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ORIGINAL ARTICLE

Mist1: a novel nuclear marker for acinic cell carcinoma of the salivary gland

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Abstract

Muscle, intestine, and stomach expression 1 (Mist1) is a transcription factor that functions in the development of granule organisation in serous exocrine cells of the gastrointestinal tract. The aim of this study was to investigate whether Mist1 can be used as a marker for acinic cell carcinoma (AciCC) of the salivary gland. Immunohistochemistry (IHC) was used to analyse Mist1 expression in AciCC ($n = 26$), secretory carcinoma (SC; $n = 18$) and 12 other types of salivary gland tumours ($n = 99$). Strong Mist1 staining was observed in normal serous acinar cells and plasma cells. Mist1 was diffusely expressed in AciCC, with the immunostaining intensity ranging from moderate (4/26) to strong (22/26). Most SC specimens were either negative (7/18) or focally and mildly positive (9/18) for Mist1. Only two SC cases demonstrated a moderate level of Mist1 staining. When moderate-to-strong Mist1 staining was used as the criterion of positivity, all the tested salivary gland tumours other than AciCC were negative for Mist1, except for two cases of SC. Previously cut tissue sections and decalcified specimens exhibited reduced Mist1 immunostaining intensity, which may give false-negative results. In summary, Mist1 is a sensitive marker for serous acinar cells of salivary glands and AciCC, and background non-tumour acinar cells and plasma cells can serve as good internal positive controls.

Keywords Mist1 · BHLHA15 · Acinic cell carcinoma · Secretory carcinoma · Salivary gland

Introduction

Muscle, intestine, and stomach expression 1 (Mist1) is also known as basic helix-loop-helix family member a15 (BHLHA15) and is a basic helix-loop-helix transcription factor that is necessary for the development of granule organisation in serous exocrine cells of the gastrointestinal tract [1–5]. High protein levels of Mist1 are detected in normal pancreatic acinar cells, serous cells of the salivary glands, chief cells of the stomach, and secretory cells of the prostate and seminal vesicle [1–3]. Mist1 is also expressed in terminally differentiated plasma cells and plasmacytic neoplasms [6, 7]. As a specific marker for gastric chief cells, Mist1 expression is reduced in intestinal metaplasia, dysplasia, and intestinal type adenocarcinoma [4]. Although the expression of Mist1 in normal salivary gland is known, the applicability of Mist1-specific immunohistochemistry (IHC) in diagnosing salivary gland tumours, especially acinic cell carcinoma (AciCC), has not been evaluated. AciCC is a malignant salivary gland tumour characterised by serous acinar cell differentiation and cytoplasmic zymogen granules [8]. In addition to cells with zymogen granules, AciCC tumours also contain intercalated duct-like cells, vacuolated cells, or clear cells according to their histological features. AciCC is therefore composed of a mixture of acinar cells and other types of cells. The proportion of acinar cells in AciCC can vary case by case. It is a diagnostic challenge when the pathognomonic serous acinar cells are rare.

Secretory carcinoma (SC), also known as mammary analogue secretory carcinoma, is a distinct salivary gland tumour with genetic changes involving chromosome rearrangements resulting in *ETV6-NTRK3* or *ETV6-RET* fusions [9–11]. SC shares many histological features with AciCC, including both tumours having duct-like cells with eosinophilic cytoplasm or vacuolated cells arranged in mixed solid, microcystic, or

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follicular patterns. Prior to the identification of the *ETV6* rearrangement, a large number of SCs were diagnosed as AciCC. Fluorescence in situ hybridisation (FISH) is currently a commonly used method to confirm an *ETV6* rearrangement in order to differentiate SC from AciCC. Immunohistochemical labelling of markers such as S100, vimentin, mammaglobin, lysozyme, DOG1, and carbonic anhydrase VI (CA6) has also been used as a screening tool to differentiate SC and AciCC [12–16]. SC typically demonstrates strong staining for mammaglobin, S100 and vimentin, and negative staining for DOG1, lysozyme and CA6. In comparison, AciCC typically demonstrates positive staining for DOG1 and CA6, but is negative for mammaglobin, S100 and lysozyme. DOG1 is currently the most commonly used marker for AciCC, which typically shows mixed apical membranous, cytoplasmic and complete membranous staining [12]. However, DOG1 is not a specific marker for acinar cells and can also present in other salivary gland tumours, including adenoid cystic carcinoma and epithelial–myoepithelial carcinoma [12]. In order to evaluate the diagnostic utility of Mist1, we evaluated the expression of Mist1 in clinical AciCC and SC specimens. We then evaluated Mist1 expression in other types of salivary gland tumours. Our findings indicated that Mist1 was a sensitive and relatively specific marker for AciCC and may be useful in the differential diagnosis of the salivary gland tumours.

