The Laryngoscope

Infiltrating Lymphocytes and Human Papillomavirus-16–Associated Oropharyngeal Cancer

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Objectives/Hypothesis: Human papillomavirus-16 (HPV-16)–associated carcinoma of the oropharynx has a favorable prognosis. Such patients have elevated CD8+ T-lymphocyte levels that correlate with response to chemotherapy and survival. Tumor-infiltrating lymphocyte (TIL) subpopulations were assessed in pretreatment biopsies from a prospective patient cohort to determine if TIL subsets differed by HPV status, clinical factors, or patient outcome or correlated with peripheral blood T-cell levels.

Study Design: Retrospective immunological correlative study of patients entered in a prospective Phase 2 clinical trial.

Methods: Measured were CD8, CD4, CD68, and Treg (FoxP3) lymphocytes by immunohistochemistry in a tissue microarray created from patients (n = 46) with advanced oropharyngeal cancer. Correlations with peripheral blood levels, HPV status, expression of epidermal growth factor receptor (EGFR), clinical tumor, and patient characteristics and outcome were determined. Median follow-up was 6.6 years.

Results: HPV-16–positive patients had improved survival ($P = .016$). Degree of T-cell infiltration did not differ by HPV status but was significantly related to disease-specific survival (DSS) and overall survival (OS). Even after adjusting for HPV status, we found that CD8, FoxP3, and total T cells were significantly associated with DSS ($P = .0236$, $P = .0040$, and $P = .0197$, respectively) and OS ($P = .0137$, $P = .0158$, and $P = .0115$, respectively). Less T-cell infiltration ($P = .0130$) and CD4 cells in particular ($P = .0792$) were associated with higher EGFR expression.

Conclusions: Improved outcomes are associated with increased TILs independent of HPV status and suggest the local immune response may be more related to factors such as tumor size, EGFR expression, or performance status than HPV status. Further study of larger numbers of patients and infiltrates combined with functional analysis of individual subsets may be necessary to detect significant differences in local immunity in HPV-16–related cancers.

Key Words: Human papillomavirus, T cell infiltrates, oropharyngeal cancer, cellular immunity.

Level of Evidence: 1b.

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INTRODUCTION

It is unclear why patients with human papillomavirus-16 (HPV-16)–associated oropharyngeal cancer have a more favorable prognosis than patients with HPV-16–negative cancers.[1–3] Patients with HPV-16–associated cancers often are younger, frequently have large, cystic, regional metastases, and often are nonsmokers.[4–6] The better prognosis of such patients is likely due to differences in tumor biology and differing oncogenesis but may also reflect the role the host immune system and tumor microenvironment play in cancer homeostasis.[7,8]

To better characterize potential differences in host cellular immune reactivity among patients with HPV-16–positive or negative cancers, we undertook a systematic analysis of T-lymphocyte subpopulations in the peripheral blood and the tumor microenvironment in a cohort of patients with advanced oropharyngeal cancer who were entered in a prospective phase II clinical trial of induction chemotherapy followed by concurrent chemoradiation.[9] We previously identified significantly higher pretreatment levels of CD8-positive T lymphocytes in the peripheral blood of HPV-16 positive patients that correlated with improved survival.[10] Higher CD8 levels were also associated with tumor response to induction chemotherapy. Others have noted low CD8 cell infiltrates associated with poor cause-specific survival in laryngeal cancer[11] and higher T-cell infiltrates in

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patients with HPV-positive cancers that were associated with improved survival only in patients with HPV-negative tumors.[12] The current investigation extends these findings to an examination of the characteristics of the T-cell subpopulations infiltrating primary oropharyngeal cancers by immunohistologic assessment of select T-cell subpopulations in a tissue microarray (TMA) created from pretreatment biopsies.

