Original Article

Activation Toll-Like Receptor7 (TLR7) Responsiveness Associated with Mitogen-Activated Protein Kinase (MAPK) Activation in HIOEC Cell Line of Oral Squamous Cell Carcinoma

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KEY WORDS

Toll-Like Receptor 7;
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ABSTRACT

Statement of the Problem: Oral squamous cell carcinoma is the most common oral malignancy. Toll-like receptor (TLR) activation led to alterations in the levels of mRNA encoding the TLR accountable for recognizing the inducing agonist and cross-regulation of other TLR.

Purpose: The purpose of this study is determination of mitogen-associated protein kinase (MAPK) activation in human immortalized oral epithelial cell (HIOEC) line via up regulating of TLR7.

Materials and Method: expression of TLR7 was measured in HIOEC and normal cells by quantitative real-time polymerase chain reaction (qRT-PCR) and samples were calibrated by β-actin.

Results: Western blot analysis discovered high expression of TLR7 and MAPK in HIOEC cell lines. TLR7 was over-expressed in HIOEC cell line. Imiquimod-induced expression of interleukin (IL)-6, IL-8, and vascular endothelial growth factor (VEGF) was inhibited by TLR7 siRNA in HIOEC cells as determined by reverse transcription polymerase chain reaction (RT-PCR). Mean fluorescence intensity of nuclear p38 expression was determined in HIOEC cell lines ($p< 0.05$). RT-PCR analysis of IL-6, IL-8, and VEGF mRNA expression in HIOEC cells stimulated with imiquimod (1 μg/ml) for indicated time points.

Conclusion: TLR7 is functionally over-expressed in HIOEC cell line of oral squamous cell carcinoma and development of resistance to cisplatin in human oral squamous cell carcinoma might occur through the mechanism involving activation of TLR7 and its dependent signaling pathway.

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Introduction

The prognosis of human oral squamous cell carcinoma (OSCC) is usually poor with a 5-year survival rate. [1-2] Recently, there has been an increasing interest in anti-

tumor functions introduced by the innate immune response. The role of toll-like receptors (TLRs) and their signaling in tumor immune escape and resistance to apoptosis is among the frontiers of exploration. [3-6] Toll-like receptors (TLRs) were primarily discovered in drosophila, in the membranes of binding pattern recognition receptors (PRRs) and were recognized as targeting a series of mechanisms causing synthesis and secretion of cytokines and activating other host defense programs, critical for initiation of innate or adaptive immunity. [7-10] Recently, it is reported that most mammalian species have 10 to 15 types of TLRs. Thirteen TLRs (TLR1 to TLR13) have been recognized in human and mice. [8-11] The extracellular domains of TLR comprise 16-28 LRRs that involve some physiologic functions. [12-14] TLRs 3, 7, 8 and 9 are entirely localized to intracellular membranes where they are preferably positioned for activation by nucleic acids of bacterial and viral origin. [8, 10] Some researchers have been reviewed TLR signaling pathways during pathogen recognition. They have described the induction of immune reactions by means of extracellular and intracellular pathways mediated by myeloid differentiation factor 88 (MyD88), nuclear factor kappa-light–chain-enhancer of activated B cells, and mitogen-associated protein kinase (MAPK). [15-16] The MyD88-independent pathway is mediated by TIR-domain-containing adapter-inducing interferon-β (TRIF). TLR3 and TLR4 use TRIF to activate interferon-regulated factor 3 (IRF3). Activation of IRF3 results in production of INF-β. [17-18] Finally, the activation of TRIF-related adaptor molecule (TRAM) is TLR4-induced and results in TRIF employment. TRAM localized to late endosomes where TAG prevents IRF3 activation and inflammatory cytokine production is prompted by TLR2, TLR7 and TLR9 ligands. Phosphorylation and nuclear translocation of IRF3 can present TBK1 or IKKi/IKK. TLR7 mediates anti-viral immunity by identifying ssRNA viruses. [11] Small molecular weight TLR7 agonists have already been accepted, or are still being assessed, for treatment of cancers or infectious diseases. [12] The proximal region of this promotor drives the transcription of the TLR7 gene. Pro-inflammatory stimuli starts TLR7 transcription by means of a MAPK binding in this region, and this instigation might be impeded by mutation of the MAPK binding site or addition of MAPK inhibitors. The purpose of this study was to determine MAPK activation dependent TLR7 in HIOEC cell lines of OSCC.