Materials and methods

Case selection

In previous studies, 26 cases of AciCC and 18 cases of SC were diagnostically confirmed using ETV6 break apart FISH at the National Taiwan University Hospital, Taipei, Taiwan, between 2000 and 2017 [16, 17]. All tumours were localised in the parotid glands. In order to evaluate Mist1 expression in different types of salivary gland tumours, additional 99 salivary gland tumours including nine mucoepidermoid carcinomas (four with *MAML2* rearrangements and five without *MAML2* rearrangements that were confirmed by FISH), 37 adenoid cystic carcinomas, 10 salivary duct carcinomas, five clear cell carcinomas (all containing *EWSR1-ATF1* fusions as confirmed by FISH), five lymphoepithelial carcinomas, five epithelial-myoepithelial carcinomas, four intraductal carcinomas (all negative for *ETV6* rearrangement), two sialoblastomas, four basal cell adenomas, eight pleomorphic adenomas, five oncocytomas, and five Warthin tumours were evaluated. These tumours arose from major salivary glands (parotid gland, 56 cases; submandibular gland, 24 cases; sublingual gland, 4 cases), minor salivary glands (buccal mucosa, 2 cases; palate, 3 cases; tongue base, 4 cases; parapharyngeal region, 2 cases), and other organs (lung, 2 cases; lacrimal gland, 1 case; nasal cavity, 1 case).

Immunohistochemistry

Tissue sections (4 μm) were deparaffinised and rehydrated. Antigen retrieval was performed by autoclaving the tissue sections at 121 °C for 10 min in epitope retrieval solution pH 6 (Leica Biosystems, Newcastle, UK). After antigen retrieval, the slides were incubated with a primary rabbit anti-human BHLHA15 polyclonal antibody (1:500 dilution; catalogue no. HPA047834, Atlas Antibodies, Bromma, Sweden) at room temperature for 1.5 h. Then, slides were stained using an immunohistochemical detection kit (UltraVision Quanto Detection System, Thermo Scientific, Waltham, MA, USA) and counterstained with haematoxylin. For negative controls, the primary antibody was replaced with 5% foetal bovine serum. Staining intensity was categorised as 0, no staining; 1+, weak nuclear staining; 2+, moderate nuclear staining; and 3+, strong nuclear staining and scored by two pathologists (M.S.H. and Y.H.L.).

Image and statistical analyses

Image analysis was performed using ImageJ (National Institutes of Health, Bethesda, MD, USA; https://imagej.nih.gov/ij/) and the Colour Deconvolution plug-in (http://www.mecourse.com/landinig/software/cdeconv/cdeconv.html). Mist1 IHC staining of AciCC and SC was analysed as follows. First, the tissue sections were imaged using an Olympus BX53 microscope (Tokyo, Japan) equipped with an Olympus DP70 camera system under a × 40 objective lens. The size of each captured image was 2040 × 1536 pixels. Then, the original RGB colour image was converted to 8-bit greyscale, and the total area of tumour nuclei in each image was measured automatically by selecting Image, Adjust, and Threshold in that order. Next, the Colour Deconvolution plug-in and its built-in vector 'H DAB' were used to generate three image windows: colour 1 (haematoxylin), colour 2 (DAB), and colour 3 (residual) [18]. The colour 2 image was used for IHC staining analysis. Each pixel in the colour 2 image had a value representing the brightness of the pixel, ranging from 0 (black) to 255 (white). Area of tumour nuclei with moderate-to-strong (2+ to 3+) IHC staining could be selected and measured using a threshold of 0 to 100, while area with at least mild (1+ to 3+) staining could be selected and measured using a threshold of 0 to 150. The values were then divided by the total area of tumour cell nuclei, resulting in the percentage area with moderate-to-strong (2+ to 3+) or mild-to-strong (at least 1+) staining (Fig. 1). Student's *t* test was used to determine differences in image analysis results between AciCC and SC. Fisher's exact test was used to determine differences in categorical data between cases of SC and AciCC. Two-tailed *p* values of less than 0.05 were considered statistically significant. Stata software,

