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# Study of Immune Effector Cells in Leukoplakia and Oral Cancer

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**Abstract** **Aim:** Immunosuppresion in oral squamous cell carcinoma (OSCC) is related to high degree of recurrence and believed to develop from premalignant lesion. Leukocytes especially T cell subsets are important in immune surveillance during malignant transformation. This study has been planned to observe changes in systemic immune response in premalignant and malignant oral lesions. **Method:** The proportions of Neutrophils, Monocytes, Lymphocytes, total T cells, T cell subsets including αβ/γδ T cells, Cytotoxic T cells, Helper T cells, Naive/ Effector/Memory T cells, Regulatory T cells and NK-T subpopulations were analysed in peripheral circulation of healthy donors (N= 49), Leukoplakia (N=20) and OSCC patients (N=100) by flowcytometry. **Results:** In comparison with healthy donors, decreased Lymphocytes, Naive Helper cells, NK T subpopulations and increased Effector cells were observed in Leukoplakia patients. Similarly, decreased Lymphocytes, NK T subpopulations and increased Neutrophils, Monocytes, Helper and Regulatory T cells were observed in OSCC patients as compared to healthy controls. Moreover, Lymphocytes were decreased and Regulatory T cells were increaed during the progression of Leukoplakia to OSCC. Further, in relation with clinicopathological parameters, Cytotoxic cells were found to be reduced with increasing histological grade. Also, Helper cells were found to be decreased in patients with tobacco and alcohol habit and also with increasing tumor size. Further, in univariate survival analysis, increased incidence of relapse was observed in patients with low γδ and NK T cells. In multivariate survival analysis, low γδ T cells emerged as poor prognosticator for disease free survival and high Regulatory T cells emerged as poor prognosticator for predicting overall survival. **Conclusion:** Altered systemic immune response was seen during malignant transformation and also found to be associated with patient's survival. Thus, investigation of circulating Leukocyte and T cell subsets seems to be useful for predicting patient's survival and to identify immunosuppressed patients who may be benefited with immunotherapy.

***Keywords:*** *immunosuppresion, OSCC, T cells, flowcytometry*

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## 1. Introduction

Oral cancer is the most common diagnosed Head and Neck cancer. The incidence of Oral Cancer has significant local variation; India and other Asian countries have higher rate of Oral Cancer than western countries. The latest findings of National Cancer Registry Programme (NCRP) of the Indian Council of Medical Research (ICMR) show that Oral Cancer is the second leading cancer site among males across all population based registries, an age-adjusted rate of Oral Cancer in India is 20 per 100,000 population and accounts for over 30% of all cancers in the country. At Gujarat Cancer and Research Institute, Head and Neck Cancer constituted 30.03% of total cancer. Further, among males, Oral Cancer is the most predominant site of cancer that constitutes 17.53% of total cancer. This particularly high prevalence in India is attributed to the habit of tobacco and chewing betel quid. Over 90% of oral cancers are squamous cell carcinomas. Majority of Oral Squamous Cell Carcinoma (OSCC) diagnosed at advance stage with spread to adjacent tissue or regional lymph node leading low treatment outcomes and poor overall survival. Oral carcinomas are believed to develop from oral premalignant lesions. About 80% of oral cancers were preceded by oral pre-cancerous lesions in India. Leukoplakia is among the most common oral premalignant lesions [1]. The prevalence of Leukoplakia in India varies from 0.2% to 5.2% [2]. Malignant transformation rates in Leukoplakia varied from 0.13% to 10% in various Indian populations [3,4].

Oral Squamous Cell Carcinomas are thought to be progress in immunocompetent hosts and therefore, it fails to elicit an effective immune response. The host immune response to malignant tumor comprises not only the local response to tumor microenvironment, but also systemic effects. The most frequent systemic alterations detected in solid tumor are neutrophilia and lymphopenia. Among the Leukocytes associated with immunosupression, T cells are

found to be one of the key factors which coordinate the host immune system to survey and eliminate cells with malignant transformation. T cells can affect tumor cells directly, or can act indirectly via the production of cytokines that amplify immune response. Circulating T cells are comprised of many phenotypically and functionally distinct subpopulations. Broadly they can be classify in Cytotoxic and Helper T cells, αβ and γδ T cells, NK T cells, Naïve and Effector / Memory cells, and Regulatory T cells.

Reports on circulating T cell proportion in Head and Neck cancer suggest extensive alteration in circulating T cells [5,6]. Further, altered proportion of T cells subpopulations were also observed in Head and Neck carcinoma and correlate with poor patient outcome [7,8]. Further, cancers originating from different sites in the Head and Neck may have different tumor biology.

Majority of the studies confirm an immune response towards malignant lesions, while observations of premalignant induced immune response are scarcer. Studies on experimental animal models revealed extensive manipulation in immune response during the progression from premalignant stages of progression. In this study, an attempt was made to understand immune dysfunction by Leukocyte subsets and various effector cells (αβ and γδ T cells, Helper T cells, Cytotoxic T cells, NK T cells) along with Regulatory T cells in patients with Oral Cancer along with Leukoplakia and healthy individuals. A better understanding of immune dysfunction in cancer will enable to design novel therapeutic approaches to overcome cancer induced immune dysfunction.

## 2. Method

In this study, patients with Oral Squamous Cell Carcinoma (OSCC; N=100, age range; 28-70 years) diagnosed and treated at Gujarat Cancer and Research Institute, Ahmedabad from February 2009 to November 2012 and homogenous Leukoplakia of buccal mucosa (N=20) diagnosed and treated College of Dental Science and Research Centre, Ahmedabad were enrolled. Peripheral blood samples were collected before surgery from all the patients as well as from age-matched normal Healthy individuals (N=49, age range; 25-55 years) in ethyelenediamine tetra acetic acid (EDTA) vacuette (BD Vacutainer K2 EDTA, BD NJ, USA). The detailed clinical history, pathological findings and treatment offered was noted from the case files maintained at Medical Record Department of the Institute. Patients provided the informed consent to use their sample for the study. This study was approved by the Institutional Scientific Review Board and Ethics Committee. The OSCC patients were followed up till the end of study period. Treatment modalities consisted of surgery, alone or combined with radiotherapy and chemotherapy. The detail clinical and pathological characteristics were given in Table 3.

### 2.1. Evaluation of Peripheral Blood Leukocyte and T Cell Subsets by Flow Cytometry

Immunophenotyping of peripheral blood Leukocyte and T cell subsets was performed according to manufacturer's protocol. For the surface markers, 20 µl of antibody was added to the whole blood (100 µl) and incubated for 15 minutes. The antibodies included CD45 (PerCP, clone 2D1) to indentify lymphocytes, CD3 (PECy7, clone SK7), CD4, (FITC, clone SK3), CD8 (PE, clone HIT8a), CD161 (FITC, clone DX12), CD56 (APC, clone NCAM16.2), αβ TCR (FITC, clone T10B9.1A-31), γδ TCR (PE, clone 11F2), CD45RA (FITC, clone HI100), CD45RO (PE, clone UCHL1) for T cell subsets. After incubation, 2 ml of erythrocyte lysing solution (1:10 dilution) was added and incubated for 15 minutes at room temperature. Then cells were centrifuged at 400g for 5 minutes and supernatant was discarded. Remaining pellet was washed twice with phosphate buffered saline (PBS) and then resuspended in 500 µl of PBS.

Human FOXP3 buffer was used to identify T-regulatory cells; whole blood (100 µl) was lysed using erythrocyte lysing solution (1:10 dilution) for 10 minutes at room temperature. Then cells were centrifuged at 400g for 5 minutes. The supernatant was discarded and pellet was incubated with 2 ml of freshly prepared human FOXP3 buffer A at room temperature for 10 minutes. After centrifugation, at 400g for 5 minutes, the supernatant was discarded and remaining pellet was washed with 2 ml of PBS and incubated for 30 minutes at room temperature with 500 µl of freshly prepared human FOXP3 buffer C (Mixture of FOXP3 buffer A and B). After incubation, cells were washed with 2 ml of PBS and incubated with respective antibodies for 30 minutes. Then cells were washed twice with 2 ml PBS, centrifuged at 400 g for 5 minutes and resuspended in 500 µl PBS. All the reagents were obtained from BD Biosciences, San Jose, CA. The samples were acquired in flowcytometer (FACS Canto II), the adjustment of detector voltage and compensation was carried out prior to acquisition using 7 color setup beads. The samples were acquired using BD Diva software, and at least 30,000 total T cells were acquired. Peripheral blood Leukocytes were identified based on their CD45 expression and side scatter characteristics (SSC). Within the Lymphocytes, T cell subsets were identified as follows (Figure 1).

