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New evidence of connections between increased O-GlcNAcylation and inflammasome in the oral mucosa of patients with oral lichen planus

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Summary

Oral lichen planus (OLP) is considered a chronic inflammatory immune-mediated disease of the oral mucosa. Immunopathogenesis of OLP is thought to be associated with cell-mediated immune dysregulation. O-GlcNAcylation is a form of reversible glycosylation. It has been demonstrated that O-GlcNAcylation promoted nuclear factor kappa B (NF-κB) signalling. Activation of NF-κB can induce expression of nucleotide-binding domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome, which is a large intracellular multi-protein complex involving an immune response. Dysregulated expression of the NLRP3 inflammasome was reported to be associated with autoinflammatory diseases. No integrative studies between O-GlcNAcylation and NLRP3 inflammasome in OLP patients have been reported. The present study aimed to determine the immunohistochemical expression of O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome in oral mucosae of OLP patients. Oral tissue samples were collected from 30 OLP patients and 30 healthy individuals. Immunohistochemical staining and analyses of immunostaining scores were performed to evaluate expression of O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome. According to observations in this study, significantly higher levels of O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome were demonstrated in OLP patients compared with control subjects ($P < 0{\cdot}001$). Positive correlations among O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome were also observed in OLP samples ($P < 0{\cdot}01$). In conclusion, the present study provides supportive evidence that increased O-GlcNAcylation is associated with increased expression of NLRP3 inflammasome via the NF-κB signalling pathway. These findings provide a new perspective on immunopathogenesis of OLP in relation to autoinflammation.

Keywords: inflammasome, inflammation, O-GlcNAcylation, oral lichen planus, pathogenesis

Introduction

Oral lichen planus (OLP) is a chronic immune-mediated inflammatory disease of the oral mucosa with unknown aetiology [1,2]. OLP is usually found in middle-aged or older women [3]. OLP is clinically heterogeneous, presenting as reticular, papular, plaque, atrophic, erosive and bullous forms with typical white striations called Wickham striae on the oral mucosa [4]. Molecular mechanisms that induce various forms of OLP have never been clarified. OLP is associated with an increased risk of malignant transformation [5]. Histopathological characteristics of OLP consist of degeneration of basal epithelial cells, basement membrane disruption and intense subepithelial infiltration of T lymphocytes [6]. Increased expressions of nuclear factor kappa B (NF-κB) signalling molecules and various inflammatory cytokines such as tumour necrosis

factor (TNF)-α and interleukin (IL)-1β have been demonstrated previously in OLP [7–9]. The current concepts of immunopathogenesis of OLP are related to cell-mediated immune dysregulation. Some mechanisms behind the dysregulated immune response have been elucidated [10,11]. Very little, however, is known about the role of post-translational modifications in relation to oral immune responses in OLP patients.

O-GlcNAcylation is a single attachment of N-acetylglucosamine (GlcNAc) to the hydroxyl site of serine or threonine residue of cytoplasmic, nuclear and mitochondrial proteins. This modification is a reversible process regulated by O-GlcNAc transferase (OGT) and O-GlcNAcase (OGA) for addition and removal of GlcNAc residue from target proteins [12]. It was reported that O-GlcNAcylation promotes transcriptional activity, nuclear translocation and DNA binding affinity of NF-κB signalling molecules [13,14]. Growing evidence suggests that O-GlcNAcylation modulates activation of NF-κB signalling in several chronic inflammatory diseases. DNA binding activity of p65, a functional component of NF-κB, is regulated by O-GlcNAcylation during inflammatory processes and tumorigenesis of the colon [15]. In addition, O-GlcNAcylation promotes transcriptional activity of NF-κB and up-regulated expression of TNF-α in pancreatitis [16]. Expressions of O-GlcNAcylated proteins (OGP) and O-GlcNAcylation associated enzymes (OGT and OGA) have never been investigated in the oral mucosae of OLP patients.

