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Title: *CRTC1-MAML2* Fusion in Mucoepidermoid Carcinoma of the Breast

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Abstract

Aims Mucoepidermoid carcinomas (MEC) are the most common malignant neoplasms of salivary glands but are uncommon in other sites. Salivary gland MEC are most frequently associated with *CRTC1-MAML2* translocations. Exceedingly rare MEC of the breast demonstrate a basal-like and often triple (estrogen and progesterone receptor, HER2) negative immunophenotype, with a single case previously reported to show *MAML2* rearrangement, although the fusion partner was not known. Comprehensive genomic studies of breast MEC are lacking. In this study, we analyzed the immunophenotype and molecular landscape of two breast MEC to elucidate the pathogenesis of these rare tumors.

Methods and Results Two breast MEC were subjected to capture-based next-generation DNA sequencing of 479 cancer-related genes. The presence of the *CRTC1-MAML2* fusion transcript was interrogated by reverse-transcriptase polymerase chain reaction. In addition, the immunoprofiles of breast MEC were compared to salivary gland MEC. Both breast MEC harbored *CRTC1-MAML2* fusions. In contrast to most triple-negative breast carcinomas of no special type, the mutational burden of MEC was very low, with one case demonstrating only an inactivating *SETD2* mutation, and the other harboring no somatic variants in genes on the panel. No copy number alterations were identified. The immunoprofiles of breast and salivary gland MEC were overlapping but not identical.

Conclusions The findings highlight MEC as a breast cancer subtype more closely related to its salivary gland counterpart than to basal-like/triple-negative breast cancers of no special type.

Keywords: Mucoepidermoid carcinoma; breast cancer; salivary gland; *CRTC1-MAML2*; cancer genetics

Introduction

Mucoepidermoid carcinomas (MEC) are the most common malignant neoplasms of the salivary gland but also infrequently arise at other sites. It is an exceedingly rare diagnosis in the breast; although the estimated incidence is 0.2-0.3% of all mammary tumors, it appears even rarer in practice.[1, 2] Less than 40 cases of MEC of the breast have been reported in the literature to date.[2-5] Primary MEC belong to an uncommon group of salivary gland-like neoplasms of the breast, which also includes secretory carcinoma, adenoid cystic carcinoma, acinic cell carcinoma, and adenomyoepithelioma, among others. Although most lack ER (estrogen receptor) and PR (progesterone receptor) expression and HER2 overexpression, prognosis is better than expected in comparison to conventional triple-negative breast cancers of no special type.[4, 6, 7]

MEC have also been reported in the lacrimal gland, esophagus, lung, pleura, thymus, thyroid, pancreas, penis, tonsils, and skin.[8-17] Morphologic features are similar regardless of site, with tumors characterized by variably-sized circumscribed cystic to solid nests and nodules of multiple cell populations and prominent extracellular mucin. Intermediate/basaloid, epidermoid/squamoid, and mucinous cells are present in varying proportions, although some authors differentiate these into four cell types.[2, 3, 5, 18] Mucinous or mucin-producing cells are usually located at the luminal aspect of the glands and nests and may be tall columnar or goblet-shaped with obvious cytoplasmic mucin or be more subtle, requiring special stains to highlight the mucin. Intermediate cells are most concentrated at the tumor periphery and are small with high nuclear-to-cytoplasmic ratio and oval hyperchromatic nuclei. Epidermoid cells are polygonal with well-defined borders and abundant eosinophilic cytoplasm.[19] Focal intercellular bridges may be observed in rare cases, but squamous pearls or individual cell keratinization should be absent. Cytologic features may also include clear cell or oncocytic change. A prominent lymphocytic infiltrate is often seen around tumor nodules, which in salivary gland is also referred to as tumor-associated lymphoid proliferation (TALP). Reports about *in situ* components vary.[5, 20] The most commonly used grading systems for MEC in the salivary gland are the Brandwein and Armed Forces Institute of Pathology (AFIP) methods. These three-tiered and point-based systems incorporate the relative proportion of cystic components and the presence of neural invasion, necrosis, mitotic rate, and nuclear anaplasia (as well as vascular and bone invasion in Brandwein) to equate to low, intermediate, or high grade.[21, 22] In breast MEC, the AFIP system and the modified Scarff-Bloom-Richardson (SBR) system appear largely interchangeable and yield similar prognostic data.[19]

The most common translocation of salivary gland MEC fuses exon 1 of *CRTC1* at chromosome 19p13 to exons 2-5 of *MAML2* at chromosome 11q21. Alternative fusions with *CRTC3* have also been described.[23] Low-grade tumors are more likely to be fusion-positive than high-grade tumors. The extent to which the presence of the *CRTC1-MAML2* translocation influences clinical outcome in salivary gland tumors is unclear.[24-26] Molecular characterization of breast MEC is scant. One case demonstrated an 11q21 deletion at the site of the *MAML2* gene, but a fusion partner was unknown.[5] Given the lack of genomic data or comparative genetic studies, the relatedness of MEC of the breast to analog tumors arising in other sites, as well as conventional basal/triple-negative breast cancers, remains uncertain.

