Human Papilloma Virus and p53 in Head and Neck Cancer: Clinical Correlates and Survival

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ABSTRACT
Recent studies have shown that p53 mutations are frequently found in cancer of the head and neck, whereas others have indicated that human papilloma virus (HPV) infection may be involved. Thus far, no studies have examined both p53 and HPV in the same patient population and correlated the results with clinical characteristics and outcome. The purpose of this study was to examine any relationship between p53 and HPV in patients with squamous cell carcinoma (SCC) of the head and neck. We also planned to correlate the experimental findings with clinical characteristics, known risk factors, and treatment outcome to determine whether any prognostic factors could be detected. Archival material from 66 patients with SCC of the head and neck were selected for study based on the availability of tissue from the primary tumors prior to treatment. A data base was constructed containing all clinical parameters at the time of diagnosis and risk factors. Genomic DNA was isolated and amplified using PCR, followed by SSCP analysis and direct genomic sequencing of all variants to detect p53 mutations. Two independent methods were used for HPV detection: (a) PCR amplification using primers homologous to the E6 region of HPV 16, 18, and 33, followed by RFLP analysis; and (b) PCR amplification with HPV L1 consensus primers, followed by triple restriction enzyme digestion. The results were entered into the data base for statistical analysis. Twenty-four percent of patients were found to have p53 mutations, and 18% were positive for HPV infection. Only one patient was positive for both. Tonsillar cancer was strongly correlated with HPV (P = 0.0001) and inversely correlated with p53 (P = 0.03). The only clinical parameter associated with p53 mutation was a trend toward a heavier smoking history. A subset analysis of the patients with tonsillar cancer revealed inverse correlations with smoking (P = 0.015) and alcohol use (P = 0.05). Also, white patients with SCC of the tonsil were more likely to be HPV positive (P = 0.015). No significant relationships with outcome were detected with either p53 or HPV in the entire population. A subset analysis of patients with stage IV disease revealed that HPV infection was correlated with overall survival. This is the largest study to date to examine both p53 and HPV in patients with SCC of the head and neck. Our results suggest that HPV may be involved in the development of these cancers in patients without traditional risk factors and that HPV-related cancers are more prevalent in the white race.

INTRODUCTION
Carcinoma of the head and neck is a locally aggressive disease that results in significant morbidity and mortality. Although these neoplasms account for a smaller proportion of cancer in the United States, they are a major cause of cancer in Southeast Asia and India (1-3). The epidemiology of head and neck cancer has been well described, and multiple risk factors have been identified. However, the molecular mechanisms responsible for the development of these neoplasms remain poorly understood.

The loss of tumor suppressor genes or their function has been implicated in the etiology and natural history of a variety of human cancers (4). For example, abnormal expression or absence of p53 has been found in many human cancers. Absent or abnormal p53 gene expression may be secondary to gene mutations, deletions, or enhanced degradation of the normal p53 gene product (5). These events could lead to deregulation of a crucial cell regulatory process (6) and may confer a proliferative advantage, with subsequent clonal expansion of tumor cells with altered or absent p53 gene function (7). Gene mutation seems to be the predominant mechanism of p53 inactivation in lung and esophageal cancer (8, 9). Alternatively, a loss of p53 gene function occurs with enhanced degradation of the gene product. This enhanced degradation can occur when certain viral proteins form a complex with the p53 gene product. The E6 protein of high-risk HPV3 viruses can associate with the cellular p53 protein and can direct an increase in the rate of p53 degradation (10). Oncogenic HPV DNA is detectable in >80% of cervical SCCs and the association of HPV with p53 protein has been suggested to play a role in the transforming ability of these viruses (11).

Recent studies in head and neck cancer have shown a relationship of head and neck cancer with the alteration of p53

Received 11/20/95; revised 12/20/95; accepted 12/26/95.
1 This work was supported in part by National Cancer Institute Grant CA-42596 and Cancer Center Core Grant CA-14599. We thank the Gerald- Norton Memorial Corporation, The Rice Foundation, The Center for Radiation Therapy, and the Chicago Tumor Institute for their continued support.

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3 The abbreviations used are: HPV, human papilloma virus; SCC, squamous cell carcinoma; SSCP, single-strand conformation polymorphism.
gene function (12–14) and with HPV infection (12, 15, 16). Yet, relatively little is known about the roles of \( p53 \) and HPV in the development and prognosis of head and neck cancer, because few reports have investigated both in the same patient population. Because the mucosal surfaces of the head and neck region are exposed to both mutagens and HPV, we decided to investigate the role of these agents further in the epidemiology and prognosis of head and neck cancer. In this study, we examined the relationship between \( p53 \), HPV, the site of the disease, traditional risk factors, clinical correlates, and outcome.

