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Mucin 4: A Sensitive Diagnostic Marker for Pediatric Mucoepidermoid Carcinoma

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ABSTRACT

Pediatric salivary gland-type neoplasms (SGTNs) pose a significant diagnostic problem due to histo-morphological heterogeneity. Previous reports have shown that Mucin 4 (MUC4) expression is associated with adult mucoepidermoid carcinoma (MEC). We hypothesize that MUC4 is also a sensitive marker for distinguishing MEC from other SGTNs in the pediatric population. A retrospective review of 74 SGTNs diagnosed between 1993–2015 at Children's Hospital Los Angeles, Boston Children's Hospital, and Rhode Island Hospital was performed. Hematoxylin and eosin-stained sections of 31 MECs were compared to 3 adenoid cystic carcinomas (AdCCs), 6 acinic cell carcinomas (AcCCs), 30 pleomorphic adenomas (PAs), 3 mammary analogue secretory carcinomas (MASCs), and one sialoblastoma (SB). Samples underwent immunohistochemical staining for MUC4, with expression score criteria: 0% positivity = 0, 1-10% = +, 11-50% = ++, 51-90% = +++, >90% = ++++. Ages of patients at time of tumor excision ranged from 2–19 years. All MECs were MUC4-positive, with 25 (80.65%) having an expression score ≥ +++. AdCCs and PAs demonstrated no to minimal MUC4-positivity. Subsets of AcCCs and MASCs were unexpectedly MUC4-positive. As a novel marker for pediatric MEC, MUC4's sensitivity = 100%, specificity = 79.41%, positive predictive value = 75.86%, and negative predictive value = 100%.

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Introduction

Salivary Gland-Type Neoplasms

Salivary gland-type neoplasms (hereafter referred to as SGTNs) are rare and pose significant diagnostic and prognostic difficulties given the histo-morphological heterogeneity (inter and intra-tumor, (Fig. 1-2), scarcity of long-term outcome data, and a lack of concrete recommendations for different age groups affected by these tumors [1-4]. Comparatively, this tumor class is better characterized in adults than it is in the pediatric population. SGTNs comprise 3-6% of all head and neck neoplasms, overall, and approximately 1% of pediatric head and neck neoplasms [5-8]. In adults, 10-25% of SGTNs are malignant, whereas 50% are malignant in children [9]. While more than 20 histologic sub-types of SGTNs have been identified in adults, fewer subtypes have been reported in children [5]. Subtypes that are known to appear in both adults and children include: mucoepidermoid carcinoma (MEC), pleomorphic adenoma (PA), adenoid cystic carcinoma (AdCC), and acinic cell carcinoma (AcCC). Overall, MEC is the histologic subtype with the highest incidence, followed by AcCC [5, 10]. In the pediatric population, specifically, the most common benign and malignant SGTNs are PA and MEC, respectively [7, 11]. The higher chance of malignancy in children vs. adults, paired with higher recurrence rates (in AdCC and AcCC), warrants further characterization of pediatric SGTNs [12]. Several SGTN subtypes have morphologic origins corresponding to parts of the tubule-acinar structure of the salivary secretory unit; AcCCs are derived from the acinus, PAs and AdCCs are derived from the intercalated duct, and MECs are derived from the excretory duct (Fig. 3) [13].

In terms of etiology of the disease, SGTNs have been associated with radiation exposure (including therapeutic radiation), genetic abnormalities (ex: Brooke-Spiegler Syndrome), and Epstein Bar

