IMMUNOHISTOCHEMICAL EXPRESSION OF TLR-4 AND ITS CORRELATION TO VARIOUS GRADES OF ORAL SQUAMOUS CELL CARCINOMA

Enas M. Omar MSc, Taissir A. Omar PhD, Sahar M. El-Sheikh PhD, Sahar E. Riad PhD, Ghada M. Mourad PhD, Marwa M. Afifi PhD

ABSTRACT

INTRODUCTION: Oral cancer is a major health problem, causing high morbidity and mortality rates. Oral squamous cell carcinoma (OSCC) accounts for 90-95% of all oral malignancies. During the last decade, significant evidence has suggested that inflammation plays an important role in tumorigenesis, and microenvironment-derived signals which are important constituents in all tumors. Toll-like receptors (TLRs) are classical signals that play a key role in the innate immune system. They drive the inflammatory response and control the cellular proliferation and survival by stimulating the immune cells and promoting integrated processes of inflammation and tissue repair. This can predict that TLR-4 may be of great significance in tumors. Hence, this study is to evaluate and correlate the expression of TLR-4 in various grades of oral squamous cell carcinoma (OSCC).

OBJECTIVES: To evaluate and correlate the expression of TLR-4 in different histopathological grades of OSCC

MATERIALS AND METHODS: An immunohistochemical (IHC) study using the TLR-4 antibody were done on 50 surgical specimens and 10 normal mucosal tissues taken from OSCC patients and healthy individuals, respectively. The staining were performed using a Labeled Strept-Avidin Biotin complex method (LSAB)

RESULTS: TLR-4 was expressed in human OSCC biopsies and the expression level was correlated with the tumor differentiation. Higher expression was detected in the well and the moderately differentiated squamous cell carcinomas than the poorly differentiated ones.

CONCLUSIONS: TLR-4 expression could be a prognostic factor in oral squamous cell carcinoma cases as expression was detected in the well and moderately differentiated higher than poorly differentiated squamous cell carcinomas.

KEYWORDS: Oral squamous cell carcinoma, TLR-4

INTRODUCTION

Cancer of the oral cavity and oropharynx is a global health burden with an estimated overall annual incidence of nearly 443,000 new cases and 241,450 deaths worldwide (1). The frequency of cancer in the middle east and north African (MENA) countries is increasing. It is currently considered the fourth leading cause of death after the cardiovascular, infectious, parasitic diseases, and finally traumatic injuries. More than 270,000 approximately die from cancer each year in this geographic area (2). Oral squamous cell carcinoma (OSCC), represents the most frequent oral neoplasm and counts for more than 90% of oral cancer (3). Toll-like receptors (TLRs) belong to the class of pattern recognition receptors and are the first line of defense from invading pathogens. TLRs recognize pathogen-associated molecular patterns (PAMPs), which activate inflammation and the innate immunity response (4). Humans express ten functional TLRs (TLR1 to TLR10). TLR phenotypes, such as TLR2, TLR3, TLR4, TLR5, TLR7 and TLR 9, are expressed in various head and neck squamous cell carcinomas in vivo and in vitro (5, 6). TLR-4 is well-known for recognizing lipopolysaccharide (LPS), which is a component present in many Gram-negative bacteria. TLR-4 activation results in the production of proinflammatory cytokines, through the myeloid differentiation primary response protein 88 (MyD88) dependent pathway, and the production of type 1 interferons, through a MyD88-independent pathway. TLR-4 constitutes an important part of the innate immunity as it is the first line of host defense. It is expressed in the normal epithelial, immune and cancer cells (7). Despite the favorable immune responses from TLR-4 expression, it appears to be a double-edged sword in cancers and has been related to both cancer inhibition and growth (8). Recently there is an increasing evidence that suggests the significance of TLRs and their ligands in many pathological conditions (9). Although there have been many reports on TLR-4 expression in different carcinomas, so far little, is known about its pathological significance in OSCC. However, targeting TLRs is now an exciting field for translational cancer research. In fact, to the best of our knowledge this is the first study to evaluate the expression of TLR-4 in OSCC cases in Egypt. The aim of the present work is to evaluate and correlate TLR-4 expression in the different OSCC histopathological grades.
MATERIALS AND METHODS
Fifty cases of OSCC were obtained from the bio-archiving bank at Oral Pathology Department, Faculty of Dentistry, Alexandria University, Egypt. The diagnosis of the biopsies was confirmed histopathologically in the Oral Pathology Department at the Faculty of Dentistry, Alexandria University. In addition, 10 specimens were taken from the normal oral mucosa of patients who were indicated for alveoloplasty served as a control group. The biopsies of the patients and bio-archiving were in compliance to the Code of Professional Ethics for Dentistry adopted by the Alexandria University, Faculty of Dentistry.

