EXPRESSION OF TLR7 IN ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH LYMPH NODE METASTASIS (AN IMMUNO-HISTOCHEMICAL STUDY)

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ABSTRACT

INTRODUCTION: Oral squamous cell carcinoma (OSCC) accounts for most of oral cancers which is about (90%) of all types. OSCC is an important cause of morbidity and mortality worldwide. Early detection; diagnosis and early treatment are still the key points for OSCC patients to improve survival rate and quality of life. Toll like receptors (TLRs), through their role in innate immunity, have been implicated in the pathogenesis of several diseases, including cancer and OSCC is one of these cancers. Nodal metastasis is playing a key role in detecting the prognosis and the outcome. TLR7 is one of the TLRS which is expressed in OSCC, the correlation between the expression of this receptor and the presence of nodal metastasis has been assessed and evaluated as TLR7 may be of great significance in tumors metastasis so can help in prognosis and aid in treatment modalities.

OBJECTIVES: The aim of the present study was to evaluate the expression of TLR7 in the specimens of primary tumor tissues of OSCC and correlate it with the lymph node involvement.

MATERIALS AND METHODS: TLR7expression was studied in 50 OSCC cases. The specimens were taken from the primary tumor of 25 cases with positively involved lymph nodes and another 25 cases with negatively involved lymph nodes and 10 normal mucosal tissues taken from OSCC patients and healthy individuals. Immuno-histochemical (IHC) staining was performed using the Labeled Strept-Avidin Biotin complex method (LSAB), using the anti-TLR7 antibody.

RESULTS: TLR7 showed high expression in OSCC cases with positive lymph node metastasis than those with negative lymph node metastasis.

CONCLUSIONS: TLR7 expression could be a guiding tool used to detect prognosis in OSCC patients and as an indicator for metastasis.

KEYWORDS: Oral squamous cell carcinoma, lymph node metastasis, TLR7, Immunohistochemistry.

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INTRODUCTION

Oral cancer, a subtype of head and neck cancer (1). It is a cancerous tissue growth located in the oral cavity (2). It may arise as a primary lesion originating from the oral tissues or by metastasis from a distant site of origin, or by extension from a neighboring anatomic structure, such as the nasal cavity (3). Approximately 30% of head and neck cancers are oral cavity cancers, with nearly 95% of the latter being oral squamous cell carcinomas (OSCC) (4). Oral squamous cell carcinoma (OSCC) is the sixth most prevalent malignancy worldwide (5). The prognosis of OSCC remains dismal because more than 50% of patients die from this disease or its complications within 5 years with current therapies (6). Lymph node (LN) metastasis is one of the most implicated and effective factors in the treatment and prognosis of patients with OSCC as LN metastasis are a strong determinant for a poor outcome and bad prognosis. It reduces the overall 5 years survival by nearly half (7, 8).

Recently, there has been a growing recognition of interest in anti-tumor functions initiated by the innate immune response. The role of Toll-like receptors (TLRs) is among the frontiers of exploration (9). Toll like receptors (TLRs) are a family of pattern recognition receptors (PRRs) that function as primary sensors of the innate immune system to recognize microbial pathogens. Toll-like receptors (TLRs) were first discovered in drosophila, in the membranes of binding PRRs and are known to target a series of mechanisms leading to the synthesis and secretion of cytokines and activation of other host defense programs that are crucial to the development of innate or adaptive immunity (10). Ten functional TLRs have been identified in humans (TLR1-10) (12). These receptors are highly expressed on the cells of the immune system as well as non-immune cells such as the epithelial cells in respiratory, genitourinary and gastrointestinal epithelium and skin as these areas are considered as the first line of defense also endothelial cells and fibroblasts. (11, 12, 13, 14).

