Increased immunoexpression of trefoil factors in salivary gland tumors

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Short running title:
Trefoil factors in patients with salivary gland tumors

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Abstract

Objective:

Very little is known about the role of trefoil factors (TFFs) in salivary gland tumors, and TFF immunoexpression has never been investigated in such tumors. The aim of this study was to evaluate TFF immunoexpression in benign and malignant salivary gland tumors.

Materials and Methods:

Benign (n=25) and malignant (n=25) salivary gland tumor specimens were included in this study, using mucocele (n=25) specimens as a control group. Immunohistochemical staining was performed to evaluate the expression of TFFs (TFF1, TFF2, and TFF3) by semi-quantitative means.

Results:

Expression of TFF1, TFF2 and TFF3 were significantly increased in benign ($p=0.001$; $p=0.005$; $p<0.001$, respectively) and malignant ($p<0.001$; $p<0.001$; $p<0.001$, respectively) groups as compared with the control group. Patterns of co-expression between TFF1/TFF2; TFF2/TFF3; and TFF1/TFF3 were different among the three groups.

Conclusions:

The present study provided new information showing that all TFFs were significantly increased in benign and malignant salivary gland tumors, and overexpression of TFFs could be associated with neoplastic transformation in salivary gland tissues.

Clinical Relevance:

Overexpression of TFFs may be useful as biomarkers in terms of differential diagnosis between salivary gland tumors and other oral neoplasms for which clinical manifestations are indistinguishable.

Keywords: immunohistochemistry; salivary gland tumor; trefoil factor

Introduction

Trefoil factors (TFFs) are secreted molecules mainly synthesized by mucin-producing epithelial cells and which constitute a family of short peptides with disulfide bonds that form a three leafed structure, also called a trefoil domain [1, 2]. Human TFFs consist of three members, TFF1, TFF2, and TFF3. TFF1 and TFF3 contain one trefoil domain, whereas TFF2 contains two trefoil domains. TFF1 and TFF3 may form dimers through a cysteine residue located near the C-terminus [1, 2]. TFFs are expressed in various human tissues and secretions [3, 4]. TFFs have been implicated in several biological functions such as cytoprotection and wound healing [1, 2]. Regarding oral compartments, salivary glands are the predominant site of TFF synthesis, with some contribution from goblet cells of the parotid ducts and oral epithelia [5-10]. There is a paucity of data demonstrating the functions and mechanism of TFFs in oral compartments. It was reported that TFF3 was a modifying factor for signaling pathways involved in cell survival, cell proliferation, and cell migration of oral keratinocytes [11, 12]. Therefore, the expression of TFF peptides in saliva and oral epithelia may be an essential factor in protection against oral mucosal tissue damage.

Besides the protective role of TFFs in mucosal tissues, it has been reported that TFFs have pleiotrophic actions in tumorigenesis [13]. It is evident that TFFs have contradictory roles as tumor suppression and tumor progression factors, likely dependent on the site of expression [14]. Altered expression of TFFs in human solid cancers such as stomach, breast, colon, and prostate cancers has been reported with loss of TFF1 and 2 in the stomach, and generally increased expression of most TFF in the latter epithelia with neoplastic progression [14-17]. Although compelling evidence from experimental and clinical studies indicates a strong association between TFFs and human cancers, very little is known about the role of TFFs in oral cancers. Our previous study demonstrated that TFF2 and TFF3 expression was significantly decreased in oral squamous cell carcinoma (OSCC) [18]. These findings suggest

a possible tumor suppressive role of TFF2 and TFF3 in OSCC. However, the involvement of TFFs in other related tumors of the oral cavity such as salivary gland tumors has never been identified.

Salivary gland tumors are relatively uncommon, and demonstrate a wide range of cell types and morphological diversity. The majority of salivary gland tumors are epithelial in origin [19]. The incidence of salivary gland tumors was reported to range from 0.4 to 13.5 cases/100,000 population per year [20]. Pleomorphic adenoma is common as benign salivary gland tumors, whereas mucoepidermoid carcinoma and adenoid cystic carcinoma are common as malignant salivary gland tumors [19]. Similar morphologic features between salivary and mammary gland tumors such as mucoepidermoid carcinoma, adenoid cystic carcinoma, and basal cell adenocarcinoma have been reported [21-23]. Moreover, previous studies demonstrated the involvement of *TFF1* and *TFF3* genes in mammary gland tumors, and these two genes were used as biomarkers for detecting disseminated malignant cells of breast cancer [24-26]. By analogy, it can be hypothesized that TFFs might also contribute to salivary gland tumor development. Therefore, the aim of the present study was to determine TFF expression patterns in benign and malignant salivary gland tumors, using an immunohistochemical staining method.

