Prognostic Significance of p16 Protein Levels in Oropharyngeal Squamous Cell Cancer


Departments of 1Medical Oncology, 2Otolaryngology, 3Therapeutic Radiology, and 4Pathology Yale University School of Medicine, New Haven, Connecticut

ABSTRACT

Purpose: Functional inactivation of p16 is an early and frequent event in head and neck squamous cell cancers. In this study, we sought to determine whether p16 expression is of prognostic importance in oropharyngeal squamous cell carcinoma.

Experimental Design: p16 protein expression was evaluated by immunohistochemistry in a tissue microarray composed of 123 oropharyngeal squamous cell cancers with a mean patient follow-up time of 33 months.

Results: p16 overexpression was associated with more advanced Tumor-Node-Metastasis stage and higher histologic grade. Despite this association with unfavorable features, p16 overexpression was associated with decreased 5-year local recurrence rates (11 versus 53%) and increased 5-year disease-free survival (62 versus 19%) and overall survival (60 versus 21%). In multivariate analysis, p16 expression status remained an independent prognostic factor for local recurrence, disease-free survival, and overall survival.

Conclusions: In patients with oropharyngeal squamous cell carcinoma, overexpression of p16 as determined by immunohistochemistry is associated with significantly improved prognosis and lower local recurrence rates.

INTRODUCTION

Over 500,000 patients worldwide are diagnosed with head and neck squamous cell carcinoma (HNSCC) each year (1). Despite numerous advances in diagnosis and treatment over the last several decades, mortality rates have not changed significantly (2). Even cured patients suffer significant speech and swallowing impairment because of treatment-related morbidity. HNSCC are characterized by extensive heterogeneity at the histologic, biological, and clinical level. Therefore, considerable interest lies in classifying patients in relation to prognosis to guide treatment decisions.

HNSCC provides an ideal model for understanding carcinogenic progression due to defined risk factors (tobacco and alcohol consumption) and emerging molecular characteristics (3, 4). According to the molecular progression model for HNSCC, loss of 9p21-22 is the most frequent event and is also present in the earliest definable lesions, including dysplasia and carcinoma in situ (5). The p16 (CDKN2) protein resides within this region. Thus, it has been hypothesized that p16 is the candidate tumor suppressor gene within this critically deleted area (6). The p16 protein exerts a tumor suppressor function by binding to the cyclin D1 CDK4/CDK6 complex preventing phosphorylation of the retinoblastoma (Rb) protein (7). P105Rb and the Rb family members p107 and p130 in turn regulate the activity of E2F transcription factors (8, 9). Complexes consisting of E2F and hypophosphorylated p105Rb repress the transcription of genes such as cyclin A required for cell cycle progression, and repression is relieved by cyclin dependent kinase (CDK)-mediated phosphorylation of p105Rb (10). Thus, p16 is a CDK inhibitor, which, by inhibition of Rb phosphorylation, preserves the integrity of the G1-S checkpoint and also regulates the transcriptional program involved in cell proliferation. Although point mutation of p16 gene in HNSCC is rare, alternative mechanisms of abrogation of p16 function such as homozygous deletions and methylation of the 5'-CpG promoter region of p16 have frequently been identified, suggesting that functional inactivation of p16 is a common event (11–13). Studies have also suggested that loss of p16 function may be associated with tobacco use (14, 15).

As loss of p16 function is quite common in HNSCC and has important functional implications, we sought to determine whether p16 expression levels, as determined immunohistochemically, were associated with clinical outcome in a cohort of 123 oropharyngeal squamous cell cancer patients with a mean follow-up of 33 months. Our study demonstrates an independent prognostic value of p16 in determining patient outcome.

