating the entire sequencing analysis on a sample extract from tissue of suspected positive samples.

Statistical Analysis. Kaplan-Meier survival probability estimates and log-rank tests were used initially to estimate and compare the survival probabilities for p16, p53, clinical, and demographic variables in relation to each outcome. A set of potential predictors significant at $\alpha = 0.20$ for each outcome was developed using these methods.

Cox regression models were used to estimate HRs and 95% CIs for p16 and p53, adjusting for other covariates for each outcome. Variables were retained in the final model if they demonstrated a $P$ less than 0.05 based on the Wald $\chi^2$ statistic. The $-2 \log$ likelihood ratio statistic was checked after removal of each covariate to confirm that the variable was not significant in the model. In addition, $\beta$ coefficients for p16 or p53 were also assessed for a change of $\geq 20\%$ after the removal of adjustment variables.

Evaluation of the proportional hazards assumption for each potential predictor was evaluated by examining the plot of the log ($-\log$) survival function versus time. We also tested the interactions of all of the potential predictors with time in the Cox regression model. p53, but not p16, was found to violate the proportional hazards assumption for all-cause mortality and cancer-specific mortality.

An interaction variable with time was created for p53 based on data presented in Figs. 1 and 2. Stratification of time at 550 days (when the survival curves cross) for all-cause mortality and cancer-specific mortality was undertaken for the p53 variable. Age was treated as a continuous variable. Indicator variables were used to denote treatment, stage, and site. To incorporate possible interaction of p16 and p53 with radiation therapy, models stratified by radiation were evaluated for each somatic marker. Patients receiving chemoradiation were excluded from these models to assess the effects of p16, p53, and prognosis after radiation without chemotherapy. All of the $P$s presented are two sided. Statistical analysis was conducted using SAS Version 8.1 (SAS Institute, Cary, NC).

RESULTS

Follow-up time ranged from 37 days to 66 months with a median of 30 months. The estimated median overall survival of the 171 patients was 42 months. A total of 70 deaths occurred during the follow-up period, 60 of which were determined to be attributable to mortality associated with SCCN, whereas 10 were attributable to cardiovascular disease. Forty-four individ-
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with increasing tobacco pack-year history. Those with moderate alcohol consumption (1–19 drinks/week on average, over the course of the individual’s lifetime) had a slight protective effect for all-cause mortality.

Comparison of p16 IHC with molecular sequencing for a subset of 15 tumors demonstrated that IHC detected all homozygous deletions of the p16 gene. IHC for p53 detected all missense mutations. Two tumors containing deletions were scored as negative for p53 protein. Thus, for our data, we estimate ~13% misclassification of p53 molecular status using IHC as a proxy.

Unadjusted estimates for p16 revealed that loss of protein expression was not prognostic of mortality for our cohort as seen in Table 3. HRs of 1.2 (95% CI, 0.63–2.27) for all-cause mortality and 1.4 (95% CI, 0.67–2.97) for disease-specific mortality were estimated. After adjustment for age, stage, and therapy, HRs of 1.5 (95% CI, 0.78–2.91) for all-cause mortality, 2.0 (95% CI, 0.93–4.25) for disease-specific mortality, and 0.86 (95% CI, 0.42–1.79) for locoregional recurrence were esti-
estimated. The addition of nodal status to models that were also adjusted for therapy and age did not change estimated HRs significantly. HR estimates of 1.14 (95% CI, 0.47–2.76) for all-cause mortality, 1.03 (95% CI, 0.42–2.54) and 0.54 (95% CI, 0.15–1.91) for locoregional recurrence were calculated.

Results for the univariate analysis of p53 may also be seen in Table 3. Increased expression of p53 protein was prognostic of mortality. Unadjusted HRs of 1.8 (95% CI, 1.10–3.03) and 1.9 (95% CI, 1.09–3.20) were estimated for all-cause and disease-specific mortality, respectively. There was also a suggestion of a relationship to disease recurrence with a HR of 1.6 (95% CI, 0.83–2.89). The addition of p16 to tumor classification did not increase prognostic ability. Tumors with alteration of both p16 and p53 demonstrated a decrease in the estimate of effect as compared with p53 alone.

