Original Article

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Chitinase 3-Like-1 Expression Is Upregulated Under Inflammatory Conditions in Human Oral Epithelial Cells

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Summary: ***Objective*:** Chitinase 3-like-1 (CHI3L1), also known as YKL-40, is a partially secreted glycoprotein and is involved in inflammatory disorders, including inflammatory bowel diseases. CHI3L1 is known to play a role in biological responses such as cell proliferation, tissue remodeling, and inflammation. CHI3L1 forms an immune complex (known as a Chitosome complex) with IL-13 receptor alpha 2 (IL-13 Rα2) and transmembrane protein 219 (TMEM219) to activate the MAPK/ERK and PKB/AKT signaling pathways. The objective of this study is to investigate how the expressions of CHI3L1 and a Chitosome complex in human oral cavity epithelial cells are linked with intraoral inflammatory diseases.

***Method*:** CHI3L1 and Chitosome complex mRNA expressions were analyzed using human oral squamous cancer cell lines, HSC3 and HSC4 cells. Signaling activation in HSC4 cells was analyzed by using the western blot technique. Immunohistological analysis was performed using surgical samples obtained from patients with benign oral cavity tumors and cysts.

***Results*:** Increased expression of CHI3L1 was observed in both HSC3 and HSC4 cells after TNFα stimulation. The expression of Chitosome complex factors increased as CHI3L1 levels increased, resulting in the activation of a downstream signaling pathway. Among the intraoral tissues, the epithelial cells from inflammatory lesions, but not benign tumors, were found to be intensively stained with the anti-CHI3L1 antibody.

***Conclusion*:** It was indicated that the formation of a Chitosome complex is induced during inflammation, leading to the activation of signaling pathways.

Keywords chitinase 3-like-1, inflammation, chitosome complex, oral epithelial cells

INTRODUCTION

Chitinase 3-like-1 (CHI3L1), also known as YKL-40 or Brp-39, is a glycoprotein with a molecular weight of approximately 40 kDa and is a member of the glycoside hydrolase family 18 (GH18). Among the GH18, CHI3L1 is a mammalian chitinase with no enzymatic activity due to a mutation in the catalytic domain [1,2]. CHI3L1 is secreted by a variety of cells such as macrophages [3], neutrophils [4], synovial cells [5], vascular endothelial cells [6] and colonic epithelial cells (CEC) [7]. CHI3L1 is strongly involved in immune responses, playing critical roles in innate and adaptive immunity. Strong associations with certain pathological conditions, including inflammation and tissue damage responses, have been reported. CHI3L1 is induced specifically in inflammatory lesions such as inflammatory bowel diseases [8,9], rheumatoid arthritis [10], and bronchial asthma [11]. Recent studies have reported that CHI3L1 forms an immune complex with an IL-13 receptor alpha 2 (IL-13 Rα2) and a transmembrane protein 219 (TMEM219), thus acti-

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Abbreviations: CEC, colonic epithelial cells; CHI3L1, chitinase 3-like-1; FFPE, formalin-fixed and paraffin-embedded; GH 18, glycoside hydrolase family 18; IHC, immunohistochemistry; IL-13Rα2, interleukin receptor alpha 2; RT-PCR, reverse transcriptase polymerase chain reaction; TMEM219, transmembrane protein 219.

vating the MAPK and AKT signaling pathways and playing roles in altered immune responses and inflammation [12-14]. Inflammations in which CHI3L1 is involved have been studied in a variety of pathological conditions.

In intraoral studies, high levels of CHI3L1 are reported in gingival crevicular fluid in periodontitis [15] and in the saliva of patients with periodontal disease [16]. However, reports on the association between CHI3L1 levels, intraoral tissues, and pathological conditions are limited. Oral inflammatory diseases caused by bacterial infections such as caries, periodontal diseases, mucositis, and chronic inflammatory lesions resulting from mechanical stimulation are frequently found in intraoral environments. Therefore, this study focused on CHI3L1 in the intraoral epithelial cells in benign tumors and cysts with or without inflammatory conditions.

MATERIALS AND METHODS

Cell Culture

Two human oral cancer cell lines, HSC3 and HSC4 cells, were cultured in Minimum Essential Media (MEM) (nacalai tesque, Kyoto, Japan), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) in an incubator set to 37°C with 5% CO_2 atmosphere.

