Immunoexpression of Tumor Infiltrating Lymphocytes (TILS) CD4+ and CD8+ in Oral Squamous Cell Carcinoma (OSCC) in Correlations with Clinicopathological Characteristics and Prognosis

Hanan Salman Wahbi*, Ahmed Al Manadili

Abstract

Objective: This study aimed to analyze CD4+ and CD8+ TILs in oral squamous cell carcinoma (OSCC) and to correlate it with histologic grade of malignancy and clinicopathologic data. Methods: The sample was composed of 43 archived specimens. Clinical features and histological grade of malignancy were obtained. The infiltrating intensity of CD4+, CD8 positive cells were assessed by immunohistochemistry. One-way ANOVA was used to study the association between CD4+, CD8+ and the grade of OSCC. The cut-off values of the proposed diagnostic indices were received from calculating the coordinates of the receiver operating characteristic (ROC) curve. For clinicopathologic data Independent-Samples T test, Pearson Correlation Coefficient, Correlation Coefficient were used clinicopathologic characteristics. Results: CD4+ and CD8+ were observed in all specimens. CD4+ were more frequent in poorly differentiated specimens (74.14) (P= 0.021<0.05). CD8+ were more frequent in well-differentiated specimens (51.18). None of these correlations were significant (P=0.454>0.05). CD4+/CD8 ratio was higher in low-grade specimens (180.28) (P=0.017<0.05). No differences between CD4+, CD8+ and CD4+/CD8 ratio between poorly-differentiated and moderately-differentiated groups ROC P value (0.370, 0.248, 0.126) respectively. there is a difference between CD4+, CD4+/CD8 ratio between poorly-differentiated and well-differentiated groups ROC P value (0.022, 0.341, 0.012) Sensitivity (0.857, 0.882), specificity (0.706, 0.857) respectively. and no differences between CD8+ poorly-differentiated and well-differentiated groups ROC P value (0.341). there is a difference between CD4+ between moderately-differentiated and well-differentiated groups ROC P value (0.038) Sensitivity (0.368), specificity (0.765). No significant correlation was obtained with clinicopathologic findings of OSCC. Conclusion: CD4+ and CD4+/CD8+ ratio are independent prognostic factor in OSCC.

Keywords: CD4+ - CD8+ - TILs- prognosis- clinicopathologic- OSCC

Asian Pac J Cancer Prev, 23 (12), 4177-4183

Introduction

Oral cancer accounts for 1% to 5% of human malignancies, and oral squamous cell carcinoma is the most frequent type in 90% of all oral cancer (Ahn et al., 2017). Smoking, Alcohol drinking, betel nut chewing, and human papillomavirus infection represent the major risk factors for OSCC (Zhou et al., 2018). Despite the improvement of therapy, the 5-year survival rate of patients with OSCC is below 50% over the past three decades (Bloebaum et al., 2014).

New evidence suggests that the infiltration of immune cell may be a potential prognostic marker in OSCC (Sales de Sá et al., 2021). In particular, diverse types of tumor infiltrating lymphocytes (TILs) produced different effects on tumor growth, recurrence and metastatic spreading (Zhou et al., 2018).

The CD8+ TILs are a subpopulation of cytotoxic lymphocytes and are most likely effector cells that enhance an efficient immunity against tumor. These cells can be involved in the immunologic surveillance, recognizing, and killing potentially malignant cells, which express peptides presented with MHC class I (dos antos Pereira et al., 2014).

CD4+ obligates further differentiation of this subtype into helper and regulatory CD4+. and, CD4+ helper T cells perform critical roles in the recruitment, activation and regulation of many facets of the adaptive immune response (Lluckheeram et al., 2012). Although most tumors cells do not express MHC class II molecules, CD4+ can effect an antitumor response in the absence of CD8+ T cells by secreting cytokines, such as interferon-γ (Qin et al.,...
2000), and TNF \( \alpha \) caused cytokine-induced senescence resulting in growth arrest of cancer cells (Poncette et al., 2022) or by activation and recruitment of effector cells such as macrophages and eosinophils (Hung et al., 1998). However, the main role of CD4+ in the immune response to cancer is to prime CD8+ and maintain their proliferation (Hiraoka et al., 2006).

Recently, the microenvironment of solid tumors including tumor-infiltrating lymphocytes has become a promising target and for a key to the development of immunotherapies and the ability to predict clinical responses (Chen et al., 2021).