Figs. 1 and 2 demonstrate a crossing of the survivor function estimates for p53 at 550 days and suggest that patients whose tumors accumulated p53 protein have decreased probability of survival as compared with those patients whose tumors did not accumulate p53 protein. Final models are presented in Table 4. After adjustment for therapy, stage, and age, patients whose tumors expressed increased amounts of p53 protein as measured by IHC had no increase in risk of dying as compared with those with baseline expression for the first 550 days (HR, 0.92; 95% CI, 0.48–1.79). However, after 550 days, patients whose tumors expressed increased amount of p53 protein were almost three times more likely to die (HR, 2.7; 95% CI, 1.07–6.66) as compared with patients whose tumors did not accumulate p53 protein. Models adjusted for nodal status, age, and therapy did not yield a significantly different estimate of effect for p53 compared with models adjusted for stage, age, and therapy.

This trend was also seen when disease-specific death was examined. After adjustment for age, stage, and therapy, no difference in survival is seen for the first 550 days. However, patients whose tumors expressed increased p53 protein had three times the risk of dying (HR, 3.1; 95% CI, 1.15–8.30) as compared with those with baseline expression after surviving 550 days. Finally, no relationship was noted for p53 protein expression and locoregional recurrence (adjusted HR, 1.18; 95% CI, 0.56–2.47).

An interesting trend was noted for mortality, radiation therapy, and p53, as seen in Table 5 and Figs. 3 and 4. Excluding patients who received both chemotherapy and radiation (n = 30), patients whose tumors were positive for p53 protein and received radiation were almost three times more likely to die from all causes (HR, 2.6; 95% CI, 1.03–6.50) after adjustment for age and stage, whereas those who received radiation but...
whose tumors who were not positive for p53 protein did not have increased mortality (HR, 0.66; 95% CI, 0.23–1.86). There was also a slight increase in risk in locoregional recurrence among those who received radiation and whose tumors demonstrated increased p53 protein (HR, 1.4; 95% CI, 0.60–3.06).

Patients with decreased p16 protein expression who received radiation were less likely to develop disease recurrence (HR, 0.54; 95% CI, 0.24–1.18) as compared with those with baseline expression. Among those whose tumors had decreased p16 protein expression and who did not receive radiation therapy, it should be noted that there were no events of disease-specific mortality or locoregional recurrence.

**DISCUSSION**

Our findings suggest that the accumulation of p53 protein can serve a prognostic role for both all-cause mortality and disease-specific mortality for patients with SCCHN. Our study represents the largest case series to date that we are aware of, and issues related to statistical power and study design may partially explain why our results are in contrast to previously published reports.

Of the 27 studies of SCCHN previously published in this area, sample size ranged from 16 to 178 cases, with the average sample size of 82 cases. As a result, the bulk of the studies conducted have been statistically underpowered to detect differences between groups. The possible inclusion of prevalent, rather than incident cases, introduces an additional source of heterogeneity. Only one study stated explicitly that incident, consecutive cases were used for the analysis (40). Finally, several studies did not use multivariable techniques or time-to-event methodology to adjust for clinical factors strongly associated with prognosis (23, 36, 37, 39, 45).

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**Table 4** p53 protein and mortality

<table>
<thead>
<tr>
<th></th>
<th>All-cause mortality</th>
<th>Disease-specific mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Survival ≤550 days</td>
<td>Survival &gt;550 days</td>
</tr>
<tr>
<td>p53 protein</td>
<td>HR 95% CI</td>
<td>HR 95% CI</td>
</tr>
<tr>
<td>Negative</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Positive</td>
<td>0.92 0.48–1.79</td>
<td>2.67 1.07–6.66</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>0.88 0.53–1.88</td>
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<tr>
<td></td>
<td>0.88 0.53–1.88</td>
<td>3.09 1.15–8.30</td>
</tr>
</tbody>
</table>

**Table 5** Prognosis based on radiation and somatic marker status

<table>
<thead>
<tr>
<th></th>
<th>All-cause mortality</th>
<th>Disease-specific mortality</th>
<th>Locoregional recurrence</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>No radiation</td>
<td>Radiation</td>
<td>No radiation</td>
</tr>
<tr>
<td>p16 protein</td>
<td>HR 95% CI</td>
<td>HR 95% CI</td>
<td>HR 95% CI</td>
</tr>
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<td>1.0</td>
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<tr>
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<td>0.35 0.09–1.41</td>
<td>1.32 0.54–3.23</td>
<td>1.13 0.46–2.81</td>
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<tr>
<td>p53 protein</td>
<td>HR 95% CI</td>
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<tr>
<td>Positive</td>
<td>0.66 0.23–1.86</td>
<td>2.59 1.03–6.50</td>
<td>0.78 0.23–2.69</td>
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Fig. 3 Survivor function estimates for those age 60 years and with late-stage disease who received radiation by p53 protein expression: all-cause mortality.
those containing a mutation in exons 5–9. Of 101 cases, those whose tumors expressed increased p53 protein were almost four times more likely not to respond to radiation therapy as compared with patients whose tumors did not accumulate p53 protein (24). Another study of 73 patients enrolled in a randomized clinical trial demonstrated that p53 protein expression was prognostic of disease-free survival after adjustment for those receiving radiation and chemotherapy, but not predictive of response to radiation as measured by tumor progression (15).