RNA extraction and Reverse Transcriptase-PCR (RT-PCR)

HSC3 and HSC4 cells (1×10^5 cells/well) were seeded in a 6-well plate and cultured for 48 hours, after which they were stimulated with 25 ng/ml human recombinant TNFα (R&D systems, Minneapolis, MN, USA) or 20 ng/ml human recombinant IL-13 (R&D systems) for 24 hours. After 24 hours of stimulation, the cells were collected and total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. cDNA was synthesized using the SuperScript First-Strand Synthesis System for reverse-transcription polymerase chain reaction (PCR) (Invitrogen) according to protocol in 20 μL total reaction. The cDNA produced was amplified in 34 cycles with the primers shown in Table 1. The amplified cDNA electrophoresed on a 3% agarose gel with 10 mg/ml ethidium bromide. After electrophoresis, it was detected using a Red Imager SA-1000 (Protein simple, Santa Clara, CA, USA).

Transfection and Western blotting

HSC4 cells (1×10^6) were reverse transfected with pEGFP-N1-CHI3L1 plasmid (a gift from T. Okada, Kurume University School of Medicine) seeded in a 6-well plate and cultured for 48 hours. The medium was then changed and stimulated with TNFα or IL-13. Total proteins were extracted with a RIPA buffer containing a protease inhibitor cocktail mix (Roche, Basel, Switzerland) and phosphatase inhibitors (Roche). Total protein from the cells were separated using a 5-20% gradient SDS-PAGE gel (FUJIFILM, Osaka, Japan) and then transferred to a nitrocellulose membrane (Trans-Blot Turbo Transfer System, Bio-Rad). After blocking the membrane with a 5% ECL Blocking Buffer (nacalai tesque) for 1 hour at room temperature (RT), the primary antibody was incubated at 4°C overnight. The primary antibodies used were total-p44/42 MAPK, phospho- p44/42 MAPK, total-AKT, phospho-AKT, total-p38 and phospho-p38 antibodies, which were all purchased from Cell Signaling Technology (Danvers, MA, USA) and used at a dilution of 1:1000. After the primary antibody reaction, the membrane was washed three times for 15 minutes with a PBS containing 0.1% Tween-20 and incubated with a secondary antibody at a dilution of 1:3000 (Biotium, Fremont, CA, USA) in Blocking One (nacalai tesque, Kyoto, Japan) for 1 hour at RT. The detection of target proteins was captured using the Odyssey instrument (LI-COR, Lincoln, NE, USA), and LI-COR-associated software (image studio lite) (LI-COR) was used for the band intensity of the protein.

TABLE 1.
Primers for Reverse-Transcription PCR Analysis

Primers	Forward	Reverse	Product size (bp)
GAPDH	5'-CGAGATCCCTCCAAAATCAA-3'	5'-TTCACACCCATGACGAACAT-3'	439
CHI3L1	5'-GAAGACTCTCTTGTCTGTCGGA-3'	5'-AATGGCGGTACTGACTTGATG-3'	510
IL-13Rα2	5'-ACCTGGCATAGGTGTACTTCT-3'	5'-CCAAATAGGGAAATCTGCATCCT-3'	134
TMEM219	5'-ACGGTCCAGACAGGAACAAGA-3'	5'-CTGAGCAGGATATGGGTGGC-3'	198

Materials for immunohistochemistry

Formalin-fixed and paraffin-embedded (FFPE) samples of oral tissues were used for hematoxylin and eosin staining as well as immunohistochemistry (IHC). Biopsied tissue specimens were obtained from patients with benign tumors and cysts in the oral cavity who were examined at the Division of Oral and Maxillofacial Surgery of the Dental and Oral Medical Center, Kurume University Hospital. The clinical information of the cases and controls used in this study is shown in Table 2. As a control, normal epithelial tissue, the safety margins of tissue resected for tongue cancer were used. Serial tissue sections used for hematoxylin and eosin staining as well as IHC staining were sliced into thin sections of 4 μm thickness.

