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TLR2, TLR4, TLR7 and TLR8 expressions in Squamous cell Carcinoma of the tongue

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ABSTRACT

Aim: To determine of the expression of Toll-like receptor (TLR) 2, TLR4, TLR7 and TLR8 in Oral squamous cell carcinoma (OSCC). **Methods:** Reverse transcription-polymerase chain reaction and Flow cytometry were used to analyze the expression of TLR2, TLR4, TLR7 and TLR8 mRNA and protein in samples from 50 cancer patients consisting of both tumor and normal tissue. This study was done in Tabriz medical University between 09.2011 till 07 2013. This study was done in Tabriz medical University between 09.2011 till 07 2013 with grant number 20139470. **Results:** A significant increase in TLR2, TLR4, TLR7 and TLR8 mRNA levels was detected in OSCC samples. Tumors exhibited high TLR protein expression, (70.1%, 72.4%, 66.7% and 78.2% for TLR2, TLR4, TLR7 and TLR8, respectively, $P < 0.05$). Nevertheless, a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells (48.3%). Tumors with high TLR2 expression in tumor cells or high TLR4 expression in mononuclear inflammatory cells were significantly associated with a higher probability of lymph node metastasis and increased depth of invasion. However, tumors with high TLR8 expression in fibroblast-like cells were associated with low probabilities of invasion and metastasis. There was no significant variation between the expression of TLR2, TLR4, TLR7 and TLR8 among different ethnic groups. **Conclusion:** TLR2, TLR4, TLR7 and TLR8 expression appears important to the biological pathogenesis of ESCC. TLRs may represent therapeutic targets for OSCC.

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INTRODUCTION

Oral cancer or mouth cancer [1] a subtype of head and neck cancer, is any cancerous tissue growth located in the oral cavity [2]. It may arise as a primary lesion originating in any of the oral tissues, by metastasis from a distant site of origin, or by extension from a neighboring anatomic structure, such as the nasal cavity. Oral or mouth cancer most commonly involves the tongue. It may also occur on the floor of the mouth, cheek lining, gingival (gums), lips, or palate (roof of the mouth). Most oral cancers look very similar under the microscope and are called squamous cell carcinoma, but less commonly other types of oral cancer occur, such as Kaposi's sarcoma. Oral squamous cell carcinoma (OSCC) is the sixth most prevalent malignancy worldwide and accounts for approximately 8–10% of all cancers in Southeast Asia [3]. The prognosis of OSCC remains dismal because more than 50% of patients die from this disease or complications within 5 years with current therapies [4]. Therefore, an improved comprehension of the cellular and molecular mechanisms which initiate tumorigenesis or promote cancer progression, was pursued to bring forth future progress of medical treatment of OSCC. The prognosis of human oral squamous cell carcinoma (OSCC) is usually poor with a 5-year survival rate of approximately 50 ~ 60%, which has generally been attributed to the insensitivity of most patients to chemotherapy [5, 6]. Recently, there has been a growing recognition of interest in anti-tumor functions initiated

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by the innate immune response. The role of toll-like receptors (TLRs) and their signaling in tumor immune escape and resistance to apoptosis, for example, is among the frontiers of exploration. 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 [7]. TLRs are present in vertebrates as well as invertebrates. Recently, it was estimated that most mammalian species have 10 to 15 types of TLRs. TLRs belong to the Toll/interleukin-1 receptor (TIR) family and all members of this family contain cytoplasmic TIR domains [8,9]. The endodomain of all TLRs differs from the interleukin (IL)-1R ectodomain in which TLR has leucine rich repeats (LRRs), whereas IL-1R possess Ig-like domains [10, 11]. The TIR domain consists of approximately 160 amino acids, and has three regions of particular importance, termed boxes 1, 2 and 3, and is essential for cellular signaling. The extracellular domains of TLR contain 16-28 LRRs which involve some physiological function [12-18]. The individual LRR module is 20-30 amino acids long and is composed of a conserved "LXX LXLXXN" motif and a variable part. TLR 1 and 2 have two shape structural transitions in the B sheet, and their LRR domains can be divided into 3 subdomains: the N terminal, center, and C terminal. The central domains have one or more α helices inserted in the convex area [19].