TLR7, a receptor that recognizes viral single-stranded RNA, is located in the endosomal compartments of plasmacytoid dendritic cells (pDCs), B lymphocytes, natural killer cells, and virally infected cells (15). It has also been reported to be expressed by a variety of cancer cells. TLR7 shows a wide behavior in the tumor micro-environment with either pro-tumorigenic or anti-tumorigenic effects. However, the distinct expression and

function of TLR7 in head and neck tumors, especially OSCC, are unclear (16).

To the best of our knowledge, no researches in literature are available for the association of TLR7 expression in OSCC and metastasis potentiality. This study was thus conducted to correlate the immune-histochemical expression of TLR7 in OSCC cases to LN metastasis and so such a protein marker may aid in treatment modalities.

MATERIALS AND METHODS

Study material

The present study was performed in the Oral Pathology Department, Faculty of Dentistry, Alexandria University after gaining the approval of the Research Ethics Committee. Fifty OSCC cases collected from the Cranio-Maxillofacial and Plastic Surgery Department were included. Biopsies were taken from the primary tumor of twenty-five cases proved to have positive lymph nodes and another twenty- five cases with negative lymph nodes. In addition, 10 specimens were taken from the healthy individuals with normal oral mucosa who were indicated for alveoloplasty served as a control group. The biopsies were confirmed histo-pathologically in the Oral Pathology Department at the Faculty of Dentistry, Alexandria University. The biopsies of the patients and bio-archiving were in compliance with the Code of Professional Ethics for Dentistry adopted by the Alexandria University, Faculty of Dentistry.

Histopathological and immuno-histochemical analysis

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). Immunohistochemical (IHC) staining using the anti-TLR7 antibody (the primary polyclonal rabbit anti-human TLR 7 antibody (ab45371; Abcam, USA) was performed using the Labeled Strept-Avidin Biotin complex method (LSAB) (17). The sections were then examined and the intensity of the immunostaining was quantified in terms of both the mean area percent (AP) and the mean optical density (OD) by the computer image analyzer ImageJ software (NIH, USA).

Statistical analysis of the Data

Statistical package for social sciences version 20.0 (SPSS 20.0, SPSS Inc., Chicago, IL, USA) was carried out for the statistical analyses and calculations of data (18). The differences in the mean OD and AP in OSCC cases with positive and negative lymph nodes were estimated and calculated using the student (t) test. Any P-values equal to or less than 0.05 were considered statistically significant. All the data are expressed as mean ± SD (standard deviation).

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%, n=26)**. This is followed by the buccal mucosa representing **(18%, n = 9)** and the alveolar mucosa **(12%, n = 6)**. Other sites included in the present work were the retromolar area **(6%, n= 3)**, the palate **(6%, n= 3)** and the floor of the mouth **(6%, n= 3)**.

Histopathological Results

The microscopical examination of OSCC revealed that **(22%, n=11)** were of the well differentiated type, **(64%, n=32)** were moderately differentiated, **(14%, n=7)** were of the poorly differentiated type.

Immuno-histochemical Results

All biopsies of normal oral mucosa (n=10) showed weak immunoreactivity for TLR7 which was limited to the basal and the parabasal layers. (Figure 1)

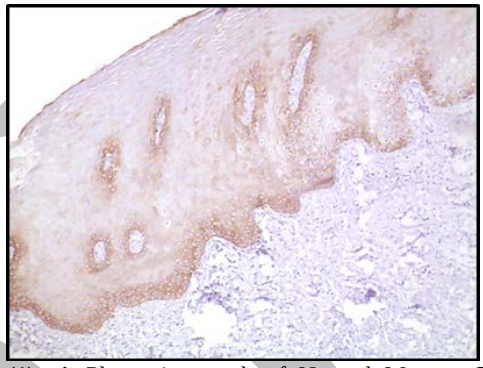

Figure (1): A Photomicrograph of Normal Mucosa Showing Weak TLR7 Immuno-signals Confined to the Basal and Para-basal Layers. (X 200)

All the tumor biopsies showed immunoreactivity to the anti-TLR7 and it was in the cytoplasm of the tumor cells as well as the nuclei with varying intensities. The expression with varying intensities ranging from weak to moderate reactions in the LN negative cases and moderate to severe reactions in the LN positive cases. (Figures 2- 4)