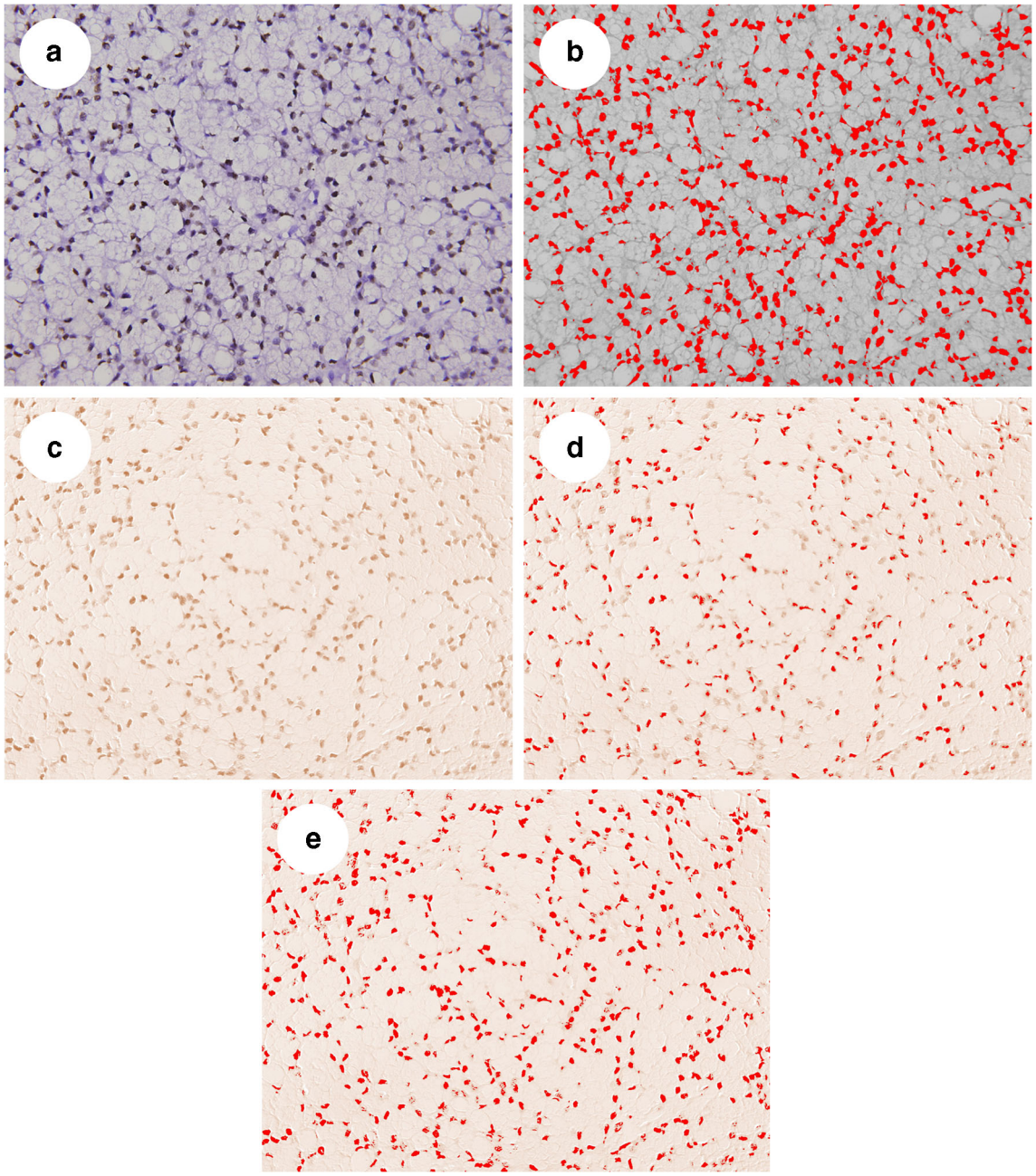

Fig. 1 Image analysis using ImageJ. **a** Original Mist1 IHC staining of one AciCC categorized as strong (3+) positivity with an image size of 2040 × 1536 pixels. **b** The total nuclear area in this image was determined using ImageJ thresholding. **c** The colour of DAB staining was separated by the ImageJ plug-in Colour Deconvolution. **d** The percentage area with moderate-to-strong staining (2+ to 3+) in this image could be selected using ImageJ thresholding (0 to 100). **e** The percentage area with mild-to-strong staining (1+ to 3+) could be selected using ImageJ thresholding (0 to 150) (original magnification: **a**–**e** × 400)

version 13.0 (Stata, College Station, TX, USA) was used for all statistical analyses.