MATERIALS AND METHODS

Tissue Microarray Construction. The cohort was assembled from patients with either primary or recurrent oropharyngeal cancer of squamous cell histology treated at Yale-New Haven hospital between 1980 and 1999. Patients were treated with primary external beam radiotherapy (EBRT) or gross total surgical resection and postoperative radiotherapy. Exclusion criteria included presentation with metastatic disease or failure to receive a full course of radiation therapy. After Institutional Review Board approval, the tissue microarray was...
constructed as previously described (16), including 123 cases that met inclusion criteria. Tissue cores were obtained from paraffin-embedded, formalin-fixed tissue blocks from the Yale University Department of Pathology archives. Slides from all blocks were reviewed by a pathologist to select representative areas of invasive tumor to be cored. The cores were placed on the recipient microarray block using a Tissue Microarrayer (Beecher Instrument, Silver Spring, MD). All tumors were represented with 2-fold redundancy. Previous studies have demonstrated that the use of tissue microarrays containing one to two histospots provides a sufficiently representative sample for analysis by immunohistochemistry (17-19). Addition of a duplicate histospot, although not necessary, does provide marginally improved reliability. Cores from human papillomavirus (HPV) type 18-positive HeLa and HPV type 16-positive Caski cell lines fixed in formalin and embedded in paraffin were selected for positive controls and included in the array. The tissue microarray was then cut to yield 5-μm sections and placed on glass slides using an adhesive tape transfer system (Instrumedics, Inc., Hackensack, NJ) with UV cross-linking.

**Immunohistochemistry.** Tissue microarray slides were deparaffinized and stained using a commercially available kit (Dako p16INK4a kit; Dako Cytomation, Carpinteria CA). In brief, slides were deparaffinized in xylene followed by absolute ethanol and subsequent rehydration in graded 95 and 70% ethanol. After a brief rinse in deionized water, heat-induced antigen retrieval was performed using the included epitope retrieval solution at 95°C for 40 minutes. Endogenous peroxidase blocking was performed using the peroxidase blocking reagent supplied for 5 minutes at room temperature. The tissue microarray slide was subsequently incubated with the included mouse monoclonal antibody against p16 at a 1:25 dilution for 30 minutes at room temperature. The negative control slide was incubated with supplied negative control reagent (mouse monoclonal antibody to Aspergillus niger glucose oxidase) at a 1:25 dilution for 30 minutes at room temperature. Secondary amplification was accomplished using the included goat antimouse dextran polymer-conjugated horseradish peroxidase visualization reagent for 30 minutes at room temperature. The antibody was subsequently visualized using the supplied 3,3′-diaminobenzidine chromagen for 10 minutes at room temperature followed by acidified hematoxylin counterstain. Slides were mounted with Cytoseal 60 (Richard-Allan Scientific, Kalama-zoo, MI). p16 status was independently determined by two investigators (M. Harigopal and P. Weinberger) and was regarded as overexpression if it was strong and diffuse (≥80% of investigator (M. Harigopal and P. Weinberger) and was regarded as overexpression if it was strong and diffuse (≥80% of tumor cells) and was regarded as nonoverexpression if absent or weak.

**Statistical Analysis.** Histospots containing <10% of a tumor were excluded from additional analysis. Disease-free survival, overall survival, and local recurrence were assessed by Kaplan-Meier analysis with log-rank score for determining statistical significance. All survival analysis was performed at 5-year cutoffs. Relative risk was assessed by the univariate and multivariate Cox proportional hazards model. Comparison of p16 overexpression with the clinical and pathological variables gender, Tumor-Node-Metastasis (TNM) stage, histologic grade, tumor type (primary versus recurrent), treatment method (primary EBRT versus primary surgical excision plus radiotherapy), oropharyngeal subsite, and drinking and tobacco usage were made using χ² analysis. All calculations and analyses were performed with SPSS 11.5 for Windows (SPSS, Inc., Chicago, IL) and, where appropriate, were two-tailed.

**RESULTS**

**Clinical and Pathological Variable Analysis**

There were 123 patients in this cohort that met inclusion criteria. Tumors were primary 93 (76%) or recurrent 27 (22%). Sixteen (13%) of patients were TNM stage II, 39 (32%) stage III, and 68 (55%) stage IV. Oropharyngeal subsites included 48 (39%) tonsillar fossae, 64 (52%) base of tongue, and 11 (9%) other oropharynx. Seventy-three (59%) patients were managed with primary EBRT and 44 (36%) with surgical excision followed by EBRT. Twenty-two (18%) patients were also treated by chemotherapy. Demographic and clinicopathological variables for the cohort are summarized in Table 1.

