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Mucoepidermoid carcinoma may be devoid of squamoid cells by immunohistochemistry: expanding the histologic and immunohistochemical spectrum of *MAML2*- rearranged salivary gland tumours

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Mucoepidermoid carcinoma may be devoid of squamoid cells by immunohistochemistry: expanding the histologic and immunohistochemical spectrum of *MAML2*- rearranged salivary gland tumours

Mucoepidermoid carcinoma (MEC) is historically defined by a mix of squamoid, intermediate, and mucous cells, but we have recently encountered several cases lacking immunoreactivity for squamous markers p40, p63, and CK5/6 despite *MAML2* fusions. This study will characterise these unique tumours. Ten MEC were collected arising from the parotid gland (n = 4), submandibular gland (n = 2), nasopharynx (n = 1), base of tongue (n = 1), bronchus (n = 1), and trachea (n = 1). Six tumours were low-grade, two intermediate-grade, one high-grade, and one demonstrated low-grade areas with high-grade transformation. Four cases were oncocytic, four had clear-cell features, two had spindle cell features, and one high-grade MEC had prominent solid, cord-like, and micropapillary features. The tumours were negative for p40 (10/10), p63 (10/10), and CK5/6 (9/9). Targeted RNA sequencing demonstrated *CRTC1::MAML2* in five cases, *CRTC3::MAML2* in two, and a novel *MAML2::CEP126* in the unusual high-grade case. In two cases with insufficient RNA, *MAML2* fluorescence *in situ* hybridisation (FISH) showed rearrangement. Genetically-confirmed MEC may lack overt squamous differentiation by histology and immunohistochemistry. While most cases harboured canonical fusions and fit within the spectra of MEC variants with oncocytic, clear cell, and/or spindle cell features, one had a novel *MAML2::CEP126* fusion and unusual morphology. In MEC without squamoid cells, the use of immunohistochemistry may hinder, rather than aid, the correct diagnosis. In such cases, *MAML2* analysis is most useful. The historical definition of MEC as a carcinoma with squamoid, intermediate and mucous cells should be revisited.

Keywords: *MAML2*, mucoepidermoid carcinoma, p40, p63, salivary gland neoplasms

Introduction

Historically, salivary gland tumour diagnosis was based primarily on routine histologic examination,

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with occasional use of histochemical stains like mucicarmine and periodic acid Schiff. Modern ancillary testing, however, has revolutionised salivary gland tumour classification. First, the introduction and widespread application of immunohistochemistry allowed pathologists to easily demonstrate differentiation of various cell types (e.g. myoepithelial, ductal, squamoid, acinar, neuroendocrine) that comprise a salivary gland tumour, facilitating more precise recognition of these elements and more accurate separation of overlapping entities. Subsequently, recognition of tumour-specific genetic alterations testing has allowed for recognition of entirely new entities (e.g. *ETV6* fusions in secretory carcinoma and *MEF2C::SS18* fusions in microsecretory adenocarcinoma) and refined the spectrum of how established neoplasms may appear (e.g. metatypical adenoid cystic carcinoma with *MYB* fusions and frankly invasive intraductal carcinoma with *RET* or *ALK* fusions).[1–5] As a result, immunohistochemistry and molecular testing have both become standard practice in evaluating salivary gland tumours.

Such ancillary testing has dramatically shaped our diagnosis and understanding of mucoepidermoid carcinoma (MEC), the most common salivary gland malignancy. Traditionally, MEC has been defined by the histologic presence of mucous, intermediate, and squamoid cells - requirements that persist in the current 2022 World Health Classification of Head and Neck Tumours.[6] While this definition is seemingly straightforward, in reality the concept of three discrete and consistent component cells belies many histologic subtleties in dealing with this tumour. MEC often includes cell types (e.g. columnar, nonspecific ductal, oncocytic), which are not part of the definition. "Intermediate cells" are not easy in practice to locate or even define. Moreover, squamous differentiation is sometimes not evident on light microscopy. As a result, immunohistochemistry for squamous immunohistochemical markers like p40, p63, and CK5/6 was often relied on to confirm definitional squamous differentiation in MEC.[7–12] The vast majority of MEC has also been found to harbour *CRCT1::MAML2* or *CRCT3::MAML2* fusions, which are regarded as specific to this entity in the salivary glands and are desirable criteria in the WHO Classification.[6,13,14] Not only can *MAML2* testing confirm a challenging diagnosis, but identification of *MAML2* fusions as a genetic gold standard has allowed for recognition of multiple variant forms of MEC, including oncocytic, ciliated, sclerosing, Warthin-like, and mucoacinar types.[10,15–18]

