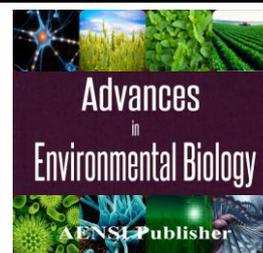




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## Activation of NF-KB Signaling Pathway Trough TLR7 Expression in Oral Squamous Cell Carcinoma

Abbas Karimi, Mohammad Bayat, Gholamreza Shirani, Hamid Mahmood Hashemi, Hossein Hosseinye Todashki, Shiva Solahaye Kahnamooui

*Oromaxillofacial Sugery Department, Tehran University of Medical Science, Tehran Iran.*

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### ABSTRACT

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. Aim of this study is determine of MAPK activation in OSCC cell lines via up regulating of TLR7. The cell line WSU-HN6, a human OSCC cell line from tongue, was obtained from the Institute Pastur of Tehran. HIOEC and HB cells were obtained from the Laboratory of Oral Oncology, Gazi Hospital of Tabriz. The expressions of TLR7 were measured by quantitative real-time polymerase chain reaction (qRT-PCR), and all samples were calibrated by b-actin. Western blot analysis revealed high expression of TLR7 and MAPK in OSCC cell lines. Results are expressed as mean  $\pm$  SD of cytokine concentrations (pg/ml) in three independent experiments (\*\*p<0.001, \*\*p<0.01, \*p<0.05, compared with the NC siRNA group). Imiquimod -induced expression of IL-6, IL-8, and VEGF was inhibited by TLR7 siRNA in HIOEC cells as determined by RT-PCR. Results are representative of three independent experiments performed with each cell line (\*p<0.05, compared with control). We found that TLR7 was expressed in human OSCC cell lines but not on normal human oral epithelial cells. The expression levels of TLR7 and MAPK in situ were correlated with tumor differentiation.

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## INTRODUCTION

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 [1, 2]. 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) and their signaling in tumor immune escape and resistance to apoptosis, for example, is among the frontiers of exploration [3, 4]. 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 [7-10]. TLRs are present in vertebrates as well as invertebrates. Recently, it was estimated that most mammalian species have 10 to 15 types of TLRs. 13 TLRs (TLR1 to TLR13) have been identified in humans and mice [8,9,11]. TLRs belong to the Toll/interleukin-1 receptor (TIR) family and all members of this family contain cytoplasmic TIR domains. 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 [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 [12]. The extracellular domains of TLR contain 16-28 LRRs which involve some physiological function [13,14]. TLRs 3, 7, 8 and 9 are almost exclusively localized to intracellular membranes where they are ideally positioned for activation by nucleic acids of bacterial and viral origin (1). TLR3 is activated in response to viral double stranded RNA (dsRNA) (1). Human TLR8 and its murine orthologue TLR7 recognize viral ssRNA as well as various synthetic imidazoquinolines, compounds with a double cyclic organic backbone, which have different affinities toward TLR7 and TLR8 (22).some researchers have reviewed TLR signaling pathways during

**Corresponding Author:** Shiva Solahaye Kahnamooui, Oromaxillofacial Sugery Department, Tehran University of Medical Science, Tehran Iran.  
E-mail: [shiva.kahnamooui@gmail.com](mailto:shiva.kahnamooui@gmail.com)

pathogen recognition; they describe in detail the induction of immune reactions via extracellular and intracellular pathways mediated by myeloid differentiation factor 88 (MAPK), nuclear factor kappa-light-chain-enhancer of activated B cells (MAPK), and mitogen-associated protein kinase (MAPK). Toll-like receptor 7 (TLR7) mediates anti-viral immunity by recognizing ssRNA viruses. Small molecular weight TLR7 agonists have been approved, or are being evaluated, for treatment of cancers or infectious diseases. Although TLR7 is predominantly expressed in a restricted set of immune cell types including plasmacytoid dendritic cells (pDCs), it is also expressed in non-native expressing cells (e.g., hepatocytes) under certain circumstances. To elucidate the molecular basis of TLR7 induction by pro-inflammatory stimulation and the subsequent cellular responses in these non-native TLR7-expressing cell types, we firstly cloned and characterized the 5'-promoter region of TLR7. The proximal region of this promoter drives the transcription of the TLR7 gene. Pro-inflammatory stimuli activated TLR7 transcription via a MAPK binding motif in this region, and this activation could be blocked by mutation of the MAPK binding site or addition of MAPK inhibitors. Aim of this study is determine of MAPK activation in OSCC cell lines via up regulating of TLR7.

