Correlation of Cellular Immunity With Human Papillomavirus 16 Status and Outcome in Patients With Advanced Oropharyngeal Cancer

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Objective: To determine whether the favorable outcome associated with human papillomavirus (HPV) 16–positive oropharyngeal cancer is related to a patient’s adaptive immunity.

Setting: Academic medical center.

Patients: Forty-seven of 66 previously untreated patients (6 of 20 patients with stage III and 41 of 46 with stage IV cancer) in a prospective clinical trial of chemoradiotherapy.

Intervention: All patients were treated with a single course of neoadjuvant chemotherapy followed by either surgery (for nonresponders) or chemoradiotherapy.

Main Outcome Measures: Pretreatment levels (percentages and absolute counts) of CD3, CD4, CD8, natural killer, and B cells and overall white blood cell counts were measured by flow cytometry. Correlations of subsets with HPV-16 status, tumor subsite, cancer stage, T class, N class, smoking status, performance status, sex, response to chemoradiotherapy, p53 mutation type, epidermal growth factor receptor expression, and disease-specific and overall survival were determined.

Results: After a median follow-up of 6.6 years, improved survival was associated with an elevated percentage of CD8 cells ($P = .04$), a low CD4:CD8 ratio ($P = .01$), low epidermal growth factor receptor expression ($P = .002$), and HPV status ($P = .02$). The percentage of CD8 cells was significantly higher ($P = .04$) and the CD4:CD8 ratio was significantly lower ($P = .02$) in HPV-16–positive patients. A higher percentage of CD8 cells was associated with response to induction chemotherapy ($P = .02$) and complete tumor response after chemoradiotherapy ($P = .045$).

Conclusion: These findings confirm previous correlations of outcome with circulating CD8 cell levels and support the conjecture that improved adaptive immunity may play a role in the favorable prognosis of patients with HPV-16–positive cancers.

tients with HPV-16–associated oropharyngeal cancer, our group prospectively measured pretreatment peripheral blood levels of T-lymphocyte subsets in patients with advanced oropharyngeal cancer as part of a prospective phase 2 clinical trial of therapeutic chemoradiotherapy. That trial included patients with HPV-16–positive and –negative cancers treated in a uniform fashion. Trial details and clinical outcomes have been reported previously.

In the present study, levels of T-cell subsets were correlated with HPV-16 status, overall and disease-free survival, and response to induction chemotherapy.

METHODS

PATIENT POPULATION

The phase 2 clinical trial included 66 patients with stage III (n=20) or IV (n=46) oropharyngeal squamous cell carcinoma. Of these, 36 cancers were located in the base of tongue, and 30 were located in the tonsil or lateral pharynx. Pretreatment tumor tissue was available from 50 patients for creation of a tissue microarray (TMA). Sufficient tumor for extraction of DNA for p53 mutational analysis was available from 42 patients. There was adequate tumor biopsy tissue to determine HPV status in 41 patients using a highly sensitive and specific quantitative method that combines polymerase chain reaction and mass spectroscopy. There were 27 HPV-positive and 14 HPV-negative patients. Clinical tumor response and survival analyses are reported for the patients with biomarker data. There were no statistically significant differences in clinical outcomes among patients with and those without tissue available for biomarker analysis. Median clinical follow-up of these patients was 6.6 years.

TREATMENT REGIMEN

Patients received induction chemotherapy as 1 cycle of cisplatin (100 mg/m²/d for 1 day) and fluorouracil (1000 mg/m²/d for 5 days). Carboplatin (area under the curve) was used in place of cisplatin in patients with renal insufficiency or hearing loss. Response to the initial chemotherapy regimen was evaluated by surface measurements at direct laryngoscopy, supplemented by radiographic imaging (computed tomography or magnetic resonance imaging) for deeply infiltrative tumors. Pretreatment tumor tissue was available from 50 patients for creation of a tissue microarray (TMA). Sufficient tumor for extraction of DNA for p53 mutational analysis was available from 42 patients. There was adequate tumor biopsy tissue to determine HPV status in 41 patients using a highly sensitive and specific quantitative method that combines polymerase chain reaction and mass spectroscopy. There were 27 HPV-positive and 14 HPV-negative patients. Clinical tumor response and survival analyses are reported for the patients with biomarker data. There were no statistically significant differences in clinical outcomes among patients with and those without tissue available for biomarker analysis. Median clinical follow-up of these patients was 6.6 years.