MATERIALS AND METHODS

Patient Population

Of 66 patients entered in the prospective clinical trial, adequate tissue for creation of a TMA was available for 50 patients, and adequate tissue for immunohistologic analysis of T-cell infiltrates on the microarray was present for 46 patients. There were 36 male and 10 female patients. Mean age was 57 years (range, 39–77 years). A total of nine patients were stage III and 37 were stage IV. A total of 15 patients were T1,2; 17 were T3; and 14 were T4. Only seven patients were N0; 10 were N1; 24 were N2; and five were N3. All patients had previously untreated, potentially resectable cancers. Eleven patients were never smokers, 18 were past smokers having quit more than a year before diagnosis, and 17 were current smokers. Karnofsky performance status was 100 in 34 patients, 90 in eight patients, and 80 in four patients. Site of primary tumor was base of tongue in 27 and tonsil in 19 patients. Adequate DNA for HPV testing was available for 38 of these cases. A total of 25 patients had HPV-16–positive cancers, and 13 were negative for HPV-16 by highly sensitive and specific quantitative method (Sensigen, Ann Arbor, MI) using combined real-time competitive polymerase chain reaction and matrix-assisted laser desorption ionization-time-of-flight mass spectroscopy testing.[13]

Clinical Treatment, Response to Chemotherapy, and Survival

Patients received induction chemotherapy consisting of one cycle of cisplatin (100 mg/m2/day for 1 day) and 5-fluorouracil (1,000 mg/m2/day 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 was evaluated by surface measurements at direct laryngoscopy, supplemented by radiographic imaging (computed tomography or magnetic resonance imaging) for deeply infiltrative tumors. Patients who demonstrated at least a 50% tumor reduction of the primary tumor were considered responders and underwent definitive concurrent chemotherapy (cisplatin 100 mg/m^2 days 1, 22, 43) and radiation therapy (70 Gy; daily 2 Gy fractions $\times$ 7 weeks). Following radiation, two cycles of adjuvant paclitaxel (175 mg/m^2) every 21 days was administered to complete responders. Nonresponders to induction chemotherapy received salvage surgery and radiation. A total of 39 patients responded to induction chemotherapy and 36 were still considered responders 3 months after completion of concurrent chemotherapy and radiation. One patient was nonevaluable for induction response. Median follow-up of the patient cohort is 6.6 years. A total of 26 patients remained alive (56%), 15 died of their cancer, and five died of other causes.

Immunohistology

Pretreatment biopsies were retrieved from 50 of 66 patients for construction of a TMA. Pathology blocks were not available from 15 patients who had outside biopsies. Briefly, TMA slides created from biopsy specimens from the patients in this trial were deparaffinized, rehydrated, and peroxidase quenched (Dako Cytomation, Glostrup, Denmark). For antigen retrieval, slides were incubated with pepsin (epidermal growth factor receptor [EGFR]; 10 minutes at 37°C) or with citrate buffer (p16 and p53; 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, CA), p16/16P04, and p53/DO1 (Lab-Vision, Fremont CA) were added for 1 hour and were probed with avidin/biotin peroxidase (ABC Kit; Vector Laboratories, Burlingame, CA). Antibody binding was scored by a pathologist who was blinded to the clinical outcome using a continuous scale (i.e., 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 for tumor staining: 1 was less than 5%; 2 was 5% to 20%; 3 was 21% to 50%; and 4 was 51% to 100% tumor staining. At least 5% of cells had to stain for a tumor core to be considered positive. Intensity was scored as 1 = no staining; 2 = low intensity; 3 = moderate; and 4 = high intensity. Scores for multiple cores from each patient were averaged. For p53 mutation, *p53* exons 4 to 9 were amplified by using specific primers. Products from two polymerase chain reactions were sequenced in both directions and were analyzed by using Mutation Surveyor version 2.61 (Soft Genetics, State College, PA) and by manual review.