## MATERIAL AND METHODS

### *Cell lines:*

The cell line WSU-HN6, a human OSCC cell line from tongue, was obtained from the Institute Pasteur of Tehran. HIOEC and HB cells were obtained from the Laboratory of Oral Oncology, Gazi Hospital of Tabriz. HIOEC is human immortalized oral epithelial cells, which had been obtained from normal oral mucosa immortalized by transfection of HPV16 E6/E7 gene. HB cell was establish from the HIOEC by induction with benzo(a)pyrene. HIOEC cells were maintained in defined keratinocyte SFM (Gibco, NY, USA) medium and other cells were maintained in DMEM supplemented with 10% heat inactivated fetal bovine serum (Gibco). All cells were cul tured in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. IMIQUIMOD was obtained from Sigma (MO, USA), and p38 specific inhibitor PD169316 was from Abcam (MA, USA).

### *Immunohistochemistry:*

Forty OSCC specimens were collected from patients who had undergone surgery between 2009 to 2012 in the Department of Oral and Maxillofacial Surgery, Tabriz Emam Reza Hospital. For immunohistochemical examination, OSCC tissues were fixed with 4% para formaldehyde and embedded with paraffin. Sections of the samples were blocked with 10% goat serum in PBS and incubated overnight at 4°C with either anti-TLR7 antibody (Imgenex, CA, USA) or with anti-MAPK antibody (Abcam) at a dilution of 1:100. After three washes with PBS, the sections were incubated with peroxidase-conjugated goat anti-mouse/rabbit antibody for 1 h, followed by incubation with 3, 3'-Diaminobenzidine (DAB) substrate for 3 min. Counter-staining was performed with hematoxylin, and then dehydration was performed with ethanol and dimethyl benzene. Slides were mounted with Permount (Santa Cruz, CA, USA) and visualized by Axio Imager (Zeiss, Oberkonchen, Germany). The IHC results in tissues were scored by two independent investigators based on the level of staining intensity as follows: none (-): 0% of stained cells; weak (+): 1-25% of stained cells; moderate (++) : 26-50% of stained cells; strong (+++): >50% of stained cells (17).

### *Quantitative real-time PCR (qRT-PCR):*

The expressions of TLR7 were measured by quantitative real-time polymerase chain reaction (qRT-PCR), and all samples were calibrated by b-actin. Briefly, total RNA was isolated from monocytes using the TRIzol (Invitrogen) isolation solution according to the manufacturer's instructions. Isolated RNA was eluted in RNase-free water and reverse-transcribed with ReverTra Ace RT-qPCR kit (TOYOBO, Osaka Boseki, Japan). The TLR7 mRNA level was quantified by qRT-PCR amplification using a 7500 fast Real-Time PCR system (Applied Biosystem, Foster, CA, USA) in a total volume of 10 µL containing 5 µL SYBER Green1 mix (Bio-Rad, Hercules, CA, USA), 0.4 µL forward and reverse primer and 0.06 µL Tag polymerase, 2.5 µL ddH<sub>2</sub>O and 2 µL cDNA templates. The recommended cycling conditions for qRT-PCR was as follows: denaturation at 94 °C for 2 min followed by 35 cycles of 94 °C 10s, 60 °C 15s, 72 °C 30 s. The specificity of the amplification products was controlled using a melting curve analysis. The copy number of objective gene or b-actin transcript in sample was calculated with the BIOSYSTEM software according to corresponding standard curves. The sequences for the primers used were as follows: TLR7 forward primer, 5'-ttgtcccgtgcaaactgcccggggagga-3', TLR7 reverse primer, 5'-aagtcctgtattactgcccgttagga-3'; b-actin forward primer, 5'-tggatcctgtggcatcatgaac-3', b-actin reverse primer, 5'-taaacgcagctcagtaaacgtccg-3'. and 5'-CTCTCCACATCCCTTCC-3' (forward) and 5'-CCGCACGTTCAAGAACAGAGA-3' (reverse) for MAPK. Each gene was amplified in triplicate.

