

Tumor protein D54: A promising marker of mucoepidermoid carcinoma

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Received: 11 June 2021 / Accepted: 10 August 2021

Abstract

A definitive diagnosis of salivary gland tumors is extremely difficult to make without evaluating the entire tumor and conducting immunohistochemical examinations. In this study, we aimed to examine and compare the expression patterns of the tumor protein (TP) D52 family, including TPD52, TPD53, and TPD54, in salivary gland tumor cells by using immunohistochemical staining. Among over 30 benign and malignant salivary gland tumors with extensive and diverse morphological features and overlapping histological similarities, we selected Warthin's tumor and pleomorphic adenoma to represent benign salivary gland tumors and mucoepidermoid carcinoma to represent malignant ones. Tumor samples were fixed in 10% buffered formalin and embedded in paraffin. Then, immunohistochemical staining was performed using antibodies against TPD52, TPD53, and TPD54. Neither the benign salivary gland tumors nor mucoepidermoid carcinoma stained for TPD52. However, the intensity of TPD53 and TPD54 staining was found to be low in the benign salivary gland tumors and high in mucoepidermoid carcinoma. TPD54 may serve as a pathological indicator of benign salivary gland tumors and mucoepidermoid carcinoma.

Key words :tumor protein D52 family, TPD54, mucoepidermoid carcinoma, immunohistochemistry

Introduction

The salivary glands are exocrine organs that produce saliva and are complex tissues composed of ductal, acinar, myoepithelial, and basal cells¹. Collectively called as luminal cells, ductal and acinar cells are present on the luminal side of the salivary duct system. Myoepithelial and basal cells are located on the basement membrane around the luminal cells and are thus called abluminal cells². In general, 3 types of acini (namely serous, mucinous, and mixed) and ducts (i.e., intercalated, striated, and excretory) are found in the salivary glands. The acini and intercalated ducts are surrounded by myoepithelial cells, whereas the striated and excretory ducts are

surrounded by basal cells³.

Tumors of the salivary glands comprise less than 1% of all neoplasms in the body⁴; however, there are more than 30 benign and malignant salivary gland tumors with extensive and diverse morphologies yet overlapping histological similarities⁴. Hence, it is extremely difficult to definitively diagnose salivary gland tumors without evaluating the entire tumor and conducting immunohistochemical examinations⁵.

Expression patterns of the tumor protein (TP) D52 family, including TPD52, TPD53, TPD54, and TPD55, have been investigated in malignant tumors. TPD52, also known as CRHSP-28m, was first identified as a chromosome 8q21 amplification target in breast cancer and was subsequently detected in lung, prostate, ovarian, endometrial, and hepatic cancers⁶. TPD53 has been identified as a novel 14-3-3-binding partner in breast cancer, with the highest level of expression observed at the G2-M transition; dysregulated expression of TPD53 results in incomplete mitosis⁷. TPD54 has been shown to affect the proliferation, adhesion, and invasion of oral cancer cells⁸; regulate the expression of pyruvate dehydrogenase⁹; and

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influence the sensitivity of breast cancer cells to metformin⁹. All members of the TPD52 family contain a coiled-coil structural motif that mediates homomeric and heteromeric interactions among them¹⁰. We have previously evaluated the role of the TPD52 family in chondrocytes and oral squamous cell carcinoma (OSCC) cells^{8, 11–13}. In OSCC cells, TPD54 is highly expressed both in the cancerous tissue and in the surrounding connective tissue, regardless of the tumor differentiation level. However, the expression of TPD52 in both highly and poorly differentiated OSCC cells is lower than that of TPD54; besides, TPD52 is barely expressed in normal tissue, whereas TPD53 is moderately expressed in cancer tissue¹². TPD54 might act as a negative regulator of tumor progression. Although the TPD52 family has been investigated in many cancers, their expression and function in salivary gland tumors still remain unclear. Several efforts have been made to identify new prognostic and diagnostic molecular markers and chemotherapeutic targets for salivary gland tumors¹⁴. While calponin is believed to be a useful and highly specific marker for identifying myoepithelial cells¹⁵, all luminal and abluminal cells of the salivary glands stain positively for pan-cytokeratin¹⁶. Nevertheless, there is no reliable immunohistochemical marker to distinguish benign salivary gland tumors from mucoepidermoid carcinoma. Here, we hypothesized that the immunohistochemical examination of the expression of the TPD52 family could be useful in the pathological diagnosis of salivary gland tumors.