PATIENTS AND METHODS

Information regarding all patients with head and neck cancer seen at the University of Chicago between 1985 and 1993 was obtained from the University of Chicago Cancer Research Center and the tumor registry. Patients with diagnoses other than SCC of the head and neck were excluded from the analysis. From this list, 66 patients were identified who had adequate pathological material available for review and analysis. Data regarding TNM stage, site, histology, smoking history, alcohol consumption, treatment, and outcome were combined with the HPV and \( p53 \) laboratory results.

Pathological material for analysis was obtained from the primary tumors at the time of biopsy or surgical excision before any therapy in 66 patients. Specimens were fixed in 10% neutral formalin and embedded in paraffin. At least five sections 5 \( \mu \)m thick were taken from each specimen. One section was stained with H&E and reviewed. The remaining sections were micro-dissected to exclude any nontumor tissue. Dissected tissue was dewaxed with two washes with xylene, followed by one wash with ethanol, and then lyophilized. The tissue was digested with proteinase K (100 \( \mu \)g/ml) in 10 \( \mu \)M Tris (pH 8.0), 1 \( \mu \)M Na\(_2\)EDTA, 75 \( \mu \)M NaCl, and 1% SDS for 24 \( h \) at 37\( ^{\circ} \)C or 8 \( h \) at 50\( ^{\circ} \)C. DNA was isolated by extracting three times with phenol:chloroform:isoamyl alcohol (24:24:1) and once with chloroform and concentrated on a Censtor-100 (Amicon) column.

\( p53 \) SSCP and Direct Genomic Sequencing. Genomic DNA was examined for alterations in DNA sequences of exons 5–11 of the \( p53 \) gene as described previously (12). Briefly, PCR amplification (17) of genomic DNA was followed by SSCP analysis (18) and direct genomic sequencing of all variants as described previously (19). Exon-specific primers were chosen for SSCP to include 10–20 \( bp \) of introns both 5' and 3' to the exon of interest. Each sample underwent electrophoresis once on a 6% nondenaturing polyacrylamide gel and once either on a similar gel with the addition of 10% glycerol or on a Mutation Detection Electrophoresis gel (AT Biochem, Inc.). Amplified DNA was generated for sequencing using a second PCR reaction with at least one new primer external to that used for SSCP analysis. Amplified DNA samples were combined with one of several different \( ^{32}P \) end-labeled primers internal to the amplified fragment. Both the coding and noncoding strands were examined. Mutations were confirmed by repeated sequencing using a separate PCR reaction.

HPV Detection and Typing. Two independent methods were used to screen all tumor specimens for the presence of HPV DNA (20, 21). Both used PCR amplification followed by RFLP analysis. The first method used oligonucleotide primers homologous to a region of the E6 gene of HPV types 16, 18, and 33 in a separate reaction to generate fragments of type-specific length (100–168 \( bp \)). After visualizing these on an ethidium bromide-stained 3.5% NuSieve/SeaPlaque (FMC Bioproducts) gel, the samples, which were identified as positive, were amplified and cut with restriction enzymes (MspI for HPV-16, HaeIII for HPV-18, and Sau3AI for HPV-33). Cut and uncut DNA were run on another 3.5% NuSieve/SeaPlaque gel to verify the HPV type. The primer sequences used to detect E6 sequences are shown in Fig. 1.

The second method used HPV L1 gene consensus primers as described by Ting and Manos (21) for amplification, followed by triple restriction enzyme digestion with HaeIII, PstI, and Rsal as described by Lungu et al. (22). This method allows the detection of 13 HPV types. Controls using known HPV-positive and -negative material were used for all extractions, amplifications, digests, and gels.

Statistical Analysis. Correlations of HPV infection and \( p53 \) mutations with patient demographic variables were performed with \( \chi^2 \) or Fisher's exact tests for categorical variables (23), exact trend tests for ordinal categorical variables (such as levels of tobacco and alcohol exposure Ref. 24), and \( t \) tests for continuous variables (23). All tests were two sided, with the exception of the one-sided trend tests. Overall survival was measured from the date of diagnosis until death of any cause or until the date of last patient contact. The time to progression was measured from the date of diagnosis until the first evidence of local progression or distant failure. Patients dying of intercurrent disease without evidence of disease were censored for the time to progression but not overall survival. The time to local failure and time to distant failure were measured from the date of diagnosis until the date of local or regional recurrence or the date of distant failure, respectively. Time-to-event measures were estimated by the method of Kaplan and Meier (25) and compared by the log rank test (26). Multivariate survival analysis was performed by Cox's proportional hazards regression (27). Forward and backward stepwise methods were used with significance set to \( P < 0.15 \) for inclusion in the predictive model. All statistical analyses were performed in either Number Cruncher statistical system (Ness; Jerry Hintze, Kaysville, UT) or Stata (Stata Corp., College Station, TX).