#### *Flow cytometry:*

TLR7 expression in cells was evaluated by flow cytometry as follows: cells were collected and then labeled with the APC-labeled mouse anti-human TLR7 antibody (eBioscience, CA, USA) for 30 min at 4°C. The cells were analyzed using Cell Quest Software. Apoptosis was measured by flow cytometry as follows: cells were harvested and washed in PBS, resuspended in pre-diluted binding buffer and stained with AnnexinV-FITC (BD Biosciences, CA, USA) for 30 min at room temperature. After being washed and resuspended in PI binding buffer, the cells were immediately subjected to apoptosis analyses by flow cytometry using Cell Quest Software.

#### *Elisa:*

Cells ( $1 \times 10^6$  cells) were cultured for 4 hours in medium containing 2% FCS and then stimulated with loxoribine or IL-1 $\beta$  for the indicated time periods. Nuclear protein extraction was performed using the Nuclear Extract Kit (Active Motif) according to the manufacturer's recommendations. Briefly, cells were incubated for 15 minutes on ice in hypotonic buffer. After addition of detergents, cell lysates were centrifuged, and supernatants were collected. Protein contents in the cell lysates were quantified using the quick start Bradford protein assay kit (Biorad) to ensure that all samples contained similar amounts of protein. Next, nuclear contents were assayed to determine the relative quantity of p50 and p65 MAPK subunits contained in the nucleus using TransAm MAPK ELISA Kit (Active Motif) according to the manufacturer's recommendations. Briefly, MAPK consensus sequence containing oligonucleotides was coated in plates before adding the nuclear extract containing activated transcription factor. After the binding of MAPK to its consensus sequence, the relative quantity of MAPK subunits was determined by colorimetric reaction. MAPK gene reporter assays. MAPK gene reporter assays were carried out with the pNIFTY2-SEAP plasmid (Invivogen) following the recommendations of the manufacturer. Briefly, A549 cells ( $5 \times 10^4$  cells) were cultured in 12-well plates and transfected using Iyovec (Invivogen) and 1  $\mu$ g/ml pNIFTY2-SEAP. At 48 hours after transfection, cells were stimulated or not with loxoribine or IL-1 $\beta$  for 24 hours. Supernatants were collected and incubated with pNpp. MAPK activity was analyzed by measuring the hydrolysis of pNpp in the supernatants with a spectrophotometer at 405 nm.

#### *Immunofluorescence microscope:*

Tumor cells deposited on glass slides were washed twice with PBS and fixed in 4% paraformaldehyde in PBS for 20 min. The cells were further permeabilized with 0.1% TritonX in PBS for 8 min, washed and blocked with 5% bovine serum albumin in PBS for 30 min, then treated with monoclonal mouse anti-p38 (Santa Cruz) antibody overnight. FITC-labeled (1:100) anti-mouse IgG served as the secondary antibody. Sections were then mounted in a medium containing Hoechst33342 for 5 min to visualize cell nuclei. Slides were evaluated with a laser scanning confocal microscope TCS SP2 (Leica, Wetzlar, Germany), and Adobe Photoshop 7.0 was used for the digital image analysis.

#### *Luciferase reporter gene assay used to indicate MAPK:*

Activity cells were cotransfected with the mixture of 200 ng p38-Luc and 10 ng pRenilla using the Lipofectamine<sup>TM</sup> 2000 Reagent (Invitrogen) according to the manufacturer's instructions. 24 h after transfection, the cells were either left untreated or stimulated with 1  $\mu$ g/ml of IMIQUIMOD. Cell lysates were assayed for expression of luciferase using a dual luciferase assay kit (Promega). Chemiluminescence, representing the expression of luciferase, was measured in a Junior LB9505 luminometer (Berthold, Wildbad, Germany). All transfection experiments were performed in two wells and repeated independently three times. The activity of controls was set at 1.0.