Data availability

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

Mist1 expression in non-neoplastic salivary gland tissue

Serous acinar cells in the salivary gland showed strong nuclear staining for Mist1 (Fig. 2a, b). Other cell types in the salivary gland tissue, including mucinous acinar cells, intercalated ductal cells, striated ductal cells, and large excretory ductal

cells, were either negative or only faintly reactive for Mist1. Mist1 expression was still preserved in areas with chronic inflammation and acinar atrophy. The background plasma cells were also positive for Mist1 staining, which could serve as an internal positive control.

Mist1 expression in AciCC and SC tumours

The IHC results for the detection of Mist1 in the 26 cases of AciCC and 18 cases of SC are summarised in Table 1. The strong nuclear expression of Mist1 in serous acinar cells located in non-tumorous tissue could be used as an internal positive control and as a standard for 'strong' staining intensity for IHC evaluation of AciCC and SC specimens. AciCC tumour cells were diffusely positive for Mist1, and the staining intensity was usually strong (22/26, 84.6%) or moderate (4/26, 15.4%). AciCC tumours were diffusely positive for Mist1, irrespective of the proportion of pathognomonic acinar cells (Fig. 2c–f). The intercalated duct-like cells and vacuolated cells in AciCC also showed diffuse nuclear staining for Mist1. One AciCC that was mostly composed of duct-like cells without zymogen granules and arranged in solid and microcystic growth structures mimicking adenocarcinoma not otherwise specified was also diffusely positive for Mist1 staining (Fig. 1e, f). In the SC group, Mist1 staining was negative in seven cases, weakly and focally positive in nine cases, and moderately positive in two cases (Fig. 3). None of the SC group demonstrated diffuse and strong nuclear staining for Mist1. Mist1 IHC typically showed heterogeneous staining in SC tumours, which ranged from negative to mildly positive staining intensity that was much weaker than that of the background, non-tumour serous acinar cells. Overall, when moderate-to-strong nuclear staining (2+ to 3+) was used as the criterion for positivity, Mist1 was a sensitive and useful marker for AciCC tumours ($p < 0.001$). Differences in Mist1 staining were confirmed by image analysis using the ImageJ

