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**ORIGINAL ARTICLE**

# Expression of CD3 Levels of Tumor Infiltrating Lymphocytes (TILs) in Various Grades of Oral Squamous Cell Carcinoma of Tertiary Care Hospitals of Peshawar

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## ABSTRACT

**Introduction:** The diagnosis of oral squamous cell carcinoma occurs late in the disease. Poor prognosis of the patient is directly correlated with the occurrence of metastatic lesions. Lymphocytes that have invaded tumors significantly influence how well cancers respond to treatment.

**Objectives:** The objective of the study was to compare the immunohistochemical expression of CD3 TILs receptor in various grades of oral squamous cell carcinoma.

**Materials and Methods:** This study was a retrospective comparative cross sectional (Analytical) study. It was conducted at Department of Pathology, Peshawar Medical College from August 2020 to August 2021. Sample size was calculated through G Power and 104 patients were included in the study. Statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) version 20.0. Chi Square test was performed for categorical variables.

**Results:** It was observed in our study that the mean age of OSCC was 59.16 ± 13.549 SD. Half of the cases were > 60 years (n= 52, 50%) and half of the cases were < 60 years (n= 52, 50%). The male to female ratio was approximately 2:1. The most common site involved by OSCC was buccal mucosa (n=39, 37.5 %) followed by tongue (n = 31, 29.8 %).

Most OSCC were well differentiated (n=54, 51.9%), followed by moderately differentiated cases (n=29, 27.9%) and poorly differentiated (n=21 cases, 20.2 %). The p value hence showed a significant difference for intratumoral and intrastromal CD3 TILs expression and grades of OSCC cases (P- value <0.0001).

**Conclusion:** Well-differentiated oral squamous cell carcinoma has the highest CD3 TILs level, followed by moderately and poorly differentiated OSCC.

**Keywords:** Tumor Infiltrating Lymphocytes, Tumor Microenvironment, CD3, Immunohistochemistry

## INTRODUCTION

Globally, oral squamous cell carcinoma is the 18th most frequent cancer. Among the head and neck malignancies, it is the most widespread malignancy in the world, with a high incidence and fatality rate (Zhou et al., 2018). It is responsible for 354,864 new cases and approximately 177,384 deaths per year (Bray et al., 2018). In Asia, it is the 12$^{th}$ most common malignancy among all cancers (Cheong et al., 2017). According to GLOBACAN 2020, oral carcinoma is ranked 2nd most common cancer in Pakistan. It causes 16,959 (10.9%) new cases and 10617 (9.1%) deaths per year in both genders of all age groups (Sung et al., 2021). According to the new number of cases, cancer of the lip and oral cavity is found more frequently in males than in females (Ferlay et al., 2018).

Tobacco smoking, alcohol, betel quid chewing, bidi, viral infection such as Epstein Bar Virus and Human Papilloma Virus and genetic susceptibility are some of the most commonly reported risk factors for OSCC (Mehdi et al., 2019). Beside these risk factors, snuff has also been linked with the risk of OSCC (Sadykov et al., 2016).Snuff usage for an extended period of time may cause highly unfavorable keratotic lesions and related epithelial abnormalities in the oral cavity. An association between tobacco-specific N‑nitrosamines in smokeless tobacco and cancers of the upper digestive tract (esophagus and stomach) and mouth is already established (Sadykov et al., 2016).

TME, which contains a variety of cell types, is a significant region of stromal tissues surrounding the tumor i.e., T lymphocytes, fibroblasts and endothelial cells that affect the cancer cells growth and the clinical sequel (Helal & Wahba, 2016). TILs are defined as lymphocytes that directly oppose and/or surround tumor cells (Scolyer & Busam, 2019). They play a role in halting tumor proliferation and metastasis that leads to improved patient survival and hence can be used as a useful prognostic marker (Nguyen et al., 2016). Immune response in squamous cell carcinoma (SCC) is mainly associated with CD3 T lymphocytes. All T cells have the receptor glycoprotein known as CD3 antigen (Helal & Wahba, 2016). The infiltration density of CD3 TILs can be evaluated by immunohistochemistry and is crucial for a potent immunogenic antitumor response in many carcinomas (Boxberg et al., 2019). Keeping in view the role of CD3 TILs and TME and in preventing tumor progression, metastasis, it is important to study CD3 expression in various grades of OSCC.

This study is designed to evaluate the immunohistochemical expression of CD3 TILs in various grades of OSCC in our population.