Growing evidence has shown that the presence of TILs is significantly associated with prognosis of various solid tumors such as head neck squamous cell carcinoma (HNSCC) (de Ruiter et al., 2017). It has been associated with a favorable prognosis, although few studies have explored the prognostic relevance of CD8+ Immunoexpression in OSCC (Lequerica-Fernández et al., 2021; Shimizu et al., 2019). The types and functional statuses of different TILs as well as their locations within the Tumor Microenvironment (TME) can determine the balance between antitumor and promotion of cancer (Zancepe et al., 2010). Therefore, the roles of CD4+ and CD8+ in OSCC remain still indecisive (Gregory et al., 2015). Thus, this study aimed to thoroughly evaluate the immune expression of CD8+ and CD4+ in the stroma of OSCC and their relationships with clinicopathological features and histological grade of malignancy.

**Materials and Methods**

**The sample**

Resection specimens of OSCC from 43 patients treated from 2014 to 2021 were taken from the archives of Damascus hospital. Clinical data (e.g., gender, age, smoking, clinical stage, tumor local, lymph node metastasis, and distant metastasis) were collected from medical records of patients. Tumor stage and clinical stage were classified as initial (I and II) or advanced (III and IV). (Table 1). Histologic grades were classified according to WHO criteria (El-Naggar et al., 2017) into three groups well-differentiated (grade I), moderately differentiated (grade II), and poorly differentiated (grade III) tumors.

**Immunohistochemistry Stain**

Paraffin-embedded specimens were cut into 4 μm thickness and mounted on glass slides positive charged slides and then dried by autoclave. Sections were deparaffinized in xylene for 3 mints followed by rehydrated with an alcohol gradient (100% then 95%, then 70%) 3 mints for each one, and then washed with H\(_2\)O. For antigen retrieval, and according to manufacturer’s instruction of the Bio SB company the sections were boiled in Immune DNA Retriever with EDTA (BSB0030-BSB 0033) by microwave for 30 mints followed by washing with (wash buffer) for 5 mints three times and then were blocked by incubation with Poly Detector Peroxidase Blocker for 10 mints, then tissue sections were washed in TBS (wash buffer) 5 or 5 mints 3 times 5 followed by incubation with the primary antibodies CD4 (Clone RBT-CD4, Bio SB), CD8 (Clone EP334, Bio SB,) for 1h at 4°C overnight and then washed by TBS (Tris-buffered saline) for 15 mints. After that the sections were incubated with the secondary antibody, Poly Detectors 15 mints followed by washing with TBS and then incubation with Poly Detectors HRP label for 15 mints followed by washing with TBS (Tris-buffered saline) for 15 mints followed by (DAB) for 15 min and then washed with H\(_2\)O. Finally, the samples were counterstained with Meyer’s hematoxylin and mounted.

**Assessment the expression of CD4+ and CD8+**

For each slide, five representative fields were selected for counted the numbers of CD4+ and CD8+ at high power (400 × ) The final density of each section was calculated as the average number of five high-power fields (HPFs). The location of CD4+ and CD8+ TILs was evaluated between tumor nests (Quan et al., 2020). All slides were evaluated by two investigators blinded to the clinical data.

**Statistical analysis**

Statistical analysis was performed using SPSS Statistics version 13.0; software. One-Way ANOVA test was used to associations expression of cd4+ and cd8+ with histologic grade of Statistical significance was defined as p<0.05. and Student’s t test was used for association the expression of cd4+ and cd8+ with clinicopathological parameters. operating characteristic (ROC) and area under curve (AUC) analyses were used to estimate the predictive value of CD4+ and CD8+ to identify the predictors of the histological grade of OSCC.

**Results**

**Patient Characteristics**

The patients were 24 men (55.8%) and 19 women (44.2%) and ranged in age from 43 to 82 years (mean 64.7, standard deviation 9.4). The tumor sub site was classified as the tongue (n = 22 = 51.2%), and another sites of oral cavity (n = 21 = 48.8) is reported in 19 (44.2%), 21 (51.2) had stage I/II, 22 22 (51.2%) had stage III/IV, Table 1.

Histopathologic degree of differentiation were calculated according to the following formulas:

\[
\text{Sensitivity} = \frac{\text{True positive}}{\text{True positive} + \text{False negative}} \\
\text{Specificity} = \frac{\text{True negative}}{\text{True negative} + \text{False positive}}
\]

\[
\text{Sensitivity} \times 100 \times \frac{\text{True positive} + \text{False negative}}{\text{True positive}} \\
\text{Specificity} \times 100 \times \frac{\text{True negative} + \text{False positive}}{\text{True negative}}
\]

Statistical analysis was performed using SPSS Statistics version 13.0; software. One-Way ANOVA test was used to associations expression of cd4+ and cd8+ with histologic grade of Statistical significance was defined as p<0.05. and Student’s t test was used for association the expression of cd4+ and cd8+ with clinicopathological parameters.
Immunoexpression of CD4+, CD8+ in Oral Squamous Cell Carcinoma.

