Molecular Profiling of Salivary Oncocytic Mucoepidermoid Carcinomas Helps to Resolve Differential Diagnostic Dilemma With Low-grade Oncocytic Lesions

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Abstract: Oncocytic mucoepidermoid carcinoma (OMEC) is a rare but diagnostically challenging variant of mucoepidermoid carcinoma (MEC). OMEC is notable for differential diagnostic considerations that are raised as a result of overlap with other benign and low-grade oncocytic salivary gland tumors. Diffuse and strong immunoreactivity of p63 protein may be useful in distinguishing OMEC from its mimics. However, focal p63 staining can be present in benign oncytomas. Presence of mucin-containing cells, mucinous cystic formation, and foci of extravasated mucin are considered a hallmark of MEC. True mucocytes may be, however, very few and hardly discernable in OMECs. Recent evidence has shown that most MECs harbor gene fusions involving *MAML2*. A retrospective review of archived pathology files and the authors' own files was conducted to search for "low-grade/uncertain oncocytic tumor," "oncocytoma," and "oncocytic carcinoma" in the period from 1996 to 2019. The tumors with IHC positivity for p63 and/or p40, and S100 negativity, irrespective of mucicarmine staining, were tested by next-generation sequencing using fusion-detecting panels to detect *MAML2* gene rearrangements. Two index cases from consultation practice (A.S. and A.A.) of purely oncocytic low-grade neoplasms without discernible mucinous cells showed a *CRTC1-MAML2* fusion using next-generation sequencing, and were reclassified as OMEC. In total, 22 cases of oncocytic tumors, retrieved from the authors' files, and from the Salivary Gland Tumor Registry, harbored the *MAML2* gene rearrangements. Presence of mucocytes, the patterns of p63 and SOX10 immunopositivity, and mucicarmine staining were inconsistent findings. Distinguishing OMEC devoid of true mucinous cells from oncocytoma can be very challenging, but it is critical for proper clinical management. Diffuse and strong positivity for p63 and visualization of hidden mucocytes by mucicarmine staining may be misleading and does not always suffice for correct diagnosis. Our experience suggests that ancillary studies for the detection of *MAML2* rearrangement may provide useful evidence in difficult cases.

Key Words: salivary gland neoplasms, mucoepidermoid carcinoma, oncocytic, *MAML2*, oncocytoma, gene fusion, *CRTC1-MAML2*

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Salivary gland carcinomas represent <10% of head and neck malignancies, the most common type being mucoepidermoid carcinoma (MEC).[1,2] MEC is a distinctive salivary gland malignancy composed of mucinous, intermediate, clear, and squamoid neoplastic cells arranged in cystic and solid growth patterns in variable proportions.[1] Morphologic features compatible with low-grade MECs include the presence of cells with true intracytoplasmic mucin, intermediate and squamoid cells, and usually combined solid and cystic growth patterns. In intermediate-grade and high-grade MECs intermediate cells may not always be readily identifiable and in high-grade MECs cells with intracytoplasmic mucin may be scarce

and difficult to identify. The diagnosis of MEC is, however, generally straightforward on histologic grounds alone. In less typical cases, the application of mucicarmine or Alcian blue (AB)/periodic acid-Schiff (PAS) or PAS-diastase stain could facilitate the identification of true intracytoplasmic mucin. There is, however, a spectrum of rare monomorphic variant tumors, such as sclerosing, oncocytic, and clear-cell MECs that differ from the conventional appearance, and in these cases a correct diagnosis can be very difficult.

Oncocytic mucoepidermoid carcinoma (OMEC) is a rare and diagnostically challenging variant of MEC.[3] Before the Weinreb et al's[3] paper, in which the authors report 12 cases, only case reports of this lesion have been published.[4–10] Most of such neoplasms were described as low grade, and having a good prognosis, but the method of their grading was not always well defined. OMEC is notable for differential diagnostic considerations that are raised as a result of overlap with other benign and low-grade oncocytic salivary gland tumors.[3,11] Diffuse and strong immunoreactivity for p63 protein may be useful in distinguishing OMEC from its mimics. Salivary oncocytomas and oncocytic carcinomas are, however, also invariably positive for p63, even if the immunopositivity seems to be largely restricted to the most peripheral or basal layers of the individual tumor nests.[12,13] Presence of mucin-containing cells, mucinous cystic formation, and foci of extravasated mucin are considered a hallmark of MEC.[1] True mucocytes, however, may be very few and hardly discernible in OMECs.

Recent evidence has shown that up to 80% of MECs harbor gene fusions involving *MAML2*, with the highest occurrence in low-to-intermediate–grade tumors.[14–19] Even though the *MAML2* alteration is specific for MEC, *MAML2* testing is generally regarded as unnecessary. The role of *MAML2* testing as a diagnostic adjunct is reserved for those tumors that differ from the conventional appearance of MEC and more closely resemble some other tumor types. In those MECs where the histologic picture is dominated by oncocytic cells (ie, oncocytic variant of MEC), the finding of a *MAML2* rearrangement is extremely useful in distinguishing the OMECs from other oncocyte-rich neoplasms including oncocytoma and oncocytic carcinoma.[11,20]

Recently, 2 index cases of low-grade oncocytic neoplasms without any mucous cells, received as consultation cases with primary diagnosis of oncocytoma/oncocytic tumor of uncertain malignancy (A.A. and A.S.), were reclassified as OMEC, based on molecular profiling. To examine the usefulness of *MAML2* testing, we collected a cohort of oncocytic neoplasms from the consultation files of the authors, and identified 22 cases, which were reclassified as OMECs. In this study, we highlight the usefulness of *MAML2* testing in the identification of challenging variants of MEC.

MATERIALS AND METHODS

The study was approved by the institutional review board. A retrospective review of Salivary Gland Tumor Registry (A.S.) was conducted to search for "low-grade/uncertain oncocytic tumor," "oncocytoma," and "oncocytic carcinoma" in the period from 1996 to 2019. The search yielded 125 salivary gland neoplasms. Clinical features and outcomes, for example age, sex, site of the primary tumor, follow-up period, recurrence, and distant metastasis, were recorded. All slides were reviewed by 3 pathologists (A.S., M.B., and O.S.) to collect the following pathologic parameters: predominant oncocytic features (>75% tumor cells), solid and cystic growth pattern (>50% solid), at least focal invasive growth, and features suspicious of malignancy, such as cellular atypia, necrosis, and/or enhanced mitotic and proliferative activity. Any unusual histologic features were documented.

All cases retrieved from archived salivary gland pathology files in Pilsen were stained with mucicarmine, PAS, and/or AB/PAS and investigated immunohistochemically (IHC) for S100 protein, SOX10, and p63/p40 proteins. This search yielded 19 oncocytic tumors with IHC positivity for p63 and/or p40, and S100 negativity (19/125). In addition, available cases of oncocytic neoplasms considered as OMECs were retrieved from the archived pathology files and personal consultation files of the coauthors. Irrespective of mucicarmine staining and SOX10/p63 immunostaining results, 30 selected neoplasms were tested by next-generation sequencing (NGS) using fusion-detection panels to detect *MAML2* gene rearrangements. The NGS results were confirmed by reverse-transcription polymerase chain reaction (RT-PCR) and/or fluorescence in situ hybridization (FISH).