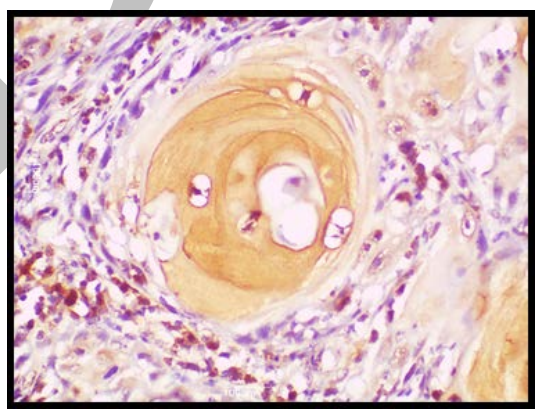

Figure (2): A Photomicrograph of a Well Differentiated OSCC Case with Negative LN Status Showing Weak TLR7Immuno-signals. (X400)

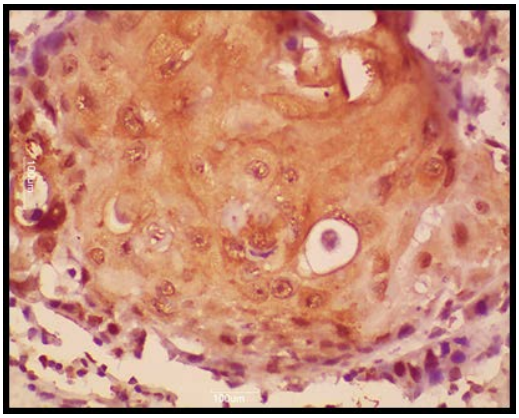

Figure (3): A Photomicrograph of a Moderately Differentiated OSCC Case with Positive LN status Showing positively Intense Cytoplasmic and nuclear TLR7 Immuno-signals. (x400)

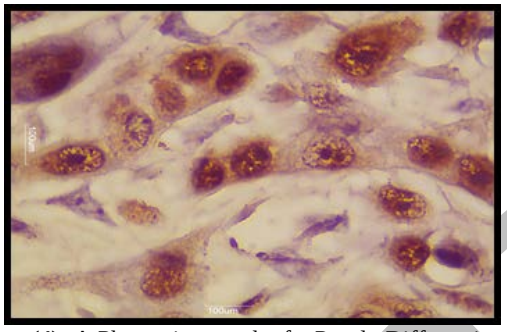

Figure (4): A Photomicrograph of a Poorly Differentiated OSCC with Positive LN status Showing Positively intense Cytoplasmic and nuclear TLR7 Immuno-signals in the malignant pleomorphic Cells. (X1000)

Correlation of TLR7 expression with the lymph node involvement in OSCC

OSCC cases with positive LNs showed higher TLR7 immunoreactivity than the negative LN cases. The expression of TLR7 immunostaining was calculated in terms of mean area percent (AP) and mean optical density (OD). The mean AP was (88.06 ± 3.39) for the LN positive group and (49.86± 13.62) for the LN negative group. The mean OD was also higher in the LN positive group (56.56± 4.89) than the LN negative group (41.63 ± 8.05). The difference between these two groups regarding both AP and OD revealed statistical significance at (P≤0.05) using the student t-test. (Tables 1&2)

Table (1): Comparison between Area Percent of TLR7 Immunostaining in Negative and Positive LN Metastasis

Mean AP	LN –ve (n=25)	LN +ve (n=25)	t	P
Min. – Max.	31.15- 71.41	82.56- 92.17	7.7421	<0.0001*
Mean	49.86	88.06		
SD	13.62	3.39		

t, p: t and p values for **Student t-test** for comparing between the two groups

*: Statistically significant at p ≤ 0.05

Table (2): Comparison between Optical Density of TLR7 Immunostaining in Negative and Positive LN Metastasis