In this study, we demonstrate for the first time the presence of the *CRTC1-MAML2* fusion transcript in MEC of the breast. In addition, we used capture-based next-generation sequencing of 479 cancer-related genes to more comprehensively characterize the genomics of two breast MEC. The findings shed light on our understanding of breast MEC biology and may help explain the favorable clinical behavior of these tumors.

Materials and Methods

Case selection

This study was approved by the institutional review boards of the University of California San Francisco (UCSF) and the University of Massachusetts Medical School-Baystate. Two breast MEC were confirmed by experienced breast pathologists (Y.C. and C.N.O.) using a combination of routine hematoxylin and eosin (H&E)-stained sections, immunohistochemistry, and genetic findings. Selected findings in case 2 were previously reported.[5]

Tissue microarray construction

Tissue microarrays (TMA) were created from low-grade MEC of salivary gland origin. Three 2-mm punch biopsy tissue cores, with each core containing the three cell types of MEC, were obtained from each tumor for analysis. Positive and negative on-slide controls consisted of normal breast, normal salivary gland, and invasive ductal carcinoma, not otherwise specified.

Immunohistochemistry

The following antibodies were used: CK7 (OV-TL12/30, 1:100, DAKO, Santa Clara, CA, USA), Cam5.2 (1:100, Becton Dickinson, Franklin Lakes, NJ, USA), CK5/6 (D5/16B4, 1:200 with anti-background, Millipore, Billerica, MA, USA), MUC4 (8G7, 1:500, Millipore), MUC5AC (MRQ-19, undiluted, Cell Marque, Rocklin, CA, USA), GATA3 (L50-823, undiluted, Ventana, Tucson, AZ, USA), mammaglobin (304-1A5, 1:4, DAKO), gross cystic disease fluid protein 15 (GCDFP-15; 23A3, undiluted, Covance, Dedham, MA, USA), p63 (4A4, undiluted, Ventana), SMM (SMMS-1, 1:300, DAKO), calponin (26A11, undiluted, Leica Biosystems, Buffalo Grove, IL, USA), SMA (alpha sm-1, undiluted, Leica Biosystems), ER (SP1, undiluted, Ventana), PR (1E2, undiluted, Ventana), HER2 (4B5, undiluted, Ventana), and androgen receptor (AR; SP107, undiluted, Cell Marque). Antigen retrieval was as follows: for SMA—none; for CK7, Cam5.2, CK5/6, MUC4, mammaglobin, GCDFP-15, p63, and SMM—Bond epitope retrieval solution 1 (Leica Biosystems); for calponin—Bond epitope retrieval solution 2 (Leica Biosystems); and for GATA3, MUC5AC, ER, PR, HER2, and AR—Cell conditioning solution 1 (Ventana). For ER, PR, and HER2, positive staining was defined according to ASCO/CAP guidelines.[27, 28] For the two breast MEC, immunohistochemistry was performed and evaluated on standard sections, whereas TMA was applied for MEC of salivary gland origin.

MAML2 fluorescence in situ hybridization

Dual-color fluorescence *in situ* hybridization (FISH) was performed using 3' *MAML2* DNA (clones CTD-2544I7, RP11-936C10, RP11-1123F20, CTD-252L1, and RP11-7D4) labeled with SpectrumGreen dUTP (Abbott Molecular/Vysis Products, Abbott Park, IL, USA) and 5' *MAML2* DNA (clones RP11-8N17, CTD-2325K3, and RP11-1056O10) labeled with SpectrumOrange dUTP (Abbott Molecular/Vysis Products). The probe set was applied to 5 micron-thick unstained formalin-fixed paraffin-embedded (FFPE) sections on glass slides, hybridized, and washed as previously described.[26] Enumeration of the fusion and break-apart signals was conducted using an Applied Imaging Workstation (Foster City, CA, USA). In each case, 100 cells were analyzed in the targeted region.

Detection of CRTC1-MAML2 fusion by RT-PCR

Total RNA was extracted from 10 micron-thick unstained FFPE sections using the RNeasy FFPE Isolation Kit (Qiagen, Hilden, Germany), followed by reverse-transcription polymerase chain reaction (RT-PCR) using SuperScript III One-Step RT-PCR System (ThermoFisher Scientific, Foster City, CA, USA) with previously reported primers CRTC1 5′-TCGCGCTGCACAATCAGAAG-3′ and MAML2 5′-GGTCGCTTGCTGTTGGCAGG-3′.[29, 30]

These products were diluted 1:50 and subjected to a nested PCR with inner primers CRTC1 5'-GAGGTCATGAAGGACCTGAG-3' and MAML2 5'-TTGCTGTTGGCAGGAGATAG-3'.[29, 30] Products were resolved by agarose gel electrophoresis and Sanger sequenced using BigDye terminator chemistry (ThermoFisher Scientific) following standard techniques.