For the immunohistologic evaluation of tumor infiltrating lymphocytes (TILs), all tests were carried out on 5-μm formalin-fixed, paraffin-embedded TMA sections. Sections were baked in a hot-air oven at 65°C overnight. Each section was dewaxed using a series of xylene, graded alcohol, and buffer immersion steps. Antigen retrieval was performed in a preheated pressure cooker. Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using DAKO-labeled avidin-biotin-peroxidase method (LSAB+) and 3,3′-diaminobenzidine as the chromogen. Deparaffinized sections were stained with five types of Mabs: CD4 1:250 (Abcam ab846), CD8 1:40 (Nova Castra VP-C320), FOXp3 1:200 (Abcam Ab20034), CD104 1:50 (Beta-4 integrin, eBioscience 439-9b), and CD68 1:100 (Dako M0814). Appropriate negative (no primary antibody) and positive controls (tonsillar tissue and various carcinomas) were stained in parallel with each set of tumors studied. Digital photomicrographs were obtained at 20$\times$ magnification. The number of positively stained TILs for CD4, CD8, FOXP3, and CD68 was assessed quantitatively by two investigators blinded to patient outcome and HPV status. Tissue cores were initially examined after CD104 (beta-4 integrin) staining to confirm the presence and location of tumor nodules in each core, and only TILs infiltrating tumor nodules were counted (Fig. 1). This was because of the abundance of lymphoid aggregates normally present in the peritumoral stroma in oropharyngeal cancer patients that might not reflect a tumor-related immune response. TILs in each tumor core on the TMA were manually counted using a 20$\times$ objective lens. Methods for counting were adapted from published methods used to analyze T-cell infiltrates in follicular lymphoma and head and neck TMAs.[14,15] Three tumor cores and one normal tissue core were provided from each patient represented on the TMA. The mean count of replicate cores for each subject was used for analysis.

Pretreatment peripheral blood was analyzed by routine automated flow cytometry for T, B, and NK cells and subpopulations of CD3-, CD4-, and CD8-positive T lymphocytes. Detailed methods have been previously described.[10,16] 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 Pathology Department. The results of correlations with survival and HPV-16 status have been previously published.[10]