#### *Western blotting analysis:*

OSCC cells were lysated in lysis buffer [50 mM Tris, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10 mM NaF, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 2 mM Na<sub>4</sub>P<sub>2</sub>O<sub>7</sub>, 1 mM phenylmethanesulfonyl fluoride (PMSF) and 'complete' protease inhibitor cocktail tablets (Roche, Basel, Switzerland)]. Cell debris was removed by centrifugation, and extracts were quantified by protein assay (Thermo Scientific NanoDrop 1000 UV-vis Spectrophotometer) and then boiled in SDS gel-loading buffer containing 10%  $\beta$ -mercaptoethanol. Proteins were separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyscreen polyvinylidene fluoride (PVDF) membranes (Perkin-Elmer, Boston, MA, USA). The membranes were blocked with 5% (w/v) non-fat dry milk 1% (v/v) Tween 20 in PBS for 1 h at room temperature and incubated overnight with commercially available anti-MAPK antibody (1:1000) (Abcam) at 4

C. Detection was performed with electrochemiluminescence (ECL), and the blots were quantified by densitometry using the image analysis program (Amersham Biosciences, San Francisco, CA, USA)

#### *Effects of TLR7-specific siRNA:*

Expression of TLR7 in the cell lines was temporarily silenced using small interfering RNA (siRNA) of TLR7. The sense and antisense strands of siRNA were: 5-GUCUAGUGGCUAAUCCUA-3 and 5-

UAGGAAUUAGCCACUAGAC-3. Briefly,  $2 \times 10^5$  tumor cells were seeded in wells of a six-well plate and cultured in DMEM. The next day, the cells were transfected with human TLR7 siRNA (GenePharma Co., Shanghai, China) at different concentrations according to the manufacturer's instructions. The negative control consisted of siRNA with no homology to known sequences from humans. Cells were incubated in complete DMEM medium at 37°C in an atmosphere of 5% CO<sub>2</sub>. Western blot was used to test TLR7 expression and further experiments were carried out.

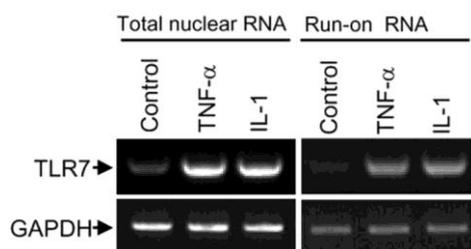
#### Statistical analysis:

All statistical analyses were performed using SPSS 17.0 statistical analysis software. Data were expressed as the mean  $\pm$  SD in text and figures. Comparisons between paired and unpaired groups were performed using the t-test or one-way ANOVA with Bon-ferroni correction  $< 0.05$  considered to be statistically significant.