Virus infection [14, 15]. In addition to being heterogeneous in terms of their histology and etiology, SGTNs are also heterogeneous in terms of their location/tissue of origin. SGTNs occur in a variety of organs, not all of which are salivary glands (despite the neoplasm's name). Diagnosis is, therefore, further complicated by the fact that SGTNs found in non-salivary-gland locations can be primary to the non-salivary-gland tissue, or metastatic from a true salivary gland [16]. Previous studies have concluded that the parotid gland is the most common site of SGTN occurrence, followed by the submandibular and sublingual glands, minor salivary glands, and then non-salivary-gland locations (including the lacrimal gland, bronchi and parabronchial glands, lung, breast, thymus, the hard and soft palates, and the floor of the mouth) [6, 16-20]. SGTNs found in the lung or bronchi are not associated with smoking, and are most commonly of the MEC or AcCC subtypes [16, 17]. SGTNs found in the breast are typically triple negative [18]. Previous reports have stated that pediatric SGTNs are more commonly low-grade and localized to their primary sites compared to adult SGTNs [3]. Being poorly differentiated/high grade, having perineural invasion, and having extracapsular spread are associated with poorer outcomes [8].

Ancillary testing, in particular immunohistochemistry (IHC) and fluorescence in situ hybridization (FISH), can be utilized to diagnose SGTNs [21]. These methods allow for the identification of tumor type-specific fusion oncogenes that are the result of characteristic chromosomal translocations; for example, the MECT1-MAML2 fusion gene (also called the CRTC1-MAML2 fusion gene, resulting from a t(11;19)(q21;p13) translocation) is associated with MEC, the PLAG1-CTNNB1 fusion gene (resulting from a t(3;8)(p21;q12) translocation) is associated with PA, the MYB-NFIB fusion gene (resulting from a t(6;9)(q22-23;p23-34) translocation) is associated with AdCC, the ETV6-NTRK3 fusion gene (resulting from a t(12;15)(p13;q25) translocation) is associated with mammary analog secretory carcinoma (MASC), the EWSR1-ATF1 fusion gene is associated with hyalinizing clear cell carcinoma, point E710D mutations in the PRKD1 gene are associated with polymorphous adenocarcinoma, translocations involving the PRKD1-3 gene are associated with cribriform adenocarcinoma of minor salivary glands, and the BCOA4-RET fusion gene (as well as molecular features often associated with invasive ductal breast carcinoma, i.e. involving HER2, TP53, PIK3CA, HRAS, and PTEN) are associated with high-grade salivary duct adenocarcinoma [1, 21].

The treatment of choice for SGTNs is surgical excision (for low to intermediate and early-stage tumors); supplemental radiotherapy may be indicated for less favorable cases, but its tumorigenic risk must be considered, especially in pediatric patients [3, 7, 9, 22, 23]. Another risk to consider, in the case of head and neck lesions, is that radiation therapy can result in xerostomia and salivary dysfunction [8]. The prognosis for SGTNs is generally favorable because the neoplasms are typically diagnosed at an early stage; however, higher-grade malignancies generally have a poor prognosis even if multimodal therapy is pursued [5, 23].

MEC Subtype of SGTN

As mentioned earlier, MEC is the most common malignant SGTN seen in the pediatric and adult populations [7, 11]. In both children and adolescents, MECs are more frequently diagnosed in females [24]. Common locations of pediatric MEC include the parotid gland and palate (both hard and soft) [9, 24]. MEC often appear grossly as a blue submucosal lump, and microscopically as a mixture of mucin-producing, intermediate-type, and squamoid cells; MEC can have areas of both cystic and solid growth patterns [8, 24].

As earlier stated, MEC is associated with expression of the MECT1-MAML2 fusion gene [8]. Traditionally, this fusion gene has been associated with low-grade MEC, specifically; however, a recent report suggests that, in the pediatric population, the MECT1-MAML2 fusion gene may actually serve as a marker for all MECs, regardless of histological grade [25]. High-grade MECs often show genetic aberrations involving HER2 and/or EGFR [8]. Currently established IHC markers for the diagnosis of MEC include CK5/6 positivity and p63 negativity [21].

Just as with other subtypes of SGTN, the treatment of choice for pediatric MEC is surgical excision, and prognosis is generally good (histologic grade of most pediatric MEC is low to intermediate, and recurrence rates are <10%) [7, 24]. Previous studies have shown that other potential therapeutic targets for MEC include basic leucine zipper and W2 domains 1 (BZW1), the interleukin (IL)-6 pathway, nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), and histone deacetylase (HDAC), all of which are involved in MEC pathogenesis [8].

