Investigation of trefoil factor expression in saliva and oral mucosal tissues of patients with oral squamous cell carcinoma

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Short running title:
Trefoil factors in patients with oral squamous cell carcinoma

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Abstract

Objectives: The aims of our study were to determine levels of trefoil factor (TFF) peptides in saliva and oral mucosal tissues from patients with oral squamous cell carcinoma (OSCC), and to evaluate whether individual members of TFFs (TFF1, TFF2, and TFF3) might act as biomarkers of disease.

Materials and Methods: Saliva samples were from 23 healthy subjects, and 23 OSCC patients. Tissue samples were collected from 32 normal oral mucosa (NOM) and 32 OSCC biopsy specimens. ELISA and immunohistochemical methods were used to evaluate the expression of TFF1, TFF2, and TFF3 in saliva and oral mucosal tissues, respectively.

Results: Expression of TFF2 and TFF3 in oral mucosal tissues of OSCC patients was strongly down-regulated when compared to healthy subjects ($p<0.001$ and $p=0.002$, respectively). However, there were no differences in levels of salivary TFF concentrations between OSCC patients and healthy subjects.

Conclusions: The present study extends previous observations, demonstrating the reduction of TFF2 and TFF3 expression in oral mucosal tissues of OSCC patients.

Clinical Relevance: These findings suggest the clinical significance of TFF2 and TFF3 molecules as negative markers of tumor progression in OSCC. Quantification of TFF levels in saliva may not be optimal in terms of diagnostic or predictive value for OSCC derived from oral mucosa.

Keywords: ELISA; immunohistochemistry; oral cancer; saliva; trefoil factors

Introduction

Trefoil factors (TFFs) belong to a family of short peptides with disulfide bonds that form a trefoil domain. Human TFFs consist of three members, TFF1, TFF2, and TFF3 [1-3]. TFF1 contains one trefoil domain and can be found as a monomer or a dimer with molecular weight of 6.5 kDa and 14 kDa, respectively. TFF2 contains two trefoil domains with approximate molecular weight of 12 kDa. Molecular weight of TFF2 can be variable due to glycosylation. TFF3 contains one trefoil domain and has a molecular weight of 6.6 kDa in a monomeric form or 13 kDa in a dimeric form [4]. The genes encoding these TFF peptides are located on human chromosome 21q22.3 [5]. TFF peptides are secreted from mucin-producing epithelial cells of the gastrointestinal tract [6] and TFFs were detected in other tissues such as brain, tear ducts, salivary glands, parotid ducts and oral mucosa, respiratory tract, lymphoid tissue, liver and gall bladder, and uterus [7-16]. Secreted TFF peptides were detected in serum, saliva, milk, gastric juice, pancreatic fluid, and urine [17-23].

Previous studies demonstrated that TFF expression was upregulated by various factors including local protein regulators such as growth factors [24, 25], growth hormones [26, 27], and several cytokines such as IL-4 and Il-13 [28]. In contrast, TFF expression was downregulated by proinflammatory cytokines such as IL-1beta and IL-6 [29] and by microbial pathogens such as *Helicobacter pylori* [30, 31]. It was suggested that the SHP2/Erk or JAK/STAT signaling pathways [32] might be involved in the upregulation of TFF expression, whereas C/EBPβ and NFκB signaling pathways were associated with downregulation of TFF expression [29]. Detailed regulation of the TFF expression has been reported elsewhere [33]. TFFs are involved in several biological functions such as cytoprotection against tissue damage [34-36], the immune response [37, 38], and tumorigenesis [39-41]. Informative data on the biological functions of TFFs in oral compartment are very limited. Recent studies reported that TFF3 was a modifying factor in

signaling pathways which were involved in cell survival, cell proliferation, and cell migration of oral keratinocytes [42, 43].