Immunohistochemistry

The expression of CHI3L1 was evaluated by IHC. All specimens were sliced into a thickness of 4 μm, dewaxed and hydrated. Tris-EDTA solution (pH 9.0) was added and heated for antigen retrieval. After cooling to room temperature, a CHI3L1 antibody (Dilution, 1: 50; bs-10215R, Bioss Antibody, Boston, MA, USA) was added, and the sections were incubated overnight at 4°C. The slides were washed three times with PBS for 15 minutes, and then incubated with biotin-labeled goat anti-rabbit IgG secondary antibody (Dilution, 1:200; Vector laboratories, Burlingame, CA, USA) for 1 hour at RT. After washing the slides, the avidin-biotin-labeled enzyme complex (Vector) was reacted at RT for 45 minutes. AEC3-amino-9-ethylcarbazole (Abcam, Cambridge, MA, USA) was reacted in an incubator at 37°C for 8 minutes. Finally, it was immersed in a hematoxylin solution (Dako, Glostrup, Denmark) for 1 minute to induce a reaction. For the negative control, PBS was used instead of the primary antibody. The stained slides were captured with an upright microscope (Axio imager2: ZEISS, Berlin, Germany) (magnification: 400 times).

RESULTS

In human oral cavity epithelial cells, the CHI3L1 expression is enhanced under the presence of inflammation, and the factors making up a Chitosome complex are expressed.

In this study, the presence of CHI3L1 in human oral cavity epithelial cells was investigated using HSC3 and HSC4 cells, and its expression was confirmed by RT-PCR. The expression of CHI3L1, despite the low level, was observed under steady state conditions in both HSC3 and HSC4 cells (Figure 1). Previous studies indicated that the expression of CHI3L1 is enhanced by TNFα stimulation in CECs [7]. In this study, therefore, we stimulated HSC3 and HSC4 cells using TNFα and confirmed the enhanced

TABLE 2.
Patient Clinical Characteristics for Immunohistochemistry

Sample No.	Diagnose	Gender	Age	WBC* (10^3 μL)	NEUT** (%)
Inflammatory odontogenic cyst					
1	Radicular cyst	Female	46	10.6	67.9
Non-odontogenic developmental cysts					
2	Postoperative maxillary cyst	Female	28	4.4	41.7
Benign epithelial odontogenic tumour					
3	Ameloblastoma	Male	35	10.1	65.2
Benign mesenchymal odontogenic tumours					
4	Cemento-ossifying fibroma	Female	67	7.2	60.1
Fibro-osseous and osteochondromatous lesions					
5	Cemento-osseous dysplasia	Male	42	6.3	63.3
Non-odontogenic benign tumor					
6	Denture fibroma	Female	76	3.7	57.1
7	Pleomorphic adenoma	Female	58	4.1	56.8
8	Schwannoma	Female	76	5.7	46.2

*WBC: White Blood Cells, **NEUT: Neutrophils.

expression of CHI3L1 in these cell lines.

For comparison, the CHI3L1 expression was assessed by stimulating the same cell lines by a Th2 cytokine, IL-13. In contrast to the cell lines stimulated by TNFα, the CHI3L1 expression decreased in HSC3 but increased in HSC4. This indicates that the CHI3L1 expression following IL-13 stimulation is cell-dependent.

It is shown that CHI3L1 forms a Chitosome complex with IL-13 Rα2 and TMEM219 to activate a downstream MARK signaling pathway [13]. To demonstrate the presence of a Chitosome complex in human oral cavity epithelial cells, the expression of its factors, IL-13Rα2 and TMEM219, was evaluated in this study. One of the factors, TMEM219, showed constant and marked expression in both cell lines, regardless of presence or absence of stimulation with TNFα or IL-13. Another factor, IL-13Rα2, also showed constant expression, although the level of expression varied depending on the cell line. Stimulating IL-13Rα2 with TNFα amplified its expression, and additional stimulation by IL-13 showed an even stronger effect in amplifying its expression. These results demonstrated that CHI3L1 increases under inflammatory conditions and induces the expression of IL-13 Rα2 and TMEM219.

