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Tumor Infiltrating Lymphocytes (TIL) and Prognosis in Oral Cavity Squamous Carcinoma: A Preliminary Study

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Abstract

Objectives—Tumor infiltrating lymphocytes (TILs) in the microenvironment reflect may tumor biology and predict outcome. We previously demonstrated that infiltrates of CD4, CD8, and FoxP3 positive lymphocytes were associated with HPV-status and survival in oropharyngeal cancers. To determine if TILs were of prognostic importance in oral cancer, TIL levels were evaluated retrospectively in 52 oral cancer patients treated with surgery and correlations with outcome determined.

Methods—Complete TIL and clinical data were available for 39 patients. Levels of CD4, CD8, FoxP3 (Treg), CD68 and NK cells were assessed by immunohistochemistry in tumor cores on a tissue microarray. Associations with clinical variables, tobacco and alcohol use and histologic features were assessed using Spearman correlation coefficient and the non-parametric Kruskal-Wallis testing. Timeto-event outcomes were determined using univariate and multivariate Cox models. Median follow up was 60 months.

Results—The ratio of CD4/CD8 (p=.01) and CD8 infiltrates (p=.05) were associated with tumor recurrence but not overall survival. Lower CD4 infiltrates were associated with alcohol use (p=.005) and poor tumor differentiation (p=.02). Interestingly, there higher levels of CD68+ macrophages were found associated with positive nodes (p=.06) and poorer overall survival (p=.07). Overall and DSS survival were significantly shorter for patients with positive nodes, extracapsular spread , or perineural invasion.

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Conclusions—Infiltrating immune cell levels in oral cavity cancer appear influenced by health behaviors and tumor characteristics. In contrast to oropharynx cancer, infiltrates of CD68 positive tumor associated macrophages may contribute to metastatic behavior and outcome in advanced oral cavity carcinoma.

Keywords

Oral Cavity Cancer; Tumor Infiltrating Lymphocytes

Introduction

Treatment results for patients with squamous carcinoma of the oral cavity have not improved significantly over the past 30 years despite technical advances in surgery and radiation, the addition of chemotherapy regimens, and application of advanced methods of reconstruction that have allowed for more aggressive surgical resections. Oral cavity primary cancers are often ulcerated and heavily infiltrated by lymphocytes and are frequently found to have early nodal micrometastases with histologic lymphovascular and/or perineural invasion patterns that are associated with increased tumor recurrence rates (1). It remains uncertain if inflammation at the primary tumor site represents a beneficial manifestation of a patient's immune response to cancer in the tumor microenvironment or actually is a carcinogenic response that enhances tumor progression through elaboration of regulatory cytokines such as IL6, IL10 and IL17(2-5). Better understanding of the immunologic characteristics of the microenvironment including numbers, location and function of tumor infiltrating lymphocytes and macrophages is necessary in order to explore and test immunotherapeutic strategies that might be beneficial to these patients (6).

Numbers, cell types and location of tumor infiltrating lymphocytes have shown to be of significant prognostic importance in patients with breast, colon, lymphoma or gastric cancers and have been associated with response to chemotherapy and prognosis (7-13). In oral carcinoma, tumor infiltration by antigen presenting cells such as macrophages, Langerhans cells and CD1a positive dendritic cells may be associated with impaired cancer surveillance and overall patient survival (14-18) however the role of these antigen presenting cells in enhancing tumor surveillance or contributing to tolerance in the microenvironment is unclear. We and others have previously studied tumor infiltrating lymphocyte subsets in mixed populations of patients with head and neck cancer of various sites and found that CD4 infiltrate levels were associated with a more favorable prognosis (19, 20) and infiltrates of tumor associated macrophages were associated with nodal metastases (21). However, more recent analyses in a more homogenous population of patients with advanced oropharyngeal cancer demonstrated that the subpopulations of regulatory CD4+, Forkhead Box Protein P3 (FoxP3), and CD8 T lymphocyte subsets were prognostically more important than macrophages and were associated with improved disease free and overall survival regardless of Human Papilloma Virus-16 status (22). Others have also suggested that higher FoxP3 infiltrates were associated with improved local/regional control in head and neck cancer (23,24) but some studies report worse survival in other solid malignancies such as breast or colon (12, 25). Some investigators report better prognosis when tumors are infiltrated by FoxP3 regulatory T cells (26,27) and decreased FoxP3

infiltrates in response to successful chemotherapy (12, 27). Understanding the role of these regulatory lymphocytes and macrophages has been considered the biggest barrier to development of effective immunotherapy strategies (28).