Mucin 4 as a Tumor-Associated Antigen

Mucins are high-molecular weight glycoproteins involved in a multitude of biological pathways [26, 27]. Mucin 4 (MUC4), specifically, is a large, type I, transmembrane mucin that is involved in epithelial homeostasis [28-30]. Under normal circumstances, MUC4 is expressed in airway epithelial cells, bodily fluids (e.g. saliva, tears, breast milk), the small and large intestine, the prostate, and the cervix [26, 27, 29, 31]. Under pathological circumstances, MUC4 drives inflammation and, thus, inflammation-associated tumorigenesis [29]. MUC4 also functions as a tumor-associated antigen that has been reported to promote tumorigenicity, therapy resistance, cell proliferation, migration/metastasis (through its interaction with the ErbB2 receptor via EGF domains), and poor outcomes in numerous epithelial carcinomas (including pancreatic, breast, colorectal, ovarian, prostate, and oral squamous cell carcinomas) 26-33].

Because MUC4 is a tumor-associated antigen, it has been shown to play a role as both a tumor marker and a therapeutic target. Previous studies have shown that MUC4 can serve as a marker for a subset of angiomatoid fibrous histiocytoma (a neoplasm seen in the pediatric population that occurs in the dermis/subcutis, and displays a characteristic t(2;22) translocation involving EWSR1 and CREB1) [34, 35]. Expression of MUC4 has also been reported to serve as a diagnostic and prognostic marker for MEC in adults [36, 37]. In the literature, it has been demonstrated that lowering expression of MUC4 reduces proliferation and metastasis of pancreatic cancer cells [32]. A MUC4β-nanovaccine has even been developed for pancreatic cancer [38].

This study aims to establish whether MUC4, given its diagnostic utility for MEC in adults, can serve as a sensitive marker for distinguishing MEC from other salivary gland-type tumors in the pediatric population. The MUC4 antibody is a clinically-validated antibody routinely used on pediatric surgical pathology cases at our institution, Children's Hospital of Los Angeles (CHLA). To our knowledge, this is the first study to investigate the utility of MEC4 as a marker for pediatric MEC.

Materials and Methods

Study Design

This study was approved by Children's Hospital Los Angeles (CHLA) Institutional Review Board (IRB). A total of 74 SGTNs diagnosed between 1993-2015 at CHLA, Boston Children's Hospital (BCH), and Rhode Island Hospital (RIH) were reviewed.

These samples included 31 MECs (25 from CHLA, 5 from BCH, 1 from RIH), 3 AdCCs (1 from CHLA, 2 from BCH), 6 AcCCs (2 from CHLA, 4 from BCH), 30 PAs (all from CHLA), 3 MASCs (1 each from CHLA, BCH, RIH), and one SB (from BCH). After review of the hematoxylin and eosin (H&E) stained sections, MUC4 immunostaining of the MEC cases was compared to that of the 5 other SGTN subtypes.

Immunohistochemistry

To assess MUC4 protein expression, each SGTN was immunostained with a MUC4 rabbit monoclonal antibody, clone 8G7 (Cell Marque, Rocklin, California). Expression was rated using a score criteria of: 0% MUC4-positivity = 0, 1-10% MUC4-positivity = +, 11-50% MUC4-positivity = ++, 51-90% MUC4-positivity = +++, and >90% MUC4-positivity = ++++. Additional immunostaining was performed for p63 and S100 (at CHLA), as well as DOG1, GCDFP-15, and mammaglobin (at RIH) for confirmation of selected MASCs and AcCCs. Histochemical staining with mucicarmine and Alcian blue (pH 2.5) served as comparative and/or confirmatory tests (both are stains for mucin) for MEC.

CHLA immunostaining assays were performed on 5-µm formalin-fixed paraffin-embedded (FFPE) tissue sections. The Leica Bond Polymer Refine Detection Kit was utilized with the Leica Bond-Max automatic immunostainer (Leica, Bannockburn, Illinois). After 30-minute incubation of the primary antibody at validated 1:200 dilution with the tissue sections, epitopes were retrieved in 25 minutes by Leica H2 buffer. Validated positive controls were utilized.