While these variants have broadened the histologic spectrum that is recognised as MEC, the immunohistochemical and molecular findings in all of these alternate morphologies still seem to meet WHO criteria, including canonical *MAML2* fusion partners and some degree of immunopositivity for p63, p40, and CK5/6 supporting squamous differentiation even when it is not evident at the histological level. For example, oncocytic MEC may be composed almost entirely of oncocytes with scattered mucinous cells, but up to this point, reported cases have consistently shown at least focal staining with squamous markers. However, we recently identified a small group of MEC that did not express squamous markers despite *MAML2* rearrangement—a phenomenon that defies current tenets of MEC classification. This study aims to describe the features of MEC that lack clear evidence of squamous differentiation and consider their implications for the definition and diagnosis of MEC.

Materials and methods

CASE SELECTION

With Institutional Review Board approval (IRB 112017–073), cases of MEC negative for squamoid cells were retrieved from the authors' surgical pathology archives and consultation files. All cases were diagnosed as MEC, and retrieved on personal recall. Each case was initially believed to be MEC based on morphology, but when faced with an unexpected absence of squamous marker immunoreactivity, molecular confirmation was sought by the original pathologist. Each MEC was submitted entirely for evaluation. All cases were reviewed centrally by the primary author, and various demographic and histologic features were tabulated.

IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed one representative block on all cases. Using standard automated protocols, staining was performed on a Ventana BenchmarkXT autostainer (Ventana Medical Systems. Tucson, AZ, USA) using antibodies for p63 (Biocare Medical, Concord, CA, USA), p40 (Biocare), and CK5/6 (Ventana). All immunohistochemical signals were visualised using the Ultra view polymer detection kit (Ventana).

FLUORESCENCE *IN SITU* HYBRIDISATION (FISH)

Break-apart FISH was performed on a subset of cases using a standard dual colour break-apart probe

(centromeric 3′-side green, telomeric 5′-side orange) for *MAML2* following the manufacturer's protocol (Abbott Molecular, Des Plains, IL, USA). Sections were deparaffinised, pretreated for 25 min at 80°C, treated with protease for 38 min at 37°C, probe and target codenatured at 80°C for 15 min, hybridised overnight (37°C), and then washed (at 74°C) for 2 min. Then slides were stained with DAPI and evaluated using epifluorescence microscope and ASI software (Applied Spectral Imaging, Chicago, IL, USA); 100 nuclei were evaluated from each slide. Cases with split signals in >12% of cells were regarded as positive.

RNA SEQUENCING

Targeted RNA sequencing was attempted on eight cases using different TruSight RNA Fusion panels or modified Pan-Cancer kits (Illumina, San Diego, CA, USA) as previously described.[3,19,20] Whole-slide tissue sections were cut at 5 μm and Qiagen AllPrep kits (Qiagen, Germantown, CA, USA) were utilised for RNA isolation. A sequencing library was constructed using a modified TruSight RNA Pan-Cancer kit (Illumina) with 1425 genes. Sequencing was performed on the NextSeq 550 (Illumina) with a minimum of 6 million mapped reads. Fusions were called using the Star-Fusion algorithm[21] and manually reviewed via the Integrated Genomics Viewer (Broad Institute, Cambridge, MA, USA).

Results

Ten cases of MEC that were negative for squamoid cells were identified, and they are summarised in Table 1. The cases arose in eight females and two males, ranging from 9 to 84 (median, 40 years). The tumours arose from the parotid gland (n = 4), submandibular gland (n = 2), base of the tongue (n = 1), nasopharynx (n = 1), bronchus (n = 1), and trachea (n = 1). Six tumours were low-grade, two were intermediate-grade, one was purely high-grade, and one demonstrated low-grade areas with high-grade transformation.