Since prognosis for oral cavity cancer patients is much worse than for patients with oropharyngeal cancer, and immune cell correlations with outcome could differ significantly depending on differing treatment modalities, we undertook a preliminary study to characterize tumor infiltrating lymphocyte subsets in a cohort of surgically treated patients with advanced oral cavity cancer and determine correlations with histologic characteristics such as nodal extracapsular spread and perineural invasion and association with overall prognosis.

Methods

Patient Population

The study cohort consisted of 52 consecutive patients with oral cavity squamous carcinoma treated by one of the senior authors (DBC). The analytic cohort consisted of 39 of these patients who had complete clinical, histologic and immunohistologic data available. This cohort included 3 patients with Stage I, 8 patients with Stage II, 8 patients with Stage III and 20 patients with Stage IV cancer. A total of 16 patients were male and 23 were female. Pretreatment clinically positive nodes were present in 17 patients. The demographics of the patient cohort is shown in Table 1. Tumor differentiation. All patients underwent primary surgical resection with clear margins. Clear margins were defined as margins tumor free greater than 5 mm. from edges of resection determined histologically from final resection pathology. Adjuvant postoperative radiation was added for patients with histologically positive regional lymph nodes. Resected specimens were used to create a tissue microarray for immunohistologic studies as previously described (22). Clinical and histologic variables assessed included T classification, nodal class, clinical and pathologic nodal status including extracapsular spread (10 patients), primary tumor differentiation (5-well, 24-moderate, 7 poor), and presence of perineural (6 patients) or perivascular invasion (4 patients). Tumor differentiation was classified using WHO formulation according to well, moderately well of poorly differentiated. Patient factors analyzed included age, gender, smoking status (3-never, 27-past, 5-current), and alcohol use (11-never, 20-past, 5-current). All patients were followed for tumor recurrence and survival. Median follow up was 60 months (range 5-60 months).

Immunohistology

Formalin-fixed, paraffin-embedded tissue blocks (FFPE) for the 39 cases were obtained from the files of the Department of Pathology, University of Michigan Medical Center, Ann Arbor, MI. The University of Michigan Institutional Review Board provided a waiver of informed consent to obtain these samples. After pathological review, a tissue microarray was constructed from the most representative area using the methodology of Nocito et al. (29). Each case was represented by three 0.7 mm diameter cores, obtained from the most representative, non-necrotic area of the tumor selected by one of our pathologists (JM). Core size of 0.7 mm is standard in our tissue core laboratories and were obtained from central

portions 9not invasive front) of the tumor selected by our head and neck pathologist (JM) without knowledge of patient treatment or outcome., Each tissue core was evaluated entirely at 200× magnification. Only areas of tumor confirmed by Beta-4 Integrin expression were counted for infiltrating immune cells.