The specimens were fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax using the conventional procedures. Serial sections of 3-4 μm thickness were placed on glass slides and stained using Hematoxylin and Eosin (H&E) for confirmation. Immunohistochemical (IHC) staining using the TLR-4 antibody (Thermofisher, USA) was also performed using the Labeled Strept- Avidin Biotin complex method (LSAB) (10). The immunostaining results were scored using an image analyzer (Image J software).

Statistical Analysis (11):
The data were fed to the computer and analyzed using IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) (12). The Kolmogorov-Smirnov test was used to verify the normality of distribution. Quantitative data were described using range (minimum and maximum), mean, standard deviation and median. In all our statistical results, a p value < 0.05 was considered significant. The F-test (ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, while the Post Hoc test (Tukey) was used for pairwise comparisons.

RESULTS
Clinical Results
The demographic data of the patients included in the current study showed that: The age of the patients ranged from (44-81 years). The mean age was found to be (61 years) while the median age was (59.5 years). Thirty-two patients (64%) were females and eighteen (36%) were males.

The most common site of occurrence was the lateral side of the tongue (52%). This is followed by the buccal mucosa representing (18%) and the alveolar mucosa (12%). Other sites included in the present work were the retromolar area, the palate and the floor of the mouth which accounted for (6%) each.

Histopathological Results
The microscopical examination of QSCC revealed that (22%) were of the well differentiated type, (64%) were moderately differentiated, (14%) were of the poorly differentiated type.

Immunohistochemical Results
All the cases of normal oral mucosa showed immunoreactivity to TLR-4. It was mainly detected in the basal and parabasal cell layers (Figure 1).

All the included OSCC cases showed immunoreactivity to TLR-4 antibody:
The well differentiated OSCC showed diffuse positive cytoplasmic immunosignals of TLR-4 in the malignant epithelial cells forming the keratin pearls, (Figure 2).

The Moderately differentiated OSCC showed positive strong cytoplasmic and membranous immunoreaction to TLR-4. Prominent nucleoli, abnormal mitosis figures and apoptotic cells were detected, (Figures 3 and 4).

Figure 1: Normal Mucosa showing Positive Immunostaining of TLR-4 in The Basal and Para Basal Cell layers (x200).

Figure 2: Well Differentiated Squamous Cell Carcinoma Showing Positive Cytoplasmic Immunosignaling of TLR-4 in The Malignant Epithelial Cells Forming the Keratin Pearls. (x400).

Figure 3: Moderately Differentiated Squamous Cell Carcinoma Showing Positive Strong Cytoplasmic Immunoreaction of TLR-4. Prominent Nucleoli are Noted (Red Arrows) Note the Abnormal Mitotic Figure (Blue arrow) (x400).
In case of the poorly differentiated OSCC positive membranous and cytoplasmic immunosignals were detected in the highly anaplastic malignant epithelial cells. However, some malignant epithelial cells were devoid from any reaction (Figure 5).

Table (1): Comparison Between the Different Histological Grades of OSCC According to The Mean of the Area Percent of TLR 4 Receptor Immunoexpression

<table>
<thead>
<tr>
<th>Mean of area percent</th>
<th>Well differentiated OSCC</th>
<th>Moderate differentiated OSCC</th>
<th>Poorly differentiated OSCC</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min. – Max.</td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>18.31 – 56.25</td>
<td>27.81 – 78.64</td>
<td>1.10 – 19.61</td>
<td></td>
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<tr>
<td>Mean ± SD.</td>
<td>39.65 ± 12.44</td>
<td>55.81 ± 11.78</td>
<td>13.89 ± 7.38</td>
<td>32.02</td>
<td>&lt;0.00 1*</td>
</tr>
<tr>
<td>Median</td>
<td>40.72</td>
<td>11.78</td>
<td>15.94</td>
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</table>

Table (2): Comparison Between Different Histological Grades of OSCC According to The Mean of the Optical Density of TLR 4 Immunoexpression