CHI3L1 activates MAPK signaling pathway in human oral cavity epithelial cells

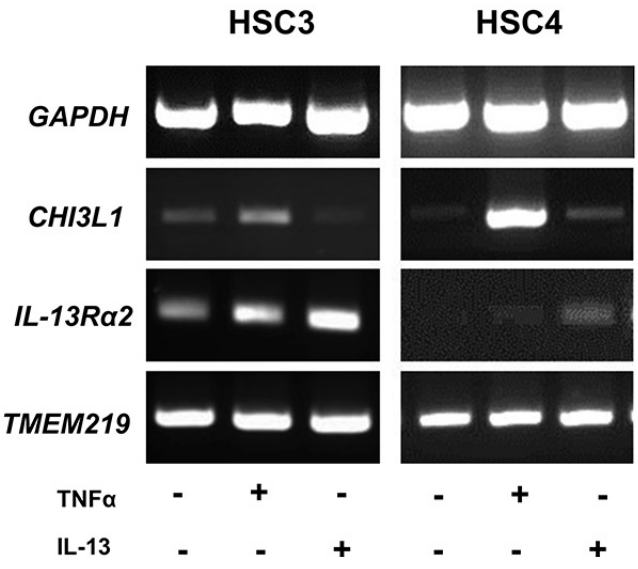

Fig. 1. RT-PCR results for 2 types of oral epithelial cell lines.
Expression of CHI3L1 is enhanced by stimulation of TNFα and decreased by stimulation of IL-13. IL-13 Rα2 and TMEM219 is constitutively expressed on all the both cell lines regardless the TNFα stimulation.

CHI3L1 is considered to play critical roles in p44/p42 MAPK, AKT/PKB and p38 MAPK signaling pathways. The expression of mRNA composing a Chitosome complex was detected in the oral cavity epithelial cells. Therefore, western blotting was performed to visualize the signal transduction induced from a Chitosome complex. The results showed that stimulation by TNFα amplified the AKT and p38 MAPK responses, whereas the stimulation by IL-13 enhanced a p44/42 MAPK response (Figure 2). These results indicate that CHI3L1 activates the signaling pathways in human oral cavity epithelial cells.

CHI3L1 expression was confirmed in tumor cells of human intraoral tissues

IHC was performed to confirm the expression of CHI3L1 in the cells of benign tumors and cysts in human oral cavities. CHI3L1 was found in the inflammatory epithelial cells of denture fibroma (Figure 3A, 3B). The fibrous connective tissue inside the fibroma was found stained (Figure 3A, 3C). In the case of a radicular cyst, caused by chronic apical inflammation, the epithelial cells of the cyst walls were intensely stained (Figure 3D, 3E). The expression of CHI3L1 was also found in inflammatory granulation tissue, where it was mainly expressed in plasma cells. (Figure 3D, 3F). Fibrous connective tissue of ameloblastoma (Figure 4B), fibroblast-rich connective tissue of ce-

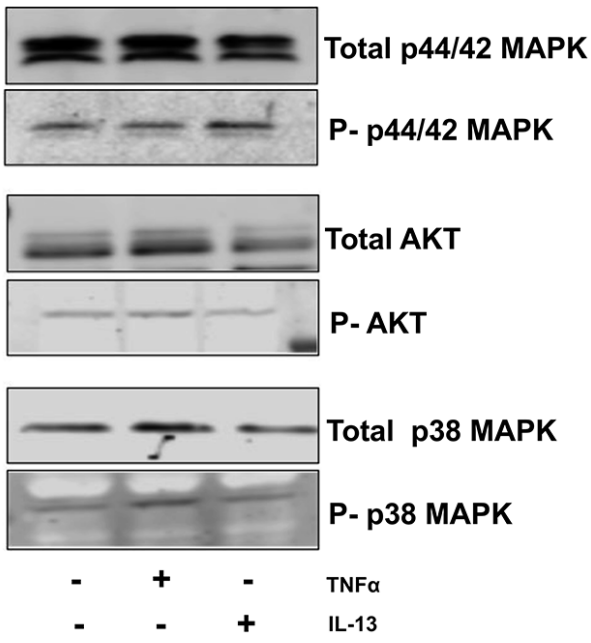

Fig. 2. Expression of major signals by Western blot using HSC4 cells.
AKT and p38 have slightly enhanced expression after stimulated with TNFα. P44/42 MAPK has enhanced expression after stimulated with TNFα.

mental ossifying fibroma (Figure 4C), epithelial cells and myoepithelial cells of pleomorphic adenoma (Figure 4E) and tumor cells derived from the Schwan cell of the nerve sheath tumor (Figure 4F) were found stained. Neither epithelial cells of a postoperative maxillary cyst (Figure 4A), cement-like hard tissue (Figure 4C), nor bone tissue showed any CHI3L1 expression (Figure 4C, 4D). These results show that CHI3L1 is expressed in fibrous connective tissues, muscle cells and nerve fibers of intraoral tumors. In inflammatory lesions of periapical cysts and denture ulcers, which are considered to be associated with chronic inflammation, an intense expression of CHI3L1 was found in epithelial cells, indicating the involvement of CHI3L1 in inflammatory changes of intraoral tissues.