Aberrant expression of TFFs in tissues of human solid tumors such as breast, lung, stomach, colon, and prostate cancers has been demonstrated, and detailed expression of TFF peptides in human solid tumors of various organs has been reviewed elsewhere [44]. In addition, changes in TFF concentrations in serum of patients with prostate cancer **has been** reported [45]. It has been suggested that TFFs have contradictory roles in human carcinogenesis as tumor suppressor and tumor progression factors, depending on the site of expression [46]. However, the clinical significance of TFF expression in **saliva** and oral mucosal tissues in patients with oral squamous cell carcinoma (OSCC) has never been identified. Therefore, the aim of the present study was to quantify TFF concentrations in saliva and determine their expression patterns in oral mucosa from patients with OSCC as compared to those in healthy subjects.

Materials and Methods

Saliva specimens

There were 46 participants in this study including 23 OSCC patients, and 23 healthy subjects. The approval of the ethical committee for the use of human subjects, Khon Kaen University (HE480239) was obtained. OSCC patients were recruited from Otorhinolaryngology Clinic, Faculty of Medicine, and Oral and Maxillofacial Surgery Clinic, Faculty of Dentistry, Khon Kaen University, Thailand. OSCC patients consisted of 7 women and 16 men with a median (range) age of 62 (26-87) years. Upon histopathologic findings, 10 cases were graded as well differentiated, 7 cases as moderate differentiation, and 6 cases as poor differentiation. Healthy individuals were recruited from Oral Diagnosis Clinic, Faculty of Dentistry, Khon Kaen University, and consisted of 10 women and 13 men with a median (range) age of 51 (42-77) years. Regarding the medical records of healthy subjects, none had systemic diseases or took medications. All participants refrained from eating or drinking one hour before saliva collection. Each participant was asked to expectorate whole saliva into a 50-mL centrifuge tube, and a final saliva volume of 3 to 5 mL was collected. All saliva samples were immediately placed on ice for transport. Samples were vortexed for 3 minutes, followed by centrifuging at 2300g for 10 minutes. Supernatants were stored at -80^{0}C until further analysis.

Oral mucosal tissue specimens

Thirty two formalin-fixed and paraffin-embedded biopsy specimens from OSCC patients and 32 normal oral mucosa (NOM) specimens from healthy subjects were retrieved from the archives of Division of Oral Pathology, Department of Oral Diagnosis, Faculty of Dentistry, Khon Kaen University, Thailand. Clinicopathologic characteristics of OSCC and clinical information of NOM are as follows. OSCC specimens were from 18 women and 14 men with a median (range) age of 65 (39-85) years. **OSCC** tissue specimens were from

buccal mucosa (n=12), tongue (n=5), floor of mouth (n=5), gingiva (n=4), retromolar area of mandible (n=4), and palate (n=2). Upon histopathologic findings, 11 cases were graded as well differentiated, 11 cases as of moderate differentiation, and 10 cases as poor differentiation. The biopsies of NOM were from the normal retromolar mucosa (no clinical sign of inflammation) of healthy individuals consisting of 20 women and 12 men undergoing impacted tooth removal with a median (range) age of 20 (18-30) years.

Measurement of salivary TFF1, TFF2 and TFF3 peptides

Salivary supernatants were placed on ice and treated with ultrasound for 10 seconds to disrupt the mucus. Total protein concentrations from saliva samples were determined to test whether any differences in TFF peptide concentrations could be ascribed to differences in total salivary protein concentrations, employing the BCA Protein Assay (PIERCE, VWR international). ELISA assays previously characterized [47] for measurement of TFF1, TFF2 and TFF3 in human saliva were performed. Briefly, the TFF1-ELISA is based on two polyclonal rabbit antibodies, the TFF2-ELISA on a monoclonal mouse antibody and a polyclonal rabbit antibody, and the TFF3-ELISA on two polyclonal rabbit antibodies. Recombinant human TFF1, TFF2, and TFF3 peptides were used as calibrators. Anti-human TFF1, TFF2, and TFF3 antibodies and recombinant human TFF1, TFF2 and TFF3 peptides were a kind gift from Lars Thim, Novo Nordisk, Denmark. Salivary supernatants were diluted with assay buffer and further dilutions were performed if the initial dilution was too high or too low to allow quantification of the TFF peptides in question. Recombinant human TFF1, TFF2, and TFF3 peptides as calibrators were diluted in assay buffer with concentrations ranging from 0.005-0.171 nmol/L for TFF1, 0.006-0.200 nmol/L for TFF2 and 0.003-0.183 nmol/L for TFF3. Imprecision as judged from serum pools run over a period of 1 month (mean nmol/L [%CV]) are as follows: TFF1 (n=6): 0.013 [12], and 0.058 [10]; TFF2 (n=6): 0.010 [17] , and 0.042 [10;] TFF3 (n=6): 0.008 [7] and 0.031 [7]. All data of salivary

