Prognostic significance of tumor-infiltrating lymphocytes in oropharyngeal cancer

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Abstract

The presence of tumor-infiltrating lymphocytes has been shown to significantly improve clinical outcomes in many types of cancer. However, their effects on outcomes in patients with oropharyngeal cancer specifically have yet to be elucidated. We conducted a retrospective study in an effort to shed light on this issue. We reviewed the records of 48 consecutively presenting patients with oropharyngeal cancer, and we performed immunohistochemistry to analyze their archived paraffin-embedded tissue samples for the presence of CD3-positive tumor-infiltrating lymphocytes. We also used real-time polymerase chain reaction testing to look for human papillomavirus type 16 (HPV-16) in the tumors. We found that patients with large numbers of tumor-infiltrating lymphocytes (CD3\textsuperscript{high}) had a significantly lower incidence of metastasis at presentation than did those with low numbers of tumor-infiltrating lymphocytes (CD3\textsuperscript{low}) (40.0 vs. 88.5%; \( p = 0.001 \)), regardless of HPV status. When HPV status was taken into account, the correlation between a high CD3 count and a lower rate of metastasis was maintained in the HPV-positive patients but not in the HPV-negative patients. We also found that the CD3\textsuperscript{high} patients had higher rates of overall survival and disease-free survival at 3 and 5 years than did the CD3\textsuperscript{low} patients; however, these differences only approached but did not reach statistical significance.

Introduction

Human papillomavirus (HPV) -associated head and neck squamous cell carcinoma (HNSCC) is a unique form of cancer. Between 15 and 23% of all HNSCCs are associated with HPV infection; as many as 115,000 cases are diagnosed each year globally.\(^1\)\(^-\)\(^5\) The most common site of HPV-associated HNSCC is the oropharynx, and the type of HPV most often identified in HPV-associated HNSCC is HPV type 16.\(^5\)\(^,\)\(^6\)

The management of this distinct type of HNSCC presents otolaryngologists with new challenges and new opportunities. The presence of viral proteins within tumor cells provides well-characterized targets for novel diagnostic, preventive, and therapeutic modalities. One such novel therapeutic modality is immunotherapy. The goal of immunotherapy is to generate an immune response capable of eliminating bulky tumors. Several therapeutic head and neck cancer vaccines are being investigated in preclinical and/or clinical trials.\(^1\)\(^,\)\(^7\) These vaccines are designed to create a tumor-infiltrating lymphocyte response that will lead to the elimination of cancerous cells.

Studies have shown that the number of tumor-infiltrating lymphocytes in a tumor is correlated with survival in several different types of cancer, including ovarian,\(^5\) pancreatic,\(^8\) skin,\(^9\) and oral\(^10\) cancer. However, to the best of our knowledge, no similar study of tumor-infiltrating lymphocytes in oropharyngeal cancer has been published.

In this article, we describe our investigation into whether the number of CD3-positive tumor-infiltrating lymphocytes is correlated with clinical outcome in a cohort of patients with oropharyngeal cancer in general and HPV-associated HNSCC in particular.

Patients and methods

We reviewed the case records of 48 consecutively presenting patients—40 men and 8 women, aged 37 to 83 years (mean: 58.8)—who had been treated surgically for squamous cell carcinoma of the oropharynx at the Hospital of the University of Pennsylvania from January 1996 through December 2001. In addition to demographic data, we recorded data on tumor site and tumor stage at presentation. Tumors were staged in accordance with American Joint Committee on Cancer criteria.
We also noted the length of overall survival and disease-free survival during follow-up (mean 38.6 mo) and the incidence of metastasis at presentation:

- Overall survival was calculated from the date of treatment to either the date of death or the date of the last documented clinical encounter.
- A recurrence was defined as the presence of a pathologically verified squamous cell carcinoma in the oropharynx after initial surgical treatment. Disease-free survival was calculated from the date of treatment to the date when a recurrent squamous cell carcinoma was documented at the primary site or in the neck.
- The presence or absence of regional lymphatic metastasis at presentation was determined (1) by histologic confirmation of a positive neck node in those cases in which a neck dissection was performed or (2) by clinical and/or radiographic analysis in the absence of a neck dissection. Patients were deemed to be N0 if findings on neck dissection were negative or if preoperative computed tomography (CT) demonstrated no pathologically enlarged nodes.