DISCUSSION

The results of this study indicated that the expression of CHI3L1 is associated with inflammatory lesions in human oral cavities as is the case with inflammatory lesions in other parts of the body. This study

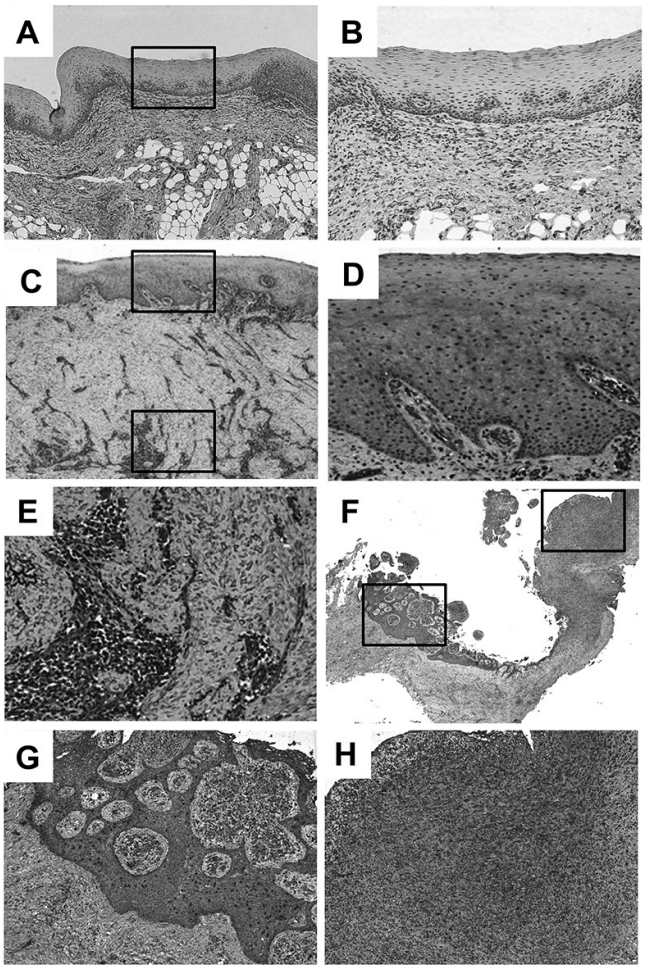

Fig. 3. Representative immunohistochemical images in oral inflammatory lesions.
CHI3L1 immunohistochemical analysis result of surgical samples obtained from the patients with non-inflammation epithelial tissue (A, B), Denture fibroma (C-E) and radicular cyst (F-H) is shown. The areas indicated by rectangles in A, C and F correspond to B, D/E and G/H, respectively. Positive CHI3L1 staining has been observed in inflamed epithelial cells (D), fibrous connective tissue inside the fibroma (E), and cyst wall (G). Diffuse and strong CHI3L1 staining has been observed in the lesion of inflammatory granulation (H). (A) objective, 10×; (C) objective, 5×; (B, D, E, G, H) objective, 20×; (F) objective, 2.5×.