Capture-based next generation DNA sequencing

Capture-based next generation sequencing was performed at the UCSF Clinical Cancer Genomics Laboratory, using an assay (UCSF500 panel) that targets the coding regions of 479 cancer-related genes, select introns from 41 genes (not including *CRTC1* or *MAML2*), and the *TERT* promoter, with a total sequencing footprint of 2.8 Mb (Supplementary Table S1). Sequencing libraries were prepared from genomic DNA of tumor and matched normal formalin-fixed, paraffin-embedded tissue extracted from macrodissected unstained sections. Target enrichment was performed by hybrid capture using a custom oligonucleotide library. Sequencing was performed on a HiSeq 2500 (Illumina, San Diego, CA, USA). Duplicate sequencing reads were removed computationally to allow for accurate allele frequency determination and copy number calling. The analysis was based on the human reference sequence UCSC build hg19 (NCBI build 37), using the following software packages: BWA: 0.7.10-r789, Samtools: 1.1 (using htslib 1.1), Picard tools: 1.97 (1504), GATK: 2014.4-3.3.0-0-ga3711, CNVkit: 0.3.3, Pindel: 0.2.5a7, SATK: 2013.1-10- gd6fa6c3, Annovar: v2015Mar22, Freebayes: 0.9.20 and Delly: 0.5.9.[31-40] Only insertions/deletions (indels) up to 100 bp in length were included in the mutational analysis. Somatic single nucleotide variants and indels were visualized and verified using Integrated Genome Viewer. Genome-wide copy number analysis based on on-target and off-target reads was performed by CNVkit and Nexus Copy Number (Biodiscovery, Hawthorne, CA, USA).

Results

Clinical data

Case 1

A 53-year-old woman presented with a circumscribed nodule in the left breast on screening mammogram. Diagnostic mammogram revealed a mildly lobulated 1 cm nodule at 12 o'clock, 2 cm from the nipple. Ultrasound demonstrated a 0.9 cm heterogeneous mass with a mildly

nodular contour. Needle core biopsy was performed; the initial diagnosis was reported as "carcinoma with mucin secretion and squamoid features." Fluorescence *in situ* hybridization was subsequently performed and was positive for *MAML2* and negative for *ETV6* gene rearrangements. An addendum diagnosis of "low-grade mucoepidermoid carcinoma" was issued. The patient underwent subsequent wire-localized partial mastectomy and sentinel node lymphadenectomy; histologic examination confirmed the diagnosis, revealing a 1.6 cm tumor with negative margins and lymph nodes. The tumor was low grade by the AFIP grading system and grade 1 by the modified SBR system. Biomarker testing was negative for ER, PR, and HER2. She was treated with partial breast radiation and alive and well at sixteen months after surgery.

Case 2

The clinical data of case 2 were previously reported.[5] Briefly, a 49-year-old woman presented with a palpable right breast mass. Mammogram demonstrated a 1.5 cm round mass at 12 o'clock, as well as a 3 cm area of increased density in the posterior medial right breast. The patient underwent modified radical mastectomy with sentinel node lymphadenectomy and axillary dissection. Histologic examination demonstrated mucoepidermoid carcinoma of at least 5 cm with one positive sentinel lymph node. The tumor was intermediate grade by the AFIP system and grade 2 by the modified SBR system. Biomarker testing was negative for ER, PR, and HER2. She was treated with chemotherapy and alive and well at 1 year after diagnosis.

Microscopic features of breast mucoepidermoid carcinomas

H&E sections of both breast MEC showed histologic features characteristic of MEC. Both tumors had broad pushing margins associated with a peripheral dense lymphoplasmacytic infiltrate (Figure 1A). The neoplastic cells were variably arranged in macrocystic or microcystic/cribriform structures and solid nests, with basophilic and/or eosinophilic secretions filling the cystic spaces (Figure 1B,C). Intermediate and epidermoid cells were well-represented (Figure 1D); mucinous cells were morphologically more subtle but could be highlighted with a mucicarmine stain (Figure 1E). In the partial mastectomy specimen of case 1, the central portion of the tumor was predominantly composed of large irregular cysts, with smaller irregular nodules scattered at the periphery (Figure 1C). Case 2 demonstrated extensive ductal carcinoma *in situ* (DCIS), with expanded lobules and ducts lined by variable numbers of intermediate, epidermoid, and mucinous cells and forming fenestrations filled with basophilic secretions (Figure 1F). There

were two foci of microinvasion consisting of small irregular nests with a desmoplastic response (ref 5, Figure 1). Lymphovascular invasion was not identified in either case.

Immunohistochemical features of breast and salivary gland mucoepidermoid carcinomas

Both breast MEC expressed low molecular weight cytokeratins (LMWCK: CK7, CAM5.2), high molecular weight cytokeratins (HMWCK: CK5/6), and p63, with immunohistochemistry differentially highlighting the various cell populations and their distribution. Intermediate cells were positive for p63 and HMWCK and negative for LMWCK; epidermoid cells reacted with both LMWCK and HMWCK; and mucinous cells preferentially expressed LMWCK (Figure 2A,B). The peripheral p63 staining of intermediate cells could be difficult to distinguish from myoepithelial cell staining (Figure 2C); however, the invasive tumor was negative for other myoepithelial cell markers, including SMM (Figure 2D), calponin, and SMA. The tumor in case 2 consists predominantly of DCIS with foci of microinvasion; the DCIS and invasive components show similar cytomorphology. This scenario illustrates the limited utility of p63 alone as a myoepithelial cell marker in this context. The staining of peripheral myoepithelial cells by p63 may be difficult to discern from the prominent co-staining of intermediate cells in DCIS, but an intact myoepithelial layer can be highlighted by positive SMM (Figure 2E) and calponin staining.