Total T cells, αβ and γδ T cells: In Lymphocyte population, $CD3^+$ cells were considered as total T cells. Among $CD3^+$ T cells, subpopulation of $CD3^+\alpha\beta TCR^+$ cells was considered as αβ T cells and $CD3^+\gamma\delta TCR^+$ cells as γδ T cells.

Cytotoxic T cells and their subsets: Among $CD3^+$ T cells, $CD3^+CD8^+$ cells were considered as Cytotoxic T cells, $CD3^+CD8^+CD45RA^+$ cells as Naive T cells and $CD3^+CD8^+CD45RA^-$ cells as Effector T cells.

Helper T cells and their subsets*:* Among $CD3^+$ T cells, $CD3^+CD4^+$ cells were considered as Helper T cells. Among $CD4^+$ T cells, $CD4^+$ $CD45RA^+$cells as Naive T cells, $CD4^+$ $CD45RO^+$cells as Memory T cells and $CD4^+CD25^{high}FOXP3^+$ cells were considered as T-regulatory cells.

NK T cells: Among $CD3^+$ T cells, subpopulations of $CD3^+161^+CD56^+$, $CD3^+161^-CD56^+$, $CD3^+161^+CD56^-$, cells were considered as NK T cells.

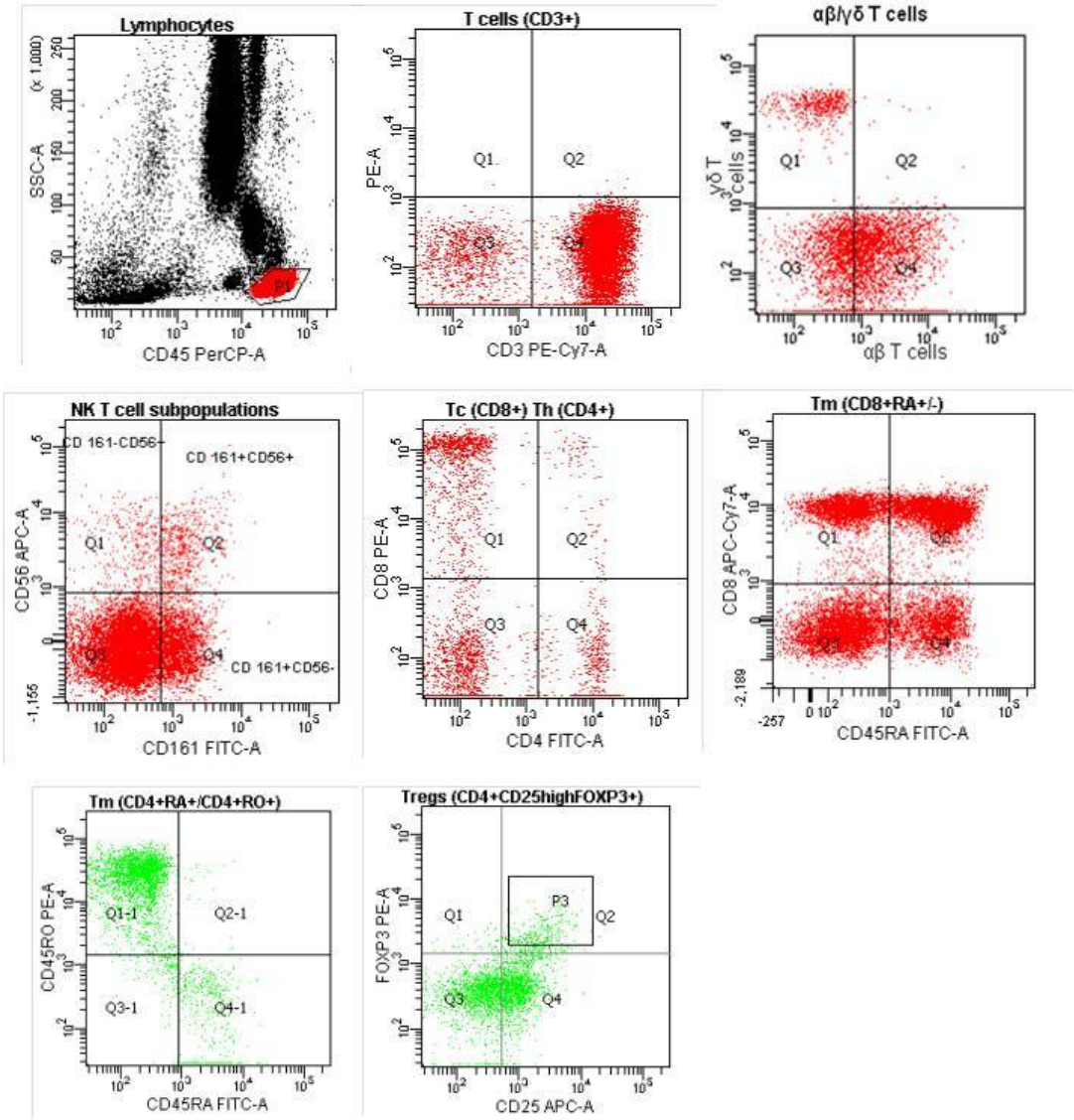


**Figure 1**. Representative dot plots of Leukocyte and T cell subsets

## 2.2. Statistical Analysis

Statistical analysis was carried out using SPSS statistical software version 19 (SPSS Inc, USA). Mean ± standard error (SE) value for Leukocytes and T cell subsets of Healthy individuals, Leukoplakia and Oral Squamous cell Carcinoma (OSCC) patients was calculated and compared using one way ANOVA and post HOC Tukey's α test, correlation with clinicopathological parameters was done by student's t test, differences with $p \leq 0.05$ were considered as significant. Univariate survival analysis was carried out using the Kaplan-Meier method and compared by the log-rank test for disease free survival and overall survival. Multivariate analysis was performed using the Cox regression model with forward step wise (likehood ratio) method.

# 3. Results

The mean percentage of Leukocyte and T cell subsets of Healthy individuals, Leukoplakia and OSCC patients were compared after adjusting with gender using one way ANOVA and post hoc Tukey's alpha test.

## 3.1. Comparison of Leukocyte subsets between Healthy individuals, Leukoplakia and OSCC Patients

Leukoplakia patients, in comparison with Healthy individuals had reduced mean percentage of Lymphocytes (P=0.04) and increased mean Neutrophils to Lymphocytes ratio (P=0.05). Further, OSCC patients, in comparison with Healthy individuals had increased mean percentage of Neutrophils (P=0.004), Monocytes (P=0.01) and reduced mean percentage of Lymphocytes (P=0.0001), as well as enhanced mean Neutrophils to Lymphocytes ratio (P=0.0001), and in comparison with Leukoplakia had reduced mean percentage of Lymphocytes (P=0.006) (Table 1).

## 3.2. Comparison of T Subsets between Healthy Individuals, Leukoplakia and OSCC Patients

T cell subsets of Leukoplakia patients when compared with Healthy individuals, increased mean percentage of Effector cells ($CD8^+CD45RA^-$; P=0.0001) and reduced

mean percentage of Naive Helper cells ($CD4^+CD45RA^+$; P=0.002), NK T subpopulations $CD161^-CD56^+$ (P=0.04) and $CD161^+CD56^+$ (P=0.001) was found. Further, a trend of increased mean percentage of Memory T cells ($CD4^+CD45RO^+$) was observed as compared to Healthy individuals (Table 2).

T cell subsets of OSCC patients when compared with Healthy individuals, increased mean percentage of Helper T cells (P=0.001) and Regulatory T cells (P=0.0001) with increased mean Helper to Cytotoxic T cell ratio (P=0.01) and decreased mean percentage of $CD161^+CD56^+$ (P=0.02) was observed. Further, trend of reduced mean percentage of $CD161^-CD56^+$ NK T subpopulation was observed in OSCC patients as compared to Healthy individuals (Table 2).

