Transcriptomic and Immunophenotypic Characterization of Tumor Immune Microenvironment in Squamous Cell Carcinoma of the Oral Tongue

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Abstract

The tumor immune microenvironment of oral tongue squamous cell carcinoma may be accountable for differences in clinical behavior, particularly between different age groups. We performed RNA expression profiling and evaluated tumor infiltrating lymphocytes (TILs) and their T-cell subsets in order to assess the functional status of oral tongue squamous cell carcinoma tumor microenvironment and detect potentially clinically useful associations. Archival surgical pathology material from sixteen oral tongue squamous cell carcinoma patients was microscopically evaluated for TIL densities. RNA was extracted from macrodissected whole tumor sections and normal controls and RNA expression profiling was performed by the NanoString PanCancer IO 360 Gene Expression Panel. Immunostains for CD4, CD8 and FOXP3 were evaluated manually and by digital image analysis. Oral tongue squamous cell carcinomas had increased TIL densities, numerically dominated by CD4 + T cells, followed by CD8 + and FOXP3 + T cells. RNA expression profiling of tumors versus normal controls showed tumor signature upregulation in inhibitory immune signaling (CTLA4, TIGIT and PD-L2), followed by inhibitory tumor mechanisms (IDO1, TGF- β , B7-H3 and PD-L1). Patients older than 44 years showed a tumor microenvironment with increased Tregs and CTLA4 expression. Immunohistochemically assessed CD8% correlated well with molecular signatures related to CD8 + cytotoxic T-cell functions. FOXP3% correlated significantly with CTLA4 upregulation. CTLA4 molecular signature could be predicted by FOXP3% assessed by immunohistochemistry ($R^2 = 0.619$, $p = 0.026$). Oral tongue squamous cell carcinoma hosts a complex inhibitory immune microenvironment, partially reflected in immunohistochemically quantified CD8 + and FOXP3 + T-cell subsets. Immunohistochemistry can be a useful screening tool for detecting tumors with upregulated expression of the targetable molecule CTLA4.

Keywords Tumor infiltrating lymphocytes · CD4-positive T lymphocytes · CD8-positive T lymphocytes · Regulatory T lymphocytes · Immunohistochemistry · Genetic transcription · Tongue · Oral cavity · Squamous cell carcinoma

Abbreviations

HPV Human papillomavirus
TILs Tumor infiltrating lymphocytes
PD-L1 Programmed death ligand 1
FOXP3 Forkhead box P3
CTLA4 Cytotoxic T lymphocyte-associated protein 4

TIGIT T cell immunoreceptor with Ig and ITIM domains
PD-L2 Programmed death ligand 2
IDO1 Indoleamine-pyrrole 2,3-dioxygenase 1
TGF- β Transforming growth factor beta
Tregs Regulatory T cells
PD-1 Programmed cell death protein 1

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Introduction

Oral tongue squamous cell carcinoma is the most common malignancy of the oral cavity, usually affecting older individuals, with a recent trend to affect younger patients,

primarily women [1]. Oral tongue squamous cell carcinoma is aggressive, with more than one third of the patients dying of the disease within 5 years, despite recent progress in treatment leading to mild improvement in survival [2]. The overall incidence of oral tongue squamous cell carcinoma has been increasing [3] and younger patients frequently have worse outcomes than older patients, despite lacking any predisposing factors such as tobacco smoking, alcohol abuse or HPV infection [4]. However, it is unclear if age has a definitive prognostic significance, with clinical treatment paradigm remaining the same between different age groups [5].

With the exceptions of increased copy number variations [6] and fewer *TP53* mutations in younger patients [7] no other significant genomic [8] or transcriptomic differences [6] have been identified between young and old patients. Since high densities of TILs [9, 10] and PD-L1 tumor expression are associated with favorable outcomes [11], studies have been querying the tumor immune microenvironment for an explanation of differences in oral tongue squamous cell carcinoma clinical behaviors. TILs can be readily assessed on routine hematoxylin and eosin (H&E) preparations and provide prognostic information relevant to the robustness of anti-tumor immune response [9]. TIL densities have been associated with increased expression of PD-L1 [12, 13], which can be targeted by checkpoint inhibitors leading to promising results in patients with carcinomas of the head and neck [11].

A recent study identified a 24 immune gene signature characterizing oral tongue squamous cell carcinomas with more aggressive clinical behavior [14]. However, it is uncertain whether immune gene expression findings can be reliably predicted by studying morphological parameters, such as densities of TILs and their subsets.

Since morphology reflects the orchestrated end-result of complex interactions occurring at molecular level [15], we performed an investigative analysis to elucidate whether TILs and immunohistochemically quantified CD4 + , CD8 + and FOXP3 + T-cell subsets correlate with the RNA expression profile of the oral tongue squamous cell carcinoma immune microenvironment.