The nucleotide oligomerization domain (NOD)-like receptor family pyrin domain containing 3 (NLRP3) inflammasomes is a large intracellular multi-protein complex consisting of three components: NLRP3, apoptosis-associated speck-like protein containing C-terminal caspase-recruitment domain (CARD) adaptor protein (ASC) and caspase-1 [17]. NLRP3 inflammasome was reported to play a role in activation of the innate immune response [18]. In addition, NLRP3 inflammasome is associated with an inflammatory form of programmed cell death known as pyroptosis [17]. Increased expression of NLRP3 inflammasomes was observed in a variety of chronic inflammatory diseases, such as Crohn's disease [19] and periodontal diseases [20,21], upon activation of the NF-κB signalling pathway [22,23]. In addition, dysregulated inflammasome-mediated expression of cytokines such as IL-1β was reported to be associated with autoinflammatory diseases [24–26]. No prior studies have reported expression of NLRP3 inflammasome in OLP. Moreover, no integrative studies between O-GlcNAcylation and NLRP3 inflammasome have been performed in OLP patients. Taking these research gaps into account, it was hypothesized that changes in expression of O-GlcNAcylation and NLRP3 inflammasome could be demonstrated at the sites of inflamed oral mucosae of OLP patients. Thus, the present study was aimed to investigate the immunohistochemical expressions of O-GlcNAcylation (OGP, OGT and OGA), NF-κB signalling molecules (NF-κB and TNF-α) and NLRP3 inflammasome (NLRP3, ASC, caspase-1 and IL-1β) in the inflamed oral mucosae of OLP patients, compared with normal oral mucosae of control subjects.

Materials and methods

Tissue specimens

This study was approved for the use of tissues from human subjects by the Institutional Human Ethics Committee of Khon Kaen University (HE602071). The diagnostic criteria of OLP in this study were based on the modified 2003 World Health Organization (WHO) diagnostic criteria [27]. The inclusion criteria for selection of biopsy specimens with histopathological diagnosis of OLP made by an oral pathologist were as follows: OLP patients with no history of systemic diseases, cigarette smoking, receiving medication or being pregnant. The exclusion criteria were as follows: OLP patients with a history of systemic diseases, cigarette smoking, receiving medications or being pregnant and OLP patients who received topical or systemic steroids for treatment of OLP in the past 3 months.

Formalin-fixed and paraffin-embedded biopsy specimens of OLP and normal oral mucosa (NOM) were collected from the archives of the Oral Pathology Division, Faculty of Dentistry, Khon Kaen University. All original haematoxylin and eosin (H&E)-stained slides of OLP and

Table 1. Demographic and clinical data of oral lichen planus (OLP) patients and control subjects

	OLP patients (n = 30)	Control subjects (n = 30)
Gender		
Male	7	13
Female	23	17
Age* (years)		
Mean ± s.d.	48·73 ± 11·82	22·27 ± 7·21
Range	32–75	17–57
Biopsy sites		
Buccal mucosa	30	–
Retromolar area of buccal mucosa	–	30
Clinical diagnoses		
Reticular OLP	4	–
Erosive OLP	13	–
Atrophic OLP	13	–
Normal oral mucosa	–	30
Duration of OLP lesions (months)		
Mean ± s.d.	15·93 ± 21·23	–
Range	0·25–84	–

*Significant differences in age between OLP patients and control subjects were observed (P = 0.001, Mann-Whitney U-test); s.d. = standard deviation.