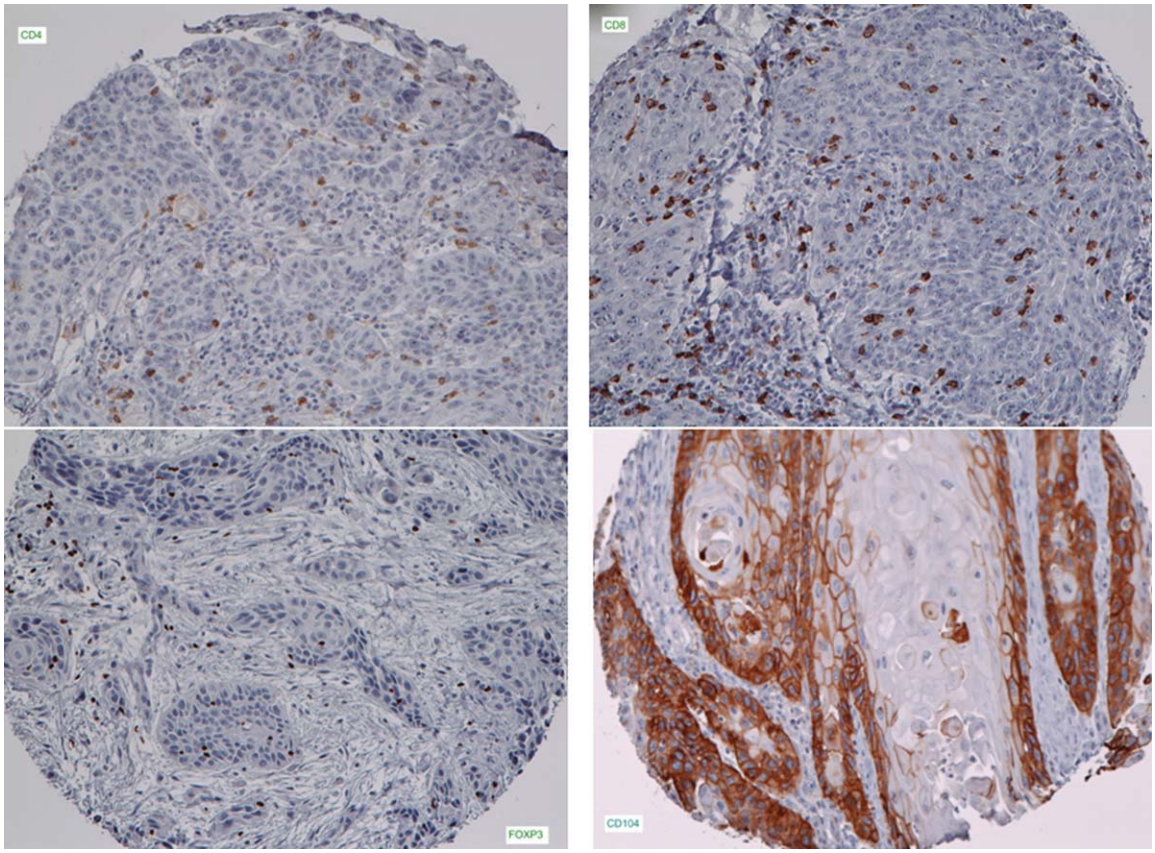

Fig. 1. Representative tissue microarray cores with immunohistochemical staining for CD4, CD8, FoxP3 cells, or beta-4 integrin (CD104). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Statistical Methods

Statistical analysis was carried out in two parts. First, all subjects who had measured infiltrate levels (n = 46) were analyzed for correlations with clinical outcomes, tumor, and patient characteristics. Second, correlations between infiltrate levels and peripheral blood lymphocyte levels (n = 34) or HPV-16 status (n = 38) were performed on only those with both sets of information.

Infiltrate levels were treated as continuous variables in all analyses. Rank-based statistical methods were used to assess univariate associations between infiltrate levels and covariates of interest. Patient level averages for EGFR score were used in this analysis. Patients were categorized as current, past (quit >12 months prior), or never smokers. Spearman rank correlation was used to assess the correlation with ordinal variables, and the Wilcoxon rank sum test was used to test variables with two groups.

Disease-specific survival (DSS) was defined as the time to death from oropharyngeal cancer. Patients who were alive at last follow-up or who died as a result of reasons other than their cancer were censored. Cox proportional hazards models were used to assess time-to-event outcomes. For each outcome, three models were constructed: a model with infiltrate level (treated as a continuous variable), a model with clinical variables, and a model with both clinical variables and infiltrate level. Likelihood ratio tests were used to compare models, and the maximum likelihood estimate for coefficients in a model was used to test significance after controlling for other variables in the model.

All statistical analyses were done in SAS version 9.2 (SAS Institute, Carey, NC). Because of the relatively small sample size, adjustments for multiple comparisons were not performed, as these analyses were retrospective and exploratory in nature. A two-tailed P value of .05 or less was considered statistically significant.

RESULTS

The major finding in this study of immune cell infiltrates in pretreatment biopsies from oropharyngeal cancer patients was the lack of significant differences in types of T-cell infiltrates among HPV-16–positive and negative patients. The pattern of T-lymphocyte subset infiltration did not differ by HPV-16 status in this group of advanced oropharyngeal cancer patients entered in this prospective, uniform treatment clinical trial. Although the mean CD4/CD8 ratio of tumor-infiltrating T lymphocytes tended to be lower in HPV-16–positive patients and the sum of CD4 and CD8 cell infiltrate counts tended to be higher, these differences were not statistically different (Table I). Likewise, no differences were noted for FoxP3 or CD68 infiltrate counts by HPV status; however, the ratio of FoxP3 cells to CD8 cells tended to be higher in HPV-negative patients ($P = .099$) primarily because of higher mean FoxP3 cell counts in HPV-negative patients. This was in contrast to the significant differences previously noted in the peripheral blood T-lymphocyte subsets in these two groups of patients where CD8 cell levels were higher and CD4 cell levels were lower in HPV-16–positive patients.[10]

TABLE I.
Mean (± Standard Error of Mean) T-Cell Subset Infiltrates in Human Papillomavirus-16–Positive and Negative Cancers.

	HPV Negative (n = 13)	HPV Positive (n = 25)
CD4	174 ± 31	189 ± 27
CD8	169 ± 37	210 ± 47
FoxP3	159 ± 36	107 ± 18
CD68	241 ± 42	250 ± 44
CD4 + CD8 sum	345 ± 62	413 ± 66
CD4/CD8 ratio	4.16 ± 2.78	1.56 ± 0.35
FoxP3/CD8 ratio	1.27 ± 0.32	0.74 ± 0.12

HPV = human papillomavirus.