Materials and Methods

Tissue specimens

There were 75 formalin-fixed and paraffin-embedded biopsy specimens in this study. All specimens were retrieved from the archives of Division of Oral Pathology, Department of Oral Diagnosis, Faculty of Dentistry, Khon Kaen University, Thailand. The specimens of the control group (n=25) were diagnosed as mucocele, those of which contain groups of normal minor salivary glands. According to WHO classification of salivary gland tumors [27], the specimens of the benign group were diagnosed as pleomorphic adenoma (n=25). The

specimens of the malignant group were diagnosed as mucoepidermoid carcinoma (n=11); adenoid cystic carcinoma (n=6); polymorphous low-grade adenocarcinoma (n=4); acinic cell carcinoma (n=3); and basal cell adenocarcinoma (n=1). The approval of the ethical committee for the use of human subjects, Khon Kaen University (HE542281) was obtained.

Immunohistochemical study

Consecutive sections of formalin-fixed and paraffin-embedded biopsy specimens were cut (5 μm thickness) and mounted on glass slides for H&E, and immunostaining. The sections were deparaffinized in xylene, hydrated through graded alcohols, and washed with phosphate buffered saline (PBS). The standard immunohistochemical method was used as previously described [28]. Briefly, endogenous peroxidase blocking was performed using Peroxo-Block™ (Invitrogen, Life Technologies Ltd, Paisley, UK). Microwave-based antigen retrieval was done with 10 mmol/l sodium citrate buffer at a pH of 6.0, followed by blocking of nonspecific antibody binding with Protein Block serum-free (DAKO, CA, USA). Antihuman TFF1, TFF2, and TFF3 polyclonal antibodies were used as previously described [29-31]. 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. Antihuman TFF1 antibody does not cross-react with human TFF2 or TFF3, nor with mouse or rat TFF1 C-terminal hapten [29]. The anti-human TFF2 antibody cross-reacts with mouse but not rat TFF2, TFF1 or TFF3 [30]. The anti-human TFF3 antibody cross-reacts with all species tested (mouse, rat, human), but not TFF1 nor TFF2 [31]. The optimal dilutions were; 1:400 for antihuman TFF1 and TFF2 antibodies, and 1:800 for TFF3 antibody. Immuno-detection system was based on HRP labeled polymer which was conjugated with secondary antibodies (DAKO EnVision+ System-HRP labeled polymer Anti-rabbit). 3,3' diaminobenzidine (DAB) was used as a substrate chromogen (DAKO). 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 original magnification 20X, the whole area of each tissue specimen was selected for analysis of positively immunostained cells. The distributions of positively stained cells in the whole area were evaluated visually by scanning the slide systematically. Assessment of distribution of positively stained cells was inferred to the 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

Characteristics of patients with mucocele, benign, and malignant salivary gland tumors were compared using the Chi-square test for categorical variables. The Kruskal-Wallis and Mann-Whitney U tests were performed to analyze differences in TFF immunostaining scores among the comparison groups. To control for the effect of age, multiple linear regression was used to evaluate the association of salivary gland tumors with the levels of TFF expression. Spearman correlation coefficient was performed to determine the correlations between the co-expresion of TFF1/TFF2; TFF1/TFF3; and TFF2/TFF3 in each studied group. **In addition, Spearman correlation coefficient was used to evaluate the correlations between TFF immunostaining scores and tumor grading in mucoepidermoid carcinoma cases**. The Two-tailed $P < 0.05$ was considered statistically significant.