Materials and Method

Cell lines

The HIOEC cell line was obtained from the Creative Bioarray Company. HIOEC is human immortalized oral epithelial cells, attained from normal oral mucosa immortalized by transfection of HPV16 E6/E7 gene. HIOEC cells were sustained in defined keratinocyte serum-free medium (Gibco, NY, USA) and normal cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (Gibco; NY, USA). Moreover, the normal oral epithelial cells were obtained from patients who undergone third molar surgeries referred to private clinic. Written informed consents were provided from all participants. All cells were cultured in a humidified atmosphere of 5% CO2 at 37°C. Imiquimod was obtained from Sigma (MO, USA), and p38 specific inhibitor PD169316 was purchased from Abcam (MA, USA).

Immunohistochemistry

For immunohistochemical (IHC) examination, HIOEC cell lines of OSCC and normal oral epithelium cells were fixed with 4% para formaldehyde and embedded with paraffin. The sample sections were blocked with 10% goat serum in PBS and incubated overnight at 4°C with either anti-TLR7 antibody (Imgenex, CA, USA) at a dilution of 1:100. After three washes with phosphate buffered saline (PBS), the sample sections were then incubated with peroxidase-conjugated goat anti-mouse/rabbit antibody for 1 h, followed by incubation with 3, 3'-Diaminobenzidine substrate for three minutes. Counter-staining was performed with hematoxylin, and then dehydration was performed with ethanol and dimethyl benzene. Slides were mounted with per mount (Santa Cruz; CA, USA) and visualized by Axio Imager (Zeiss; Oberkochen, Germany). The IHC results in tissues were recorded by two independent examiners regarding the level of staining intensity scored as none (−) for 0%of stained cells; weak (+) for 1-25% of stained cells; moderate (++) for 26-50% of stained cells; and finally strong (+++) for more than 50% of stained cells. [17]

Quantitative real-time polymerase chain reaction (qRT-PCR)

The expressions of TLR7 were assessed by qRT-PCR,

and all samples were attuned by β-actin. Isolated RNAs from the HIOEC cell lines were eluted in RNase-free water and reverse-transcribed with ReverTra Ace qRT-PCR kit (TOYOBO; Osaka Boseki, Japan). The TLR7 mRNA level was counted by qRT-PCR amplification using a 7500 fast Real-Time PCR system (Applied Bio system; Foster, CA, USA) in a total volume of 10 µL containing 5µL SYBER Green1 mix (Bio-Rad, Hercules, CA, USA), 0.4µL forward and reverse primer and 0.06 µL Tag polymerase, 2.5µL ddH20 and 2µL cDNA templates. The recommended cycling conditions for qRT-PCR were denaturation at 94^{o}C for 2 minutes followed by 35 cycles of 94^{o}C for 10s, 60^{o}C for 15s and 72^{o}C for 30s. The specificity of the amplification products was controlled by employing a melting curve analysis. The copy number of objective gene or β-actin transcript in sample was calculated with the Biosystem software based on the corresponding standard curves. The sequences for the employed primers were in the following manner: TLR7 forward primer, 5¢-ttgtcccgtgcaaacttgccggggag-ga-3¢, TLR7 reverse primer, 5¢aagtcccgttattacttgccgg-ttagga-3¢; b-actin forward primer, 5¢-tggaatcctgtggca-tccatgaaac-3¢, βactin reverse primer, 5¢-taaaacgcagct-cagtaacagtccg-3¢. and 5'-CTCCTCCACATCCCTTCC-3' (forward) and 5'-CCGCACGTTCAAGAACAGAGA-3' (reverse) for MAPK. Each gene was amplified in triplicate.