For conventional microscopy, the excised tissues were fixed in formalin, processed routinely, embedded in paraffin (FFPE), cut, and stained with hematoxylin andeosin.

For IHC analysis, 4-μm-thick sections were cut from paraffin blocks and mounted on positively charged slides (TOMO; Matsunami Glass IND, Japan). Sections were processed on a BenchMark ULTRA (Ventana Medical System, Tucson, AZ), deparaffinized and then subjected to heat-induced epitope retrieval by immersion in a CC1 solution at pH 8.6 at 95°C. All primary antibodies used are summarized in Table 1. The bound antibodies were visualized using the ultraView Universal DAB Detection

TABLE 1. Antibodies Used for IHC Study

Antibody Specificity	Clone	Dilution	Antigen Retrieval/ Time	Source
MIA	113-1	1:100	CC1/36 min	BioGenex
S100 protein	Polyclonal	RTU	CC1/20 min	Ventana
CK7	OV-TL 12/30	1:200	CC1/36 min	DakoCytomation
CK 14	SP53	RTU	CC1/64 min	Ventana
CK 5/6	D5/16B4	1:50	CC1/36 min	Dako
p63	4A4	RTU	CC1/64 min	Ventana
p40	Polyclonal	RTU	CC1/52 min	Biocare Medical
SOX10	Polyclonal	1:100	CC1/64 min	Cell Marque
MIB1	30-9	RTU	CC1/64 min	Ventana

CC1 indicates EDTA buffer, pH 8.6; RTU, ready to use.

Kit (Roche, Basel, Switzerland) and ultraView Universal Alkaline Phosphatase Red Detection Kit (Roche). The slides were counterstained with Mayer hematoxylin. Appropriate positive and negative controls were used.

Where available, clinical follow-up information was obtained from hospitals records, the patients, their physicians, or from referring pathologists.

MOLECULAR GENETIC STUDY

Sample Preparation for NGS and RT-PCR

For NGS and RT-PCR analysis, 2 to 3 FFPE sections (10 μm thick) were macrodissected to isolate tumor-rich regions. Samples were extracted for total nucleic acid using Agencourt FormaPure Kit (Beckman Coulter, Brea, CA). The RNA integrity was evaluated using PreSeq RNA QC Assay as was previously described.[21]

RNA Integrity Assessment and Library Preparation for NGS

Unless otherwise indicated, 250 ng of FFPE RNA was used as input for NGS library construction. To assess RNA quality, the PreSeq RNA QC assay using iTaq Universal SYBR Green Supermix (Biorad, Hercules, CA) was performed on all samples during library preparation to generate a measure of the integrity of RNA (in the form of a cycle threshold [C_t] value). Library preparation and RNA QC were performed following the Archer FusionPlex Protocol for Illumina (ArcherDX Inc., Boulder, CO). The Archer FusionPlex Solid Tumor Kit or *ETV6-EWSR1-MAML2* custom Archer Fusion Plexkit were used. Final libraries were diluted to 1:100,000 and quantified in a 10 μL reaction following the Library Quantification for Illumina Libraries protocol and assuming a 200 bp fragment length (KAPA, Wilmington, MA). The concentration of final libraries was around 200 nM. Threshold representing the minimum molar concentration for which sequencing can be robustly performed was set at 50 nM.

NGS Sequencing and Analysis

Libraries were diluted to 4 nM and sequenced on a NextSeq sequencer (Illumina, San Diego, CA). The optimal number of raw reads per sample was set to 3 million. Library pools were diluted to 1.6 pM library stock with 20% 1.8 pM PhiX and loaded in the NextSeq cartridge.

The fusion and other rearrangement detection algorithm in Archer Analysis relies on the specificity of the gene-specific primers used in the amplification steps in the AMP process. The resulting FASTQ files were analyzed using the Archer Analysis software (version 5.1.7; ArcherDX Inc.)

RNA Preparation for CRTC1-MAML2 Fusion Polymerase Chain Reaction

The RNA from FFPE tissue samples was reverse transcribed using Transcriptor First Strand cDNA Synthesis Kit (Roche) according to manufacturer´s instructions. RNA integrity was determined by control polymerase chain reaction (PCR) comprising 2 μL of cDNA, 12.5 μL of HotStarTaq PCR Master Mix (QIAGEN, Hilden, Germany), 10 pM of each primer, and distilled water up to 25 μL. The amplification program comprised 95°C for 14 minutes, then 40 cycles of denaturation at 95°C for 1 minute, annealing at 60°C for 0.5 minute, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes. Samples with RNA integrity below 133 bp (showing <2 desired bands on agarose gel) were excluded from further analysis.

TABLE 2. Names of Primers Used for the Detection of *CRTC1-MAML2* Fusion by PCR

Name of Primer	Sequence 5′-3′
CRTC1A (outer)	TCGCGCTGCACAATCAGAAG
CRTC1B (inner)	GAGGTCATGAAGGACCTGAG
MAML2A (outer)	GGTCGCTTGCTGTTGGCAGG
MAML2B (inner)	TTGCTGTTGGCAGGAGATAG

Detection of CRTC1-MAML2 Fusion by PCR

Two microliters of cDNA was added to the PCR reaction consisting of 12.5 μL of HotStarTaq PCR Master Mix (QIAGEN), 10 pmol of each outer primer complementary to *CRTC1-MAML2* fusion transcripts (Table 2), and distilled water up to 25 μL. The amplification conditions comprised denaturation at 95°C for 14 minutes and then 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds.

The PCR product from the first run was diluted with distilled water at a ratio of 1:50. One microliter of the diluted product was added to a second run of the same PCR with the same reagents as in the first run except for the inner primers (Table 2).

Successfully amplified PCR products of the *CRTC1-MAML2* fusion gene were purified using Agencourt AMPure (Agencourt Bioscience Corporation, A Beckman Coulter Company). Then, the PCR products were both sides sequenced using the BigDye Terminator Sequencing kit (Applied Biosystems, CA), purified using Agencourt Clean SEQ kit (Agencourt Bioscience Corporation) and run on an automated genetic analyzer ABI Prism 3130xl (Applied Biosystems) at a constant voltage of 13.2 kV for 20 minutes and compared with the GenBank sequence database.

FISH Analysis of MAML2 Rearrangement

Four-micrometer-thick FFPE sections were placed onto positively charged slides. Hematoxylin and eosin–stained slides were examined for the determination of areas for cell counting.

