EXPRESSION OF TLR4 IN ORAL SQUAMOUS CELL CARCINOMA AND ITS CORRELATION WITH LYMPH NODE METASTASIS (An Immunohistochemical Study)

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ABSTRACT

INTRODUCTION: Oral squamous cell carcinoma (OSCC) accounts for more than 90% of all oral cancers with high mortality and morbidity rates. Lymph node (LN) metastasis is one of the most important factors in the treatment and prognosis of patients with OSCC. It reduces the overall survival by nearly half. Toll like receptors (TLRs) are evolutionarily conserved proteins and major type of receptors involved in both innate and adaptive immunities and defense against pathogens. TLR4 was the first member of the TLR family to be discovered. It was verified that TLR4 is expressed in many types of tumors including OSCC. The correlation between the expression of this receptor and the presence of nodal metastasis has been studied. Involvement of TLR4 in the invasion and metastasis potentials may thus suggest the use of this protein as a prognostic marker for OSCC and may provide a new insight in the treatment strategy.

OBJECTIVES: The aim of the present study was to assess the expression of TLR4 in the primary tumor of OSCC and correlate it with the lymph node status.

MATERIALS AND METHODS: TLR4 expression was calculated in 30 OSCC cases. The specimens were taken from the primary tumor of 15 cases proved to have positive lymph nodes and another 15 cases with negative lymph nodes. Immunohistochemical (IHC) staining was performed using the Labeled Strept-Avidin Biotin complex method (LSAB), using the anti-TLR4 antibody.

RESULTS: TLR4 was expressed in the OSCC cases and was significantly higher in cases with positive lymph node metastasis than those without.

CONCLUSIONS: TLR4 expression could be used as a prognostic marker for OSCC as an indicator for metastasis.

KEYWORDS: Oral squamous cell carcinoma, TLR4, lymph node metastasis.

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INTRODUCTION

Oral cancer is the sixth most common cancer worldwide (1) with squamous cell carcinoma (SCC) accounting for more than 90% of all oral cancers (2, 3). Oral cavity cancer and oropharyngeal cancer (OCC-OPC) rank among the top 20 cancers in the Middle East and North Africa region with an estimated 1% death of all human malignancies (4). Oral squamous cell carcinoma (OSCC) remains one of the most difficult malignancies to control because of its high propensity for local invasion and cervical lymph node dissemination (5).

Lymph node (LN) metastasis is one of the most important factors in the treatment and prognosis of patients with OSCC (6). At the time of diagnosis, LN metastasis is a strong determinant of a poor outcome with a worse survival rate and a higher rate of local and regional recurrences (7). It reduces the overall 5 years survival by nearly half (8).

Toll like receptors (TLRs) are evolutionarily conserved proteins and major type of receptors involved in both innate and adaptive immunities and defense against pathogens. They recognize broad but highly conserved structural patterns on different pathogens called pathogen associated molecular patterns (PAMPs). Hence, they are considered among the first line of defense against pathogens (9). They are mammalian homologues of Toll, a receptor found in the fruit fly (drosophila) (10, 11). 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 located near the host-environment boundary including the oral mucosa (13).

TLR4 was the first member of the TLR family to be discovered, by Medzhitov et al in 1997 (14). It was verified that TLR4 is expressed in many types of tumors, such as hepatocellular carcinoma, glioblastoma, lung, breast, ovarian and colon cancers as well as melanoma (15-21). OSCC was also among the tumors that highly express this receptor (22-25).

It was suggested that TLR4 genes may have an important role in the metastatic potential of head and neck squamous cell carcinoma (HNSCC) cells (26). However, studies done to correlate the immunoexpression of TLR4 in OSCC cases to LN metastasis are limited. Involvement of TLR4 in the invasion and metastasis potential may suggest

the use of this protein as a prognostic marker for OSCC and may provide a new insight in the treatment strategy.

The aim of the present study was to assess the expression of TLR4 in the primary tumor of OSCC and correlate it with the lymph node status.

MATERIALS AND METHODS

The present study was performed in the Faculty of Dentistry, Alexandria University after gaining the approval of the Research Ethics Committee. Thirty OSCC cases collected from the Cranio-Maxillofacial and Plastic Surgery Department were included. Biopsies were taken from the primary tumor of fifteen cases proved to have positive lymph nodes and another fifteen cases with negative lymph nodes. The biopsies were confirmed histopathologically in the Oral Pathology Department at the Faculty of Dentistry, Alexandria University. Ten cases of normal oral mucosa from patients who were indicated for alveoloplasty served as the control group in this study. 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.