Materials and methods

1. Tissue samples

The present study was approved by the Institutional Ethics Committee of Showa University Hospital (Permit Number: 2016-002). We used pleomorphic adenoma of the parotid gland (5 cases) and Warthin's tumor of the parotid gland (5 cases) as representatives of benign salivary gland tumors and mucoepidermoid carcinoma of the parotid (2 cases) and minor salivary glands (8 cases) as a representative of malignant salivary gland tumors. Cancer-free submandibular gland tissue excised during radical neck dissection for OSCC (5 cases) and minor salivary glands of the lip (5 cases) were selected as normal salivary gland tissue specimens.

2. Immunohistochemical analysis of paraffin-embedded tissue sections

The specimens were fixed in 10% buffered

formalin and then embedded in paraffin wax. Serial sections (4 µm thick) collected from the tissue blocks were placed on silane-coated glass slides. Antigen retrieval was carried out by incubation with citrate-phosphate buffer (0.01 M, pH 6.0) at 121°C for 20 min. Endogenous peroxidases were blocked by incubating the samples with 10% hydrogen peroxide for 10 min. The sections were incubated at 4°C overnight with the following primary rabbit polyclonal antibodies: anti-TPD52 antibody (1: 50; orb 100564; Biorbyt, Cambridge, UK), anti-TPD53 antibody (1: 200; 14732-1-AP; Proteintech, Rosemont, IL USA), and anti-TPD54 antibody (1: 200; 11795-1-AP; Proteintech, Rosemont, IL USA). Anti-calponin 1 (1: 100, rabbit monoclonal; ab46794; Abcam, Cambridge, UK) and anti-pan-cytokeratin (1: 250, mouse monoclonal; ab7753; Abcam, Cambridge, UK) antibodies were also used for the immunohistochemical staining of calponin and pan-cytokeratin, respectively. The sections were incubated with secondary antibodies (EnVision + system-HRP labeled polymer anti-rabbit / anti-mouse; Dako, Glostrup, Denmark) prior to color development with a 3,3'-diaminobenzidine peroxidase substrate kit (Dako, Glostrup, Denmark) and were subsequently counterstained with hematoxylin. Immunohistological processes were conducted manually without using a slide preparation system. All images were captured using the Olympus BX51 microscope and Olympus cellSens Standard software (Olympus Corporation, Tokyo, Japan). Each immunohistochemical specimen was evaluated by 2 independent dentists and quantitatively scored based on the staining intensity (i.e., 0, no staining; 1, weak staining; 2, moderate staining; and 3, strong staining). The samples were evaluated twice at different times¹⁷.

3. Statistical analysis

The scores of immunohistochemical staining for TPD52, TPD53, and TPD54 were compared by one-way analysis of variance. The Tukey-Kramer method was used to determine which means among a specific group of means were statistically different. All calculations were performed using the R package, with the level of statistical significance set at $P < 0.01$. Statistically significant results are marked by an asterisk (*) in graphs.

Results

We used a squamous cell carcinoma specimen as the positive control (Fig. 1), as reported previously¹².

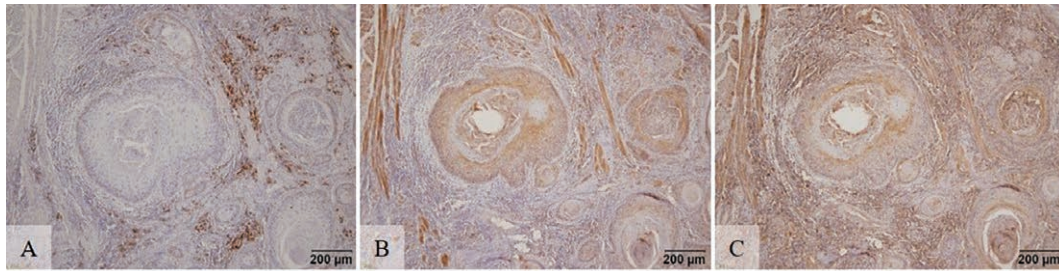


Fig. 1. Expression of the TPD52 family in differentiated OSCC cells
Expression of TPD52 (A), TPD53 (B), and TPD54 (C) in OSCC cells. TPD54 was highly expressed in cancer tissue and in the surrounding connective tissue, regardless of the tumor differentiation level. The expression of TPD52 in both highly and poorly differentiated OSCC cells was lower than that of TPD54; TPD52 was barely expressed in normal tissue. TPD53 was moderately expressed in cancer tissue.