Enzyme-linked immunosorbent assay (ELISA)

HIOEC cell lines of OSCC and normal oral epithelium cells (1×106 cells) were cultured for 4 hours in a medium containing 2% fetal calf serum (FCS) and then stimulated with loxoribine or IL1-β for the designated times. Nuclear protein extraction was done by using the Nuclear Extract Kit (Active Motif) considering the manufacturer's suggestions. Cells were incubated for 15 minutes on ice in hypotonic buffer. After adding detergents, cell lysates were centrifuged, and supernatants were collected. Protein contents in the cell lysates were measured using the quick start Bradford protein assay kit (Biorad) to ensure that all samples included comparable amounts of protein. Subsequently, nuclear contents were evaluated to define the comparative quantity of p50 and p65 MAPK subunits contained in the nucleus using TransAm MAPK ELISA Kit (Active Motif) considering the manufacturer's instructions. Briefly, MAPK consensus sequence comprising 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 verified by colorimetric reaction.

Flow cytometry

TLR7 expression in cells was evaluated by flow cytometry as it follows. HIOEC cell lines of OSCC and normal oral epithelium 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 it follows. Cells were harvested and washed in PBS, resuspended in prediluted 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 instantly analyzed for apoptosis by flow cytometry using Cell Quest Software.

Immunofluorescence microscope

HIOEC cell lines of OSCC and normal oral epithelium 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 Hoechst for 5 min to visualize cell nuclei. Slides were assessed with a laser scanning confocal microscope TCS SP2 (Leica; Wetzlar, Germany), and Adobe Photoshop 7.0 was utilized for the digital image analysis. Luciferase reporter gene assay used to indicate MAPK activity cells were co-transfected with the mixture of 200ng p38-Luc and 10 ng pRenilla using the LipofectamineTM 2000 Reagent (Invitrogen) regarding the manufacturer's recommendations.

One day (24 h) after transfection, the cells were left either untreated or stimulated with one µg/ml of imiquimod. Cell lysates were assayed for expression of luciferase by utilizing a dual luciferase assay kit (Promega). Chemiluminescence, representing the expression of luciferase, was measured in a Junior LB9505 luminometer (Berthold; Wildbad, Germany). All transfection

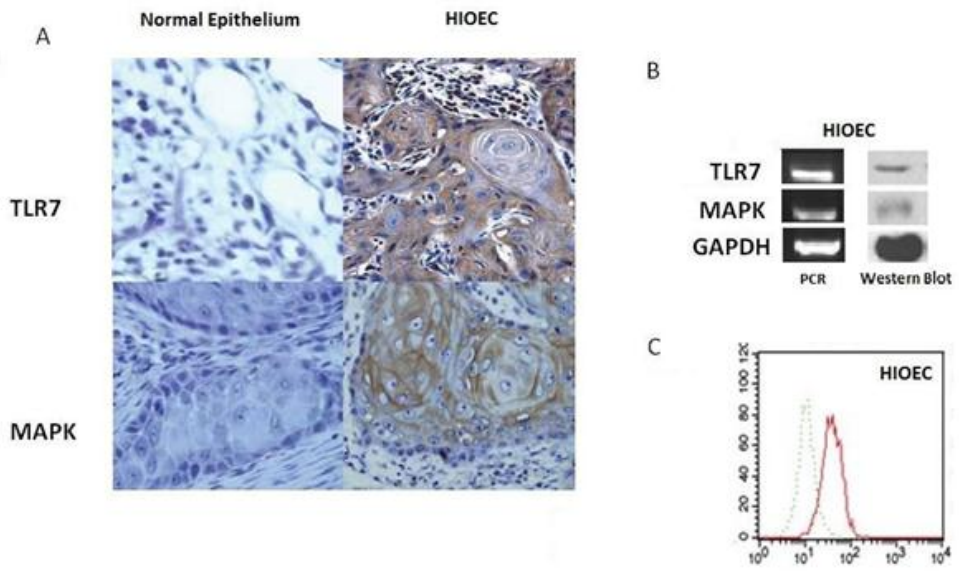

Figure 1a: IHC examination of the expression of TLR7 and MAPK in HIOEC cell lines of OSCC and normal oral epithelium cells. The blue cells show TLR7 activation in the cells (Magnification× 400). **b:** TLR7 and MAPK expression in HIOEC analyzed by RT-PCR and Western blot. Western blot analysis revealed high expression of TLR7 and MAPK in HIOEC cell lines of OSCC. **c:** Flow cytometry. Green line-covered regions represent isotype controls and red regions represent the detection of TLR7 with mAb.

experiments were done in two wells and repeated independently three times. The activity of controls was set at 1.0.