Materials and Methods

Case Selection and Clinicopathologic Review

We identified patients with available archival surgical pathology material and comprehensive clinical information, diagnosed with and treated for oral tongue squamous cell carcinoma between years 2003–2018. We randomly selected patients younger than 44 years old and stage-matched older patients. Formalin fixed paraffin embedded tissue blocks and corresponding hematoxylin and eosin (H&E) slides were

evaluated. A representative block with tumor away from the surgical margin was selected and 4 µm unstained slides were obtained for additional studies. Areas of tumor and non-neoplastic tissue away from the mass were annotated for macrodissection from the same tissue block. Non-neoplastic tissue consisted of non-dysplastic squamous mucosa and underlying skeletal muscle and minor salivary gland tissue. Clinicopathologic data were obtained through chart review.

Tumor Infiltrating Lymphocytes

TILs were manually evaluated by two surgical pathologists, per established guidelines [16] and were reported as continuous variables [17]. More specifically, we evaluated stromal TILs inside the tumor borders on whole tumor sections. After scanning slides at low magnification to determine tumor boundaries ($\times 40$) we assessed the percentage of stromal area occupied by lymphocytes and plasma cells to the total area of tumor stroma at intermediate magnification ($\times 100$). Immune cells other than lymphocytes and plasma cells were excluded. Areas of necrosis were also dismissed. The final reported percentage represented the average percentage of stromal TILs, after incorporating both TIL “hot-spots” as well as less cellular areas [16].

RNA Expression Profiling

RNA was extracted from matched normal and tumor macrodissected formalin fixed paraffin embedded samples with the PAXgene Tissue RNA kit (PreAnalytix GmbH, Hombrechtikon, Switzerland) via the automated QIAcube instrument (QIAGEN, Germantown, Maryland, USA). RNA integrity check included quantification with optical density via NanoDrop Spectrophotometer (Thermo Fisher Scientific, Waltham, Massachusetts, USA) and quality check with RNA Integrity Number by Bioanalyzer 2100 (Agilent, Santa Clara, California, USA).

RNA was plated in manufacturer’s cartridges and processed in the nCounter platform for NanoString PanCancer IO 360 Gene Expression Panel (NanoString Technologies, Seattle, Washington, USA). Expression data from 750 genes (Supplementary Table 1) included in the panel were analyzed with the nSolver version 4.0 software (NanoString Technologies, Seattle, Washington, USA) and data normalization included two steps. Depending on whether a gene participated in tumor inflammation signature, which measures intratumoral adaptive immune response [18], the first step of data normalization used a different denominator. More specifically, genes included in tumor inflammation signature were normalized by using the ratio of expression value (numerator) to the geometric mean of housekeeper genes present in tumor inflammation signature (denominator), while for genes not included in the tumor inflammation

signature normalization was performed by using the ratio of expression value (numerator) to the geometric mean of all housekeeper genes present in the panel (denominator). The second step of data normalization included the ratio of the housekeeper-normalized values and a standard run of the panel in the same cartridge used for the studied samples. Finally, housekeeper and panel standard normalized data were log2 transformed. Group analysis for differential expression of gene signatures was performed with the limma package in R, using a linear analysis model with t-tests for differences in means and the False Discovery Rate adjustment to account for correlations [19]. Relatedness among signature scores between each sample was assessed through unsupervised hierarchical clustering.

Immunohistochemistry

Immunohistochemistry was performed on 4 µm formalin fixed paraffin embedded whole thickness tumor sections of 15/16 cases with sufficient tissue, using the automated immunostaining system [Ventana M.S. Inc. Tuscon, Arizona, United States of America (USA)] of the institutional clinical lab, with antibodies against: PD-L1 (clone 22C3, Dako North America Inc., Carpinteria, California, USA); CD4 (clone SP35, Ventana M.S. Inc., Tuscon, Arizona, USA); CD8 (clone C8/144B, Agilent, Santa Clara, California, USA) and FOXP3 (clone 236A/E7, Abcam Inc., Cambridge, Massachusetts, USA). A list of the tissues used as positive and negative controls is presented in Supplementary Table 2. CD4, CD8 and FOXP3 were evaluated by digital image analysis. A subset of randomly selected cases was also independently evaluated by “eyeballing” to assess for the degree of agreement between digital and manual scoring. Two surgical pathologists independently scored the percentage of positive lymphocytes to the total population of tumor infiltrating lymphocytes present in the area of interest on the immunostained section. PD-L1 was evaluated by the combined positive score, which is calculated by dividing the number of immunoreactive cancer and immune cells by the number of viable cancer cells present on the immunostained slide. A score ≥ 1 classified the tumor as positive for PD-L1, while a score < 1 as negative [20].

Digital Image Analysis

Immunostains were evaluated using QuPath open source software for digital pathology [21, 22]. Slides were scanned using the MoticEasyScan digital slide scanner (Motic North America, Richmond, British Columbia, Canada) and digitalized slides were imported into QuPath. The areas of interest, consisted of neoplastic area and the peritumoral lymphoid cuff, were manually outlined using combination of the Rectangle, Polygon and Brush tool. The Cell detection tool was

used to detect every cell in the annotated area and the Positive cell detection tool to detect positive cells. Finally, each annotated area was computed and numbers of positively and negatively stained cells were automatically counted. The proportion of lymphocytes positive for CD4, CD8, or FOXP3 immunostains was defined as the ratio of positively stained cells to the total number of counted cells (neoplastic and non-neoplastic).