The unstained slides were deparaffinized and incubated in the 1× Target Retrieval Solution Citrate pH 6 (Dako, Glostrup, Denmark) at 95°C for 40 minutes and subsequently cooled for 20 minutes at room temperature in the same solution. Slides were washed in deionized water for 5 minutes and digested in protease solution with pepsin (0.5 mg/mL) (Sigma Aldrich, St. Louis, MO) in 0.01 M HCl at 37°C for 35 to 60 minutes according to the

sample conditions. Slides were then placed into deionized water for 5 minutes, dehydrated in a series of ethanol solution (70%, 85%, 96% for 2 min each), and air-dried.

For the detection of *MAML2* rearrangement, the commercial probe ZytoLight SPEC MAML2 Dual Color Break-Apart Probe (ZytoVision GmbH, Bremerhaven, Germany) was used.

An appropriate amount of mixed and premixed probes was applied on specimens, covered with a glass coverslip and sealed with rubber cement. Slides were incubated in the ThermoBrite instrument (StatSpin/Iris Sample Processing, Westwood, MA) with codenaturation at 85°C/8 minutes and hybridization at 37°C/16 hours. Rubber-cemented coverslip was then removed and the slide was placed in a posthybridization wash solution (2×SSC/0.3% NP-40) at 72°C/2 minutes. The slide was air-dried in the dark, counterstained with 4′,6′-diamidino-2-phenylindole DAPI (Vysis/Abbott Molecular), cover slipped, and immediately examined.

FISH Interpretation

The sections were examined with an Olympus BX51 fluorescence microscope (Olympus Corporation, Tokyo, Japan) using a ×100 objective and filter sets triple bandpass (DAPI/SpectrumGreen/SpectrumOrange), dual bandpass (SpectrumGreen/SpectrumOrange) and Single Bandpass (SpectrumGreen or SpectrumOrange).

For *MAML2* break-apart probe, 100 randomly selected nonoverlapping tumor cell nuclei were examined for the presence of yellow or green and orange fluorescent signals. The yellow signals were considered negative; separate orange and green signals were considered as positive. Cutoff values for break-apart probes were set to >10% of nuclei with chromosomal breakpoint (mean+3 SD in normal non-neoplastic control tissues).

RESULTS

Molecular Genetic Findings

Thirty selected cases of low-grade oncocytic neoplasms were analyzed by NGS using the Anchored Multiplex PCR (AMP) technique (ArcherDX), and 22 of them were reclassified as OMEC. The remaining 8 cases were excluded from the study, and interpreted in 4 cases as oncocytic metaplastic pleomorphic adenoma (PLAG1 gene break detected by FISH), in 3 cases as benign oncocytoma with extensive mucinous metaplasia, and in 1 case as oncocytic benign myoepithelioma.

Thirteen OMEC cases (59%) presented the classic *CRTC1-MAML2* fusion (Fig. 1), and in 1 case alternative *CRTC3-MAML2* fusion was detected (4.5%). Five OMEC cases were negative on NGS (23%) but *MAML2* rearrangement was confirmed by FISH in all cases (Fig. 2). Two tumors were not analyzable (9%) and in 1 case the tissue was not available for NGS testing (4.5%). Molecular genetic findings of all 22 OMEC cases are summarized in Table 3.

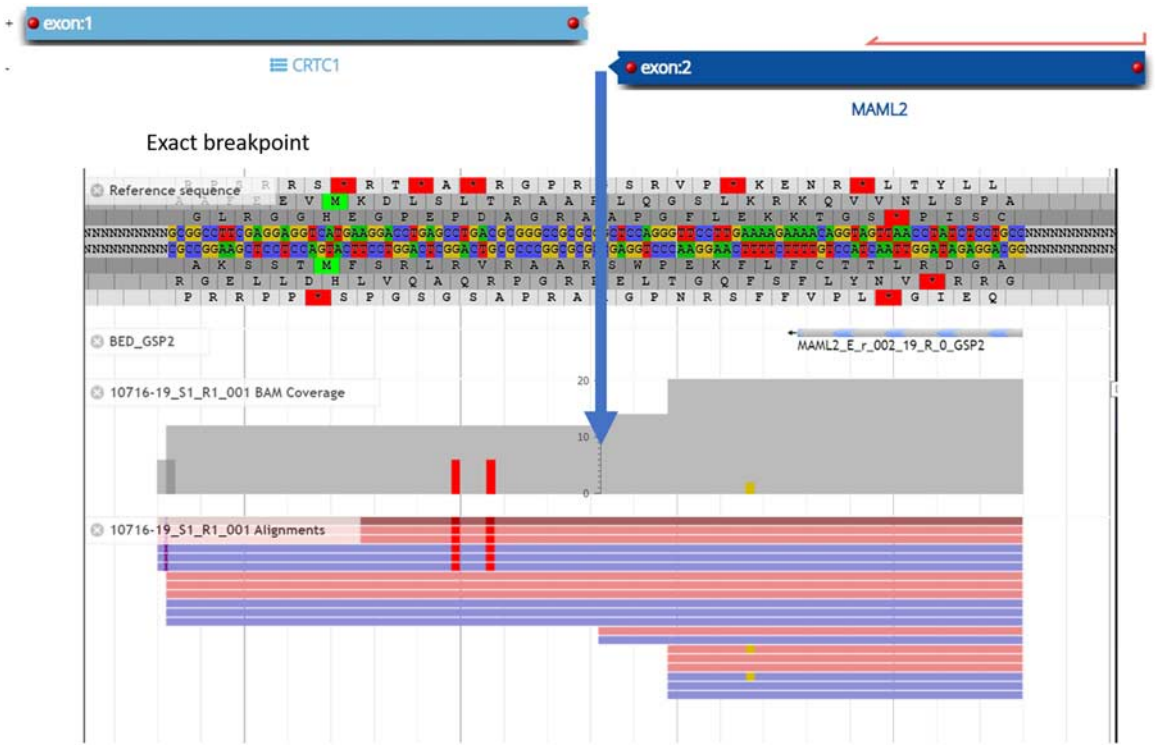

FIGURE 1. Detection of specific fusion transcript *CRTC1-MAML2* identified by ArcherDX assay. Schematic representation of the *CRTC1-MAML2* fusion transcript as revealed by NGS. The upper section: the scheme of joining of exon 1 of *CRTC1* gene and exon 2 of *MAML2* gene. The lower section: the sequencing coverage of point of fusion is indicated in gray. Several unique reads in red and blue, respectively. Blue arrow shows the exact breakpoint.