The TMA slides created from biopsy specimens from the patients were deparaffinized, rehydrated, and peroxidase quenched (Dako Cytomation, Glostrup, Denmark). For antigen retrieval, slides were incubated with pepsin or with citrate buffer and were blocked with horse serum (30 minutes at 25°C). Immunohistochemical staining was performed on the DAKO Autostainer (DAKO, Carpinteria, CA) using diaminobenzadine (DAB) as the chromogen and the antibodies, dilutions, detection systems and epitope retrieval systems as described below. Appropriate negative (no primary antibody) and positive controls were stained in parallel with each set of tumors studied. All tests were carried out on 5 micron formalin fixed paraffin embedded TMA sections. Sections were baked in 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 (DAB) as the chromogen. Deparaffinized sections were stained with six types of monoclonal antibodies: 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), CD68 1:100 (Dako M0814) and p16 (Zymed 16P04). 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 magnification. The number of positively stained TILs for CD4, CD8, FOXP3, and CD68 was assessed quantitatively by two investigators blinded to patient outcome and p16 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. This was to account for the variability in amount of peritumoral stroma in oral cavity cancer sections which might not reflect a tumor related immune response. TILs in each tumor core on the tissue microarray were manually counted using a 20× objective lens. Methods for counting were adapted from published methods used to analyze T cell infiltrates in follicular lymphoma and head and neck cancer TMAs (7,10,30). The mean count of replicate cores for each subject was recorded and used in analysis.

Statistical Methods

Multiple core counts on the tissue microarray for each patient were averaged to yield a mean count for each marker. Ratio of CD4/CD8 count and FoxP3/CD8 count were calculated after averaging of individual marker counts. Associations of mean subset levels and proportions with clinical variables, health behaviors, pathologic staging, and histologic features were assessed using the Spearman correlation coefficient and the non-parametric Kruskal-Wallis test. Sample means and sample standard errors were reported for description. Associations with time-to-event outcomes were determined using univariate and multivariate Cox models. . Disease specific survival was defined from date of initial diagnosis to death from

oral cancer malignancy; patients who did not die from disease were censored at date of death from other cause or last known alive status.

Results

TIL levels and survival

In this small patient cohort of surgically treated patients, higher CD8 infiltrate counts (Figure 1, p=.05) were associated with tumor recurrence but not with overall or disease specific survival (DSS). Mean tumor infiltrating CD8 levels added to prognosis when the strong clinical prognosticator of nodal extracapsular spread was also considered (p=.07). Mean TIL counts for all of the T lymphocyte subsets analyzed and the sum of CD4 plus CD8 counts tended to be lower in patients who died (Figure 2) and in patients who recurred (p=.05). The CD4/CD8 ratio of infiltrating cells tended to be higher in patients with better overall (p=.08) and DSS (p=0.10) but was not statistically significant. The ratio of CD4/CD8 (p=.01) was associated with tumor recurrence. However, this result was likely influenced by ratios in which the cell counts were zero and the resulting hazard ratio near 1. When repeated by ranking the ratios, these relationships were clearly not significant.

Clinical Characteristics, TIL Levels and Overall Survival

Clinical factors that were analyzed included T and N class, tumor stage, nodal extracapsular spread (ECS), perineural invasion, tumor differentiation, perivascular invasion, patient age, sex, smoking history and alcohol use. As expected, even in this small sample size, the clinical characteristics that predicted poorer overall survival included positive nodes (p=.0023), extracapsular spread (p=.02) , perineural inivasion (p=.03), and higher nodal stage (p=.001). Of the various infiltrating immune cell populations, mean counts of CD68 macrophages were higher in patients with histologically positive nodes (p= .05; Table 1). Levels of FoxP3 (Treg) cells infiltrating the tumors were higher in early T staged cancers (p=.05) and as a result, the ratio of FoxP3 to CD8 cells in the tumor was lower in patients with advanced (T3,T4) primary tumors (p=.02) and remained significantly lower after log-transformation of the values (p=.006) but neither clinical (T class) or this immune infiltrate ratio significantly predicted clinical outcome.

Disease Specific Survival

Clinical factors associated with shorter DSS included ECS (p=.02), positive nodes (p=.003), perineural invasion (p=.007), and nodal stage (p=.002). None of the TIL population counts significantly predicted disease specific survival except for the CD4/CD8 ratio which added prognostic significance after ECS was considered. As expected, tumor recurrence was associated with ECS (p=.01), perineural invasion (p<.0001) and nodal stage (p=.002). Of the various infiltrating lymphocyte subsets, mean CD8 cell counts were higher in patients with recurrence (p=.05, Figure 1) and these counts directly correlated with mean CD68 cell counts (p=.03).