Statistical Analysis

Continuous variables were assessed for normality by the Shapiro–Wilk test. For differences between mean values, t-test was used for variables following normal distribution and the non-parametric Mann–Whitney U test for non-normally distributed variables. For frequency distribution differences, we used Chi-square or Fisher’s exact test, as indicated. Pearson’s r or Spearman’s Rho correlation coefficients were calculated to identify correlations between continuous variables. Statistically significant correlations were evaluated with the Durbin–Watson test for autocorrelation. For non-autocorrelated variables (Durbin–Watson test values between 1.5 and 2.5), a linear regression analysis was performed and R^2 coefficient of determination was calculated. In order to adjust for multiple tests we calculated adjusted p values according to the Holm–Bonferroni method [23]. For grouping purposes, median values were used as cutoffs, with the exception of age where the more clinically relevant cutoff of 40 years was used [4]. Cronbach’s alpha was used to evaluate the internal consistency of immunohistochemical results reported by digital image analysis software and the independent observer. Statistical significance was set at $\alpha = 0.05$. IBM SPSS v. 25 (IBM, Armonk, New York, USA) statistical analysis software was used.

Ethical Considerations

The study was approved by the Institutional Review Board (application number 18-002756).

Results

Clinicopathologic Characteristics

Archival surgical pathology material and comprehensive clinical information was available from 16 patients diagnosed with and treated for oral tongue squamous cell carcinoma between years 2003–2018. A summary of basic clinicopathologic characteristics is presented in Table 1. Most of the patients were women (11/16 or 68.8%), of young age (median 32.5 years), non-smokers (15/16 or 93.75%), diagnosed with a mass confined to the lateral oral

Table 1 Clinicopathologic characteristics of patients with squamous cell carcinoma of the oral tongue

	N	%
Patients	16	100
Age	Years	SD
Median	32.5	
Mean	43.75	24.18
Min–Max	19–97	
Gender	N	%
Male	5	31.3
Female	11	68.8
Smoker		
Yes	1	6.25
No	15	93.75
Laterality		
Right	5	31.3
Left	11	68.8
Treatment		
Surgery alone	6	37.5
Surgery + RT	7	43.7
Surgery + RT + CT	3	18.8
Margins		
Negative	10	62.5
Close	6	37.5
Mass size	mm	SD
Median	18	
Mean	20	10.23
Min–Max	4 to 50	
Depth of invasion	mm	SD
Median	9	
Mean	9.2	5.47
Min–Max	3–20	
Perineural invasion	N	%
Present	7	43.8
Absent	9	56.2
Lymphovascular invasion		
Present	3	18.8
Absent	13	81.2
Grade		
Well differentiated	3	18.8
Moderately differentiated	7	43.8
Poorly differentiated	6	37.5
HPV status		
Positive	0	0
Negative	16	100
pT stage		
1	6	37.5
2	8	50
3	2	12.5
pN		
0	5	31.3
1	4	25

Table 1 (continued)

	N	%
2	5	31.3
N/A	2	12.5
Extracapsular extension		
Present	2	12.5
Absent	7	43.75
Not applicable	7	43.75
AJCC stage 8th edition		
I	4	25
II	3	18.8
III	4	25
IV	5	31.3
Follow up	Months	SD
Median	39.5	
Mean	43.69	35.95
Mix–Max	6–135	
Recurrence	N	%
Yes	4	25
No	12	75
Vital status		
Alive and free of disease	13	81.2
Dead of the disease	3	18.8

SD standard deviation, RT radiotherapy, CT chemotherapy

tongue (left: 11/16 or 68.8%; right: 5/16 or 31.2%) and treated with a combination of surgical resection in clear margins and adjuvant radiotherapy (7/16 or 43.7%) or surgery alone (6/16 or 37.5%), with a minority of patients receiving adjuvant chemotherapy (3/16 or 18.8%). The tumors were mostly small in size (median 18 mm) and moderately differentiated (7/16 or 43.8%), but deeply infiltrative (median 9 mm), showing perineural invasion in almost half (7/16 or 43.8%) and lymphovascular invasion in a minority (3/16 or 18.8%). Tumor size correlated with depth of invasion ($r=0.798$, $p=0.001$), but neither had a significant association with positive nodal status (depth of invasion $p=0.056$; tumor size $p=0.333$). All of the tumors (16/16 or 100%) were negative for HPV either by p16 immunohistochemistry or HPV DNA in situ hybridization. Neck dissection was performed in most patients (14/16 or 87.5%) based on the clinical suspicion for nodal involvement and a majority (9/14 or 64.3%) had positive lymph nodes, although extracapsular extension was present in only two patients (2/9 or 22.2%). Based on American Joint Committee on Cancer 8th edition [24] more than half of the patients had stage III or IV disease (9/16 or 56.25%) and were followed for a median of 39.5 months (range 6–135). During follow-up, four patients (4/16 or 25%) had recurrence of oral tongue squamous cell carcinoma and three patients (3/16 or 18.8%) died of the disease.