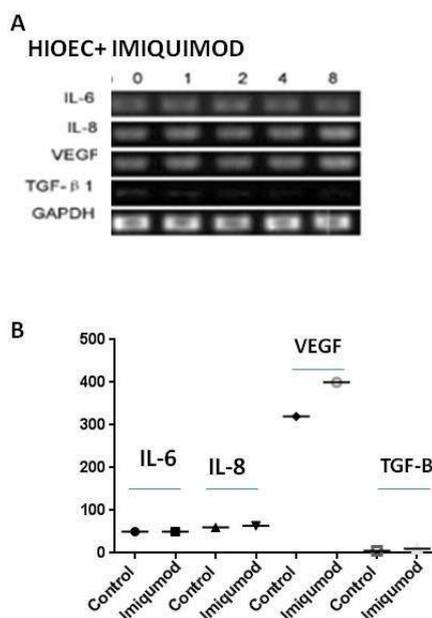
## RESULTS AND DISCUSSIONS

#### Expression of TLR7 in human OSCC tissues and cell lines:

Immunohistochemical examination of the expression of TLR7 and MAPK in OSCC tissues and adjacent non-malignant epithelia cells were shown in figure 1-A. This figure discussed about adjacent non-malignant epithelia and OSCC tissue (magnification  $\times 400$ ). Figure 1- B-D show the TLR7 and MAPK in HIOEC and OSCC cell lines were analyzed by RT-PCR also we used Western blot and flow cytometry. Green line-covered regions represent isotype controls and red regions represent the detection of TLR7 with mAb. Western blot analysis also revealed high expression of TLR7 and MAPK in OSCC cell lines (Figure 1C). Consistent with RT-PCR and Western blot results, FACS analysis demonstrated that TLR7 expression in OSCC cell lines was quite high, while the expression of TLR7 in HIOEC was very low (Figure 1D). Thus, the different expression levels of TLR7 between HIOEC and OSCC cell lines suggest that TLR7 may be functionally important in human OSCC cells.



**Fig. 1:** TLR7 in human OSCC tissues and cell lines.



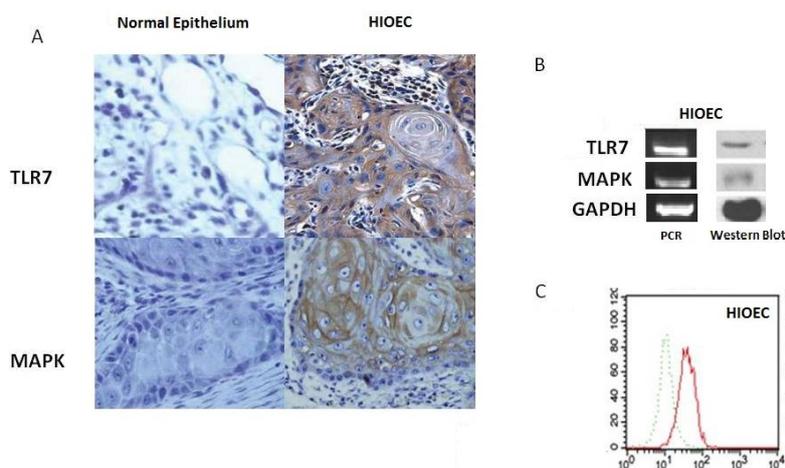
**Fig. 2:** IL-6, IL-8 and VEGF through TLR7.

*IMIQUIMOD* increased the expression and secretion of IL-6, IL-8 and VEGF through TLR7 in a human OSCC cell line instead of HIOEC:

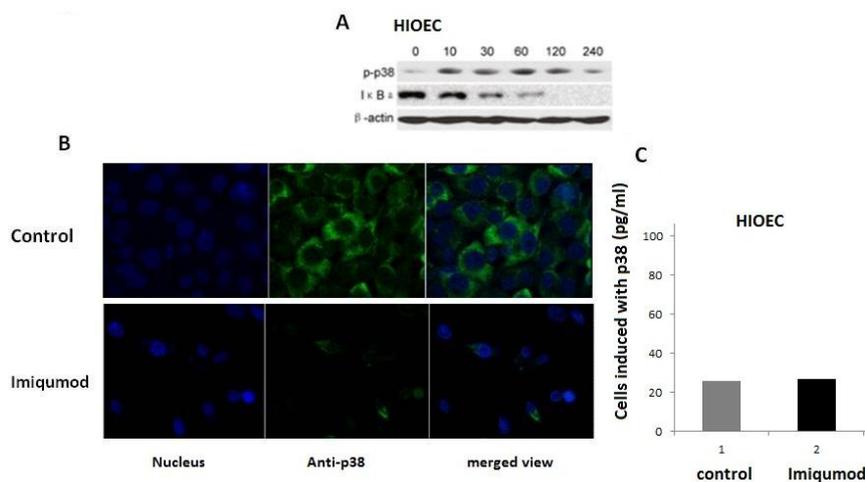
HIOEC cells ( $5 \times 10^5 \text{ml}^{-1}$ ) were stimulated with Imiquimod ( $1 \mu\text{g/ml}$ ) for 24 h, and then cytokines and chemokines in the supernatants were assayed using sandwich ELISA. Protein levels (pg/ml) are expressed as mean  $\pm$  SD in three independent experiments (\*\* $p < 0.001$ , \* $p < 0.01$ ,  $p < 0.05$ , compared with control). RT-PCR analysis of IL-6, IL-8, and VEGF and TGF- $\beta$  mRNA expression in HIOEC cells stimulated with Imiquimod ( $1 \mu\text{g/ml}$ ) for indicated time points (Fig 2-B).

