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Brief communication (Original)

Trefoil factor expression by immunohistochemistry in patients with oral lichen planus

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Background: Oral lichen planus (OLP) is a chronic immune-mediated inflammatory disease that usually causes oral mucosal damage. OLP has been considered to be a potentially malignant disorder associated with an increased risk for oral cancer. Trefoil factors (TFFs) are mainly synthesized by mucin-producing epithelial cells. Expression of TFFs in oral epithelia is thought to be an essential factor in protection against oral mucosal damage. However, very little is known about the connection between tissue damage of oral mucosa in OLP patients and expression of TFFs.

Objectives: To determine levels of TFF expression in oral mucosa from patients with OLP.

Methods: Tissue samples were collected from 35 patients with normal oral mucosa (controls) and 35 patients with OLP. An immunohistochemical method was used to determine the expression of the 3 TFFs (TFF1, 2, and 3) in mucosal tissues of the oral cavity.

Results: TFF2 and TFF3 expression in oral epithelia were significantly decreased in OLP patients (Mann–Whitney *U* test; $P = 0.006$, and $P = 0.002$, respectively). There were no significant differences in TFF1 expression between OLP patients and control subjects.

Conclusion: The present study demonstrated reduced expression of TFF2 and TFF3 in oral epithelia of OLP patients. These outcomes support our previous observations that chronic inflammation may play a major role in downregulation of TFF expression, which may be associated with oral mucosal damage in OLP patients.

Keywords: Chronic inflammation, immunohistochemistry, oral lichen planus, trefoil factor

Trefoil factors (TFFs) are small and stable molecules mainly derived from mucin-producing epithelial cells and constitute a family of short polypeptides linked by 3 disulfide bonds that form a stable trefoil motif. Human TFFs comprise three members, TFF3, TFF2, and TFF1 [1]. TFFs have been implicated in several physiological and pathological functions including cytoprotection and wound healing, the immune response, and tumorigenesis [1, 2]. TFF expression can be regulated by various signaling pathways. NF-κB signaling pathways and proinflammatory cytokines such as interleukin (IL)-1β and IL-6 are implicated in the downregulation of TFF [3], whereas Src homology 2 domain-containing Src homology tyrosine phosphatase (SHP2)/extracellular signal-regulated kinase (Erk) signaling pathways are involved in stimulation of TFF expression [4]. TFFs are detected in various oral compartments, including the oral mucosa, gingival tissues, and saliva [5, 6]. Salivary TFFs originate mainly from salivary glands, with minor contributions from parotid duct component goblet and epithelial cells [7-11]. TFF3 is a regulatory factor for signaling pathways involved in cell proliferation, migration and proliferation, of keratinocytes from the oral mucosa [12, 13]. These findings suggest that expression of TFFs in saliva and oral epithelial cells may be essential in protection against oral mucosal tissue damage. Several pathological conditions, such as chronic inflammation, can induce damage of the oral mucosa. Whether there is any connection between chronic inflammation and altered TFF expression in oral tissues is poorly understood.

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Oral lichen planus (OLP) is a chronic immune-mediated inflammatory disease that usually causes mucosal damage, resulting in ulcerations of oral mucosa. Histopathological characteristics of OLP include intense subepithelial infiltration of T-lymphocytes, degeneration of basal keratinocytes, and epithelial basement membrane disruption [14, 15]. Altered expression of surviving and heat shock protein 90 occurs in OLP and is associated with chronic inflammation [16]. OLP is considered to be a potentially malignant disorder associated with an increased risk for oral cancer [17]. The malignant transformation risk of OLP has been investigated in terms of oxidative and nitrative DNA damage and oxidative stress, cell apoptosis, and cell proliferation [18-21]. However, the exact mechanisms of chronic immune-mediated inflammation and malignant transformation of OLP remain unclear. Our recent study demonstrated reduced TFF3 and TFF2 in the oral mucosa of patients with oral squamous cell carcinoma, which were associated with chronic inflammation [5]. In addition, we found the reduction of TFF3 in inflamed gingival tissues of patients with chronic periodontitis [6]. According to our observations and previous studies, chronic inflammation may play a major role in downregulation of TFF expression. It would be of interest to determine if there is any association between chronic inflammation and alteration of TFF expression in oral mucosal diseases such as OLP because this may clarify the transition from OLP to malignancy. The present study aimed to extend our previous observations on TFF expression under the pathological condition of chronic inflammation by examining TFF expression in the oral mucosa from patients with OLP compared with controls.