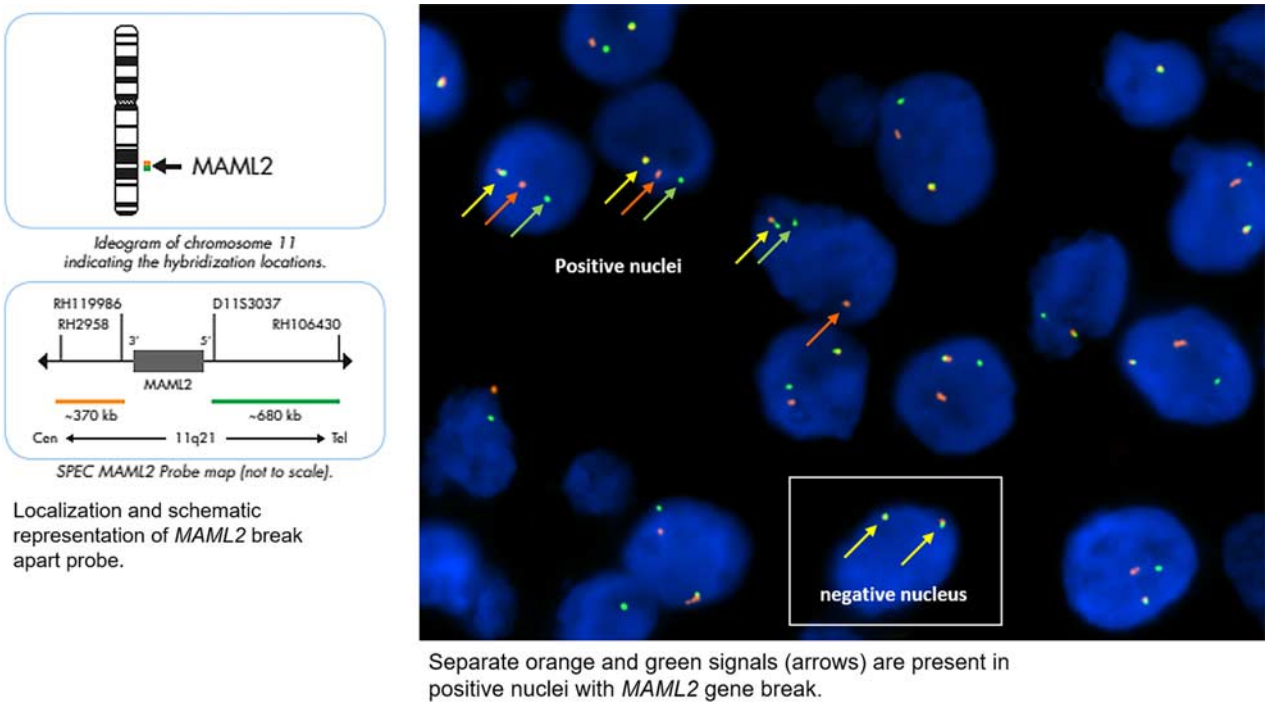

FIGURE 2. Detection of breakpoint of *MAML2* gene by FISH. FISH analysis using MAML2 break-apart probe with "classic" break-apart design. The figure shows several nuclei while yellow arrows indicate signals of an intact *MAML2* gene region.

Clinical Findings

The clinicopathologic data for the 22 identified OMEC cases are summarized in Table 4. There were 12 females and 10 males aged between 13 and 83 years, with a mean of 57 years. Fifteen tumors occurred in the parotid gland (68%), 2 cases each in the sublingual gland (9%), in the minor salivary gland of the palate (9%), and in the mucosa of the tongue (9%), and 1 in the submandibular gland (5%). The size of the tumors ranged from 0.9 to 3.5 cm (average of 2.6 cm).

The tumors were low grade (16 cases), and intermediate grade (6 cases) using the Armed Forces Institute of Pathology (AFIP) system[22]; while they were low grade (5 cases), intermediate grade (12 cases), and high grade (5 cases) using the modified Brandwein system.[23] Most of the tumors were upgraded 1 category using the Brandwein system as compared with the AFIP system. However, 2 tumors were upgraded even 2 categories being grade 1 using the AFIP system and grade 3 using the Brandwein system.

Follow-up was available for 18 patients ranging from 2 to 184 months with an average of 38.3 months. Seventeen patients showed no evidence of disease at latest control (94%). Only 1 patient developed regional lymph node metastasis, and died of disease at 20 months. The remaining 4 patients were lost to follow-up.

Index Case

A 48-year-old woman presented with a tumor mass of 7 months duration in the right parotid gland. Magnetic

TABLE 3. Molecular Genetic Findings of 22 Cases of OMEC

Case No.	FISH *MAML2*	RT-PCR *CRTC1-MAML2*	NGS FusionPlex Solid Tumor Kit Archer
1	+	ND	*CRTC1-MAML2*
2	+	ND	*CRTC1-MAML2*
3	+	ND	*CRTC1-MAML2*
4	+	Neg	*CRTC1-MAML2*†
5	+	*CRTC1-MAML2*	*CRTC1-MAML2*
6	+	ND*	ND*
7	+	Neg	NA
8	+	Neg	Neg
9	+	ND	*CRTC1-MAML2*
10	+	ND	Neg
11	+	Neg	*CRTC1-MAML2*†
12	+	Neg	Neg
13	+	*CRTC1-MAML2*	*CRTC1-MAML2*
14	+	ND	*CRTC1-MAML2*
15	+	ND	*CRTC1-MAML2*
16	+	*CRTC1-MAML2*	NA
17	+	ND	*CRTC3-MAML2*
18	+	ND	*CRTC1-MAML2*
19	+	ND	Neg
20	+	ND	*CRTC1-MAML2*
21	+	ND	Neg
22	+	ND	*CRTC1-MAML2*

*Tissue not available.
†Custom panel *ETV6-EWSR1-MAML2* using ArcherDx kit.
+ indicates FISH detected break in *MAML2* gene; NA, not analyzable; ND, not done; neg, no fusions found.

TABLE 4. Clinicopathologic Features of 22 Cases of OMEC

Case No.	Age (y)/Sex	Original Diagnosis	Site	AFIP Grade	Brandwein Grade	Outcome (mo)
1	48/female	Oncocytic neoplasm of uncertain malignant potential	Parotid	G1	G2	7 NED
2	53/male	Oncocytic neoplasm	Parotid	G2	G2	*
3	45/female	Recurrent oncocytoma	Sublingual	G1	G3	18 NED
4	74/male	Oncocytic neoplasm, possibly OMEC	Parotid	G2	G3	*
5	83/female	PA with oncocytic metaplasia	Parotid	G1	G2	*
6	41/female	Oncocytic neoplasm of uncertain malignant potential	Parotid	G1	G3	8 NED
7	46/male	Benign mucinous oncocytoma	Parotid	G1	G2	34 NED
8	71/female	Multifocal oncocytoma	Parotid	G1	G1	31 NED
9	13/female	OMEC	Palate	G1	G2	184 NED
10	59/female	Adenocarcinoma oncocytic NOS	Parotid	G2	G3	96 NED
11	13/male	Cystic sialometaplasia	Parotid	G1	G2	48 NED
12	74/female	Adenocarcinoma oncocytic, low grade	Parotid	G1	G1	60 NED
13	51/female	Oncocytic neoplasm	Palate	G2	G2	48 NED
14	72/male	Myoepithelial carcinoma	Tongue	G2	G3	20 DOD, lymph node metastasis
15	67/female	OMEC, low grade oncocytic tumor	Sublingual	G1	G2	24 NED
16	74/male	OMEC	Submandibular	G1	G1	*
17	57/male	Clear cell oncocytoma/PA/MEC	Parotid	G1	G1	2 NED
18	63/female	OMEC without mucinous cells	Parotid	G1	G2	2 NED
19	71/male	OMEC	Parotid	G1	G2	5 NED
20	70/female	Cystic intranodal oncocytic inclusion/cystic sialometaplasia	Parotid	G1	G1	72 NED
21	54/male	OMEC	Parotid	G2	G2	24 NED
22	56/male	Oncocytoma benign	Base of tongue	G1	G2	6 NED