TP, tumor protein; OSCC, oral squamous cell carcinoma.

Table 1. Details of specimens from normal and tumorous salivary gland tissue

	Type	Gland	Number
Normal	Normal salivary gland	Minor salivary glands	5
		Submandibular gland	5
Benign	Pleomorphic adenoma	Parotid gland	5
	Warthin's tumor	Parotid gland	5
Malignant	Mucoepidermoid carcinoma	Minor salivary glands	8
		Parotid gland	2

All the samples were collected during salivary gland tumor resection (Table 1).

1. Normal salivary glands

When evaluating the normal salivary gland tissue (Fig. 2A), we observed high-intensity staining for calponin in myoepithelial cells at the periphery of the acini, intercalated ducts, and striated ducts (Fig. 2B); moderate-intensity staining for pan-cytokeratin in ductal cells (Fig. 2C); negative staining for TPD52 in acinar and ductal cells (Figs. 2D & 2G); high- and moderate-intensity staining for TPD53 in mucinous and serous acini, respectively (Figs. 2E & 2H); and moderate-intensity staining for TPD54 in mucinous acini and ductal cells (Figs. 2F & 2I).

2. Pleomorphic adenoma

In the case of pleomorphic adenoma (Fig. 3A), myoepithelial cells showed diffuse, moderate-intensity staining for calponin (Fig. 3B). Duct-like cells displayed diffuse, high-intensity staining for pan-cytokeratin (Fig. 3C). Pleomorphic adenoma cells were negative for TPD52 staining (Figs. 3D & 3G). Mucinous acini and ductal cells—positively stained for calponin—were moderately stained for TPD53 (Figs. 3E & 3H). TPD54 staining intensity was moderate

and diffuse in both ductal cells and interstitial cellular components, including epithelioid and spindle-shaped cells (Figs. 3F & 3I).

3. Warthin's tumor

Warthin's tumors (Fig. 4A) did not stain for calponin (Fig. 4B), whereas high-intensity pan-cytokeratin staining was observed in the epithelial bilayer—consisting of a layer of tall, columnar, luminal cells encompassed by cuboidal oncocytic cells (Fig. 4C). Warthin's tumors exhibited negative staining for TPD52 (Figs. 4D & 4G). The intensity of staining for TPD53 (Figs. 4E & 4H) and TPD54 (Figs. 4F & 4I) was found to be moderate in oncocytic cells on the outer side of the double layer of epithelial cells resting on the dense lymphoid stroma.

4. Mucoepidermoid carcinoma

According to the Armed Forces Institute of Pathology (AFIP) grading system¹⁸, mucoepidermoid carcinoma is classified into 3 types: low, intermediate, and high-grade (Table 2).

Immunohistochemical analysis of mucoepidermoid carcinoma (Fig. 5A) revealed extremely low-intensity staining for calponin in squamous cells (Fig. 5B) as well as low-intensity pan-cytokeratin