Tumor Infiltrating Lymphocytes, Subsets and PDL1 Immunohistochemistry

The basic descriptive characteristics of morphologically assessed TILs and immunohistochemical studies for CD4, CD8, FOXP3 and PDL1 are presented in Table 2. Tumors had brisk densities of TILs (median 40%, range 10–80%) (Fig. 1a, c) and were classified as PD-L1 positive (14/15 or 93.3%) with high combined positive score (median score 31.15) (Fig. 1b). Combined positive score correlated significantly with FOXP3% ($r=0.603$, $p=0.017$). A single tumor was PD-L1 negative (score < 1) (Fig. 1d). CD4 + T-cells predominated (median 20.41%, range 7.28–36.85%) (Fig. 2a, d), followed by CD8 + T-cells (median 11.68%, range 0.11–42.8%) (Fig. 2b, e) and FOXP3 + T-cells (median 5.22%, range 1.15–8.88%) (Fig. 2c, f). As expected, FOXP3 + T-cells were a fraction of CD4 + T-cells, with the FOXP3:CD4 ratio ranging from 6 to 65% (Table 2). The distribution of CD4%, CD8% and FOXP3% per case is illustrated in Supplementary Fig. 1. Evaluation of CD4, CD8 and FOXP3 by “eyeballing” correlated well with the output of the digital image analysis software (Spearman’s $\rho=0.736$, $p=0.002$) and showed high levels of reliability (Cronbach’s $\alpha=0.838$).

Table 2 Tumor infiltrating lymphocytes, subsets and PD-L1 expression in patients with squamous cell carcinoma of the oral tongue

Tumor infiltrating lymphocytes	%	SD
Median	40	
Mean	42.19	23.24
Min–Max	10.00–80.00	
CD4	%	SD
Median	20.41	
Mean	19.51	10.27
Min–Max	7.28–36.85	
CD8	%	SD
Median	11.68	
Mean	14.43	12.45
Min–Max	0.11–42.80	
FOXP3	%	SD
Median	5.22	
Mean	4.81	2.84
Min–Max	1.15–8.88	
FOXP3:CD4 ratio	%	SD
Median	25.41	
Mean	26.83	15.56
Min–Max	6–65	
PD-L1	CPS	SD
Median	31.15	
Mean	43.27	37.63
Min–Max	0.75–126.50	

SD standard deviation, CPS combined positive score

RNA Expression Profiling

Based on the RNA expression from 750 genes (Supplementary Table 1) we obtained 43 molecular signatures, classified into 8 groups based on functional relevance (Supplementary Table 3). As shown in the gene expression heatmap (Fig. 3a), unsupervised hierarchical clustering showed differential expression of several molecular signatures between tumors and their normal controls. As depicted in the forest plot (Fig. 3b), expression upregulation was more common in tumors (22/43 signatures or 51.16%) than downregulation (4/43 signatures or 9.3%) in comparison with their normal controls. In keeping with the brisk TIL response described above, most of the upregulated molecular signatures belonged to the immune cell population abundance functional category, followed by anti-tumor immune activity, inhibitory tumor mechanisms, inhibitory immune signaling and tumor immunogenicity and sensitivity to immune attack. The highest magnitude of overexpression was seen in the inhibitory immune signaling group (signatures for CTLA4, TIGIT and PD-L2, average Log2 fold change 1.23), followed by the inhibitory tumor mechanisms group (signatures for IDO1, TGF- β , B7-H3 and PD-L1, average Log2 fold change 1.20). Interestingly, signatures related to endothelial cells, mast cells, arginase 1 and glycolytic activity were significantly downregulated in tumors compared to normal.

Correlations Between Tumor Infiltrating Lymphocytes, Their Subsets and RNA Expression Signatures

A summary of correlations between molecular signature Log2 fold change with TILs, CD4, CD8 and FOXP3 percentages is presented in Table 3. TIL% and CD4% did not correlate significantly with any of the studied molecular signatures. CD8% showed a high number of correlations with signatures across multiple categories, including tumor inflammation signature ($r=0.867$, $p=0.02$), Lymphoid ($r=0.865$, $p=0.021$), Cytotoxicity ($r=0.834$, $p=0.022$), IDO1 ($r=0.836$, $p=0.026$), PD-L2 ($r=0.777$, $p=0.025$) and immune cell abundance signatures (T-cells, Exhausted CD8). FOXP3% showed strong correlation with CTLA4 ($r=0.787$, $p=0.026$).

