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# AUGMENTATION OF TIL-CD3 IN ORAL CANCER: VARIATIONS IN RELATION TO STAGE AND GRADE OF DISEASE

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**Key words:**

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**ABSTRACT:** Oral squamous cell carcinoma (OSCC) is often associated with a lymphocytic infiltrate that is believed to represent an in vivo immune reaction to the tumor cells. In this study, the tumor-infiltrating lymphocytes (TIL) associated with primary OSCC were characterized in hundred newly-diagnosed patients to determine the nature of the immune response to the primary tumor and to correlate it with stage and grade of the disease in order to derive its significance. TILs were estimated in surgically removed oral cancer tissue, histopathologically and with immunohistochemistry, using antibody against CD3 (Dakopatts, Denmark). TIL were estimated in intratumoral and peritumoral areas and arbitrarily graded in to four groups. Site wise CD3 infiltration was found maximum in buccal mucosa and minimum in tongue, rest of the cases being distributed in other locations and the distribution showed statistical significant in intratumoral location. There was no significant correlation between tumor size and CD3 infiltration. Further CD3 infiltration was not related to either lymph node enlargement or bone involvement, however it was more in intratumoral than in peritumoral region and this was statistically not significant. OSCC initiates immune response as indicated by increase in CD3 cells infiltrating and surrounding the tumor, which persists or abates in an inconsistent manner with the growth of tumor size, grade and stage of the disease with time lapse.

**INTRODUCTION:** Squamous cell carcinoma is by far the most common type of cancer of the oral cavity, representing more than 90% of all oral cancers. Despite refinement of surgical techniques and adjuvant therapies, the prognosis for patients with oral squamous cell carcinoma remains poor. Identification of prognostic factors related to tumor biology might improve this assessment.

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Tumor-infiltrating lymphocytes (TILs) are often found in tumors, presumably reflecting an immune response against the tumor [1]. How exactly all the cells function and change their functions with differing stimuli, so as to give differing response; is far from clear.

The significance of tumor-infiltrating lymphocytes (TIL) has attracted much attention in relation to the prognosis of cancer patients. Human solid tumors progress and metastasize, whereas TIL, generally present in substantial numbers in or around these tumors, fails to restrict or arrest tumor progression. Immune cells in the tumor microenvironment not only fail to exercise antitumor effectors functions,

but they are co-opted to promote tumor growth [2]. The tumor microenvironment is created by the tumor and dominated by tumor-induced interactions. Although various immune effectors cells are recruited to the tumor site, their anti-tumor functions are down regulated, largely in response to tumor-derived signals. The result is tumor escape from the host immune system. Tumor escape is accomplished through the activation of one or several molecular mechanisms that lead to inhibition of immune cell functions or to apoptosis of anti-tumor effectors cells.

The ability to block tumor escape depends on a better understanding of cellular and molecular pathways operating in the tumor microenvironment. Early attempts at analyzing TILs in oral SCC yielded some provocative but mixed results, and ultimately were inconclusive due to the limitations described [3, 4, 5, 6]. No definitive studies exist either supporting or dismissing the prognostic relevance of TIL analysis in oral squamous cell carcinoma. As with many studies on human cancer, a limited number of samples and diverse stages and sub sites cloud the interpretation of the available data.

The present study is aimed to quantitate the tumor infiltrating $CD_3$ positive T-lymphocytes, in different grades of oral squamous cell carcinoma in Intratumoral as well as peritumoral locations using monospecific antibody staining of formalin-fixed, paraffin-embedded tissue sections.

## MATERIALS AND METHODS:

One hundred consecutive cases of histologically proven primary oral squamous cell carcinoma (OSCC) of different grades of differentiation and who consented to participate in the study were registered in the present study **Table 1**.

**TABLE 1: STUDY CHARACTERSTICS OF PATIENTS:**

| **Age distribution of patients** | |
|---|---|
| **Number (%)** | |
| ≤ 30 yrs | 07 (07%) |
| 31-40 yrs | 26 (26%) |
| 41-50 yrs | 31 (31%) |
| 51-60 yrs | 28 (28%) |
| 61-70 yrs | 07 (07%) |
| >70 yrs | 01 (01%) |
| **Sex wise distribution of patients** | |
| Male | 84 (84%) |
| Female | 16 (16%) |
| **Site of lesions in oral cavity** | |
| Alveolus | 12 (12%) |
| Buccal mucosa | 40 (40%) |
| Floor of mouth | 02 (02%) |
| Gingivobuccal sulcus | 14 (14%) |
| Hard Palate | 02 (02%) |
| Lip | 12 (12%) |
| Mandible | 01 (01%) |
| Retromolar Trigone | 03 (03%) |
| Tongue | 14 (14%) |
| **Histological grading of patients** | |
| Well differentiated tumors | 78 (78%) |
| Moderately differentiated tumors | 21 (21%) |
| Poorly differentiated tumors | 01 (01%) |
| **Clinical staging of patients** | |
| Stage I | 23 (23%) |
| Stage II | 49 (49%) |
| Stage III | 07 (07%) |
| Stage IV | 21 (21%) |