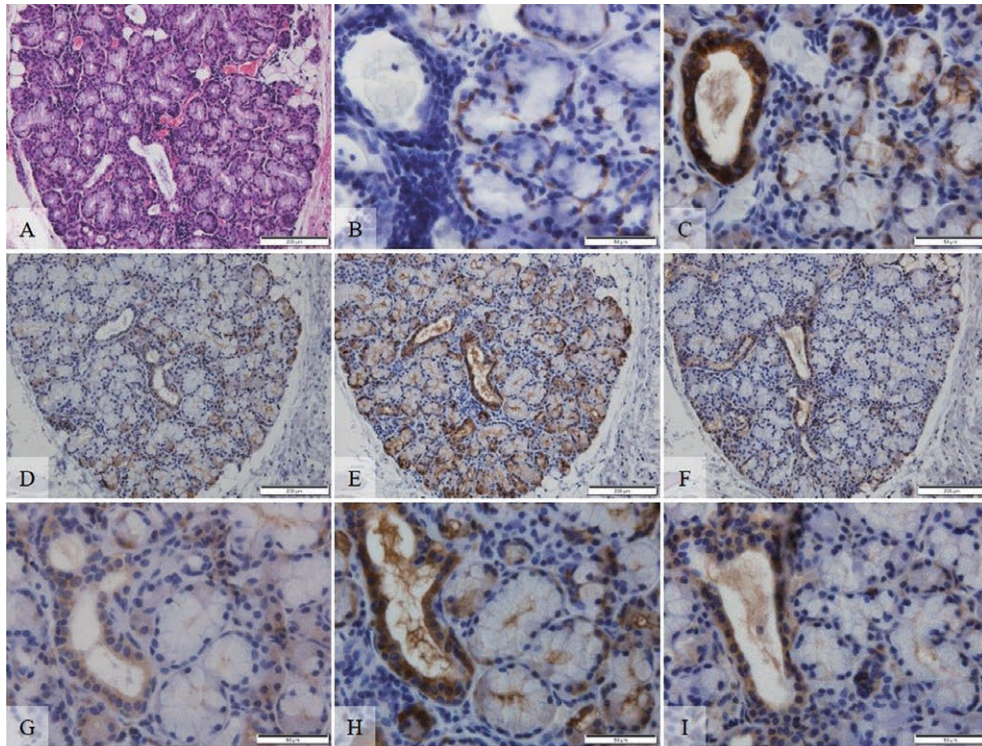


Fig. 2. Expression of the TPD52 family in normal salivary gland tissue

(A) Hematoxylin and eosin staining. (B) High-intensity calponin staining in myoepithelial cells at the periphery of the acini, intercalated ducts, and striated ducts. (C) Ductal cells showing moderate-intensity pan-cytokeratin staining. (D) and (G) Negative staining for TPD52. (E) and (H) High-intensity TPD53 staining in acini cells and low-intensity TPD53 staining in ductal cells. (F) and (I) Moderate-intensity staining for TPD54 in acini and ductal cells.
TP, tumor protein.

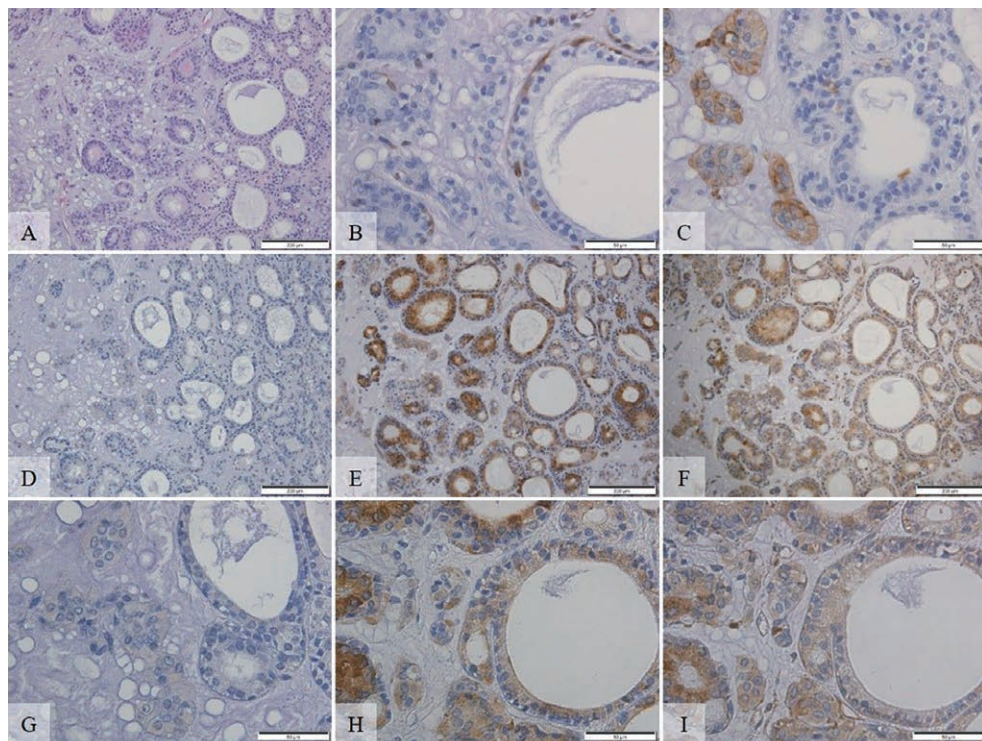


Fig. 3. Expression of the TPD52 family in pleomorphic adenoma

(A) Hematoxylin and Eosin staining. (B) Diffuse, moderate-intensity calponin staining in myoepithelial cells and periphery cell nests. (C) Diffuse, high-intensity pan-cytokeratin staining in duct-like cells. (D) and (G) Negative staining for TPD52 in pleomorphic adenoma. (E) and (H) Mucinous acini and intercalated duct cells stained moderately for TPD53. (F) and (I) Diffuse, moderate-intensity TPD54 staining in both ductal cells and interstitial cellular components consisting of epithelioid and spindle-shaped cells.