T cell subsets of OSCC patients, when compared with Leukoplakia patients, decreased mean percentage of Effector T cells ($CD8^+CD45RA^-$; P=0.001), increased mean percentage of Naive Helper T cells ($CD4^+CD45RA^+$; P=0.001) and Regulatory T cells (P=0.01) was observed (Table 2).

## 3.3. Correlation of Leukocytes and T Cell Subsets with Clinicopathological Parameters of OSCC Patients

In relation with clinicopathological parameters, increased mean percentage of Neutrophils was found in patients with buccal mucosa lesion as compared to other anatomic sites (P=0.03). Further, increased mean percentage of Monocytes was found in patients whose mucosal margins involved by tumor as compared to without involvement (P=0.01). The mean percentage of Lymphocytes did not found alter in any clinicopathological parameters (Table 3).

Further, regarding total T cells, increased mean percentage of total T cells was found in females than males (P=0.0001). Similarly, a trend of increased mean percentage αβ T cells was found in females than males. The mean percentage of γδ T cells and NK T subpopulation did not found alter in any clinicopathological parameters (Table 4).

Regarding Cytotoxic T cells and their subsets, reduced mean percentage of Cytotoxic T cells was found in grade II and grade III tumors compared to grade I tumor (P=0.03). Further, decreased mean percentage of Naive T cells ($CD8^+CD45RA^+$) and increased mean percentage of Effector T cells ($CD8^+$ $CD45RA^-$) was observed in patients with lip lesion as compared to other sites (P=0.02) (Table 5).

Regarding Helper T cells and their subsets, decreased mean percentage of Helper T cells was observed in patients with habit as compared to patients without habit (P=0.006) and in $T_3$ tumors as compared to other tumor size (P=0.05). Further, decreased mean percentage of Naive T cells ($CD4^+CD45RA^+$) was seen in patients whose mucosal margin was not involved by tumor as compared to margin involvement (P=0.01) (Table 6).

Regarding Regulatory T cells, increased mean percentage was observed in patients with lip lesion as compared to other anatomic site (P=0.04) (Table 6).

## 3.4. Correlation of Leukocytes and T Cell Subsets with Disease Status of OSCC Patients

In univariate survival analysis, in relation to DFS, high incidence of disease relapse with reduced mean months of DFS was observed in patients with low percentage γδ T (37%, 21/57; 23.0 ± 2.8 months) as compared to high percentage γδ T (21%, 09/43; 28.2 ± 2.2 months, P= 0.05). Moreover, higher incidence of disease relapse with reduced mean months of DFS was observed in patients with low $CD161^+CD56^-$ NK T cell subpopulation (36%, 20/55; 18.7 ± 2.0 months) as compared to high $CD161^+CD56^-$ NK T cell subpopulation (22%, 10/45; 32.2 ± 2.6 months, P= 0.05) (Figure 2a,b).

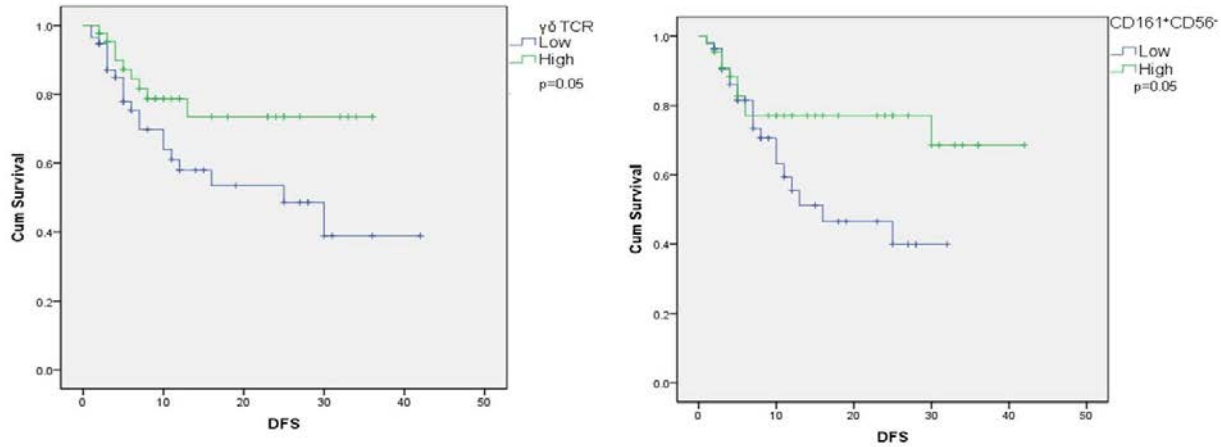


**Figure 2**. Kaplan-Meier plot for disease free survival (DFS). a) high incidence of relapse with reduced DFS was seen in patients with low γδ T cells than high γδ T cells. b) high incidence of relapse with reduced DFS was seen in patients with low $CD161^+CD56^-$ NK T cells than high $CD161^+CD56^-$ NK T cells

Further, in relation to OS, a trend of high incidence of death with reduced OS was observed in patients with low αβ T cells (26%, 13/52; 26.6 ± 2.1 months) as compared to high αβ T cells (15%, 07/48; 31.7 ± 1.9 months, P= 0.08), and patients with increased Neutrophils to Lymphocytes ratio (35%, 18/51; 22.0 ± 2.3 months) as compared to decreased Neutrophils to Lymphocytes ratio (25%, 12/49; 29.2 ± 2.9 months), respectively.

Multivariate survival analysis using Cox regression model with forward stepwise (likelihood ratio) method was performed to evaluate prognostic significance of peripheral blood Leukocyte subsets and T cell subsets

along with clinicopathological parameters. Vascular permeation was entered at step 1 (Wald statistic=6.7, df=1, Exp(B)=6.7, P=0.009), low percentage of γδ T cells was entered at step 2 (Wald statistic=3.8, df=1, Exp(B)=0.2, P=0.04) and margin involvement was entered at step 3 (Wald statistic=3.3, df=1, Exp(B)=10.5, P=0.06) for predicting DFS. Further, high percentage of Regulatory T cells was found as independent prognostic factor for predicting OS (Wald statistic=1.0, df=1, Exp (B) =72.6, P=0.29).

## 4. Discussion

Immune suppression is an early event in tumorigenesis that continues during progression to metastatic disease. The number, phenotype and functional status of Leukocytes specifically T cells can be altered in circulation via immunosuppressive factors released by tumor cells, moreover, the identification and enumeration of T cell subsets in peripheral circulation of patients with Leukoplakia and Oral Squamous Cell Carcinoma (OSCC) is primary requirement for understanding the host immune responses. Further, very few studies have examined T cell subsets in both premalignant and malignant oral lesions [9] [10]. In present study, we analysed Leukocyte and T cell subsets in both premalignant and malignant lesions of oral cavity.

In comparison with Healthy individuals, decreased Lymphocytes with increased Neutrophils to Lymphocytes ratio was observed in Leukoplakia patients, and decreased Lymphocytes, increased Neutrophils and Monocytes with enhanced Neutrophils to Lymphocytes ratio was observed in OSCC patients. Furthermore, OSCC patients had decreased Lymphocytes as compared to Leukoplakia patients. The increase in Neutrophils, Monocytes and decrease in Lymphocytes and high Neutrophils to Lymphocytes ratio in OSCC and Leukoplakia patients indicate ongoing systemic inflammation in these patients as explained by Zahorec et al [11]. Further, depletion of Lymphocytes in Leukoplakia and OSCC patients in present study indicates deprived cellular immune response during carcinogenesis. Studies on experimental animal model [12] and primary immunodeficient patients showed the association of lymphopenia with increased risk for developing Head and Neck Squamous Cell Carcinoma (HNSCC) [13,14].

Further, Leukoplakia patients showed increased Effector T cells ($CD8^+CD45RA^-$), Memory T cells ($CD4^+CD45RO^+$) and decreased Naive T cells ($CD4^+CD45RA^+$) as compared to Healthy individuals and OSCC patients. Similar to our findings, Charazinska et al [15] demonstrated decreased Naive T cells and increased Memory T cells in patients with Oral lichen planus. Moreover, study by Johannisson et al [16] showed that transition of Naive ($CD45RA^+CD45RO^-$) to Memory ($CD45RA^-CD45RO^+$) phenotype is accompanied by proliferation and activation of T cells, thus decreased Naive T cells ($CD4^+CD45RA^+$), increased Effector ($CD8^+CD45RA^-$) and Memory T cells ($CD4^+CD45RO^+$) in Leukoplakia patients in present study indicate rapid turnover of Naive T cells and activation of systemic immune response in these patients. Also, some study groups have observed shift from Naive to Memory T cell phenotype in HNSCC patients as compared to Healthy individuals [8,17,18], which was not observed in the present study suggesting defective T cell activation during carcinogenesis.