Tissue samples of oral cancer patients were fixed in formalin 10% and paraffin embedded tissue blocks were prepared. Three-micron sections of tissue were cut & stained with Haematoxylin – Eosin (HE

stain). HE stained sections were scanned in high power resolution & confirm the malignancy. After the malignancy was confirmed, thin sections were cut from tissue blocks of oral cancer patients and were captured on tissue bond coated slides (Biocare, USA) and stained using monospecific antibody against CD3 phenotype (Dakopatts, Denmark). Automated immunohistochemistry staining protocols were employed for staining. Briefly; de-parafinization of tissue sections were done by heating over hot plate at $60^0$C. Rehydration of tissue sections was done in graded alcohol (100%, 70%, and 50%), five minute each. Blocking of endogenous peroxidase was done by $H_2O_2$ in methanol, followed by antigen retrieval. Tissue sections were incubated with primary antibody at $4^o$C overnight in moist chamber.

Slides were washed in tris buffer three times, the incubation with polymer based secondary antibody was done at $37^o$C for 1hr. Slides were again washed with tris buffer as previously. Exposure with peroxidase substrate (DAB) solution was done for 10-30 sec and slides were washed and counterstained with hematoxilin. Tissue section of tonsil was used as positive control and primary antibody replaced by dd$H_2O$ was used as negative control in the study. Finally stained slides were dehydrated, mounted, observed.

All the slides were evaluated by two independent observers.

**Microscopic evaluation of Immuno-histochemistry slides:**

Lymphocytes that reacted positively to the CD3 antibodies were semi-quantitatively assessed under a light microscope at a high power magnification (63 x objectives). A total 10 spots, five visual fields (hot spots) for intratumoral and five for peritumoral were randomly selected and the numbers of CD3 positive cells were counted to define intra-tumoral / peri-tumoral / metastatic tissues as feasible & applicable. One field corresponded to 0.08 $mm^2$. Average positive lymphocytes infiltration in intra-tumoral and peri-tumoral area was counted, in hot spots and was converted to scale of positive cells per square millimeter for the purpose of homogenicity and international comparison. The degree of infiltration for CD3 positive cells was arbitrarily classified into four scales based on the number of infiltrating cells per $mm^2$ area of the tissue section: Scale I [<2000 cells /$mm^2$]; Scale II [2000-4000 cells /$mm^2$]; Scale III [4000-6000 cells /$mm^2$] and Scale IV [>6000 cells /$mm^2$].

**RESULTS AND DISCUSSION:** The tumors of oral cavity patients display a wide variation in the number of tumor infiltrating CD3 positive T cells. A detailed examination of the anti tumor response was done in a group of 100 patients of oral cavity cancer. The number of CD3 positive T cells present in stroma or between epithelial tumor cells was counted to assess the tumor specific immunity in individual patients and its association with stage and grade of the tumor.

In poorly differentiated tumors, mean number of intratumoral infiltration of CD3 positive T cells that infiltrated tumors of all patients was only of Scale I and peritumoral infiltration of CD3 positive cells could not be seen or very scanty.

In moderately differentiated tumors the intratumoral infiltration was mostly Scale II & III and peritumoral infiltration was also seen in almost all the cases between Scale II & III. In well differentiated tumors the major distribution was intratumoral higher scale and peritumoral infiltration wherever seen was of Scale II except in few cases, where it was of Scale IV. Peritumoral infiltrate was less common in well differentiated tumors (**Fig.1a).**

The mean value of intratumoral CD3 infiltration is stage I tumors was 5130 cells/$cumm^2$ showed a marked difference with peritumoral infiltration ie 2502 cells/$cumm^2$. Mean CD3 infiltration values for stage II & III tumors in intratumoral infiltration (4332 & 3662 cells/$cumm^2$) were not much variant from peritumoral infiltration (3271 & 2739 cells/$cumm^2$). While the status for stage IV was 3532 & 2932 cells/$cumm^2$ again showed marked difference.