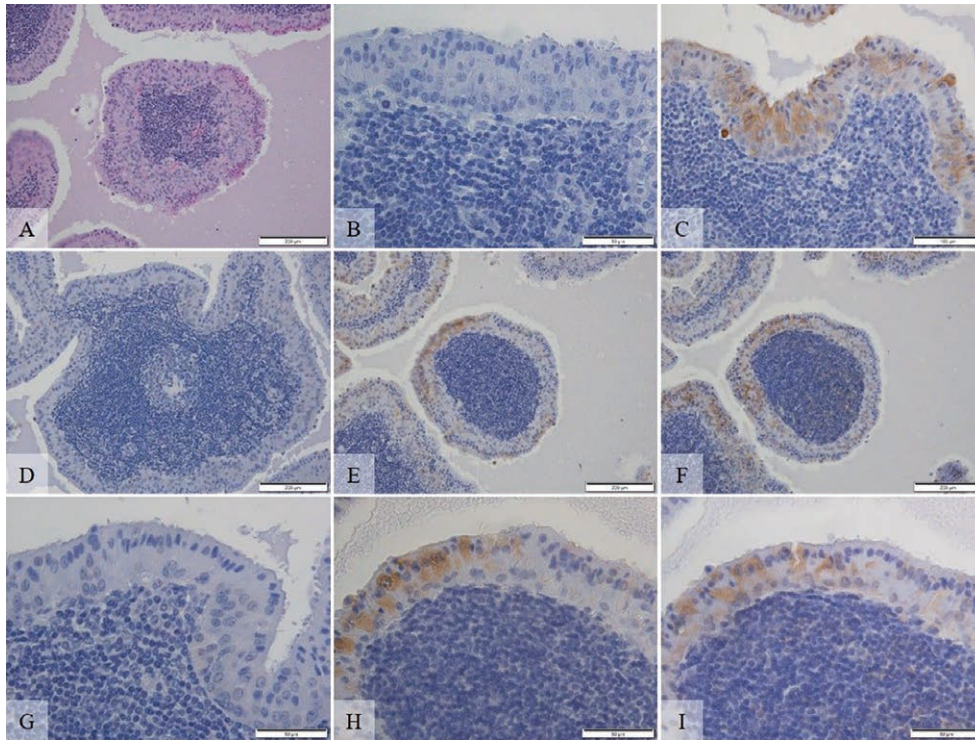


Fig. 4. Expression of the TPD52 family in Warthin's tumor

(A) Hematoxylin and eosin staining. (B) Negative calponin staining in Warthin's tumor. (C) High-intensity pan-cytokeratin staining in the epithelial bilayer composed of a layer of tall, columnar, luminal cells and cuboidal oncocytic cells on the outer side. (D) and (G) Negative staining for TPD52 in Warthin's tumor. (E) and (H) Moderate-intensity staining for TPD53 in the double layer of epithelial cells resting on the dense lymphoid stroma. (F) and (I) : Moderate-intensity staining for TPD54 in the double layer of the epithelial cells resting on the dense lymphoid stroma.

TP, tumor protein.

Table 2. Histological evaluation of mucoepidermoid carcinoma

Case No.	Intracystic component	Neural invasion present	Necrosis present	Mitosis (4 or more per 10 HPF*)	Anaplasia present	Grade
1	2	2	0	0	0	Low
2	0	0	0	0	0	Low
3	0	0	0	0	4	Low
4	2	0	0	0	4	Intermediate
5	0	0	0	3	4	High
6	2	0	0	3	4	High
7	2	0	0	0	4	Intermediate
8	0	0	3	0	0	Low
9	2	0	0	0	0	Low
10	0	0	0	0	0	Low

HPF, high-power field.

staining in mucus-secreting and squamous cells (Fig. 5C). Mucoepidermoid carcinoma cells did not stain for TPD52 (Figs. 5D & 5G). High-intensity staining for TPD53 (Figs. 5E & 5H) and TPD54 (Figs. 5F & 5I) was observed in mucous cells.