TFF concentrations were normalized for total protein concentrations and analyzed as nmol TFF/g protein. According to statistical characteristics of the data, Mann- Whitney test was selected to compare differences in levels of salivary TFF1, TFF2, and TFF3 peptides between OSCC patients and healthy subjects. Significance was established at a P-value < 0.05.

Immunohistochemical study

From each biopsy specimen, serial sections 5 μm thick were cut and mounted on glass slide. Antihuman TFF1, TFF2, and TFF3 polyclonal antibodies were used as previously described [15, 48, 49]. **The specificity of the antisera has been tested by lack of cross-reactivity with other family trefoils (both hapten and full length recombinant protein) in immunoassay, Western blotting and immunohistochemistry, as well as the same trefoil from other species. Anti-human TFF1 antibody does not cross-react with human TFF2 or TFF3, nor with mouse or rat TFF1 C-terminal hapten [48]. The anti-human TFF2 antibody cross-reacts with mouse but not rat TFF2, but not TFF1 or TFF3 [15]. The anti-human TFF3 antibody cross-reacts with all species tested (mouse, rat, human), but not TFF1 nor TFF2 [49].** Sections were deparaffinized in xylene, hydrated through graded alcohols, and washed with phosphate buffered saline (PBS). HistoStain-Plus kit (Invitrogen, Carlsbad, CA, USA) was used for immunostaining detection. Endogenous peroxidase activity was quenched for 5 minutes. The sections were then washed in PBS. Microwave-based antigen retrieval was performed for 5 min with 10 mmol/l sodium citrate buffer at a pH of 6.0. The optimal dilutions of primary antibodies were; 1:2000 for antihuman TFF1 antibody, 1:1000 for TFF2 antibody and 1:4000 for TFF3 antibody. Immuno-detection was performed with biotinylated anti-rabbit immunoglobulin, followed by peroxidase labelled streptavidin and aminoethyl carbazole (AEC) as substrate chromogen. All incubations were done in a humidified chamber at room temperature. Gastric and colon cancer tissues were used as positive controls. Biopsy specimens derived from OSCC and NOM were carried out by

substituting primary antibodies with PBS and these specimens were used as negative controls for each group. Upon microscopic examination at original magnification 10X, the distributions of positively stained cells were observed in OSCC and NOM. Semi-quantitative immunostaining scores, based on degree of distribution of positively stained cells in OSCC and NOM, were graded as: 0 = no immunostained cells; 1 = less than 25% positively stained cells; 2 = 25% to < 50% positively stained cells; 3 = 50% to < 75% positively stained cells; 4 $\geq$ 75% positively stained cells. OSCC and NOM specimens were compared for differences in TFF1, TFF2, and TFF3 immunostaining scores. According to the statistical characteristics of our investigated data, Mann-Whitney U test was selected to compare the immunostaining scores between these two groups. Significance was established at a P-value < 0.05.

Results

Levels of salivary TFF peptides in OSCC patients and healthy subjects

The normalized concentrations (nmol/g protein) of salivary TFF1, TFF2, and TFF3 peptides are presented in Table 1. In healthy subjects and OSCC patients, TFF3 was the most abundant TFF peptide in saliva followed by TFF1 and with very little TFF2 detected. No significant differences of TFF peptides were observed between healthy subjects and OSCC patients. There were no significant correlations between levels of salivary TFFs and histopathological grading of OSCC.

