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Toll-like receptor 2 activation implicated in oral squamous cell carcinoma development

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ABSTRACT

Recent studies have revealed that Toll-like receptors (TLRs) are highly expressed and activated in many types of cancer. Physiologically, TLR2 recognizes bacteria and other microorganisms in the oral cavity; however, the role of TLR2 in oral squamous cell carcinoma (OSCC) is unclear. In this study, we demonstrated that TLR2 is highly expressed in OSCC in comparison with adjacent non-malignant tissue. TLR2 was also expressed in OSCC-derived cell lines, and its expression was activated by ligands derived from bacteria and mycoplasma. Furthermore, to elucidate the mechanism of OSCC progression via TLR2 signal transduction, we focused on microRNAs (miRNAs) that are induced by TLR2 activation. Interestingly, ligand activation of TLR2 induced the expression of miR-146a and we found that downregulation of caspase recruitment domain–containing protein 10 (*CARD10*) mRNA in OSCC-derived cell lines. Moreover, knockdown of *CARD10* induced resistance to cisplatin-induced apoptosis in OSCC cells. These findings suggest that the activation of TLR2 by bacterial components can enhance the progression of OSCC and may be implicated in acquired resistance to cisplatin-induced apoptosis through regulation of the miR-146a pathway.

1. Introduction

The oral cavity is constantly exposed to numerous and varied microbial species. It has been reported that chronic periodontitis is an independent risk factor for head and neck cancer [1], and oral cancer surface biofilms contain significantly increased levels of aerobes and anaerobes at the tumor sites [2]. Furthermore, Binder Gallimidi et al. reported that chronic infection of periodontal pathogens *Porphyromonas gingivalis* and *Fusobacterium nucleatum* promote OSCC progression via direct interaction with cancerous oral epithelial cells, through the activation of epithelial TLRs [3]. We therefore hypothesized that bacteria contribute to the progression of OSCC, and focused on TLRs, which specifically recognize the presence of invading microorganisms in the host.

TLRs are pattern recognition receptors that play important roles in innate immune responses, as well as the subsequent induction of adaptive immune responses [4]. TLR family induces well-characterized signalling cascades that result in activation of the downstream transcription factor NF-κB [5,6]. Recently, several TLRs were shown to be expressed not only on immune cells but also on a variety of cancer cells [7], and many studies reported that TLRs may also play an important role in carcinogenesis and tumor progression via activation of the NF-κB pathway [8–13]. Among the TLRs, TLR2 and TLR4 play crucial roles in the recognition of various pathogens derived from bacteria [14]. TLR2 forms a heterodimer with its coreceptors TLR1 or TLR6, and together can recognize lipoproteins, lipoteichoic acid, and peptidoglycan molecules derived from Gram-positive bacteria [15]. TLR4 which is necessary for recognizing lipopolysaccharides (LPS) was reported to promote tumor development in head and neck squamous cell carcinoma [16] and be involved in the resistance to cisplatin-induced apoptosis in OSCC [17]; however, the precise roles of TLR2 in OSCC progression remain unclear.

Micro RNAs (miRNAs) are small noncoding RNAs (19–22 nucleotides in length) that regulate protein-coding gene expression by binding to the 3′-untranslated regions (UTRs) of target messenger RNAs (mRNAs), typically resulting in translational repression or the cleavage of mRNA in a sequence-specific manner

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[18]. Studies demonstrated that miRNAs are expressed at high levels in numerous human cancers and play crucial roles in oncogenesis, cancer progression, and metastasis [19]. miRNAs also function as pivotal regulators and fundamental players in molecular networks that regulate TLR-signalling pathways [20] but it is unknown whether miRNAs induced by TLR-activating signals contribute to the progression of OSCC.

In this study, we first analysed the expression patterns of TLR2 and its coreceptors TLR1 and TLR6 in clinical OSCC tissue specimens, as well as their signalling pathways in OSCC cultured cells. To determine whether TLR2 signalling contributes to the progression of OSCC, we focused on miRNAs that were induced by TLR2 activation and analysed the function of these miRNAs and their target mRNAs. We anticipate that our results will lead to the identification of bacteria involved in the malignant progression of OSCC.

2. Materials and methods

2.1. Clinical specimens of oral squamous cell carcinoma

Twenty-three pairs of primary tumor and adjacent non-malignant tissue specimens were obtained from patients with OSCC who underwent surgery at the Tokyo Medical University Hospital from 2014 to 2016. Patients' background information and clinicopathological characteristics are presented in Table 1. This study was approved by the Ethics Committee at the Tokyo Medical University (approval ID: 2842). All patients signed informed consent forms.

