# Tumor Infiltrating Lymphocytes as Immunebiomarkers in Oral Cancer: An Update

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**Abstract:** The high morbidity and mortality associated with oral cancer has necessitated the exploration of newer diagnostic and prognostic biomarkers. In recent decades, targeting immune landscape has emerged as a newer approach as aggressive tumor biology and therapy resistance are influenced by the interplay between tumor and immune cells. A reciprocal association between chronic inflammation and carcinogenesis is well established and tumor infiltrating lymphocytes (TILs) represent inflammatory milieu of tumor microenvironment (TME). The varied T-cell phenotypes in different stages of cancer influence the prognostic and predictive response of the patients. Along with the conventional treatment options, Immunotherapy has evolved as a suitable alterative for oral carcinoma patients especially with recurrent and metastatic disease (R/M) but response is still unpredictable. Tumor microenvironment (TME) plays a key role to either lessen or boost up immune responses. There is an urgent need for extensive studies to be undertaken to better understand how tumor cells escape immune surveillance and resist immune attack. This review is an attempt to elucidate the concept of immune infiltrate in oral squamous cell carcinoma (OSCC) and thus, understanding the role of immunoscore as an adjunct to TNM staging to guide patient treatment.

**Keywords:** Immunotherapy, Immune markers, Oral cancer, Precision Medicine, Prognosis, Tumor infiltrating lymphocytes.

## INTRODUCTION

Indian fact sheet of GLOBOCAN 2020 reported a significant rise in the incidence of lip and oral cavity cancer since 2012; with an incidence of 16.1% and 4.8% in males and females respectively [1,2]. Oral squamous cell carcinoma (OSCC) accounts for over 90% of Head and neck squamous cell carcinoma (HNSCC) and presents a significant challenge due to its locally invasive nature, aggressive growth pattern and dismal 5-year survival rates [3]. The high recurrence rate, increased metastasis and therapy resistance with current standard-of-care suggest that an incorrect paradigm might be followed [4]. With Precision medicine and Targeted care taking the forefront; the scientific community's concept of cancer has changed dramatically from cancer cell biology centric approach in particular genetics and epigenetic factors to dynamic tumor microenvironment (TME) and immune contexture [5]. This review is an attempt to highlight the association of expression profiling of tumor infiltrating lymphocytes (TILs) with oral cancer outcome thus substantiating their role as immune biomarkers.

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### Immune-Landscape Reflecting Cancer Biology

The immune cells infiltrating within the tumor and surrounding microenvironment participate in the pathogenesis of malignancy thus influencing tumor evolution, metastasis and behavior [6]. Many components of the adaptive and the innate immune systems (cellular components, cytokines, growth factors and check point inhibitors) drive the immune response to tumor biology [7]. The term "Immune contexture" is given to the relative fraction, localization and functional orientation of various immune cell subsets within the TME [8]. The constructive impact of the immune infiltrate has been proven in esophageal, colorectal, breast, ovarian, bladder, urothelial, prostatic, pancreatic, cervical, hepatocellular, gastric and head and neck cancer and melanoma [9]. The subpopulation of Tumor infiltrating lymphocytes (TILs) include CD3+ T cells (pan- T cell marker), CD4+ T cells (helper T cells), CD8+ T cells (cytotoxic T cells) and CD4+CD25+ regulatory T cells (Treg cells; forkhead box protein P3 [FOXP3] cells) [10]. TILs have also been classified into intratumoral (iTILs) and stromal lymphocytes (sTILs) depending on the tumor region assessed [11]. Thus quantification and sub typing of TILs affect the disease outcome by reflecting the tumor immune response. Literature review has revealed conflicting results regarding the relationship between CD3, CD8, CD4, FOXP3+ TILs and prognosis but many have agreed that "The elevated CD3 or CD8 expression exhibited by

ISSN: 1929-2260 / E-ISSN: 1929-2279/23 

TILs is considered as an independent factor for favorable outcome (local progression-free, distant metastases-freeand overall survival) [7,10]." TILs have also been translated into a therapeutic agent in adoptive cell transfer (ACT) based treatments of cancer, mostly of solid malignancies [12].

Conventional cancer classification tools include tumor staging and grading which involve assessment of various clinical and pathological parameters. The prognostic categorization provided by the TNM (tumor, node, metastasis) staging system has not drastically improved by any gene or genomic signature in over 80 years [13]. It has been observed that tumors with same stage have varying prognosis and exhibit diversity in context to aggressiveness and treatment response [14]. As cited by previous studies approximately 20-25% of TNM I/II stage patients have reported with relapse, rapid tumor progression and mortality, where as some patients have shown advanced cancer stage remaining stable for longer period of time. It would have been better if we could do risk stratification of patients especially based on immune profile, thereby improving adjuvant treatment selection. It would curb under/over treatment of patients and help avoid unnecessary toxicities without compromising outcomes [15].