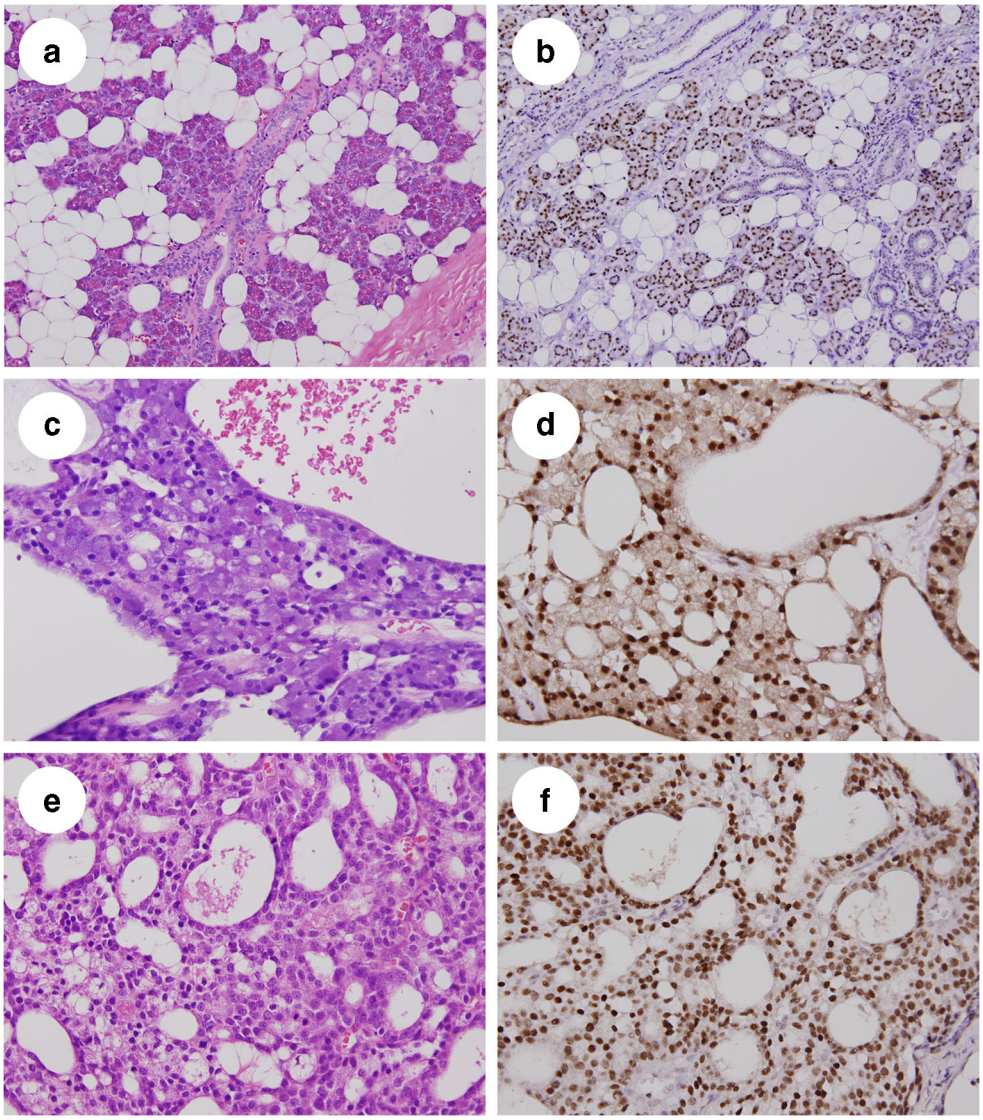

Fig. 2 **a** Normal serous acini and ductal cells in the parotid gland. **b** Serous acinar cells demonstrated strong nuclear staining for Mist1, whereas ductal cells were negative for Mist1 staining. **c** Acinic cell carcinoma (AciCC) with a high proportion of acinar cells demonstrated diffuse and strong nuclear staining for Mist1 (**d**). **e** One AciCC with areas composed of pure duct-like cells also demonstrated diffuse Mist1 staining (**f**) (original magnification: **a**, **b** × 200; **c**–**f** × 400)

Table 1 Comparison of Mist1 staining intensity between acinic cell carcinoma (AciCC) and secretory carcinoma (SC)

Intensity[a]	AciCC ($n = 26$)	SC ($n = 18$)
0	$n = 0$	$n = 7$ (38.9%)
0–1+	$n = 0$	$n = 9$ (50.0%)
2+	$n = 4$ (15.4%)	$n = 2$ (11.1%)
3+	$n = 22$ (84.6%)	$n = 0$

The $p < 0.001$ when using moderate-to-strong nuclear staining (2+ to 3+) as the criterion for positivity

[a] 0, no staining; 1+, weak nuclear staining; 2+, moderate nuclear staining; and 3+, strong nuclear staining

plug-in Colour Deconvolution followed by thresholding. The percentages of moderate-to-strong (2+ to 3+) and mild-to-strong (1+ to 3+) nuclear staining in each case are shown in Fig. 4. The percentages of moderate-to-strong (2+ to 3+) and mild-to-strong (1+ to 3+) nuclear staining in AciCC were significantly higher than those of SC ($p < 0.001$). The detailed results are provided in Online Resource 1.

Mist1 expression in other salivary gland tumours

The Mist1 IHC results using moderate-to-strong nuclear staining as the criterion for positivity are summarised in Table 2. Mist1 was positive in all 26 AciCCs (100%) and in 2 of 18 SCs (11.1%). All the remaining salivary gland tumours evaluated were negative for Mist1 staining (Online Resource 2).