## MATERIAL AND METHODS

This descriptive cross-sectional study was conducted at the department of Pathology, Peshawar Medical and Dental College, Peshawar. Sample size was measured by using G power software version (3.1.9.7), with an effective size of 0.5, alpha 0.05, power 80% and degree of freedom 2%. Sampling was done with non-probability convenient sampling technique. The research was conducted following the approval by the Institutional Review Board (IRB), Peshawar and IRB approval number is Prime/IRB/2020/236. Already diagnosed 104 cases of OSCC were included. Cases with poor formalin fixation where antigen retrieval issues may arise were excluded. Blocks of the patients receiving chemotherapies were also excluded. Cases with previous records were collected at Department of Oral Pathology, Peshawar Medical College (PMC), Department of Oral and Maxillofacial Surgery, Peshawar Dental College and Hospital (PDC), Khyber College of Dentistry, Peshawar and stained for immunohistochemistry at Department of Pathology, Peshawar Medical College, Riphah International University. Laboratory procedures were done in Department of Pathology, Peshawar Medical and Dental College, Peshawar. Already Diagnosed formalin fixed, paraffin embedded OSCC tissue blocks were taken and sections were cut for both H&E staining and IHC procedure. Four to five microns (µm) thin paraffin embedded OSCC tissue sections were stained with Heamatoxylin and Eosin (H&E) by using standard protocols. Microscopic examination of H&E slides was done for the confirmation of clinical diagnosis of OSCC and selection of IHC staining using CD3 antibody. CD3 positive TILs count was recorded in the tumor and stroma as follows: (1-25 cells) = 1, (26-50 cells) = 2, (≥51 cells) = 3. Positive TILs up to 25 cells were considered as low levels of TILs count (1)

and more than 25 cells (i.e. 2 and 3) were considered as high levels of TILs count.

**Immunohistochemistry (IHC):** Indirect immuno-histochemistry technique was adopted. Formalin-fixed and paraffin embedded tissues were deparaffinized. Antigen retrieval was accomplished by soaking the samples in citrate buffer solution and then heating them in a microwave oven at 95-100 degrees Celsius for 20 minutes. The slides were allowed to cool at ambient temperature, for 15 to 20 minutes. Phosphate buffer saline (PBS) and distilled water were used to wash the slides. Peroxidase blocking solution (PBS) was applied to the sections of the slides and incubated at room temperature for 10 minutes. Slides have been rinsed in PBS for 6 mins. To expose the colour of the antibody, chromogen substrate was added and slides were incubated in peroxidase substrate solution. Slides were cleaned again after allowing colour to develop for less than 5 minutes. After that, slides were submerged in Haematoxylin counterstaining solution for 1-2 minutes. Slides were cleaned for another 15 minutes under running tap water. Alcohol was used to dehydrate tissue slides for 5 minutes. The slides were cleaned with three changes of xylene and a cover slip was applied with mounting solution before being kept at room temperature.

## RESULTS

Data has been observed and analyzed using SPSS software (version 25). Descriptive analysis has been performed. Chi square test was used to analyze categorical data. The P-value less than or equal to 0.05 is considered as significant. The quantitative variables like age were measured as range, mean and standard deviation. The qualitative/ categorical variables like gender and count of CD3 TILs in intratumoral and intrastromal areas were measured as frequency and percentages and compared among three categories of OSCC.

In 104 cases of OSCC, average age of patients was 59.16 ±13.549 years. Over all age range of patients was 26 - 85 years (min – max). Male to female ratio was 2:1. The most common site of development of OSCC was Buccal mucosa (p= 0.01). Most OSCC were Well Differentiated (n=54, 51.9%), followed by Moderately differentiated cases (n=29, 27.9%) and poorly differentiated (n=21 cases, 20.2 %) (Table I).

The p value hence showed a significant difference for intratumoral and intrastromal CD3 TILs expression and grades of OSCC cases (P- value <0.0001) (Table II).

Figure-I shows the Heamatoxylin and Eosin staining of well differentiated OSCC. High count ((≥51 cells) of CD3 TILs expression has been seen in well differentiated OSCC cases (Figure-II).

Table 1: Clinico-pathologic Parameters for Oral squamous Cell Carcinoma Cases

| Gender | | Percentage |
|---|---|---|
| Male | 57 | 55 % |
| Female | 47 | 45 % |
| M: F | 2:1 | |
| AGE | | |
| <60 | 52 | 50 % |
| >60 | 52 | 50 % |
| Mean Age | 59.16 ± 13.549 | |
| Histological Grades Of Oral Squamous Cell Carcinoma | | |
| Well Differentiated | 54 | 51.9 % |
| Moderately Differentiated | 29 | 27.9 % |
| Poorly Differentiated | 21 | 20.2 % |