Histologically, none of the cases included overtly squamoid cells, which are usually defined in MEC as having a polygonal shape and abundant homogenous eosinophilic cytoplasm. Nevertheless, nine of the cases had morphology that was otherwise recognisable within the established spectrum of variant MEC, albeit without any of the typical squamous marker immunoexpression. Four cases were oncocytic variants, predominated by solid nests and sheets of tumour cells with abundant granular eosinophilic cytoplasm and round nuclei with prominent nucleoli, separated by bands of fibrosis (Figure 1A,B). These cases were infiltrative into nearby adipose tissue, and had focal areas of duct formation with intraluminal mucinous secretions (Figure 1C,D). Two of the four oncocytic MECs demonstrated tumour-associated lymphoid proliferation (TALP). Four cases had prominent clear-cell features, ranging from pale eosinophilic cytoplasm to completely "water clear" cytoplasm with prominent cell borders (Figure 2A,B). One of the clear-cell MECs was the case that demonstrated high-grade transformation, with an abrupt transition to a tumour with increased nuclear pleomorphism and mitotic activity (Figure 2C,D). Two MECs had vague tumour cell spindling and swirling; one MEC had both clear-cell and spindled cells (Figure 3). The clear-cell and/or spindled tumours were all punctuated by ducts and mucinous cells. Each was overtly invasive, and four of the five clear-cell and/or spindled cases demonstrated TALP.

The single purely high-grade MEC had an unusual histopathologic appearance. It was predominantly micropapillary, with other solid nests, cord-like growth, and only occasional duct formation and mucin production. The tumour cells had eosinophilic cytoplasm and pleomorphic nuclei with prominent nucleoli but lacked an overtly apocrine appearance. Mitotic figures were numerous, broad zones of comedonecrosis were present, and there was extensive lymphatic invasion. (Figure 4).

Per inclusion criteria, the tumours were uniformly negative for the squamous markers p40 (10 of 10) and p63 (10 of 10) as well as CK5/6 in all cases tested (nine of nine) (Figure 5). In addition, all cases tested were negative for mammaglobin (n = 8), S100 (n = 7), SMA (n = 7), SOX10 (n = 6), androgen receptor (n = 4), TTF1 (n = 4), GCDFP (n = 4), DOG1 (n = 3), calponin (n = 2), PAX8 (n = 2). The unusual-appearing high-grade case was also negative for CK20, CDX2, Her2, oestrogen receptor, and progesterone receptor. By molecular analysis, five cases harboured *CRTC1::MAML2* and two cases had *CRTC3::MAML2*. The atypical high-grade MEC was found to harbour a novel *MAML2::CEP126* fusion. Finally, in two cases MAML2 break-apart FISH was positive, but there was insufficient tissue to perform RNA sequencing.

Discussion

Salivary gland tumour classification has drastically evolved over the last decade as emerging molecular

Table 1. Clinical and pathologic characteristics of mucoepidermoid carcinomas lacking squamoid differentiation

Case	Age	Sex	Location	Grade	Histologic type	p40	p63	CK5/6	*MAML2* FISH	RNA-seq	Additional negative IHC
1	9	F	Nasopharynx	Intermediate	Clear cell	–	–	–	ND	*CRTC1::MAML2*	S100, SOX10, DOG1, SMA, calponin
2	14	F	Trachea	Low	Oncocytic	–	–	ND	+	ND	S100, mammaglobin, SMA, PAX8, GCDFP, TTF1
3	22	F	Submandibular gland	Low	Clear cell and spindle cell	–	–	–	ND	*CRTC3::MAML2*	S100, SOX10, DOG1, mammaglobin
4	24	F	Bronchus	Low	Oncocytic	–	–	–	ND	*CRTC1::MAML2*	S100, mammaglobin, PAX8, GCDFP, TTF1
5	38	F	Submandibular gland	Low with high-grade transformation	Clear cell	–	–	–	+	*CRTC1::MAML2*	S100, mammaglobin, SMA, calponin
6	42	F	Parotid gland	Low	Oncocytic	–	–	–	+	ND	SOX10, mammaglobin; AR, SMA
7	58	F	Parotid gland	High	Solid, cord-like, micropapillary features	–	–	–	ND	*MAML2::CEP126*	Her2, AR, CDX2, CK20, GCDFP, mammaglobin, ER, PR, TTF
8	58	F	Parotid gland	Low	Clear cell	–	–	–	ND	*CRTC1::MAML2*	S100, SOX10, DOG1, mammaglobin
9	76	M	Base of tongue	Intermediate	Spindle cell	–	–	–	ND	*CTRC3::MAML2*	S100, SOX10, TTF1, SMA, p16
10	84	M	Parotid gland	Low	Oncocytic	–	–	–	+	*CRTC1::MAML2*	SOX10, mammaglobin; AR, SMA