Assessment of immunohistochemical staining of TFFs in biopsy specimens

Semi-quantification of TFF expression by immunohistochemistry in oral mucosal tissue specimens was presented in Table 2 and Figure 1. Expression of TFF1, TFF2, and TFF3 was detected in normal oral mucosa (NOM) of control subjects and oral mucosal tissues of OSCC patients (Figure 2). TFF1 expression in NOM was mainly detected in the cytoplasm and surface membrane of oral epithelial cells in basal and suprabasal layers, whereas TFF1 expression in OSCC was mainly detected in the cytoplasm of malignant cells (Figure 2). There were no significant differences in the TFF1 immunostaining scores between controls and OSCC patients. TFF2 expression in NOM was confined mostly to the cytoplasm of oral epithelial cells in basal and suprabasal layers (Figure 2). Most biopsy specimens in OSCC (22 of 32 cases) revealed no demonstrable immunostaining for TFF2 (Table 2). Significant differences in immunostaining scores of TFF2 expression between NOM and OSCC were demonstrated (Mann-Whitney U test; $p < 0.001$), with a marked reduction in TFF2 expression in OSCC compared to NOM (Table 2). TFF3 expression in NOM was mainly detected in the cytoplasm and surface membrane of oral epithelial cells in basal and suprabasal layers, whereas mild TFF3 expression in OSCC was mainly detected in

cytoplasm of malignant cells (Figure 2). Significant differences in immunostaining scores of TFF3 expression between NOM and OSCC were demonstrated (Mann-Whitney U test; p = 0.002), with a marked reduction in TFF3 expression in OSCC compared with NOM (Table 2). There were no significant correlations between TFF expression and histopathological grading of OSCC.

Discussion

In this study, we measured the concentrations of TFF1, TFF2, and TFF3 peptides in whole saliva and determined the expression of TFF peptides in oral mucosal tissues derived from healthy subjects and patients with OSCC. **It should be noted that a marked difference in age between the two investigated groups was observed. The majority of control subjects in our study were young adults while OSCC patients were older. Thus, it is possible that a marked difference in age may affect our findings. To our knowledge, there is one recent study reporting correlations between age and levels of salivary TFF2 and TFF3 in children [50].** Secreted TFFs have been previously detected in various human body fluids [10, 17-23]. Among human serum TFF peptides, TFF1 is the most abundant, followed by TFF3 and TFF2, respectively [17, 18]. In human saliva, our findings demonstrated that TFF3 was predominant, followed by TFF1 and TFF2. A previous study demonstrated increased levels of all three TFF peptides in plasma of patients with advanced prostate cancer [45]. In contrast, no differences in salivary TFF concentrations between healthy subjects and patients with OSCC were demonstrated. TFF peptides in saliva are mainly derived from salivary glands with some contribution from epithelial and goblet cells of the parotid duct or squamous epithelium of oral mucosa [10-12]. Saliva samples in the present study were collected from patients with OSCC lesions of the oral mucosa which did not involve the salivary glands. Only small amounts of secreted TFFs from oral epithelial cells or malignant cells from OSCC lesions likely contributed to total salivary TFFs. Therefore, quantification of TFF levels in saliva may not be optimal in terms of diagnostic or predictive value for pathological condition of OSCC derived from oral mucosa. However, it may be of interest to quantify salivary TFF peptide secretion under pathological conditions such as salivary gland tumors, where TFF expression may be useful biomarkers of disease progression and prognosis.