RIH immunostaining assays were performed on 4-µm FFPE tissue sections using the Ventana Discovery system and DAB MAP detection kit (Ventana Medical Systems, Tucson, AZ). The IHCs used for the MASCs included Mammaglobin (rabbit/mouse antibody, clone 304-1A5 and 31A5; Cell Marque, Rocklin, CA), Gata-3 (mouse, clone L50-823; Biocare Medical), P63 (mouse, 4a4; Biocare Medical), and SOX-10 (rabbit antibody; Cell Marque, Rocklin, CA).

Fluorescence in Situ Hybridization (FISH)

Fluorescence in situ hybridization (FISH) analyses were performed (both at CHLA and at the Mayo Clinic's Histology Laboratory) for the t(12;15)(p13;q25) ETV6-NTRK3 translocation in MASCs, and for the t(11;19)(q1421;p1213) MECT1-MAML2 translocation in one case of (bronchial) MEC.

Statistical Analysis

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were calculated for MUC4-positivity as a diagnostic marker for pediatric MEC (as well as for MUC4-negativity as a diagnostic marker for pediatric PA). As we were comparing SGTN subtype (AcCC, AdCC, MASC, MEC, PA) vs. MUC4 expression score (0, +, ++, +++, ++++) counts – IBM SPSS Statistics 26 was utilized to perform a Freeman-Halton extension of Fisher's Exact Test, as well as a Kruskal-Wallis test with Bonferroni-adjustment for pairwise comparisons.

Results

Demographics

The 74 tumor samples came from 22 males (29.73%) and 28 females (37.84%); one sample came from an individual of unknown sex, and 11 of the samples (all of which were MECs) represented recurrent/re-excised cases (Table 1). MECs and PAs comprised the majority of our SGTN samples (41.89% and 40.54%, respectively). Median age of individuals at the time of (first) tumor excision was 14 years, with an interquartile range (IQR) of 10.25-15 years. The most common primary tumor site for SGTNs was the parotid gland (34, 45.95%), followed by the submandibular gland (9, 12.16%), the bronchus (5, 6.76%), the palate and maxillary bone (4, 5.41%), the neck (4, 5.41%), the orbit (3, 4.05%), and finally the minor salivary glands (i.e. 1 isolated minor salivary gland tumor was found in a patient's lip, 1.35%). It is worth noting that one of the submandibular gland neoplasms had extended into local minor salivary glands (but we have categorized this sample as a primary submandibular gland neoplasm in our tabulations). It is also worth noting that we did not have any sublingual gland tumors in our cohort. For those cases for which excised sample dimensions were in the database (N = 51), the median tumor sample size (going by the largest dimension measured – length, width, or depth) was 2.3 cm (IQR: 1.8 – 3.15 cm). In terms of tumor grade, 22 (29.73%) SGTNs were low-grade, 1 (1.35%) was intermediate grade, none were high grade, 48 (64.86%) were unknown, and 3 (4.05%) were simply listed as recurrent. In regard to metastatic disease, 5 samples (6.76%) had lymph node metastases, 17 (22.97%) did not have lymph node metastases, and 52 cases (70.27%) either were unknown or did not have lymph nodes evaluated. Perineural invasion was absent for 10 (13.51%) tumors, present in 5 (6.76%) tumors, and unknown or not assessed in 59 (79.73%) tumors.