LYMPHOCYTE SUBPOPULATIONS

In a total of 47 patients, pretreatment peripheral blood samples were analyzed by routine automated flow cytometry for T, B, and natural killer cells and for subpopulations of CD3, CD4, and CD8 T lymphocytes. Detailed methods have been described previously. Determinations were made using commercially available monoclonal antibody reagents by an indirect immunofluorescent technique and were performed in the clinical laboratories of the University of Michigan Department of Pathology. Correlations of percentages and absolute numbers of the various subpopulations with HPV status, primary tumor response, and overall survival were determined.

HPV-16 STATUS, EPIDERMAL GROWTH FACTOR RECEPTOR EXPRESSION, AND p53 MUTATION

Pretreatment biopsy specimens were retrieved from 50 of 66 patients for construction of a TMA. Blocks were not available from 15 patients who had undergone biopsy outside our institution. One patient had no tumor left on biopsy specimen and was so not included. In 42 of 50 biopsy specimens, sufficient tumor was present for DNA isolation and HPV analysis, although 1 patient had insufficient DNA quality for HPV analysis. There was no residual tumor after TMA construction in 8 cases.

The HPV type and copy number were assessed with a sensitive, specific quantitative method that involved real-time competitive polymerase chain reaction and MALDI-TOF (matrix-assisted laser desorption ionization–time-of-flight) mass spectroscopy separation of products on a matrix-loaded silicon chip array. Internal standards allowed quantification of HPV copies per cellular genome copy. Primers designed to amplify the E6 region distinguished 13 discrete high-risk HPV types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -56, -58, -59, and -68). Our group previously measured and reported levels of epidermal growth factor receptor (EGFR) expression and p53 mutation status in this patient cohort using methods previously described. Briefly, TMA slides created from biopsy specimens from the patients in this trial were deparaffinized, rehydrated, and peroxidase quenched (DakoCytomation, Glosstrup, Denmark). For antigen retrieval, slides were incubated with pepsin (EGFR;10 minutes at 37°C) or with citrate buffer (pH 6.0; 30 minutes at 92°C) and were blocked with horse serum (30 minutes at 25°C). Primary antibody, EGFR/31G7 (Zymed Laboratories, South San Francisco, California), p16/16P04, and p53/D01 (Laboratory-Vision, Fremont California) were added for 1 hour and were probed with avidin-biotin peroxidase (ABC Kit; Vector Laboratories, Burlingame, California). Antibody binding was scored by a pathologist (K.C.) who was blinded to the clinical outcome and used a continuous scale (10%, 30%, 90%, etc) for the proportion of EGFR-positive tumor cells in each core. For p16 and p53, a scale of 1 to 4 was used: 1 indicated less than 5%; 2, 5% to 20%; 3, 21% to 50%; and 4, 51% to 100% tumor staining. Intensity was scored as 1 (no staining), 2 (low intensity), 3 (moderate intensity), and 4 (high intensity). Scores for multiple cores from each patient were averaged. For p33 mutation, p33 exons 4 to 9 were amplified by using specific primers. Products from 2 polymerase chain reactions were sequenced in both directions and were analyzed by using Mutation Surveyor version 2.61 software (Soft Genetics, State College, Pennsylvania) and by manual review. Although limiting the mutational analysis to exons 4 to 9 may be inadequate, data were also analyzed by p53 expression and obtained similar results. However, the study was not powered to determine the impact of p53 or EGFR on response to therapy. Correlations of lymphocyte subpopulations with EGFR and p53 status were determined.

STATISTICAL ANALYSIS

Pretreatment levels (percentages and absolute counts) of CD3, CD4, CD8, natural killer, B cells, and overall white blood cell (WBC) counts were treated as continuous variables for the assessment of univariate associations with survival in Cox proportional hazards models. To illustrate survival rates, quartiles were plotted using the Kaplan-Meier method. Log-rank tests were used to test the homogeneity of survival across groups. Cutoffs explored in separate tests included quartiles, the—
lymphocytes and previously determined cutoff points from healthy individuals to classify "high" and "low" levels of CD3, CD4, and CD8 cells and high and low CD4:CD8 ratios.5

Rank-based statistical methods were used for the assessment of univariate associations between continuous lymphocyte levels and covariates of interest. Patient-level averages for EGFR and the titer of HPV-16 (log transformed) were used in this analysis. Patients were categorized as current, past (having quit >6 months ago), or never smokers. Spearman rank correlation was used to assess the correlation of subsets with performance status, response to therapy, smoking status, cancer stage, T class, N class, and EGFR expression. The Wilcoxon rank sum test was used to compare the distributions of subsets by HPV-16 status, tumor subsite, and p53 mutation type.