CD8 and FOXP3 Percentages Predict Log2 Fold Changes of Certain Molecular Signatures

Linear regression (Table 4) CD8% was highly predictive of the tumor inflammation signature ($R^2=0.751$, $p=0.007$) and Cytotoxicity signature ($R^2=0.696$, $p=0.008$), and moderately predictive of T-cell signature ($R^2=0.58$, $p=0.009$) and CTLA4 ($R^2=0.368$, $p=0.032$.) FOXP3% was stronger predictor of CTLA4 ($R^2=0.619$, $p=0.026$) than CD8%.

Fig. 1 Tumor infiltrating lymphocytes and PD-L1 expression in oral tongue squamous cell carcinoma. **a** Brisk tumor infiltrating lymphocytes (score 75%, H&E×200); **b** Low density of tumor infiltrating

lymphocytes (score 10%, H&E×200); **c** High expression of PD-L1 (combined positive score: 126,×100); **d** Case negative for PD-L1 (combined positive score: 0.75,×100)

Subgroup Analysis by Outcome, Age and TIL Densities

We studied the differences between selected clinicopathologic variables and expression signatures in different subgroups and the results are depicted in Table 5. At the end of the follow-up period, three patients (3/16 or 18.8%) had died of the disease. In comparison with living patients, those who died of the disease had larger masses ($p = 0.043$), significantly lower CD4 + T-cell percentages ($p = 0.01$), and higher expression of the following gene signatures: Antigen presenting machinery ($p = 0.014$), apoptosis ($p = 0.014$) and TGF- β ($p = 0.002$). No statistically significant differences were identified between patients who died of the disease and living patients regarding FOXP3 + percentages (t-test $p = 0.844$) or FOXP3:CD4 ratio (Mann–Whitney U Test $p = 0.295$). Patients older than 44 years had significantly higher percentages of FOXP3 + T-cells ($p = 0.005$) and higher expression levels of signatures related to anti-tumor immune activity (Interferon- γ , tumor inflammation signature, interferon downstream), inhibitory immune

signaling (CTLA4, PDL2) and inhibitory tumor mechanisms (IDO1). By using the median value as a cutoff we studied the subgroups of high ($\geq 40\%$) and low ($< 40\%$) TILs. Tumors with low TILs were associated with lymphovascular invasion ($p = 0.036$), lower CD8 + T-cell percentages ($p = 0.045$), lower signature expression for PD-L2 ($p = 0.016$) and IDO1 ($p = 0.02$) and higher for apoptosis ($p = 0.001$) and TGF- β ($p = 0.024$).

Discussion

Oral tongue squamous cell carcinoma is a unique cancer that is spanning a wide spectrum of age groups with unclear prognostic significance between age groups [5]. Our study reveals correlation of immunohistochemically quantified CD8 + and FOXP3 + T-cell subsets with the tumor immune microenvironment. In addition, age related changes were noted with immune related gene expression signatures correlating with more advanced age patients.

We showed that oral tongue squamous cell carcinomas have brisk TILs, in agreement with Heikkinen et al. who

Fig. 2 Immunohistochemical assessment of CD4+, CD8+ and FOXP3+ T-cell subsets in oral tongue squamous cell carcinoma. High densities of CD4+ (27.97%) (a), CD8+ (14.15%) (b)

and FOXP3+ (7.22%) (c) T-cells. Contrast with low densities of CD4+ (10.9%) (d), CD8+ (0.11%) (e) and FOXP3+ (2.77%) (f) T-cells

classified most of oral tongue squamous cell carcinomas as TIL-high [9]. Increased TILs were associated with absence of lymphovascular invasion, as previously seen in breast cancer [25]. CD4+ T-cells predominated over CD8+ T-cells, a finding recently observed, although not further discussed, by Meehan et al. [14]. As expected, FOXP3+ cells were representing a fraction of CD4+ cells. FOXP3+ Tregs differentiate from naïve T cells in response to cytokines (TGF- β) or tumor antigens [26] and they facilitate tumor immune escape and growth by inhibiting the anti-tumor immune response and promoting an overall inhibitory immunosuppressive tumor microenvironment [27]. Tregs express inhibitory receptors and produce and secrete a variety of cytokines most importantly interleukin-10, interleukin-35 and TGF- β , which hinder tumor antigen presentation by dendritic cells, disrupting the development of anti-tumor CD8+ T-cells and eventually leading to immune exhaustion by T-cell transcriptomic reprogramming [28]. Depletion of Tregs, phenotype switch to pro-inflammatory or checkpoint inhibition on Tregs have been subjects of intensive research in cancer immunotherapy [27]. CD4 and CD8 are $\alpha\beta$ T cell

receptor co-receptors, binding with major histocompatibility complex class II (CD4) or class I (CD8) molecules and co-expressed in immature thymocytes [29]. An early lineage determination mediated by transcription factors ThPOK and Runx3 commits T-cells to retain only CD4 or CD8, dividing them into CD8+ cytotoxic cells and CD4+ cells [30]. CD4+ T-cells show plasticity for further differentiation into several subsets, including FOXP3 expressing Tregs [31].