Further, in comparison with Healthy individuals, OSCC patients had increased Helper T cells and Regulatory T cells with increased Helper to Cytotoxic T cell ratio. In our study,

Helper T cells included combined value of Th1 and Th2 type of Helper T cells. The Th1 type of cells are characterized by the production of interferon (IFN)-γ, interleukin (IL) 2, IL12 and IL18, whereas Th2 type of cells are characterized by the production of IL4, IL6, IL10 and IL13. Also, Regulatory T cells in peripheral circulation are known to be linked with the pro inflammatory cytokines such as IL10 [7,19]. The increased Helper T cells along with enhanced Helper to Cytotoxic ratio and increased Regulatory T cells in this study may indicate the activation of Th2 phenotype in OSCC patients. However, this result needs to be confirmed by expression of activation markers along with pro inflammatory cytokines on Helper T cells.

Furthermore, increased Regulatory T cells in peripheral circulation in OSCC patients in present study is in accordance with other published reports of HNSCC [7,8,19,20,21], which may be linked with the down regulation of immune response and defective antigen presentation of dendritic cells [22].

Further, Leukoplakia and OSCC patients had decreased $CD161^+CD56^+$, $CD161^-CD56^+$ NK T subpopulation as compared to Healthy individuals. The relationship of NK T cells with Leukoplakia condition is not clear as no study has evaluated its role in Leukoplakia till date, yet, the study from Molling et al [23] demonstrated low level of circulating NK T cells in HNSCC patients than Healthy individuals, which were not restored even after treatment, suggesting that low number of circulating NK T cells may precede the cancer development. In view of this observation, decreased NK T cells in Leukoplakia patients in our study might reflect the defective innate immune response. However, some reports have also demonstrated association of reduced $CD161^+CD3^+$ cells with inflammatory diseases [24,25]. Therefore, the reduction of NK T subpopulations in Leukoplakia patients can also be linked with inflammatory condition. Moreover, in accordance with other study group's findings, we observed low NK T cells in circulation of OSCC patients [20,23,26]. This alteration may result from NK T cell death, impaired NK T cell proliferation, or an accumulation of NK T cells in the tumor tissue [23,27]. Moreover, Tahir et al [28] demonstrated that NKT cells in cancer patients are functionally impaired and produce less IFNγ.

Further, in present study, similar percentage of Cytotoxic and total T cells was found in OSCC patients as compared to Healthy individuals. In accordance with our findings, few study groups have observed similar percentage of Cytotoxic T cells [8,29,30] and total T cells [6,8] in HNSCC patients as compared to Healthy individuals. However, in contrast to our findings, some study groups showed decreased percentage of total T cells, αβ and γδ T cells in cancer patients [17,31,32]. Also, percentage of total T cells, αβ and γδ T cells, Cytotoxic T cells, Helper T cells and NK T subpopulations was

comparable between Leukoplakia and OSCC patients. Similar to our findings, Lee et al [10] also observed no change in Cytotoxic and Helper T cells between Leukoplakia and OSCC patients. However, they have not analysed the other T cell subsets.

In relation to clinicopathological parameters, high Neutrophils in patients with buccal mucosa and high Monocytes in patients with margin involvement suggesting increased systemic inflammation in patients with buccal mucosa cancer and margin involvement.

Further, reduced percentage of total T and αβ T cells in males than females was observed in our study, which is in agreement with study of Saxena et al [33], who analysed circulating T cell subsets in Indian population and observed low percentage of $CD3^+$ T cells in Indian males than females.

Regarding Cytotoxic T cells and subsets, decreased Cytotoxic T cells was seen in histological grade II and III tumors as compared to grade I tumor, which could be due to apoptosis of circulating Cytotoxic T cells in poorly differentiated tumors as these cells are more sensitive to apoptosis as demonstrated by Hoffman et al [31]. In contrast to our findings, Boucek et al [8] observed increased mean percentage of circulating Cytotoxic T cells with increasing histological grade. Further, decreased Naive ($CD8^+CD45RA^+$) T cells and increased Effector T cells ($CD8^+CD45RA^-$) in patients with lip lesion as compared to other anatomic sites suggesting Naive to Memory shift in patients with lip lesion. Patients with Squamous Cell Carcinoma of lip usually have different biological behavior from the oral cavity because of the associated etiology and high infiltrate of inflammatory cells [34,35]. Moreover, Zancope et al [36] demonstrated increased tumor infiltrating $CD8^+$ cells in lip squamous cell carcinoma than oral cavity. Regarding Helper T cells and subsets, decreased Helper T cells was found in patients with habit of tobacco and alcohol and in advance tumor size as compared to its counterparts. Manchanda et al [32] also observed tobacco related depletion of $CD4^+$ cells in OSCC. Moreover, Mortaz et al [37] showed that cigarette smoke suppress the proliferation of Helper T cells via unknown mechanism. In contrast to our findings, study by Mirllud et al [38] demonstrated increased Helper T cells in patients with advanced tumor status of HNSCC. However, they carried of study in 20 patients only and none of the patient in their study had <2 cm tumor size. Further, increased Naive ($CD4^+CD45RA^+$) T cells was observed in patients with positive mucosal margin indicates rapid turnover of T cells in these patients. Also, increased Regulatory T cells in patients with lip lesion demonstrate high inflammatory infiltration in lip lesion.

In univariate survival analysis, in relation to disease free survival (DFS), a trend of high incidence of disease relapse with reduced DFS was seen in patients with elevated Neutrophils to Lymphocytes ratio. Neutrophilia is considered as independent prognostic factor, associated with reduced survival in various human cancers [39,40,41]. Moreover, increased pre treatment Neutrophils to Lymphocyte ratio is found to be associated with reduced survival in colorectal and ovarian cancer [42,43].

Further, high incidence of disease relapse with reduced DFS was observed in patients with low percentage of γδ T cells and NK T subpopulation. In contrast to our findings, Bas et al [44] observed increased γδ T cells in recurrent HNSCC. However, study by Roden et al [45] has revealed that increase in circulating γδ T has been associated with activation of γδ T cells and activated γδ T cells are known to kill cancer cell by releasing Interferon γ and Tumor Necrotic Factor (TNF) α [46,47]. In view of this observation high incidence of relapse in low γδ T subgroup in present study may indicate γδ T cells as incompetent and lead to immune suppression. Moreover, activated autologous γδ T cells can also be used as immunotherapy for recurrent non small cell lung cancer patients [48]. In accordance with our study, report of Molling et al [27] suggesting low level of NK T cells predicts poor outcome of HNSCC patients. Moreover, pre treatment measurement of NK T cells may be useful for selecting patients for glycolipid α-galactosylceramide (α-GalCer)/DC therapy which enhance immune system by activating NK T cells [49].

With respect to overall survival (OS), a trend of high incidence of death with reduced OS was observed in patients with low αβ T cells. This observation indicates down regulation of immune status in OSCC patients and furthermore, immune status in such patients may be restored with adaptive T cell immunotherapy [17].

In multivariate survival analysis, presence of vascular permeation entered at step 1, followed by decreased γδ T cells at step 2 and mucosal margin involvement at step 3 as independent prognostic factors for predicting reduced DFS and increased Regulatory T cells was found as independent prognostic factor for predicting reduced OS.

The prognostic significance of Regulatory T cells in HNSCC is controversial. According to Loose et al [50] increased Regulatory T cells is linked to good prognosis, while some studies [8,19] showed the increase is associated with worse prognosis in HNSCC patients. Similar to our findings, Alhamarneh et al [7] have not observed significant correlation of circulating Regulatory T cells in univariate survival analysis, however, multivariate survival analysis have not been carried out by this study group. Moreover, presence of increased Regulatory T cells has been associated with high death hazard and reduced survival in ovarian carcinoma [51] which supports our finding that increased Regulatory T cells were associated with poor OS in multivariate survival analysis. Moreover, prevalence of Regulatory T cells helps to identify high risk patients, who are benefited with selective elimination of Regulatory T cells by targeting therapy like denileukin diftitox [52].