<table>
<thead>
<tr>
<th>Mean of optical density</th>
<th>Well differentiated OSCC</th>
<th>Moderate differentiated OSCC</th>
<th>Poorly differentiated OSCC</th>
<th>F</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Min. – Max.</td>
<td></td>
<td></td>
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<tr>
<td>38.09– 43.22</td>
<td>62.26 – 77.85</td>
<td>18.75 – 32.67</td>
<td></td>
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<tr>
<td>Mean ± SD.</td>
<td>39.70 ± 2.02</td>
<td>67.50 ± 3.64</td>
<td>25.14 ± 4.41</td>
<td>510.0</td>
<td>&lt;0.00 1*</td>
</tr>
<tr>
<td>Median</td>
<td>39.14</td>
<td>67.36</td>
<td>25.55</td>
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F: F value for ANOVA test, pairwise comparison bet. each 2 groups were done using Post Hoc Test (Tukey) p: p value for comparing between the different groups p1: p value for comparing between well differentiated SCC and moderate differentiated SCC p2: p value for comparing between well differentiated SCC and poorly differentiated SCC p3: p value for comparing between moderate differentiated SCC and poorly differentiated SCC *: Statistically significant at p ≤ 0.05

Figure 4: Moderately Differentiated Squamous Cell Carcinoma Exhibiting Membranous TLR-4 Immunosignals. The Nuclei are Devoid from Any Reaction. Note the Apoptotic cell (Red arrow) (x400).

Figure 5: Poorly Differentiated Squamous Cell Carcinoma Revealing Positive Membranous and Cytoplasmic Immunosignals in the Anaplastic Epithelial Cells. Few Malignant Epithelial Cells are Negative (Arrows) (x400).

TLR-4 Immunoexpression in Correlation to the Histological Grading of OSCC:
Comparing the different grades of OSCC according to the area percent of TLR 4 immunoexpression, the greatest mean value was in the moderately differentiated squamous cell carcinoma (55.81 ± 11.78), while the lowest value was detected in the poorly differentiated grade (13.89 ± 7.38) (Table 1). In regards to the optical density, the greatest mean value was detected in the moderately differentiated squamous cell carcinoma (67.50 ± 3.64) and the lowest value in the poorly differentiated OSCC (25.14 ± 4.41) (Table 2). The difference in the mean TLR-4 area percent and mean optical density between the different grades revealed statistical significance at (P≤0.05) (Figure 6).
expressed in the normal human oral cells. It seems that the difference in the results of many studies are likely to be that TLRs are mostly involved in ligand binding and they do not last long enough to stimulate the immune system. This may be because the basal epithelial layer, is found at the junction between the epithelium and the underlying lamina propria and could play a role in activating the humoral and cellular immune systems. Therefore, this specific basal layer acts as a barrier if the outermost layers are not stimulated (26).

In the current work, TLR-4 immunoeexpression was evaluated using the image analyzer. All the examined OSCC cases revealed positive expression, that was observed in the cytoplasm as well as the membrane of the malignant epithelial cells. Our examined cases (moderate and well differentiated types) showed higher statistically significant expression of TLR-4 than the poorly differentiated grade. This is in accordance with Sun et al. (25). They observed high expression of TLR-4 in the well and the moderately differentiated types, whereas it was weak in the poorly differentiated tumors. Furthermore, this agrees with Kotrashetti et al. (23) study which reported that TLR-4 is highly expressed in the well-differentiated carcinomas, and the expression level is closely associated with the degree of oral epithelial dysplasia and the distribution of cancer cells. Moreover, Maikenin et al (27) and Pakdel et al (28) investigations revealed that TLR-4 was expressed in all the primary, recurrent and metastatic tumors of OSCC. However, Park et al. (29) investigated the expression of TLR 2, 3, 4, 5, 7 and 9 in OSCC cell lines and on tissue sections, and found high expression of TLR5,7,9 and weak expression of TLR 2,3,4. In addition, Rydberg et al. (30) studied various types of TLR in head and neck squamous cell carcinoma cell lines; and they barely detected TLR-4 expression.

The difference in the expression patterns also varies greatly according to the physiological or pathological conditions prevailing in the host system. Variation in TLR expression can be explained by two mechanisms (9). Up-regulation of TLRs can be of a great benefit to the tumor owing to its anti-apoptotic activity, which permits the tumor cell survival. In addition, TLR may help the immune system to act as a defensive mechanism against the malignant transformation of the cells. Therefore, TLR agonists frequently have been used as immune adjuvants in anti-cancer immunotherapy (31,32).

CONCLUSION
TLR-4 expression could be a prognostic factor in oral squamous cell carcinoma cases since its expression was detected in the moderately and the well differentiated more than the poorly differentiated squamous cell carcinoma.

CONFLICT OF INTEREST
The authors declare that they have no conflicts of interest.

REFERENCES
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