Table 1: Demographics for SGTN Samples

Variable	All SGTNs (*N* = 74)	AcCCs (*N* = 6)	AdCCs (*N* = 3)	MASCs (*N* = 3)	MECs (*N* = 31)	PAs (*N* = 30)	SBs (*N* = 1)
Sex of patient – no. (%)							
Male	22 (29.73)	1 (16.67)	*0 (0)*	*0 (0)*	9 (29.03)	12 (38.71)	*0 (0)*
Female	28 (37.84)	1 (16.67)	1 (33.33)	2 (66.66)	6 (19.35)	18 (58.06)	*0 (0)*
Unknown	13 (17.57)	4 (66.67)	2 (66.67)	1 (33.33)	5 (16.13)	*0 (0)*	1 (100)
Recurrent/re-excised §	11 (14.86)	*0 (0)*	*0 (0)*	*0 (0)*	11 (35.48)	*0 (0)*	*0 (0)*
MUC4 Expression Score – no. (%)							
0	30 (40.54)	1 (16.67)	1 (33.33)	*0 (0)*	*0 (0)*	28 (93.33)	*0 (0)*
+	11 (14.86)	5 (83.33)	2 (66.67)	*0 (0)*	2 (6.45)	2 (6.67)	*0 (0)*
++	5 (6.76)	*0 (0)*	*0 (0)*	*0 (0)*	4 (12.90)	*0 (0)*	1 (100)
+++	9 (12.16)	*0 (0)*	*0 (0)*	1 (33.33)	8 (25.81)	*0 (0)*	*0 (0)*
++++	19 (25.68)	*0 (0)*	*0 (0)*	2 (66.67)	17 (54.84)	*0 (0)*	*0 (0)*
Age of patient at time of (first) tumor excision (yrs.)							
Median (IQR) †	14 (10.25-15)	15 (14.5-15.5)	-- ‡	12 (10.5-13.5)	12 (9-15) §	15 (12-15)	-- ‡
Mean (±SD)	12.72 (±3.75)	15 (±1.41)	--	12 (±4.24)	11.33 (±4.39)	13.47 (±3.32)	--
Range	2-19	14-16	--	9-15	2-17	5-19	--
Primary tumor site – no. (%)							
Parotid gland	34 (45.95)	2 (33.33)	*0 (0)*	2 (66.67)	15 (48.39)	15 (50)	--
Submandibular glandΦ	9 (12.16)	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	9 (30)	--
Bronchus	5 (6.76)	*0 (0)*	*0 (0)*	*0 (0)*	5 (12.90)	*0 (0)*	--
Palate, maxillary bone	4 (5.41)	*0 (0)*	*0 (0)*	*0 (0)*	4 (3.23)	*0 (0)*	--
Neck	4 (5.41)	*0 (0)*	*0 (0)*	*0 (0)*	1 (16.13)	3 (10)	--
Orbit, eyebrow	3 (4.05)	*0 (0)*	1 (33.33)	*0 (0)*	*0 (0)*	2 (6.67)	--
Minor salivary glandsΦ	1 (1.35)	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	1 (3.33)	--
Unknown	14 (18.92)	4 (66.67)	2 (66.67)	1 (33.33)	6 (19.35)	*0 (0)*	--
Tumor size (cm)Ψ							
Median (IQR) †	2.3 (1.8-3.15)	1.65 (1.48-1.83)	--	3.20 (2.50-3.90)	2.1 (1.25-2.65)	2.6 (2-3.5)	--
Mean (±SD)	2.59 (±1.38)	1.65 (±0.49)	--	3.20 (±1.98)	2.09 (±1.00)	2.92 (±1.51)	--
Range	0.5-7.6	1.3-2	--	1.8-4.6	0.6-4	0.5-7.6	--
Tumor grade – no. (%)							
Low	22 (29.73)	2 (33.33)	1 (33.33)	*0 (0)*	19 (61.29)	*0 (0)*	*0 (0)*
Intermediate	1 (1.35)	*0 (0)*	*0 (0)*	*0 (0)*	1 (3.23)	*0 (0)*	*0 (0)*
High	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*	*0 (0)*
Listed as Recurrent	3 (4.05)	*0 (0)*	*0 (0)*	*0 (0)*	3 (9.68)	*0 (0)*	*0 (0)*
Unknown	48 (64.86)	4 (66.67)	2 (66.67)	3 (100)	8 (25.81)	30 (100)	1 (100)
Lymph node metastases – no. (%)							
Yes	5 (6.76)	*0 (0)*	*0 (0)*	1 (33.33)	4 (12.90)	*0 (0)*	*0 (0)*
No	17 (22.97)	1 (16.67)	*0 (0)*	1 (33.33)	8 (25.81)	7 (23.33)	*0 (0)*
Unknown or not evaluated	52 (70.27)	5 (83.33)	3 (100)	1 (33.33)	19 (61.29)	23 (76.67)	1 (100)
Perineural invasion – no. (%)							
Yes	5 (6.76)	1 (16.67)	*0 (0)*	*0 (0)*	4 (12.90)	*0 (0)*	*0 (0)*
No	10 (13.51)	1 (16.67)	*0 (0)*	1 (33.33)	8 (25.81)	*0 (0)*	*0 (0)*
Unknown, not evaluated, or NA	59 (79.73)	4 (66.67)	3 (100)	2 (66.67)	19 (61.29)	30 (100)	1 (100)

