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# Proportion of CD4 and CD8 tumor infiltrating lymphocytes predicts survival in persistent/recurrent laryngeal squamous cell carcinoma

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ABSTRACT

Tumor infiltrating lymphocytes (TILs) have been shown to be an important prognostic factor in patients with previously untreated head and neck cancer. After organ preservation therapy for laryngeal cancer and subsequent persistence/recurrence, the prognostic value of TILs is unknown. Our goal was to determine if TILs have value as a prognostic biomarker in patients with surgically salvageable persistent/recurrent laryngeal squamous cell carcinoma.

Levels of TILs were quantified on tissue microarrays from 183 patients undergoing salvage total laryngectomy for persistent/recurrent laryngeal cancer after radiation or chemoradiation between 1997 and 2014. Demographic and clinical data were abstracted. Immunohistology evaluation included CD4, CD8, PDL-1, p16, CD31, Vimentin, EGFR, and p53.

Elevated levels of either CD8 or CD4 positive TILs were associated with improved disease specific survival (CD8: HR 0.46, 95% CI 0.24–0.88, CD4: HR 0.43; 95% CI 0.21–0.89) and disease free survival (CD8: HR 0.53, 95% CI 0.29–0.94, CD4: HR 0.52; 95% CI 0.27–0.99). Levels of CD8 (HR 0.74; 95% CI 0.47–1.17) or CD4 (HR 0.66; 95% CI 0.40–1.08) TILs were not significantly associated with overall survival. In bivariate analysis, patients with elevated CD4 and/or CD8 TILs had significantly improved disease specific survival (HR 0.42; 95% CI 0.21–0.83) and disease free survival (HR 0.45; 95% CI 0.24–0.84) compared to patients with low levels of CD4 and CD8. PDL-1, p16, CD31, Vimentin, EGFR, and p53 were not significant prognostic factors. On multivariate analysis, elevated CD8 TILs were associated with improved disease specific survival (HR 0.35; 95% CI 0.14–0.88, p = .02) and disease free survival (HR 0.41; 95% CI 0.17–0.96, p = .04).

CD8, and possibly CD4, positive TILs are associated with favorable disease free and disease specific survival for recurrent/persistent laryngeal cancer.

## Introduction

Recurrent and persistent head and neck squamous cell carcinoma is becoming an increasingly important cohort for head and neck cancer providers. While curative treatment is achieved in many instances, approximately 25–50% of patients with head and neck squamous cell carcinoma will experience recurrence, and the overall median survival for those with recurrent disease who undergo treatment is less than 22 months [1]. Treatment for recurrence tends to be difficult, as these tumors are often resistant to standard therapy, complication rates are higher, and prognosis is guarded [2]. In patients who are not eligible for salvage therapy with curative intent, median survival is less than 12 months [1].

Laryngeal cancer in particular is increasingly being treated initially with radiation (RT) or chemoradiation (CRT) organ-preservation protocols [3]. However, while a significant proportion of these patients

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achieve cure, a non-trivial proportion experience persistent/recurrent disease. For these patients, salvage surgery is currently the only method for cure, with less than ideal overall survival rates in patients requiring salvage laryngectomy (ranging from 29 to 66%) [4–7]. Additionally, as with other head and neck cancers, salvage surgery results in high rates of complications due to poor healing rates and the increased difficulties of operating in a radiated field [8].

As a result, researchers have attempted to identify biomarkers that will allow for better patient treatment stratification and prognostication. One of the more promising areas of investigation involves the evaluation of biomarkers that reflect the patient's immune system. It is increasingly appreciated that the immune system plays a key role in head and neck cancer tumorigenesis, progression, and response to therapy. Immunologic signatures have been determined in some subsets of primary head and neck cancers to predict improved survival [9–11]. It is unknown, however, whether immunologic biomarkers may demonstrate utility in predicting survival and potentially stratifying salvage treatment options for recurrent/persistent head and neck cancer. Identifying prognostic biomarkers in recurrent head and neck cancer may be useful in providing counseling for patients, considering escalation or de-escalation of care, or adding adjuvant immunotherapeutic agents. Thus, the purpose of this study was to investigate the prognostic role of a panel of immunologic biomarkers in patients with recurrent/persistent laryngeal squamous cell carcinoma after initial RT or CRT.