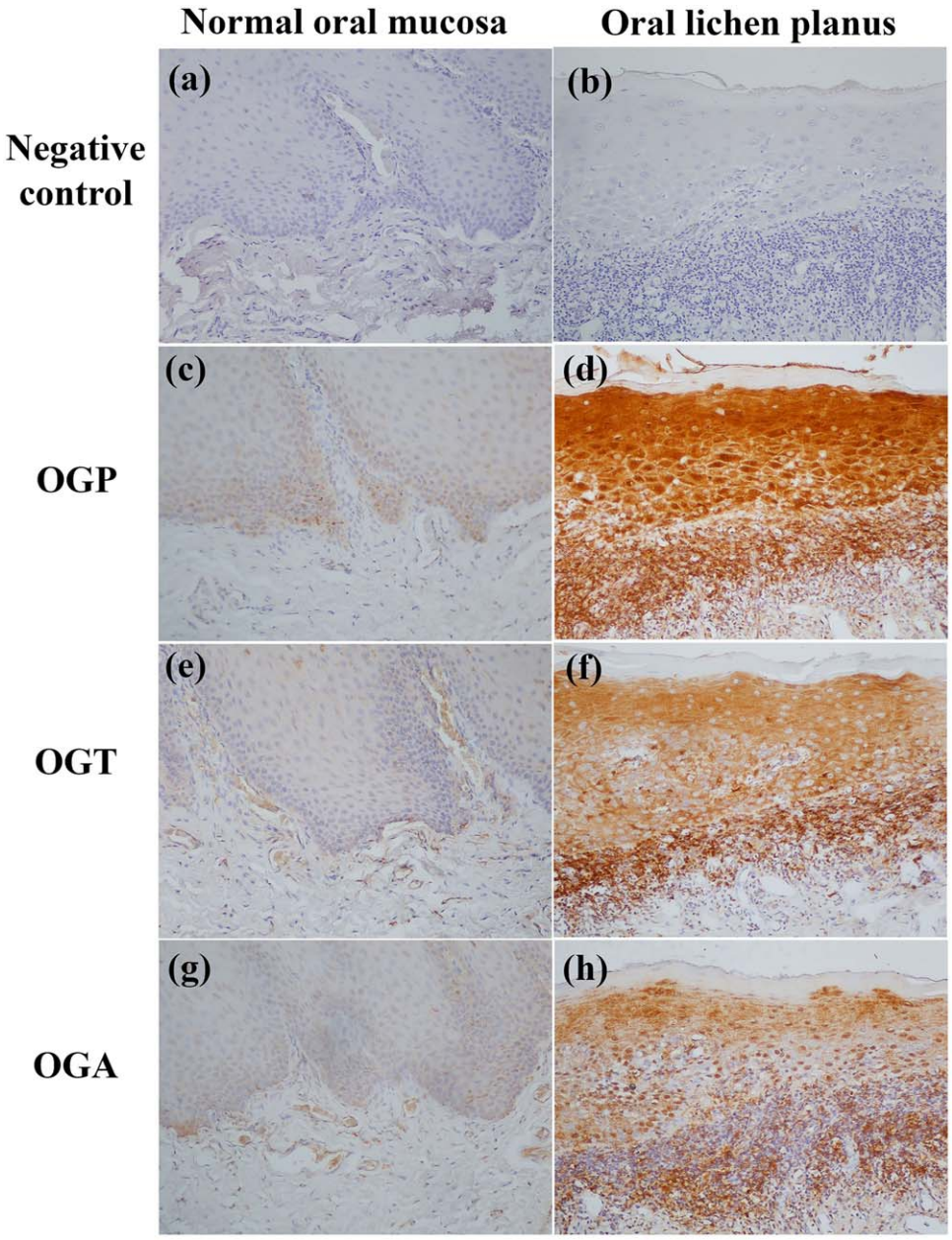

Fig. 1. Expression of O-GlcNAcylated protein (OGP), O-linked N-acetylglucosamine (GlcNAc) transferase (OGT) and O-GlcNAcase (OGA) in normal oral mucosae (c,e,g) and oral lichen planus (OLP) lesions (d,f,h). (a,b) Negative controls. Stronger immunoreactivities of OGP, OGT and OGA are observed in OLP lesional mucosae. Original magnification ×200. [Colour figure can be viewed at wileyonlinelibrary.com]

NOM from 2007 to 2016 were examined under a light microscope for the quality and suitability of oral mucosal tissue. OLP specimens demonstrating epithelial dysplasia were excluded. Then, OLP (n = 30) and NOM (n = 30) with intact epithelia and connective tissue were selected for immunohistochemical staining.

In the control group, tissue specimens were obtained from oral mucosae located at the retromolar area of the surgical procedure of tooth extraction due to the ethical limitations in collecting normal tissue samples at the same sites of surgical biopsy and with ages matching those in the OLP group. Control subjects had no history of systemic diseases, cigarette smoking, receiving medication or being pregnant. Selected biopsy specimens in the control group were diagnosed clinically and histopathologically as having normal oral mucosa.

Immunohistochemical method

The procedure of immunostaining was modified from previous studies by the present authors [28,29]. Briefly, all biopsy specimens were fixed in 10% neutral buffered formalin upon collection, embedded in paraffin wax, sectioned at 5-μm thicknesses and incubated in a hot-air oven at 60°C for 24 h. Deparaffinization of tissue specimens was performed. Antigen retrieval was performed by heating the tissue sections, which were immersed in sodium citrate buffer pH 6.0 in a microwave for 10 min. Endogenous

peroxidase activity was eliminated by 3% hydrogen peroxide. Non-specific bindings were blocked with protein block free serum (Dako, Carpinteria, CA, USA). Sections were incubated with primary antibodies in a humidifying chamber overnight at 4°C. Details of primary antibodies (OGP, OGT, OGA, NF-κB, TNF-α, NLRP3, ASC, caspase-1 and IL-1β) used in this study are shown in Supporting information, Table S1. Sections were incubated with the second antibodies (Dako EnVision + system-horseradish peroxidase (HRP)-labelled polymer anti-rabbit or anti-mouse) and then with 3,3'-diaminobenzidine (DAB; Dako) and counterstained with Mayer's haematoxylin. The slides were dehydrated in ethanol, cleared in xylene and mounted. Biopsy specimens derived from patients with periodontitis, colon cancer and oral squamous cell carcinoma were used as positive controls. Negative controls were established by incubating with phosphate-buffered saline (PBS) solution instead of primary antibodies.