If we examine the $CD_3$ cell counts as such which for ease for comparison and interpretation, we grouped arbitrarily in to a scale of I for 1-2000cells/$cumm^2$, scale II for 2001-4000 cells/$cumm^2$, scale III from 4001-6000 cells/$cumm^2$

and scale IV >6001 cells/cumm$^2$ and correlated with WHO grade of the disease. In our study group, 21 cases were moderately and one was poorly differentiated while remaining 78 were well differentiated. As only one case was poorly differentiated, hence, it was assessed combined with moderately differentiated cases. We find that, for well differentiated tumors the intratumoral CD3 cells ranged from 9598-1137 cells/cumm$^2$, with a mean of 4594.9 cells/cumm$^2$ and poorly and moderately differentiated group CD3 cells ranged from 8080-1067 cells/cumm$^2$, with a mean of 4196.7 cells/cumm$^2$ which was not statistically significant (p=0.459).

While in peritumoral tumoral locations CD3 cells in well differentiated tumors was ranged from 7946 -1038 cells/cumm$^2$ with a mean of 2474.5 cells/cumm$^2$ in intratumoral and 6785-1256 cells/cumm$^2$, with a mean of 4653.4 cells/cumm$^2$ showing significantly higher mean ratio in well-differentiated cases as compared to that of poorly/moderately differentiated cases (p=0.001).

There were different types of morphological appearances of SCC in our study. In some of the cases intact and normal looking epithelium was located whereas in other cases, this lining epithelium itself was proliferative and that had larger and deeply growing retipegs. Some of these retipegs were showing marked cellular proliferating activity. In still other groups of cases the tumor was seen arising from the lining epithelium itself (**Fig.1b**). In those cases, where normal or moderately proliferating epithelium was seen, there was a clear sub-epithelial zone before the tumor proliferation could be seen. This group of tumors shows concentration of CD3 lymphocytes in between the epithelium and tumor and this was taken as best example of peritumoral infiltration **(Fig.1c**).

In many of these sections some salivary glands as well as muscle tissue could also be seen. Although sparse but CD3 cell were seen in these locations as well (**Fig.1d**).

In cases of the above subgroup, where tumor was poorly differentiated or in advanced stage, $CD_3$ cell infiltration was only of low scale (**Fig.1e**).

The other morphological pattern in our study group comprised cases where sections at the far end of surgical resection showed only tumor. These were the cases where classical peritumoral infiltration of CD3 cells could not be assessed. However, in these cases groups and lobules of tumor cells were seen and some cells surrounding these lobular areas as a whole or in between interlobular areas could be seen. CD3 count in these areas also differed from classical densely infiltrated peritumoral areas.

The classical intra lobular infiltration of CD3 cells constitutes the real intratumoral infiltration (**Fig.1f),** but in wider perspective all the cells which are seen either within a single lobule or within the diffused undefined tumor area have been taken as intratumoral. The intratumoral infiltration itself varied in intensity and has been grouped from scale I-IV. In some of the cases there was dense collection of CD3 cells around the blood vessels and these areas were really adjacent to well formed tumors.