Materials and methods

Tissue specimens from the oral mucosa

After approval of this study by the institutional human ethics committee of Khon Kaen University (HE522010), we obtained 35 biopsy specimens from OLP patients and 35 normal control biopsies from patient participants who had signed written informed consent for their participation in the study. Tissue samples were anonymized with respect to patient identity, fixed in formalin, and embedded in paraffin. Modified World Health Organization diagnostic criteria [22] and criteria proposed by Gandolfo et al. [23] were used for the diagnosis of OLP. Severity of OLP lesions was assessed clinically according to the criteria set by Thongprasom et al. [24]. Tissue biopsy specimens were confirmed by histopathologic examination. Oral mucosal tissue from the buccal mucosa of OLP patients (27 women and 8 men, with a mean age of 51 years (range 31–78 years)) were examined. The biopsies of normal oral mucosa were from the retromolar mucosa of healthy individuals consisting of 26 women and 9 men who had undergone impacted tooth removal (mean age of 20 years (range 18–30)). All OLP patients presented with a burning sensation and pain, and were classified into an atrophic-ulcerative category. No OLP patients took medications, smoked cigarettes, or had systemic diseases. All control individuals demonstrated normal oral mucosa. Likewise none had took medication, smoked cigarettes, or had systemic diseases.

Immunohistochemistry

Serial sections with 5 μm thickness were cut from each biopsy, and were mounted on glass slides. Sections were deparaffinized in xylene, hydrated through a graded series of alcohol concentrations, and washed with phosphate buffered saline (PBS). Endogenous peroxidase activity was quenched by Peroxo-Block (Invitrogen Life Technologies, Paisley, UK). Antigen retrieval by microwave for 5 min was performed in 10 mmol/L sodium citrate buffer at a pH of 6.0, followed by blocking of nonspecific antibody binding sites with Protein Block serum-free (DAKO, Carpinteria, CA, USA). Monoclonal anti-human TFF1 (Sigma-Aldrich, St. Louis, MO, USA), TFF2 (R&D Systems, Minneapolis, MN, USA), and TFF3 (R&D Systems) antibodies were used in this study. Primary antibodies were used at the following dilutions; 1:100 for TFF3 antibody, 1:50 for TFF2 antibody, and 1:100 for anti-human TFF1 antibody. The immunodetection system was based on a horseradish peroxidase (HRP)-labeled polymer, which was conjugated with secondary antibodies (DAKO EnVision+ System-HRP labeled polymer anti-rabbit). 3,3′-Diaminobenzidine (DAB, DAKO) was used as the substrate chromogen. The sections were counterstained with hematoxylin and then were dehydrated, cleared, and mounted. Gastric and colon cancer tissues were used as positive controls, and negative controls were achieved by omitting primary antibodies and substituting with PBS. Upon microscopic examination at an original magnification of 20×, the whole area of oral epithelial layers was selected for analysis of immunoreactive

(positively-stained) cells. The distributions of positively-stained cells were evaluated visually by scanning the slide systematically. Assessment of the distribution of positively-stained cells was inferred from immunostaining scores as: 0 = no immunostained cells; 1 = low distribution (less than 25% positively-stained cells); 2 = moderate distribution (25% to 50% positively-stained cells); 3 = high distribution (>50% positively stained cells).

Statistical analysis

Comparison of TFF immunostaining scores between OLP and control groups was conducted using a Mann–Whitney *U* test. Correlations between TFF immunostaining scores and severity of OLP lesions were assessed using Spearman's rank correlation coefficient. Significance was established at $P < 0.05$.