The immunohistochemical staining scores for each specimen are summarized in Table 3. The signal intensity of TPD53 and TPD54 did not have any significant relationship with the histological grades of mucoepidermoid carcinoma.

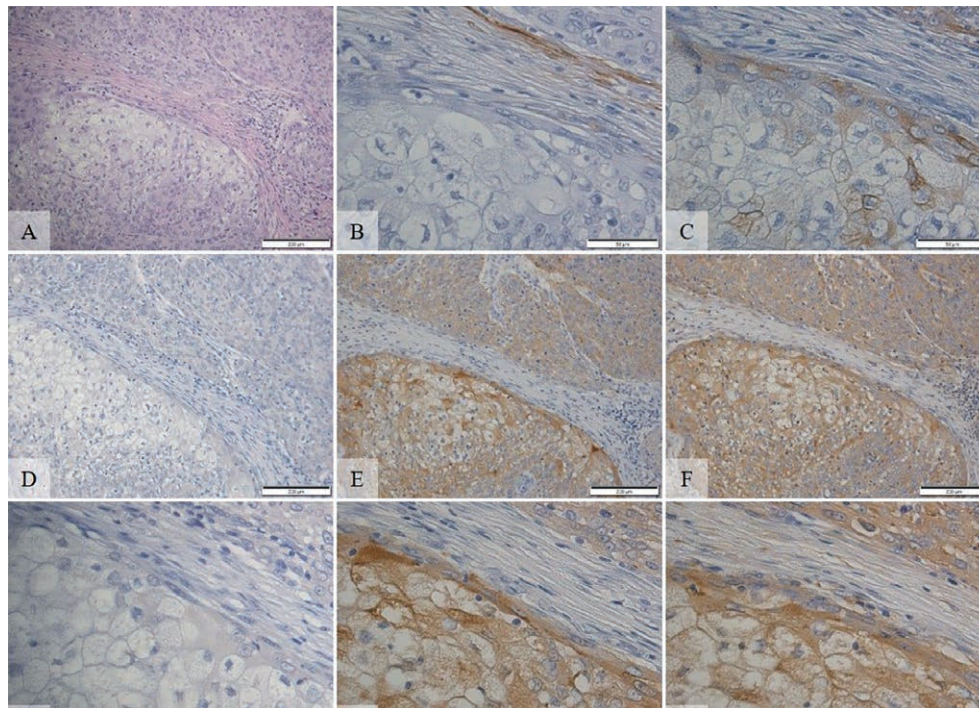


Fig. 5. Expression of the TPD52 family in mucoepidermoid carcinoma

(A) Hematoxylin and eosin staining. (B) Low-intensity calponin staining in squamous cells. (C) Low-intensity pan-cytokeratin staining in mucus-secreting and squamous cells. (D) and (G) : Negative staining for TPD52 in mucoepidermoid carcinoma. (E) and (H) : High-intensity staining for TPD53 in squamous and mucin-secreting cells. (F) and (I) : High-intensity staining for TPD54 in squamous and mucin-secreting cells.

5. Comparison of immunohistochemical staining scores

The mean score of TPD52 staining was 0.5 ± 0.53 for normal tissue, 0.9 ± 0.88 for benign salivary gland tumors, and 0.6 ± 0.52 for mucoepidermoid carcinoma. The mean score of TPD53 staining was 2.7 ± 0.48 for normal tissue, 2.2 ± 0.63 for benign salivary gland tumors, and 2.4 ± 1.16 for mucoepidermoid carcinoma. The mean score of TPD54 staining was 1.3 ± 0.68 for normal tissue, 1.9 ± 0.32 for benign salivary gland tumors, and 2.5 ± 0.53 for mucoepidermoid carcinoma. Only the TPD54 staining score of mucoepidermoid carcinoma differed significantly from that of the normal salivary gland tissue ($P < 0.01$) (Fig. 6).