Lower CD4/CD8 ratios were also associated with alcohol use (p=.005) and poor tumor differentiation (p=.02). This was primarily due to lower CD4 levels associated with poorly differentiated tumors (p=.02) and with alcohol use (p=.06). Interestingly, higher levels of

CD68 positive infiltrating macrophages were associated with clinically positive nodes (p=.06), and poorer overall survival (p=.07). The FoxP3/CD8 ratio was lower in patients with higher clinical (p=.06) and pathologic T stage (p=.02).

Correlations among TILs

Mean TIL levels for CD4, CD8, and FoxP3 cells were significantly correlated with each other and were higher in surviving patients (Figure 2). Tumors that had higher levels of CD4 and CD8 cell infiltrates added together also had higher CD68 cell counts (p=.01). Individually, CD8 cell counts were directly associated with mean CD68 cell levels (p=.03). Levels of CD4 infiltrates were positively associated with levels of FoxP3 cells (p=.01) which reflects the fact that FoxP3 cells are a subset of CD4 lymphocytes. All tumors expressed beta-4 integrin (CD104). Patterns of expression were classified into three groups according to prior methods that demonstrated a correlation of high alpha-6 beta-4 integrin expression with prognosis (31). Only four (10 %) tumors showed positive intensity and proportion for immunohistologic expression of p16. Two p16 positive patients were node negative Stage II and two were node positive Stage IV. Three were female and only one patient was a never smoker. Neither p16 nor beta-4 integrin expression correlated with any of the TIL levels or with disease free or overall survival.

Discussion

The major observation in this series of surgically treated patients with oral cavity carcinoma was the lack of correlation of individual T lymphocyte subset tumor infiltrates with overall prognosis. In particular, the CD8 subset which has been shown to be important in breast and colon cancers (8) only showed a borderline significantly higher mean level in patients who recurred even though CD8 counts added some prognostication when the important clinical variable extracapsular nodal extension was examined. The CD8 population includes cytotoxic T lymphocytes thought to be the major subpopulation responsible for immune mediated tumor regression and implicated in contributing to response to chemotherapy in breast cancer and melanoma. It was found that lower overall lymphocyte infiltration (sum of CD4 and CD8 cells) tended to be associated with higher recurrence rates supporting the contention that the balance of immune mediated cellular infiltrates may be more important in host immunity to a specific cancer.

A second important finding was confirmation of the potential importance of tumor associated macrophage infiltration related to lymph node metastases and poor prognosis in oral cavity cancer patients. Tumor associated macrophages have been implicated in cancer development and progression by influencing angiogenesis, invasion and metastases (32, 33). Presence of CD68 positive cells has been associated with increased tumor vascularity and poor prognosis in breast, ovarian, melanoma and non-small cell cancers (32-35). A three marker immune signature of high CD68 along with high CD4 and low CD8 infiltrates in the tumor microenvironment has been associated with reduced survival in breast cancer (9). In experimental models of colorectal cancer, tumor associated macrophages have been implicated in recruitment of regulatory FoxP3 T cells to the tumor microenvironment (36). Macrophages are thought to include both immune enhancing (M1) macrophages and

tolerogenic or suppressive macrophage (M2) populations that are primarily tumor associated and are felt to contribute to tumor progression (37). Tumor associated macrophages produce pro-angiogenic factors such as VEGF, TNF-alpha, GM-CSF, IL1 and IL6 that enhance tumor growth. Intratumoral macrophages have also been implicated in epithelial-mesenchymal transition in solid malignancies such as non-small cell lung cancer (38) CD68 positive monocytes are primarily activated tissue macrophages that also function in antigen presentation, but there is evidence that some tumor associated fibroblasts (TAF) may also stain with CD68 antibodies (39). Other forms of tumor infiltrating dendritic cells such as Langerhans and CD1a cells have been associated with improved prognosis in head and neck cancers (14,16-18, 40). The potential importance of better understanding of the roles of such antigen presenting cells is reflected in widespread interest in dendritic cell based vaccines which may or may not be of therapeutic benefit in head and neck cancer patients (41).