Results

Assessment of TFF immunohistochemical staining in biopsies

Examination of the distribution of TFF immunostaining in biopsy specimens from control subjects and OLP patients demonstrated that TFFs were detected predominantly in the cytoplasm of oral epithelial cells of the basal and suprabasal layers (**Figures 1 and 2**). In some OLP specimens, expression of TFFs was also demonstrated in inflammatory cells located in

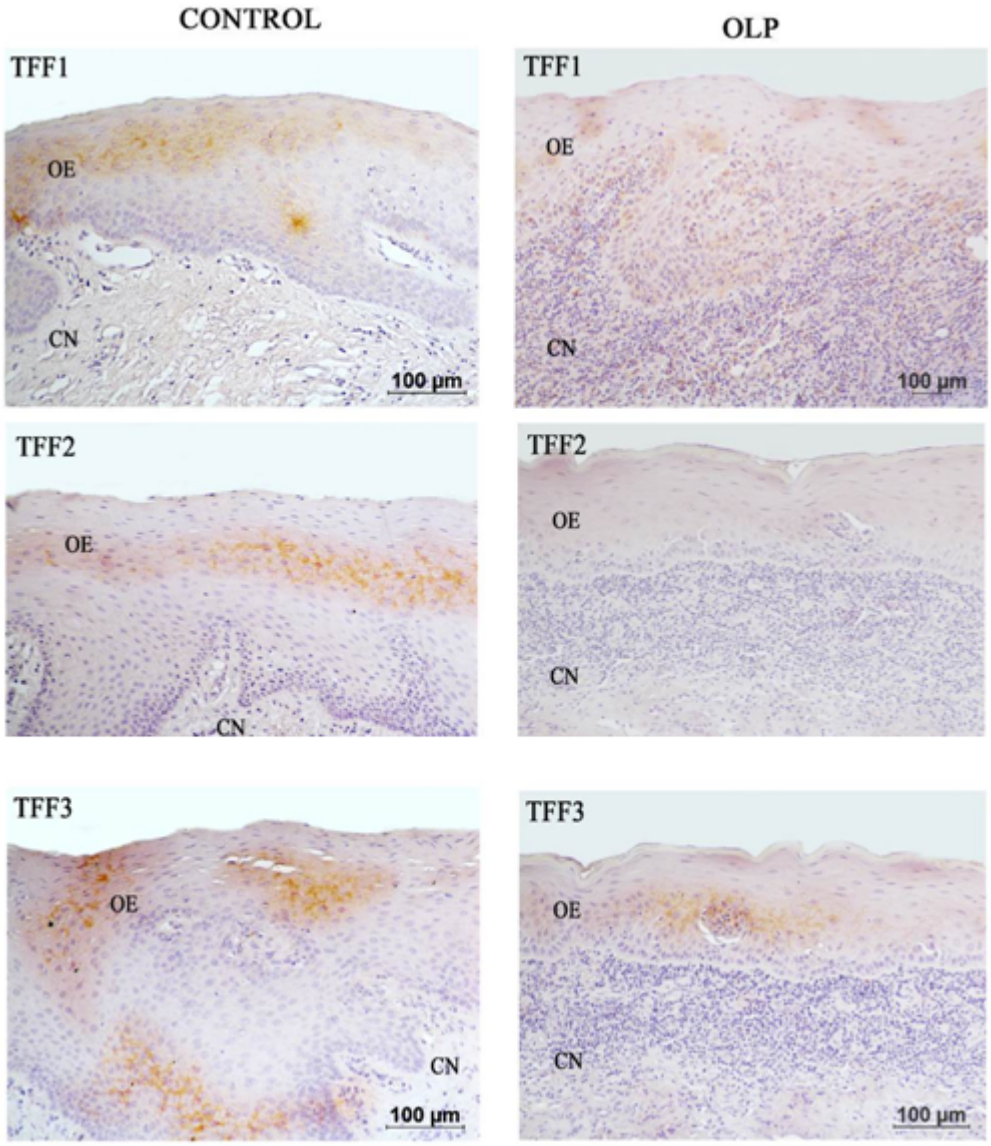

Figure 1. Immunohistochemical examination (original magnification 20×) of the distribution of trefoil factor (TFF)1, TFF2, and TFF3 in normal oral mucosa from control subjects and oral mucosal tissues from patients with oral lichen planus (OLP). Distribution of TFF1 expression in control and OLP is evident in oral epithelia (OE). In OLP, expression of TFF1 is also demonstrated in inflammatory cells located in connective tissues (CN). Distribution of TFF2 expression is demonstrated in the oral epithelia of the controls, whereas no TFF2 expression is observed in OLP. Distribution of TFF3 expression in control and OLP is seen in oral epithelia.

connective tissues. TFF expression in oral epithelia as detected by immunostaining was evaluated semiquantitatively with outcomes presented in **Table 1**. There was more apparent variability in TFF1 expression, as compared with TFF2 and TFF3, between control subjects and OLP patients. However, no significant differences in overall TFF1 expression were demonstrated between the two groups (**Table 1**). For TFF2 expression, most control specimens demonstrated low, but consistent levels of TFF2, whereas most OLP cases showed no TFF2 expression. Immunostaining scores of TFF2 expression were significantly decreased in OLP patients as compared with control subjects (Mann–