In summary, marked systemic inflammation was seen in Leukoplakia and OSCC patients. Moreover, deprived systemic immune response was observed in OSCC patients, which was mainly mediated through Regulatory T cells. Further, altered Neutrophils to Lymphocytes ratio along with low γδ T cells, NK T cell in circulation predicts poor disease free survival and increased Regulatory T cells predicts poor overall survival of OSCC patients. Leukoplakia patients showed effective systemic immune response by activated T cells. However, altered systemic immune response including noticeable lymphopenia, defective T cells proliferation and activation was seen during malignant transformation. Thus, investigation of circulating Leukocyte and T cell subsets seems to be useful predictors of survival and also help to identify immunosuppressed patients prior to surgery for immunotherapy.

**Table 1. Comparison of leukocyte subsets between healthy controls, Leukoplakia and OSCC patients**

| Leukocyte subsets | Healthy controls (N=49) Mean ± SE | Leukoplakia (N=20) Mean ± SE | OSCC (N=100) Mean ± SE |
|---|---|---|---|
| Neutrophils | 54.0 ± 1.13‡ | 59.0 ± 2.20 | 60.0 ±1.06‡ |
| Monocytes | 3.8 ± 0.20§ | 4.0 ± 0.45 | 5.0 ± 0.18§ |
| Lymphocytes | 28.0 ± 0.81*‖ | 24.0 ± 1.60* | 19.0 ± 0.61‖ |
| Neutrophils/Lymphocytes ratio | 1.9 ± 0.08†** | 2.8 ± 0.35† | 3.5 ± 0.15** |

OSCC = Oral Squamous Cell Carcinoma, P value ≤ 0.05 is significant *=0.04, †=0.05, ‡=0.004, §=0.01, ‖=0.0001, **=0.0001

**Table 2. Comparison of leukocyte subsets between healthy controls, Leukoplakia and OSCC patients**

| T cell subsets | Healthy controls (N=49) Mean ± SE | Leukoplakia (N=20) Mean ± SE | OSCC (N=100) Mean ± SE |
|---|---|---|---|
| Total T cells | | | |
| $CD3^+$ | 73.0 ± 1.1 | 70.4 ± 1.6 | 71.6 ± 0.9 |
| αβ T cells and γδ T cells | | | |
| $CD3^+\alpha\beta\ TCR^+$ | 54.0 ± 2.6 | 42.4 ± 5.7 | 47.0 ± 2.2 |
| $CD3^+\gamma\delta\ TCR^+$ | 7.2 ± 0.7 | 6.0 ± 0.7 | 5.8 ± 0.3 |
| Cytotoxic T cells and subsets | | | |
| $CD3^+CD8^+$ | 42.0 ± 1.4 | 40.5 ± 2.4 | 39.5 ± 0.8 |
| Naive / Effector T cells | | | |
| $CD8^+CD45RA^+$ | 24.3 ± 1.4 | 22.0 ± 2.6 | 23.1 ± 0.7 |
| $CD8^+CD45RA^-$ | 21.4 ± 0.9* | 33.8 ± 3.0*a | 19.0 ± 0.8a |
| Helper T cells and subsets | | | |
| $CD3^+CD4^+$ | 45.0 ± 1.4‖ | 49.0 ± 2.5 | 52.1 ± 0.9‖ |
| Naive helper / Memory T cells | | | |
| $CD4^+CD45RA^+$ | 29.0 ± 1.5† | 16.5 ± 1.9†b | 29.2 ± 1.5b |
| $CD4^+CD45RO^+$ | 68.3 ± 1.7 | 76.0 ± 2.9 | 71.5 ± 1.3 |
| Regulatory T cells | | | |
| $CD4^+CD25^{high}FOXP3^+$ | 3.0 ± 0.1†† | 4.0 ± 0.5c | 6.0 ± 0.2††c |
| Helper / Cytotoxic T cells ratio | | | |
| $CD4^+/CD8^+$ | 1.1 ± 0.06** | 1.3 ± 0.16 | 1.4 ± 0.06** |
| NK T cells | | | |
| $CD3^+CD161^+CD56^-$ | 12.0 ± 0.7 | 9.2 ± 1.0 | 14.5 ± 0.6 |
| $CD3^+CD161^-CD56^+$ | 10.0 ± 1.0‡ | 6.3 ± 0.8‡ | 7.6 ± 0.6 |
| $CD3^+CD161^+CD56^+$ | 5.0 ± 0.4§‡‡ | 2.5 ± 0.3§ | 3.0 ± 0.2‡‡ |

OSCC = Oral Squamous Cell Carcinoma, P value ≤ 0.05 is significant, *=0.0001, †=0.002, ‡= 0.04, §= 0.001, ‖=0.001, **=0.0001, ††=0.0001, ‡‡=0.02, a=0.001, b=0.001, c=0.01

**Table 3. Correlation of Leukocyte subsets with clinicopathological parameters**

| Parameter | Patients N (%) | Neutrophils Mean ± SE | Monocytes Mean ± SE | Lymphocytes Mean ± SE |
|---|---|---|---|---|
| Age | 100 | | | |
| ≤45 | 59 (59) | 61.5 ± 1.4 | 4.7 ± 0.2 | 19.5 ± 0.8 |
| >45 | 41 (41) | 59.0 ± 1.4 | 4.8 ± 0.2 | 18.0 ± 2.1 |
| Gender | 100 | | | |
| Male | 91 (91) | 60.7 ± 1.1 | 4.8 ± 0.1 | 19.5 ± 0.6 |
| Female | 09 (09) | 56.6 ± 3.0 | 4.7 ± 0.9 | 19.4 ± 2.1 |
| Anatomic site | 100 | | | |
| Buccal mucosa | 45 (45) | 63.4 ± 1.0* | 5.0 ± 0.2 | 20.7 ± 0.8 |
| Tongue | 36 (36) | 59.0 ± 2.3* | 4.5 ± 0.3 | 17.8 ± 1.0 |
| Lip | 8 (08) | 56.3 ± 4.8* | 4.1 ± 0.7 | 19.2 ± 2.7 |
| Alveolus | 7 (07) | 59.0 ± 2.4* | 5.1 ± 0.4 | 20.7 ± 2.0 |
| Floor of mouth | 4 (04) | 50.6 ± 2.4* | 5.2 ± 1.1 | 22.6 ± 2.9 |
| Habit | 100 | | | |
| No Habit | 7 (07) | 53.0 ± 5.4 | 4.2 ± 1.0 | 17.0 ± 1.7 |
| Habit | 93 (93) | 61.0 ± 1.1 | 4.7 ± 0.1 | 19.6 ± 0.6 |
| Type of habit | 93 | | | |
| Tobacco chewer | 66 (66) | 62.2 ± 1.0 | 5.0 ± 0.2 | 20.0 ± 0.7 |
| Smoker | 13 (13) | 57.0 ± 3.7 | 4.3 ± 0.4 | 15.7 ± 1.9 |
| Chewer & smoker | 12 (12) | 57.0 ± 4.2 | 4.0 ± 0.5 | 19.0 ± 1.9 |
| Alcohol | 2 (02) | 63.5 ± 1.5 | 5.0 ± 1.0 | 19.5 ± 2.5 |
| Tumor size | 99 | | | |
| $T_1$ | 32 (32) | 58.1 ± 2.5 | 4.4 ± 0.3 | 20.0 ± 1.2 |
| $T_2$ | 40 (40) | 60.4 ± 1.8 | 4.7 ± 0.3 | 19.3 ± 0.9 |
| $T_3$ | 08 (08) | 59.8 ± 2.7 | 5.2 ± 0.5 | 20.4 ± 1.7 |
| $T_4$ | 19 (19) | 63.8 ± 1.5 | 5.0 ± 0.3 | 20.0 ± 1.3 |
| Nodal status | 100 | | | |
| $N_0$ | 60 (60) | 60.2 ± 1.6 | 4.7 ± 0.2 | 20.0 ± 0.8 |
| $N_1$ | 20 (20) | 60.9 ± 2.3 | 4.3 ± 0.4 | 18.7 ± 1.4 |
| $N_2$ | 20 (20) | 60.7 ± 2.1 | 5.0 ± 0.4 | 19.0 ± 1.1 |
| Stage | 100 | | | |
| I | 23 (23) | 56.4 ± 3.2 | 4.2 ± 0.3 | 20.0 ± 1.5 |
| II | 22 (22) | 62.6 ± 2.4 | 5.0 ± 0.4 | 19.5 ± 1.3 |
| III | 25 (25) | 60.8 ± 2.1 | 4.5 ± 0.3 | 19.2 ± 1.2 |
| IV | 30 (30) | 61.6 ± 1.6 | 4.9 ± 0.3 | 19.4 ± 1.0 |
| Histological grade | 100 | | | |
| I | 32 (32) | 58.0 ± 2.3 | 4.6 ± 0.3 | 19.0 ± 1.2 |
| II | 65 (65) | 61.6 ± 1.3 | 4.7 ± 0.2 | 19.6 ± 0.7 |
| III | 03 (03) | 62.0 ± 2.6 | 5.0 ± 1.1 | 23.3 ± 2.0 |
| Lymphatic permeation | 93 | | | |
| Absent | 70 (73) | 59.3 ± 1.5 | 4.6 ± 0.2 | 19.4 ± 0.8 |
| Present | 23 (27) | 63.3 ± 1.6 | 4.7 ± 0.4 | 19.8 ± 0.9 |
| Vascular permeation | 90 | | | |
| Absent | 82 (90) | 59.5 ± 1.3 | 4.6 ± 0.2 | 19.5 ± 0.7 |
| Present | 08 (10) | 64.8 ± 1.8 | 4.7 ± 1.0 | 19.6 ± 2.6 |
| Neural invasion | 58 | | | |
| Absent | 38 (66) | 61.5 ± 1.7 | 5.1 ± 0.2 | 20.6 ± 1.1 |
| Present | 20 (34) | 59.5 ± 2.8 | 4.4 ± 0.3 | 19.2 ± 1.3 |
| Margin involvement | 97 | | | |
| Not involved | 86 (90) | 60.4 ± 1.3 | 4.5 ± 0.1† | 19.2 ± 0.6 |
| Involved | 11 (10) | 60.2 ± 2.7 | 6.1 ± 0.7† | 21.6 ± 2.4 |