**Immunohistochemistry for p16 Expression**

Of the oropharyngeal tumors meeting inclusion criteria, 120 of 123 (98%) were interpretable for p16 staining. Seven of eight cell-line positive controls were interpretable for p16 staining. Tumors displayed one of three distinct immunostaining phenotypes (Fig. 1): intense diffuse cytoplasmic and nuclear...
staining, weak diffuse cytoplasmic and nuclear staining, and absence of immunostaining. In keeping with previous investigators’ methods, p16 overexpression was defined as tumors that displayed intense diffuse cytoplasmic and nuclear staining in >80% of tumor cells. There were 24 of 120 (20%) tumors designated as p16 overexpressors, as were 7 of 7 (100%) cell line-positive controls. No staining was observed on any spots for the negative control reagent. Comparisons were made between clinical/pathological variables and p16 status by analysis. There was a trend for p16-overexpressing tumors to be associated with primary versus recurrent, as 22 of 24 (92%) of p16-overexpressing tumors were primary tumors, but this did not reach statistical significance (P = 0.055). p16 overexpression was associated with more advanced TNM stage, with 20 of 24 (83%) of p16-overexpressing tumors being stage IV (P = 0.002), p16 overexpression was also associated with higher histologic grade with 14 of 24 p16-overexpressing tumors being poorly differentiated (P = 0.001). There was no association between p16 status and the other clinical/pathological variables. These results are summarized in Table 2.

Survival Analysis

Local Recurrence. The expression status of p16 as determined by immunohistochemistry was evaluated for association with local recurrence using Kaplan-Meier survival analysis with log-rank statistic for determining significance. This analysis (Fig. 2A) demonstrated that p16 overexpression is associated with decreased 5-year local recurrence rates. Patients with p16 overexpression had a local recurrence rate of 11.1% compared with 53.0% for p16-nonoverexpressing patients (log rank = 8.0, P = 0.0046). Univariate Cox analysis revealed a hazard ratio for p16 nonoverexpressors of 6.1 (P = 0.013).

Disease-Free Survival. The expression status of p16 was also evaluated for association with disease-free survival. Kaplan-Meier survival analysis (Fig. 2B) demonstrated that p16 overexpression is associated with improved disease-free survival. Patients with p16 overexpression had a 5-year disease-free survival of 62.2% compared with 18.5% for p16 nonoverexpressors (log rank = 8.2, P = 0.0042). Univariate Cox analysis revealed a hazard ratio for p16 nonoverexpressing patients of 2.8 (P = 0.006).

Overall Survival

The expression status of p16 was also evaluated for association with overall survival. Kaplan-Meier survival analysis (Fig. 2C) demonstrated that p16 overexpression is associated with improved overall survival. Patients with p16 overexpression had a 5-year overall survival of 59.8% compared with
21.4% for p16 nonoverexpressors (log rank = 5.7, P = 0.0174). Univariate Cox analysis revealed a hazard ratio for p16-nonoverexpressing patients of 2.4 (P = 0.021). Results for univariate survival analysis of local recurrence, disease-free survival, and overall survival are summarized in Table 3.

Multivariate Analysis

Using the Cox proportional hazards model, we performed multivariate analysis to assess the independent predictive value of p16 expression for 5-year disease-free survival, overall survival, and local recurrence. The following prognostic variables were also included: tumor type (primary versus recurrent), management (primary EBRT versus surgery plus EBRT), TNM stage, and histologic grade. For disease-free survival, only p16 expression status remained an independent prognostic factor (P = 0.01). For local recurrence, both p16 status and management were independent prognostic factors (P = 0.029). For overall survival, both p16 status and TNM stage were independent prognostic factors (P = 0.038). The results for multivariate survival analysis are summarized in Table 4.

DISCUSSION

In this study, we found that p16 expression status in the tissue of patients with oropharyngeal cancer is a strong independent prognostic factor for local recurrence as well as disease-free and overall survival. Patients with p16-nonoverexpressing tumors had a 6-fold increase in risk of local recurrence and an almost 3-fold increase in risk of death from any cause. In multivariate analysis, p16 expression status remained an inde-
pendent prognostic factor. This result is striking, given that p16 overexpression was also associated with poor prognostic features such as advanced TNM stage and high histologic grade.