AR, androgen receptor; GCDFP, gross cystic disease fluid protein; F, female; FISH, fluorescence *in situ* hybridisation; IHC, immunohistochemistry; M, male; ND, not done; RNA-seq, targeted RNA sequencing; SMA, smooth muscle actin.

findings have refined the histologic boundaries of many well-established entities.[1] This is especially true for MEC, the most common salivary gland carcinoma, which now has several well-recognised histologic variants, including oncocytic, ciliated, sclerosing, Warthin-like, and mucoacinar. For each variant, the presence of *MAML2* rearrangement, which is regarded is specific for MEC among salivary gland tumours,[22] confirmed that the tumours were MEC despite unusual histologic features. Consequently, the histologic spectrum of MEC widened beyond what previously could have been imaginable. For example, the presence of acinar differentiation was long regarded as pathognomonic for acinic cell carcinoma, but *MAML2*-rearranged mucoacinar MECs are now defined by this finding.[23] Far from being a molecular-only diagnosis, each described MEC variant has also refined the morphologic boundaries between MEC and other tumours. For example, it is now recognised that very bland, cystic cases previously regarded as Warthin tumours that lack a well-formed bilayer of oncocytes are usually Warthin-like MEC.[16,17,24] While many of these subtypes do not show squamoid differentiation morphologically, all previous variants of MEC have demonstrated evidence of squamous differentiation at least at an immunohistochemical level. Here, we expand the immunohistochemical spectrum of MEC with 10 cases that unexpectedly were negative for p63 and p40 despite confirmation of the presence of *MAML2* fusions.

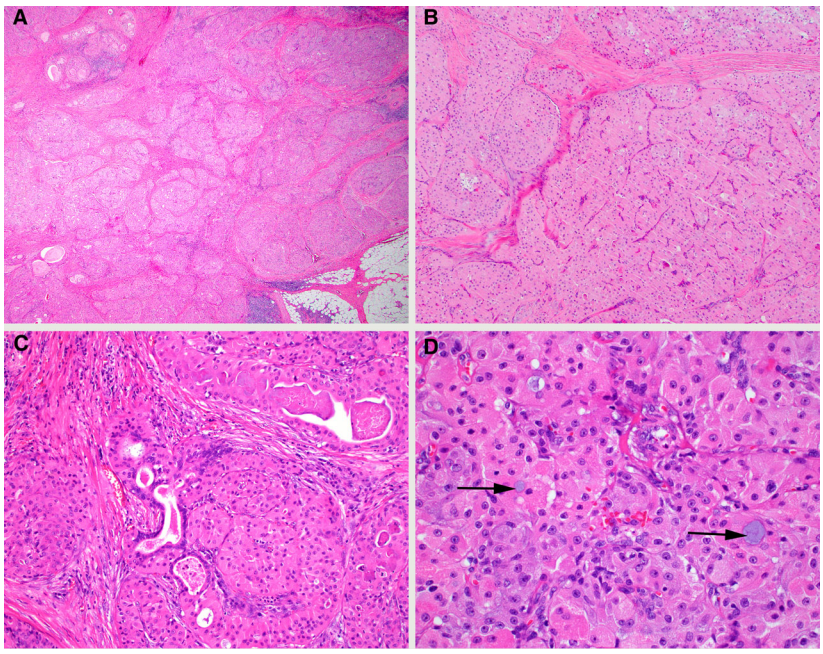

Figure 1. Four of the mucoepidermoid carcinomas were oncocytic. The tumours were infiltrative into fat and separated by bands of fibrosis (**A**). They consisted of solid, back-to-back nests of oncocytes (**B**), with focal ducts (**C**), and mucin droplets (**D**, arrows).