Parameters shows normal distribution, P value ≤ 0.05 is significant, *=0.03, †=0.01

**Table 4. Correlation of Total T cells, αβ and γδ T cells with clinicopathological parameters**

| Parameter | Patients N (%) | Total T cells Mean ± SE | αβ T cells $CD3^+\alpha\beta TCR^+$ Mean ± SE | γδ T cells $CD3^+\gamma\delta TCR^+$ Mean ± SE | NK T cells $CD161^+CD56^-$ Mean ± SE | NK T cells $CD161^-CD56^+$ Mean ± SE | NK T cells $CD161^+CD56^+$ Mean ± SE |
|---|---|---|---|---|---|---|---|
| Age | 100 | | | | | | |
| ≤45 | 59 (59) | 71.1 ± 1.3 | 49.1 ± 2.8 | 5.7 ± 0.4 | 15.7 ± 1.0 | 7.1 ± 0.6 | 3.7 ± 0.2 |
| >45 | 41 (41) | 72.2 ± 1.4 | 47.0 ± 4.0 | 6.0 ± 0.6 | 15.0 ± 0.7 | 8.8 ± 1.2 | 3.8 ± 0.4 |
| Gender | 100 | | | | | | |
| Male | 91 (91) | 70.4 ± 1.0* | 46.4 ± 2.3 | 5.9 ± 0.3 | 15.0 ± 0.6 | 7.8 ± 0.6 | 3.8 ± 0.2 |
| Female | 09 (09) | 84.2 ± 1.7* | 61.3 ± 7.9 | 5.0 ± 0.5 | 19.0 ± 2.0 | 6.2 ± 1.2 | 2.4 ± 0.5 |
| Anatomic site | 100 | | | | | | |
| Buccal mucosa | 45 (45) | 69.8 ± 1.6 | 49.0 ± 3.2 | 5.6 ± 0.5 | 15.0 ± 1.1 | 7.8 ± 0.3 | 3.5 ± 0.3 |
| Tongue | 36 (36) | 72.8 ± 1.5 | 48.8 ± 3.7 | 6.0 ± 0.5 | 16.1 ± 1.1 | 3.9 ± 0.4 | 3.9 ± 0.4 |
| Lip | 8 (08) | 70.2 ± 2.1 | 41.7 ± 9.3 | 4.7 ± 0.6 | 13.8 ± 1.8 | 4.1 ± 0.7 | 4.1 ± 0.7 |
| Alveolus | 7 (07) | 77.7 ± 3.6 | 42.7 ± 12.2 | 5.2 ± 1.1 | 15.5 ± 1.9 | 4.0 ± 1.0 | 4.0 ± 1.0 |
| Floor of mouth | 4 (04) | 73.6 ± 0.9 | 42.4 ± 13.8 | 5.5 ± 3.3 | 14.5 ± 2.1 | 3.2 ± 2.2 | 3.2 ± 2.2 |
| Habit | 100 | | | | | | |
| No Habit | 7 (07) | 78.2 ± 5.0 | 55.8 ± 7.1 | 5.4 ± 1.0 | 18.0 ± 2.4 | 3.6 ± 1.4 | 2.6 ± 0.6 |
| Habit | 93 (93) | 71.3 ± 1.1 | 46.9 ± 2.5 | 5.9 ± 0.3 | 15.2 ± 0.7 | 7.7 ± 0.6 | 3.8 ± 0.2 |
| Type of habit | 93 | | | | | | |
| Tobacco chewer | 66 (66) | 70.0 ± 1.2 | 47.0 ± 2.8 | 6.0 ± 0.4 | 15.0 ± 0.6 | 8.2 ± 0.8 | 3.8 ± 0.3 |
| Smoker | 13 (13) | 70.3 ± 2.4 | 42.5 ± 5.3 | 5.0 ± 0.7 | 13.4 ± 1.2 | 4.0 ± 1.4 | 3.3 ± 0.5 |
| Chewer & smoker | 12 (12) | 77.0 ± 2.1 | 51.8 ± 7.9 | 5.9 ± 0.9 | 18.4 ± 2.8 | 9.5 ± 1.1 | 4.1 ± 0.6 |
| Alcohol | 2 (02) | 80.5 ± 6.5 | 51.5 ± 3.5 | 6.0 ± 2.0 | 11.5 ± 6.5 | 8.0 ± 1.2 | 2.0 ± 1.4 |
| Tumor size | 99 | | | | | | |
| $T_1$ | 32 (32) | 73.0 ± 1.9 | 53.3 ± 4.2 | 5.5 ± 0.5 | 17.1 ± 1.0 | 7.3 ± 0.9 | 3.7 ± 0.3 |
| $T_2$ | 40 (40) | 70.3 ± 1.4 | 44.9 ± 3.8 | 6.0 ± 0.5 | 14.5 ± 1.2 | 7.2 ± 1.0 | 3.9 ± 0.3 |
| $T_3$ | 08 (08) | 67.0 ± 4.7 | 31.3 ± 5.3 | 4.7 ± 1.0 | 12.6 ± 2.6 | 11.4 ± 2.9 | 3.6 ± 1.6 |
| $T_4$ | 19 (19) | 74.7 ± 2.2 | 55.0 ± 5.5 | 6.6 ± 1.1 | 15.1 ± 0.9 | 7.2 ± 1.1 | 4.0 ± 0.6 |
| Nodal status | 100 | | | | | | |
| $N_0$ | 60 (60) | 70.1 ± 1.2 | 48.4 ± 2.9 | 6.2 ± 0.5 | 15.3 ± 0.9 | 7.3 ± 0.8 | 3.5 ± 0.3 |
| $N_1$ | 20 (20) | 74.6 ± 2.3 | 48.9 ± 5.6 | 5.2 ± 0.5 | 15.0 ± 1.3 | 6.6 ± 1.1 | 4.2 ± 0.6 |
| $N_2$ | 20 (20) | 72.4 ± 1.0 | 46.6 ± 5.9 | 5.2 ± 0.6 | 16.1 ± 1.1 | 9.0 ± 1.2 | 3.7 ± 0.4 |
| Stage | 100 | | | | | | |
| I | 23 (23) | 71.6 ± 2.0 | 52.2 ± 4.8 | 5.9 ± 0.6 | 16.6 ± 1.2 | 7.1 ± 1.1 | 3.3 ± 0.4 |
| II | 22 (22) | 67.6 ± 1.8 | 43.3 ± 4.9 | 6.0 ± 0.8 | 15.0 ± 2.0 | 7.2 ± 1.5 | 3.5 ± 0.4 |
| III | 25 (25) | 72.1 ± 2.0 | 46.4 ± 4.5 | 5.1 ± 0.5 | 14.5 ± 1.3 | 7.7 ± 1.1 | 4.3 ± 0.6 |
| IV | 30 (30) | 73.6 ± 2.1 | 49.8 ± 4.8 | 6.3 ± 0.8 | 15.7 ± 0.8 | 7.9 ± 0.5 | 3.7 ± 0.5 |
| Histological grade | 100 | | | | | | |
| I | 32 (32) | 71.2 ± 2.0 | 52.2 ± 4.5 | 5.5 ± 0.4 | 16.7 ± 1.4 | 7.3 ± 1.0 | 3.0 ± 0.4 |
| II | 65 (65) | 72.2 ± 1.3 | 46.0 ± 2.8 | 6.3 ± 0.5 | 15.1 ± 0.6 | 8.0 ± 0.8 | 4.2 ± 0.3 |
| III | 03 (03) | 63.0 ± 5.6 | 62.5 ± 9.6 | 5.7 ± 3.4 | 15.7 ± 4.6 | 5.6 ± 3.2 | 3.6 ± 0.3 |
| Lymphatic permeation | 93 | | | | | | |
| Absent | 70 (73) | 71.6 ± | 46.4 ± 2.8 | 5.9 ± 0.4 | 15.0 ± 0.8 | 7.5 ± 0.7 | 3.7 ± 0.3 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1.2 | | | | | |
| Present | 23 (27) | 72.8 ± 2.3 | 50.8 ± 5.4 | 5.3 ± 0.6 | 16.1 ± 1.3 | 8.1 ± 1.0 | 3.9 ± 0.4 |
| Vascular permeation | 90 | | | | | | |
| Absent | 82 (90) | 71.1 ± 1.1 | 46.8 ± 3.4 | 5.8 ± 0.3 | 15.1 ± 0.7 | 7.5 ± 2.7 | 3.8 ± 0.3 |
| Present | 08 (10) | 79.1 ± 2.5 | 60.2 ± 9.1 | 5.0 ± 0.7 | 17.6 ± 2.7 | 8.8 ± 2.7 | 3.1 ± 0.7 |
| Neural invasion | 58 | | | | | | |
| Absent | 38 (66) | 70.9 ± 1.6 | 46.9 ± 3.4 | 6.1 ± 0.6 | 16.5 ± 1.3 | 7.3 ± 0.8 | 3.1 ± 0.3 |
| Present | 20 (34) | 71.7 ± 3.1 | 41.8 ± 5.9 | 5.7 ± 0.6 | 15.1 ± 1.1 | 7.6 ± 1.4 | 3.9 ± 0.4 |
| Margin involvement | 97 | | | | | | |
| Not involved | 86 (90) | 72.1 ± 1.2 | 47.0 ± 2.7 | 5.8 ± 0.3 | 15.6 ± 7.5 | 7.5 ± 0.6 | 3.8 ± 1.2 |
| Involved | 11 (10) | 70.1 ± 2.0 | 50.6 ± 6.7 | 6.7 ± 1.7 | 13.8 ± 1.9 | 5.4 ± 1.2 | 4.0 ± 0.1 |