**DNA extraction from paraffin sections.** Tissue samples were obtained in accordance with a protocol approved by the Institutional Review Board of the University of Pennsylvania.

Paraffin-embedded tissue was taken from each of the tumor samples in 7- to 10-mm sections, and the sections were mounted on glass slides. On the slides, areas of squamous cell carcinoma were outlined on a hematoxylin- and eosin-stained slide by a board-certified pathologist (E.E.). DNA was extracted by scraping each slide with an 18-gauge needle in the area that the pathologist had labeled tumor. The QIAamp DNA Mini Kit (Qiagen; Valencia, Calif.) was used to purify the DNA.

Briefly, the protocol was carried out thus:

The samples were deparaffinized with xylene. The cells then were resuspended in Buffer ATL and digested with the Proteinase K solution provided in the kit. The samples were then incubated at 56°C for 3 hours on a rocking platform to ensure inactivation of the Proteinase K. Protein was precipitated with Buffer AL and incubated at 70°C for 10 minutes. Ethanol (100%) was then added to the sample to precipitate the DNA. The mixture was applied to a QIAamp spin column in a 2-ml collection tube and centrifuged at 20,000 × g (14,000 rpm) for 1 minute. The filtrate was discarded, 500 μL of Buffer AW1 was added to the column in a clean 2-ml collection tube, and the tube was centrifuged at 20,000 × g for 1 minute. Next, the filtrate was discarded, 500 μL of Buffer AW2 was added to the column in a clean 2-ml collection tube, and the tube was centrifuged at 20,000 × g for 3 minutes. Finally, the sample was rehydrated with Buffer AE. All samples were stored at 4°C for later use.

**Polymerase chain reaction.** The integrity of the DNA was verified by conventional polymerase chain reaction (PCR) assay with 2 sets of primers. Samples were amplified with primers (PC04/GH20) specific for the human β globin gene. The amplicon size was 268 base pairs.

The forward primer sequence was:

GH20 (GGG GAA GAG CCA AGG ACA GGT AC).

The reverse primer sequence was:

PC04 (GGG CAA CTT CAT CCA CGT TCA CC).

Samples were also amplified with primers specific for the *BRAF* gene, which encodes a ubiquitous serine/threonine kinase. The amplicon size was 280 base pairs.

The forward primer sequence was:

5′-GGC CAA AAA TTT AAT CAG TGG A-3′.

The reverse primer sequence was:

5′-TCA TAA TGC TTG CTC TGA TAG GA-3′.

Only those DNA samples that were of sufficient quality to be amplified by both sets of primers were included in this study.

**Quantitative real-time PCR for HPV-16 detection.** Quantitative PCR was performed with an ABI Prism 7900HT Sequence Detection System (Applied Biosystems; Foster City, Calif.).

The forward primer sequence was:

E7 5′-GGG TGA AAT AGA TGG TCC AGC TG-3′.

The reverse primer sequence was:

E7 5′-CAC TTG CAA CAA AAG GTT ACA ATA TTG-3′.

The probe sequence was:

E7 5′-FAM-ACA AGC AGA ACC GGA CAG AGC CCA TT-TAMRA-3′.

The E7 primers and probe were used to amplify HPV-16 E7 from 10 ng of each sample DNA in triplicate. We also used DNA from the CaSki cell line (600 viral copies of HPV-16/cell) in our analysis. In order to standardize the DNA, we ran each of these samples with the β globin primers and probe (Applied Biosystems) simultaneously with the E7 primers. Using serial dilutions of CaSki DNA (50 ng, 5 ng, 0.5 ng, 0.05 ng, and 0.005 ng), standard curves were
developed for HPV copy number as well as for β globin. The HPV copy number is determined by comparing the data generated by our unknown samples with that of the CaSki DNA. An HPV copy number greater than 0.1/cell was considered positive for HPV-16.

**Tissue microarray construction.** The microarray was made at the Pathology Core of the Children’s Hospital of Philadelphia. In brief, 0.6-mm core samples were taken from the paraffin-embedded tumor tissues and assembled on a recipient paraffin block with a micrometer-precise coordinate system for assembling tissue cores on a block. Six cores from each tissue sample were placed at 1-mm intervals on the block. After construction, multiple 5-μm sections were prepared with a microtome for immunohistochemical analysis.