Morphometric analysis

Immunostained slides were examined and immunostaining scoring was performed under a light microscope at ×200 magnification by one examiner (T. T. D.) after calibrating with an expert (P. C.). The whole area of oral epithelial layers and the lamina propria beneath the basement membrane were selected for immunohistochemical analyses. Immunostaining scores were established for semiquantitative analyses as follows: 0 = no immunostained cells; 1 = fewer than 25% positively stained cells, 2 = 25–50% positively stained cells, 3 = 50–75% positively stained cells and 4 = more than 75% positively stained cells. All specimens were assessed, and immunostaining scores were conducted twice with an interval of 2 weeks.

Statistical analyses

Statistical analyses were performed using SPSS Statistics version 17.0. The Mann–Whitney *U*-test was performed to compare differences in age and differences in immunostaining scores of OGP, OGT, OGA, NF-κB, TNF-α, NLRP3, ASC, caspase-1 and IL-1β between OLP patients and control subjects. Ordinal logistic regression was performed to eliminate confounding effects of age. Spearman's correlation coefficient was used to evaluate correlations among these investigated molecules in each group. A two-tailed *P*-value less than 0·05 was considered statistically significant.

Search Tool for Interacting Chemicals (STITCH) analyses

STITCH version 5.0 [30,31] was used to predict relationships among investigated molecules, including OGT, NF-κB, TNF-α, NLRP3, ASC, caspase-1 and IL-1β.

Results

Demographic and clinical data

Details of OLP patients and control subjects are shown in Table 1.

Agreement levels in the judgement of immunostaining scores

The level of agreement on immunostaining scores for intra-observer reliability by the kappa statistics was 0·819.

Expression of OGP, OGT and OGA in the oral mucosae of OLP patients and control subjects

According to immunohistochemical analyses, higher expressions of OGP, OGT and OGA were observed in oral epithelia and connective tissues of OLP patients compared with control subjects (all $P < 0{\cdot}001$) (Fig. 1) (Table 2). Due to a marked difference in age between OLP patients and control subjects, multivariable analyses after adjusting for age were performed and the results confirmed that expressions of OGP, OGT and OGP in the OLP group were significantly higher than those in the control group [OGP: odds

Table 2. Immunostaining scores of O-GlcNAcylated proteins (OGP), O-GlcNAc transferase (OGT), O-GlcNAcase (OGA), nuclear factor kappa B (NF-κb), tumour necrosis factor (TNF)-α, nucleotide-binding domain-like receptor family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), caspase-1 p10, interleukin (IL)-1β in normal oral mucosae (NOM) from control subjects ($n = 30$) and inflamed oral mucosae from oral lichen planus (OLP) patients ($n = 30$)

Investigated molecules		Immunostaining scores					*P*-values*
		0	1	2	3	4	
OGP	OLP	0	4	7	6	13	0·002
	NOM	0	23	4	3	0	
OGT	OLP	0	6	6	6	12	0·027
	NOM	1	18	10	1	0	
OGA	OLP	0	7	8	7	8	0·001
	NOM	2	22	6	0	0	
NF-κB	OLP	2	7	9	7	5	0·001
	NOM	20	10	0	0	0	
TNF-α	OLP	1	5	8	8	8	0·004
	NOM	5	23	1	1	0	
NLRP3	OLP	1	7	8	5	9	0·012
	NOM	1	25	3	1	0	
ASC	OLP	0	4	6	5	15	< 0·001
	NOM	1	27	2	0	0	
Caspase-1	OLP	0	10	9	4	7	0·011
	NOM	0	29	1	0	0	
IL-1β	OLP	0	5	7	5	13	0·002
	NOM	0	17	11	2	0	

**P*-values were established using logistic regression to eliminate confounding effects of age.

ratio (OR) = 16·94, 95% confidence interval (CI) = 2·67–90·74 and $P = 0·002$; OGT: OR = 6·47, 95% CI = 1·24–33·85 and $P = 0·027$; OGA: OR = 19·12, 95% CI = 3·13–116·62 and $P = 0·001$].