*Transcriptional regulation of TLR7 expression:*

Cells were treated with TNF- $\alpha$  or IL-1 for 4 h. Induction of TLR7 expressions in these cells were analyzed by RT-PCR. Nuclear run-on reaction was performed to determine the transcription rate of TLR7 following TNF- $\alpha$  or IL-1 stimulation. TLR7 transcripts in the total nuclear RNA pool, and the run-on RNA pool were determined by RT-PCR. In these PCR reactions, amplification of GAPDH was performed to control for equivalent amounts of cDNA used as template (fig 3).



**Fig. 3:** Transcriptional regulation of TLR7.



**Fig. 4:** P38 MAPK pathways through TLR7.

*IMIQUIMOD* induced activation of p38 MAPK pathways through TLR7 in OSCC cell lines:

phosphorylation and I- $\kappa$ Ba degradation for indicated time points in OSCC cell lines. B. HIOEC and OSCC cells plated overnight were stimulated with Imiquimod ( $1 \mu\text{g/ml}$ ) for 8 h. 200 cells were randomly counted. Results are representative of three independent experiments performed with each cell line ( $p < 0.05$ , compared with control). Figure 4 showed that the Mean fluorescence intensity (MFI) of nuclear p65 expression was determined in OSCC cell lines. Results are expressed as mean  $\pm$  SD of MFI for three independent experiments performed with each cell line ( $p < 0.05$ , compared with control). Determination of the Imiquimod -activated, MAPK-dependent transcriptional activity in HIOEC cells. The cells were transiently cotransfected with p38-Luc

and pRenilla. Then after 24 h, cells were either left untreated or stimulated with 1 µg/ml of Imiquimod for various times. Luciferase activity was assessed in the cells (\* $p < 0.05$ , compared with control). TLR7 was effectively silenced as determined by Western blot and TLR7 siRNA suppressed the activation of p38 MAPK and MAPK pathways in OSCC cell lines treated with IMIQUIMOD. MAPK-dependent transcriptional activity was inhibited by TLR7 siRNA in HIOEC cells. HIOEC cells were transiently transfected with NC siRNA and TLR7 siRNA, then cotransfected with p38-Luc and pRenilla. 24 h later, the cells were either left untreated or stimulated with 1 µg/ml of IMIQUIMOD for various time. Luciferase activity was assessed in the cells stimulated with or without IMIQUIMOD. (\* $p < 0.05$ , compared with NC siRNA) (Figure-4)