Overall survival was defined as time to death from any cause. The Kaplan-Meier method and the log-rank test were used to compare the homogeneity of overall survival and disease-specific survival across quartiles using the median as a cutoff point and using cutoff points from healthy patients established in previous work.7 Disease-specific survival was defined as the time to death from oropharyngeal cancer. Patients who were alive at last follow-up or who had died for reasons other than oropharyngeal cancer were censored. Cox proportional hazards models were used to assess time-to-event outcomes. For each time-to-event outcome, 3 models were constructed: a model with subset levels (treated as a continuous variable), a model with clinical variables, and a model with both clinical variables and subset levels. Likelihood ratio tests were used to compare models.

All statistical analyses were performed in SAS version 9.2 software (SAS Institute Inc, Cary, North Carolina). A 2-tailed P ≤ .05 was considered statistically significant.

RESULTS

LYMPHOCYTE SUBSETS AND HPV-16 STATUS

Of 66 patients entered in the prospective clinical trial, pretreatment lymphocyte subsets were available in 47. The mean percentage of CD8 cells was significantly higher (P = .04) and the CD4:CD8 ratio was significantly lower (P = .02) in patients with HPV-16–positive cancers (Figure 1). There were no significant differences in other T-cell or B-cell levels among HPV-16–positive or –negative patients. The overall WBC count was higher in HPV-16–negative patients and in smokers (P = .02 and .051, respectively), and hemoglobin levels were lower in smokers compared with past or never smokers. All the HPV-16–negative patients studied were smokers. The percentages of CD8 cells were significantly and similarly elevated in HPV-16–positive smokers and nonsmokers compared with HPV-16–negative smokers (P = .04, Figure 2). There were no significant differences in the proportions of lymphocyte subset levels among smokers or nonsmokers despite overall lower WBC counts among HPV-16–positive patients, suggesting that differences in CD8 levels were more closely associated with HPV status than with smoking history.

LYMPHOCYTE SUBSETS AND RESPONSE TO CHEMOTHERAPY

All patients received a single cycle of induction chemotherapy, and clinical tumor response was assessed by direct laryngoscopy and radiologic imaging to allow bioselection for subsequent treatment according to the protocol. An elevated percentage of CD8 cells (P = .02) and a low CD4:CD8 ratio (P = .02) were significantly associated with complete and partial clinical tumor response to neoadjuvant chemotherapy (Figure 3). This was true when we used...
both mean levels and our previously established cutoff points for normal or elevated levels. In fact, although HPV-16 status was also a predictor of response to neoadjuvant chemotherapy ($P = .10$, Fisher exact test), the CD8 levels were more predictive of response than was HPV-16 status alone ($P = .04$). Higher percentages of CD8 cells were also significantly associated with ultimate tumor response after chemoradiotherapy ($P = .045$, Kruskal-Wallis test, and $P = .04$, Fisher exact test). Smoking status was not found to have a significant correlation with response to neoadjuvant chemotherapy or to overall response after chemoradiotherapy.

**LYMPHOCYTE SUBSETS, p53 MUTATION, AND EGFR EXPRESSION**

Among the other biomarkers examined, HPV-16 status, EGFR expression, WBC count, and smoking status were significantly associated with survival as single variables. Both HPV-16 status ($P = .02$) and intensity of EGFR overexpression ($P = .002$) were significantly associated with disease-free and overall survival consistent with our previous analysis in the larger cohort of patients. Intensity of EGFR was a strong predictor of overall survival even after controlling for HPV-16 ($P = .01$) and smoking ($P = .02$) status. However, there were no significant correlations of any lymphocyte subset with EGFR overexpression. Likewise, p53 mutations were found in only 5 of our 33 patients (15%) and were not significantly predictive of overall survival ($P = .13$). The study was not powered to adequately determine the impact of p53 status on patient outcome. The correlation of p53 status and EGFR expression with clinical outcomes was previously reported. In all, 21 of 22 HPV-16-positive cancers (96%) had wild-type p53 and 7 of 11 HPV-negative cancers (64%) had wild-type p53. Because WBC count tended to be higher in patients with HPV-16–negative tumors, WBC count was also higher in patients with mutated p53, but we did not find any correlation of individual lymphocyte subset levels with p53 mutation status.