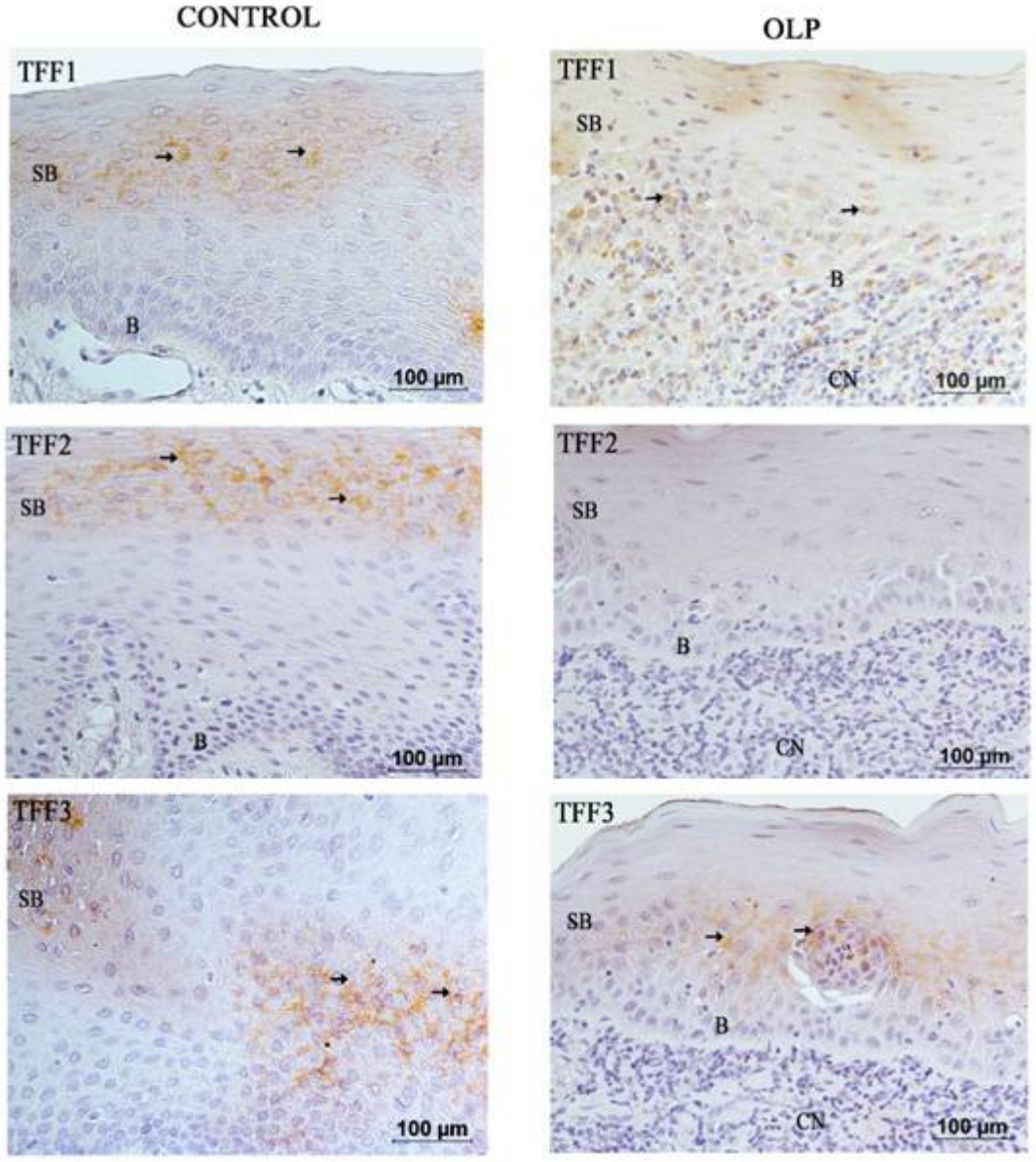

Figure 2. High power view (original magnification 40×) of the cellular distribution of trefoil factor (TFF)1, TFF2, and TFF3 in normal oral mucosa from control subjects and oral mucosal tissues from patients with oral lichen planus (OLP). In the control group, expression of TFF1, TFF2, and TFF3 were mainly demonstrated in the cytoplasm (arrows) of epithelial cells in the suprabasal (SB) layer. In the OLP group, expression of TFF1 is seen in the cytoplasm (arrows) of epithelial cells in the suprabasal (SB) and basal (B) layers. TFF1 expression is also demonstrated in inflammatory cells located in connective tissues (CN). No expression of TFF2 is seen in OLP. Expression of TFF3 in OLP is demonstrated in the cytoplasm (arrows) of oral epithelial cells in the suprabasal (SB) layer.

Whitney *U* test; *P* = 0.006) (**Table 1**). All specimens from control subjects demonstrated expression of TFF3, which varied from low to high numbers of TFF3 positively-stained cells. By contrast with the control group, three cases of OLP demonstrated no TFF3 expression and most OLP specimens showed a low distribution of TFF3 positively stained cells. Expression of TFF3 was significantly decreased in OLP patients as compared with control subjects (Mann–Whitney *U* test; *P* = 0.002) (**Table 1**). There were no significant correlations between TFF immunostaining scores and severity of OLP lesions.

Discussion