Results

Characteristics of tissue specimens

Demographic characteristics of the investigated specimens are shown in Table 1. The control group was significantly younger ($p<0.001$) than those in benign and malignant groups. No differences in age of patients were observed between benign and malignant groups. Although no significant differences in gender were demonstrated among the three groups, females seemed to be predominant in benign and malignant salivary gland tumors within this study. The labial mucosa was the major site for biopsy in the control group, whereas the palate was the major site for biopsy in the benign group. In the malignant group, palate and maxilla were the major sites for biopsy. Most benign and malignant salivary gland tumors were derived from minor salivary glands. Only a few cases were derived from major salivary glands, such as the parotid and sublingual glands. **Tumor grading was performed in mucoepidermoid carcinoma cases. There were one case with low grade, 5 cases with intermediate grade, and 5 cases with high grade.**

Immunoexpression of TFFs in salivary gland biopsy specimens

TFF expression in mucocele

Examination of the cellular distribution in mucocele specimens demonstrated that TFF1, TFF2, and TFF3 were present in mucous acini and salivary ducts. TFF immunoexpression was mostly seen in the cytoplasm. Low levels of TFF1, TFF2, and TFF3 were mainly detected in acinar cells, whereas moderate or high levels of TFF1, TFF2, and TFF3 were observed in ductal cells (Figure 1). No staining or low levels of TFF1 and TFF3 were demonstrated in most mucocele specimens with few cases revealing moderate or high expression. More variations in TFF2 expression were observed as compared with TFF1 and TFF3 (Table 2). Co-expression between TFF1/TFF3 was significantly correlated ($r=0.597$;

p=0.01). In contrast, no significant correlations between TFF1/TFF2 nor TFF2/TFF3 were demonstrated.

TFF expression in benign salivary gland tumors

TFF1, TFF2, and TFF3 expression in pleomorphic adenoma specimens was observed in both non-tumorigenic and tumorigenic areas (Figure 2). In non-tumorigenic areas, low levels of TFF1 and TFF2 and moderate levels of TFF3 were demonstrated in acinar cells, whereas moderate or high levels of TFF1, TFF2, and TFF3 were observed in ductal cells. In tumorigenic areas, high levels of TFF1, TFF2, and TFF3 were observed (Figure 2). Expression of TFF1, TFF2 and TFF3 was significantly increased in pleomorphic adenoma as compared with the control group (p=0.001; p=0.005; p<0.001, respectively) (Table 2). Co-expression between TFF1/TFF2 was significantly correlated (r=0.808; p=0.01). In contrast, no significant correlations between TFF1/TFF3 nor TFF2/TFF3 were demonstrated.

TFF expression in malignant salivary gland tumors

All types of malignant salivary gland tumors in this study demonstrated the expression of TFF1, TFF2 and TFF3 (Table 3, Figure 3). More than 70% of malignant salivary gland tumor specimens demonstrated high expression of TFF1, TFF2, and TFF3 (Table 2). Expression of TFF1, TFF2 and TFF3 was significantly increased in malignant salivary gland tumors as compared with the control group (p<0.001; p<0.001; p<0.001, respectively) (Table 2). In addition, expression of TFF2 in malignant salivary gland tumors was significantly higher than the benign group (p=0.015) (Table 2). Co-expression between TFF1/TFF2 (r=0.441; p=0.05), and TFF2/TFF3 (r=0.650; p=0.01) was significantly correlated. In contrast, no significant correlations between TFF1/TFF3 were observed. **There were no significant correlations between the expression of TFFs and tumor grading in mucoepidermoid carcinoma.**

Discussion

The present study demonstrated, for the first time, that TFF1, TFF2, and TFF3 were significantly increased in benign and malignant salivary gland tumors. It should be noted that all biopsy specimens in the benign group were pleomorphic adenoma. Thus, obtaining salivary gland tissues from individuals with other benign salivary gland tumors would strengthen an analysis of TFF immunoexpression. Our results are in agreement with previous studies demonstrating overexpression of TFFs in a variety of human cancers [32-36]. However, the present findings are in contrast with our previous observations [18] demonstrating the reduction of TFF2 and TFF3 in oral squamous cell carcinoma (OSCC) lesions of oral mucosa. These findings and the clear inhibition of TFF1 and TFF2 in gastric cancer [37, 38], suggest diverse regulation and function of TFF proteins under tumorigenic conditions in different luminal compartments. In the condition of OSCC of oral mucosa which is usually associated with chronic inflammation, it could be postulated that inflammation-mediated signaling transduction such as NFκB signaling pathway might be associated with downregulation of TFF2 and TFF3 [18, 39]. In the condition of salivary gland tumors which is not normally associated with inflammation, it remains unclear which regulatory mechanisms are associated with overexpression of TFFs. It would be of interest to investigate which molecular mechanisms control the expression of TFFs in benign and malignant conditions. On the other hand, it is important to investigate whether overexpression of TFFs could accelerate the rate of neoplastic transformation in salivary gland tissues.