Surrounding the tumor islands as compact clusters within the tumor, and some were distributed sparsely.

Among T cells, CD4+ subpopulation was a few more than CD4+ TILs and few more than CD8+ T cells Figures 1, 2, 3 and 4. CD4+ were significantly associated with poorly-differentiated tumors (74.14) (P= 0.021<0.05).

CD8+ were not significantly associated with histological grade of OSCC (P=0.454>0.05) Table 2.

No differences between CD4+, CD8+ and CD4+/CD8 ratio between poorly-differentiated group and moderately-differentiated groups ROC p value (0.370, 0.248, 0.126) respectively. There is a differences between CD4+ TILs, CD4+/CD8 ratio between poorly-differentiated and well-differentiated groups ROC p value (0.022, 0.341, 0.012) Sensitivity (0.857, 0.882), specificity (0.706, 0.857) respectively. and no differences between CD8+ poorly-differentiated and well-differentiated groups ROC p value (0.341) Figures 5 and 6; Tables 2 and 4.

The value of Sensitivity and specificity of CD4+ and CD4+/CD8 as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

The best value between sensitivity and specificity of CD4+ was at the value of 69.5, where the sensitivity value was 0.857 and the specificity value was equal to 0.706, and therefore we conclude that the value 69.5 can be determined as a standard value for the CD4+ as a prognostic for poorly-differentiated squamous cell carcinoma in the research sample, Table 5.

The best fit between sensitivity and specificity of CD4+/CD8+ was at the value of 148.26, where the sensitivity value was 0.857 and the specificity value was equal to 0.882, and therefore we conclude that the value 148.26 can be determined as a standard value for the CD4+/CD8+ as a prognostic for poorly-differentiated squamous cell carcinoma in the research sample, Table 5.

The value of Sensitivity and specificity of CD4+ and CD4+/CD8+ as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

The best value between sensitivity and specificity of CD4+ was at the value of 69.5, where the sensitivity value was 0.857 and the specificity value was equal to 0.706, and therefore we conclude that the value 69.5 can be determined as a standard value for the CD4+ as a prognostic for poorly-differentiated squamous cell carcinoma in the research sample, Table 5.

The best value between sensitivity and specificity of CD4+/CD8+ was at the value of 148.26, where the sensitivity value was 0.857 and the specificity value was equal to 0.882, and therefore we conclude that the value 148.26 can be determined as a standard value for the CD4+/CD8+ as a prognostic for poorly-differentiated squamous cell carcinoma in the research sample, Table 5.

The best value between sensitivity and specificity of CD4+ as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

The best value between sensitivity and specificity of CD4+ and CD4+/CD8+ as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

The best value between sensitivity and specificity of CD4+ and CD4+/CD8+ as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

The best value between sensitivity and specificity of CD4+ as a prognostic for poorly-differentiated OSCC in the group of well-differentiated and poorly differentiated.

Table 1. Clinicopathologic Features of Oral Squamous Cell Carcinoma (n = 43)

<table>
<thead>
<tr>
<th>Clinicopathologic Features</th>
<th>Number (n=43)</th>
<th>percentage %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>43-82</td>
<td>-64.7</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>24</td>
<td>-55.8</td>
</tr>
<tr>
<td>Female</td>
<td>19</td>
<td>-44.2</td>
</tr>
<tr>
<td>Smoking</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yes</td>
<td>19</td>
<td>-44.2</td>
</tr>
<tr>
<td>No</td>
<td>24</td>
<td>-55.8</td>
</tr>
<tr>
<td>Clinical stage</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I/II</td>
<td>21</td>
<td>-48.8</td>
</tr>
<tr>
<td>III/IV</td>
<td>22</td>
<td>-51.2</td>
</tr>
<tr>
<td>Lymph node</td>
<td></td>
<td></td>
</tr>
<tr>
<td>N0</td>
<td>27</td>
<td>-62.8</td>
</tr>
<tr>
<td>N1</td>
<td>9</td>
<td>-20.9</td>
</tr>
<tr>
<td>N2</td>
<td>7</td>
<td>-16.3</td>
</tr>
<tr>
<td>Metastasis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M0</td>
<td>0(0)</td>
<td>0</td>
</tr>
<tr>
<td>M1</td>
<td>0(0)</td>
<td>0</td>
</tr>
<tr>
<td>Location</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tongue</td>
<td>22</td>
<td>51.2</td>
</tr>
<tr>
<td>Other sities</td>
<td>21</td>
<td>48.8</td>
</tr>
</tbody>
</table>

classified according to WHO, OSCC were 17(39.5%) well-differentiated, 19 (44.2%) moderately and 7 (16.3%) poorly-differentiated.

