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Computer-aided automated evaluation of MAML2 and EWSR1 rearrangements in five cases of mucoepidermoid carcinoma: A single-institution study using fluorescence in situ hybridization

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Abstract : Background: Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumor. Histological diagnosis of MECs that exhibit classical histological features is not difficult. However, diagnosis of some histological variants or high-grade MEC tumors can be challenging because of overlaps in histological features with other diseases. Recently, chromosomal translocations in salivary gland tumors have been recognized, and the detection of *MAML2* rearrangements can support the pathological diagnosis of MECs. Recently, MECs without *MAML2* rearrangements might be reclassified as hyalinizing clear cell carcinoma (HCCC) based on the detection of *EWSR1*-ATF1 gene fusions. The present study aimed to investigate the presence of *MAML2* and *EWSR1* rearrangements in MECs via fluorescence in situ hybridization (FISH).

Materials and methods: We retrospectively analyzed tumor samples from five MEC patients diagnosed in our hospital between 2008 to 2015. A total of six tumors from the five cases were pathologically evaluated and subjected to FISH analysis, which was performed using a MetaSystems image analysis system.

Results: The median age of the patients was 75 years (range: 32-79), with the majority of patients being male (male to female ratio, 4:1). The localization and pathological classification (histological grade, histological variant) of the six tumors were parotid gland/low grade/classical histology (n=2), submandibular gland/low grade/clear cell variant (n=1), lung/high grade (n=1), lung/low grade/classical (n=1), and maxillary sinus/low grade/clear cell variant (n=1). Less than 10% of the background non-neoplastic cells showed split signals of *MAML2*. Positive *MAML2* rearrangements were observed in two parotid tumors and one maxillary sinus tumor because each tumor showed over 90% of *MAML2* split signals. The other tumors appeared to show equivocal results for *MAML2* rearrangement. No *EWSR1* rearrangements were detected in all cases, suggesting that HCCC was not present.

Conclusion: FISH was found to be effective for obtaining conclusive MEC diagnoses with small biopsy samples. However, this method may have a high rate of returning equivocal results, thereby limiting its use in confirming the presence of gene rearrangements.

Keywords : Mucoepidermoid carcinoma, *MAML2* rearrangement, *EWSR1* rearrangement, fluorescence in situ hybridization

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Introduction

Mucoepidermoid carcinoma (MEC) is the most common malignant salivary gland tumor affecting children and young adults. MEC is characterized by cystic and solid growth of mucinous, intermediate, and squamous tumor cells.[1] MECs have variable histological subtypes, including the oncocytic, clear-cell, and sclerosing variants. MECs are classified into three histological grades, namely, low, intermediate, and high.[1] High-grade MECs are more aggressive than low- to intermediate-grade MECs.[1] The presence of typical histological features of MEC usually leads to the correct diagnosis. However, histological variants and high-grade MECs can be difficult to diagnose because of overlaps in histological features with other tumor types. For example, the characteristics of high-grade MECs can be very similar to those of adenosquamous carcinoma.

The presence of chromosomal translocations in MECs was first reported by Tonon et al. in 2003.[2] Most MECs are genetically characterized by a t(11;19)(q21;p13) translocation and a *CRTC1-MAML2* gene fusion, t(11;15)(q21;q26) translocation and *CRTC3-MAML2* gene fusion, or t(6;22)(p21;q12) translocation and *EWSR1-POU5F1* gene fusion.[1] The translocation generating a *CRTC1-MAML2* fusion gene is detected in 34% to 82% of MECs, with an average detection rate of 52% (356/679 cases).[3-13] Other translocations are rare, and a recent study reported only a 6% incidence of *CRTC3-MAML2*-positive MECs (6/101 cases).[14] Recently, *EWSR1-ATF1* gene fusion has been recognized as a novel marker for hyalinizing clear cell carcinoma (HCCC) in the salivary glands.[15] HCCC is characterized by sheets or nests of clear, glycogen-rich tumor cells and is histologically similar to MEC because of high mucin production.[16] *MAML2* fusion-negative MECs may be reclassified as HCCC if the tumors are positive for *EWSR1-ATF1* gene fusion.[16]