#### Discussion:

We found that TLR7 was expressed in human OSCC cell lines but not on normal human oral epithelial cells. The expression levels of TLR7 and MAPK in situ were correlated with tumor differentiation. TLR7 and MAPK were highly expressed in well-differentiated and moderately-differentiated tumors, but weakly expressed in poorly differentiated tumors. Cisplatin is widely used for chemotherapy of many malignancies, especially for OSCC. However, the efficiency of cisplatin in the treatment of recurrent tumors is limited because of acquired or intrinsic resistance. In our study, ligation of IMIQUIMOD to TLR7 can protect cisplatin-induced apoptosis in OSCC cell lines but not in HIOEC cells. IMIQUIMOD activated NF-κB and p38 MAPK pathways and triggered target gene transcription. IL-6, IL-8 and VEGF were found to be elevated in OSCC cell lines upon IMIQUIMOD stimulation. Therefore, OSCC cell lines could respond to oral bacteria via the derived IMIQUIMOD, which may lead to apoptotic resistance to cisplatin. It is well known that IL-6 production and increased circulating level have been emerged as biomarkers of poor prognosis in many human cancers. In ovarian cancer, increased levels of IL-6 in patients' sera are linked to tumor progression, resistance to apoptosis and chemoresistance [14]. IL-6 is also able to promote tumor angiogenesis and invasion [15-17]. In clinical investigations, high IL-6 levels in the sera of patients with colon carcinoma correlate with tumor size [18]. The effect of IL-6 on hepatocyte proliferation depends on the balance between its pro- and anti-proliferative arms, after the integration of the effects of other transcription factors acting on the same genes as IL-6 [19]. IL-8 is a proangiogenic cytokine/chemokine and anti-apoptotic molecule that can promote tumor metastasis and death resistance. IL-8 and VEGF are involved in the malignant transformation process. It has been reported that IL-8 is over expressed in ovarian cancer and its level is associated with decreased patient survival and poor clinical outcome [20, 21]. VEGF, in addition to inducing angiogenesis, is also an immunosuppressive cytokine that promotes ascite formation through stimulation of vascular permeability [20, 22]. The expression of VEGF is negatively correlated with DC numbers in the tumor tissue and peripheral blood of cancer patients [23, 24]. Also, VEGF has an inhibitory effect on DC differentiation in patients with non-small-cell lung cancer [25]. Thus, our results show that the elevated production of IL-6, IL-8 and VEGF in OSCC cell lines by IMIQUIMOD stimulation may lead to the development of resistance to cisplatin in human OSCC. It is well established that MAPK is an anti-apoptotic transcriptional factor upon tumor cell stimulation with IMIQUIMOD, radiation, and some chemotherapeutic agents. MAPK has also been previously linked to upregulation of anti-apoptotic protein expression and an increase in cell proliferation. Due to the anti-apoptotic properties of activated NF-κB, its high expression levels in tumor cells are associated with tumor progression and induction of chronic inflammation in the tumor microenvironment. Activation of MAPK has been shown to induce resistance through the expression of the MDR1 gene. It was also reported that IMIQUIMOD could induce MAPK activation in colon cancer cells and pancreatic cancer cells. Silencing of TLR7 expression in SKOV3 cells resulted in sensitization of SKOV3 cells to PTX-induced apoptosis, and this sensitization was IMIQUIMOD-triggered TLR7 signaling usually activates two different pathways: the NF-κB signal pathway and the p38 MAPK signal pathway. In the cancer cells, researcher demonstrated that activation of MAPK induced by IMIQUIMOD was responsible for the production of cytokines such as IL-6, IL-8 and VEGF in SKOV3 cells [11]. Triggering of TLR7 by IMIQUIMOD induced tumor promotion by the induction of proliferation, activation of NF-κB, p65 binding to DNA, and resistance to NK cell-mediated cytotoxicity accompanied by the increased production of proinflammatory cytokines (IL-6 and IL-8), VEGF [10]. The p38 MAPK pathway has also been shown to be involved in IMIQUIMOD-induced IL-6 secretion in pituitary adenomas and bladder cancer cells. However, up to now, the mechanisms by which TLR7 activation with IMIQUIMOD induces resistance to chemotherapy have not been fully understood. One report showed that inhibition of the MAPK pathway can significantly attenuate IMIQUIMOD-induced apoptosis resistance [9]. In our present study, we found that TLR7 ligation can activate both p38 MAPK and MAPK pathways in human OSCC cell lines, and inhibition of TLR7 by siRNA can significantly attenuate IMIQUIMOD-induced NF-κB and p38 MAPK activation. We also found that the secretion of cytokines was significantly abrogated by p38 MAPK specific inhibitor, which proved that p38 MAPK was responsible for IMIQUIMOD induced production of IL-6, IL-8 and VEGF in OSCC cells. IMIQUIMOD pretreatment can decrease cisplatin-induced cell death and apoptosis through TLR7 signaling pathway. On the contrary, HIOEC cells, which have very low expression levels of TLR7 and MAPK, did not show these effects. These findings strongly suggest that IMIQUIMOD can provide a survival benefit to OSCC

cell lines and alter their sensitivity to cisplatin through activation of p38 MAPK and MAPK signaling pathway via TLR7. Present study conclusions indicated that TLR7 was functionally expressed in human OSCC cells and development of resistance to cisplatin in human OSCC might occur through the mechanism involving activation of TLR7 and its signaling pathway. Suppression of TLR7 and its signaling pathway might thus elevate sensitivity to cisplatin and potentially help improve the prognosis of patients with OSCC.

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