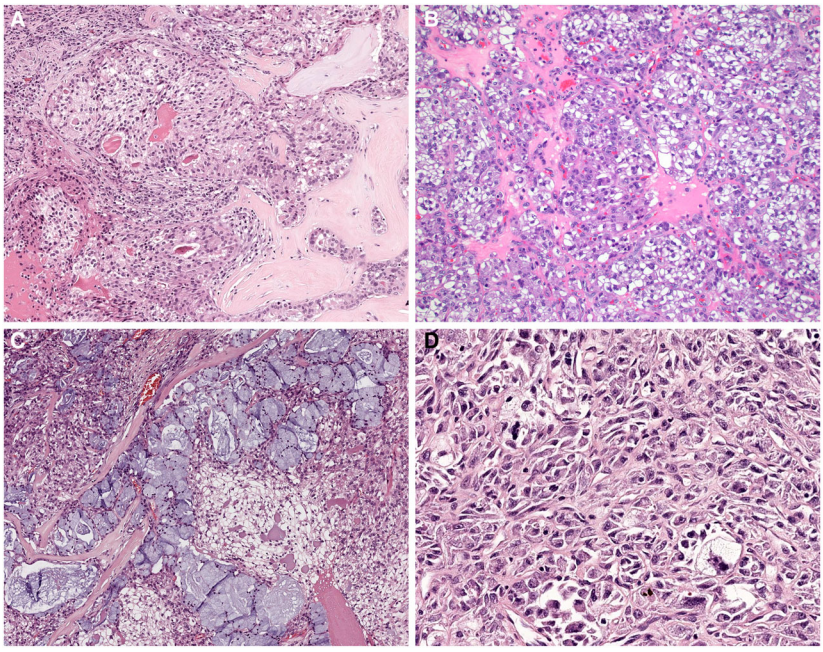

Figure 2. Four of the cases had prominent clear-cell features. The clear cells varied from those with pale eosinophilic (**A**) to optically clear cytoplasm (**B**). One of the clear-cell mucoepidermoid carcinomas had both low-grade (**C**) and high-grade (**D**) areas, indicating high-grade transformation.

MEC has for decades been defined as a malignant salivary gland neoplasm that has squamoid, intermediate, and mucinous cells—a definition that persists in the 2022 WHO Classification of Head and Neck Tumours.[6] The term "epidermoid" in "mucoepidermoid" refers to these squamoid cells, emphasizing their traditional importance in the diagnosis. Corresponding immunohistochemical positivity for squamous markers p63, p40, and CK5/6 is a consistent feature of MEC that has likewise become an important diagnostic tool for confirming an MEC diagnosis. Given the histologic overlap between various cell types in MEC, immunohistochemistry is frequently relied upon as the gold standard for squamous differentiation. In particular, diffuse p63 or p40 expression is particularly helpful for

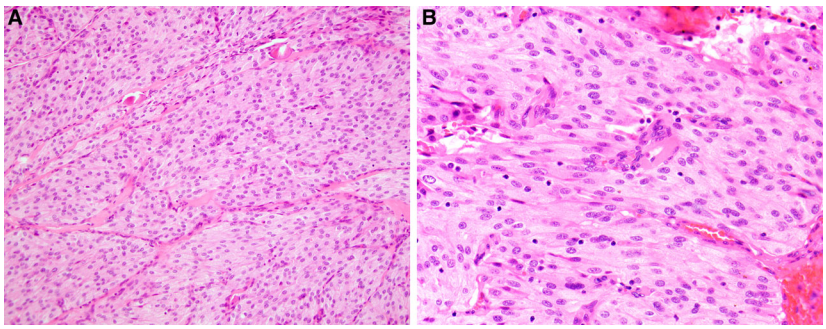

Figure 3. Two of the mucoepidermoid carcinomas demonstrated vague cell spindling (**A**); one of them had clear-cell features as well (**B**). [Color figure can be viewed at wileyonlinelibrary.com]