Our analysis confirmed that HPV-16 status and EGFR expression were the most significant prognostic factors for overall survival in this study ($P = .0455$, hazard ratio: 0.32, 95% confidence interval [CI]: 0.12-0.85; and $P = .0083$, hazard ratio: 1.89, 95% CI: 1.18-3.04, respectively). When T-cell infiltrates were analyzed with respect to overall survival, the infiltrate CD4/CD8 ratio ($P = .0056$) and sum of CD4 and CD8 mean infiltrate counts were significant ($P = .0154$) predictors of overall survival and were still important after adjusting for HPV status ($P = .034$ and $P = .0298$, respectively, Cox regression). After controlling for EGFR, these T-cell infiltrate measures remained significant ($P = .008$ and $P = .04$, respectively, Cox regression). Overall survival tended to be better in patients with higher tumor infiltrates of CD8 cells ($P = .0615$), and the mean CD8 infiltrate count added prognostic significance after adjusting for HPV status ($P = .0137$, likelihood ratio test). Mean infiltrate levels according to whether patients survived or were dead are shown in Table II. Likewise, higher FoxP3 infiltrate counts were also significantly associated with better overall survival after adjusting for HPV status ($P = .029$, Cox regression).

DSS was significantly associated with EGFR expression ($P = .015$, hazard ratio: 1.95, 95% CI: 1.14-3.35), HPV status ($P = .026$, hazard ratio: 0.24, 95% CI: 0.07-0.82), peripheral blood CD4/CD8 ratio ($P = .047$) and smoking history ($P = .003$). Of the various infiltrate markers, only the infiltrate CD4/CD8 ratio, the FoxP3/CD8 ratio, and sum of CD4 plus CD8 cell infiltrates were significantly associated with DSS ($P = .0058$, $P = .0457$, and $P = .0244$, respectively) (Fig. 2 and 3).

TABLE II.
Mean (± Standard Error of Mean) T-Cell Infiltrates by Disease Status.

	Alive	Dead
CD4	203 ± 27	152 ± 21
CD8	253 ± 50	130 ± 22
FoxP3	149 ± 27	98 ± 19
CD68	267 ± 43	209 ± 36
CD4 + CD8 sum	494 ± 70	276 ± 35
CD4/CD8 ratio	1.15 ± 0.22	3.78 ± 1.86
FoxP3/CD8 ratio	0.72 ± 0.10	1.09 ± 0.24

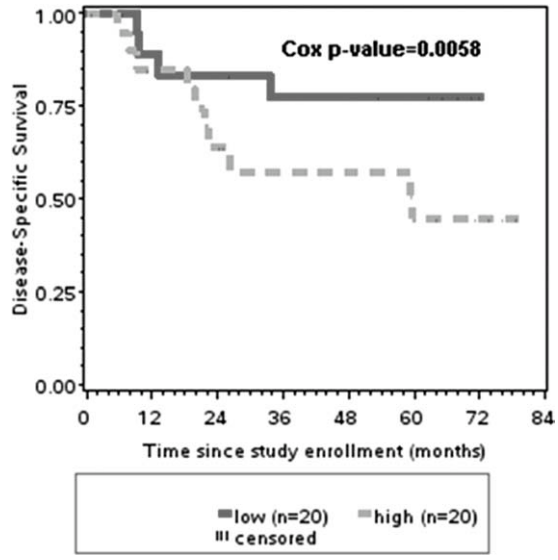

Fig. 2. Disease-specific survival was significantly better in patients with low CD4/CD8 ratio in tumor infiltrates compared to patients with higher (>median) ratio ($P = .0058$).

However, after adjusting for HPV-16 status, individual levels of CD8 and FoxP3 infiltrates added significant prognostic information ($P = .0236$ and $P = .004$, Likelihood ratio test), and EGFR intensity remained an important factor (Table III).