Discussion

The proportion of acinar cells is variable in AciCC and can be inconspicuous in some cases. AciCC with a high proportion of acinar cells has a basophilic appearance by light microscopy under low power due to the zymogen granules in the tumour cells. In AciCC with a low proportion of acinar cells, the tumour is primarily composed of intercalated duct-like cells or vacuolated cells, which results in a relatively eosinophilic colour at a scanning power. AciCC with predominant intercalated duct-like cells may have microcystic, cystic or follicular patterns and morphologically are very similar to SC. Currently there are several IHC markers which are strongly positive in SC tumours but usually negative in AciCC tumours, such as S100, vimentin, mammaglobin and lysozyme [12–15]. However, positive IHC markers for AciCC are fewer, and DOG1 is the most commonly used one. DOG1 is a calcium-activated chloride channel that is expressed in normal acinar cells and intercalated ductal cells and is positive in AciCC tumours demonstrating a mixed apical membranous and cytoplasmic staining pattern [12]. Nevertheless, DOG1 is not AciCC-specific and can also be positive in adenoid cystic carcinoma, epithelial–myoepithelial carcinoma, and even some SCs [12, 16]. In our previous study, we demonstrated

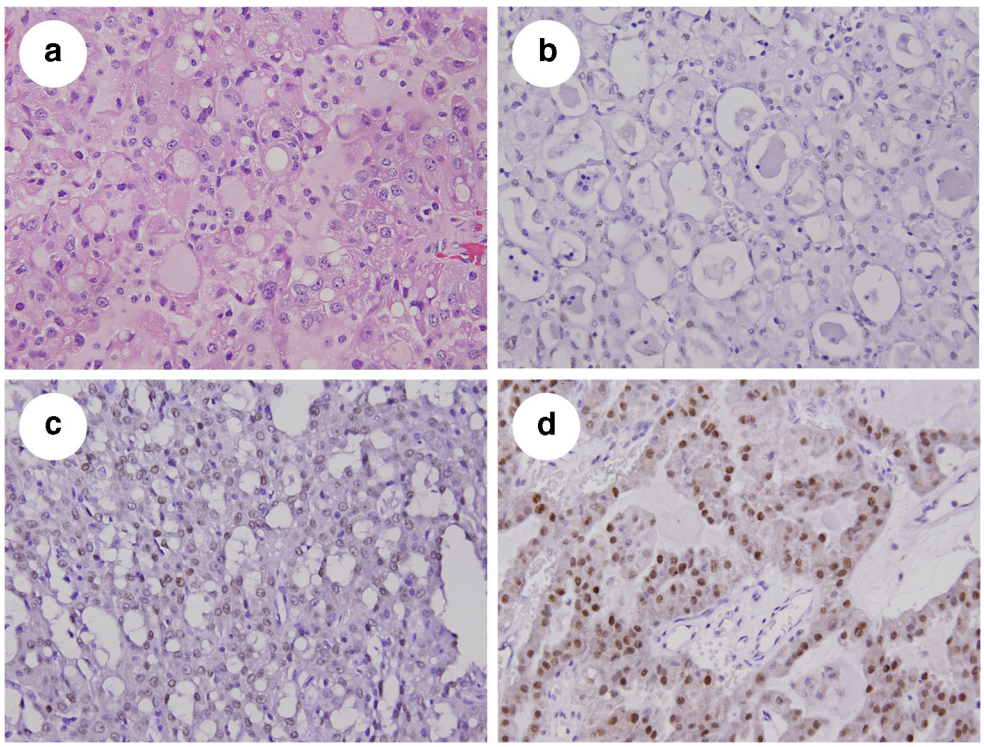

Fig. 3 **a** Secretory carcinoma (SC) cells typically showed eosinophilic cytoplasm and were arranged in microcystic or cystic-papillary structures. SC either stained negative for Mist1 (**b**) or demonstrated focal and weak Mist1 staining (**c**). **d** Two SC cases in this study (2/18, 11.1%) demonstrated moderate Mist1 staining (2+) (original magnification: × 400)