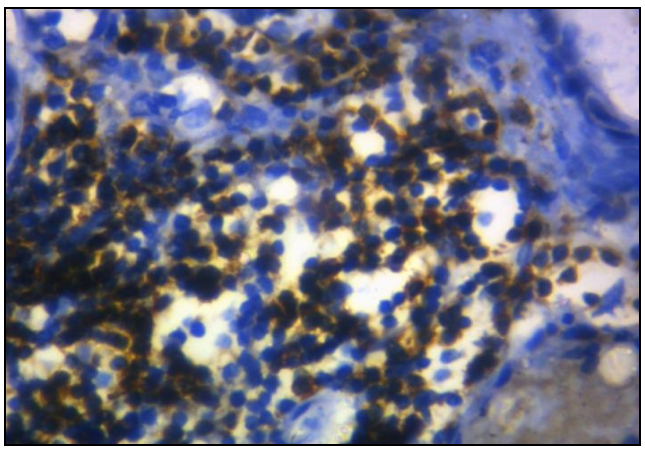

**FIG. 1 a**

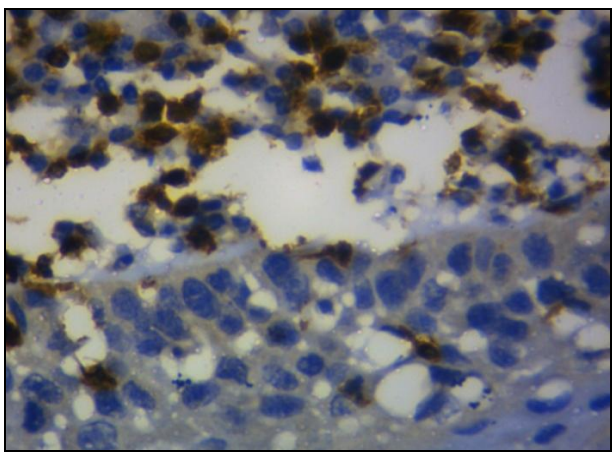

**FIG. 1 b**

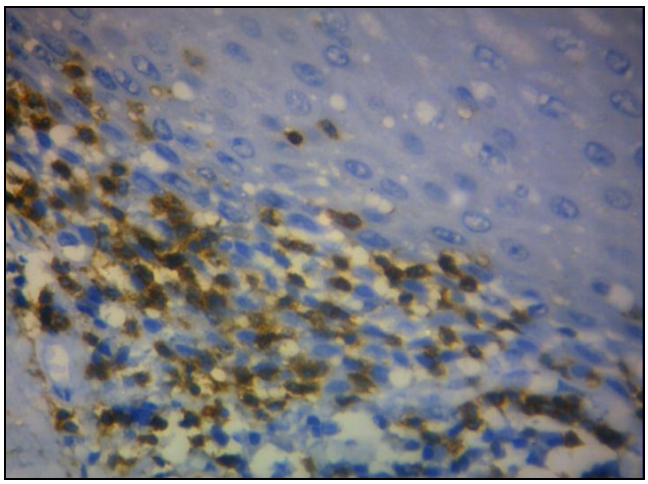

**FIG. 1 c**

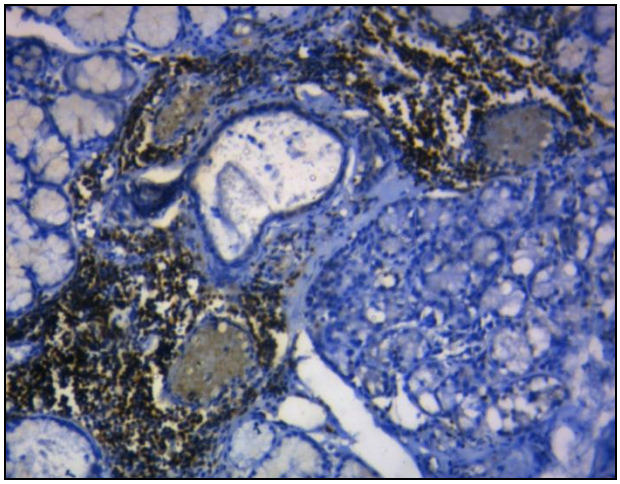

**FIG. 1 d**

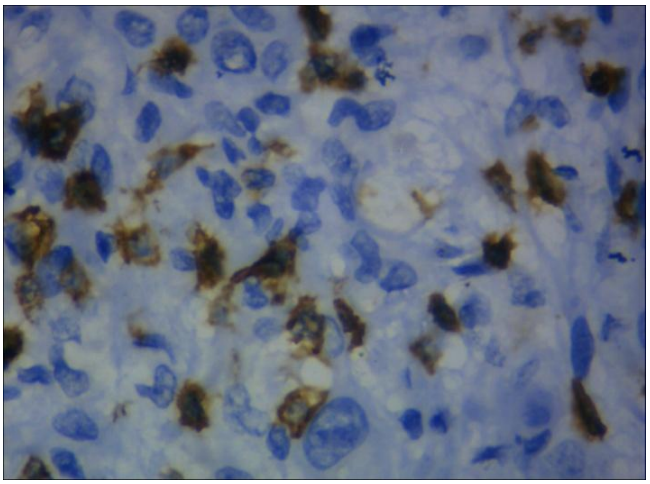

**FIG. 1 e**

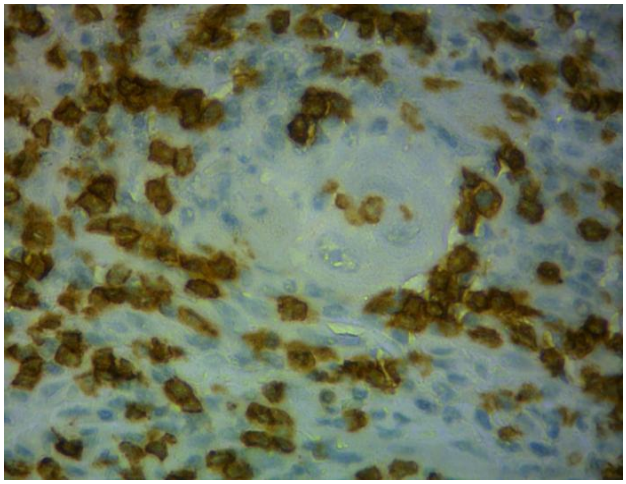

**FIG. 1 f**

Oral cancer in India is almost always tobacco related (eating, chewing, snuffing or bidi & cigarette smoking) and or also associated with alcohol consumption; and the newest causative factor appears to be HPV related oral cancer which is most common in Western Countries. In India no studies have been taken to sub classify the ateopathogenesis in relation to immunology, genetics or as such molecular biology parameters. This is important because the mechanisms which are now coming up are pointing towards basic genetic alterations caused by any factor.