Although TFF peptides are predominantly expressed by epithelial cells of the gastrointestinal tract [6], one member of the family, TFF3 has been shown to be expressed in normal epithelial cells of parotid duct and oral squamous epithelium [12]. The present study extends these observations, showing that all three TFF peptides are expressed in the normal oral squamous epithelium. The differences in immunostaining results of TFF expression in normal oral mucosa may be due to confounding variables such as fixation, antigen retrieval techniques, types of primary antibodies, and detecting systems. In recent years, association between TFFs and carcinogenesis has been investigated. Either increased or decreased expression of TFFs has been reported in human cancers [44]. Our immunohistochemical results demonstrated a significantly decreased expression of TFF2 and TFF3 in oral squamous epithelium of OSCC patients in comparison to healthy subjects, whereas no significant differences of TFF1 expression between these two groups were observed. The present findings suggest a possible tumor suppressive role of TFF2 and TFF3 in OSCC as has been shown in the stomach [48, 51, 52]. However, such a role needs to be verified using suitable *in vivo* genetic models and cell lines. In addition, the apparent pro-carcinogenic role of some TFF peptides in non-gastric sites, suggests that trefoil factors may have context-specific functions in different mucosae.

There were no significant correlations between the histopathological grading of OSCC and levels of salivary TFF concentrations or levels of TFF immunostaining scores. The explanation may be due to the semi-quantitative method for the histopathological grading of OSCC. It is possible that the semi-quantitative method is not sufficiently sensitive to detect such correlations between histopathological grading of OSCC and levels of salivary TFF concentrations or levels of TFF expression in oral mucosal tissues. In addition, we could not exclude the possibility that TFF expression may not be directly affected by the pathological condition of oral carcinogenesis. One previous study

reported that inflammation-mediated signaling transduction such as NFκB signaling pathway was associated with downregulation of TFF expression [29]. Therefore, TFF expression might be affected by the inflammatory responses in oral mucosal compartments. Further investigations are needed to validate the connection between chronic inflammatory responses in OSCC and regulation of TFF2 and TFF3 expression in oral mucosal compartments.

In conclusion, as for previous reports of altered expression of TFF peptides in patients with various cancers, the present study provided additional information of reduced TFF2 and TFF3 expression in oral mucosal tissues from patients with OSCC. These findings need to be extended in order to determine their clinical significance in OSCC. Therefore, further investigations at a molecular level are needed to clarify mechanisms regulating TFF expression in the oral compartment and the role of TFFs in the development of oral cancer.

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Conflicts of Interest Statement

The authors declare that they have no conflicts of interest.

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Table 1 Levels of salivary TFF peptides in healthy subjects and patients with oral squamous cell carcinoma (OSCC)

	normalised concentrations of TFF peptides (nmol/g proteins)			
	median	interquartile range	minimum	maximum
TFF1				
Healthy subjects (n=23)	2.61	1.46	0.98	8.39
OSCC (n=23)	2.39	3.80	0.36	38.50
(Mann-Whitney U test; p = 0.362)				
TFF2				
Healthy subjects (n=23)	0.07	0.07	0.01	0.38
OSCC (n=23)	0.08	0.13	0.01	0.33
(Mann-Whitney U test; p = 0.852)				
TFF3				
Healthy subjects (n=23)	11.90	9.65	4.27	25.91
OSCC (n=23)	9.76	19.26	2.77	47.10
(Mann-Whitney U test; p = 0.462)				

Table 2 Expression of TFF1, TFF2, and TFF3 in normal oral mucosa (NOM) from healthy subjects and oral tissues from patients with oral squamous cell carcinoma (OSCC).