An important therapeutic backbone in various solid malignancies already include anti-PD-1 (programmed death protein) agents which have proven beneficial in platinum-refractory recurrent/metastatic HNSCC[16]. PD-1/PD-L1 axis (programmed death ligand 1) attenuates antigen-specific T-cell immunologic response thus substantiating the hypothesis that PD-1/PD-L1 blockade may be an effective cancer immunotherapy [17]. However, not all patients are benefitted and responses are unpredictable to anti-PD-1/PD-L1 antibody treatment. Researchers have observed that tumor regression induced by PD-1/PD-L1 blockade is dependent on a PD-L1 status, pre-existing TIL density and cytokines level [18]. An immune microenvironment model comprised of TIL status (presence or absence) and PD-L1 expression status (positive or negative) is established for immunotherapy prediction and concept of immunogenic (hot) TME or a nonimmunogenic (cold) TME is being developed (Table **1**) [19]. The classification of cancer into immunogenic "hot" and "cold" tumors is an impending advancement in immunological context of tumors reflecting the patient's immune profile [20]. The current research is therefore focused on typifying immune signatures, to evaluate tumor receptiveness to immunotherapy, also to explicate the mechanisms of immune evasion, immunosurveillance and immunoediting [21].

## Protocols for TIL Evaluation

The assessment of TILs has been attempted by various researchers using different methodologies. It has been recommended to develop a simple and accurate method for TILs evaluation in HNSCCs and OSCCs which if used during routine histopathological reporting could be useful for the pathologist and the clinician by providing valuable prognostic information [22]. Histopathology based studies, which employ conventional H&E staining and immunohistochemistry (IHC) along with descriptive, semiquantitative and quantitative scoring methods have been used to evaluate TILs in tissue sections (Table **2**) [23]. A standardized approach to evaluate density of TILs in

**Table 1: Concept of Hot and Cold Tumor Predicting Response to Immunotherapy**

| S. No | HOT TUMOR | COLD TUMOR |
|---|---|---|
| 1. | higher CD8+ TIL density (Th1), less immunosuppressive cells | Lower CD8 + TIL density (Th1), more immunosuppressive cells |
| 2. | high level of IFN/IFN-γ | lower level of IFN,IFN-γ |
| 3. | increased cytokines /chemokines | decreased cytokines /chemokines |
| 4. | high level of immune checkpoints | low level of immune checkpoints |
| 5. | high tumor mutational burden | low mutational load |
| 6. | high MHC class I expression | low MHC class I expression |
| 7. | good or favorable gut bacteria | Unfavourable gut bacteria |
| 8 | Activated NF κB signaling | Activated Wnt/β Catenin Pathway |
| 9. | better ICI efficacy | lesser response to ICI |
| 10. | Metabolically non-compromised | Metabolically compromised |

IFN-γ -Interferon gamma, MHC-Major histocompatiblity Complex, NFκB- Nuclear factor kappa B, ICI-Immune checkpoint Inhibitors.