**Immunohistochemical staining.** The paraffin-embedded slides were then stained for the expression of CD3 in the Pathology Core at the University of Pennsylvania. (CD3 is a complex of four transmembrane signaling chains that is present on most lymphocytes. CD3 polyclonal rabbit antihuman antibody was provided by the Core.) The slides were deparaffinized and hydrated with xylene and a graded alcohol series. Slides were then heated with 1× citrate buffer (10 mM; pH: 6.0; Lab Vision; Fremont, Calif.) for 8 minutes in a 1,200-W oven at 70% power. Slides were then cooled for 20 minutes. Primary antibody was applied to the slides for 30 minutes. The slides were washed and incubated with biotinylated goat secondary IgG for 30 minutes at room temperature. Then the slides were washed again and processed with the hors eradish peroxidase-based EnVision System (Dako-Cytomation; Carpinteria, Calif.). The slides were washed again, counterstained with hematoxylin, and dehydrated with a graded alcohol series.

The slides were then examined by the pathologist, who determined the degree to which CD3-positive cells were present in the tumor (figure 1). Samples were graded on a scale of 0 to 3+: 0 (no positively staining cells), 1+ (few), 2+ (a moderate amount), or 3+ (many). All the slides were read at one sitting in a blinded fashion.

Each of the cores corresponding to a patient’s tumor was graded individually, and the scores were averaged so that a representative score could be assigned to each tumor. Tumors were classified as either CD3<sup>low</sup> (mean score: ≤1) or CD3<sup>high</sup> (>1).

**Statistical analysis.** Prognostic covariates included in the statistical analysis were the degree of CD3 infiltration, the stage of disease at presentation, HPV status, and rates of overall survival, disease-free survival, and metastasis. Correlation between CD3 infiltration and HPV status with the other prognostic variables was evaluated according to Fisher’s exact test or the chi-square test for trend. Analysis of differences in overall survival between groups was based on the log-rank statistic and Kaplan-Meier analysis.

### Results

**CD3 status.** Of the 48 patients, 22 were designated CD3<sup>high</sup> (45.8%) and 26 were CD3<sup>low</sup> (54.2%).

**Tumor site.** The most common sites of oropharyngeal cancer were the tonsils (68.8% of cases) and the tongue base (22.9%) (table 1).

**Tumor stage.** Almost two-thirds of tumors (64.6%) were stage IV (table 1). It is interesting that when data were analyzed according to T category, 10 of 25 CD3<sup>low</sup> patients (40.0%) presented with a category T4 lesion, compared with only 3 of the 22 CD3<sup>high</sup> patients (13.6%) (figure 2).

The difference approached statistical significance (p = 0.0561). (For 1 of the CD3<sup>low</sup> patients, sufficient data were not available to accurately assign a T category, although we...
know that the patient did have stage IV disease because of documented cervical metastases.)

**HPV-16 status.** Among the entire group of 48 patients, 33 patients (68.8%) were HPV-positive. Of these 33, 17 (51.5%) were CD3<sup>high</sup> and 16 (48.5%) were CD3<sup>low</sup>. Of the 15 HPV-negative patients, 5 (33.3%) were CD3<sup>high</sup> and 10 (66.7%) were CD3<sup>low</sup>.

Survival. The CD3<sup>high</sup> patients had a higher overall survival rate than did the CD3<sup>low</sup> patients at 3 years (77 vs. 64%) and at 5 years (77% vs. 51%), but the differences were not statistically significant \((p = 0.152)\) (figure 3, A).

Disease-free survival rates were also higher in the CD3<sup>high</sup> patients at 3 years (71 vs. 44%) and at 5 years (71 vs. 37%). These differences approached but did not reach statistical significance \((p = 0.09)\) (figure 3, B).

Metastasis. Of the 48 patients, 46 had undergone either a neck dissection or CT that objectively documented the presence or absence of lymphatic disease. Among these 46 patients, we found a strong correlation between the degree of lymphocytic infiltration and the presence or absence of metastasis at presentation. Among the 20 CD3<sup>high</sup> patients, only 8 (40.0%) had documented metastasis (table 2). By contrast, metastasis was documented in 23 of 26 CD3<sup>low</sup> patients (88.5%). The difference was statistically significant \((p = 0.001)\).