Overall sample size for each SGTN subtype is listed in the top row (i.e. *N* = #). *Abbreviations: no. = number, % = percentage, yrs. = years, IQR = interquartile range, SD = standard deviation, cm = centimeters, SGTN = salivary gland-type neoplasm, AcCCs = acinic cell carcinoma, AdCC = adenoid cystic carcinoma, MASC = mammary analogue secretory carcinoma, MECs = mucoepidermoid carcinoma, PA = pleomorphic adenoma, SB = sialoblastoma.* Demographic data for each category listed was not available for all

patients in our database (see further details below).

† Distribution is non-normal, so median and IQR better describe the data than do mean and SD.

§ Two females each gave 2 samples; another female gave 7 samples; one male gave 2 samples, and another male gave 3 samples. Therefore, there were 11 recurrent/re-excised cases, all of which were MECs.

‡ Only 1 of the 3 AdCC samples had age listed in our database (i.e. 8 years old). The age of the patient from whom the SB was derived was unknown. For this reason, descriptive statistics regarding age were not performed for these 2 SGTN subtypes.

Φ In terms of differentiating neoplasms of the submandibular, sublingual, and minor salivary glands: one of the submandibular gland neoplasms had extended into local minor salivary glands (we have categorized this sample as a primary submandibular gland neoplasm in our tabulations, however). We did not have any sublingual gland tumors in our study cohort.

Ψ Tumor size data represents the largest dimension measured (i.e. length, width, or height) for each sample. Tumor dimension data was missing for 4 AcCCs, all AdCCs (3), 1 MASC, 13 MECs, 1 PA, and all SBs (1).

-- = Data is not known or is inappropriate to calculate given the sample size.

Expression of MUC4 and Other Markers

All 31 MECs expressed MUC4 (Figs. 4-6). Over half of the MECs (17, 54.84%) had a MUC4 expression score of ++++ (the maximum, meaning that >90% cells of the cells observed expressed MUC4). Additionally, 8 of the MECs (25.81%) had an expression score of +++ (meaning that 51-90% of the cells observed expressed MUC4), 4 of the MECs (12.90%) had an expression score of ++ (meaning that 11-50% of the cells observed expressed MUC4), and 2 of the MECs (6.45%) had an expression score of + (meaning that 1-10% of the cells observed expressed MUC4). Thus, 25 of the MECs (80.65%) had an expression score of +++ or greater.