The immunoprofiles of the two breast MEC were compared to seven salivary gland MEC analyzed by TMA (Table 1 and Figure 2F-H). Both breast and all seven salivary gland MEC were negative for ER, PR, and HER2 by ASCO/CAP guidelines. Breast and salivary gland MEC expressed CK5/6 and MUC4 and showed no to minimal expression of GCDFP-15 and AR (0-5% staining). Both breast MEC showed patchy or diffuse GATA3 and mammaglobin staining, in contrast to salivary gland MEC, in which staining for these markers was absent or focal (≤10%) in most cases (7/7 and 6/7, respectively). In contrast, 5/7 salivary gland MEC but neither of the breast MEC showed patchy MUC5AC expression. Of note, while cytokeratins and p63 demonstrated differential expression depending on the tumor cell types, other markers did not exhibit this staining pattern.

Identification of CRTC1-MAML2 translocation in breast mucoepidermoid carcinomas

FISH was positive for *MAML2* gene rearrangement in both breast MEC (Figure 3A-C). Both tumors demonstrated one green/orange fusion signal, reflecting a normal *MAML2* locus. Case 1 additionally revealed one separate green and one separate orange signal (Figure 3B), indicative of a translocation event involving one *MAML2* gene. In case 2, one green signal was

accompanied by loss of the orange signal (Figure 3C), indicating deletion of the 5' portion of the *MAML2* locus. RT-PCR analysis revealed *CRTC1-MAML2* fusion transcripts in both cases (Figure 4A), which were confirmed by Sanger sequencing (Figure 4B).

Next generation DNA sequencing of breast mucoepidermoid carcinomas

Both breast MEC were subjected to targeted sequencing of 479 cancer-related genes. The mean target sequencing coverage was 724 and 507 unique reads per target interval in cases 1 and 2, respectively. No non-silent single nucleotide variants or indels were detected in case 1. Case 2 demonstrated a solitary pathogenic nonsense mutation in *SETD2* (p.S543*). No copy number alterations were identified in either case. No pathogenic germline variants associated with increased cancer risk were identified in either patient.

Discussion

In this study, we describe the morphologic features, immunophenotype, and detailed genetic landscape of two breast MEC. The characteristic histologic features of MEC are distinctive in the breast. However, as pathologists may not be familiar with this rare subtype of breast cancer, MEC can be confused with various benign and malignant conditions when assessing limited material on core biopsy or fine needle aspiration, depending on the architectural pattern and dominant cell type (Table 2). Partially sampled MEC with macrocystic architecture could easily be misinterpreted as simple cysts. Microcystic-predominant architecture may raise consideration of cribriform pattern DCIS. On the other hand, the heterogeneous cell populations with bland cytology and irregular fenestrations can mimic usual ductal hyperplasia, which may be further confounded by positive CK5/6 staining. Epidermoid cells and the immunophenotype (positive CK5/6 and p63 and triple-negative) may also be confused with squamous metaplasia or a squamous metaplastic carcinoma. Lastly, low-grade MEC could be mistaken for secretory carcinoma, another rare salivary gland-type carcinoma.[41] Both tumors can have a prominent cystic component, abundant PAS-D positive secretory material, and cytologically bland cells which may have eosinophilic, clear, or vacuolated cytoplasm. In addition, both are immunohistochemically positive for mammaglobin, MUC4, and CK5/6 and negative for ER, PR, and HER2. However, in contrast to secretory carcinoma, MEC is consistently positive for p63 and is usually negative or only focally positive for S100. Distinction can be definitively established by FISH, using *MAML2* and *ETV6* break-apart probes, as in case 1.

Breast MEC and its salivary gland counterpart share histopathologic features and have an overlapping immunophenotype. However, although our study is limited by the small number of cases of these rare tumors, we note subtle differences in the morphology and immunohistochemical profile of tumors from these two sites. Mucinous cells in the two breast MEC lacked tall columnar or goblet cytology, which is often observed in salivary gland MEC and has been noted in a previous report.[19] Although MEC from both sites demonstrate a basal-like immunophenotype (ER, PR, and HER2 negative and CK5/6 positive) and express MUC4, breast MEC show strong GATA3 and mammaglobin expression, whereas the salivary gland tumors are negative or only minimally positive for these latter markers. In contrast, MUC5AC expression, which has been previously reported in salivary gland MEC,[42] is absent or scant in breast MEC. In this context, it is interesting to note that a prior study of lung MEC found lack of expression of the pulmonary markers TTF-1 and napsin A in these tumors,[8] suggesting that MEC arising in some but not all sites may retain tissue-specific expression patterns, despite otherwise similar morphologic and genetic features. Analysis of more cases is necessary to confirm these observations.