**LYMPHOCYTE SUBSETS, HPV-16 STATUS, AND SURVIVAL**

In the subset of patients included in this study, HPV-16 status was a significant prognostic factor, consistent with our previous analysis of the entire study population ($P = .005$, Figure 4). Although mean levels of the various lymphocyte subsets did not differ significantly among patients by overall survival status, the mean percentage of CD8 cells tended to be higher ($P = .13$) and the CD4:CD8 ratio lower ($P = .06$) in surviving patients assuming unequal variances in mean levels. Using previously established cutoff points for elevated CD8 levels (>24%) and low CD4:CD8 ratio (<2.0), we noted a trend for improved overall survival in patients with a high percentage of CD8 cells and a low CD4:CD8 ratio ($P = .14$ and $P = .11$, respectively, Figure 5). In assessing other traditional prognostic factors, there were no significant correlations of T-lymphocyte subsets with Karnofsky performance status, sex, T class, N class, tumor stage (III vs IV), or tumor subsite. Specifically, there were no significant differences in CD8 or CD4 levels among patients with tonsil or base of tongue primary tumors, although CD8 levels tended to be higher ($P = .054$) and the CD4:CD8 ratio tended to be lower ($P = .08$) in patients with stage III cancer.

**COMMENT**

In this retrospective analysis of a prospective study, we evaluated the relationship of pretreatment peripheral...
blood lymphocytes with HPV-16 status, tumor response to chemoradiotherapy, and overall survival. The most significant finding in the present study was that elevated percentages of CD8 cells and low CD4:CD8 ratios were associated with HPV-16 status, improved disease-free interval, and overall survival. Improved overall survival was also significantly associated with low EGFR expression, positive HPV-16 status, and nonsmoking history. These various factors, except for HPV-16 status, appeared to be independent of lymphocyte subset levels.

Our data suggest that alterations in cellular immune response in patients with HPV-16–positive cancers may be partially responsible for the better prognosis associated with HPV-16–positive cancers. T cells appear to be important in the pathogenesis of HPV-16–associated infections because there is an increased incidence in T-cell–immunosuppressed patients. Although memory cytotoxic T cells directed against an HPV-16 E7-encoded epitope have been demonstrated in patients with HPV-16–positive cervical lesions, little is known about T-cell levels in HPV-16–associated oropharyngeal cancer. Previously, CD8 T cells specific for HPV-16 E7 epitopes and for wild-type p53 peptide sequences have been described in a small number of patients with oropharyngeal cancers, but correlations with outcome have been lacking. Our results in this larger cohort of patients with advanced disease support these findings and suggest that circulating CD8 cell levels may be an important prognostic factor.

The measurement of peripheral blood lymphocyte subpopulations represents a reflection of immune homeostasis and is not a direct functional measure of overall immune competence. Many studies, however, have demonstrated the functional relevance of peripheral CD8 T-cell frequency to tumor immunity and autoimmunity. Mice with mutated or depleted CD8 cells challenged with HPV-16–positive tumor cells showed greatly decreased tumor regression compared with controls, demonstrating that antitumor effects are CD8 dependent. The mechanism and significance of increased T lymphocytes in the peripheral blood of HPV-16–positive patients has not been elucidated. One of many possible explanations is that HPV-16–positive tumors have increased antigenicity through the E7 antigen, causing enhanced stimulation of the immune response. The expression of E7 in dysplastic tumor cells may allow the immune system to more readily identify tumor cells as foreign. This is supported by DNA vaccine evidence from a mouse model using HPV-16 E7/heat shock protein 70 genes that induced a stronger E7-specific CD8 T-cell immune response and resulted in a more significant therapeutic effect against E7-expressing tumor cells. In addition, in an animal model of HPV-16–transformed murine tumors, intralymphatic immunization with E7 peptide resulted in expansion of E7-specific CD8 cells responsible for tumor regression. In that study, the effect could be enhanced by removal of immunosuppressive T-regulatory cells. Taken together, these findings demonstrate that clearance of HPV-16–positive cells is antigen dependent and immune mediated and offers support for novel approaches to immunotherapy of HPV-16–related disease.