Expression of NF-κB signalling molecules and NLRP3 inflammasome in oral mucosae of OLP patients and control subjects

NF-κB and TNF-α were localized in the nuclei and cytoplasm of oral epithelial cells and infiltrated immune cells (Fig. 2). Expressions of NF-κB and TNF-α were significantly higher in OLP patients compared to control subjects ($P < 0·001$) (Table 2). Multivariate analyses after adjusting for age demonstrated that expressions of NF-κB and TNF-α in the OLP group were significantly higher than those in the control group (NF-κB: OR = 27·68, 95% CI = 3·70–206·43 and $P = 0·001$; TNF-α: OR = 16·05, 95% CI= 2·46–95·87 and $P = 0·004$).

Expressions of NLRP3, ASC, caspase-1 and IL-1β were increased significantly in oral epithelia and connective tissues of OLP patients compared to those in control subjects ($P < 0·001$) (Table 2) (Fig. 3). After adjusting for age, multivariate analyses demonstrated that expression of NLRP3, ASC, caspase-1 and IL-1β in the OLP group were significantly higher than those in the control group (NLRP3: OR = 9·66, 95% CI = 1·66–56·32 and $P = 0·012$; ASC: OR = 74·67, 95% CI = 7·94–702·05 and $P < 0·001$; caspase-1: OR = 54·38, 95% CI = 4·16–710 and $P = 0·011$; IL-1β: OR = 8·80, 95% CI = 1·64 – 47·18 and $P = 0·002$).

Correlations among expression of O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome in OLP patients

Positive correlations among O-GlcNAcylation (OGP, OGT, OGA), NF-κB signalling molecules (NF-κB and TNF-α) and NLRP3 inflammasome (NLRP3, ASC, caspase-1 and IL-1β) were observed in OLP patients ($P < 0·01$). No correlations of these investigated molecules were demonstrated in control subjects. Details of Spearman's rank correlation analyses among these investigated molecules in OLP patients are demonstrated in the Supporting information, Table S2. STITCH analyses revealed predictive relationships among OGT, NF-κB, TNF-α, NLRP3,