Previously, we did not find a significant correlation of CD68 cell infiltrates with outcome in a study of patients with advanced oropharyngeal cancers who were uniformly treated with chemotherapy and radiation (22). However, in a prior small study from our group that included both oral and oropharyngeal cancer patients treated with surgery, upregulation of monocyte chemoattractant protein 1 in tissue specimens was found along with a significant correlation of tumor associated macrophages with lymph node metastases(21) but not with overall prognosis. These findings were similar to the results in the current study. Others have confirmed increased macrophage numbers and function in patients with nodal metastases (42). Whether the lack of correlation in patients with oropharyngeal cancers is related to the biology of these cancers, particularly when the association with human papilloma virus is considered, or is due to differences in definitive treatment modality is unknown. In breast cancer, complete response to induction chemotherapy has been reported to be higher in patients with lower tumor CD68 cell levels (9) and FoxP3 infiltrates (13). In the current study, infiltrating CD68 cell counts were higher in patients with positive nodes and those with evidence of extracapsular spread, which could explain associations with poor outcome. It was unexpected to see a direct association of CD68 counts with CD8 cell infiltrates, but is consistent with the higher mean CD8 cell infiltrates seen in patients who recurred since both immune cell populations are known to have suppressive qualities in the tumor microenvironment.

The expected clinical prognosticators such as tumor stage, extracapsular spread, perineural invasion, and nodal status were confirmed in this patient cohort. It was unexpected to observe an association of tumor T cell infiltrates with health behaviors such as alcohol use. However, this was a small study cohort and alcohol use was only characterized by current or past users versus never users. Lower CD4 cell infiltrates may have been associated with alcohol use simply because of purported immunosuppressive effects of alcohol induced liver disease, but it was also seen that lower infiltrates were found in more poorly differentiated cancers which tend to be less immunogenic.

A more recently described population of regulatory T cells (FoxP3), which are a subset of CD4 cells, has been implicated as a positive prognostic factor in breast and colon cancer, (13, 26, 42) and that decreases in levels are associated with complete response to neoadjuvant chemotherapy (43, 44). In head and neck cancer patients, peripheral blood

levels of FoxP3 have been shown to be elevated (45-48) and like tumor macrophages, associated with secretion of IL10 and Transforming growth factor beta1 which can cause immune suppression in the microenvironment. Compared to surgical treatment, chemoradiotherapy has been associated with prolonged increases in levels of these regulatory cells in the circulation of head and neck cancer patients(47). We have shown, however, that higher peripheral blood levels of this subset were associated with improved survival in oropharyngeal cancer patients (49). Other investigators have reported tumor infiltrate findings consistent with our current results suggesting better survival with increased FoxP3 infiltrates in head and neck cancer(20), gastric adenocarcinoma(12) and lymphoma (3). Infiltrates of FoxP3 cells tended to be sparse in the oral cavity cancers we studied and were higher in our survivors. The levels were directly associated with the other T cell subsets suggesting a more general inflammatory response may be beneficial rather than a specific recruitment of these cells to the tumor site. However, levels of these cells were higher in early staged tumors in our cohort which could suggest that their suppressive role might be more important in early cancer growth rather than regulating immune response in advanced cancers. Some studies have suggested that these cells are suppressive and that eliminating such cells might allow a more robust beneficial immune response to cancer (4,45).

Unfortunately, the retrospective nature of this small correlative study did not allow immune cell isolation and functional assays of the various T cell infiltrating subsets. Thus, we can only infer from correlations of disease outcome and TIL morphologic characteristics and density counts, what the potential function of such cells might be in the microenvironment. It is likely that balance among the infiltrates is important as suggested by ratios of CD4 to CD8 cells and FoxP3 to CD8 ratios. A consistent finding in most studies however has been the negative impact of high infiltrates of tumor associated macrophages (12,21,50). Further functional studies of TILs in homogenous cohorts undergoing standardized treatment regimens will be needed in order to determine how modifications of infiltrates might be beneficial. This is of great importance since successful therapeutic manipulation of immune cytokines and blockade of immune cell interactions in the microenvironment has recently been reported in melanoma and pancreatic cancer patients (51-54). Serial tumor specimens from well designed trials of immunotherapy will be even more valuable to assess how changes in infiltrates might mediate clinical benefit in patients with oral squamous carcinoma.