Many patients with HPV-16–positive cancers are nonsmokers. Smoking was an important and independent prognostic factor in this study. Interestingly, there were no observed effects of smoking status on the relative frequency of CD4 or CD8 cells. Smoking was significantly associated with an increase in mean WBC count only. This increase is possibly a result of nonspecific inflammation related to smoking. Our group previously reported the profound negative impact of smoking status on overall survival in head and neck cancer and in HPV-16–related oropharyngeal cancer. A smoking habit has many potential detrimental effects, including causing gene mutations, epigenetic silencing of tumor-suppressor genes, and increased mucosal inflammation resulting in production of immunosuppressive inflammatory cytokines. In the present study, WBC levels were higher in smokers than nonsmokers but were not directly related to alterations in the percentage of cells among the T-lymphocyte subsets. Surprisingly, levels of CD8 cells were similarly elevated in HPV-16–positive smokers and nonsmokers. However, the study population had few never smokers, and all the HPV-16–negative patients were smokers. Because smokers with HPV-16–positive cancers tend to have a poorer prognosis compared with nonsmokers, CD8 level is likely one of several related factors associated with better outcomes in HPV-16–associated cancers.
In this cohort of patients, HPV-16 status and lymphocyte subsets were independent markers of response to chemotherapy and radiotherapy. Elevated percentages of CD8 cells and low CD4:CD8 ratios were more predictive of chemotherapy response than was HPV-16 status alone. Similarly, percentages of CD8 cells and CD4:CD8 ratios were significantly associated with overall response to chemoradiotherapy. These observations are consistent with in vivo results in mice, in which HPV-16–positive tumors had complete responses but HPV-16–negative tumors showed only partial responses to cisplatin therapy. Furthermore, the same treatment was administered to immunodeficient mice that were found to be unable to clear HPV-16–positive tumor cells as effectively as immunocompetent mice. These combined findings are consistent with the hypothesis that a robust immune response can augment the antitumor activity of therapeutic chemotherapy and radiotherapy.

Lymphocyte subset alterations were independent of other important biomarkers, such as EGFR expression and p53 mutation. Overexpression of EGFR has been associated with poor prognosis independent of HPV-16 status in oropharyngeal cancer and also has been directly associated with CD3 T-cell infiltration of oropharyngeal tumors. However, CD3 infiltration was found to be of prognostic significance only in patients with HPV-16–negative cancers. Typically, patients with head and neck cancers and p53 mutations tend to have a poorer prognosis. We found no significant relationship of lymphocyte subsets with EGFR expression or p53 mutation. The failure to find a significant prognostic effect of p53 or correlations of p53 mutation with lymphocyte subsets was not surprising because most of the patients studied had cancers with wild-type p53. Only 5 patients had p53 mutations, and these patients tended to have a poorer prognosis (P = .13).

Although there is no proven causal relationship between HPV-16 status and T-cell subsets, our data suggest that measures of adaptive immunity could be useful in the clinical setting. Cutoff points for percentage of CD8 cells and CD4:CD8 ratio previously established from a large group of age-matched smoking control subjects could be applied in clinical scenarios to predict prognosis. In the present study, levels of lymphocyte subsets were measured by automated flow cytometry in routine pathology department clinical laboratories. Patients with a percentage of CD8 cells greater than 24% and a CD4:CD8 ratio less than 2.0 tended to have a higher tumor response rate and improved overall survival compared with patients with a low percentages of CD8 cells and a high CD4:CD8 ratio. This is in agreement with our previous data in surgically treated patients who also exhibited significantly improved outcomes using these same clinical laboratory cutoff points.

Our overall findings of significant alterations in peripheral T-lymphocyte subsets that correlate with HPV-16 status and prognosis in patients with oropharyngeal cancer help to better characterize and confirm the importance of adaptive immunity in these patients. Functional analyses of these lymphocyte subsets are clearly needed to better understand the mechanistic role of these cells in the biological mechanisms of HPV-16–related cancer. Longitudinal studies of changes in subset levels after therapy may provide insight into the design and monitoring of secondary cancer–prevention strategies for these patients. Detailed analysis of tumor-infiltrating lymphocytes in HPV-16–positive and –negative patients may also help provide a better understanding of the relationship between increased levels of circulating T cells, tumor-infiltrating lymphocytes, host immune reactivity, and outcome in the unique setting of HPV-16–related oropharyngeal cancer.
Additional Contributions: This study would not have been possible without the generosity and participation of our patients and the dedicated assistance and teamwork of the members of the Head and Neck Oncology Program at the University of Michigan who enrolled and cared for the patients in this study.

REFERENCES