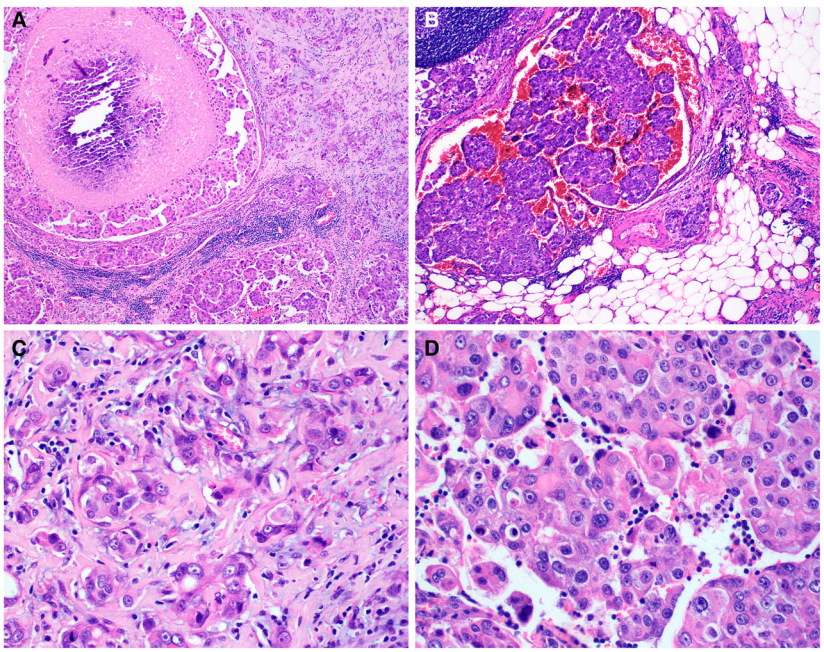

Figure 4. One of the squamoid cell-negative cases was high-grade, with comedonecrosis (**A**), micropapillary growth with prominent lymphatic invasion (**B**), and cord-like growth with limited duct formation (**C**). The tumour cell nuclei were pleomorphic (**D**). This tumour harboured a novel *MAML2::CEP126* fusion. [Color figure can be viewed at wileyonlinelibrary.com]

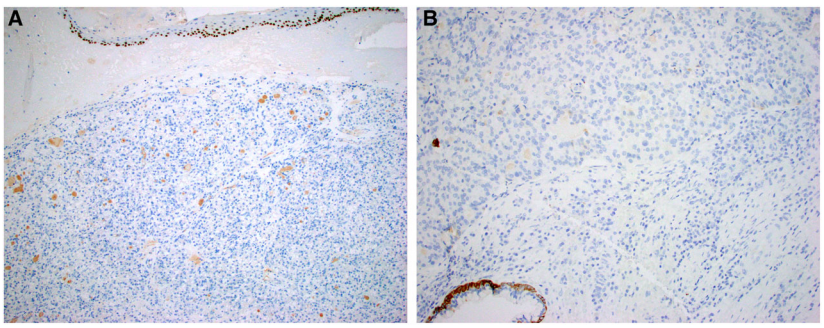

Figure 5. All cases were negative for p40 and p63, and all nine cases tested were negative for CK5/6. Normal squamous epithelium (**A**, top) and native myoepithelial cells (**B**, bottom left) served as useful internal controls. [Color figure can be viewed at wileyonlinelibrary.com]

differentiating variant forms of MEC, such as oncocytic MEC from morphologic mimics that have more limited or biphasic expression.[8,10,15,25–27] However, the 10 salivary gland tumours in this study harboured the *MAML2* rearrangements diagnostic for MEC, yet entirely lacked p63, p40, or CK5/6 expression and had no overt squamoid component histologically. There are several potential explanations for this phenomenon. First, this unexpected finding reasonably calls into question whether *MAML2* rearrangements are truly specific for MEC among salivary gland tumours. None of these cases,

however, fit well into any other salivary gland tumour category. Rather, 9 of the 10 cases fit very well within the previously recognised spectrum of MEC, which is well-established to have prominent oncocytic, clear-cell, and or spindled features.[10,15,18,28–30] Conversely, this absence of the definitional squamoid elements could be theoretically conceived as evidence of dedifferentiation, but most cases were actually low or intermediate grade and retained other classic histologic cell types of MEC. Ultimately, the tumours that lack p63 or p40 expression and overt squamoid cells most likely represent the extreme ends of the spectra of those particular MEC in which the variant morphology completely predominates to the exclusion of any histologic or immunohistochemical evidence of squamous differentiation.

This absence of squamous differentiation highlights the limitations of overemphasizing immunohistochemistry to identify salivary gland tumours. When evaluating a challenging oncocytic or clear-cell-predominant salivary gland neoplasm, the unexpected absence of p63 or p40 positivity could be a major pitfall that causes a pathologist to miss a diagnosis of MEC, even if the morphology is overall concordant with this diagnosis. As with all immunohistochemical markers, p63 and p40 are not infallible and should not be regarded as 100% sensitive for recognition of MEC. On the other hand, the main alternative diagnoses of each of those variants would also be expected to have some expression of squamous markers. Oncocytoma and nodular oncocytic hyperplasia, the main considerations in the differential diagnosis of oncocytic MEC, very consistently have a patchy, peripheral pattern of staining with p40, p63, and CK5/6.[8,25–27] Hyalinizing clear-cell carcinoma, a mimic of clear-cell MEC, is characteristically diffusely positive for p63 and p40.[31,32] Paradoxically, a complete absence of squamous marker immunoreactivity in a salivary gland tumour with prominent oncocytic or clear-cell features may actually point to, rather than away from, a diagnosis of MEC. Of course, molecular analysis for *MAML2* rearrangements can help resolve these diagnostic dilemmas. Importantly, it remains essential that application and interpretation of these ancillary tests in salivary gland neoplasms continue to be guided by morphologic clues.