Although the roles of TFFs in tumorigenesis have been investigated intensively, the functional role of TFFs in salivary gland tumors has never been elucidated. Previous studies demonstrated that TFF1 and TFF3 enhanced cell proliferation and promoted cell migration and invasion in mammary carcinoma cell lines [40, 41], whereas TFF2 stimulated cell

migration and inhibited apoptosis in breast adenocarcinoma cell lines [42, 43]. It has been reported that several salivary gland tumors share similar morphologic features with mammary gland tumors [21-23]. It would be of interest to investigate the functional role of TFFs in salivary gland tumors as compared with the mammary gland tumors in order to evaluate whether TFFs may have context-specific functions in different tissue compartments. According to our observations, the level of TFF2 expression was significantly higher in malignant salivary gland tumors as compared with pleomorphic adenoma. Moreover, co-expression patterns between TFF2 and other TFFs were different between pleomorphic adenoma and malignant salivary gland tumors. These findings suggest that TFF2 may have its own expression profile in response to various pathologic conditions between pleomorphic adenoma and malignant salivary gland tumors. Although pleomorphic adenoma is classified as a benign salivary gland tumor, it has a potential for malignant transformation. Thus, overexpression of TFF2 might be a possible oncogenic factor that enhances malignant transformation in pleomorphic adenoma. However, further studies would be essential to validate the proposed hypothesis.

In the oral cavity, TFFs are mainly produced by salivary glands. Our previous study demonstrated no significant differences in salivary TFF concentrations between OSCC patients and control subjects. These findings imply that production of TFFs by salivary glands is not affected by OSCC lesions of oral mucosa [18]. Based on the present observations, it is tempting to hypothesize that overexpression of TFFs in salivary gland tumors might lead to increased TFF amounts in saliva, and higher levels of salivary TFF concentrations in the patients may be suggestive of salivary gland tumors. In addition, measurements of secreted TFFs in mucosal fluids and serum have been reported for their potential use as diagnostic markers [44]. Thus, quantification of salivary TFF concentrations in patients with oral tumors may be useful for development of biomarkers in terms of

differential diagnosis between salivary gland tumors and other oral neoplasms which clinical manifestations are indistinguishable. **Therefore, further investigations such as comparative studies on the levels of salivary TFF concentrations before and after treatment of patients with salivary gland tumors and other oral neoplasms would help to confirm the clinical significance of TFFs.**

In conclusion, the present study provided new information of increased TFF immunoexpression in patients with benign and malignant salivary gland tumors. However, there remain many research gaps in our knowledge about the connection between the functional roles of TFFs and neoplastic transformation in salivary gland tissues. Therefore, intensive studies at a molecular level are needed to clarify regulatory mechanisms for TFF expression in salivary glands under the tumorigenic conditions. In addition, further investigation between overexpression of TFF2 and enhancement of malignant transformation in salivary glands would be of importance.

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Conflicts of Interest Statement

The authors declare that they have no conflicts of interest.

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Table 1 Demographic characteristics of the investigated specimens with salivary gland pathology

	a control group (n=25)
Age (mean±,SD; range)	26.60±12.89; 10-58 years
Gender (male/female)	13/12
Sites of biopsy	
labial mucosa	n=23
buccal mucosa	n=2
Type of salivary gland pathology	
mucocele	n=25
	a benign group (n=25)
Age (mean±SD; range)	45.36±20.16; 13-83 years
Gender (male/female)	8/17
Sites of biopsy	
palate	n=18
parotid gland	n=3
buccal mucosa	n=3
labial mucosa	n=1
Type of salivary gland pathology	
pleomorphic adenoma	n=25
	a malignant group (n=25)
Age (mean±SD; rangc)	45.60±14.33: 18-72 years
Gender (male/female)	10/15
Sites of biopsy	
palate	n=9
maxilla	n=7
buccal mucosa	n=4
retromolar area of mandible	n=2
sublingual gland	n=2
vestibular area	n=1
Types of salivary gland pathology	
mucoepidermoid carcinoma	n=11
adenoid cystic carcinoma	n=6
polymorphous low-grade adenocarcinoma	n=4
acinic cell carcinoma	n=3
basal cell adenocarcinoma	n=1