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-TLR4 antibody (Thermofisher, USA, Catalog number MA5-16216) was also performed using the Labeled Strept-Avidin Biotin complex method (LSAB) (27). The sections were then examined and the intensity of the immunostaining (cytoplasmic) 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

The difference in the mean optical density (OD) as well as the mean area percent (AP) in OSCC cases with positive and negative lymph nodes were estimated using the student (t) test. A (P) value less than 0.05 was considered significant. The values were given as a mean value ± SD (standard deviation).

RESULTS

Clinical Results

In the present study, 30 patients with OSCC were included. The patients' age ranged between 44 and 75 years (59.9 ± 9.48). Eighteen patients (60%) were females and twelve patients (40%) were males.

The most common site of occurrence was the lateral side of the tongue (60%) while the least occurring site was the palate (5%).

Histopathological Results

The microscopical examination revealed that more than half of the cases included in the study (60%, n=18) were moderately differentiated squamous cell carcinoma. Nine cases (30%) were well differentiated and only 10% were poorly differentiated (n=3).

Immunohistochemical Results

All biopsies of normal oral mucosa (n=10) showed weak immunoreactivity for TLR4 which was limited to the basal and the parabasal layers. (Figure 1)

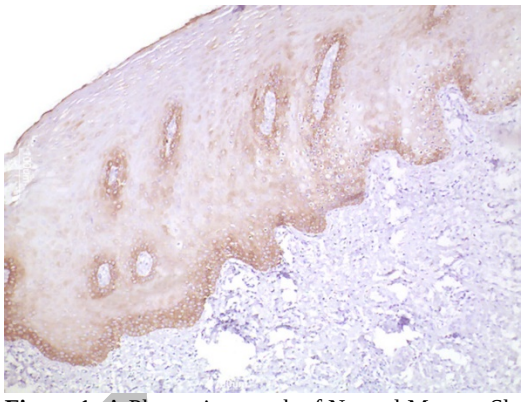

Figure 1: A Photomicrograph of Normal Mucosa Showing Weak TLR4 Immunosignals Confined to the Basal and Parabasal Layers. (X100)

All the studied cases showed immunoreactivity to the anti-TLR4 antibody with varying intensities ranging from weak to moderate reactions in the LN negative cases to moderate and severe reactions in the LN positive cases. The expression was evident in the cytoplasm of the tumor cells with absence of immunosignals from the nucleus. (Figures 2, 3 and 4)

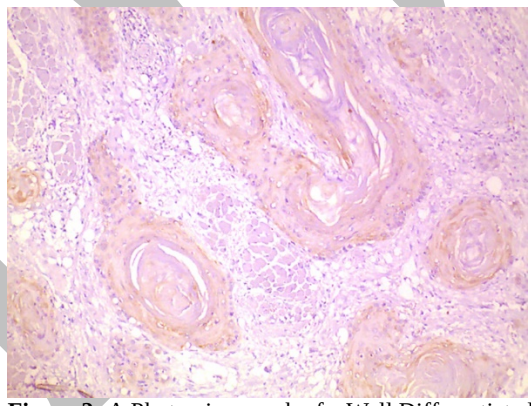

Figure 2: A Photomicrograph of a Well Differentiated SCC Case with Negative LN Status Showing Weak TLR4 Immunosignals. (X100)

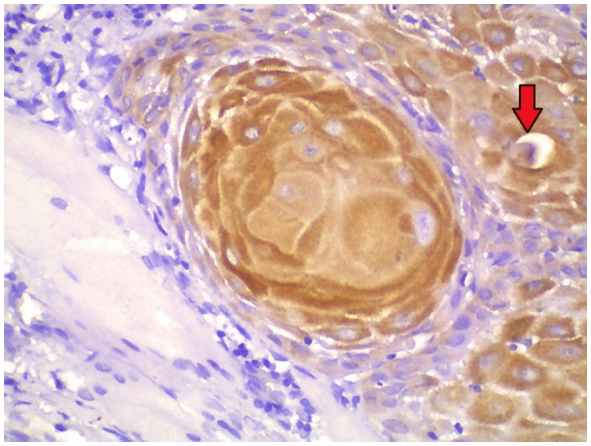

Figure 3: A Photomicrograph of a Moderately Differentiated SCC Case with Positive LN Status Showing Intense Cytoplasmic TLR4 Expression with Absence of Immunosignals from the Nuclei. Note the Apoptotic cell (arrow). (x400)

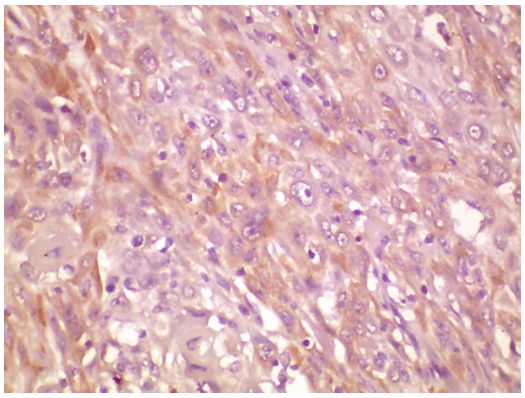

Figure 4: A Photomicrograph of a Poorly Differentiated SCC with Positive LN Showing Positive Cytoplasmic TLR4 Expression in the Pleomorphic Cells with Absence of Immunosignals from the Nuclei. (X400)