Ours is the first study to demonstrate the presence of *CRTC1-MAML2* fusion typical of salivary gland MEC in breast MEC and to analyze the genomics of these rare tumors using next-generation sequencing of a large panel of cancer-related genes. Both of our cases harbored *MAML2* rearrangement by FISH and demonstrated the *CRTC1-MAML2* fusion transcript by RT-PCR. Both tumors showed a simple genome with no copy number alterations and demonstrated a very low mutational burden of genes on the panel, with an inactivating *SETD2* mutation in one case as the only identified somatic nonsynonymous variant. *SETD2* is a tumor suppressor gene that encodes a histone methyltransferase responsible for trimethylation of lysine 36 of histone H3 (H3K36me3). Most prevalent in clear cell renal cell carcinoma, inactivating mutations have been described in multiple cancers.[43, 44] *SETD2* mutations have been rarely reported in breast tumors (at most ~3% of cases), notably only in luminal A cancers and phyllodes tumors but not in triple-negative breast carcinomas.[45-50] No *SETD2* alterations have been reported to date in MEC of the salivary gland,[51] and the significance of this isolated finding in one breast MEC is uncertain.

Comprehensive molecular studies of triple-negative breast carcinomas have shown a heterogeneous mutational milieu with frequent *TP53* and *PIK3CA* alterations.[45-49, 52-56] However,

these studies have generally not included triple-negative salivary gland-like tumors that can arise in the breast, including secretory carcinomas, acinic cell carcinomas, and adenoid cystic carcinomas, as well as MEC and even rarer types.[1, 5, 7, 19, 57-59] Recent studies suggest that secretory carcinomas and adenoid cystic carcinomas of the breast are genetically more similar to their respective salivary gland counterparts than they are to other triple-negative carcinomas.[57, 60] In contrast, acinic cell carcinomas arising in the breast harbor frequent *TP53* mutations and complex patterns of copy number alterations, which are not present in salivary gland acinic cell carcinomas, suggesting that these two tumors are not related despite their histologic similarity.[61, 62] The molecular landscape of salivary gland MEC beyond *CRTC1-MAML2* rearrangement have only been explored recently.[51, 63] Wang et al utilized a panel of 315 cancer-related genes to interrogate 48 salivary gland MEC, including seven low-grade tumors. Alterations in common oncogenic drivers such as *TP53*, *PIK3CA*, *CDKN2A/B*, *BAP1, ERBB2,* and *BRCA1/2* were frequently detected in intermediate- or high-grade MEC, but were absent or each present in only single cases of low-grade tumors.[63] Kang et al performed whole exome sequencing on 18 salivary gland MEC, including nine low-grade tumors. Although *TP53* was frequently mutated in intermediate- and high-grade MEC, none of the low-grade tumors had *TP53* mutations, with the only recurrent mutation in these tumors being *POU6F2* (in three tumors). In addition, most tumors had a low mutational burden with many (6/9) showing no copy number alterations.[51] The breast MEC in our study lacked *TP53* or *PIK3CA* mutations or complex copy number profiles typical of high-grade triple-negative carcinomas of no special type and indeed showed no or only isolated genetic abnormalities aside from *CRTC1-MAML2* fusions. *POU6F2* is not included on the UCSF500 panel and could not be analyzed in our study. Our findings suggest that breast MEC, like other fusion gene-driven special breast cancer subtypes, are genetically more similar to their counterparts in the salivary gland than they are to other primary breast cancers.

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G.R.B., G.K., and Y.C. designed the research study, analyzed the data, and wrote the manuscript. C.N.O. and S.C. contributed one of the two breast cases and edited the paper. D.A.S., J.J.G., and J.V.Z. contributed expertise with the molecular results and edited the paper. A.V.Z. contributed the salivary gland cases and edited the paper.

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Table 1: Immunophenotypes of breast and salivary gland mucoepidermoid carcinomas.

	Breast MEC	**Salivary gland MEC**

Immunostain*	1	2	1	2	3	4	5	6	7
GATA3	90	50	<1	5	0	0	1	0	<1
Mammaglobin	60	40	0	5	<1	10	15	<1	10
GCDFP15	0	1	0	<1	0	0	<1	0	0
ER	<1	<1	0	0	0	0	0	0	0
PR	0	0	0	0	0	0	0	0	0
HER2	0	0	0	0	1	0	0	0	0
AR	1	2	<1	1	0	0	1	5	<1
CK5/6	90	90	70	90	40	90	90	50	70
MUC4	20	80	80	100	60	70	80	90	80
MUC5AC	0	<1	20	1	70	5	50	50	70

*Values are percentages of positive-staining tumor cells, except for HER2 where values represent staining intensity as defined by the ASCO/CAP guidelines.

Table 2: Morphologic mimics of breast mucoepidermoid carcinoma.

Differential diagnosis	Features that may overlap with MEC	Features helpful in differential diagnosis
Simple cysts	Macrocystic architecture with one to few cell layers; mucoid material alone on limited sampling	Radiologic-pathologic correlation against simple cysts in MEC; presence of mucinous cells in MEC; negative SMM/calponin around cysts of MEC

UDH	Heterogeneous cell population with bland cytology and irregular microcystic spaces; strong positive CK5/6	Presence of mucinous and epidermoid cells in MEC; ER patchy positive in UDH and ER negative in MEC
DCIS, cribriform pattern	Rounded ductal contours with microcystic/cribriform architecture; well-defined cellular borders	Lack of nuclear polarization around spaces in MEC; positive CK5/6 and negative ER in MEC; negative CK5/6 and diffuse strong ER in DCIS
Metaplastic SCC	Squamoid tumor cells with overlapping immunophenotype (triple negative, positive CK5/6 and p63)	Circumscribed nodules of MEC versus infiltrative growth of SCC; multiple cell types in MEC; lack of true keratinization in MEC
Secretory carcinoma	Prominent cystic components; PASD-positive secretory material; cytologically bland tumor cells with overlapping immunophenotype (triple negative, positive mammaglobin and MUC4)	Positive p63 in MEC; positive S100 in secretory carcinoma; positive *MAML2* break-apart FISH in MEC; positive *ETV6* break-apart FISH in secretory carcinoma