**Table 2: Methods for Tumor Infiltrating Lymphocytes (TILs) Evaluation**

| S. No. | Author | Technique | Assessment Methodology and Scoring Criteria |
|---|---|---|---|
| 1. | Rajjoub S *et al.* (2007) [11] | Tissue microarray (TMAs) and Immunohistochemistry (IHC) | Expression rate of the CD3+ cells present in the tumor was assessed on a scale of 0 to 3. Tumors were classified in to two groups CD3 low or CD3 high based on the mean score value. |
| 2. | Prestcher D *et al.* (2009) [45] | Tissue microarray (TMAs) and IHC | The image analysis software COUNT was used for semiautomatic evaluation and average number of lymphocytes was evaluated for quantification. |
| 3. | Zancope E *et al.* (2010) [42] | Immunohistochemistry (IHC) | Quantified the CD8+ and NK cells present in the peritumoral and intratumoral region of OSCC by calculating the percentage and the density of the cells using an integration graticule. |
| 4. | Balermpas P *et al.* (2014) [10] | Immunohistochemistry (IHC) | The densities of TILs were assessed in intraepithelial compartment, the stroma and the tumor periphery semiquantitatively using the criteria given by Dahlin *et al* on a scale of 1-4. |
| 5. | Ohman J *et al.* (2015) [36] | Immunohistochemistry (IHC) | Digitalized images of sections, were retrieved with a light microscope and positively stained nucleated cells on the selected epithelium and the connective tissue compartments were quantified. |
| 6. | Nguwen N (2016) [39] | Tissue microarray (TMAs) | Digital images were taken using specific softwares. CD4, CD8, FoxP3, and CD68 antibodies stained TILs were quantitated manually. |
| 7. | Brahambhatt B *et al.* (2016) [47] | Immunohistochemistry (IHC) | CD8+, CD4+ and regulatory T cells (FOXP3+) infiltrated in different tumor compartments were counted in three different areas under ×40 microscopic field. Mean value for each area was calculated, and results were expressed as number of T cells infiltrated in each tumor compartment. |
| 8. | Xu Q *et al.* (2017) [37] | Hematoxylin and eosin (H&E) | Proportion of TILs was assessed in different stromal compartments which were further categorized as cell-rich(carcinoma cells or tumor nest occupy more than 70% of the tumor area), moderate(the intermediate situation) and stroma-rich types(more than 70% is stromal area).<br>TILs were evaluated and score was calculated as the total stromal area divided by the lymphocyte-occupied area. |
| 9. | Ou D *et al.* (2017) [30] | Immunohistochemistry (IHC) | Whole slides were digitized and image analysis was conducted for CD8 and FoxP3 stained cells using specific softwares. TILs were quantified as average number of stained cells per mm square of the selected tumoral and control areas. |
| 10. | Fang J *et al.* (2017) [31] | Immunohistochemistry (IHC) | Images were taken and semi-automatically evaluated using computer assisted image analysis software. The specific markers (CD4, T-bet, CD8, CD57 and CD68) were used to identify and quantify immune cells. |
| 11. | Steele KE *et al.* (2018) [26] | Immunohistochemistry (IHC) | Digitization of immunostained slides was done. Digital images were viewed and annotated manually to mark tumor regions. |
| 12. | Chen WY *et al.* (2018) [48] | Immunohistochemistry (IHC) | TILs stained with specific markers were counted and quantified. The median number was used as the cutoff point to define high or low expression. |
| 13 | Diao P *et al.* (2021) [29] | Immunohistochemistry (IHC) | TIls were quantitatively evaluated using specific markers using digital analysis |
| 14 | Caruntu A *et al.* (2021) [49] | Immunohistochemistry (IHC) | CD4+, CD8+ and CD56+ lymphocytes were evaluated as the number of cells per high power field (HPF) both in the intratumor location and at the front of invasion (in the hotspot by counting 10 adjacent HPFs). Pattern of distribution was also seen (absent, nodular, diffuse). |
| 15. | Dasgupta S *et al.* (2022) [22] | Hematoxylin and eosin (H&E) | In the stromal and intratumoral area of the tumor mononuclear cells were assessed and the TIL level was classified a %TILs. th stromal TIL and TIL levels were noted in each case. |
| 16 | Almangush A *et al.* (2022) [24] | Hematoxylin and eosin (H&E) | TILs scoring was done as per the criteria proposed by the International Immuno-Oncology Biomarker Working Group. The percentage of stromal area occupied by infiltrating lymphocytes represented Stromal TILs. |

breast cancer was recommended by the International TILs Working Group in 2014, by measuring the area occupied by mononuclear cells over the stromal area. The density of TILs was determined on H-E-stained sections as %age of stromal TILs [24]. The consensus guidelines for TILs assessment in breast cancer have been adapted for colorectal carcinoma, other solid malignancies and head and neck squamous cell carcinomas, demonstrating significant results [25]. In the last two decades approaches for TIL evaluation has changed from conventional to digital. Automated image analysis methods using different softwares focus on certain morphological or spatial aspects of the TME [26]. Digitization of histology slides and automation has made image analysis of tissue sections feasible, where the softwares can classify and estimate the immunostained cells [27,28]. Although these softwares do have certain issues as the softwares only distinguishes the cells based on different depth of color, so by varying the range of the depth of color, density of TILs may be overestimated or underestimated.