Correlation of TLR4 expression with the lymph node status of OSCC

OSCC cases with positive LNs showed higher TLR4 immunoreactivity than the negative LN cases. The expression of TLR4 immunostaining was calculated in terms of mean area percent and mean optical density. The mean AP was (72.238 ± 25.633) for the LN positive group and (19.635 ± 8.116) for the LN negative group. The mean OD was also higher in the LN positive group (55.861± 13.981) than the LN negative group (32.089± 11.343). 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 TLR4 Immunostaining in Negative and Positive LN Metastasis

Mean AP	LN –ve (n=15)	LN +ve (n=15)	t	P
Min. – Max.	3.715 - 34.107	22.242- 95.897	22.242	<0.0001*
Mean	19.63496	72.23792		
SD	8.115832	25.63347185		

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 TLR4 Immunostaining in Negative and Positive LN Metastasis

Mean OD	LN –ve (n=15)	LN +ve (n=15)	T	P
Min. – Max.	18.24 - 51.02	29.99 - 73.76	6.6524	<0.0001*
Mean	32.09	55.8612		
SD	11.34	13.98085		

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

*: Statistically significant at p ≤ 0.05

DISCUSSION

Numerous researches exploring the mechanisms of occurrence, development and metastasis of cancers have been conducted (28). In recent years, numerous studies have been focusing on the genetic predispositions and tumor markers of OSCC which could be used as predictors of tumor aggressiveness and thus the treatment approach (29).

The role of TLR4 in the activation of innate immune response, defense against invading pathogens, cytokine production, and development of adaptive immune responses is well known (30). However, it was also suggested that TLR4 expressed on tumor cells may contribute to tumor progression and metastasis (23, 29).

The present study evaluated the possible relationship between the expression of TLR4 in OSCC tissues and the presence or absence of lymph node metastasis.

In the current study, the expression of TLR4 protein was significantly higher in the cases that were associated with metastatic lymph nodes compared to those without LN metastasis. This suggests an association between the expression of TLR4 in the tumor cells and their ability for invasion and metastasis.

Several studies were done to clarify the association between TLR4 expression and the tumor aggressiveness. In a study by Ren et al (31), the authors they demonstrated that high levels of TLR4 expression in OSCC cases were associated with less tumor differentiation as well as short survival rate. However, they didn't study the correlation between TLR4 expression and LN metastasis.

Later on, and in accordance with our findings, Makinen et al (22) in a study on tongue SCC (OTSCC) found that TLR4 as well as other TLRs (TLR2 and TLR9) were expressed in primary tumors, neck metastases as well as in recurrent tumors. They hypothesized that these receptors seem to play a role in the development, progression and the invasive potential of tongue carcinoma. Hence, they investigated the effect of a TLR4 antagonist on the invasive capacity of an OTSCC cell line in an experimental model. Regardless of their finding that this antagonist failed to inhibit the invasion of the OTSCC cells in the experimental model, they found that the expression levels of TLR4 were more prominent in the invasive cells at the deeper level than in the cells close to the surface.

Moreover, Yang et al (29) in a study on OSCC mentioned that TLR4 expression was significantly correlated with the primary tumor stage, LN status, tumor differentiation, depth of invasion, and pTNM (pathological TNM) stage.

Similar studies done on thyroid, lung, breast, cervical and gastric cancer found a similar correlation between TLR4 expression and LN metastasis as well as the tumors' ability to progress and invade (18, 32-35).

On the other hand, Simiantonaki et al (36) found that the reduced expression of TLR4 is correlated with the metastatic status of human colorectal cancer. They hypothesized that loss of TLR4 is associated with lack of antitumoral immune response and that signaling via TLR4 induces anticancer immunity through maturation of antigen-presenting cells and cytokine induction.

In accordance with this point of view, Davis et al concluded that administration of a TLR4 agonist improves the antitumor responses in head and neck SCC and melanoma tumor models (37).

Moreover, Lamrani et al (38) in their study on a breast cancer model found that TLR4/IFNγ pathways induce tumor regression rather than progression.

Despite these contradicting results, there is a growing body of evidence supporting the role of TLR4 in cancer invasion and metastasis. The emergence of TLR4 as a prognostic biomarker for OSCC aggressiveness may affect the prognosis and the therapeutic approach of this tumor.

CONCLUSION

TLR4 expression was significantly correlated with the lymph node status and presents a potentially useful prognostic marker for OSCC.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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