Case 16 has been published earlier.[5]
*Not known.
NED indicates no evidence of disease; NOS, not otherwise specified; PA, pleomorphic adenoma.

resonance imaging showed a solid lesion of 30×22×26 mm involving the superficial and deep portions of the gland giving clear and polycystic profiles. The lesion extended around the branch of the mandible and into the proximity of the masseter muscle, from which it was separated by a rim of normal parotid parenchyma. Furthermore, there was dislocation of the external carotid artery and the retromandibular vein, but they appeared free of tumor.

On clinical examination preoperative facial motility was normal. On palpation the lesion appeared firm and it was not tender. The overlying skin was not infiltrated. Intraoperatively, the lesion could be separated from the surrounding normal parotid tissue but not from the nerve branches. The lesion and the nerves were fused in a whitish fibrous tissue.

Radical parotidectomy and a super selective neck dissection of levels IIA, IIB, and III were performed and were followed by radiotherapy. The tumor was staged as pT4aN0. The patient is alive with no signs of recurrence or metastatic spread after 7 months of follow-up. The original histopathologic diagnosis was an oncocytic neoplasm of uncertain malignant potential.

Histopathologic and IHC Findings

Histologically, at low power magnification the tumors showed nested and multinodular growth patterns composed of oncocytic cells (Fig. 3A) with focal and occasionally prominent stromal sclerosis (Fig. 3B). Hyalinized and focally interrupted tumor capsule was present in 7 cases, while 15 neoplasms showed invasive growth into the surrounding tissues. Perineural and lymphovascular invasion were detected in 6 and in 2 cases, respectively. In most cases, the oncocytic change of neoplastic cells was extensive, ranging between 75% and 100% of all tumor cells. Foci of conventional non-OMEC were not seen in any case. In 1 case, multiple large communicating cystic spaces were seen and the tumor showed extracellular keratinization (Fig. 3C). Another macrocystic tumor was lined by a complete layer of mucinous cells, and it contained intracapsular solid nests of oncocytes devoid of intracellular mucin (Fig. 3D).

Five cases were essentially solid, composed of nests and sheets of large oncocytic cells, and were completely devoid of true mucocytes (Fig. 4A). Focal tubular structures containing dense intraluminal secretions lined by fully developed oncocytes without any intracellular mucin were observed in these 5 cases (Fig. 4B). In 15 cases, mucin-containing cells were identified, but they were very scarce and hardly discernible on hematoxylin and eosin staining (Fig. 4C). In these cases, mucin-containing cells were identifed on mucicarmine or AB/PAS staining. Mucinous cyst formation was seen in 4 cases with 2 containing <5% cystic component (Fig. 4D). In only 1 case, the area of mucinous cysts amounted to about 20% of tumor tissue. Small foci of extravasated mucin were identified in another case. Hyalinized and focally interrupted tumor capsule was present in 6 cases, while 14 neoplasms showed invasive growth into the surrounding

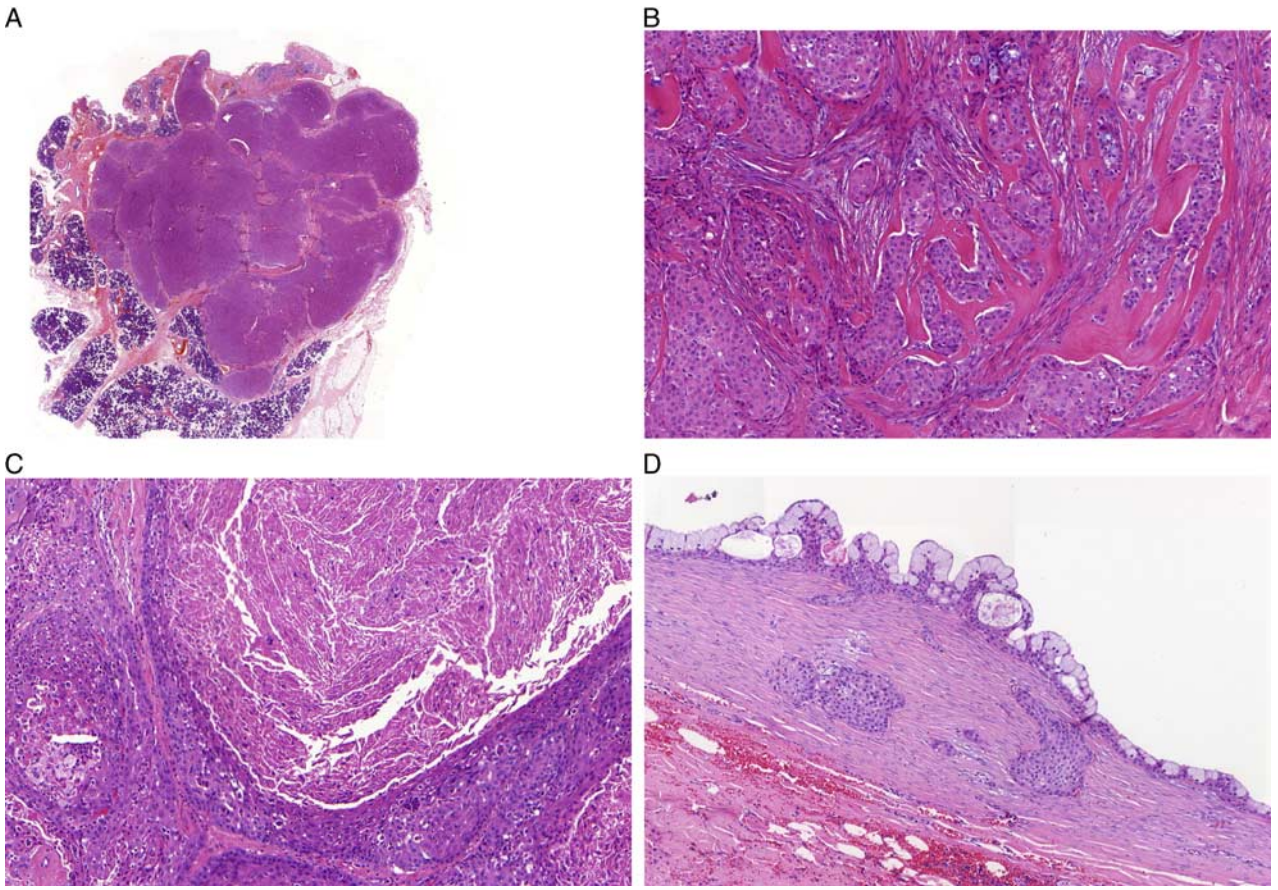

FIGURE 3. OMEC. At low power, the tumor shows nested and multinodular growth pattern (A) composed exclusively of oncocytic cells with focal prominent stromal sclerosis (B). Multiple large communicating cystic spaces with evidence of keratinization were found (C) and still another macrocystic tumor was lined by a complete layer of mucinous cells, and contained intracapsular solid nests composed of oncocytes devoid of intracellular mucin (D).

salivary gland parenchyma. Three cases contained foci of squamoid cell differentiation. In one of them multiple small squamous eddies, and in the other 2 squamous metaplasias due to previous fine needle aspiration injury were seen.