RESULTS

Sixty-six patients with SCC of the head and neck were included in this study. The pathological material from all 66
patients received a variety of treatments, including radiation therapy alone (4 patients), surgery alone (9 patients), chemotherapy alone (3 patients), and combined modality therapy (49 patients). One patient died prior to treatment. At the time of analysis, 34 patients were alive and free of disease, with a median follow-up of 53.8 (range, 24–111) months.

**p53 and HPV.** Archival tissue from all 66 patients was examined for p53 mutations as well as HPV infection by the techniques listed above. Overall, 24% (16 of 66) specimens tested positive for p53 mutations, and 18% (12 of 66) specimens were found to have HPV infection, as shown in Table 3. Seven mutations were single-bp substitutions (four transversions and three transitions). Three mutations were single-bp insertions resulting in a frame shift. Six mutations were deletions that resulted in a frame shift (three single bp and three multiple bp). Twelve of the tumor specimens contained oncogenic HPV DNA. All 12 patients were positive for E6, and 9 were positive for L1 as well. Eleven of the HPV-positive specimens contained HPV-16 DNA, and one specimen contained HPV-33 DNA.

Only one patient in this series was found to have HPV infection and a p53 mutation. This patient was the only patient with tonsillar carcinoma found to have a p53 mutation.

The number of patients with p53 mutations and/or HPV infection according to disease site is shown in Table 4. Mutations in p53 were found in 15–43% of patients based on the site
of the primary tumor, and no correlations were observed. Subsite analysis revealed a negative correlation for p53 and tonsillar carcinoma (P = 0.03). In contrast to p53, 83% (10 of 12) of the HPV-positive cases were found in patients with primary tumors of the oropharynx, for which a strong positive correlation was observed (P = 0.005). This correlation reflected the high rate of HPV infection in patients with SCC of the tonsil, of whom 50% (9 of 18) were found to have HPV (P = 0.0001). Only 12.5% (1 of 8) of oropharyngeal cancers excluding tonsils were positive for HPV compared with 5% (2 of 40) of nonoropharyngeal cancers, in contrast to the distribution of p53 mutations.

Clinical Correlations. All clinical features in Table 1 were examined to determine whether any correlations with p53 mutation or HPV infection could be detected. A trend toward a heavier smoking history (P = 0.08) was noted in the patients with p53 mutations. No other clinical features were found to be associated with p53 mutation. This is in contrast to patients with HPV infection, for whom several highly significant correlations were observed. In the overall population, HPV infection was significantly correlated with a more advanced stage at diagnosis (P = 0.0008), oropharyngeal primary lesions (P = 0.005), a higher nodal stage (P = 0.006), and more poorly differentiated tumors (P = 0.02). Unlike p53 mutation, for which a trend toward a heavy smoking history was found, an inverse relationship with smoking history was seen in patients with HPV infection (P = 0.05).

Because patients with head and neck cancer tend to be a heterogeneous population with differing primary sites, stages, and prognoses, a subset analysis was performed based on site. Nine of the 12 HPV infections were in the subset of 18 patients with tonsillar cancer. Thus, a risk factor analysis of patients with tonsillar primary tumors was performed, and the results are shown in Table 5. Histological differentiation, nodal stage, and overall stage were no longer significantly correlated with HPV infection. There was a significant inverse negative relationship with smoking history (P = 0.015). Also, there was a modest inverse correlation with alcohol use (P = 0.05) and a correlation of HPV infection with race (P = 0.015), because white patients with tonsillar carcinoma were more likely to be HPV positive.

Except for a trend toward increased tobacco exposure in patients with p53 mutations, there were no other correlations when testing the entire group of 66 patients. Because p53 was inversely correlated with tonsillar cancer, we postulated that the 48 patients without carcinoma of the tonsil might represent a more homogeneous group and repeated the risk factor analysis on that subset to determine whether any correlations could be detected. The results are shown in Table 6. There was no demonstrable correlation of p53 mutations with race, smoking, or alcohol consumption in this cohort.

Stepwise Cox regression analysis was performed to determine which clinical features were significant independent predictive factors of survival, time to progression, distant failure, and local failure. Factors tested were age, sex, overall stage, tumor stage, nodal stage, tumor differentiation, site of the primary tumor, and p53 mutation status. All patients with HPV infection presented with stage IV disease and could not be tested independently. Therefore, separate stepwise Cox regression analyses were performed on the subset of patients who presented with stage IV disease to evaluate all the above clinical features along with HPV status.