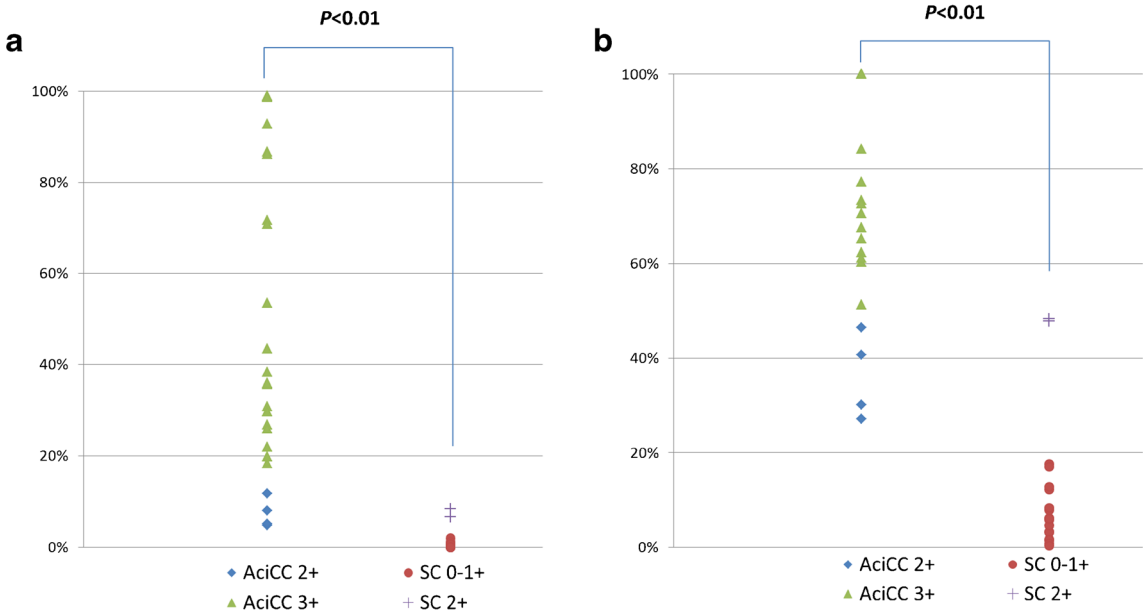

Fig. 4 Percentages of total tumour nuclei in AciCC and SC with **a** at least moderate (including 2+ to 3+) Mist1 nuclear staining using a threshold of 0 to 100 and **b** at least mild (including 1+ to 3+) Mist1 nuclear staining using a threshold of 0 to 150. Images were analysed with ImageJ. In both conditions, AciCC exhibited more Mist1 staining than SC ($p < 0.01$)

that CA6 is a specific marker for serous acinar cells and AciCC with an equal sensitivity as that of DOG1 [16]. Immunostaining for CA6 is positive only for cells with evident serous differentiation and shows strong cytoplasmic granular localisation. Depending on the number of acinar cells in an AciCC tumour, IHC analysis of CA6 may result in different staining patterns such as scattered CA6-positive cells, clustered CA6-positive cells or sheet-like CA6-positive cells [16]. Therefore, CA6 may be considered as a marker for terminally differentiated serous cells with basophilic granular cytoplasm, but it is not expressed in cells without conspicuous zymogen granules, such as intercalated duct-like cells or vacuolated cells. Consequently, results from immunostaining for CA6 must be carefully evaluated in order to not miss scattered CA6-positive cells in small specimens, such as core biopsy tissue.

In the current study, Mist1 was confirmed to be a novel marker for AciCC. Mist1 is more specific than DOG1 since

Table 2 Mist1 immunostaining in salivary gland tumours

Diagnosis	Positive[a] (*n*)		Negative[b] (*n*)		Total (*n*)
	3+	2+	0–1+	0	
Acinic cell carcinoma	22	4	0	0	26
Secretory carcinoma	0	2	9	7	18
Intraductal carcinoma	0	0	0	4	4
Mucoepidermoid carcinoma	0	0	0	9	9
Adenoid cystic carcinoma	0	0	0	37	37
Salivary duct carcinoma	0	0	0	10	10
Clear cell carcinoma	0	0	0	5	5
Lymphoepithelial carcinoma	0	0	0	5	5
Epithelial–myoepithelial carcinoma	0	0	0	5	5
Sialoblastoma	0	0	0	2	2
Basal cell adenoma	0	0	0	4	4
Pleomorphic adenoma	0	0	0	8	8
Oncocytoma	0	0	0	5	5
Warthin tumour	0	0	0	5	5