Cytologically, the individual oncocytes showed a low nuclear-cytoplasmic ratio, centrally placed nuclei, and abundant granular eosinophilic cytoplasm filled by the mitochondria (Fig. 5A). The tumor cells were bland looking in most cases, but a moderate degree of nuclear atypia with enlarged and irregular nuclei was seen in 2 cases (Fig. 5B). Five cases showed a clear cell phenotype with tumor cells having watery clear cytoplasm and distinct small nuclei (Fig. 5C). In places, oncocytic tumor cells with clear cell and eosinophilic phenotypes were interspersed (Fig. 5D).

Immunohistochemically, tumor cells in all studied cases were positive for CK7 (17/17), CK 14 (6/6), CK 5/6 (7/7), and antimitochondrial antigen MIA (16/16). In most cases, there was a strong nuclear staining for p63 (18/21) and p40 proteins (9/12). Nuclear staining for p63 was distributed throughout the solid oncocytic nests in an abluminal position of glandular/tubular structures (Fig. 6A). This staining pattern for p63 was seen in most cases. In other cases, OMEC displayed double-layered tubular structures where p40 and p63 stainings were concentrated in the peripheral cells of the nests, in a pattern similar to that reported previously for oncocytomas[12] (Fig. 6B). In 6 tumors, there was remarkable SOX10 immunopositivity. In 2 of these cases, the staining was strong, and distributed within the whole tumor in diffuse pattern (Fig. 6C), while in another 4 cases it was focal. S100 protein was negative in all but 1 case (Fig. 6D). Interestingly, this S100 protein and SOX10-positive case harbored a *CRTC3-MAML2* fusion. Proliferative activity in our tumors was predominantly low with MIB1 index ranging from 1 to 10% (average: 5.4%). Only 1 case showed a high hotspot type proliferative activity with a focal MIB1 index of 30%.

DISCUSSION

OMEC is a rare variant of MEC, composed predominantly of oncocytic cells, although the percentage of oncocytic cells justifying this diagnosis has not been defined.[1,3] To define OMEC in this study, we used an arbitrary cutoff of $>75\%$ of oncocytic cells. The real importance of recognizing OMEC is in distinguishing it from

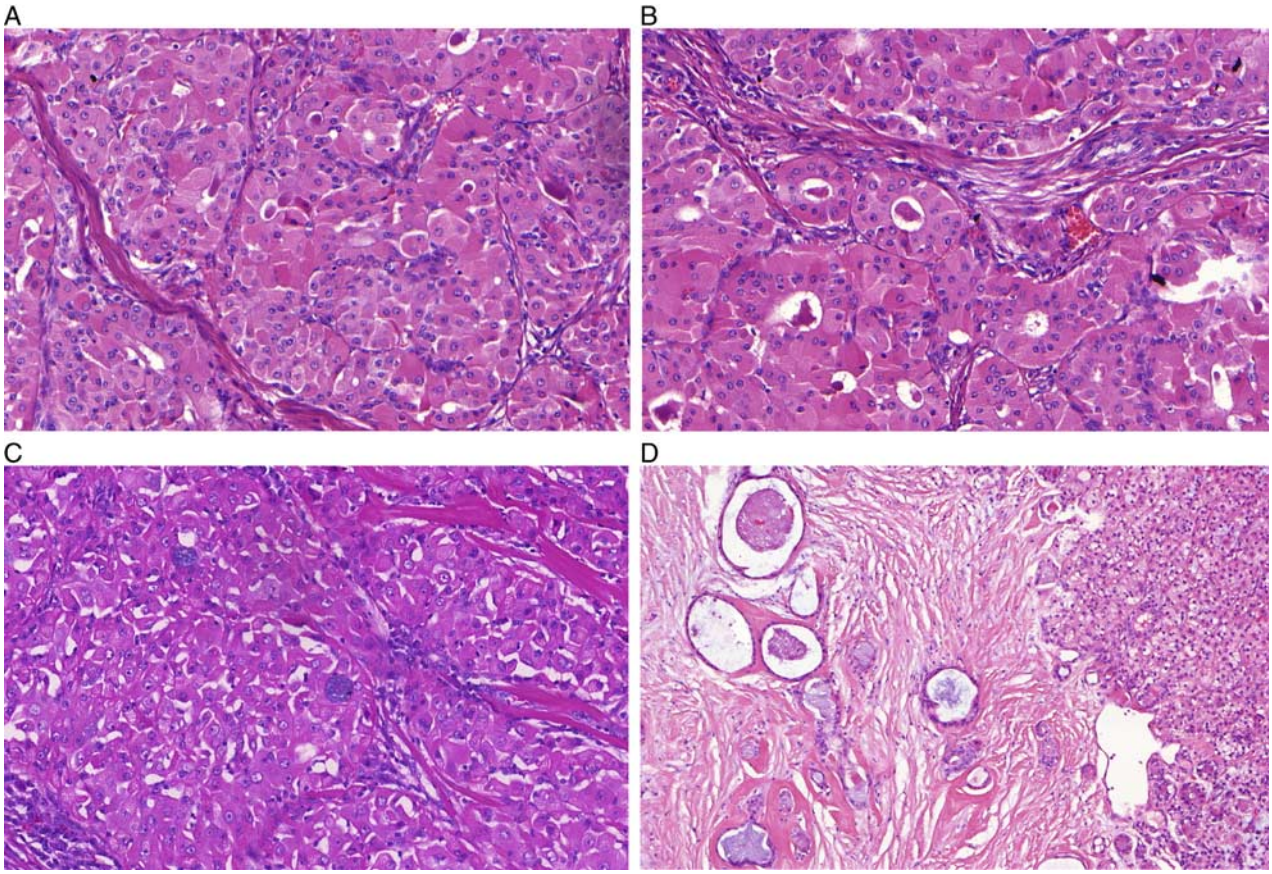

FIGURE 4. OMEC. A, OMEC is composed solid sheets of large oncocytic cells and completely devoid of true mucocytes. B, Focal tubular structures containing dense intraluminal secretions lined by fully developed oncocytes without any intracellular mucin are observed. C, Mucin-containing cells are rare and hardly discernible. D, Mucinous cyst formation.