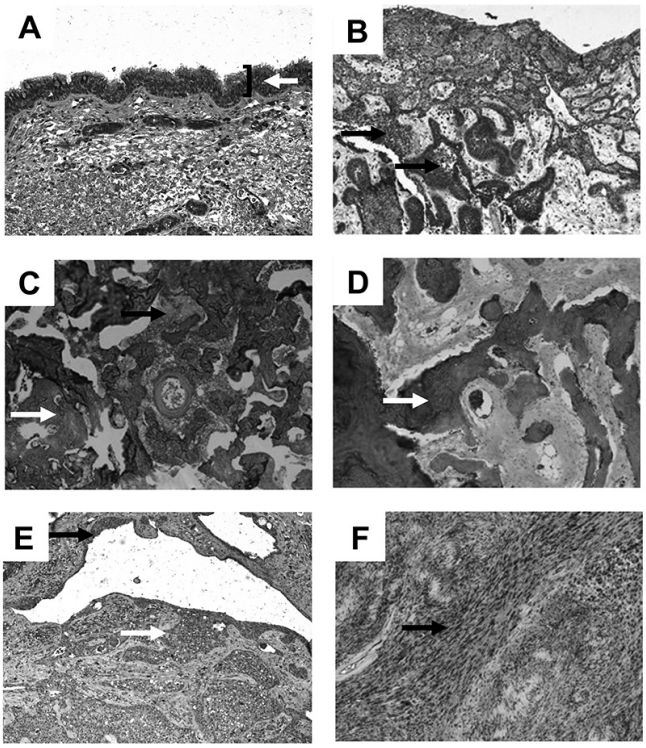

Fig. 4. Representative immunohistochemical images in oral being tumors.
CHI3L1 immunohistochemical analysis result of surgical samples obtained from the patients. (A) Postoperative maxillary cyst (Non-odontogenic developmental cysts). Pseudostratified ciliated columnar epithelium (white arrow) is not stained. (B) Ameloblastoma (Benign epithelial odontogenic tumor). Fibrous connective tissue (black arrows) is not stained. (C) Cemento ossifying fibroma (Benign mesenchymal odontogenic tumors). Fibroblast-rich connective tissue (black arrows) is stained. Bone tissue (white arrow) is not stained. (D) Cemento Osseous dysplasia (Fibro-osseous and osteochondromatous lesions). Bone tissue (white arrow) is not stained. (E) Pleomorphic adenoma (Non-odontogenic benign tumor). Exocrine epithelial tissue (black arrow) and myoepithelial tissue (white arrow) are stained. (F) Schwannoma (Non-odontogenicbenign tumor). Schwann cell-derived tumor cells (black arrow) are stained. (A-F) objective, 10×.

showed staining of mixed types of cells, including epithelial cells, and presumably macrophages and neutrophils, of CHI3L1 in oral lesions. The expression of CHI3L1 was observed in human oral cavity epithelial cells as well as in the epithelial tissues of benign tumors and cysts. The mRNA expression of IL13Rα2 and TMEM219, which are factors composing a Chitosome complex, was also confirmed, demonstrating the activation of the transduction of signals involved in a Chitosome complex. CHI3L1 functions to regulate cell proliferation, adhesion, migration, and tissue remodeling. CHI3L1 is produced by a variety of cells, including immunocompetent cells, such as macrophages and neutrophils, vascular endothelial cells, synovial cells, and human chondrocytes [17]. Its presence in the exocrine epithelial cells, nerve cells, vascular endothelial cells and mesenchymal cells has been shown by IHC analysis of intact tissues [18]. The results of this study also showed the CHI3L1 expression in fibrous connective tissue, myoepithelial cells, and Schwan cell-derived tumor cells of parenchyma of benign tumors. Given that CHI3L1 is known to play a role in regulating cell inflammation, apoptosis, and tissue remodeling [19], CHI3L1 is considered to be involved in cell proliferation and infiltration.

Protein-level elevation of CHI3L1 is observed in disorders such as rheumatoid arthritis, systemic lupus erythematosus [10], bronchial asthma [20], and inflammatory bowel diseases [8,9]. In intraoral inflammatory lesions, the protein-level of CHI3L1 has been elevated, which is presumably associated with continuous bacterial infection [21] and external mechanical forces [22]. Further study will be required to prove the association with these factors.

CHI3L1 functions as a ligand of IL-13 Rα2, and does not compete with IL-13. By forming an immune complex with IL-13 Rα2 and TMEM219, it activates the MAPK/ERK and PKB/AKT signaling pathways [12,13]. Thus, CHI3L1 is known to be involved in cell proliferation and tissue remodeling [11-13]. This signaling activation may also be involved with inflammatory lesions in human oral cavities.

The involvement of CHI3L1 in the process of carcinogenic change of epithelial cells under chronic inflammation has also been reported [23]. In the CEC, CHI3L1 causes exacerbation of colonic inflammation by promoting bacterial adhesion and infiltration [7,24]. Enhanced expression of CHI3L1 is related with chronic colitis-induced neoplastic changes in CEC [25,26]. Chronic inflammatory lesions of bacterial origin, or long-term mechanical stimulation in the oral cavity, can cause neoplastic changes of oral epithelial cells including oral dysplasia and oral squamous cell carcinoma. Since this study has been focusing only on the expression of CHI3L1 in oral non-malignant diseases, further studies will be necessary to elucidate its association with neoplastic transformation in oral epithelial cells.