[a] Positive: moderate (2+) to strong (3+) nuclear staining

[b] Negative: no staining (0) to weak (1+) nuclear staining

Mist1 is not expressed in adenoid cystic carcinoma or epithelial–myoepithelial carcinoma. In addition, Mist1 is more sensitive than CA6 because Mist1 is expressed in all types of tumour cells in AciCC irrespective of the presence or absence of zymogen granules. In this study, there was one AciCC with large areas comprising purely duct-like cells that mimicked adenocarcinoma not otherwise specified and stained diffusely positive for Mist1 (Fig. 1e, f). AciCC may have high-grade transformation, which is characterised by the presence of a poorly differentiated high-grade component at a variable proportion [19]. Histologically, the high-grade component may be solid undifferentiated carcinoma or adenocarcinoma not otherwise specified [19]. The applicability of Mist1 in AciCC with high-grade transformation needs further study because no high-grade transformed cases were enrolled in the current study.

Table 3 summarises the IHC markers currently used for the differential diagnosis of AciCC and SC. Mist1 and CA6 are two markers that are specific for serous acinar cells. Compared to the other markers which show cytoplasmic or membranous staining patterns, the nuclear staining pattern of Mist1 is more easily evaluated. In addition, the expression of nuclear transcription factors is usually retained in less well-differentiated tumour cells, unlike the expression of terminal differentiation markers, making nuclear transcription factors more sensitive for determining cell lineage.

AciCC and SC are considered low-grade salivary gland cancers and occasionally exhibit similar histologic features such as microcystic structures. It is worth noting that both tumours are positive for SOX10, a transcription factor essential for the development of secretory units of the salivary gland including acinar and intercalated duct cells [20–22]. AciCC and SC possibly arise from cells of the secretory units of the salivary gland through different tumorigenic mechanisms. SC exhibits the pathognomonic *ETV6* rearrangement, while a subset of AciCC shows recurrent *MSANTD3* rearrangement [23]. In this study, a total of 14 different salivary gland tumours were evaluated, and only AciCC and SC exhibited Mist1 staining (Table 2). The different staining intensities between AciCC and SC correlated well with their cytologic characteristics, as Mist1 is an important transcription factor in the development of granule organisation in serous exocrine cells.

Table 3 Immunohistochemical markers commonly used in the diagnosis of acinic cell carcinoma (AciCC) and secretory carcinoma (SC)

Marker	AciCC	SC
Mist1	Positive	Negative or weak/focal
CA6	Positive (in zymogen$^+$ cells)	Negative
DOG1	Positive	Mostly negative
Mammaglobin	Negative	Positive
S100	Mostly negative	Positive
Vimentin	Mostly negative	Positive
SOX10	Positive	Positive

The staining intensity of some antibodies may decrease if tissue sections that have been stored long-term are used for IHC. This effect is called ‘slide aging’ [24]. It is important to note that the slide aging effect was observed in Mist1 IHC. The originally strong Mist1 staining observed in freshly cut tissue sections decreased, or even became absent, in tissue sections cut more than 6 months prior to IHC. We also observed that the staining intensity of Mist1 could be markedly decreased in specimens treated with decalcifying agents. Therefore, using older tissue sections or decalcified tissue sections may give false-negative results. The background non-tumour serous acinar cells and plasma cells can serve as good internal positive controls in evaluating the IHC results.

In conclusion, Mist1 is a sensitive marker for AciCC. Except for a minority of SC, no other salivary gland tumours have moderate to strong Mist1 staining. Because Mist1 is a transcription factor that demonstrates nuclear staining, it is appropriate for use and results in easy observation. The background normal serous acinar cells and plasma cells can serve as good internal controls for evaluating the quality of the IHC staining. Mist1 appears to be superior to other AciCC markers, including DOG1 and CA6.

Author contributions Min-Shu Hsieh, Yi-Hsuan Lee and Yung-Ming Jeng performed the research. Min-Shu Hsieh and Yung-Ming Jeng designed the research study and analysed the data. Min-Shu Hsieh and Yung-Ming Jeng wrote the paper.

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Compliance with ethical standards

This study was approved by the Research Ethics Committee of National Taiwan University Hospital (201504071RINC) and conducted in accordance with the Declaration of Helsinki.

Conflict of interest The authors declare that they have no conflict of interest.

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