its mimickers. The distinction of OMEC from other oncocytic and oncocytoid tumors is particularly challenging in cases composed entirely of oncocytes[24] or in cases of MEC with a significant proportion of oncocytic cells devoid of mucous cells. In our experience, the most difficult differential diagnosis was the distinction of OMEC from a benign oncocytoma/low-grade oncocytic tumor of uncertain malignancy and from a cellular pleomorphic adenoma and/or myoepithelioma with extensive mucinous and oncocytic metaplasia.[25] Another differential diagnostic consideration was the oncocytic variant of a benign cystadenoma. Cystadenoma of salivary glands is a rare benign tumor characterized by predominantly multicystic growth in which the epithelium demonstrates adenomatous proliferation.[26] Extensive oncocytic change may be seen, and these lesions are classified as oncocytic cystadenomas.[27–29] A diagnostically challenging case of oncocytic papillary cystadenoma with prominent mucinous differentiation has been reported recently.[30]

According to the study of Weinreb et al,[3] diffuse p63 immunopositivity may be a reliable marker for the diagnosis of OMEC. Indeed, most OMEC cases of our study showed p63 immunostaining. However, a diffuse and strong p63 positivity was not seen in all cases. In 2 tumors the staining was focal, while another 2 showed abluminal positivity similar to that described in oncocytomas.[12,13] Finally, in 3 cases p63 protein was completely negative. Contrary to this, other salivary tumor types including pleomorphic adenomas, myoepitheliomas, and Warthin tumors show extensive p63 immunostaining. The identification of mucous cells in OMEC can be useful, but oncocytic cystadenomas, metaplastic Warthin tumors, and oncocytic metaplastic pleomorphic adenomas may also have mucous cells. Moreover, true mucocytes may be very few and hardly discernible in OMECs.

Another possible pitfall in the differential diagnosis of OMEC relates to the SOX10 immunopositivity, which we encountered in a subgroup of OMECs. In the normal salivary gland, expression of SOX10 is limited to acinar cells and the intercalated ducts (both luminal and abluminal cells).[31] Until recently, it was proposed that salivary gland tumors can be divided into a subgroup of SOX10-positive, including acinic cell carcinoma, secretory carcinoma (mammary analogue), adenoid cystic carcinoma, epithelial-myoepithelial carcinoma, myoepithelioma, myoepithelial carcinoma, and pleomorphic adenoma, while a subgroup of SOX10-negative tumors comprised salivary duct carcinoma, MEC, oncocytic carcinoma, oncocytoma, and Warthin

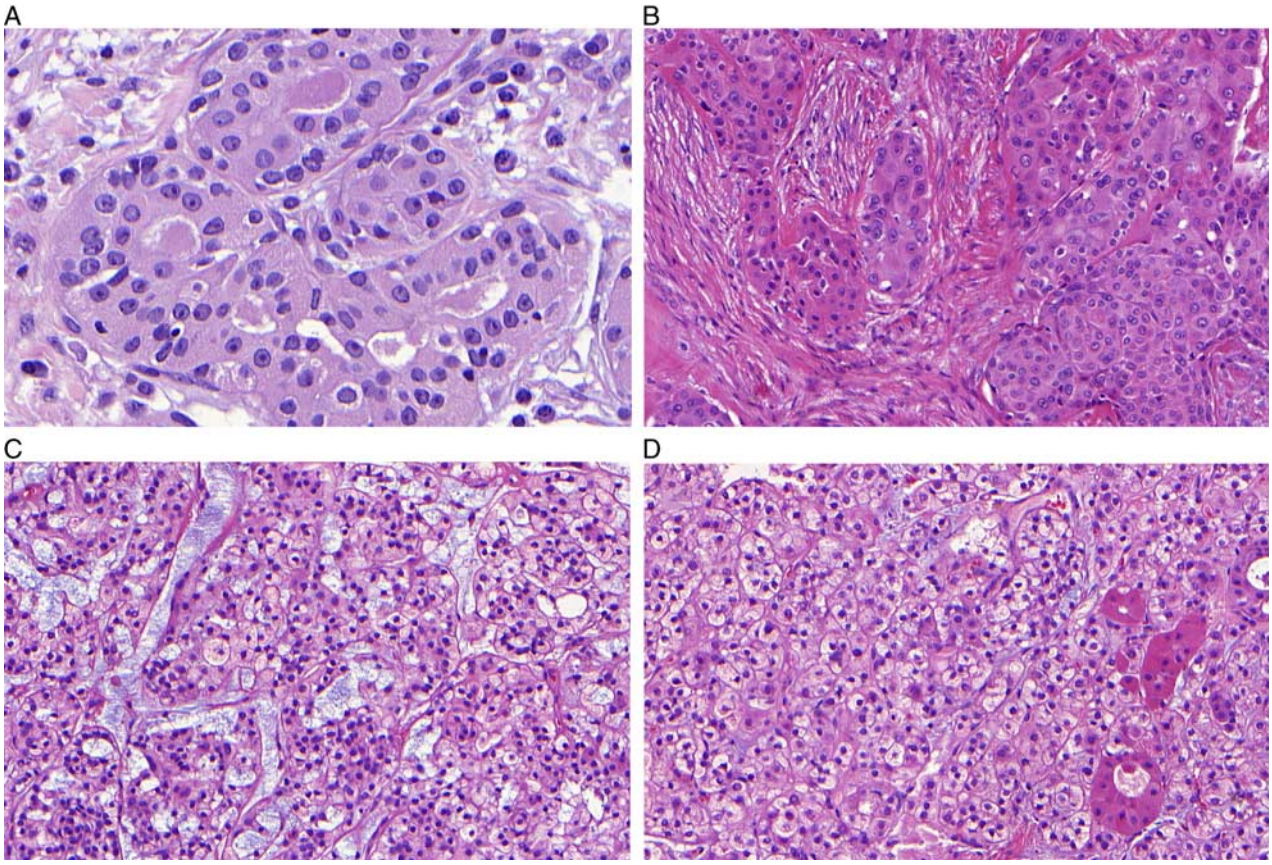

FIGURE 5. OMEC. Oncocytes show centrally placed nuclei with abundant granular eosinophilic cytoplasm, and a low nuclear-cytoplasmic ratio (A). The tumor cells show a moderate degree of nuclear atypia with enlarged and irregular nuclei (B). Oncocytes with a watery clear cytoplasm and distinct small nuclei are seen (C). In places, neoplastic oncocytic cells with clear and eosinophilic phenotype are intermingled (D).

tumor.[31] Agreeably SOX10 is very useful in distinguishing myoepithelial tumors, mammary analogue secretory carcinoma, and acinic cell carcinoma from ductal cell-derived salivary neoplasms, but it cannot be used for the diagnosis of MEC because of a subgroup of SOX10-positive MECs.[32] In our series, 6 of 20 OMECs were positive for SOX10.