MATERIALS AND METHODS

This study was done between 09.2011 till 07 2013. A total of 50 tongue tissue specimens from patients with tongue carcinoma who had not received pre-operative radiotherapy or chemotherapy were blocked with formalin-fixed and paraffin-embedded; all specimens were processed at our private pathology laboratory. The borderline tumor tissues were used as controls. Each specimen was histologically examined, and the tumor was graded by at least two experienced pathologists. The main characteristics of OSCC patients, including tumor grade, stage, and lymph node status of the tumor, were categorized according to the TNM guideline (American Joint Committee of Cancer). Among the 50 specimens 20 were well-differentiated tumors, 19 moderately differentiated tumors, and 11 poorly differentiated tumors. 23 patients had lymph node metastases. In addition, 40 frozen biopsies that included 20 normal tongue epithelia and 20 OSCC samples were subjected to RT-PCR for the detection of TLR2, TLR4, TLR7 and TLR8 mRNA expression. All patients were enrolled by written informed consent.

To determine TLR expression at the mRNA level, reverse transcription-PCR (RT-PCR) was performed. Tumor cells (2×10^6) were harvested, washed in PBS, cryopreserved, and stored in -80°C . After cell thawing and washing, total RNA was isolated using the Trizol reagent (Invitrogen). RT-PCR was performed according to the manufacturer's instructions. Reverse transcription was performed with random primers using Omniscript RT kit (Qiagen). The set of primers used to detect TLR2, TLR4, TLR7, TLR8 were previously designed by us: TLR2, 5'-GTGCCAGAACTTCCCATGT-3' (forward) and 5'-CTTCCAATTGCGTGAAAACA-3' (reverse); TLR4, 5'-CTGCAATGGATCAAGGACCA-3' (forward) and 5'-TCCCATTCCAGGTAAGTGTT-3'(reverse); TLR7, 5'-CCTCAGCCACAACCAACTG-3' (forward) and 5'-TTGTGTGCTCCTGGCCCC-3(reverse); TLR8, 5'-AACTTGAGCCACAACAACATTT-3(forward) and 5'-ATCTCCAATGTACAGGTGC-3' (reverse). The PCR was performed using an Expand High Fidelity PCR System (Roche) under the following conditions: denaturation temperature of 95°C for 45 s, annealing temperature of 60°C for 45 s, and extension temperature of 72°C for 1 min. mRNA for β -actin was used as a normalization control in RT-PCR and as a loading control in conventional PCR. TLR2,4,7,8 expressions in cells were evaluated by flow cytometry as follows: cells were collected and then labeled with the APC-labeled mouse anti-human TLR2,4,7,8 antibodies (eBioscience, CA, USA) for 30 min at 4°C . The cells were analyzed using Cell Quest Software.

All statistical analyses were performed with the SPSS statistical software package (version 17.0). The chi-square test was used to compare the differences in cumulative TLR2, TLR4, TLR7 and TLR8 expression between normal and OSCC groups, and to determine whether the clinicopathologic variables were associated with the levels of TLR2, TLR4, TLR7 and TLR8 as well as compare the mRNA expression in fresh frozen OSCC tissues with that of normal samples as determined by RT-PCR. P values < 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Results:

RT-PCR and immunostaining results showed that TLRs are expressed on tumor cells. The RT-PCR results showed the highest level of mRNA for TLR4 was detected in the tumor cells (Fig. 1-a). Also results showed TLR2, TLR7 and TLR8 mRNA expression was increased in OSCC tissues. TLR2 and TLR7 gene expression was quantified in 15 OSCC and 3 normal tissues. TLR4 mRNA expression was higher in OSCC samples than in normal controls after normalization to β -actin expression. A significant increase in TLR2, TLR4, TLR7 and TLR8 mRNA levels was detected in OSCC samples. Tumors exhibited high TLR protein expression, (70.1%,

72.4%, 66.7% and 78.2% for TLR2, TLR4, TLR7 and TLR8, respectively, $P < 0.05$) (fig1-b). Immunofluorescence confirmed TLR4 protein expression in all tumor cell lines (Fig. 2). TLR4 was also detected in tumor tissues, as previously reported [17]. The results showed that the positive rates of TLR2, TLR4, TLR7 and TLR8 expression in the normal Tongue surface epithelium were higher in OSCC lesions compare with healthy tissue.

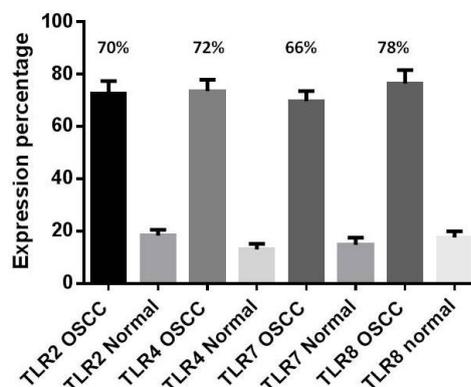


Fig. 1-A: mRNA expression patterns of Toll-like receptor 2, Toll-like receptor 4, Toll-like receptor 7 and Toll-like receptor 9. M: 100-600 bp marker ladder. Lanes 1 to 4 show the expression of Toll-like receptors, and lane 5 shows the expression of β -actin.