The p16 locus has been shown to be inactivated in many human cancers such as lung (50), breast (51), melanoma (52), pancreatic (53), and brain (54) and in >80% of SCCHN tumors (7). Loss of p16 protein expression was not found to give any prognostic information related to all-cause mortality or disease-specific mortality for our cohort.

Only one other study of p16 and prognosis for SCCHN has been published, and the authors reported decreased p16 protein expression to have an association with survival (9). However, 55% of their tumor specimens demonstrated decreased p16 expression, which is closer to the expected proportion of alterations based on molecular analysis of all forms of gene alteration, including methylation of the promoter region, deletion, and point mutation (7).

Strengths of our study include the validation of IHC, verification of outcomes using a national database, and minimal losses to follow-up. Misclassification exists when using protein accumulation as a surrogate marker for mutational status of the p53 locus (35, 41). Reported misclassification for p53 mutational status when using IHC ranges from 33% false negatives to 30% false positives for breast cancer (55) and 28% false negatives to 49% false positives for SCCHN (35). Our study used standardization of IHC methods and scoring, as well as validation of IHC for underlying mutational status of the gene with only 13% misclassification of p53.

An accumulation of p53 protein has been shown to occur through other mechanisms besides mutation of the locus. Wild-type p53 protein may be stabilized by other proteins such as p300, poly(ADP-ribose) polymerase (PARP), Wilms’ tumor suppressor gene WT1, hypoxia-inducible factor 1α (14) and by viral exposures such as large T cell antigen SV40 and HPV type 16 (35). HPV infection has been suggested as a risk factor for SCCHN (56, 57). Thus, it may not be necessary to make the assumption that p53 protein accumulation represents an underlying genetic alteration.

We did not adjust for comorbid conditions in our cohort despite a recent study that has suggested that comorbid conditions such as cardiovascular disease and other cancers may play a role in prognosis for elderly men and women diagnosed with head and neck cancer (58). The decision to not adjust for comorbidity was based on two observations. First, the findings of Reid et al. (58) were estimated using SEER data. Individuals that increased p53 protein accumulation does increase risk of death among those who receive radiation therapy after adjustment for age and stage.

Nine studies have examined the relationship of p53 protein expression and prognosis among patients receiving radiation therapy (49). Only four of the studies used survival as an outcome, and among those only one study had more than 100 cases (49). Of 101 cases, those whose tumors expressed increased p53 protein were almost four times more likely not to respond to radiation therapy as compared with patients whose tumors did not accumulate p53 protein (24). Another study of 73 patients enrolled in a randomized clinical trial demonstrated that p53 protein expression was prognostic of disease-free survival after adjustment for those receiving radiation and chemotherapy, but not predictive of response to radiation as measured by tumor progression (15).
with other types of cancer or HIV/AIDS were included in the Reid cohort, whereas individuals were considered not eligible to participate in our study if they had other cancers or HIV/AIDS. Secondly, it has been suggested that atherosclerosis and cancer share common molecular pathways of disease development and progression (59). Adjusting for comorbid conditions such as cardiovascular disease may not be appropriate when studying the effect of acquired somatic mutations on survival because adjustment for casual intermediates can introduce bias in estimates of effect (60).

Although our study is among the largest, our estimates of the associations of somatic mutational status and treatment as related to prognosis are imprecise. The site of tumor had no relationship to any of our defined outcomes; however, an increase in sample size would allow for site-specific models, as well as an examination of the effects of chemoradiation, p53 and p16, and survival. In addition, our study would also be strengthened by the sequencing of all of the tumors rather than just a random subset.

Because mortality remains a significant burden for both those diagnosed with SCHHN and those administering treatment, future population-based studies are necessary to explore hypotheses related to somatic mutations and survival. Methodological issues related to time-varying effects of p53 protein expression and prognosis need to be evaluated among larger cohorts. In addition, studies examining the role of other somatic markers such as fragile histidine triad (FHIT) are indicated.

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