The relationship between p16 expression levels and prognosis is controversial. Loss of p16 expression has been found to be associated with worse prognosis in several cancers, including non-small cell lung cancer, melanoma, nasopharyngeal, hepatocellular, and colorectal carcinoma (15, 20–24). This is in contrast to reports that overexpression of p16 is associated with poor survival in prostate cancer and neuroblastoma (25–27). In HNSCC, conflicting data also exists regarding the prognostic significance of p16. Loss of p16 expression has been found to be associated with worse prognosis in carcinoma of the anterior tongue (28). In a similar fashion, p16 overexpression has been correlated with improved outcome in tonsillar carcinoma (29). To the contrary, other investigators have found no prognostic significance of p16 in HNSCC (30, 31). To our knowledge, our study is the largest of its kind in evaluating the prognostic significance of p16 expression in oropharyngeal tumors.

Oropharyngeal cancers are a heterogeneous group in terms of etiology, pathogenesis and clinical behavior. Besides tobacco and alcohol use, evidence has accumulated that some of the oncogenic HPVs, especially high-risk HPV types 16 and 18, are associated with a subset of patients with oropharyngeal tumors (32, 33). The molecular events in cervical carcinogenesis, which is known to be HPV associated, have been described extensively. During carcinogenic progression, integration of the viral DNA into the host cell genome often disrupts the gene that encodes the E2 protein, the main viral transcription/replication factor (34–37). The E2 protein functions as a transcriptional repressor of the HPV E6 and E7 oncogenes. Loss of the E2 protein results in dysregulated expression of the E6 and E7 genes. These genes encode oncoproteins that neutralize the p53 and Rb tumor suppressor pathways, respectively (38–40). Rb acts as a negative regulator of p16 expression at the transcriptional level (41, 42). It has been demonstrated in a wide variety of cancers that p16 and Rb inactivation are almost always mutually exclusive (43). Khleif et al. (44) studied cervical cancer cell lines and demonstrated that p16 protein is expressed in cells that contain a wild-type Rb functionally inactivated by E7. Additionally, the authors found that inactivation of Rb was tightly correlated with subsequent up-regulation of p16, confirming a feedback loop between p16 and Rb.

Overexpression of p16 has been repeatedly reported in HPV-associated cancers. In cervical and genital lesions, levels of p16 protein expression have been correlated with HPV oncogenic potential (45, 46). A recent study of lymph node metastases in HNSCC reported that p16 overexpression is a surro-

### Table 3 Univariate Cox regression analysis by p16 expression levels

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpressor</td>
<td>1.675 (0.8–3.7)</td>
<td>0.20</td>
</tr>
<tr>
<td>Nonoverexpressor</td>
<td>0.407 (0.2–0.97)</td>
<td>0.043*</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpressor</td>
<td>1.412 (0.8–2.4)</td>
<td>0.20</td>
</tr>
<tr>
<td>Nonoverexpressor</td>
<td>0.965 (0.6–1.6)</td>
<td>0.90</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Overexpressor</td>
<td>5.293 (1.2–23.6)</td>
<td>0.029*</td>
</tr>
</tbody>
</table>

### Table 4 Multivariate analysis by Cox regression

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard ratio (95% confidence interval)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Local recurrence</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type (primary/recurrent)</td>
<td>1.675 (0.8–3.7)</td>
<td>0.20</td>
</tr>
<tr>
<td>Management (EBRT/surgery + EBRT)</td>
<td>0.407 (0.2–0.97)</td>
<td>0.043*</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1.142 (0.8–2.4)</td>
<td>0.20</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>0.965 (0.6–1.6)</td>
<td>0.90</td>
</tr>
<tr>
<td>p16 overexpressor</td>
<td>5.293 (1.2–23.6)</td>
<td>0.029*</td>
</tr>
<tr>
<td>Disease-free survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type (primary/recurrent)</td>
<td>1.616 (0.9–3.0)</td>
<td>0.13</td>
</tr>
<tr>
<td>Management (EBRT/surgery + EBRT)</td>
<td>0.581 (0.3–1.1)</td>
<td>0.08</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1.453 (1.0–2.1)</td>
<td>0.06</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>1.033 (0.7–1.5)</td>
<td>0.87</td>
</tr>
<tr>
<td>p16 overexpressor</td>
<td>3.000 (1.3–7.0)</td>
<td>0.010*</td>
</tr>
<tr>
<td>Overall survival</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor type (primary/recurrent)</td>
<td>1.648 (0.9–3.1)</td>
<td>0.12</td>
</tr>
<tr>
<td>Management (EBRT/surgery + EBRT)</td>
<td>0.648 (0.4–1.2)</td>
<td>0.14</td>
</tr>
<tr>
<td>TNM stage</td>
<td>1.679 (1.1–2.5)</td>
<td>0.014*</td>
</tr>
<tr>
<td>Histologic grade</td>
<td>0.984 (0.7–1.5)</td>
<td>0.94</td>
</tr>
<tr>
<td>p16 overexpressor</td>
<td>2.449 (1.1–5.7)</td>
<td>0.038*</td>
</tr>
</tbody>
</table>