The fusion status can serve as a reliable prognostic marker for detecting MECs because *CRTC1-MAML2* fusion-positive MECs tend to be low to intermediate grade and have lower risk of local recurrence, metastases, disease-free survival, or tumor-related death than those of fusion-negative MEC.[4, 7-12, 17] Moreover, *CRTC1-MAML2* and *CRTC3-MAML2* often remain undetected in normal salivary glands and other salivary gland tumors.[3, 5, 7, 17, 18] Thus, detection of *MAML2* rearrangements is expected to be useful for MEC diagnosis. Fluorescence in situ hybridization (FISH) and reverse transcriptase-polymerase chain reaction (RT-PCR) have been used for detecting MAML2 rearrangements. Previous FISH studies have shown that tumors with *MAML2* split signal cut-offs ranging from 10% to 20% are classified as MEC;[3, 7, 19] and cut-off values for defining positive gene rearrangements have not been established for FISH.

In the present study, we performed FISH to evaluate the presence of *MAML2* and *EWSR1* rearrangements in samples from MEC patients diagnosed or treated in our hospital. The diagnostic value of FISH for MEC detection was evaluated using a MetaSystems image analysis system.

Materials and Methods

This case study was approved by Japanese Red Cross Kochi Hospital Institutional Review Board (No. 218). We retrospectively analyzed five MEC cases diagnosed or surgically diagnosed in the Japanese Red Cross Kochi Hospital between 2008 and 2015. A total of six tumors from the five cases were clinicopathologically and genetically evaluated. Schwarz et al. classified tumors as 'classical' based on equal proportions of the three cell types or the dominance (≥ 50%) of mucous cells together with at least one other cell type.[8] On the other hand, tumors were classified as ‘variant’ if ≥ 80% of the tumors were single non-mucous cell type.[8] The histological grading system of MEC given by Goode et al. was used.[20] In case 5, HCCC and epithelial-myoepithelial carcinoma

were candidates for diagnosis, and a conclusive diagnosis could not be made without FISH.

FISH for MAML2 and EWSR1 and automated analysis of the FISH signals

We performed FISH analysis for *MAML2* and *EWSR1* gene rearrangements. Formalin-fixed and paraffin-embedded (FFPE) tumors, which had been prepared from five surgically resected tumors and one biopsy sample, were cut into 4-μm-thick sections, placed on glass slides, pre-treated with a VP-2000 Processor (Abbott Molecular, Tokyo, Japan), and incubated with a *MAML2* Dual-Color Break-Apart Probe (ZytoVision, Bremerhaven, Germany) or an LSI *EWSR1* Dual-Color Break-Apart Probe (Abbott Molecular, Des Plaines, IL, USA). Hybridization was performed using a ThermoBrite system (Abbott Molecular, Tokyo, Japan). Slides were placed in a post-hybridization wash solution, counterstained with DAPI (Vysis, Inc., Downers Grove, IL, USA), and examined immediately after processing. Fluorescence images were obtained using an Axio Imager 2 Upright Microscope (Zeiss, Tokyo, Japan). Data were automatically analyzed using the Metafer 4 version 3.10.4 imaging system (MetaSystems, Altlussheim, Germany). At least 100 nuclei were automatically scored. Cells with two fusion signals (one orange and one green fluorochrome) were defined as normal. Cells harboring *MAML2* rearrangements had one normal fusion signal, one orange, and one green signal.

Results

Clinicopathological data from the five patients are summarized in Tables 1 and 2. The median age of the patients was 75 years (age range; 32-79). Five tumors were classified as low-grade MECs, while one tumor was classified as high-grade MEC. In three low-grade MECs (two parotid MECs and one maxillary sinus MEC), over 90% of the tumor cells showed split signals of *MAML2* with FISH (Figure 1) and were thus considered positive for *MAML2* rearrangement. In the other three MECs, 17% to 20% of the tumor cells showed split signals, which was considered as equivocal based on internal validation of the background non-neoplastic tissues and a previously reported cut-off value of MECs.[7] *MAML2* rearrangement was not detected in the high-grade pulmonary MEC. No *EWSR1* rearrangements were detected in all cases.

Discussion

In the present study, FISH was conducted to evaluate the chromosomal abnormalities in

Table 1. Clinical data of the five mucoepidermoid carcinoma cases.