Smoking status was also a significant prognostic factor for both overall survival ($P = .01$) and DSS ($P = .003$) and remained significant for DSS after controlling for HPV-16 status ($P = .009$). The sum of mean CD4 and CD8 cell infiltrates was significantly higher in nonsmokers ($P = .047$), and FoxP3 infiltrates also tended to be higher in nonsmokers ($P = .081$). After controlling for

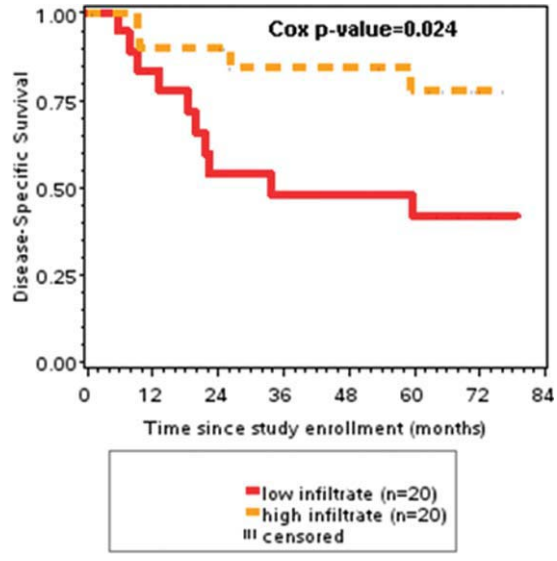

Fig. 3. Disease-specific survival was significantly better in patients with higher CD4 and CD8 T-cell tumor infiltrates compared to patients with lower (<median) T-cell infiltrates ($P = .024$). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

TABLE III.
P Values for the Association of Infiltrate Subsets With Disease-Specific Survival by Cox Regression.

	Univariate	Adjusted for HPV-16 Status*
HPV-16†	.0263	—
EGFR expression	.0150	.0470
CD4/CD8 ratio	.0058	.0554
CD4 + CD8 sum	.0244	.0197
FoxP3/CD8 ratio	.0457	NS
CD8	.0848	.0236
FoxP3	.0824	.0040

*Likelihood ratio test.
†Log transformed.
HPV = human papillomavirus; EGFR = epidermal growth factor receptor; NS = not significant.

smoking, the CD4/CD8 infiltrate ratio, the FoxP3/CD8 ratio, and the sum of CD4 and CD8 infiltrates were still prognostically important for overall survival ($P = .046$, $P = .0372$, and $P = .029$, respectively). After controlling for smoking and EGFR intensity, the CD4/CD8 infiltrate ratio was still prognostically important for overall ($P = .026$) and DSS ($P = .030$). For DSS, EGFR intensity, HPV status, peripheral blood CD8 levels, and the CD4/CD8 infiltrate ratio were significant prognostic factors after controlling for smoking. For overall survival, after controlling for both smoking and HPV status, both CD4/CD8 ratio and infiltrate sum showed a trend for prognostic significance ($P = .07$ for each).

When clinical features were analyzed, there were no significant differences in overall T-cell infiltrates with respect to tumor stage, response to induction chemotherapy, or overall tumor response after concurrent chemoradiation. Overall survival and DSS did not differ by tumor stage ($P = .9016$) or T class ($P = .1727$). However, overall and DSS differed significantly by N class ($P = .0237$, $P = .0032$ respectively). Mean CD8 infiltrates and total CD4 and CD8 counts were higher in node-positive patients ($P = .011$, Wilcoxon for both), yet CD8 infiltrates declined with increasing T class ($P = .003$, Spearman rho = −0.424). Because CD8 infiltrates increased with N stage and were associated with improved survival, we analyzed N class with respect to overall survival and DSS. We found that contrary to what is typical for other tumor sites, increasing N class was not a negative prognostic factor in this cohort of oropharyngeal cancer patients. This was primarily due to better DSS in patients with advanced (N2) neck disease. This is likely related to patients with HPV-16–positive cancers who often have small primary tumors and multiple small lymph nodes or a single large cystic node. Recurrence rates were highest in patients with N0 disease who had larger primary cancers. Interestingly, CD8 infiltrates were also significantly higher in association with better patient Karnofsky performance status ($P = .003$, Spearman rho = 0.43), and the infiltrates of CD4 and FoxP3 cells also tended to be increased in those patients ($P = .10$, $P = .18$, Spearman, respectively), which resulted in the sum of T-cell infiltrates (CD4 plus CD8) being significantly higher when performance status was better ($P = .004$, Spearman rho = 0.45).