In the present study, we determined TFF expression in the oral mucosa from patients with OLP and control subjects using immunohistochemistry. There was a marked age discrepancy between the comparator groups, so that OLP patients tended to be older than the control subjects. Thus, a marked difference in age might affect our findings because a previous study demonstrated the correlation between aging and TFF expression [25]. According to our observations, there were no significant differences in TFF1 expression, whereas TFF2 and TFF3 expression was reduced in oral epithelia of OLP patients. These findings suggest that regulatory mechanisms for the expression of TFF2 and TFF3 in response to chronic inflammation may be different from those for TFF1 expression in oral epithelia of OLP patients. Inflammation-mediated signalling via NF-κB is associated with inhibition of TFF expression [3]. In addition, NF-κB expression is increased in the oral mucosa of OLP patients [26]. Considering these findings, it is tempting to hypothesize that chronic inflammation in OLP mediates the decreased expression of TFF2 and TFF3 in oral epithelia via the activation of the NF-κB signaling pathway, resulting in the inhibition of protective mechanisms of these trefoil factors in the oral mucosa of OLP patients. Nonetheless, TFF1 may have its own expression profile in response to activation of NF-κB. This issue would be of great interest and certainly warrants further investigation of the association between NF-κB signaling pathway and regulation of TFF expression in response to chronic inflammation and oral mucosal damage in OLP patients.