Abbreviations: MEC – mucoepidermoid carcinoma; UDH – usual ductal hyperplasia; DCIS – ductal carcinoma in situ; SCC – squamous cell carcinoma; PASD – Periodic acid-Schiff, diastase; FISH – fluorescence in situ hybridization

Figure 1. Morphologic features of breast mucoepidermoid carcinomas.

(A) Low-power view of case 1 demonstrates variably-sized cystic nodules and nests with a prominent lymphoid infiltrate at the edge of the tumor. (B) Cystic architecture is prominent in the low-grade lesion. (C) Small irregular solid nests are noted focally. (D) Intermediate, epidermoid, and mucinous cells are present in variable proportions with basophilic and eosinophilic intraluminal secretions. (E) Mucicarmine stain highlights mucinous cells with cytoplasmic mucin vacuoles. (F) *In situ* component shows similar cytomorphology to invasive tumor as well as a periductal lymphoid infiltrate.

Figure 2. Immunohistochemical profile of mucoepidermoid carcinomas of the breast and salivary gland.

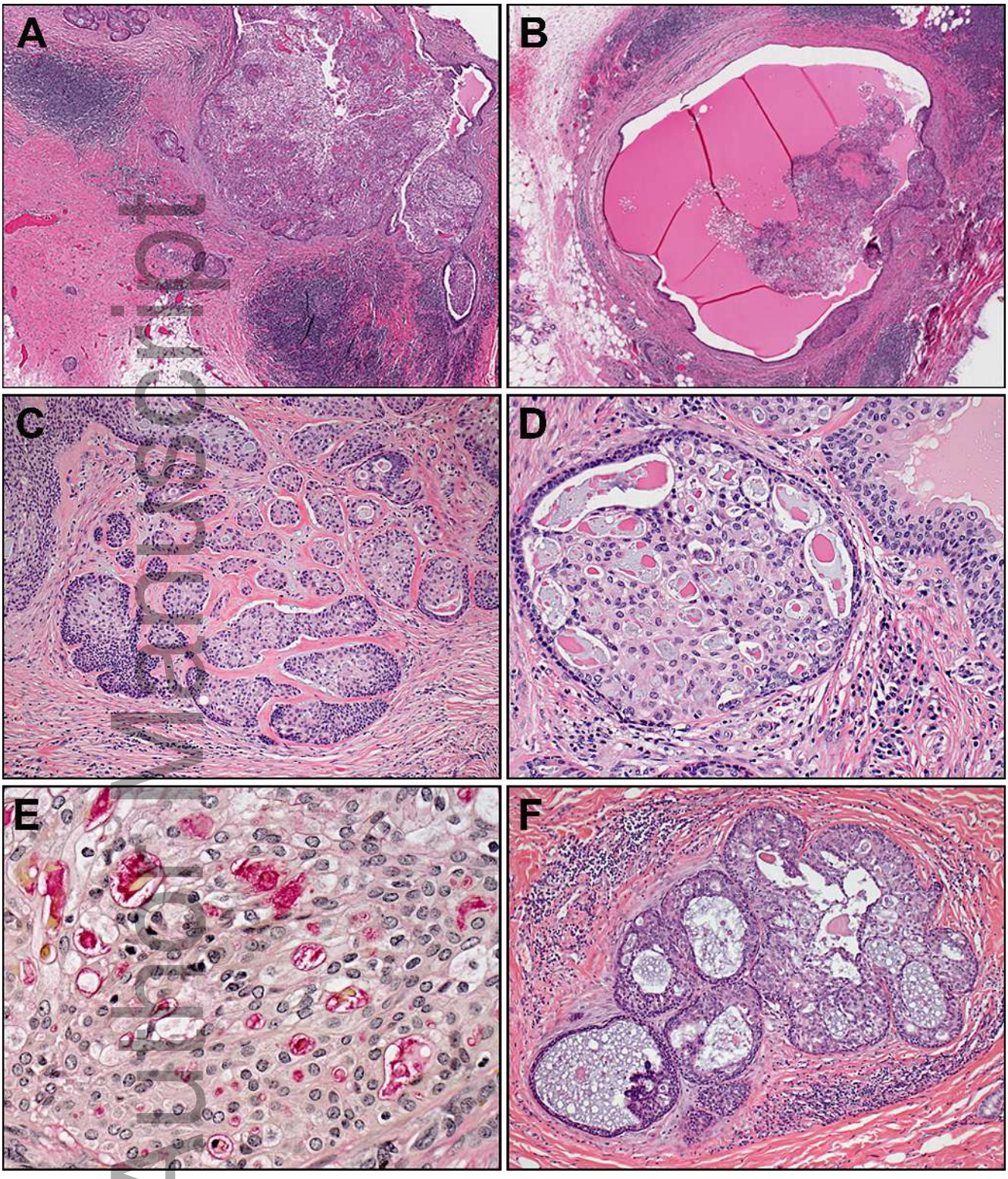
Immunohistochemical stains for cytokeratins differentially highlight the cell types of breast MEC, often in a zoning pattern with their spatial distribution. (A) LMWCK such as CAM5.2 preferentially stains mucinous and epidermoid cells. (B) HMWCK such as CK5/6 highlights intermediate and epidermoid cells. (C) p63 also shows a zoning pattern with staining of peripherally-situated intermediate cells which can be confused with myoepithelial cell staining. (D) Other myoepithelial markers such as SMM are negative. (E) In contrast, *in situ* carcinoma shows peripheral SMM staining, while p63 (*inset*) highlights both intermediate and myoepithelial cell populations. (F-G) Mammary specific markers are positive in breast MEC, compared to MEC in the salivary gland (*insets*). (F) GATA3 shows patchy to diffuse staining in breast MEC, but not salivary gland. (G) Mammaglobin staining is also more diffuse in breast MEC than in salivary gland. (H) MUC4 demonstrates positive staining in both.