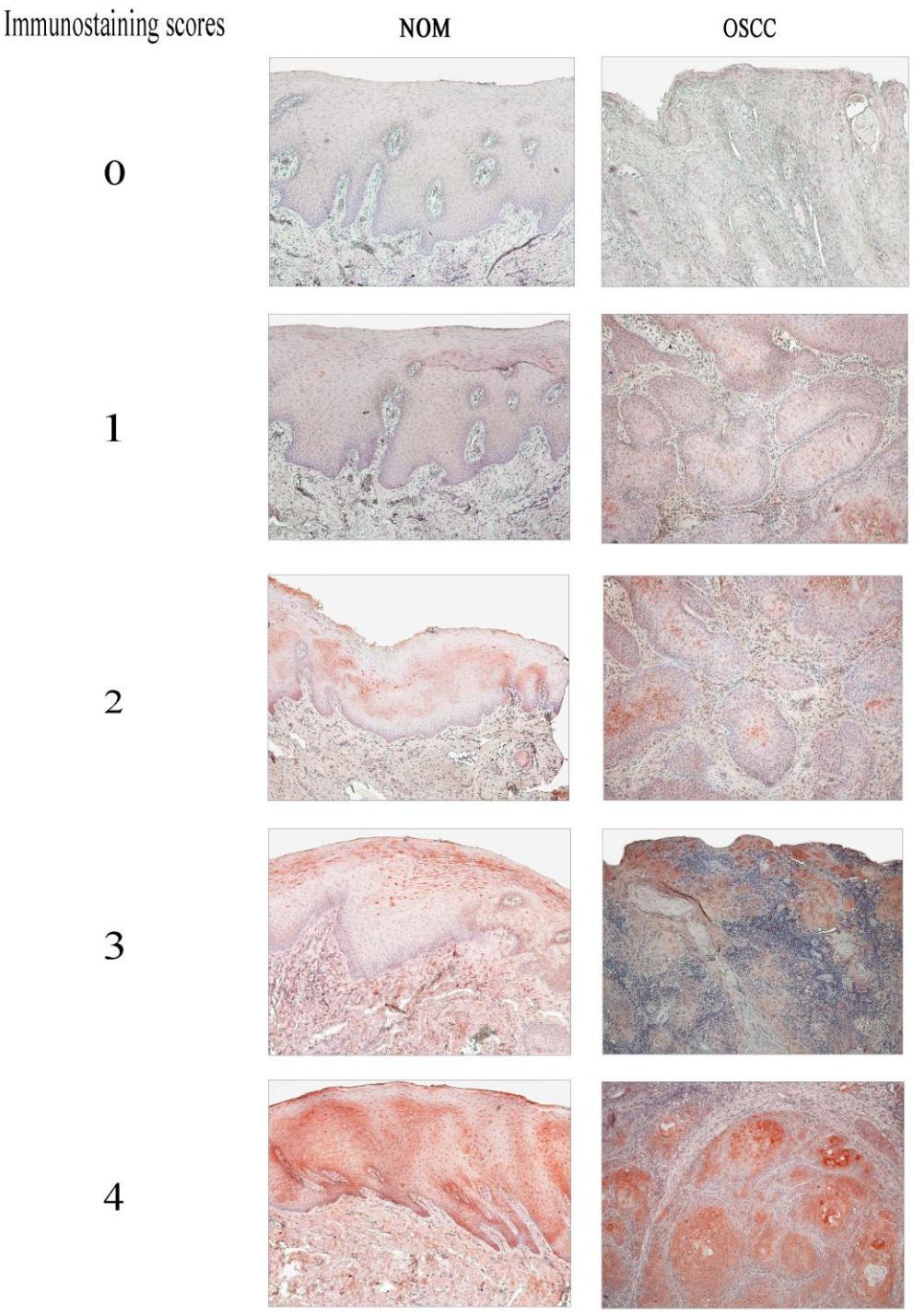
Immunostaining score[a]	TFF1		TFF2*		TFF3**	
	NOM (n = 32)	OSCC (n = 32)	NOM (n = 32)	OSCC (n = 32)	NOM (n = 32)	OSCC (n = 32)
0	-	1	8	22	-	9
1	14	20	19	9	12	14
2	10	5	5	1	12	4
3	7	2	-	-	6	5
4	1	4	-	-	2	-