Table 2 Levels of TFF immunostaining scores in salivary gland biopsy specimens

Salivary gland tissues	TFF immunostaining scores[a]											
	TFF1[*]				TFF2[**]				TFF3[***]			
	0	1	2	3	0	1	2	3	0	1	2	3
Control group[b] (n=25)	10	11	3	1	9	6	6	4	10	12	2	1
Benign group[c] (n=25)	5	4	2	14	1	8	7	9	-	5	4	16
Malignant group[d] (n=25)	-	3	4	18	2	2	2	19	-	3	-	22

[a]TFF 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).

[b]A control group consisted of 25 cases of mucocele.

[c]A benign group consisted of 25 cases of pleomorphic adenoma.

[d]A malignant group consisted of 11 cases of mucoepidermoid carcinoma; 6 cases of adenoid cystic carcinoma; 4 cases of polymorphous low-grade adenocarcinoma; 3 cases of acinic cell carcinoma; and one case of basal all adenocarcinoma.

[*]Levels of TFF 1 immunoexpression are significantly increased in benign ($p=0.001$) and malignant ($p<0.001$) groups as compared with a control group.

[**]Levels of TFF2 immunoexpression are significantly increased in benign ($p=0.005$) and malignant ($p<0.001$) groups as compared with a control group.

[***]Levels of TFF3 immunoexpression are significantly increased in benign ($p<0.001$) and malignant ($p<0.001$) groups as compared with a control group.

Table 3 Levels of TFF immunostaining scores in malignant salivary gland tumors

	TFF immunostaining scores[a]											
Salivary gland tumors	TFF1				TFF2				TFF3			
	0	1	2	3	0	1	2	3	0	1	2	3
mucoepidermoid carcinoma (n=11)	-	1	1	9	2	-	1	8	-	2	-	9
adenoid cystic carcinoma (n=6)	-	2	2	2	-	1	1	4	-	-	-	6
polymorphous low-grade adenocarcionma (n=4)	-	-	-	4	-	-	-	4	-	-	-	4
acinic cell carcinoma (n=3)	-	-	1	2	-	1	-	2	-	1	-	2
basal cell adenocarcinoma (n=1)	-	-	-	1	-	-	-	1	-	-	-	1

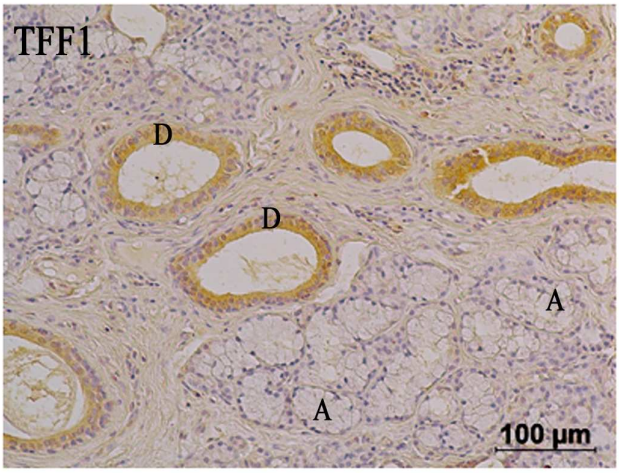
[a] TFF immunostaining scores were graded as: 0 = no immunostained cells; 1 = low distribution (less than 25% positively stained cells); 2 = moderate distribution (25% to 50% positively stained cells); 3 = high distribution (> 50% positively stained cells).