Figure 3. *MAML2* translocation in breast mucoepidermoid carcinomas.

Fluorescence *in situ* hybridization with (A) a *MAML2* break apart probe demonstrating (B) one separate orange and one separate green signal in case 1 and (C) one green signal and loss of the orange signal in case 2.

Figure 4. *CRTC1-MAML2* fusion transcript in breast mucoepidermoid carcinomas.

(A) Amplified RT-PCR products resolved by agarose gel electrophoresis, with 100 bp DNA ladder (*lane 1*), breast MEC case 1 (*lane 2*), breast MEC case 2 (*lane 3*), salivary gland MEC positive control (*lane 4*), and water negative controls (*lanes 5-6)*. The expected RT-PCR product is 95 bp. (B) Direct (Sanger) sequencing of amplified RT-PCR product confirms presence of *CRTC1-MAML2* fusion.



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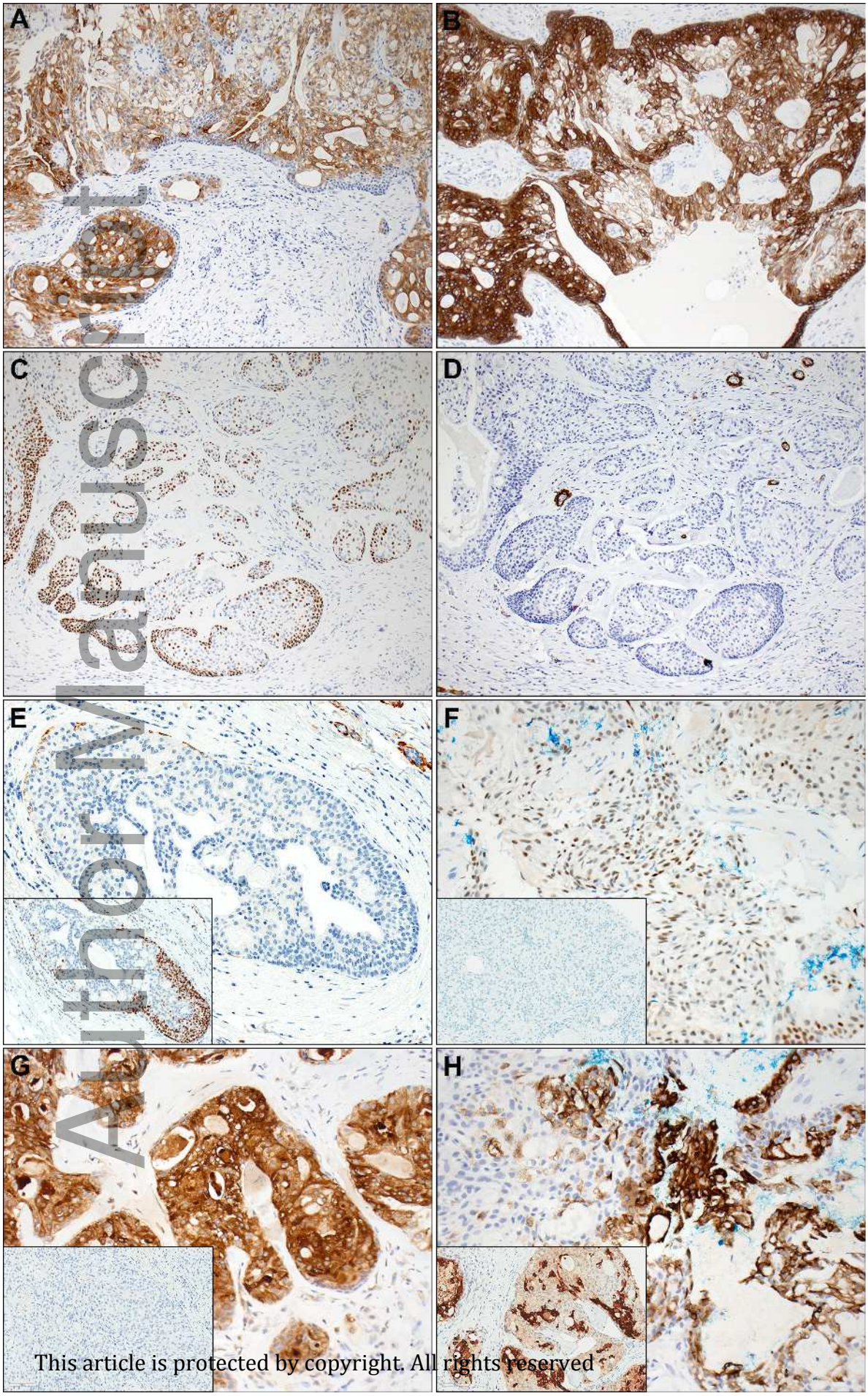