The two clinical features that were predictive of poorer survival in a stepwise Cox model were advanced stage (P = 0.13) and hypopharyngeal primary tumor (P = 0.085; median survival, 15.7 months). In a subset analysis of stage IV patients, the only significant predictor of survival was HPV infection (P = 0.054). Stage IV patients with HPV infections had better
survival (median, 76.6 months) than those who did not (median, 32.9 months) as shown in Fig. 2.

Stepwise Cox regression analyses were repeated for the time to progression at any site, time to distant failure, and time to local failure. The best model to predict the time to progression for the entire group included advanced stage ($P = 0.031$) and oropharyngeal primary tumors ($P = 0.093$). Those patients with tumors of the oropharynx had favorable prognoses. The most significant factors associated with the development of distant metastases were advanced nodal stage ($P = 0.028$) and hypopharyngeal tumors ($P = 0.068$). The only factor associated with local failure was advanced tumor stage ($P = 0.069$). Although HPV infection was associated with more favorable overall survival in subset analysis of the stage IV patients, it was not a significant predictor of the time to progression, time to distant failure, or local control. There were no significant predictors of treatment failure in stage IV patients.

### DISCUSSION

Head and neck cancer is largely a disease stemming from environmental insults, because it occurs almost exclusively among those with prolonged exposure to tobacco and alcohol. A complex, multistep process is likely in carcinogenesis of the aerodigestive tract epithelium (2). There is evidence that aberrations of oncoproteins and tumor suppressor genes are probably essential for this process (28). Abnormal p53 function has been detected in 33–100% of head and neck cancer specimens, depending on the source of tissue and the method of detection (12, 29–34). Several studies have addressed the question of p53 status and the possible correlation with tumor stage and nodal involvement (12, 13, 31, 32). The results of these studies suggest that p53 abnormalities occur relatively early in the neoplastic process and may increase in frequency with disease progression. However, the above studies have found no other association with other prognostic factors of age, sex, performance status, or differentiation.

In addition to gene mutation, abrogation of p53 function can also occur via complex formation with certain oncoproteins, including the protein product of the E6 gene of oncogenic HPVs, particularly types 16, 18, and 31 (11). The E6 protein produced by oncogenic HPVs has been shown recently to bind to wild-type p53, shortening its half-life and inactivating its function in infected cells (10, 35, 36). The link between certain HPV types and benign head and neck lesions has been known for some time (37, 38). All laryngeal papillomas contain HPV DNA and are usually HPV 6 or 11. Although p53 mutations occur frequently in head and neck cancer, it is not a universal finding. Because the mucosal surfaces of the head and neck region are exposed to both mutagens and HPV, it is reasonable to determine whether tumors without p53 mutations harbor HPV, because oncogenic HPV could be involved in the development of a portion of the p53-negative cancers. The reported incidence of HPV infection in head and neck SCCs ranges from 5 to 46% (12, 15, 16). Although HPV infection and p53 mutations have been reported to occur in head and neck cancer, few studies have attempted to detect both HPV subtypes as well as p53 mutations within the same tumor specimens and to correlate the results with clinical risk factors and outcome.

In our series, 24% of patients were found to have p53 mutations. These results are slightly lower than other series in the literature. p53 mutation has been linked to a history of heavy tobacco and alcohol exposure (39, 40). One explanation is that fewer p53 mutations were found in our study, because the patients tended to have histories of less exposure. There was no correlation with the site of the primary tumor, except for a strong negative correlation with tonsilar cancer, of which 5.5% contained mutations in p53. This compares with a 26–43% mutation rate in sites other than the tonsil. Except for the negative correlation with tonsilar primary tumors, no other correlations with the clinical factors tested were detected. The p53 mutations were not significantly correlated with traditional risk factors within this small group of patients. Also, p53 mutations were not correlated with survival, disease-free survival, local failure, and distant failure.