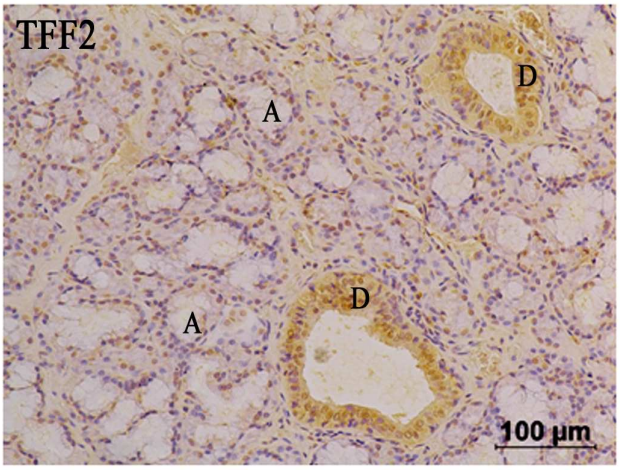
Figure legends

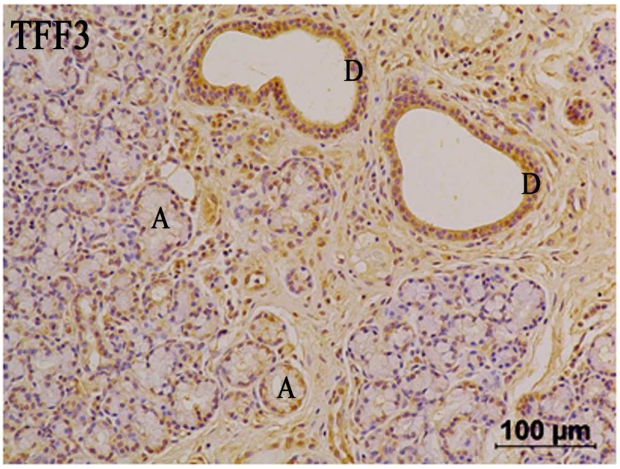
Figure 1 Immunohistochemical detection of trefoil factors (TFF1, TFF2, and TFF3) in minor salivary glands of specimens diagnosed with mucocele. Immunostaining of TFFs was markedly positive in acinar cells (A) and ductal cells (D). Expression of TFFs was mostly seen in cytoplasm.

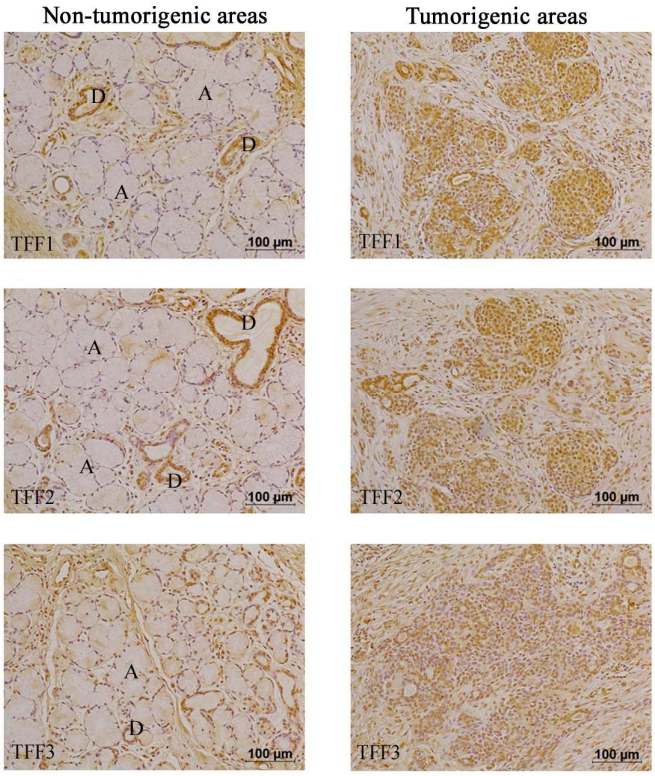
Figure 2 Immunohistochemical examinations of trefoil factors (TFF1, TFF2, and TFF3) in non-tumorigenic and tumorigenic areas of pleomorphic adenoma. Expression of TFFs was mostly seen in cytoplasm of acinar (A) and ductal (D) cells in non-tumorigenic areas. Increased expression of TFFs was demonstrated in tumorigenic areas of pleomorphic adenoma.