Thus, due to these differential diagnostic difficulties, ancillary diagnostic testing to support a diagnosis of MEC is desirable. Here, we have evaluated the utility of testing for *MAML2* rearrangement using FISH and NGS to confirm the diagnosis of OMEC. MECs are known to harbor *CRTC1-MAML2* or *CRTC3-MAML2* as characteristic gene fusions in up to 80% of cases.[14–19] While the prognostic value of *MAML2* fusions has been questioned recently,[16,18,19,33] the finding of recurrent translocations seems to be a useful diagnostic "gold standard" for confirming the diagnosis of MEC in difficult cases.[11,20]

Last but not least, diversity in clinical presentation and variability of histologic appearances has made MEC challenging to grade, stage, and treat. Multiple histopathologic grading systems for MEC have been created to stratify patients better for prognostication and treatment, namely the AFIP system,[22] the Brandwein system,[23] and the system by Katabi et al.[34] All these grading systems have 3 grades: low, intermediate, and high. However, applying the different systems to the same tumor may result in different grades, so that the Brandwein system tends to assign higher grades and the AFIP system lower grades. This is particularly true for OMEC because of its typical solid and nested growth pattern. In our series of 20 OMECs, use of the Brandwein system resulted in upgrading of 11 tumors by 1 grade and 2 tumors by 2 grades as compared with the AFIP system. These OMECs had an uneventful clinical follow-up. The important consequence is that the patients may receive more aggressive management schemes in such cases. Low-grade MECs generally require complete surgical excision while high-grade MECs are also treated with adjuvant radiotherapy with or without chemotherapy.[35] Intermediate-grade carcinomas may behave similar to low-grade carcinomas, and treatment with surgical excision alone has been considered.[36,37] While the Brandwein high grade is likely the best predictor of aggressive behavior, upgrading of OMECs from low-to-intermediate grade because of the mere presence of isolated solid growth seems not warranted.[19]

In our series, follow-up was available for 16 patients ranging from 2 to 184 months, and all but 1 were alive at the latest follow-up. Only 1 patient (grade 2 by the AFIP

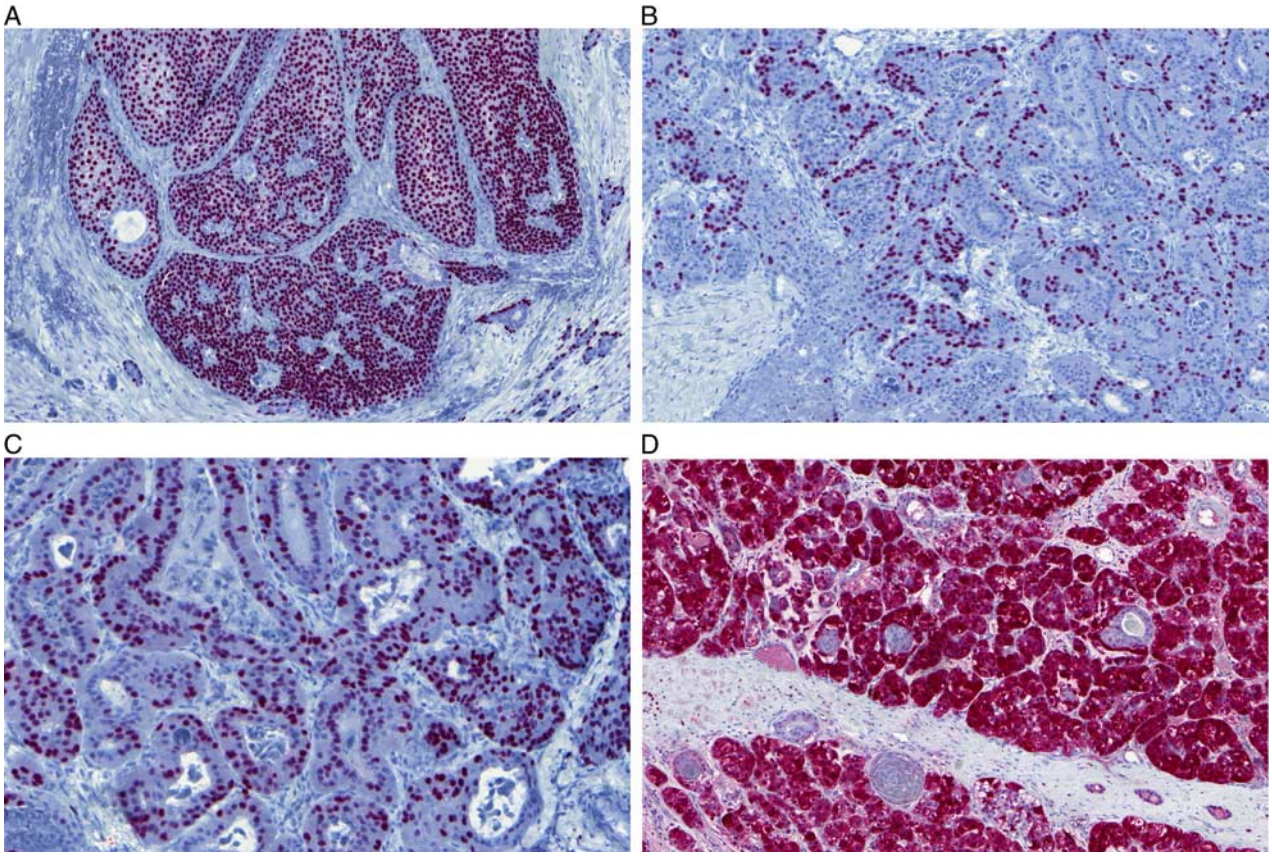

FIGURE 6. IHC stainings in OMEC. Nuclear staining for p63 was distributed throughout the solid oncocytic nests in abluminal position to glandular/tubular structures in most OMECs (A). Less common pattern of p63 nuclear staining concentrated in the peripheral cells of the nests (B). In 6 tumors, there was remarkable SOX10 immunopositivity (C). S100 protein was expressed only in 1 case (D). Interestingly, this tumor was also strongly positive for SOX10 and harbored *CRTC3-MAML2* fusion.

system and grade 3 by the Brandwein system) developed regional lymph node metastases and died of disease at 20 months. Until the present study, the largest series of OMEC with follow-up information was presented by Weinreb et al.[3] Compiling all published cases with follow-up information, including the review of literature in Weinreb et al's[3] report, only 2 recurrences have been documented. None of the cases has shown lymph node metastases or death due to disease. Generally, it seems that OMEC is biologically a low-grade malignancy with potential for locally aggressive disease. In our study, however, we document the first OMEC with metastatic behavior and death of the patient due to disease. This case presented with lymph node metastases at the time of diagnosis, and was distinguished by a focally abrupt (hotspot) Ki-67 index of 30% centered around vessels.

In conclusion, we have described a series of 22 OMECs which is the largest so far. The novel finding in our series is a description of 5 OMECs that were essentially solid, composed of nests and sheets of large oncocytic cells and completely devoid of true mucocytes. Our study highlighted the usefulness of *MAML2* testing in the identification of OMEC and its distinction from mimickers.

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