Our results indicate that p53 mutation occurs in a percentage of squamous cell head and neck cancers without a predilection for any specific site, in agreement with the literature. Unlike p53, HPV infection demonstrated a predilection for patients with tonsilar carcinoma. This is in contrast to the results of Clayman et

### Table 6  Clinical correlations of p53 mutation

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<th>Risk factor</th>
<th>Patient</th>
<th>p53-positive</th>
<th>$P$</th>
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<tr>
<td>Race</td>
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<td></td>
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<tr>
<td>African-American</td>
<td>26</td>
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</tr>
<tr>
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<td>&lt;20 pack/yr</td>
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<td>20-40 pack/yr</td>
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* AJCC, American Joint Committee on Cancer.
al. (15), in which a high rate of HPV infection was detected in patients with laryngeal and hypopharyngeal cancer. However, patients with tonsilar primary tumors were not included in this study. One small series examined biopsy material from patients with tonsilar carcinoma and severe tonsillitis (16). In this series, all 10 patients with carcinoma contained HPV DNA, whereas the cases of tonsillitis were negative. These results suggest that HPV infection may be involved in the development of cancer of the tonsil, because those patients with tonsillitis did not contain HPV DNA.

No significant correlations were found between clinical risk factors and p53 mutation, whereas a number of significant correlations were found in patients with HPV infection. When the cohort of patients with carcinoma of the tonsil was analyzed, the patients with HPV infections did not have the traditional risk factors associated with head and neck cancer. In fact, inverse correlations were found, because the HPV-infected patients tended to be nonsmokers (P = 0.015) and nondrinkers (P = 0.05). An unexplained finding was the tendency to find HPV infections in white patients (P = 0.015).

Studies of cervical cancer have demonstrated that patients with HPV-positive tumors (as opposed to those with p53 mutations) seem to have better prognoses (41, 42). These results are similar to our findings. Although the stage was the single most important predictor of overall survival, a subset analysis of patients with stage IV cancer revealed that the most significant predictor of improved survival was HPV infection. These results are in contrast to those of Clayman et al. (15), who found that those patients with HPV infection, vascular invasion, and nodal status were the most significant predictors of survival using log rank analysis. However, the results must be interpreted with caution, because log rank testing would not take into account possible interactions. Our stepwise analysis, which controlled for risk factors and clinical characteristics, including tumor stage, nodal stage, and site, demonstrated that patients with HPV infection fared better than those who were HPV negative. Nevertheless, these results could be spurious, because this was a heterogeneous population that underwent a variety of treatments. The small sample size did not allow for inclusion of treatment parameters in the statistical analysis.

Only the traditional characteristics of stage, nodal stage, and tumor stage were significantly correlated with time to progression, time to metastases, and time to local failure, respectively. No significant correlation of any disease progression parameter was found with HPV infection. There is no obvious explanation of why HPV was correlated with survival but not treatment failure. The small sample size may help explain this finding. An alternate explanation of this finding could be the fact that those patients with HPV infection had less exposure to tobacco and alcohol. Thus, the HPV-positive patients would be at a lower risk of dying of intercurrent disease. Whether HPV infection would be a significant predictor of the time to progression with a larger sample size requires further investigation.

Traditionally, the treatment and prognosis of SCC of the head and neck have been based on clinical parameters of the stage and site of the primary tumor. Although this information is easily obtained at the time of diagnosis, it may not be the best method for basing treatment decisions. A recent study by Brennan et al. (43) demonstrated that molecular assessment of histopathological specimens in head and neck SCC can predict an increased risk of local tumor recurrence. These authors were able to examine the surgical margins and lymph nodes of patients with p53 mutations using molecular probes. The results demonstrated positive margins in >50% of the patients who were thought to be completely resected after histopathological assessment. Patients who had positive margins based on molecular analysis were at a higher risk for local failure.
Our results suggest that oncogenic HPV infection may be responsible for a portion of those cases without p53 mutations, and that HPV may be involved in patients with carcinoma of the tonsil who do not have traditional risk factors. The data further suggest a racial predilection, because white patients were more likely to be HPV positive. Although all patients with HPV infection presented with advanced stage IV disease, they had better overall survival than those stage IV patients who were HPV negative, which is similar to the findings in cervical cancer. Although HPV seems to be sexually transmitted in cervical and anal cancer, the method of transmission in head and neck cancer is unknown and requires further investigation.

Advances in molecular biology may someday result in a sophisticated battery of tests to design treatment and assess prognosis. HPV and p53 may eventually prove useful as part of initial tumor assessment along with stage. Individual studies usually examine one marker in a cohort of patients. As new molecular markers are identified, they should be tested simultaneously and should be correlated with known clinical parameters to determine what interrelationships, if any, exist. Careful analysis and correlation with clinical information present at the time of diagnosis, treatment, and outcome are necessary to form valid conclusions, which will enable us to tailor treatment and improve results more precisely.

ACKNOWLEDGMENTS

We thank Claire Powers for the initial chart review; Leonid Buzya for cutting the blocks and staining the slides; Phil Means and Alan Houghton for assistance with the computer data base; and Laimonis Lamins, who provided advice and positive controls for HPV 16, 18, and 31.

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