Only one case in this series fell beyond the recognised histologic spectrum of MEC—the high-grade parotid gland tumour with micropapillary architecture. Most high-grade MEC are predominantly squamoid, with only focal mucinous differentiation, but this particular case exclusively comprised markedly anaplastic, presumably ductal cells. In the absence of both classic histologic features of MEC and any p63 or p40 expression, it was virtually impossible to identify this tumour as MEC without molecular analysis. Given that this tumour also harboured a novel *MAML2::CEP126* fusion, it even more strongly raises questions whether they represent something other than MEC. Outside the salivary glands, *MAML2* is no longer regarded as specific for MEC, as there are rare examples of tumours such as cutaneous poroid neoplasms, lymphomas, and low-grade gliomas that show *MAML2* fusions to various partners, including *YAP1*, *KMT2A*, and *MYB*.[33–36] This tumour was, however, clearly centreed in salivary gland parenchyma without evidence of disease elsewhere. Moreover, it was clearly a carcinoma that did not fit well into any non-MEC designation. Although the micropapillary architecture resembled salivary duct carcinoma, it was not obviously apocrine and was negative for the androgen receptor, features that most experts regard as obligatory for a diagnosis of salivary duct carcinoma.[37] While high-grade adenocarcinoma, not otherwise specified, could be considered, it is difficult to relegate a tumour with a *MAML2* fusion to this wastebasket category, as rare alternate fusion partners are generally accepted in other common salivary tumours as long as one canonical gene is present.[38–41] At this point, even a novel fusion involving *MAML2* points to an MEC diagnosis, regardless of unusual histologic features. More cases with *MAML2::CEP126* or other partners will be needed to better understand how variant fusions should be classified more definitively. It is difficult to speculate on the biochemical similarity of *MAML2::CEP126* to *CRTC1::MAML2* or *CRTC3::MAML2*; this novel fusion retains the area of *MAML2* thought to interact with intracellular Notch signalling.

While molecular testing has provided an important tool to facilitate salivary gland tumour diagnosis, it also has created new challenges when unexpected conflicts between histologic, immunohistochemical, and molecular findings emerge, calling traditional definitions for tumour entities into question. Moving forward, head and neck pathologists will have to evaluate whether salivary gland neoplasms should be defined by molecular, immunohistochemical, or traditional histopathologic features—or some combination of all of these findings. Most of the cases in this series that show recognisable MEC features and canonical fusions despite a lack of squamous differentiation suggest that the latter option is optimal, providing an example of how molecular testing can refine our

understanding of the acceptable immunohistochemical features as well as histologic boundaries of common salivary entities. However, the high-grade tumour with micropapillary morphology and variant *MAML2::CEP126* fusion raises thornier questions of what minimum evidence is necessary to fit an unusual tumour into an established salivary tumour category. At the very least, it is clear that the historical definition of MEC requiring squamoid elements must be revisited. It is already well accepted that many firmly-established variants of MEC do not exhibit squamoid cells histologically, and now it is evident that it may be lacking at the immunohistochemical level as well. Perhaps a more fitting, updated definition is a carcinoma with conventional features (admixed squamoid, intermediate, and mucous cells) and/or evidence of *MAML2* rearrangement. A thoughtful and nuanced approach will be essential to overcome the classification challenges raised by conflicting ancillary studies across anatomic sites.

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JAB and LMR designed the study and prepared the article; JAB, LMR, LDRT, BS, MM, and RDC contributed to data collection and analysis; JG analysed sequencing data and interpreted the results. All authors edited the article and read and approved the final article.

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Conflict of interest

All authors certify that they have no affiliations with or involvement in any organisation or entity with any financial interest or nonfinancial interest in the subject matter or materials discussed in this article.

Ethics approval statement

All procedures performed in this retrospective data analysis involving human participants were in accordance with the ethical standards of the Institutional Review Board (UT Southwestern IRB 112017–073), which did not require informed consent.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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