PD-1/PD-L1 interaction is an important mechanism of tumor immune escape targeted by checkpoint inhibitors [32]. Tumors frequently upregulate PD-L1 and bind PD-1 on intratumoral CD8+ T-cells [32]. PD-L1 or PD-L2 upregulation can occur in various ways, such as a secondary event in the cascade of activating oncogenic mutations in *EGRF*, *MAPK* or *PIK3CA* [33], epigenetically [34], induction by cytokines released by TILs [32], or by viral infection such as in the case of PD-L1 upregulation induced by Epstein-Barr virus in nasopharyngeal carcinomas [35]. Binding of PD-L1 to PD-1 induces a metabolic shift to T-cells from oxidative phosphorylation to aerobic glycolysis dependent on fatty acid breakdown and oxidation, eventually leading to a more

Fig. 3 Transcriptomic profiling of oral tongue squamous cell carcinoma. **a** Gene expression heatmap illustrating unsupervised hierarchical clustering accounting for the differential expression of several

molecular signatures between tumors and their normal controls; **b** Forest plot illustrating upregulated, unchanged and downregulated gene expression signatures between tumors and their normal controls

Table 3 Correlations of morphologically assessed tumor infiltrating lymphocytes and their subsets with gene expression signature Log2 fold changes in oral tongue squamous cell carcinoma

Molecular signature expression gradient (tumor vs normal)	Log2 FC	p-value	TILs %		CD4%		CD8%		FOXP3%	
			R	p-value	R	p-value	R	p-value	R	p-value
Anti-tumor immune activity										
TIS	1.069	0.002	0.504	0.1104*	0.52	0.874*	0.867	0.02*	0.548	0.816*
Lymphoid	0.7425	<0.001	0.679	0.075*	0.667	0.144*	0.865	0.021*	0.43	0.1
Cytotoxicity	0.6873	0.03	0.642	0.154*	0.48	0.065	0.834	0.022*	0.27	0.31
Immune cell population abundance										
T-cells	0.7401	0.038	0.657	0.115*	0.626	0.276*	0.761	0.023*	0.28	0.29
Exhausted CD8	0.6623	0.024	0.684	0.078*	0.549	0.726*	0.875	0.024*	0.244	0.38
Inhibitory immune signaling										
CTLA4	1.74	<0.001	0.33	0.2	0.665	0.15*	0.606	0.224*	0.787	0.026*
PDL2	0.5066	0.039	0.566	0.418*	0.541	0.74*	0.777	0.025*	0.47	0.072
Inhibitory tumor mechanisms										
IDO1	1.468	0.001	0.602	0.26*	0.666	0.156*	0.836	0.026*	0.45	0.089

Bold values are statistically significant

FC fold change

*Adjusted for multiple tests

Table 4 Linear regression of CD8 and FOXP % percentages to Log2 fold change in tumor molecular signatures

Molecular signature	CD8%		FOXP3%	
	R-square ^a	p-value*	R-square ^a	p-value*
Anti-tumor immune activity				
TIS	0.751	0.007		
Cytotoxicity	0.696	0.008		
Immune cell population abundance				
T-cells	0.58	0.009		
Inhibitory immune signaling				
CTLA4	0.368	0.032	0.619	0.026

*Adjusted for multiple tests

^aValues presented for significantly correlated variables with Durbin-Watson test values between 1.5 and 2.5

oxidative cellular environment, which ultimately depletes T-cell functionality in chronic diseases and cancer [36]. Targeting the PD-1/PD-L1 interaction partially reverses these effects boosting the effectiveness of anti-tumor immune response [32].

Most of our patients had tumors positive for PD-L1 by immunohistochemistry. A female gender predilection for PD-L1 expression in oral tongue squamous cell carcinoma has previously been highlighted by Satgunaseelan et al. [37]. We identified a statistically significant correlation of PD-L1 combined positive score with FOXP3%, an observation previously highlighted in squamous cell carcinomas of the floor of mouth by Stasikowska-Kanicka et al. [38]. PD-L1 can recruit, maintain and expand the Treg intratumoral population [39], leading to immunosuppression by functionally and numerically depleting cytotoxic CD8 + T-cells [31]. PD-L1 expression by oral tongue squamous cell carcinoma has been associated with high levels of TILs [12], CD4 + and CD8 + subsets [40] and tumor associated macrophages [41]. PD-L1 as assessed by immunohistochemistry has been reported as a potentially useful biomarker of favorable prognosis in oral tongue squamous cell carcinoma [11], although this finding has not been validated in a series of subsequent studies [12, 40, 42, 43] and even conflicting results showing association of high PD-L1 expression with worse outcomes have been published [44, 45]. Therefore, conclusive evidence regarding the prognostic role of PD-L1 expression in oral tongue squamous cell carcinoma is lacking at this time.

Among the RNA expression signatures, interferon- γ showed the highest magnitude of upregulation, reflecting an immune microenvironment rich in CD4 + and CD8 + T-cells. Interferon- γ acts through the JAK-STAT signaling pathway [46] and is primarily expressed by Natural Killer cells, CD8 + cytotoxic T-cells and CD4 + Th1-cells, promoting an anti-tumor immune response [47].