## Methods

### *Patient population*

We performed a single-institution retrospective case series informed by a prospectively maintained database of patients with head and neck cancer. The University of Michigan Hospital and Health Systems IRB approved the protocol (HUM00081554). Inclusion criteria stipulated: (1) biopsy-proven laryngeal squamous cell carcinoma; (2) persistent/recurrent disease at the primary site after radiation (for early stage tumors) or chemoradiation (for advanced stage tumors); (3) total laryngectomy with neck dissections for surgical salvage; and (4) tumor tissue available for creation of tissue microarray. Patients were excluded if they had a second primary tumor necessitating surgery. There were 183 patients who met inclusion criteria, and demographics are shown in Table 1. Patients were staged in accordance to the 7th edition American Joint Committee on Cancer (AJCC) Staging System [10].

### *Immunohistology*

Formalin-fixed paraffin-embedded (FFPE) tissue blocks from salvage surgery and representative hematoxylin-eosin stained slides were reviewed for greater than 70% cellularity. The tissue microarray was constructed with triplicate 0.7 mm diameter cores for each patient [11].

TMA sections were incubated in hot-air oven at 65 °C overnight, deparaffinized, rehydrated with xylene, graded alcohols, and buffer immersion steps. Antigen retrieval was carried out by heat-induced epitope retrieval method. The slides were incubated in a preheated pressure cooker with Citrate buffer pH6 or Tris-EDTA buffer pH9 and blocked with horse serum (30 min at 25 °C). Immunohistochemical staining was completed on a DAKO autostainer using liquid streptavidin biotin horseradish peroxidase and DBA (DAKO labeled avidin-biotin-peroxidase kits) as chromogens. Deparaffinized sections were stained with eight monoclonal antibodies at the following titrations: CD4-1:250 (Abcam Ab846); CD8-1:40 (Nova Castra VP-C320); CD31 – 1:50 (Dako M0823); Vimentin – 1:100 (Dako M0725); PDL1 – 1:200 (Cell signaling E1L3N); p16 – predilute (Ventana 725-4713); EGFR – 1:50 (Invitrogen 2,80,005); and p53 – 1:50 (Cell Marque SP5).

The TMA slides were digitally imaged, scanned, and retrieved with Aperio ImageScope v.12 software. Only cores consisting of greater than 50% tumor parenchyma were counted. The CD4, CD8, PDL1, p16,

**Table 1**
Patient characteristics.

| | Patient characteristics (n = 183) |
|---|---|
| *Gender* | |
| Male | 153 (83.6) |
| Female | 30 (16.4) |
| *Ethnicity* | |
| White | 161 (88.0) |
| Black/other/unknown | 22 (12.0) |
| **Initial cancer** | |
| Age at initial tumor | 58.63 |
| Initial site | |
| Glottis | 109 (59.6) |
| Supraglottis | 71 (46.4) |
| Other/unknown | 3 (1.6) |
| *Initial cT stage* | |
| cT1 | 46 (25.1) |
| cT2 | 61 (33.3) |
| cT3 | 44 (24.0) |
| cT4 | 18 (9.8) |
| Unk | 14 (7.7) |
| *Initial cN status* | |
| cN0 | 141 (77.0) |
| cN1 | 15 (8.2) |
| cN2+ | 14 (7.7) |
| Unk | 13 (7.1) |
| *Initial stage* | |
| I | 46 (25.1) |
| II | 54 (29.5) |
| III | 44 (24.0) |
| IV | 25 (13.7) |
| Unk | 14 (7.7) |
| *Initial treatment* | |
| RT | 112 (61.2) |
| CRT | 71 (38.8) |
| **Recurrent cancer** | |
| Age at recurrence (yrs) | 60.87 |
| Time to recurrence (mo) | 23.48 |
| *Recurrent site* | |
| Glottis | 99 (54.1) |
| Supraglottis | 81 (44.2) |
| Other/unknown | 3 (1.6) |
| *Recurrent cT stage* | |
| cT1 | 8 (4.4) |
| cT2 | 80 (43.7) |
| cT3 | 51 (27.9) |
| cT4 | 44 (24.0) |
| *Recurrent cN status* | |
| cN0 | 159 (86.9) |
| cN1 | 10 (5.5) |
| cN2+ | 14 (7.7) |
| *Recurrent cStage* | |
| I | 7 (3.8) |
| II | 76 (41.5) |
| III | 49 (26.8) |
| IV | 51 (27.9) |
| **Pathologic data** | |
| *Recurrent pT stage* | |
| pT1 | 6 (3.3) |
| pT2 | 60 (32.8) |
| pT3 | 53 (29.0) |
| pT4 | 64 (35.0) |
| *Recurrent pN status* | |
| pN0 | 137 (74.9) |
| pN1 | 15 (8.2) |
| pN2+ | 31 (16.9) |
| *Recurrent pStage* | |
| I | 6 (3.3) |
| II | 53 (29.0) |
| III | 48 (26.2) |
| IV | 76 (41.5) |