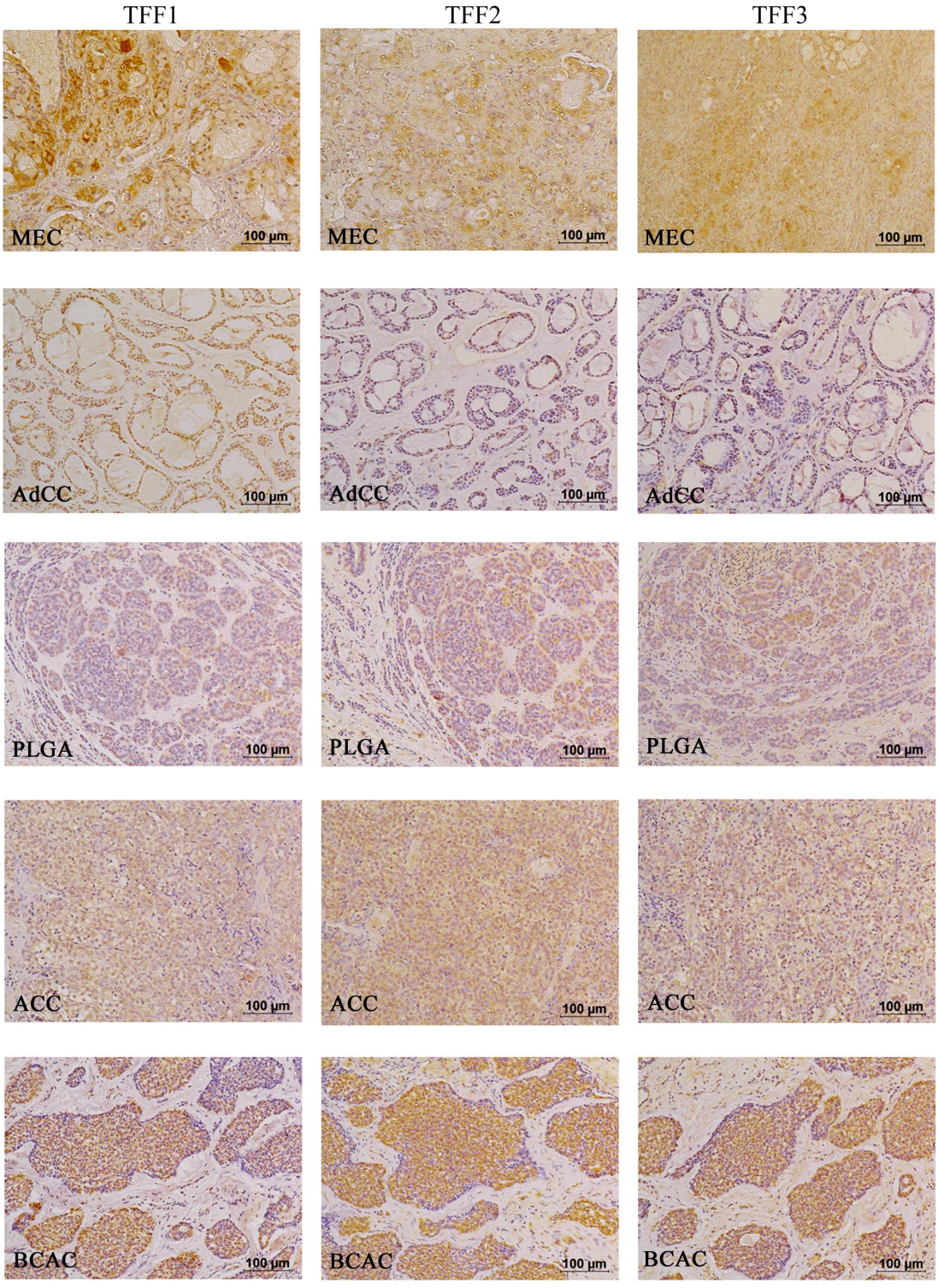
Figure 3 Immunohistochemical examinations of trefoil factors (TFF1, TFF2, and TFF3) in various types of malignant salivary gland tumors including mucoepidermoid carcinoma (MEC); adenoid cystic carcinoma (AdCC); polymorphous low grade adenocarcinoma (PLGA); acinic cell carcinoma (ACC); and basal cell adenocarcinoma (BCAC). Variations in immunoexpression of TFFs were observed in all malignant salivary gland tumors.


TFF1
D
D
A
A
100 µm


TFF2
D
A
D
A
100 µm


TFF3
D
D
A
A
100 µm


Non-tumorigenic areas
Tumorigenic areas
D
A
D
A
TFF1
100 µm
TFF1
100 µm
A
D
A
D
TFF2
100 µm
TFF2
100 µm
A
D
TFF3
100 µm
TFF3
100 µm


TFF1
TFF2
TFF3
MEC
AdCC
PLGA
ACC
BCAC
100 μm