Table 2: Relation of CD3 TILs expression in OSCC cases with Grades of OSCC:

| Grades of OSCC | n (%) | CD3 TILs Intratumoral | | p- value <0.0001 | CD3 TILs Intrastromal | | p-value <0.0001 |
|---|---|---|---|---|---|---|---|
| | | Low count | High count | | Low count | High count | |
| Well differentiated OSCC | 54 (51.9%) | 0 | 54 (100%) | | 3 (6%) | 51 (94%) | |
| Moderately differentiated OSCC | 29 (27.9%) | 0 | 29 (100%) | | 4 (14%) | 25 (86%) | |
| Poorly differentiated OSCC | 21(20.2%) | 19 (90%) | 2 (10%) | | 20 (95%) | 1 (5%) | |

Table 3: Frequency of cases in relation to scores of CD3 TILs in OSCC

| CD3 TILs Count ( Intratumoral) | | | CD3 TILs Count (Intrastromal) | | |
|---|---|---|---|---|---|
| High Count | Low Count | Total | High Count | Low count | Total |
| 85 (81.7%) | 19 (18.3%) | 104 (100%) | 77 (74%) | 27 (26%) | 104 (100%) |
| *low (1-25%), *high (26-50%) (≥ 50%) | | | | | |

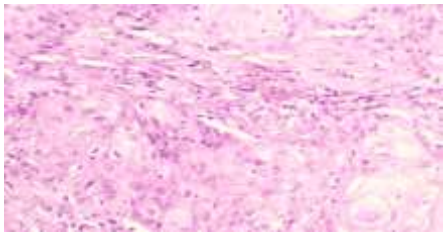

Figure 1: Well Differentiated OSCC (H&E Mag 40X)

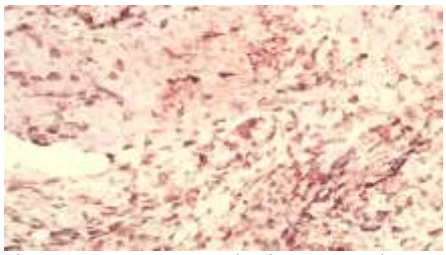

Figure-2: CD3 TILs Expression (IHC Mag 40x)

## DISCUSSION

**Demographics of Oral Squamous Cell Carcinoma:** In our study male to female ratio of OSCC cases was 2:1. In accordance with our results Sarfaraz et al., 2020 in a study on OSCC carried at Rehman Medical College, Peshawar showed a higher frequency of OSCC in male patients as compared to females. Maruse et al., 2018 from Japan in a study on OSCC also showed male predominance. Khan et al., 2020 from Pakistan also reported a male predominance.

A study by Sahaf et al., 2017 from Pakistan showed a slight male predominance in OSCC (M: F 1.3:1). A study conducted by Asif et al., 2020 in Pakistan also showed a slight male predominance (M: F 1.4:1). In contrast to our study, an international study by Troeltzsch et al., 2017 from Germany reported equal number of cases (M: F 1:1).

The mean age in our study population was 59.16 ± 13.549 which is in accordance with the studies by Goncalves et al., 2017 (mean age 55 years), Sahaf et al.,2017 (mean age : 51.88 ±15.18 years) and Qureshi et al.,2020 ( mean age : 55.73±11.7 years). Multiple international studies also reported OSCC in 5th and 6th decade of life with mean ages of 61.34 years, 64.6 years, 65.7 years, 57 years respectively (Troeltzsch et al., 2017,Weber et al., 2017; Tojyo et al., 2019 ; Vicente et al., 2019).

However, a few studies from Karachi, Pakistan reported mean age of OSCC in comparatively younger age group of 4th decade i.e., 47.1±12.22 years, 47.62±12.18 and 48.91±11.7 years (Alamgir et al., 2016; Anwar et al., 2020; Khan et al., 2020) probably because of a widespread use of gutka, pan, areca nut in the population from a very young age.

The most common site involved by OSCC was found to be Buccal Mucosa (n=42, 40.4 %) followed by Tongue (n = 35, 33.7 %). Similar to our observation a study by Khan et al., 2020 from Pakistan showed buccal mucosa to be the most common site for OSCC. Qureshi et al., 2020 also reported buccal mucosa as the most common site involved in OSCC while Maruse et al., 2018 and Vicente et al., 2019 showed tongue to be the most common site for Oral Squamous Cell Carcinoma which is contrary to the results in our study. These variations in site involvement in OSCC from different geographic areas point towards the differences in risk factors, exposure and different genetic makeup in these populations.