Case	Age/ Sex	Tumor location	Maximum size	UICC Stage at diagnosis	Treatment	Metastasis/ recurrence	Follow-up (months)
1	32/M	Parotid gland	32 mm	NA	Surgery	NA	NA
2	74/M	Parotid gland	12 mm	T1N0M0	Surgery	No	AWOD (27)
3	79/F	Maxillary sinus	70 mm	T4N0M0	Palliative care	No	AWD (8)
4	75/M	Submandibular gland	25 mm	T2N0M0	Surgery, and postoperative radiotherapy (total 50 Gy)	No	AWOD (53)
5	76/M	Lung, left upper (main tumor)	50 mm	T4N0M1	Surgery, and postoperative chemotherapy	Lung metastasis present at surgery and lung recurrence was present after surgery	DOD (66)
		S8 tumor (metastatic lesion)	10 mm				

Abbreviations: M, male; F, female; UICC, Union for International Cancer Control; NA, not available; Gy, gray; AWOD, alive without disease; DOD, died of disease; AWD, alive with disease

Table 2. Pathological data of the five mucoepidermoid carcinoma cases.

Case	MEC, histological subtype	Cystic component <20%	Neural invasion	Necrosis	Four or more mitoses/10 HPFs	Anaplasia	Histological grade	MAML2 FISH (rate of split signals)
1	Classical	No (30%)	Present	Absent	Absent (1/10 hpf)	Absent	Low grade	94%
2	Classical, TALP present	No (30%)	Absent	Absent	Absent (1/10 hpf)	Absent	Low grade	94%
3	Clear cell variant	Yes (0%)	Absent	Absent	Absent (0/10 hpf)	Absent	Low grade	98%
4	Clear cell variant	Yes (10%)	Present	Absent	Absent (1/10 hpf)	Absent	Low grade	17%
5	Main lung tumor: squamoid variant	Yes (<5%)	Absent	Present	present (8/10 hpf)	Present	High grade	20%
	S8 tumor and recurrent tumors: classical	Yes (0%)	Absent	Absent	Absent (<3/10 hpf)	Absent	Low grade	18%

Abbreviations: MEC, mucoepidermoid carcinoma; TALP, tumor-associated lymphoid proliferation; hpf, high power field; FISH, fluorescence in situ hybridization

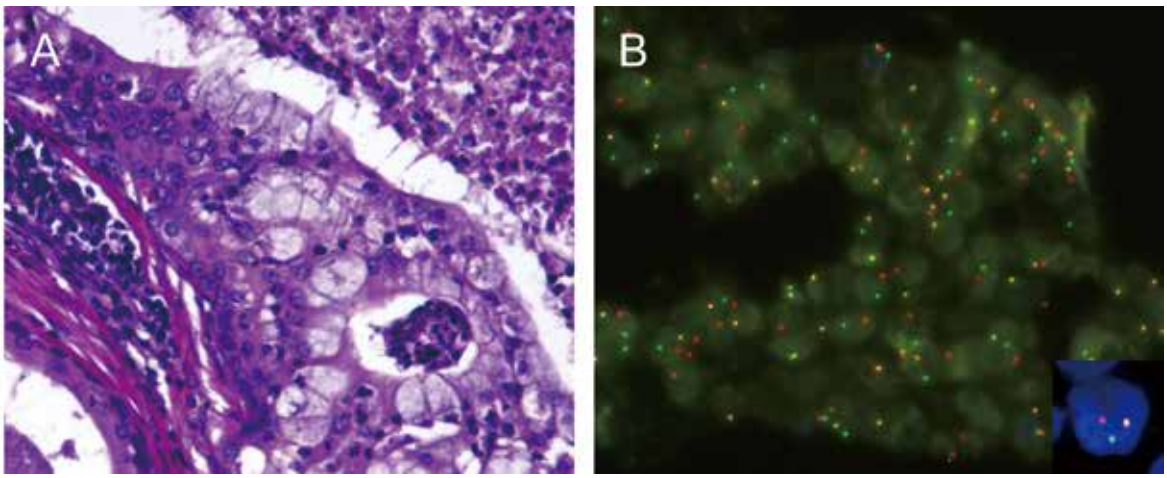

Figure 1. Histological and fluorescence in situ hybridization results for a classical, low-grade mucoepidermoid carcinoma.
A. Hematoxylin-and-eosin staining of a classical, low-grade mucoepidermoid carcinoma showing a mixture of mucinous, intermediate, and/or squamoid cells (400×). B. Fluorescence in situ hybridization of the MAML2 gene shows that most tumor cells have split signals, including isolated red signals and green signals. The inset shows a typical split signal in a tumor nucleus.