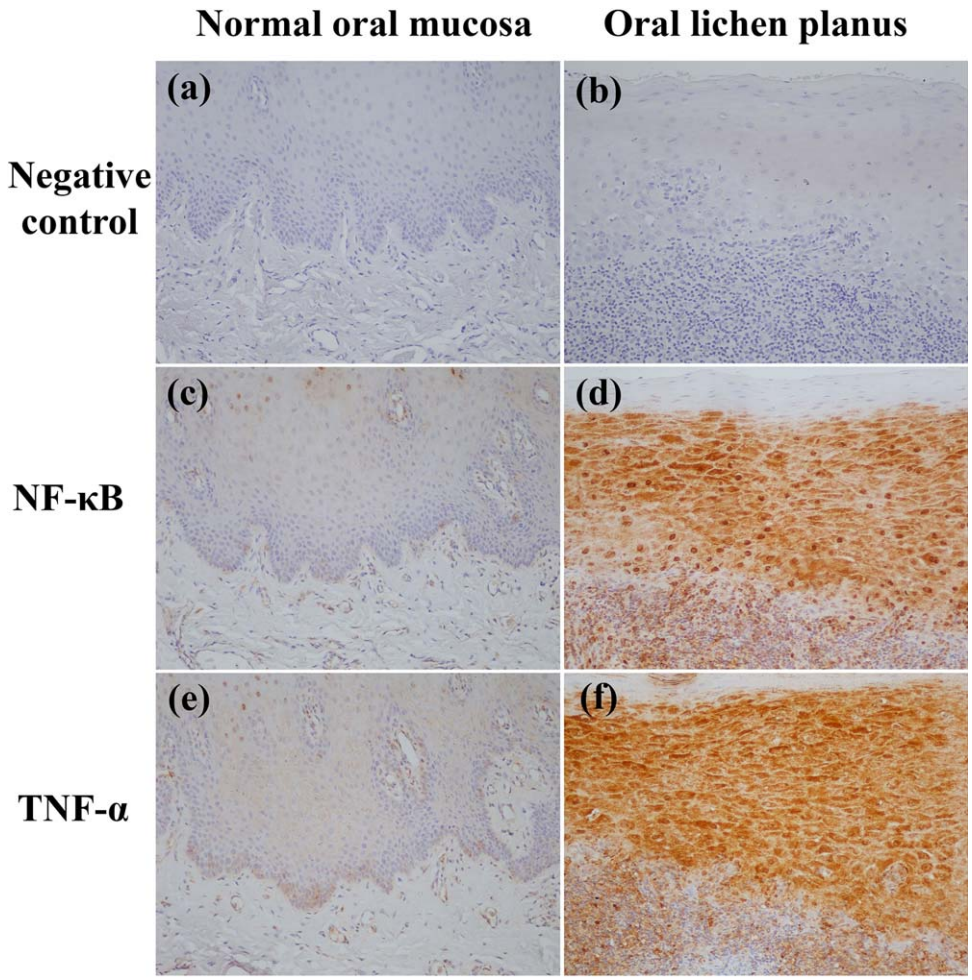

Fig. 2. Expression of nuclear factor kappa B (NF-κB) and tumour necrosis factor (TNF)-α in normal oral mucosae (c,e) and oral lichen planus (OLP) lesions (d,f). (a,b) negative controls. Stronger immunoreactivities of NF-κB and TNF-α are observed in OLP lesional mucosae. Original magnification ×200. [Colour figure can be viewed at wileyonlinelibrary.com]