Individual T-cell subset infiltrates were not directly associated with either EGFR expression or p53 mutation status. T-cell subset levels in the peripheral blood were available in 34 of the 46 patients with cancers analyzed for T-cell tumor infiltrates. Type and degree of specific T-cell subset infiltration was not related to peripheral blood levels of the various subsets except for CD4 infiltrates, which tended to be higher when peripheral blood natural killer cell levels were low ($P = .0133$, rho = −0.439). Levels of each of the infiltrate subsets except for CD68 cells tended to be directly correlated with each other ($P < .0001$) and with total T cells. In particular, levels of FoxP3 cells correlated significantly and directly with both CD4 ($P = .0364$) and CD8 ($P < .0001$) infiltrates. Levels of CD8 and FoxP3 cells were inversely correlated with the CD4/CD8 ratio ($P < .0001$ and $P = .012$, respectively).

DISCUSSION

This is the first study to correlate pretreatment peripheral blood T-lymphocyte levels with T-cell tumor infiltrates in patients with advanced oropharyngeal cancer and to examine the association with HPV-16 status and other known prognostic factors such as EGFR expression, smoking status, tumor characteristics, and performance status. A major finding was the lack of association of T-cell infiltrates with HPV status or with EGFR expression. This is particularly interesting since our prior study of peripheral blood T-cell levels demonstrated that increased CD8 cell levels were directly associated with HPV-16 status, lower EGFR expression, response to induction chemotherapy, and favorable prognosis.[10] This suggests that peripheral blood markers of adaptive immunity are not surrogates for immune parameters in the primary tumor microenvironment. Despite the lack of association with HPV status, the lower tumor infiltrate CD4/CD8 ratio and higher mean sum of CD4 and CD8 infiltrate were predictive of overall survival and DSS, indicating that an immune response in the local tumor microenvironment may be an important factor in overall prognosis. Generally, TILs are thought to represent a host immune response. It would be expected that infiltrates would be beneficial.[17] However, even in HPV-related cervical cancer, large polyclonal infiltrates of T cells are seen that appear to be functionally inactive.[18] Animal model studies suggest that multiple subtypes of CD4 and CD8 are necessary to generate tumor reactive CD8 functional activity and that TILs can be both immune reactive and immunosuppressive.[19,20] Relative expansion of infiltrates of FoxP3 regulatory T cells (Treg) and cytotoxic T cells have been described in head and neck cancer patients, and either no association with outcome or improved locoregional tumor control was found.[15,21–23] In many other types of cancer such as breast, colorectal, hepatocellular, gastric, and pancreatic, high levels of tumor-infiltrating Treg cells have been associated with poor outcome.[24] These were studies primarily of adenocarcinoma patients in

whom histology and immune reactivity differ from patients with squamous carcinoma.

In our study, the associations of T-cell infiltrates with survival remained significant after adjusting for EGFR expression and HPV status. This important observation suggests that the local immune response and its impact on outcome may be independent of other important prognostic markers and not directly related to peripheral blood measures of cellular immunity, which previously were shown to differ by HPV status.[10] Recently, gene signatures of adaptive immunity have been shown to outperform HPV status in prognostication.[21] Increased levels of CD3-positive tumor-infiltrating T cells in general have been reported in oropharyngeal cancer patients who were without regional metastases that were related to HPV-16 status.[12,22] Others have reported that higher levels of activated CD4 and FoxP3 cells in tumor stroma are associated with improved locoregional control and survival[23] in a patient cohort consisting of multiple sites of head and neck cancer. CD4+ FoxP3+ regulatory T cells recovered from the peripheral blood of head and neck cancer patients have been associated with local immune suppression mediated by IL-10 and TGF-beta.[25] However, higher levels of such suppressive cells in the blood were found in patients with no evidence of disease after therapy.[25,26,27] Further, when analyzed in human tumor sections, higher Treg cell levels were associated with improved prognosis,[23,28] which was consistent with our findings. Levels of these cell populations may be less important than the balance of cells within tumor or stroma. Improved survival has been reported for higher ratios of CD8 to Treg cells in both tumor and stroma of oral cancer.[29] Our results are consistent with these observations and indicate that this association is primarily due to differences in levels of Treg cells rather than CD8 cells. Also, understanding the function of such cells in the local tumor environment and their influence on the inflammatory response will be critical to determining in what situations the local immune response and ratio of effectors is beneficial or detrimental to outcome.