MAML2 and *EWSR1* genes in five MEC cases. Based on FISH results, three MECs showed high rates (> 90%) of split signals in the *MAML2* gene and were thus considered positive for *MAML2* rearrangement. The FISH method significantly contributed to obtaining conclusive diagnosis of the maxillary sinus tumor (case 5), which could not be diagnosed using biopsy specimens.

Consistent with the results of previous studies, all tumors that were positive for *MAML2* rearrangement were low-grade tumors. The rate for positive *MAML2* rearrangement in salivary gland MECs was higher in low- or intermediate-grade tumors than that in high-grade tumors [low grade, 57% (169/298 cases); intermediate grade, 48% (35/73 cases); high grade, 23% (38/164 cases)].[4, 7-12] Similar results were obtained for pulmonary MECs [low to intermediate grade 60% (33/55 cases) and high grade 23% (5/22 cases)].[17, 19, 21] In a previous study on adenosquamous carcinoma, all 36 cases did not show *MAML2* rearrangement. Thus, although high-grade MECs had low rates of

MAML2 rearrangement, further genetic studies on high-grade MECs will be useful to distinguish between high-grade MECs and adenosquamous carcinoma.[17, 22] Seethala et al. suggested that translocation-negative high-grade MECs may be more appropriately categorized as a different tumor type, such as adenosquamous carcinoma.[7] The clear cell variant of MECs shows a high frequency of *MAML2* rearrangement,[8] and one of two MECs classified under the clear cell variant in our study was positive for gene rearrangement. However, further study with a higher number of cases will be required.

In the present study, less than 10% of the background non-neoplastic cells showed split signals of *MAML2*, suggesting that three MECs with a split signal rate of 17% to 20% were positive for *MAML2* rearrangement. However, the split signal rate of these three MECs was notably lower than that of the other three MECs; previous FISH studies have shown that tumors with an *MAML2* split signal cut-off of 20% are classified as MEC.[7] Thus, the equivocal results of our study were validated, and we suggest that intratumoral heterogeneity might be present in these tumors. Further validation with other techniques, such as RT-PCR, will be required if equivocal results are obtained with FISH.

Detection of *MAML2* rearrangements is expected to contribute to histological diagnosis of MECs. However, Warthin tumors can also be positive for *MAML2* rearrangements. Tirado et al. reported that 36% (4/11 cases) of Warthin tumors were positive for *CRTC1-MAML2* fusion transcript based on RT-PCR.[5] On the other hand, Seethala et al. reported that none of 24 cases of Warthin tumor in their sample were negative for the translocation based on FISH and RT-PCR [7]. Fehr et al. reported that tumors with the t(11;19)(q21;p13) rearrangement favored a diagnosis of MEC rather than Warthin tumor because fusion-positive Warthin tumors could be reclassified as MEC.[18] Rotellini et al. suggested that *MAML2* fusion-positive Warthin tumors represented a molecular link between the two entities because fusion-positive Warthin tumors presented squamous metaplasia.[23] Thus, pathologists should consider a diagnosis for Warthin tumor even if the translocation is present in MECs. In our study, Warthin tumors were not included in all cases.

No *EWSR1* rearrangements were detected, suggesting that HCCC was not included in our cases. Hsieh et al. reported that 18% (3/17 cases) of *MAML2*-fusion-negative MEC tumors could be reclassified as HCCC with confirmation of *EWSR1-ATF1* fusion and proposed that MEC cases arising from minor salivary glands or without *MAML2* fusion should be distinguished from HCCC.[16]

Conclusion

The use of FISH in detecting *MAML2* rearrangements is effective for obtaining a conclusive MEC diagnosis using small biopsy specimens. However, *MAML2* FISH might be less effective for confirming the presence of *MAML2* rearrangements in FFPE blocks of MEC tumors.

Declarations

Competing interests: The authors declare no competing interests.

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