TILs role in HNSCC and OSCC is still uncertain because of small cohort sizes, retrospective approaches, heterogeneous populations, univariate analyses, lack of standardized methodology for TIL quantification, varied anatomic sub-site, tumor compartment differences (intra-tumoral vs. stromal) and HPV-positive ($HPV^+$) compared to HPV-negative ($HPV^-$) status [29,30]. The immune infiltrate is analyzed in various tumor compartments, e.g., the invasive margin, the tumor periphery or the tumor nests, but the exact definition and demarcation of these compartments itself is not clear. In addition, tissue samples obtained for evaluation have been very inconsistent and non-uniform [11] and based on the preference or availability, resection samples, tissue microarrays, punch biopsy or incisional biopsy samples have been used [31]. Preliminary analysis on a tissue sample may be perplexed by the pre/post treatment dynamics, prognostic significance of which needs to be evaluated further [32]. In OSCC, many immune biomarkers have been studied retrospectively, using tumor samples from the archive and thus may not depict the spatial and temporal intratumoral heterogeneity impact [33]. Also, a series of complex inflammatory targets are implicated in the progression from potentially malignant disorders to OSCC, which have not been elucidated yet [34]. Therefore, elaborate knowledge of dynamic interaction between immune contexture and TME is critical for both selection of and understanding the reaction to treatment [35]. Due to these issues, a standardized, uniform immune stratification system based on the immune contexture needs to be delineated for OSCC along with standard cut off values, to speed up the process of quantification thus facilitating routine diagnostic pathology [20].

## Immunoscore(IS) a Future Concept

TIL subsets can guide disease progression into different directions based on a “agonist or antagonist” relation [36]. International validation and acceptance of the IS labeled as TNM-I (TNM-Immune); as a new indicator for classifying and stratifying the cancer is under investigation. The tool has already been validated for breast, melanoma and colorectal cancer and could have a promising role in HNSCC too. Till date, in HNSCC, a programmed ‘IS’ evaluation on the whole cross sections of the tumor tissue has not been applied [37]. In the literature, studies have documented that the immune system may boost chemotherapy and radiation approaches as pre-existing immunologic response has been associated with enhanced effects of these treatment modalities [38,39]. A high IS in HNSCC is associated with lower levels of Tregs, increased PD-L1 and MHC type I expressions in tumor cells, signifying its ability to identify anti-PD-1/PD-L1 therapy sensitive subset of tumors [40].

## Potential Challenges and Future Perspectives

In order to establish the precision medicine-informed immunotherapy there are few challenges that needs to be addressed and some concepts need further clarification. The need of hour is to study the mechanisms that inhibit or impede the growth of OSCC by reinstating immunehomeostasis at the premalignant stage [41]. Advanced techniques like digital analysis, quantitative IHC and quantitative multiplex immunofluorescence can be used for the scoring practice. In OSCC, TILs need to be analyzed in patients undergoing radio/chemotherapy to comprehend the effect of changes in infiltrates on the clinical outcome [42]. The novel IS parameter has not yet completely elucidated the role of cancer cell factors that transform tumor immunogenicity. The impact of cancer cell autonomous interferon gamma (IFN-γ) signaling in inciting an “inflamed” microenvironment is still ambiguous in HNSCC as compared to other malignancies. It would be essential to determine complex set of genes which are associated with a cytotoxic T-cell response [43]. In addition, separate evaluation of potential biomarkers sans other factors, could miscalculate the intricacy of immune response. A

dynamic communication between the immune system and TME is facilitated by the complex oral and intestinal microbiota, ultimately regulating the effectiveness of cancer therapies. Future research should consider the promising immunomodulatory effects of the microbiota, as a predictive immune biomarker in OSCC [44].

## CONCLUSION

Adopting immunoscore as an adjunct to TNM staging will better stratify the patients, guide the treatment and positively impact the outcome. The understanding of molecular mechanisms that channel a favorable "inflamed" TME can increase the patient's response to novel immunotherapeutic agents. High-throughput molecular profiling of the TME would open up new therapeutic options as immunomodulatory agents have brought altogether new approach to treat cancer patients after multiple other modalities have been exhausted. The future of immunotherapy will rest on standardization and increased clinical utility of immunemarkers and evaluation of these agents in early-stage disease.

## FUNDING

Self Funded.

## CONFLICT OF INTEREST

Authors declare no conflict of interest.

## CONTRIBUTIONS MADE BY AUTHORS

**Concept & Design:** Dr. Deepti Sharma, Dr. George Koshy and Dr. Abi M Thomas.

**Definition of intellectual content**: Dr. Deepti Sharma and Dr. George Koshy.

**Literature Search:** Dr. Deepti Sharma and Dr. Dr. George Koshy.

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Received on 07-05-2023 Accepted on 15-06-2023 Published on 07-07-2023

https://doi.org/10.30683/1929-2279.2023.12.6