We showed significant CTLA4 upregulation, in agreement with Meehan et al. [14]. CTLA4 is a member of the immunoglobulin superfamily [48], highly expressed by Tregs, which negatively regulates T-cell activation [49]. The

role of Tregs in oral squamous cell carcinoma is discussed in detail by O'Higgins et al. who highlight the population expansion of CTLA4 + Tregs, seen more often in patients with poor outcomes [50]. Ipilimumab is a monoclonal antibody directed against CTLA4 that has been used in the treatment of melanoma [51]. Its utility in head and neck squamous cell carcinoma is currently the subject of several clinical trials [52]. Anti-CTLA4 treatment may be more relevant in treating older patients, as we demonstrated that they have higher FOXP3 + T-cell percentages and significant upregulation of relevant gene expression signatures—most importantly CTLA4—compared with younger patients. These observations validate the findings reported by Maroun et al. [53].

TGF- β can be produced by virtually any cell in the body, including tumor and stromal cells [54]. Initially TGF- β acts as tumor suppressant but tumor cells quickly acquire TGF- β resistance, leading to increased stromal and tumor cell TGF- β expression stimulating motility, invasiveness and immunosuppression [54, 55]. Looking into the subgroups of those oral tongue squamous cell carcinoma patients (3/16 or 18.8%) who died of the disease, we identified significantly higher expression of TGF- β , apoptosis and antigen presenting machinery signatures, and a lower percentage of CD4 + T-cells, but without significant variations in FOXP3 + percentages or FOXP3:CD4 ratio in relationship to living patients. Interpretation of these findings is challenging, as it is impossible to distinguish if the higher expression of apoptosis and antigen presenting machinery signatures is attributed to tumor cells, immune cells, or both. Antigen presenting machinery signature reflects the abundance of genes expressed in the major histocompatibility class I pathway and would thus be indicative of a strong anti-tumor immune response [56]. However, it is hard to explain the concurrent higher expression of TGF- β , a pro-tumor anti-inflammatory signature [54, 55]. Given the lower percentage of CD4 + T-cells in patients who died, but without a concurrently lower FOXP3 + population, we can hypothesize

Table 5 Subgroup comparison of clinicopathologic characteristics and tumor immune microenvironment expression profile

	Vital status			Age group (years)			TIL group		
	Dead	Alive		Age ≥ 44	Age < 44		TILs ≥ 40%	TILs < 40%	
N	3	13		5	11		10	6	
Age (years)									
Mean	32	48.46	p = 1.000				48.8	35.33	p = 0.238
Mass size (mm)									
Mean	32.7	17.2	p = 0.043	19	20.55	p = 0.824	18.1	23.33	p = 0.427
Depth of invasion (mm)									
Mean	11.5	8.73	p = 0.800	10	8.78	p = 0.727	7.67	12.5	p = 0.149
Perineural invasion									
Present	2	5	p = 0.550	4	3	p = 0.106	5	2	p = 0.633
Absent	1	8		1	8		5	4	
Lymphovascular invasion									
Present	1	2	p = 0.489	0	3	p = 0.509	0	3	p = 0.036
Absent	2	11		5	8		10	3	
TILs									
Mean	23.33	46.54	p = 0.122	51	38.18	p = 0.323			
CD4%									
Mean	9.36	22.04	p = 0.01	27.71	16.52	p = 0.058	22.18	15.5	p = 0.23
CD8%									
Mean	10.47	15.41	p = 0.559	23.72	11.05	p = 0.08	19.04	7.51	p = 0.045
FOXP3%									
Mean	4.5	4.88	p = 0.844	7.91	3.68	p = 0.005	4.32	5.54	p = 0.435
PDL1 CPS*									
Mean	64.75	37.9	p = 0.285	49.19	41.12	p = 0.728	35.63	54.73	p = 0.354
	Average score			Average score			Average score		
Anti-tumor immune activity									
IFN-γ	6.87	6.48	p = 0.735	7.97	5.91	p = 0.017	7.1	5.65	p = 0.095
TIS	7.65	7.33	p = 0.611	8.07	7.08	p = 0.046	7.66	6.94	p = 0.147
IFN downstream	6.48	5.92	p = 0.353	6.66	5.73	p = 0.013	6.18	5.76	p = 0.395
Inhibitory immune signaling									
CTLA4	8.13	8.19	p = 0.906	8.84	7.88	p = 0.016	8.18	8.18	p = 0.994
PDL2	4.91	5.06	p = 0.578	5.81	4.68	p = 0.003	5.38	4.46	p = 0.016
Tumor immunogenicity									
APM	7.18	6.17	p = 0.014	6.3	6.38	p = 0.851	6.3	6.44	p = 0.711
Tumor sensitivity to immune attack									
Apoptosis	5.34	4.86	p = 0.014	4.75	5.04	p = 0.10	6.3	6.44	p = 0.001
Inhibitory tumor mechanisms									
IDO1	4.49	5.45	p = 0.97	6.33	4.79	p = 0.022	5.78	4.43	p = 0.02
TGF-β	7.52	6.03	p = 0.002	6.08	6.41	p = 0.481	5.96	6.89	p = 0.024