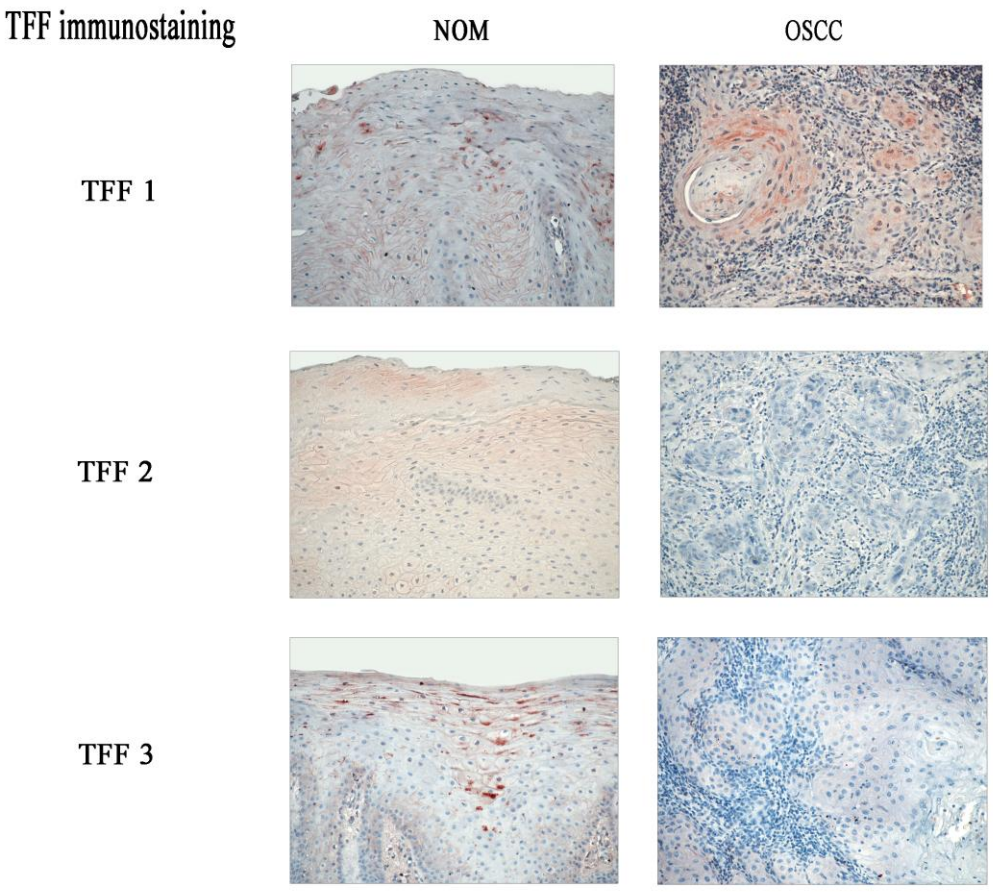
[a]Immunostaining score was graded as: 0 = no immunostained cells; 1 = < 25% positively stained cells; 2 = 25% to < 50% positively stained cells; 3 = 50% to < 75% positively stained cells; 4 $\geq$ 75% positively stained cells. * The asterisk indicates a statistically significant difference of TFF2 immunostaining scores (Mann-Whitney U test; $p < 0.001$) between in NOM and OSCC. ** The asterisks indicate a statistically significant difference of TFF3 immunostaining scores (Mann-Whitney U test; $p = 0.002$) between in NOM and OSCC.

Figure legends

Figure 1 **Semi-quantification of TFF1 expression by immunohistochemistry in oral mucosal tissue specimens of normal oral mucosa (NOM) from healthy subjects and oral mucosal tissues from oral squamous cell carcinoma (OSCC) patients (original magnification 10X). The levels of immunostaining scores are designated as follows: 0 = no immunostained cells; 1 = less than 25% positively stained cells; 2 = 25% to < 50% positively stained cells; 3 = 50% to < 75% positively stained cells; 4 ≥ 75% positively stained cells.**

Figure 2 **Immunostaining of TFF1, TFF2, and TFF3 in biopsy specimens of normal oral mucosa (NOM) from healthy subjects and oral mucosal tissues from oral squamous cell carcinoma (OSCC) patients (original magnification 20X).**


Immunostaining scores
NOM
OSCC
0
1
2
3
4


TFF immunostaining
NOM
OSCC
TFF 1
TFF 2
TFF 3

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