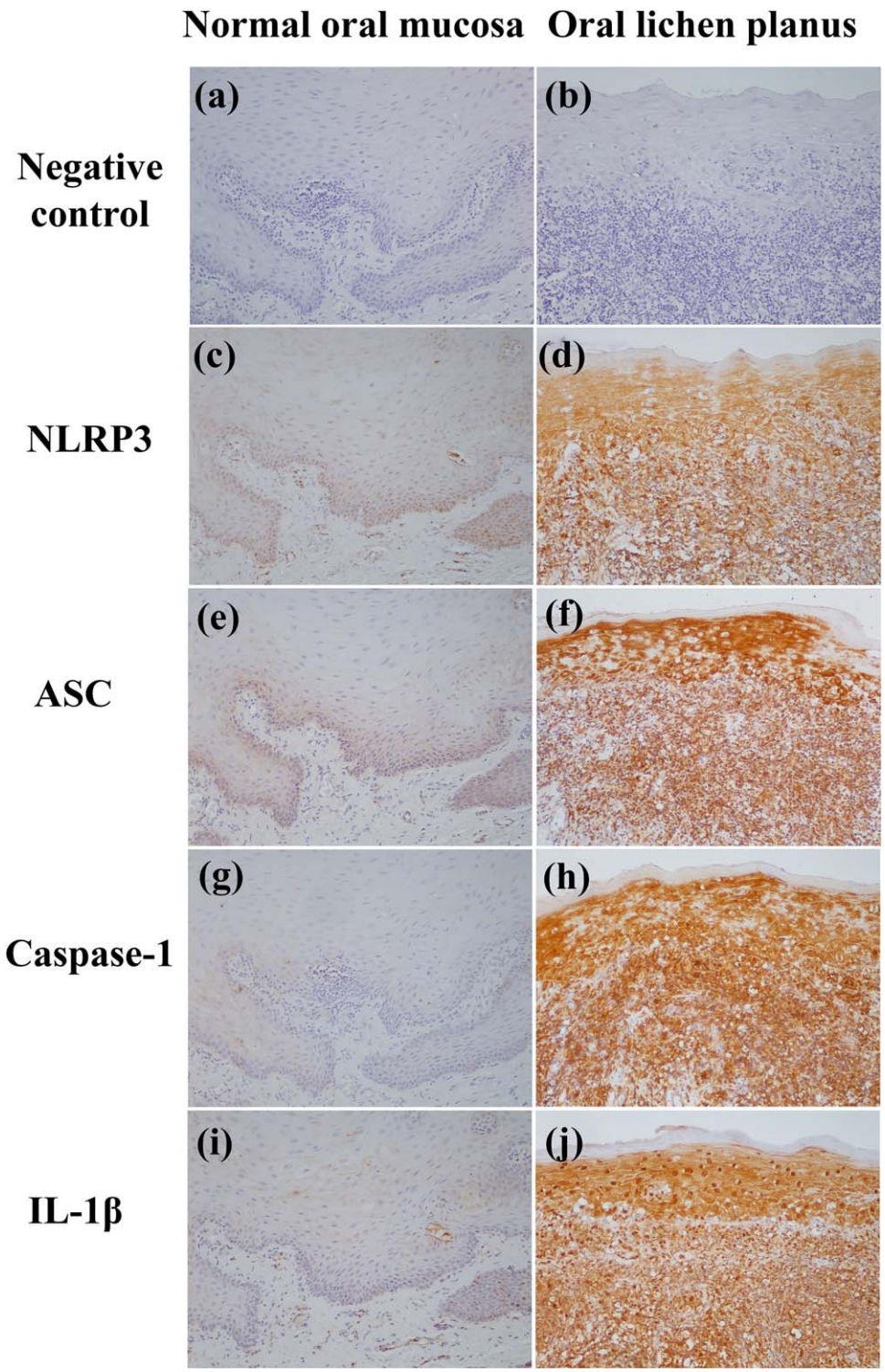

Fig. 3. Expression of nucleotide-binding domain-like receptor family pyrin domain containing 3 (NLRP3), apoptosis-associated speck-like protein containing a C-terminal caspase recruitment domain (ASC), caspase-1 and interleukin (IL)-1β in normal oral mucosae (c,e,g,i) and oral lichen planus (OLP) lesions (d,f,h,j). (a,b) Negative controls. Stronger immunoreactivities of NLRP3, ASC, caspase-1 and IL-1β are observed in OLP lesional mucosae. Original magnification ×200. [Colour figure can be viewed at wileyonlinelibrary.com]

ASC, caspase-1 and IL-1β (Fig. 4). In addition, these investigated molecules are associated with danger associated molecular pattern (DAMP) molecules such as heat shock protein (HSP)60 and HSP90. Details of these analysed molecules are demonstrated in the Supporting information, Table S3.

Discussion

In the present study, increased immunohistochemical expression of O-GlcNAcylated proteins (OGP) and O-GlcNAcylation associated enzymes (OGT and OGA) was detected in OLP lesional mucosae. One previous study

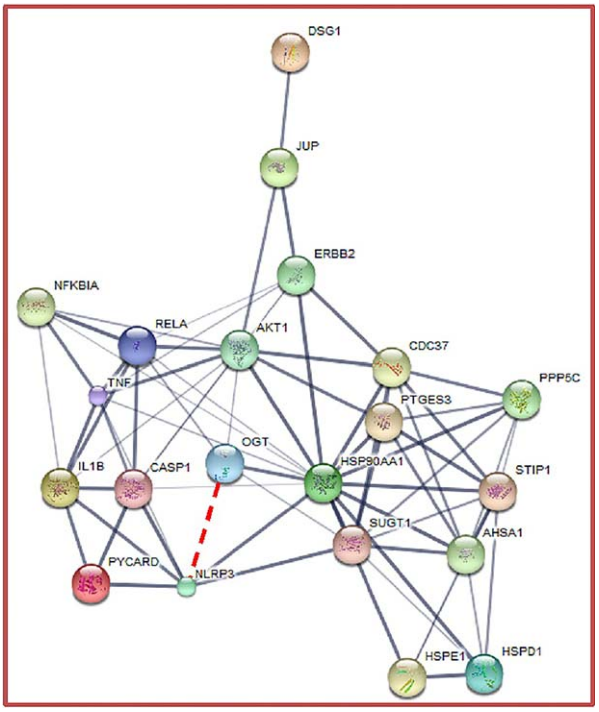

Fig. 4. Data analysis by Search Tool for Interacting Chemicals (STITCH) version 5.0 for prediction of relationships among: O-GlcNAcylation (OGT: O-GlcNAc transferase); nuclear factor kappa B (NF-Kb) signaLling (RELA: transcription factor p65; TNF: tumour necrotic factor); danger-associated molecular patterns [hear shock protein (HSP)D1: HSP60; HSP90AA1: HSP90]; nucleotide-binding domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome [NLRP3, cASP1: caspase-1, interleukin (IL)-1β)]; epithelial cell differentiation and cell adhesion (DSG1: desmoglein1; JUP: junction plakoglobin) (http://stitch.embl.de/cgi/network.pl?taskId=1t51EemYpTt0). The red dashed line was established to demonstrate the correlation between OGT and NLRP3 according to the immunohistochemical analyses. [Colour figure can be viewed at wileyonlinelibrary.com]

demonstrated that an increase in O-GlcNAcylation in tissue specimens from patients with cholangiocarcinoma was due to increased expression of OGT but decreased expression of OGA [32]. These findings suggest that regulation of O-GlcNAcylation is dynamic and may depend not only on the expression levels of its cycling enzymes (OGT and OGA), but also on its enzymatic activities [12]. O-GlcNAcylation was reported to play a role in epithelial cell differentiation and cell adhesion [33–35]. Regarding the clinicopathogenesis of OLP, it would be beneficial to identify specific O-GlcNAcylated proteins at the sites of the OLP lesional mucosa and to investigate whether changes in O-GlcNAcylated proteins during chronic inflammation might interfere with homeostasis of oral epithelial cell differentiation and cell adhesion, contributing to clinical heterogeneity of OLP. In addition, further studies are needed to clarify the role of O-GlcNAcylation in activation of immune responses at the site of inflamed oral mucosa.