Mean OD	LN –ve (n=25)	LN +ve (n=25)	t	p
Min. – Max.	28.96- 58.39	48.62- 62.51	7.0895	<0.0001*
Mean	41.63	56.56		
SD	8.05	4.89		

t, p: t and p values for **Student t-test** for comparing between the two groups

*: Statistically significant at p ≤ 0.05

DISCUSSION

Up till now, early detection, diagnosis and early treatment are still the key points for OSCC patients to improve survival rate and quality of life. Besides routine clinical examination, laboratory and image examinations are considered to be useful for early detection and early diagnosis (19). With advances in molecular biology and immunology, researches on tumor markers in tissues have been gradually deepened. Their detection and correlation with OSCC have been suggested to be potentially useful for clinical diagnosis and prognostic monitoring. Tumor-derived markers are genes or proteins that play a direct role in carcinogenesis, and may sometimes also be used as biomarkers to improve cancer detection, predict disease outcome or response to therapy (20, 21).

TLRs function as double-edged swords, with both pro and anti-tumor consequences. However, the exact mechanisms by which TLRs interact with tumor cells and how these cells are able to escape immunological eradication have only recently started to unravel. Therefore, understanding the roles of TLRs in tumor biology may pave the way for the discovery of novel therapeutic targets in cancer therapy (22-24). TLR7 has a role in innate immune response and at the same time contribute to tumor progression.

To the best of our knowledge and after expanded search and exploration in the literature, no studies were available for the association of TLR7 expression in OSCC and metastasis potentiality therefore, this study was conducted to correlate the immune-histochemical expression of TLR7 in OSCC cases to LN metastasis in Egyptian population and so such a protein marker may aid in treatment modalities.

In the current study, the immuno-histochemical expression of TLR7 protein was significantly higher in the cases that were associated with metastatic LNs compared to those without LN metastasis. This suggests an association between the expression of TLR7 in the tumor cells and their ability for invasion and metastasis. In contrast to these findings, Farzaneh et al. (25) and Shiva et al. (26) postulated in their studies that significant increase in TLR2, TLR4, TLR7 in OSCC samples in the tongue was (70.1%, 72.4%, 66.7%) respectively and tumors with high TLR2 and TLR4 expression only were significantly associated with a higher probability of lymph node metastasis and increased depth of invasion also. In accordance to these results also Ilyar et al. (27) evaluated the expression of TLR 3, 4, 7, 9 in esophageal squamous cell carcinoma and he found that TLR4 also is the only one associated with a higher probability of lymph node metastasis and increased depth of invasion.

Julien et al. (28) stated that stimulation and triggering of TLR7 expressed by human lung cancer cells induces cell

survival and chemo-resistance. In agreement to this finding Saradiya et al. (29) confirmed the fact that TLR7 promotes tumor progression, chemotherapy resistance, and poor clinical outcomes in non–small cell lung cancer.

TLR7 agonists are under investigation for their ability to enhance antitumor immune responses. Mee et al. (30) investigated that imiquimod, TLR7 agonist, inhibits the proliferation of oral cancer cells. However, Marion et al. (31) stated that these agonists can also stimulate TLR7 expressing tumor cells so high TLR7 expression in the primary tumor confers poor clinical outcome and resistance to chemotherapy in lung cancer patients. This pro-tumorigenic effect of TLR7 has been validated in murine models of lung carcinoma.

Multiple parameters could underlie this apparent discrepancy, such as the type of tumor, the level of TLR7 expression, the downstream function of TLR7 signaling in particular tumor cells, or chemotaxis of suppressive cells into the tumor. These major pathways of TLR7 stimulation could act either directly or indirectly on both immune and tumor cells, converging on cancer patient outcome and may affect the prognosis and the therapeutic approach of this tumor.

CONCLUSION

TLR7 expression was significantly correlated with the lymph node involvement and so can be used as a prognostic tool in OSCC cases.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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