In future, it is going to be an immune reaction for/or against the tumor and this also will be different in different individuals. Although we are still far away at individualistic approach in immunopathologic or immunotherapeutic level, some broad generalizations have to be made which was the basic idea of the present study.

In the present study which comprise a series of 100 oral cavity SCCs in which we have been able to establish certain facts with confidence. The first major observation has been that, all the tumors which have well differentiated tumor morphology and without distant metastasis showed marked increase in CD3 infiltration, in both locations intratumoral and peritumoral. This distribution was different in various stages of tumors. Peritumoral infiltration was of high grade in moderately differentiated tumors whereas intratumoral infiltration was more in well differentiated tumors. Perhaps this distribution of $CD_3$ cells in tumors could be related to tumor microenvironment and secretions by T/B lymphocytes which allow the penetration and localization of more T lymphocytes within the tumor. In one case which was poorly differentiated, the infiltration was of scale I and at intratumoral location and only a shade better than that is of scale II in peritumoral locations. When we further stratified our data according to tumor size:

we grouped tumors/size in to three groups-small(>0.5-1cm), moderate (2-3 cm) and large size (4->4cm).

Very few workers have described as larger a series as ours, comprising only oral cavity tumors, that too SCCs. This has facilitated us to divide tumors into eight different sites group from which it arose. When we correlated our results according to tumor site maximum number of tumors were seen arising from buccal mucosa (40), followed gingivobuccal sulcus and tongue (14 each), alveolus and lip (12each), retro molar trigone (3), floor of the mouth (2), hard palate (2) and mandible (1). None of the associations were significant statistically.

Ravindra Uppaluri et al; 2008 [7] in his review described that the first studies examining specific lymphocytes were performed in 1980s using newly available monoclonal antibodies for specific T cells [8]. Using a selected cut off number of cells per high power field (HPF), Wolf and Colleagues identified a survival benefit for patients who had intratumoral CD4+ T cell, but not CD8+ T cell, infiltration. However, the significant limitations in the study included a 9.5 month average follow-up (range 2-25 month), limited tumor numbers and heterogeneous tumor sites.

The largest subset of tumors in their 40-patient study was a group 10 patient with oral cavity tumors. In contrast Guo and colleagues [9] examined 26 patients, again from diverse sub sites, and found a trend towards improved survival with increased numbers of all T lymphocytes subsets but statistical significance was not achieved. Finally, Snydermann et al [10] performed a fluorescence activated cell sorter (FACS) based assay on TIL preparations from 16 patients with various stages of HNSCC. These investigators identified a better prognosis that had CD4/CD8 ratio less than one and therefore suggested that the lack of CD8 cells in the tumor may have led to poorer outcomes.

In a study by TV Shibuya et al. 2002[11] who also mainly worked on CD3 response was seen in poorly differentiated cancers and these patients had a higher incidence of recurrence. He also mentioned that CD3 response was the single greatest prediction of reduced disease free survival.

We have slightly different experience with our un-to-date limited time follow-up of one poorly differentiated, five moderately differentiated, and eight well differentiated tumors who had a node metastasis. All these cases had scale II- IV TIL either intratumoral or peritumoral infiltration. None of these cases showed recurrence in our limited time follow-up. This shall be better explained when results of other markers are available for conclusive correlation and explanations.

**CONCLUSIONS:** Every patient is different and so is its immune response is different like genetic susceptibility and the concept of personalized medicine for each person on similar lines. Individualistic immune response will, very soon, have to be taken into consideration as to how cancer will behave in the particular host.

So is the TIL story, which depends upon a number of factors in an individual like genetic makeup, immune system strength, prexposure to a stimulant and present status of the intensity of the stimulus like intensity and dose of infection. Another very important factor will be the time lapse after the tumor development, and when we got the tumor tissue after surgery. This time lapse itself will be responsible for intensity of infiltration besides other factors.

In immediate future, we shall need the tools to measure the above mentioned status in patients. Only then, may we be a position to predict the course of disease and outcome precisely.

**CONFLICT OF INTEREST:** There is no conflict of interest to disclose.

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