Parameters shows normal distribution, P value ≤ 0.05 is significant, *=0.0001

**Table 5. Correlation of Cytotoxic T cells and their subsets with clinicopathological parameters**

| Parameter | Patients N (%) | Cytotoxic T cells $CD3^+CD8^+$ Mean ± SE | Naive T cells $CD8^+CD45RA^+$ Mean ± SE | Effector T cells $CD8^+CD45RA^-$ Mean ± SE |
|---|---|---|---|---|
| Age | 100 | | | |
| ≤45 | 59 (59) | 39.0 ± 1.0 | 23.0 ± 2.0 | 18.8 ± 1.1 |
| >45 | 41 (41) | 40.3 ± 1.2 | 23.0± 1.0 | 19.5 ± 1.3 |
| Gender | 100 | | | |
| Male | 91 (91) | 39.7 ± 0.8 | 22.8 ± 0.7 | 19.7 ± 0.9 |
| Female | 09 (09) | 37.8 ± 1.3 | 25.7 ± 1.7 | 15.7 ± 2.4 |
| Anatomic site | 100 | | | |
| Buccal mucosa | 45 (45) | 40.1 ± 1.2 | 23.3 ± 1.1† | 18.0 ± 1.2‡ |
| Tongue | 36 (36) | 38.0 ± 1.3 | 22.5 ± 1.1† | 17.1 ± 1.4‡ |
| Lip | 8 (08) | 39.2 ± 4.4 | 17.5 ± 2.6† | 29.0 ± 0.4‡ |
| Alveolus | 7 (07) | 40.0 ± 3.2 | 29.1 ± 2.3† | 19.1 ± 3.3‡ |
| Floor of mouth | 4 (04) | 42.0 ± 1.4 | 29.0 ± 1.5† | 17.0 ± 2.1‡ |
| Habit | 100 | | | |
| No Habit | 7 (07) | 32.1 ± 2.4 | 21.0 ± 2.2 | 15.7 ± 3.6 |
| Habit | 93 (93) | 40.1 ± 0.9 | 23.4 ± 1.0 | 19.3 ± 0.9 |
| Type of habit | 93 | | | |
| Tobacco chewer | 66 (66) | 40.3 ± 1.0 | 23.5 ± 0.9 | 19.2 ± 1.0 |
| Smoker | 13 (13) | 36.6 ± 2.6 | 20.0 ± 1.7 | 19.3 ± 2.3 |
| Chewer & smoker | 12 (12) | 39.0 ± 2.0 | 25.0 ± 2.4 | 20.2 ± 2.9 |
| Alcohol | 2 (02) | 38.5 ± 3.5 | 24.5 ± 1.5 | 16.5 ± 2.5 |
| Tumor size | 99 | | | |
| $T_1$ | 32 (32) | 37.4 ± 1.3 | 21.5 ± 1.4 | 19.1 ± 1.6 |
| $T_2$ | 40 (40) | 40.0 ± 1.4 | 22.2 ± 1.1 | 20.0 ± 1.4 |
| $T_3$ | 08 (08) | 45.1 ± 3.1 | 26.1 ± 1.8 | 19.4 ± 2.8 |
| $T_4$ | 19 (19) | 40.0 ± 2.0 | 26.5 ± 1.7 | 19.1 ± 2.5 |
| Nodal status | 100 | | | |
| $N_0$ | 60 (60) | 39.2 ± 1.0 | 23.6 ± 1.0 | 19.3 ± 1.2 |
| $N_1$ | 20 (20) | 37.2 ± 2.0 | 20.1 ± 1.2 | 18.4 ± 2.2 |
| $N_2$ | 20 (20) | 42.5 ± 2.0 | 24.4 ± 1.5 | 20.0 ± 1.8 |
| Stage | 100 | | | |
| I | 23 (23) | 37.8 ± 1.7 | 22.0 ± 1.7 | 19.1 ± 2.1 |
| II | 22 (22) | 40.8 ± 1.7 | 23.3 ± 1.7 | 19.7 ± 1.8 |
| III | 25 (25) | 38.7 ± 1.8 | 20.8 ± 1.2 | 19.0 ± 1.8 |
| IV | 30 (30) | 40.7 ± 1.5 | 26.2 ± 1.2 | 19.5 ± 1.6 |
| Histological grade | 100 | | | |
| I | 32 (32) | 42.8 ± 1.4* | 24.3 ± 1.4 | 22.0 ± 1.7 |
| II | 65 (65) | 38.2 ± 1.0* | 22.9 ± 0.8 | 18.4 ± 1.0 |
| III | 03 (03) | 37.0 ± 5.7* | 17.2 ± 3.6 | 16.0 ± 5.9 |
| Lymphatic permeation | 93 | | | |
| Absent | 70 (73) | 40.0 ± 1.1 | 23.5 ± 0.9 | 18.7 ± 1.0 |
| Present | 23 (27) | 39.0 ± 1.5 | 21.4 ± 1.4 | 21.2 ± 2.0 |
| Vascular permeation | 90 | | | |
| Absent | 82 (90) | 39.8 ± 0.9 | 23.0 ± 0.8 | 19.0 ± 1.0 |
| Present | 08 (10) | 39.2 ± 2.8 | 22.1 ± 2.3 | 24.0 ± 3.5 |
| Neural invasion | 58 | | | |
| Absent | 38 (66) | 40.8 ± 1.3 | 23.3 ± 1.1 | 19.3 ± 1.5 |
| Present | 20 (34) | 40.9 ± 2.1 | 23.7 ± 1.4 | 21.2 ± 2.1 |
| Margin involvement | 97 | | | |
| Not involved | 86 (90) | 39.7 ± 0.9 | 23.0 ± 0.8 | 19.0 ± 0.9 |
| Involved | 11 (10) | 37.4 ± 2.7 | 22.9 ± 1.9 | 19.8 ± 3.7 |