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Highlights

Immune responses within the tumor microenvironment are increasingly important predictors of tumor biology and outcome. Numbers of tumor infiltrating lymphocytes (TILs), their function, and location in the microenvironment of head and neck squamous cancers appear important and may differ by tumor site and extent. This report describes preliminary findings of the association of tumor infiltrating immune cells with clinical characteristics and prognosis in a uniformly treated cohort of oral cavity cancer patients with long follow up. Infiltrating immune cell levels in oral cavity cancer appear influenced by health behaviors and tumor characteristics. In contrast to prior findings in oropharynx cancer patients, infiltrates of potentially immunosuppressive CD68 positive tumor associated macrophages may be important in metastatic behavior and outcome for patients with advanced oral cavity carcinoma.

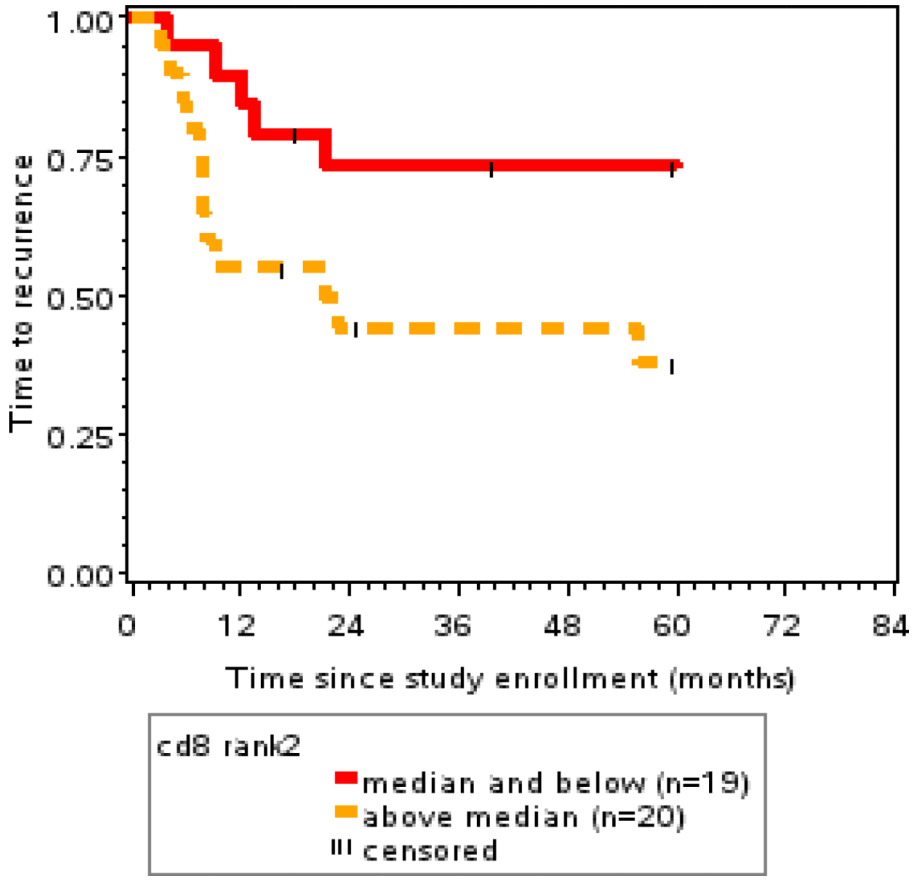

Figure 1.