Discussion

Salivary gland tumors display significant morphological diversity. Pleomorphic adenoma is the most common benign salivary gland tumor that consists of epithelial and mesenchymal cell components with wide variety in morphology. On the other hand, mucoepidermoid carcinoma is the most frequently occurring malignant salivary gland tumor involving mucus-secreting and squamous cells¹⁹. The differential diagnosis of mucoepidermoid

carcinoma is broad and depends on tumor grade and morphology (e.g., the presence of oncocytes or clear cells)². Warthin's tumor, also known as adenolymphoma or cystic papillary adenoma, is a benign salivary gland tumor that involves the lymphoid stroma and glandular epithelium with a characteristic eosinophilic cytoplasm²⁰. Neoplastic myoepithelial cells show diverse patterns in salivary gland tumors. Therefore, there is a need to identify novel biomarkers for the differential diagnosis of salivary gland tumors.

TPD52 is a member of a small protein family including TPD52, TPD53, TPD54, and TPD55^{10, 21, 22}, which were identified in 1995²³, 1996²⁴, 1998²⁵, and 2006²⁶ respectively. TPD52 is normally overexpressed in exocrine cells that contain large secretory granules²⁷. Furthermore, it regulates exocytotic secretion and vesicle trafficking, which may play an important role in the calcium-dependent membrane trafficking necessary for cytokinesis in rapidly proliferating malignant cells^{10, 21}. Animal studies have shown that TPD52 is expressed along with early endosomal markers in rat pancreatic acinar cells²⁸. TPD52 is predominantly localized in the cytoplasm of ovarian carcinoma cells and is frequently observed in mucinous and clear cell carcinomas⁶. The

Table 3. Immunohistochemical results of the expression of the TPD52 family in normal and tumorous salivary glands

	Pathological Diagnosis	No.	Organ	TPD52	TPD53	TPD54
Normal	Normal salivary gland	1	Submandibular	0	3	2
		2	Submandibular	0	3	2
		3	Submandibular	1	3	3
		4	Submandibular	0	3	1
		5	Submandibular	0	3	1
		6	Minor salivary gland	1	2	1
		7	Minor salivary gland	1	3	1
		8	Minor salivary gland	0	2	1
		9	Minor salivary gland	1	3	1
		10	Minor salivary gland	1	2	0
Benign	Pleomorphic adenoma	1	Parotid	0	3	2
		2	Parotid	0	2	2
		3	Parotid	0	2	2
		4	Parotid	2	3	2
		5	Parotid	2	3	2
	Warthin's tumor	1	Parotid	1	1	1
		2	Parotid	0	2	2
		3	Parotid	1	2	2
		4	Parotid	2	2	2
		5	Parotid	1	2	2
Malignant	Mucoepidermoid carcinoma	1	Parotid	1	3	3
		2	Palate	0	3	2
		3	Floor of oral cavity	0	3	3
		4	Palate	1	0	2
		5	Lip	1	3	3
		6	Buccal mucosa	1	2	2
		7	Palate	0	3	3
		8	Buccal mucosa	0	1	2
		9	Buccal mucosa	1	3	2
		10	Buccal mucosa	1	3	3

TP, tumor protein.

gene encoding TPD52 is located at chromosome 8q21.13 (i.e., a frequently amplified region in breast cancer), which is related to cell survival, proliferation, migration, and invasion⁶. Clinically, TPD52 is associated with a poor prognosis in breast cancer patients²⁹. It has also been reported that TPD52 expression is influenced by posttranscriptional modifications that independently affect messenger ribonucleic acid stability¹³. TPD53 is implicated in membrane trafficking because of its ability to bind to 14-3-3 proteins—a family of multifunctional, cytoplasmic molecules that negatively regulate the G2-M transition during mitosis. TPD53 is a cell

cycle regulator that is highly expressed during the G2 to M transition¹⁰. TPD54 is found on numerous small vesicles throughout the cell, functioning as a membrane trafficking protein²⁸. It not only affects cell proliferation, adhesion, and invasion⁸, but also serves as a negative regulator of cell growth in breast cancer and OSCC⁹. In addition, TPD54 inhibits anchorage-independent growth and cell migration in vitro and attenuates tumor growth in vivo; therefore, TPD54 may act as a negative regulator of tumor progression in OSCC cells¹². The expression of TPD55 is restricted to the testes, indicating a role in testis development and spermatogenesis²⁶. Hence,