Parameters shows normal distribution, P value ≤ 0.05 is significant, *=0.03, †=0.02, ‡=0.02

**Table 6. Correlation of Helper T cells and their subsets with clinicopathological parameters**

| Parameter | Patients N (%) | Helper T cells $CD3^+CD4^+$ Mean ± SE | Naive T cells $CD4^+CD45RA^+$ Mean ± SE | Memory T cells $CD4^+CD45RO^+$ Mean ± SE | Regulatory T cells $CD4^+CD25^{high}FOXP3^+$ Mean ± SE |
|---|---|---|---|---|---|
| Age | 100 | | | | |
| ≤45 | 59 (59) | 52.3 ± 1.2 | 32.0 ± 2.2 | 71.5 ± 1.6 | 6.2 ± 0.3 |
| >45 | 41 (41) | 50.3 ± 1.4 | 26.1 ± 2.0 | 72.0 ± 2.4 | 5.6 ± 0.4 |
| Gender | 100 | | | | |
| Male | 91 (91) | 51.0 ± 1.0 | 28.1 ± 1.5 | 71.2 ± 1.4 | 5.8 ± 0.2 |
| Female | 09 (09) | 56.5 ± 1.6 | 31.0 ± 4.1 | 74.3 ± 3.8 | 7.0 ± 1.0 |
| Anatomic site | 100 | | | | |
| Buccal mucosa | 45 (45) | 50.2 ± 1.3 | 29.0 ± 2.0 | 71.0 ± 1.9 | 5.6 ± 0.7§ |
| Tongue | 36 (36) | 53.9 ± 1.5 | 28.2 ± 2.1 | 68.8 ± 2.3 | 6.0 ± 0.5§ |
| Lip | 8 (08) | 54.1 ± 4.4 | 32.8 ± 10.4 | 74.5 ± 6.7 | 8.8 ± 1.7§ |
| Alveolus | 7 (07) | 54.0 ± 2.0 | 21.6 ± 5.3 | 80.8 ± 4.8 | 5.7 ± 0.7§ |
| Floor of mouth | 4 (04) | 47.0 ± 5.3 | 27.8 ± 3.1 | 81.2 ± 2.8 | 5.4 ± 1.0§ |
| Habit | 100 | | | | |
| No Habit | 7 (07) | 61.7 ± 2.6* | 32.8 ± 6.3 | 79.2 ± 4.2 | 6.7 ± 0.7 |
| Habit | 93 (93) | 51.4 ± 0.9* | 29.0 ± 1.6 | 71.1 ± 1.4 | 5.8 ± 0.3 |
| Type of habit | 93 | | | | |
| Tobacco chewer | 66 (66) | 49.7 ± 1.1 | 28.0 ± 1.4 | 70.5 ± 1.6 | 5.7 ± 0.3 |
| Smoker | 13 (13) | 53.9 ± 2.8 | 27.0 ± 5.6 | 70.4 ± 4.1 | 6.0 ± 0.7 |
| Chewer & smoker | 12 (12) | 52.7 ± 1.8 | 31.0 ± 6.3 | 76.7 ± 3.0 | 6.4 ± 0.8 |
| Alcohol | 2 (02) | 55.5 ± 3.5 | 30.0 ± 6.0 | 65.5 ± 3.5 | 9.0 ± 2.0 |
| Tumor size | 99 | | | | |
| $T_1$ | 32 (32) | 55.0 ± 1.3† | 26.8 ± 2.1 | 72.9 ± 2.2 | 5.8 ± 0.4 |
| $T_2$ | 40 (40) | 52.0 ± 1.6† | 30.8 ± 3.2 | 71.4 ± 2.1 | 6.0 ± 0.4 |
| $T_3$ | 08 (08) | 45.0± 3.2† | 29.3 ± 2.5 | 70.5 ± 5.1 | 5.7 ± 0.7 |
| $T_4$ | 19 (19) | 50.1 ± 2.0† | 30.7 ± 2.3 | 67.8 ± 4.0 | 6.0 ± 0.6 |
| Nodal status | 100 | | | | |
| $N_0$ | 60 (60) | 50.6 ± 1.4 | 31.5 ± 2.1 | 69.4 ± 1.7 | 5.6 ± 0.3 |
| $N_1$ | 20 (20) | 52.2 ± 1.2 | 28.8 ± 3.5 | 71.0 ± 3.3 | 6.6 ± 0.7 |
| $N_2$ | 20 (20) | 53.0 ± 9.3 | 23.0 ± 2.2 | 77.2 ± 2.8 | 6.2 ± 0.6 |
| Stage | 100 | | | | |
| I | 23 (23) | 54.1 ± 1.7 | 26.4 ± 2.4 | 72.4 ± 2.6 | 5.6 ± 0.4 |
| II | 22 (22) | 49.4 ± 1.7 | 37.5 ± 4.9 | 69.5 ± 2.5 | 5.5 ± 0.5 |
| III | 25 (25) | 53.4 ± 1.5 | 27.6 ± 2.7 | 71.4 ± 2.6 | 6.0 ± 0.5 |
| IV | 30 (30) | 52.1 ± 0.9 | 27.1 ± 2.1 | 71.3 ± 3.1 | 6.3 ± 0.5 |
| Histological grade | 100 | | | | |
| I | 32 (32) | 50.6 ± 1.4 | 29.5 ± 3.3 | 71.2 ± 2.6 | 5.8 ± 0.5 |
| II | 65 (65) | 52.2 ± 1.2 | 28.5 ± 1.6 | 72.0 ± 1.6 | 6.0 ± 0.3 |
| III | 03 (03) | 53.0 ± 9.3 | 28.0 ± 11.8 | 71.5 ± 11.0 | 6.7 ± 1.6 |
| Lymphatic permeation | 93 | | | | |
| Absent | 70 (73) | 51.6 ± 1.1 | 29.8 ± 2.0 | 71.5 ± 1.7 | 5.7 ± 0.3 |
| Present | 23 (27) | 53.3 ± 2.0 | 26.5 ± 2.5 | 72.2 ± 2.9 | 6.4 ± 0.7 |
| Vascular permeation | 90 | | | | |
| Absent | 82 (90) | 52.0 ± 1.0 | 29.0 ± 1.8 | 72.0 ± 1.6 | 5.9 ± 0.3 |
| Present | 08 (10) | 54.0 ± 2.6 | 26.0 ± 2.9 | 71.6 ± 3.9 | 6.4 ± 0.6 |
| Neural invasion | 58 | | | | |
| Absent | 38 (66) | 51.3 ± 1.5 | 30.4 ± 2.9 | 71.3 ± 2.2 | 6.1 ± 0.5 |
| Present | 20 (34) | 52.7 ± 2.0 | 24.6 ± 2.8 | 73.6 ± 3.2 | 6.0 ± 0.5 |
| Margin involvement | 97 | | | | |
| Not involved | 86 (90) | 52.6 ± 1.0 | 27.0 ± 1.5‡ | 72.3 ± 1.5 | 5.9 ± 0.3 |
| Involved | 11 (10) | 50.8 ± 3.2 | 40.0 ± 6.0‡ | 68.2 ± 3.2 | 5.8 ± 0.7 |

Parameters shows normal distribution, P value ≤ 0.05 is significant, *=0.006, †=0.05, ‡=0.01, §=0.04,


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