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Reevaluation of *MAML2* fusion–negative mucoepidermoid carcinoma: a subgroup being actually hyalinizing clear cell carcinoma of the salivary gland with *EWSR1* translocation☆,☆☆

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Summary Hyalinizing clear cell carcinoma (HCCC) is a rare salivary gland tumor with a specific *EWSR1-ATF1* fusion gene and can have mucin production. Mucoepidermoid carcinoma (MEC) with a clear cell component is its morphologic mimic. Using *MAML2* fluorescence in situ hybridization (FISH), a total of 49 MEC cases were separated into *MAML2* fusion–positive (32 cases) and *MAML2* fusion–negative groups (17 cases). This study used *EWSR1* FISH to investigate *MAML2* fusion–negative cases to identify previously unrecognized HCCC. Among 17 *MAML2* fusion–negative cases, 3 had rearrangement of the *EWSR1* gene and were reclassified as HCCC. Including 5 previously diagnosed HCCC cases, these 8 HCCC cases had a male-to-female ratio of 1:7, and most (7/8) tumors arose from oral minor salivary glands in the oral cavity (tongue base and palate). *EWSR1-ATF1* fusion was confirmed by FISH in all 8 HCCC cases. The histologic features between genetically confirmed HCCC and MEC were compared. HCCC was significantly associated with minor salivary gland involvement, a discrepancy between low-grade cytology and intermediate- to high-grade histology using the MEC grading system, and absence of both epidermoid cells with abundant cytoplasm and goblet cells lining cysts or forming clusters. Clear cells and a hyalinized stroma were not specific for HCCC. HCCC may be erroneously classified as MEC because clear cells may be a minor histologic component and mucin production is not uncommon. Previously diagnosed MEC cases should be reevaluated, especially those arising from minor salivary glands or without *MAML2* fusion. Careful histologic evaluation with supporting molecular testing can facilitate pathologic diagnoses.

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1. Introduction

Hyalinizing clear cell carcinoma (HCCC) is a rare salivary gland tumor first described by Milchgrub et al [1] in 1994. HCCC most often arises from minor salivary glands in the head and neck, especially those of the palate and tongue [2]. HCCC also reportedly arises from submucosal glands of the bronchus, nasopharynx, and nasal cavity [2-6]. Morphologically, HCCC is characterized by small monomorphic cells with pale eosinophilic to clear cytoplasm, arranged in cords or nests in a hyalinized and fibrocellular stroma [1,5,7-10]. Antonescu et al [7] first identified a recurrent gene fusion of the Ewing sarcoma breakpoint region 1 (*EWSR1*) gene and the activating transcription factor 1 (*ATF1*) gene in HCCC. More than 80% of HCCC tumors harbor this unique *EWSR1-ATF1* mutation, which distinguishes HCCC from other salivary gland tumors [7-9]. Identification of this specific *EWSR1* gene rearrangement refined our knowledge of HCCC. Based on molecularly verified cases, HCCC mucin production is not uncommon and is no longer an exclusion criterion in pathologic diagnoses [5,8,9,11]. Antonescu et al [7] observed mucin in 10 (44%) of 23 HCCC tumors, and mucin distribution varied from focal to diffuse. Clinically, HCCC is considered a low-grade malignancy with excellent prognosis; nevertheless, a few HCCC patients developed delayed recurrence or distant metastasis [2,7,12].

HCCC shares common morphologic features with mucoepidermoid carcinoma (MEC), because both tumors can have mucin and clear cells. The small HCCC monomorphic cells with pale eosinophilic cytoplasm are similar to intermediate MEC cells. Therefore, the differential diagnosis between HCCC and MEC sometimes can be very difficult [5,8,9,11]. With the discovery of characteristic fusion genes in salivary gland tumors, molecular tests can be a powerful tool [13].