2.2. Cell lines

The human OSCC-derived cell lines, HSC3 (Riken Cell Bank), HSC3-M3 (JCRB Cell Bank) were cultured in 10% FBS contained Minimum Essentials Medium Eagle (Sigma-Aldrich), SCC9 and SCC25 (American Type Culture Collection) were cultured in 10% FBS contained DMEM-F12 HAM (Sigma-Aldrich).

2.3. Immunohistochemistry

Acetone fixed OSCC frozen sections were incubated with anti-TLR2 antibody (ab9100, Abcam) or anti-TLR1 antibody (NBP 1-77244, Novus Biologicals) or anti-TLR6 antibody (sc-5657, Santa Cruz Biotechnology).

2.4. Fluorescence immunocytostaining

After fixation with acetone and blocking with 1% goat serum, cells were incubated with anti-TLR2 antibody (ab9100, Abcam) or anti-TLR1 antibody (NBP 1-77244, Novus Biologicals) or anti-TLR6 antibody (sc-5657, Santa Cruz Biotechnology). And then, samples were incubated with Alexa Fluor 594 IgG (Life Technologies) or Alexa Fluor 594 IgG.

2.5. Western blotting

Cells were lysed with TNE Buffer. Ten μg of proteins were separated by SDS-polyacrylamide gel electrophoresis and then transferred onto PVDF membranes. After blocking, the membranes were incubated with the following antibodies: anti-TLR2 antibody (ab108998, Abcam), anti-TLR1 antibody (ab22057, Abcam), anti-TLR6 antibody (sc-5657, Santa Cruz Biotechnology), anti-caspase-3 antibody (D3R6Y, Cell Signaling Technology), or anti-GAPDH antibody (D16H11, Cell Signaling Technology). Proteins were detected by ECL Select Western Blotting Detection System (GE Healthcare Life Sciences).

2.6. Luciferase reporter gene assay

HSC3 and HSC3-M3 cells were transfected with 10 μg of plasmid containing pGL 4.32 (luc2P/NF−κB-RE/Hygro; Promega). After transfection, the cells were added Pam3CSK4 (Novus Biologicals) or FSL-1 (Invivogen). The cells were harvested, and luciferase assays were performed using the Luciferase Assay System (Promega).

2.7. Micro RNA microarray analysis

Microarray analysis were performed using the 3D-Gene miRNA microarray platform (Toray). At each 24-hr time point after the addition of Pam3CSK4 (100 ng/mL) or FSL-1 (100 ng/mL) to cells, total RNA was isolated using Isogen reagent (Nippon Gene) and

Table 1
Clinicopathological characteristics of the OSCC patients.

Patient no	Age	Sex	Location	Tumor	Nodes	Metastasis	Stage	Differentiation
1	71	M	Gingiva	2	1	0	III	Well
2	72	M	Oral flloor	4a	2c	0	IVA	Moderate
3	70	M	Oral flloor	4a	0	0	IVA	Moderate
4	39	M	Gingiva	4a	0	0	IVA	Well
5	64	F	Gingiva	4a	2b	0	IVA	Moderate
6	67	M	Tongue	1	0	0	I	Moderate
7	64	M	Gingiva	4a	0	0	IVA	Well
8	83	M	Tongue	2	0	0	II	Well
9	69	F	Gingiva	2	2b	0	IVA	Well
10	64	F	Tongue	4a	1	0	IVA	Poor
11	67	M	Buccal mucosa	2	0	0	II	Well
12	85	F	Buccal mucosa	4a	2b	0	IVA	Poor
13	73	F	Tongue	1	0	0	I	Well
14	78	F	Gingiva	2	2b	0	IVA	Moderate
15	63	M	Oral flloor	2	0	0	II	Moderate
16	85	M	Gingiva	2	0	0	II	Well
17	50	F	Oral flloor	4a	0	0	IVA	–
18	73	M	Gingiva	4a	2b	0	IVA	–
19	68	M	Gingiva	3	2b	0	IVA	–
20	46	M	Tongue	4a	1	0	IVA	Well
21	74	F	Gingiva	2	0	0	II	Moderate
22	50	F	Buccal mucosa	2	1	0	III	–
23	71	M	Oral flloor	2	0	0	II	Moderate

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