It is remarkable and encouraging that we found significant associations of lymphocyte infiltrates with outcome in this study, which utilized the small tissue cores present on a tumor microarray created from pretreatment samples. Tumor-bearing tissue in the biopsies was specifically selected by the pathologist in creating the arrays; however, there was no effort to specifically select any particular area of the tumor, leading to the possibility of sampling errors for areas representative of a local tumor immune response. Also, many of these tumors had associated lymphoid components because of the normal lymphoid tissue present in the tonsil and tongue base sites, suggesting the possibility that some of the infiltrates could represent resident normal lymphoid aggregates. To minimize this potentially confounding possibility, the tissue cores were also stained with Beta-4 integrin (CD104) to specifically identify tumor within the cores samples, and only T lymphocytes present in the tumor nodules were counted to minimize this confounding variable.

This is the first analysis to look at response to induction chemotherapy and local tumor immune parameters in patients with oropharyngeal cancers. We previously found that higher systemic CD8 cell levels were associated with tumor response to chemotherapy. In this further analysis, we found that tumor response was not associated with any of the T-lymphocyte infiltrates. This was consistent with other reports of CD4+ lymphocyte infiltrates measured in the stroma of a heterogeneous cohort of head and neck patients[24] and contrasts findings of higher tumor infiltrating CD8+ T cells in metastatic lymph nodes from patients with favorable outcomes.[15] It was disappointing that tumor response to chemotherapy was not associated with T-lymphocyte infiltrates, as has been reported in breast cancer.[30]

The consistent correlations of higher T-cell infiltrates with favorable outcome are also reflected in the association of higher CD8 infiltrates in patients with lower T classifications and better performance status. These patients would be expected to have a better outcome. Infiltrate levels of CD4, CD8, and FoxP3 cells were also associated with the important prognostic factor of smoking status, and a trend for improved survival with higher infiltrates was present even after adjusting for smoking habit. The potential effects of smoking on tumor initiation, progression, epigenetic gene methylation and inflammation are probably some of the factors influencing T-cell levels and outcome in oropharyngeal cancer patients beyond simple HPV status. The relative importance of these various factors related to the attraction and retention of immune cells to the tumor microenvironment remains largely unknown. Our findings extend those of others[8,17,31] and suggest that immunologic methods to increase immune reactive, cytotoxic CD8 cell levels in the peripheral blood or in the tumor microenvironment may be beneficial in both HPV+ and HPV− cancer patients. Evidence from studies in head and neck[23,31,32] and other cancer types such as breast,[33] ovarian,[34] or colon,[35] support the conjecture that there are differences in numbers and function of T lymphocytes, and Treg cells in particular, in intratumoral versus peritumor stromal locations and that the association of regulatory T cells with outcome differs by tumor type.[36] Thus, tumor-specific and site-specific studies in homogeneous populations of head and neck cancer patients are necessary to better understand how peripheral blood levels and tumor infiltration by immune reactive cells affect prognosis. It remains to be seen if experimental vaccination schemes can modulate levels of both regulatory and cytotoxic T cells in the tumor microenvironment of patients with HPV-positive cancers.

CONCLUSION

Improved outcomes are associated with increased TILs independent of HPV status and suggest the local immune response in oropharyngeal cancer may be related in part to factors such as tumor size, EGFR expression, smoking history, performance status, or innate immunity rather than HPV status. Assessment of

TILs in tissue microarrays is difficult due to small core sample size and variation in tumor representation in tissue cores. Further study of larger numbers of patients and infiltrates in whole tumor sections combined with functional analysis of individual subsets may be necessary to detect significant differences in local immunity in HPV-16–related cancers. The lack of differences in infiltrating T cells in patients with oropharyngeal cancer regardless of HPV status suggest that immunotherapy regimens that are effective in HPV-positive cancer patients should also be explored in patients with HPV-negative cancer.

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