Associations between CD4+, CD8+ TILs Density and clinicopathologic features

CD4+ and CD8+ TILs infiltration was not significantly associated with age, gender and smoking, and stage, Table 3.

Associations between CD4+, CD8+, TILs Density and histological grade of OSCC

Most infiltrating CD4+,CD8+ TILS were distributed

Figure 1. Expression of CD4+ and CD8+ based on IHC Cell Count Analysis in Well–Differentiated OSCCA( H&E) Staining the Tumor Cells Nests (the blue arrow ) and the immune cells), B the infiltration of CD4+ ( the yellow arrow) in stroma off OSCC between tumor nests ,C the infiltration of CD8+ ( the yellow arrow) in stroma of OSCC between tumor nests.
to 0.368 and the specificity value was equal to 0.765, and therefore we conclude that the value 72.5 can be determined as a standard value for the CD4+ rate as a predictor of moderately-differentiated OSCC in a sample search, Table 6.

**Discussion**

The presence of TILS represent the host immune response against tumor cells and could be a valuable predictor of patients prognosis (Shimizu et al., 2019). This antitumor immunity is represented by various subsets of lymphocytes, such as CD8+ and CD4+ (dos antos Pereira et al., 2014).

In this study, we assessed the correlation between CD4+, CD8+, and CD4+/CD8+ TILS and clinicopathologic features and histological grades of OSCC. We found that the number of CD4+, CD8+ TILS were not significant with clinicopathologic features. Thus in disagreement with results that are consistent with other reports (Cho et al., 2011; Zancope et al., 2010), it is due to, the size of samples, the diverse scoring systems used to calculate TIL expression, and the different techniques of tissue analysis and lymphocyte characterization, could result in the discrepancies of the results of these studies.

Previous studies have reported that D8+ TILs have favorable effects on the survival of patients with various tumors including head and neck cancer (de Ruiter et al., 2017; OSCC (Cho et al., 2011; Shimizu et al., 2019; Wolf et al., 2015; Zancope et al., 2010) and (Shota

---

### Table 2. Average Numbers of cd4+, cd8+, cd4+/cd8+ According to Histological Grades of OSCC

<table>
<thead>
<tr>
<th>OSCC grades</th>
<th>Number of patients</th>
<th>CD4+ Mean± SD</th>
<th>P-value</th>
<th>CD8+ Mean± SD</th>
<th>P-value</th>
<th>CD4+/CD8+ Mean± SD</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Well-differentiated</td>
<td>17</td>
<td>63.94±9.13</td>
<td>0.021</td>
<td>51.18±9.89</td>
<td>0.454</td>
<td>128.92±30.63</td>
<td>0.017</td>
</tr>
<tr>
<td>Moderately-differentiated</td>
<td>19</td>
<td>71.84±10.52</td>
<td></td>
<td>50.74±11.68</td>
<td></td>
<td>147.16±33.98</td>
<td></td>
</tr>
<tr>
<td>Poorly-differentiated</td>
<td>7</td>
<td>74.14±7.22</td>
<td></td>
<td>45.00±13.95</td>
<td></td>
<td>180.28±60.87</td>
<td></td>
</tr>
</tbody>
</table>

cd4+, cd8+, cd4+/cd8+. P indicate P-value and correlation coefficient from one-way ANOVA test; significant P-value < 0.05