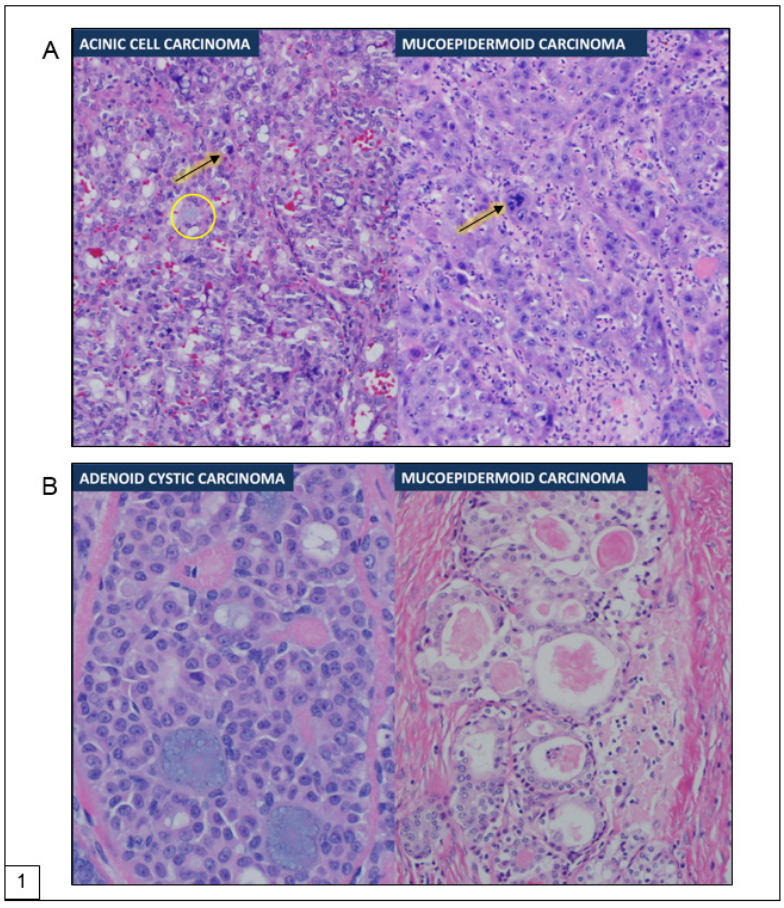

Figure 1: Tumor heterogeneity and morpho-histological overlaps: MEC vs. AcCC and AdCC. (**A**): AcCC and MEC can be hard to distinguish based on H&E stain alone. As seen here, AcCCs can have mucus cells (see yellow **circle**). In both we see mitotic figures and nuclear pleomorphism (see **arrows**). (B): MEC can also mimic the appearance of AdCC. *AcCC = acinic cell carcinoma*, *MEC = mucoepidermoid carcinoma*, *H&E = hematoxylin and eosin*, *AdCC = adenoic cystic carcinoma*.