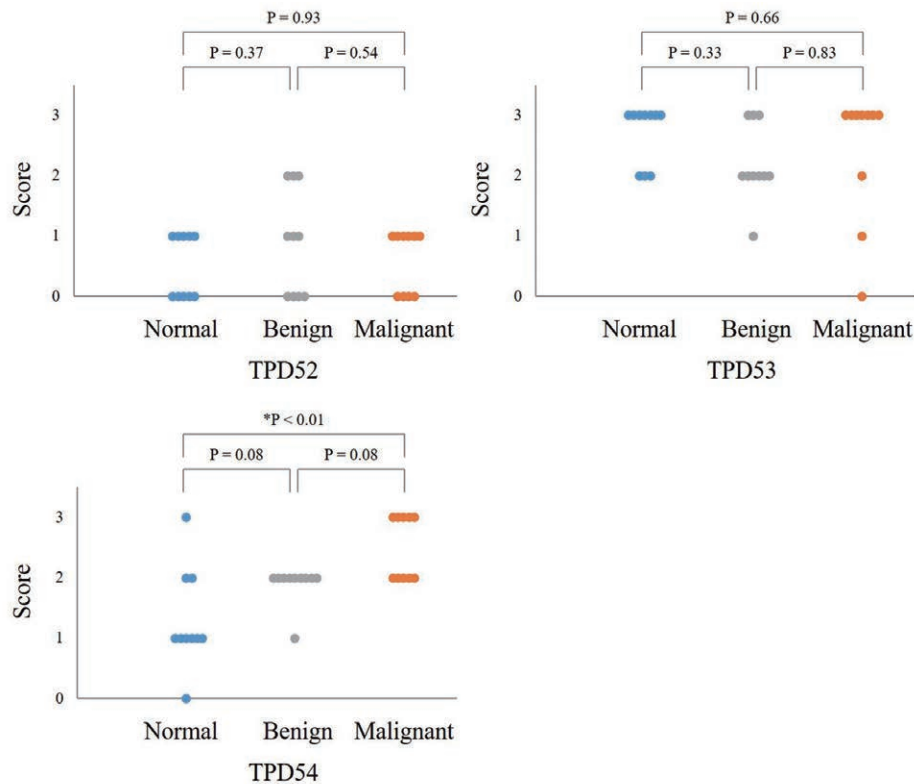


Fig. 6. Distribution of immunohistochemical staining scores
Scores of immunohistochemical staining for TPD52, TPD53, and TPD54 are plotted. Each circle represents one sample. The data were evaluated by the Tukey–Kramer method. Statistically significant results are marked by an asterisk (*) in each graph.
TP, tumor protein.

we did not include TPD55 in this study. Based on the distribution of the TPD52 family members in human cells, we selected TPD52, TPD53, and TPD54 for immunohistochemical evaluation in salivary gland tumors.

TPD54 expression may be a novel prognostic marker for cancer, because TPD54 has been shown to be upregulated in several types of solid malignant tumors³⁰. Moreover, it has been demonstrated that the knockdown of TPD54 in OSCC cell lines inhibits cell growth, promotes cell apoptosis, and inhibits extracellular matrix-dependent cell migration and attachment in OSCC patients¹². Here, we observed that TPD52 was expressed neither in normal nor in tumorous salivary gland tissue. The salivary glands are involved in exocytotic secretion and cell vesicle trafficking; however, TPD52 may not contribute to these roles.

To the best of our knowledge, this is the first study to demonstrate the expression of TPD54 in mucoepidermoid carcinoma. TPD54 is upregulated in several types of solid malignancies and is a tumor protein responsible for the high proliferation

rate of cancer cells. The expression of TPD54 has been reported to be significantly upregulated in prostate cancer tissue compared to the adjacent normal prostate tissue, indicating its critical role in the progression of prostate cancer³⁰. In this study, however, there was no relation between the expression of TPD54 and the grade of mucoepidermoid carcinoma. Thus, to elucidate the role of the TPD52 family in salivary gland tumors, future studies should follow up disease recurrence and metastasis for a substantial period of time. Moreover, knockdown and overexpression analyses in salivary gland tumor cell lines are required to understand the biological function of the TPD52 family in salivary gland tumors.

There was no relationship between the AFIP grades, assigned using the AFIP grading system recommended by the World Health Organization, and the intensity of staining for the TPD52 family. Nonetheless, we believe that TPD54 may be a useful and promising marker for the differential diagnosis of salivary gland tumors.

Conclusions

In the TPD52 family, immunohistochemical staining for TPD54 may hold the potential for the pathological differential diagnosis of benign and malignant salivary gland tumors.

Ethical approval

This study was conducted in accordance with the Declaration of Helsinki and was approved by our institutional review board.

Conflict of interests disclosure

None.

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