Regarding histologic grades of OSCC, we observed majority of the cases were Well Differentiated (54 %), followed by Moderately Differentiated cases (29 %) and then Poorly Differentiated cases (21 %). In accordance with our study Sarfaraz et al., 2020 and Memon et al., 2014 showed Well Differentiated to be the most common grade for OSCC cases. A study by Khaleel et al., 2015 also showed Well Differentiated as the commonest grade for OSCC. Contrary to our Results Mehdi et al., 2019 from Pakistan showed equal number of moderately differentiated and poorly differentiated cases and slightly less number of well differentiated cases. Lin et al., 2020 from Taiwan also showed moderately differentiated grade as the most common grade for OSCC.

Out of 57 males, 27 were snuff users and 30 were snuff nonusers. Out of 47 females, 24 were snuff users and 23 were snuff nonusers (0.708). There was no significant relation between age and history of snuff use and nonuse (p 0.169).

**CD3 TILs count in Oral Squamous Cell Carcinoma:** To evaluate the tumor-specific immunity in individual patients and its relationship with tumor grade, the number of CD3 TILs present between epithelial tumor cells (intratumoral region) and stromal cells (intrastromal region) was counted. We found that the number of CD3 TILs is maximum in intratumoral region (85%) than intrastromal region (77%). Our findings are in accordance with Tiwari et al., 2016 from India who showed increased infiltration of CD3 TILs in intratumoral region than intrastromal region.

According to our observation there was a statistical significance between intrastromal CD3 TILs count and age of the patients. However, there was no association between intratumoral CD3 TILs count and age of the patient. There was no significant association between intratumoral and intrastromal CD3 TILs count and gender of the patients. However, there was higher count of CD3 TILs in female patients as compared to males (Intratumoral 83%, Intrastromal 81% Vs Intratumoral 81%, Intrastromal 68 %). In accordance with our study Zhou et al., 2018 from China and Huang et al., 2020 from Taiwan found no relation between CD3 TILs count and Gender of the patient. In contrast to our study Saeed et al., 2017 showed a significant association between CD3 TILs count and gender of the patients (p=0.0001). In accordance to our study Saeed et al., 2017 showed highly significant association between CD3 TILs count and age of the patients i-e (p=0.0048).

According to our findings there was a statistically significant difference between site and CD3 TILs count. CD3 TILs count was found maximum in buccal mucosa followed by tongue, lip, alveolar ridge, gingiva and soft palate. There was a significant association between site and intratumoral CD3 TILs count (p=0.011). There was no statistical association between site and intrastromal CD3 TILs count (p=0.144). In accordance with our study Tiwari et al., 2016 showed maximum CD3 TILs in buccal mucosa which was statistically significant in intratumoral region.

In our study a statistically significant association was found between the CD3 TILs count and grades of OSCC (Table II). A higher intratumoral and intrastromal CD3 TILs count was seen in Well Differentiated cases i-e (n = 54, 100 %) and (n= 51, 94 %) followed by Moderately Differentiated (n=29, 100%) and (n=25, 86%) and Poorly Differentiated (n=2, 10 %) and n=1, 5%) respectively (Table II). Our findings are consistent with a 2016 study from Egypt by Helal & Wahba, which demonstrated a significantly higher CD3 TILs count in well-differentiated tumors (mean 112.44 ± 2.49) compared to moderately (102.05 ±1.82) and poorly (101.50 ± 2.09) differentiated tumors.

An Indian study in 2016 by Tiwari et al. revealed that well-differentiated cases had greater CD3 TIL counts than moderately and poorly differentiated ones. The idea that improved immunity has a protective effect on cancer patients is supported by these studies. According to a German study by Balermpas et al. 2014, SCC patients with high levels of CD3+ and CD8+ T cells have an excellent prognosis. Compared to cases with low CD3+ and CD8+ T lymphocytes, patients had a good prognosis and fewer metastatic tumors.

According to our results CD3 TILs count in the intratumoral region was significantly more (81.7%) than intrastromal region (74%) (Table III). Identical to our study Karimi et al., 2020 from Iran showed higher count of CD3 TILs in the intratumoral region than the intrastromal region. Tiwari et al., 2016 also showed higher count of CD3 TILs in intratumoral areas than in intrastromal areas.

## CONCLUSION

1 CD3 TILs expression in intratumoral and intrastromal regions in various grades of OSCC showed a statistically significant association.

2 Although a higher expression of CD3 TILs was found in intratumoral than intrastromal region in OSCC patients but it was not statistically significant.

**Limitations:** A comparatively small sample size.

Limited time available.

Financial constraints.

**Recommendations for further work:** Future studies with a larger sample size including follow up may be designed to substantiate our findings.

CD3 TILs expression in OSCC patients may be used as a prognostic marker.

OSCC patients with a low CD3 TILs expression may need a closer management and care.

**Conflict of Interest:** Authors declare no conflict of interest.

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