Because metastasis was the most significant clinical parameter we found, we extended our analysis to examine its correlation with CD3 and HPV status. Of the 17 patients who were CD3<sup>high</sup> and HPV-positive, accurate lymphatic staging was available for 16. Of these 16, only 6 (37.5%) had metastatic disease at presentation, compared with 14 of 16 CD3<sup>low</sup> and HPV-positive patients (87.5%) (table 3). This difference was statistically significant \((p = 0.009)\).

A high CD3 count provided no such significant benefit in HPV-negative patients. The CD3<sup>high</sup> HPV-negative patients for whom accurate metastasis data were available had a much higher rate of metastasis (3 of 4; 75.0%) than did the CD3<sup>high</sup> HPV-positive patients (37.5%). Among the CD3<sup>low</sup> patients, 9 of 10 HPV-negative patients (90.0%) had metastatic disease at presentation, a rate comparable to that among the HPV-positive patients (87.5%). The difference between rates of metastasis between CD3<sup>high</sup> and CD3<sup>low</sup> HPV-negative patients was not statistically significant \((p = 0.5055)\) (table 3).

Finally, we compared rates of metastasis according to HPV status in CD3<sup>high</sup> patients alone. We expected that the difference would not be statically significant and, indeed, the \(p\) value was 0.6090 (data not shown).
Discussion

In this study, we showed that the presence of large numbers of tumor-infiltrating lymphocytes was inversely correlated with the incidence of nodal metastasis in our cohort of patients with oropharyngeal squamous cell carcinoma, particularly those who were HPV-positive. This finding illustrates the important role that the immune system may play in influencing the course of oropharyngeal cancer. The importance of the immune system in controlling other types of cancer is well documented. For example, immunocompromised patients are known to be at high risk for different types of lymphomas. With regard to head and neck cancer, the evidence is more indirect, but it is still convincing. It has been shown that many head and neck cancers probably evade immune surveillance by downregulating key immunologic molecules and by creating a tumor microenvironment hostile to immune function.

Two reasons can be postulated to explain the relationship between tumor-infiltrating lymphocytes and metastasis:

• First, it is conceivable that the presence of these lymphocytes helps to limit tumor volume and tumor invasiveness, which may prevent metastasis. We saw far more T4 lesions in CD3\textsuperscript{low} patients than in CD3\textsuperscript{high} patients.

• Second, the presence of tumor-infiltrating lymphocytes in the tumor may reflect the presence of tumor-specific lymphocytes in the draining cervical lymph nodes. Tumor-specific lymphocytes in the lymph nodes may then target metastatic tumor cells and eliminate them at a higher rate in CD3\textsuperscript{high} patients. Studies are under way to help confirm the presence of tumor-specific lymphocytes in the lymph nodes in certain oropharyngeal cancer patients.

Most of the oropharyngeal cancer patients in our cohort were HPV-16–positive. It is well documented that HPV-positive patients tend to have better clinical outcomes than do HPV-negative patients, and we attempted to determine whether tumor-infiltrating lymphocytes may play a role in that difference. Our finding that a large number of tumor-infiltrating lymphocytes was correlated with a lower rate of metastasis in HPV-positive tumors but not in HPV-negative tumors must be examined in light of the fact that the size of our sample of HPV-negative patients was small. However, this trend begs an important question: Are the tumor-infiltrating lymphocytes in HPV-positive tumors better able to limit tumor progression because of a stable tumor antigen, which is not present in HPV-negative tumors? There is evidence that HPV-specific lymphocytes are present in the circulation of HPV-associated oropharyngeal cancer patients, and studies are under way in our laboratory to determine what percentage of lymphocytes in the tumor are HPV-specific. If we can show that the presence of HPV-specific tumor-infiltrating lymphocytes correlates with better clinical outcomes, we would provide further justification for efforts to develop immunotherapeutic strategies that generate these tumor-infiltrating lymphocytes.

In conclusion, our results show a correlation between a high degree of lymphocyte tumor infiltration and a low rate of metastasis in oropharyngeal cancer patients. This correlation was maintained in the subset of HPV-positive patients, but not in HPV-negative patients. This finding has direct implications on the future prognostic and immunotherapeutic modalities for this clinical entity. Further investigation of the effect of HPV on tumor-infiltrating lymphocytes and the tumor microenvironment in general is warranted.

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