* Significant at the 0.05 level.
gate marker for oropharyngeal origin and HPV association (47). This confirms previous reports associating p16 overexpression with HPV positivity in oropharyngeal squamous cell carcinomas (29, 48, 49).

If p16 overexpression is a surrogate marker of HPV association, then our finding of a 20% incidence of p16 overexpressors would be inconsistent with most studies reporting an incidence of 50% HPV positivity in oropharyngeal squamous cell carcinomas (32, 50, 51). An alternative explanation of our results would be that p16 overexpression defines a subgroup of HPV-positive oropharyngeal tumors with a more favorable prognosis. The latter explanation is justified by the fact that the presence of HPV DNA in tumors, although necessary, is not sufficient evidence for a causal role of the virus in disease pathogenesis. Furthermore, recent studies have raised the probability that not all HPV-positive head and neck cancers have a causal association with HPV (52). Thus, there may be a subgroup of HPV-positive p16-nonoverexpressing oropharyngeal tumors of multifactorial pathogenesis; in this model, tobacco may induce loss of p16 function and negate the favorable impact that the virus would have on prognosis. This hypothesis is also supported by the conflicting data in the literature regarding the impact of HPV on prognosis of patients with oropharyngeal cancer. Although most studies have found HPV-positive head and neck cancers to have improved prognosis (32, 53–55), some have demonstrated no difference or worse prognosis (56–58). It is possible that the results of these studies are affected from the incidence of the p16 overexpressors within the group of HPV-positive tumors examined. Molecular studies in our laboratory are under way to investigate this model.

In our cohort, preservation of p16 function was such a powerful favorable prognostic factor that it overcame the potentially adverse impact of high TNM stage and poor histologic grade. Previous studies have reported similar findings. Gillison et al. (32) reported in their cohort of oropharyngeal tumors an association of HPV positivity with poorly differentiated tumor grade yet an overall improved prognosis. In a recent case-control study of 120 HNSCC patients and their matched controls, presence of anti-HPV type 16 antibodies in serum was significantly associated with poorly differentiated tumor status (59). A study of oral cavity and oropharyngeal tumors found that among women, HPV positivity was associated with advanced TNM stage (55). Taken together, these findings underscore the inadequacy of the current clinicopathological factors in classifying patient prognosis and the necessity of using molecular technologies to discover novel prognostic factors.

Our finding is important for two reasons: first, it identifies p16 as a strong, independent prognostic indicator in patients with oropharyngeal cancers. Second, it raises the possibility of p16 status guiding clinicians in tailoring therapy. For example, in tumors with loss of p16, replacement of p16 or cdk4 inhibitors may improve therapeutic outcome. p16INK4A adenovirus-mediated gene therapy has been successfully applied against HNSCC in vivo and appears promising in patients with recurrent and metastatic tumors (60–62). Alternatively, cdk4 inhibitors, in combination with chemotherapy, are currently in clinical trials and show promising activity (63–65). Most importantly, if indeed p16 overexpressors represent a unique class of HPV-positive head and cancers with a HPV-causal etiology, then p16 overexpression may identify those cancers that would benefit from HPV-targeted therapies such as vaccines or antiviral pharmacotherapy.

REFERENCES


Prognostic Significance of p16 Protein Levels in Oropharyngeal Squamous Cell Cancer


Updated version
Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/10/17/5684

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.