Western blotting analysis

HIOEC cell lines of OSCC and normal oral epithelium cells were lysated in lysis buffer [50mM Tris, 150mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 10mM NaF, 1mM Na3VO4,2mM Na4P2O7, 1mM phenylmethanesulfonyl fluoride (PMSF) and 'complete' protease inhibitor cocktail tablets (Roche, Basel, Switzerland)]. Cell debris was removed by centrifugation, and extracts were measured by protein assay (Thermo Scientific NanoDrop 1000 UV–vis Spectrophotometer) and then boiled in SDS gel-loading buffer comprising 10% β-Mercaptoethanol. Proteins were then separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyscreen polyvinylidene fluoride (PVDF) membranes (Perkin-Elme; Boston, MA, USA). The membranes were blocked with 5% (w⁄v) non-fat dry milk 1% (v⁄v) Tween 20 in PBS for 1 hour at room temperature. Subsequently, they were incubated overnight with commercially available anti-MAPK antibody (1:1000) (Abcam) at 4^{O}C. Electrochemiluminescence (ECL) was used for detection process, and the blots were computed by densitometry, utilizing the image analysis program (Amercontrol Biosciences; San Francisco, CA, USA)

Statistical analysis

All statistical analyses were performed using SPSS version 17.0. Paired and unpaired groups were compared by using the t-test or one-way ANOVA with Bonferroni correction. *p* Values< 0.05 were considered statistically significant.

Results

Expression of TLR7 in human OSCC tissues and cell lines

IHC examination of the expression of TLR7 and MAPK in HIOEC cell lines of OSCC and normal oral epithelium cells are illustrated in Figure 1a. Figure 1b and c show TLR7 and MAPK expression in HIOEC analyzed by reverse transcription polymerase chain reaction (RT-PCR), 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 HIOEC cell lines of OSCC (Figure 1b). Consistent with RT-PCR and Western blot results, fluorescence-activated cell sorting (FACS) analysis demonstrated that TLR7 expression in HIOEC cell lines of OSCC was high (Figure 1c). Thus, the over-expression levels of TLR7 in HIOEC cell lines of OSCC suggest that TLR7 might be functionally important in human OSCC cells. Imiquimod increased the expression and secretion of IL-6, IL-8, and VEGF through TLR7 in a h-

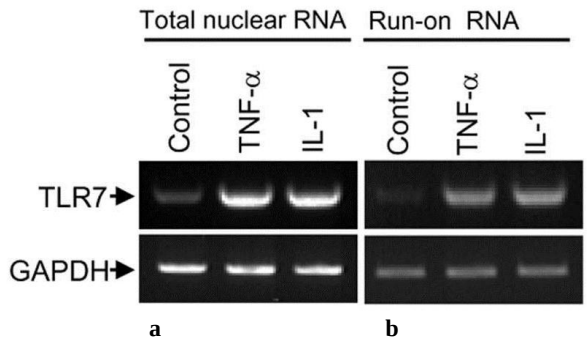

Figure 2a: RT-PCR analysis of IL-6, IL-8, and VEGF and TGF-β mRNA expression in HIOEC cells stimulated with imiquimod (1 μg/ml) for indicated time points, **b:** The levels of each cytokine in ELISA, protein levels (pg/ml) are expressed as mean ± SD (p <0.05).

uman OSCC cell line instead of HIOEC. HIOEC cell lines of OSCC and normal oral epithelium cells (5×105ml) were stimulated with imiquimod (1 μg/ml) for 24 h, and then cytokines and chemokines in the supernatants were assayed using sandwich ELISA. RT-PCR analysis of IL-6, IL-8, and VEGF and TGF-β mRNA expression were done in HIOEC cells stimulated with imiquimod (one μg/ml) for indicated time points (Figure 2).