Bold values are statistically significant

CPS combined positive score

that the antitumor response is hindered by lower counts of anti-tumor CD4 + FOXP3- T-cells and higher expression of TGF-β, despite the higher expression of abundant major histocompatibility type I molecules. Although this hypothesis is subject to future validation, there is evidence that CD4 + FOXP3- T-cells have significant anti-tumor activity by activating dendritic cells and expanding the subset of

myeloid cells producing anti-tumor cytokines [57]. Unfortunately, our study is limited by the small number of cases (N = 16) and uncensored events (N = 3), which hinders further outcome analysis [58].

IDO1 is an enzyme of tryptophan metabolism expressed by antigen presenting, endothelial, epithelial and tumor cells, which facilitates expansion of the Treg population [59]. Foy

et al. reported upregulation of IDO1 in oral squamous cell carcinoma, associated with high PD-L1 expression and dense T-cell infiltrate [60]. We validated those potentially clinically important findings in our cohort. Results from administration of pembrolizumab (anti-PD1) plus epacadostat (anti-IDO1) in patients with head and neck squamous cell carcinoma are very promising [61].

We also demonstrated significantly upregulated checkpoint signatures, such as TIGIT, PD-L1 and PD-L2. TIGIT is a member of the immunoglobulin superfamily expressed by Tregs, memory and activated T-cells [62], which binds to CD155, a high affinity ligand present on dendritic cells, endothelial cells, fibroblasts and neoplastic cells. The TIGIT-CD155 interaction downregulates the anti-tumor immune response by inducing secretion of interleukin-10, an anti-inflammatory cytokine [63]. Upregulation of TIGIT in oral squamous cell carcinoma has been reported by several investigators [14, 53, 64]. Experimental blockade with monoclonal antibodies reverses T-cell exhaustion, enhancing anti-tumor immune response [65]. Yearley et al. showed that expression of PD-L2, also a ligand for PD-1, correlates with expression of PD-L1 in head and neck squamous cell carcinoma, although some PD-L2 expressing tumors can be negative for PD-L1—a finding potentially expanding the pool of patients eligible for treatment with pembrolizumab [66].

Decrease in *ARG1* expression was previously reported by Meehan et al., who discuss the depleting effects of arginase 1 function on T-cells. They favor the possibility that *ARG1* downregulation is related to tumor cell dependence on arginine for proliferation, suggesting arginine depletion as a hypothetical treatment strategy in oral tongue squamous cell carcinomas [14].

Downregulated tumor glycolytic activity is harder to explain, since tumors commonly show glycolysis upregulation despite the presence of oxygen [67, 68]. Decreased glycolysis signature expression may be due this increased glucose demand, which can quickly deplete tumor glucose supplies [67].

Concurrent downregulation of mast cell and endothelial cell signatures is not surprising, given the promoting role of mast cells in neoangiogenesis [69]. Interaction between mast cells and Tregs is complex, with mast cells counteracting the inhibitory role of Tregs and Tregs suppressing mast cell functions, including secretion of CD8+ T-cell recruiting chemokines [70]. Since Tregs can also inhibit maturation of dendritic cells [71], the downregulation of both mast cell and DC signatures seen in oral tongue squamous cell carcinomas could reflect the functional dominance of Tregs in the tumor immune microenvironment, a hypothesis needing further exploration given the adverse prognostic role of low mast cell densities in patients with oral squamous cell carcinoma [72].

Study Strengths and Limitations

In this study we performed an in-depth investigative analysis of the tumor immune microenvironment of oral tongue squamous cell carcinoma. Novel in the present study is the correlation of transcriptomic findings with morphologically or immunohistochemically assessed TILs and their subsets. Among the limitations of the study is the small number of cases, which puts some objective limits in the generalization of results and hinders further subgroup analysis and characterization. A certain amount of selection bias may be affecting our results because of the case selection process, since we identified patients younger than 44-year-old and staged-matched older patients. Validation of results in a larger randomly selected patient group is therefore necessary. Although we identified significant gene expression differences between tumors and matched non-neoplastic tissues, a limitation worth mentioning is that according to the field cancerization model pre-neoplastic changes may be present at the molecular level in the tissues located at the proximity of a tumor [73].

Conclusion

RNA expression profiling of oral tongue squamous cell carcinoma shows a complex inhibitory tumor immune microenvironment, which is partially reflected by the relative proportions of T-cell subsets as determined through immunohistochemistry and digital image analysis. Immunostains for CD8 and FOXP3 can be informative in deciphering the functionality of the underlying anti-tumor immune response, and in potentially predicting response to systemic therapy. Further prospective studies are needed to validate these findings.

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