The present study did not demonstrate any significant correlation between levels of TFF immunostaining scores and severity of oral mucosal tissue damage in OLP patients. It is possible that the semiquantitative methods for evaluating expression of TFFs in oral epithelia and for grading oral mucosal tissue damage in OLP lesions are not sufficiently sensitive to detect such correlations. However, accumulated evidence supports a functional role of TFFs in cytoprotection against tissue damage. TFF3 increases migration of oral epithelial cells [12]. TFF3 can serve as a potential tool for the prevention and healing of mucosal ulcerations [27, 28]. Considering these results, TFF3 may enhance oral mucosal repair mechanisms and may have future therapeutic implications for the treatment of patients with oral ulcerations. However, the safety of using TFF3 must be taken into account because of their capability to induce cell proliferation, angiogenesis, and invasiveness in some tissues [29].

Table 1. Immunostaining scores of trefoil factor (TFF) expression in oral epithelia of normal oral mucosa (NOM) from control subjects and oral mucosa from patients with oral lichen planus (OLP).

Immunostaining scores[a]	TFF1		TFF2*		TFF3**	
	NOM (n = 35)	OLP (n = 35)	NOM (n = 35)	OLP (n = 35)	NOM (n = 35)	OLP (n = 35)
0	1	2	7	18	–	3
1	16	19	23	15	15	24
2	10	5	5	2	12	5
3	8	9	–	–	8	3

[a]Immunostaining scores were graded as: 0 = no immunostained cells; 1 = low distribution (less than 25% positively stained cells); 2 = moderate distribution (25% to 50% positively stained cells); 3 = high distribution (>50% positively stained cells). *P = 0.006, **P = 0.002.

Reduced immunoreactivity of TFF2 and 3 in the oral epithelia of OLP patients as demonstrated in the present study, is in agreement with our previous observations showing a similar inhibition of these trefoils in inflamed mucosal tissues of patients with oral squamous cell carcinoma [5]. These findings imply a possible connection between inflamed oral epithelia of patients with OLP and a malignant transformation risk for oral squamous cell carcinoma. It could be hypothesized that, under chronic inflammation of OLP, the reduction of TFF2 and TFF3 expression may affect cytoprotective mechanisms and interfere in the balance between cell proliferation and apoptosis in oral epithelia, leading to the initiation of malignant transformation. Thus, reduction of TFF2 and TFF3 expression may be useful for development of biomarkers for evaluating malignant transformation in the oral mucosa of patients with OLP. However, we cannot exclude the possibility that confounding factors such as difference in age; variations in tissue fixation and processing; epitope retrieval methods; types of primary antibodies; immunohistochemical detecting systems; and criteria for grading immunostains might cause discrepancies in the interpretation of the main outcomes. Therefore, standardization of immunohistochemical methods and establishment of criteria for collecting tissue from patients with OLP are of importance for validating TFF2 and TFF3 as risk indicators for malignant transformation. Suitable in vitro and in vivo models, and substantial prospective studies are needed to verify this hypothesis.

In conclusion, the present study provides additional information about chronic inflammation and alteration of trefoil factor disposition in mucosal tissues of the oral cavity. The present findings are consistent with our previous observations by demonstrating reduced reduction in expression of TFF2 and 3 in inflamed oral epithelia of patients with OLP as detected by immunoreactivity. Such inhibition of these key regulatory trefoil factors may affect cytoprotective mechanisms in the oral mucosa of patients with OLP. Further molecular investigations are needed to verify regulation of TFF expression in response to chronic inflammation and oral mucosal tissue damage in patients with OLP. In addition, investigations on the functional roles of TFF2 and 3 against oral mucosal tissue damage could be of importance for the future treatment of patients with OLP.

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The authors declare that they have no conflicts of interest.

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