Disease free interval according to level of tumor infiltrating CD8+ cells. Patients with levels above the group median tended to earlier tumor recurrence (p = 0.05)

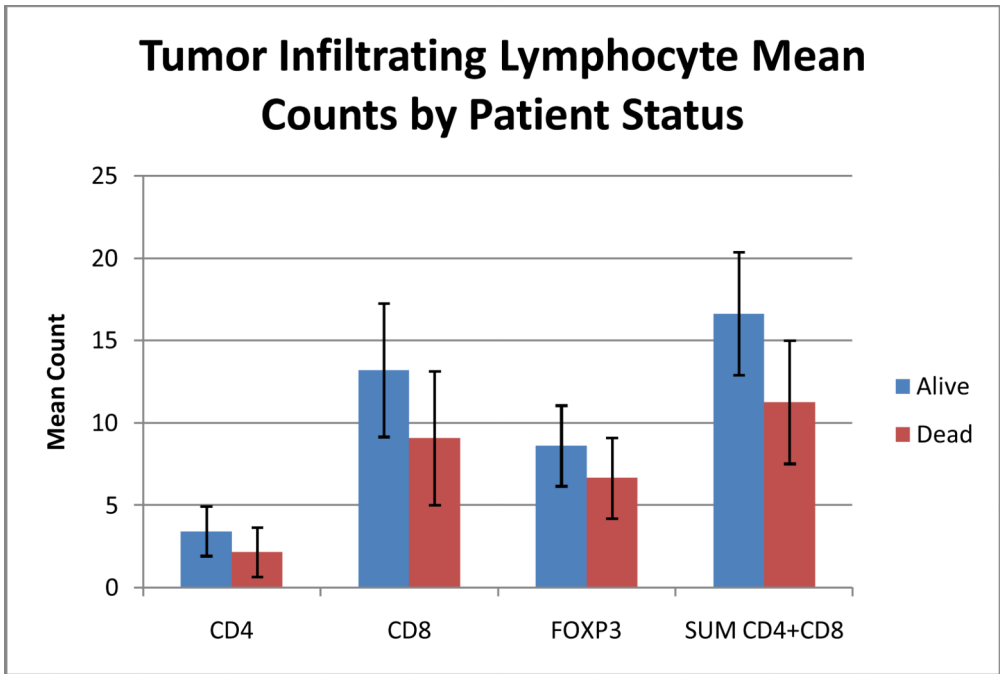

Figure 2.

Mean counts for all T lymphocyte subsets were lowere in patients who died. Levels werr also lowere in patient who recurred (data not shown p = 0.05)

Table 1

Demographics of the Patient Cohort

Characteristic		N	(%)	Mean (std)
Age at Diagnosis				57.7 (16.8) years
Gender	Male	16	41%	
	Female	23	59%	
Disease Site	Lateral Tongue	15	38%	
	Anterior Tongue	3	8%	
	Upper Alveolus	2	5%	
	Floor of Mouth	14	36%	
	Hard Palate	1	3%	
	Buccal Mucosa Retromolar	4	10%	
Cancer Stage	I	3	8%	
	II	8	21%	
	III	8	21%	
	IV	20	51%	
T Stage	T1	3	8%	
	T2	11	28%	
	T3	7	15%	
	T4	18	49%	
N Stage	N0	22	56%	
	N1	10	26%	
	N2	7	18%	
Tobacco Use*	Current	5	14%	
	Former	27	77%	
	Never	3	9%	
Alcohol Use*	Current	5	14%	
	Former	20	56%	
	Never	11	31%	
Median Follow-up				60 months
Min-Max Time to Death				4.8 - 26.0 months
Min-Max Follow-up among alive				5.9 - 60.0 months

% may not add to 100% due to rounding.

* **n does not add to 39 due to missing data.**

Table 2

Mean (+/− SE) TIL Cell Counts for Node Positive and Node Negative Patients

Cell Type	Node Negative (N=17)	Node Positive (N=22)
CD4	2.4 +/− 0.97	3.4 +/− 0.84
CD8	8.2 +/− 1.91	14.4 +/− 4.92
FoxP3	8.8 +/− 1.95	7.2 +/− 1.25
CD68	6.2 +/− 1.47	10.3 +/− 1.46