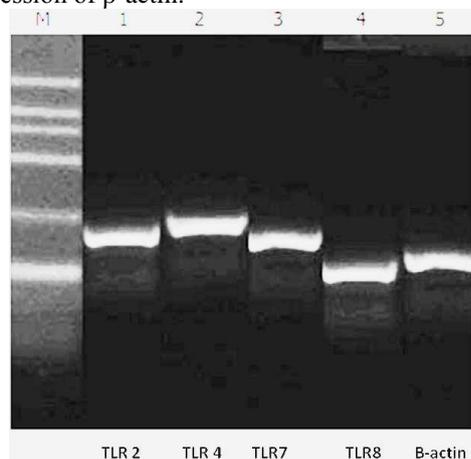


Fig. 1-B: The expression of TLRs in OSCC compare with normal value.

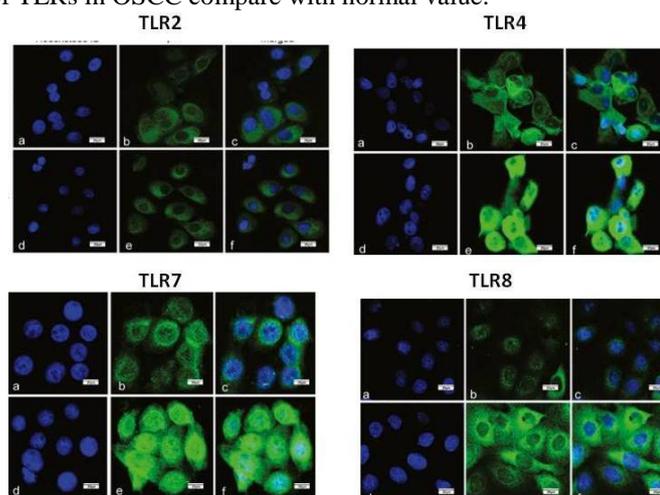


Fig. 2: Immunoflowcytometry staining of Tongue lesions with Toll-like receptor-specific mAbs. A1 to D1 show the expression of Toll-like receptor (TLR) 2, TLR4, TLR7 and TLR8 in normal tongue epithelium, respectively. A2 and C2 show positive staining for TLR2 and TLR7 in tongue squamous cell carcinoma cells. B2 shows positive TLR4 staining in tumor cells and D2 shows positive TLR9 staining in tumor cells.

Discussion:

Current evidence indicates that TLR play a pivotal role in the activation of innate immunity against invading pathogens, cytokine production, and development of adaptive immune responses [21]. In contrast to the protective role of TLR against pathogen infections, this study suggests that TLR expressed on tumor cells contribute to tumor progression [22]. The present study evaluated the possible relationship between the expression of TLRs in tumor cells and the clinicopathologic characteristics of OSCC including tumor stage, histological grade, lymph node metastasis, and depth of invasion. TLR2 expression in tumor cells was significantly associated with depth of invasion and lymph node metastasis. TLR4 expression in tumor cells was significantly associated with lymph node metastasis. TLR7 expression in tumor cells was significantly associated with tumor grade. TLR8 expression was found to gradually increase with worsening histopathological grade ($P < 0.005$). However, the TLR8 IF staining scores did not correlate with the depth of invasion and lymph node metastasis. We analyzed the association between the expression of TLR4 and TLR8 in tumor cells and poor prognostic indicators because a significant percentage of tumors also exhibited TLR4 expression in mononuclear inflammatory cells and TLR8 expression in fibroblast like cells.

We found that carcinoma patients with higher TLR4 expression in the stromal compartment had a significantly higher risk of disease progression. That show may TLR4 expression in mononuclear inflammatory cells were significantly associated with the depth of invasion and lymph node metastasis. These findings indicated that increased TLR protein expression may interfere with normal TLR signaling pathways and function and may represent useful markers of the malignant transformation of cancer cells. In addition, cancer cells activated by TLR signals may release cytokines and chemokines that in turn recruit and stimulate immune cells to release additional cytokines and chemokines. This process results in immune tolerance, cancer progression, and propagation of the tumor microenvironment. In this study, we observed the expression of TLR2, TLR4, TLR7 and TLR8 in tumor cells as well as their association with the clinicopathologic characteristics of OSCC. In this study, TLR2, TLR4, TLR7 and TLR8 expression appeared important to the biological pathogenesis of Tong cancer. However, different phenotypes of TLR expression in cancer cells can lead to different results and as a series of candidate prognostic factors, the function of these markers in ESCC should be further investigated.

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