Transcriptional regulation of TLR7 expression

HIOEC cell lines of OSCC and normal oral epithelium cells were treated with TNF- α or IL-1 for four hours. Expressions of TLR7 in these cell lines were analyzed by RT-PCR. Nuclear run-on reaction was performed to determine the transcription rate of TLR7 following TNF-α or IL-1 stimulation. TLR7 transcripts in the total nuclear RNA pool, and the run-on RNA pool were verified by RT-PCR. In these PCR reactions, amplification of GAPDH was performed to control for equivalent amounts of cDNA used as template (Figure 3). Imiquimod induced activation of p38 MAPK pathways through TLR7 in OSCC cell lines. HIOEC cell lines of OSCC and normal oral epithelium cells plated overnight were stimulated with imiquimod (1 μg/ml) for 8 hour. Figure 4-B showed that the mean fluorescence intensity (MFI) of nuclear p38 expression was determined in HIOEC cell lines. Imiquimod activated MAPK-dependent transcriptional activity was determined in HIOEC cells. The cells were transiently co-transfected with p38-Luc .Then after 24 hours, cells were either left untreated or stimulated with 1 μg/ml of imiquimod for various times. Luciferase activity was assessed in the

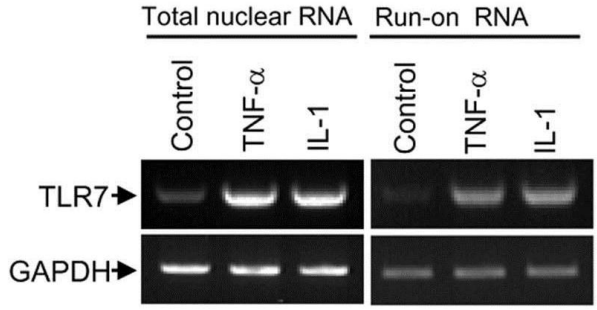

Figure 3: Nuclear run-on reaction to determine the transcription rate of TLR7 following TNF-α or IL-1 stimulation. Amplification of GAPDH to control equivalent amounts of cDNA used as template.

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 HIOEC cell lines treated with imiquimod. Luciferase activity was assessed in the cells with or without stimulation imiquimod (p< 0.05, compared with NC siRNA) (Figure 4c).

Discussion

MAPKs operate as transducers of extracellular signaling through tyrosine kinase–growth factor receptors and G-protein–linked receptors to elements regulating transcription. [14] Regulation of MAPK is consigned to a cascade of protein kinases, culminating in dual specificity kinases that phosphorylate MAPK. [14-15] An essential signaling mechanism of inflammation utilizes the TLR family, an extremely preserved family of transmembrane proteins that identifies a broad spectrum of microbial agents in addition to endogenous macromolecules released by injured tissue. [16-17] Ligand activation of TLRs is the vanguard of inflammatory response since it recruits key signaling pathways in the regulation of innate and adaptive immunity, as well as tissue repair and regeneration, and is tightly regulated under normal conditions. [18] However, when these inflammatory processes go away, the dysfunction of TLR pathways cause emergence of chronic inflammatory diseases, and therefore, presents potential therapeutic targets in pathologic conditions such as septic shock, stroke, diabetes, and cancer. [14-15, 18] In cancer, the TLRs mediate both pro- and anti-tumorigenic pathways, and hence; participate in forming the tumor microenvironment. [19] Activated TLR signals on tumor cells may endorse cancer progression, anti-apoptotic activity