---

### Table 3. Average Numbers of cd4+, cd8+, cd4+/cd8+ and Correlation with Clinicopathologic Features of OSCC According to Histological Grades of OSCC.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>OSCC grades</th>
<th>Sex P-value</th>
<th>Age P-value*</th>
<th>Smoking P-value</th>
<th>Clinical stage I/II AND III/IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>well-differentiated</td>
<td>0.522</td>
<td>0.626</td>
<td>0.315</td>
<td>0.704</td>
</tr>
<tr>
<td></td>
<td>Moderately-differentiated</td>
<td>0.449</td>
<td>0.282</td>
<td>0.092</td>
<td>0.545</td>
</tr>
<tr>
<td></td>
<td>poorly-differentiated</td>
<td>0.663</td>
<td>0.782</td>
<td>0.254</td>
<td>0.882</td>
</tr>
<tr>
<td>CD8+</td>
<td>well-differentiated</td>
<td>0.592</td>
<td>0.362</td>
<td>0.734</td>
<td>0.438</td>
</tr>
<tr>
<td></td>
<td>Moderately-differentiated</td>
<td>0.616</td>
<td>0.168</td>
<td>0.327</td>
<td>0.306</td>
</tr>
<tr>
<td></td>
<td>poorly-differentiated</td>
<td>0.188</td>
<td>0.553</td>
<td>0.962</td>
<td>0.064</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>well-differentiated</td>
<td>0.843</td>
<td>0.367</td>
<td>0.387</td>
<td>0.277</td>
</tr>
<tr>
<td></td>
<td>Moderately-differentiated</td>
<td>0.349</td>
<td>0.500</td>
<td>0.92</td>
<td>0.084</td>
</tr>
<tr>
<td></td>
<td>poorly-differentiated</td>
<td>0.215</td>
<td>0.340</td>
<td>0.802</td>
<td>0.223</td>
</tr>
</tbody>
</table>

cd4+, cd8+, cd4+/cd8+. P indicate P-value and correlation coefficient from T student test; m Tstudent test; Asterisk (*) indicates P-value and correlation coefficient from Pearson test. Statistically significant p < 0.05 values are in bold.
Shimizu ET AL reported that cd8+ is an indicator of tumor recurrence and prognosis in OSCC (Shimizu et al., 2019) and cd8+ was associated with high grade malignancy (dos antos Pereira et al., 2014).

In this study cd8+ was not associated with the grade of malignancy, possibly due to the complexity of its correlation with OSCC microenvironment such as signaling through receptors including CTLA-4 and...
Table 5. The Sensitivity and Sensitivity Values of PD1, PDL-1/TILS and PDL-1/TC between the the Groups of Well-Differentiated and Poorly-Differentiated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Ratio of CD4+ to CD4+/CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>0.857</td>
<td>0.706</td>
<td>69.5</td>
</tr>
<tr>
<td>CD4+/CD8+</td>
<td>0.857</td>
<td>0.882</td>
<td>148.26</td>
</tr>
</tbody>
</table>

Table 6. The Sensitivity and Sensitivity Values of PD1, PDL-1/TILS and PDL-1/TC between the the Groups of Well-Differentiated and Moderately-Differentiated.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Ratio of CD4+ to CD4+/CD8+</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD4+</td>
<td>0.368</td>
<td>0.765</td>
<td>72.5</td>
</tr>
</tbody>
</table>

PD-1 on cd8+ cells may inhibit their cytotoxic activity (Chatzopoulos et al., 2021). This results was agreed with Zancope et al (Zancope et al., 2010) who did not found significant differences between CD8+ with histologic grade of OSCC.

CD4+ cells may play an important role in initiating and maintaining anticancer immune responses. In addition, CD4+ can inhibit tumor growth in the absence of CD8+ cells by lysing MHC class II positive tumor cells or by enhancing the recruitment of other effector cells (Luckheeram et al., 2012). Previous studies did not found a significant differences histological grade or survival (dos antos Pereira et al., 2014; Lequerica-Fernández et al., 2021). In disreagreement this study, the number of CD4+ and cd4+/cd8+ had significant prognostic value with Poorly differentiated OSCC. This finding suggests that cd4+ cells have different functions in the host immune responses and may play a central role in initiating and maintaining anticancer immune responses against OSCC.

Author Contribution Statement

Collecting data, research and writing the manuscript: Hanan Wahbi, Manuscript revision: Ahmad AL Manadili.

Acknowledgements

General: we acknowledged professor Amirah ALnour (head of oral pathology department) for her help in this study and we acknowledged the lab technicians in Damascus hospital for the facilities they presented in collecting the samples.

Funding Statement

This study was funded by faculty of dentistry, Damascus university.
Asian Pacific Journal of Cancer Prevention, Vol 23

Immunoeexpression of CD4+, CD8+ in Oral Squamous Cell Carcinoma.


Qin Z, Blankenstein T (2000). CD4+ T cell-mediated tumor rejection involves inhibition of angiogenesis that is dependent on IFN gamma receptor expression by nonhematopoietic cells. Immunity, 12, 677-86.


This work is licensed under a Creative Commons Attribution-Non Commercial 4.0 International License.