Regarding the expression of NF-κB signalling molecules, these results are similar to previous studies [7–9], demonstrating an increase in expression of NF-κB and TNF-α in OLP. Moreover, increased levels of NLRP3 inflammasome (NLRP3, ASC, caspase-1 and IL-1β) in OLP are in agreement with previous studies in other chronic inflammatory diseases [19–21,36]. Correlations between NF-κB signalling and NLRP3 inflammasome activation have been described in some studies [22,37]. It was suggested that NF-κB signalling was important for correct activation of the NLRP3 inflammasome [23]. In other studies, ASC and caspase-1 were reported to modulate NF-κB activation [38–40]. Dysregulated NLRP3 inflammasome expression was reported to be associated with autoinflammatory diseases [24,25,41]. Considering these observations, an increased expression of NLRP3 inflammasome in oral epithelial cells and infiltrated immune cells at the sites of OLP lesional mucosae possibly reflects cellular responses to pathological stimuli. DAMP molecules such as HSPs have been demonstrated to play a role in NLRP3 inflammasome activation [42,43]. Previous studies have demonstrated aberrant expression of HSP60 and HSP90 in OLP lesions [44,45]. Taking these findings into account, it is temping to postulate that altered expression of DAMP molecules such as HSP60 and HSP90 due to pathological stimuli may induce activation of the NLRP3 inflammasome in OLP. Future studies should elucidate the role of HSP60, HSP90, O-GlcNAcylation and NLRP3 inflammasome in the pathogenesis of OLP.

Regarding correlations among O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome, previous studies have demonstrated that increased O-GlcNAcylation enhanced the activation of NF-κB, and promoted an inflammatory response in several chronic inflammatory diseases [14,15]. In addition, it was reported that O-GlcNAcylation played a role in activation of T lymphocytes [46,47], and OGT activity was necessary for functions of effector T cells [48]. Previous studies have also demonstrated NLRP3 inflammasome-regulated activation of T helper cells [49,50]. The current study demonstrated increased levels of OGT and NLRP3 inflammasome in infiltrated lymphocytes at the sites of OLP lesions. Thus, it is possible that O-GlcNAcylation and NLRP3 inflammasome may be associated with activation of T lymphocytes in OLP. In addition, NLRP3 inflammasome was reported to be involved in pyroptosis, which was shown to be a form of programmed cell death under an inflammatory condition [51]. This molecular mechanism could be one possible explanation for the degeneration of basal epithelial cells in OLP.

In conclusion, there are two important findings from these current observations. First, increased levels of O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome in OLP lesional mucosae were demonstrated. Secondly, positive correlations among O-GlcNAcylation, NF-κB signalling molecules and NLRP3 inflammasome were also observed in OLP. The present findings provide supportive evidence of a connection

between O-GlcNAcylation and NLRP3 inflammasome in relation to autoinflammation. In addition, this study provides incitement for innovative future research to unravel the immune-mediated inflammatory signalling processes in the oral mucosal environment that would help to clarify the immunopathogenesis of OLP.

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Author contributions

T. T. D. contributed to collection of tissue samples and clinical data, design of experiments and analysis of the data. C. P., V. C. and A. S. contributed to design of the experiments and analysis of the data. P. C. contributed to design of the experiments, analysis of the data and interpretation of the results. T. T. D. and P. C. wrote the manuscript. All the authors critically reviewed the manuscript and approved the final version for submission.

Ethical clearance

This study was approved for the use of tissues from human subjects by the Institutional Human Ethics Committee of Khon Kaen University (HE602071).

Disclosure

The authors declare that they have no disclosures related to this manuscript.

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Supporting information

Additional Supporting information may be found in the online version of this article at the publisher's web-site:

Table S1. List of antibodies used for immunohistochemical staining.

Table S2. Analyses of correlations among the expression of O-GlcNAcylation, nuclear factor kappa B (NF-κB) signalling molecules and nucleotide-binding domain-like receptor family pyrin domain containing 3 (NLRP3) inflammasome in the oral lichen planus (OLP) group using SPSS Statistics version 17.0.

Table S3. Full names and functions of investigated molecules analysed by Search Tool for Interacting Chemicals (STITCH) version 5.0.