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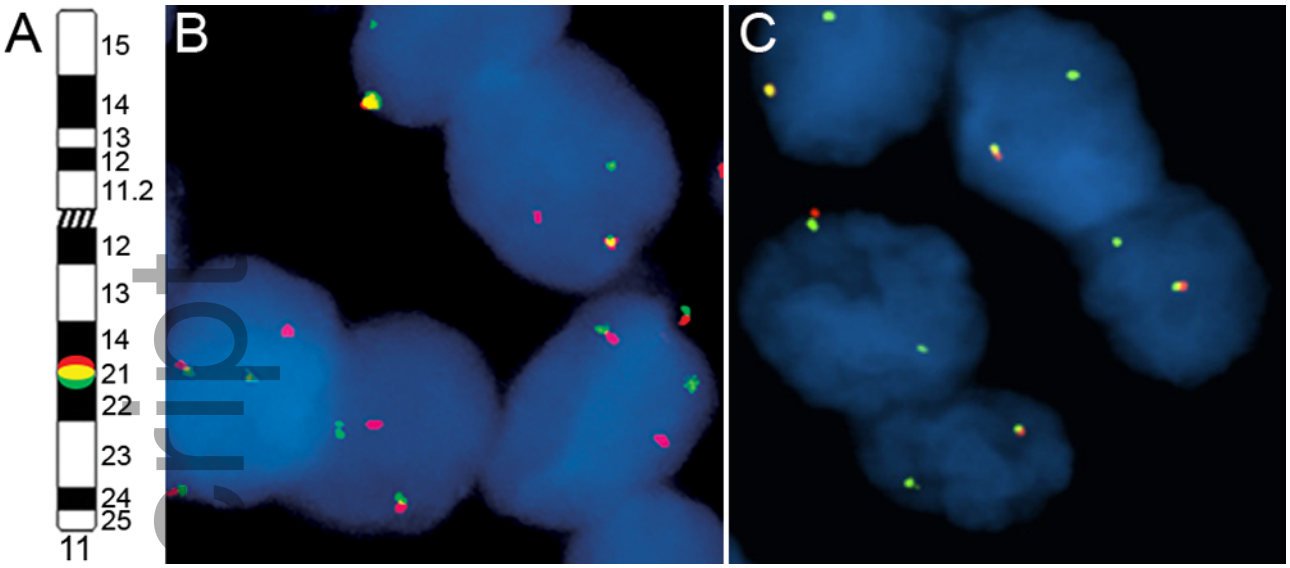

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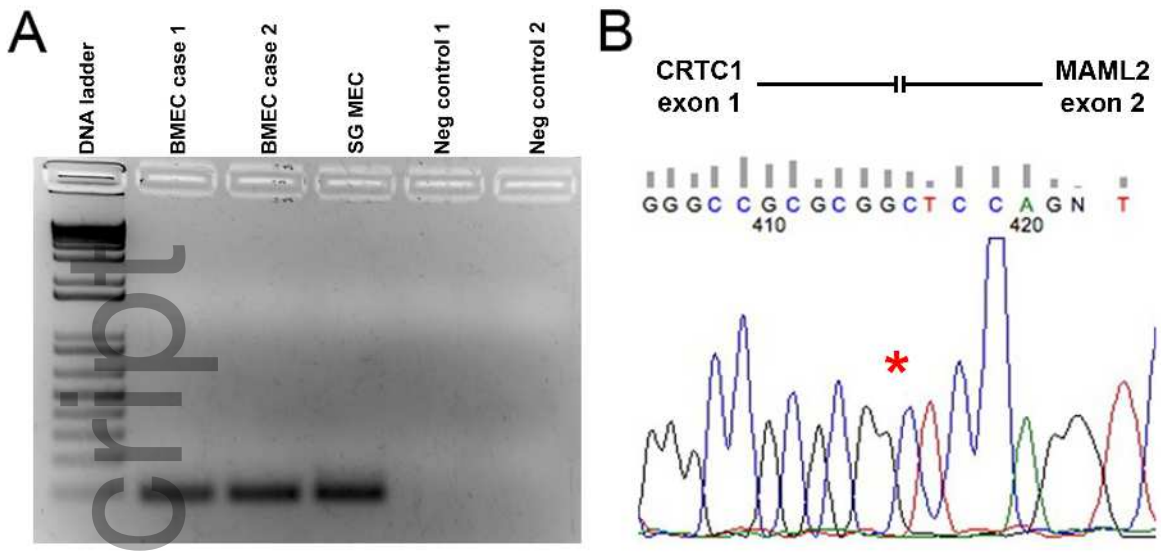

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