CONCLUSION

CHI3L1 presumably plays an important role as an inflammation-associated inducible factor in intraoral tissues. It is possible that factors composing a Chitosome complex may be associated with the induction and perpetuation of the CHI3L1 expression in human oral epithelial cells.

CONFLICTS OF INTEREST: The author declares no conflicts of interests.

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SUPPLEMENTARY METHODS

Confirmation of Expression of CHI3L1 in mock plasmid

Human oral cancer cell lines, SAS cell, were cultured in Dulbecco's Modified Eagle Medium (DMEM) (nacalai tesque), supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, St. Louis, MO) in an incubator set to 37 °C with 5% CO_2 atmosphere.

SAS cells (1×10^6) were reverse transfected with pEGFP-N1-CHI3L1 plasmid and pEGFP-N1 empty (mock) plasmid and seeded into 6-well plate and cultured for 48 hours. Total proteins were extracted with RIPA buffer containing protease inhibitor cocktail mix (Roche, Basel, Switzerland) and phosphatase inhibitors (Roche). Total protein from the cells were separated using 5-20% gradient SDS-PAGE gel (FUJIFILM) and then transferred to nitrocellulose membrane (Bio-Rad). After blocking the membrane with 5% ECL Blocking Buffer (nacalai tesque) for 1 hour at room temperature (RT), the primary antibody was incubated at 4°C overnight. The primary antibodies used was mouse anti-GFP (Roche) at dilution of 1:3000. After the primary antibody reaction, the membrane was washed three times for 15 minutes with PBS containing 0.1% Tween-20 and incubated with secondary antibody at dilution of 1:3000 (Biotium) in Blocking One (nacalai tesque) for 1 hour at RT. The detection of target proteins was captured using the Odyssey instrument (LI-COR), and LI-COR-associated software (image studio lite) (LI-COR) was used for the band intensity of the protein (Fig. S1).

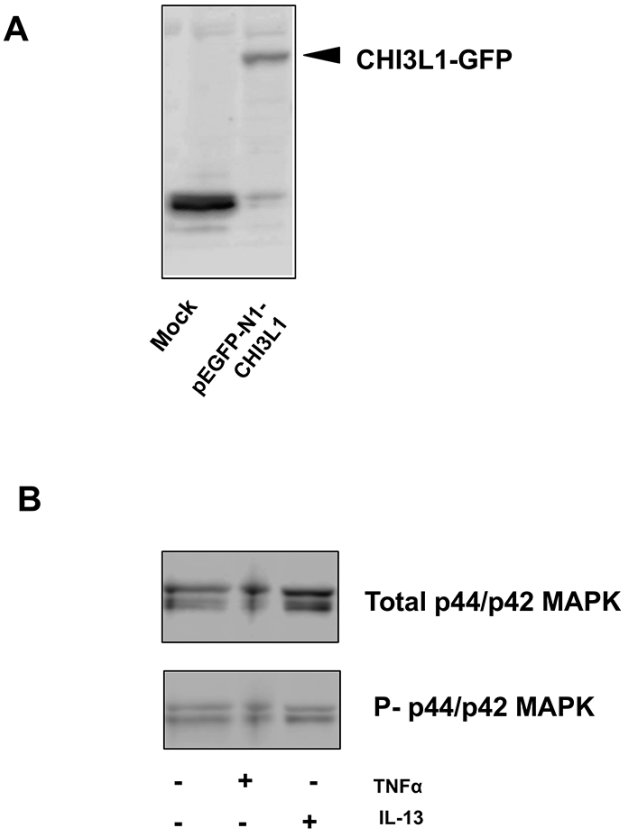

Fig. S1.
(A) CHI3L1 expression in SAS cells with Mock and pEGFP-N1-CHI3L1
CHI3L1 expression in an empty vector (shown as mock) is not detected.
(B) Expression of p44/42 MAPK signals by Western blot in HSC4 cells with pEGFP-N1 empty plasmid transfection. No signal can be detected after pEGFP-N1 empty plasmid transfection.