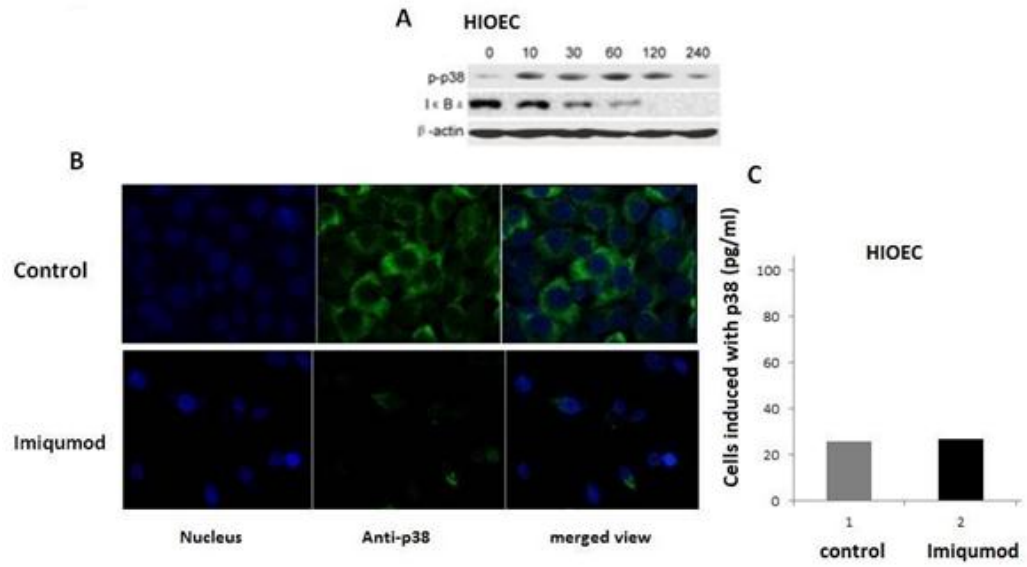

Figure 4a: TLR7 was effectively silenced as determined by Western blot and TLR7 siRNA suppressed the activation of p38 MAPK and MAPK pathways in HIOEC cell lines treated with imiquimod. **b:** Mean fluorescence intensity (MFI) of nuclear p38 expression in HI-OEC cell lines. **c:** Luciferase activity in the cells with or without stimulation of imiquimod.

and host immune resistance. [19] Accordingly, TLR expression may deliver reliable tumor biomarkers and their selected targeting may be therapeutically beneficial. The current study found that TLR7 was over-expressed in human HIOEC cell lines. Imiquimod activated NF-κB and p38 MAPK pathways and triggered target gene transcription.

IL-6 may promote tumor angiogenesis and invasion. [20] Some studies reported that the high IL-6 levels in the sera of patients with colon carcinoma correlate with tumor size. [15] The influence of IL-6 on proliferation of tumoral cells requires the balance between its pro- and anti-proliferative arms following the combination of the effects of other transcription factors influencing IL-6 genes. [16] IL-8 is a proangiogenic cytokine/chemokine and anti-apoptotic molecule that can stimulate tumor metastasis and death resistance. [18] IL-8 and VEGF are implicated in the course of malignant transformation. VEGF not only induces angiogenesis, but also acts as an immunosuppressive cytokine, which promotes ascite formation through stimulation of vascular permeability. [21-23] The results of the present study showed that the elevated production of IL-6, IL-8 and VEGF in HIOEC cell lines by imiquimod stimulation might lead to the development of resistance to cisplatin in human HIOEC cells. It is well established that MAPK is an anti-apoptotic transcriptional factor on tumoral cell stimulation with imiquimod (Imidazoquinolines are synthetic toll-like receptor 7 and 8 agonists). [24-25] MAPK has been proposed to be responsible in up-regulation of anti-apoptotic protein expression and an increase in cell proliferation. [22-23] Concerning the anti-apoptotic properties of activated NF-κB, its high expression levels in tumoral cells is allied to tumor progression and inducing chronic inflammation in the tumoral microenvironment. [23-24] Activation of MAPK has been shown to induce resistance by means of the expression of the MDR1 gene. [22-25] It was also reported that imiquimod could induce MAPK activation in colon cancer cells and pancreatic cancer cells. 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) and VEGF. [24] 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 completely recognized. The present study showed that TLR7 ligation could activate both p38 MAPK and MAPK pathways in human HIOEC cell lines. Moreover, the current study showed that the secretion of cytokines was significantly revoked by p38 MAPK specific inhibitor. This would possibly

prove that p38 MAPK could be responsible for imiquimod-induced production of IL-6, IL-8 and VEGF in HIOEC cells. Imiquimod pretreatment can decrease cisplatin-induced cell death and apoptosis through TLR7 signaling pathway. [25] These findings strongly suggest that imiquimod might provide a survival benefit to HIOEC cell lines and alter their sensitivity to cisplatin through activation of p38 MAPK and MAPK signaling pathway via TLR7 activity.

Conclusion

The current study indicated that TLR7 was functionally over-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. Thus, suppression of TLR7 and its signaling pathway might elevate sensitivity to cisplatin and potentially improve the prognosis of patients with OSCC.

Conflict of Interest

The authors declare that they have no conflict of interest.

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