The mastermind-like transcriptional coactivator 2 (*MAML2*) translocation is considered a specific genetic change in MEC that can be identified in 55% to 66% of cases [13-17]. This translocation is more commonly found in low- to intermediate-grade MEC and is associated with a better clinical prognosis [13-17]. It has been proposed that MEC may be classified according to its *MAML2* fusion status as high grade; *MAML2* fusion–negative cases would actually not represent MECs but rather other carcinomas with overlapping morphologic features [18-20].

Antonescu et al [7] suggested that some "clear cell variant MEC" may be HCCC. We wanted to prove this hypothesis that some past diagnosed MEC cases are actually HCCC, especially those without *MAML2* translocation. Using fluorescence in situ hybridization (FISH), we first identified MEC cases that were *MAML2* fusion negative. We then used *EWSR1* FISH to investigate these cases to identify previously unrecognized HCCC. We further compared clinicohistologic parameters and immunohistochemistry between molecularly confirmed cases of HCCC and MEC to find useful parameters to facilitate pathologists' selections of cases that receive confirmatory FISH testing.

2. Materials and methods

2.1. Case selection

We searched the archives of the Department of Pathology at National Taiwan University Hospital and identified 49 cases with an original diagnosis of MEC and 5 HCCC cases that were surgically resected between 1999 and 2015. All cases had available formalin-fixed and paraffin-embedded specimens. MEC cases were separated into *MAML2* fusion–positive and *MAML2* fusion–negative groups according to their *MAML2* FISH results. *MAML2* fusion–negative ones were selected for *EWSR1* FISH testing. All 5 HCCC cases had *EWSR1* gene translocation that was confirmed by *EWSR1* FISH at our hospital. Two HCCC cases were initially diagnosed as clear cell variant MEC at other hospitals. The clinical information for each patient, including age, sex, tumor location, tumor size, lymph node status, and local recurrence or distant metastasis during clinical follow-up, was collected from medical records. Hematoxylin and eosin–stained slides were reviewed by 2 pathologists (M. S. H. and Y. H. L.). The proportion of clear cells in each tumor was recorded. This study was approved by the Research Ethics Committee of National Taiwan University Hospital.

2.2. Detection of *MAML2*, *EWSR1*, and *ATF1* gene translocations and *EWSR1-ATF1* gene fusion by FISH

Commercial ZytoLight SPEC *MAML2* Dual Color Break Apart Probe (Zytovision, Bremerhaven, Germany) and Vysis *EWSR1* Dual Color Break Apart FISH Probe (Abbott Molecular, Des Plaines, IL) were used to assess *MAML2* and *EWRS1* gene translocation, respectively. Dual-color FISH to test *ATF1* gene rearrangement was carried out using bacterial artificial chromosome FISH probes (Empire Genomics, Buffalo, NY) RP11-831J22, RP11-3M16, and RP11-73M17 labeled with Spectrum Orange, and RP11-139D22, RP11-1056L15, and RP11-368D17 labeled with Spectrum Green, which map to the centromere and telomere sides of the *ATF1* gene, as previously described [7] (Supplementary Fig. S1). Dual-color FISH to confirm *EWSR1-ATF1* fusion was carried out using bacterial artificial chromosome FISH probes (Empire Genomics) RP11-945 M21, RP11-965D15, and RP11-77 M13 labeled with Spectrum Orange, which map to the centromere side of the *EWSR1* gene, and RP11-139D22, RP11-1056 L15, and RP11-368D17 labeled with Spectrum Green, which map to the telomere side of the *ATF1* gene [7] (Supplementary Fig. S1).

Briefly, 4-μm-thick paraffin-embedded tissue sections were deparaffinized in xylene (3 times, 10 minutes each), followed by two 5-minute washes in 100% ethanol. Sections were then treated with pretreatment reagent (Abbott Molecular) at 80°C for 30 to 50 minutes, after which sections were treated with protease mixed with a protease buffer. Sections were hybridized using specific FISH probes.