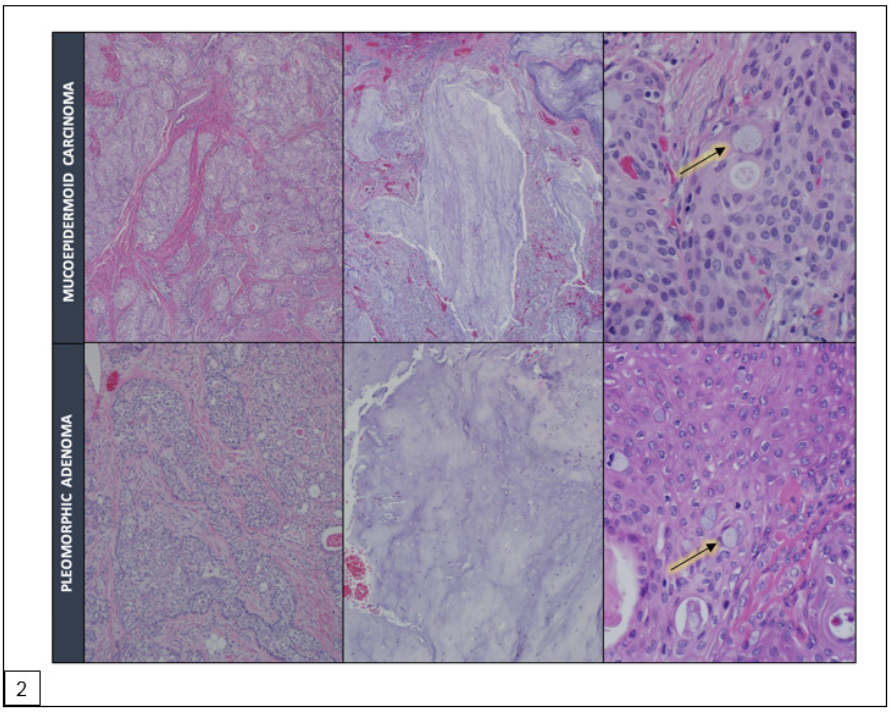

Figure 2: Tumor heterogeneity and morpho-histological overlaps: MEC vs. PA. Three different MEC samples are shown (in the top row) here, each with a partnered PA (in the second row) that appears morphologically similar. Because MEC is made up of 3 main cell types, and can display various patterns, it can mimic PA (which is, itself, polyphenotypic). This can be an issue particularly with small biopsies. Mucus cells can be seen in both subtypes (see **arrows**). *MEC = mucoepidermoid carcinoma*, *PA = pleomorphic adenoma.*

A minority of the PAs (2, 6.67%) showed patchy, cytoplasmic, weakly positive staining (i.e. expression score of +), while the rest of the PAs (28, 93.33%) were negative for MUC4 (Fig. 7). Among the AcCCs, 1 was negative (16.67%), and the rest (5, 83.33%) were weakly-positive (score: +) with patchy cytoplasmic staining (Fig. 8). One of the AdCCs tested negative for MUC4 (33.33%), while the other 2 (66.67%) were weakly positive (score: +) with patchy cytoplasmic staining (Fig. 9). The MASCs were all positive for MUC4 with scores of ≥ +++. MASCs were also: strongly positive for mammaglobin, strongly positive for GCDFP15, strongly positive for S100, weakly to moderately positive for Gata-3, negative for p63, and moderately positive for SOX-10 (Fig. 10). In terms of FISH analysis, one MASC was positive for the ETV6-NTRK3 translocation, and one MEC was positive for the MECT1-MAML2 translocation (FISH images not shown).

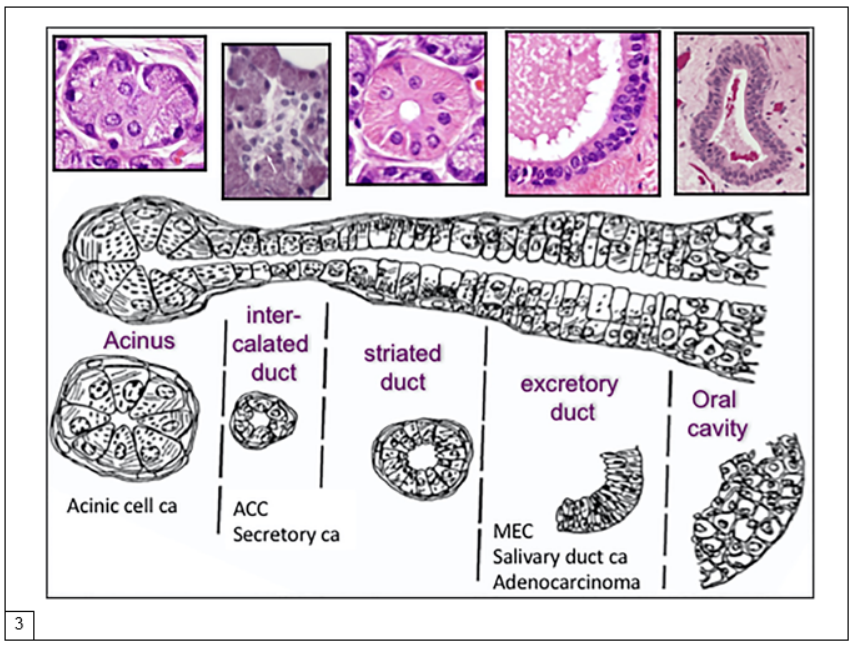

Figure 3: "Schematic representation of the salivary gland with histological images and examples of the most common malignancies arising in each structure of the acini." Image is reproduced from: Schvartsman et al. (2019) *Head Neck*. *Ca = carcinoma*, *ACC = adenoid cystic carcinoma*, *MEC = mucoepidermoid carcinoma*.

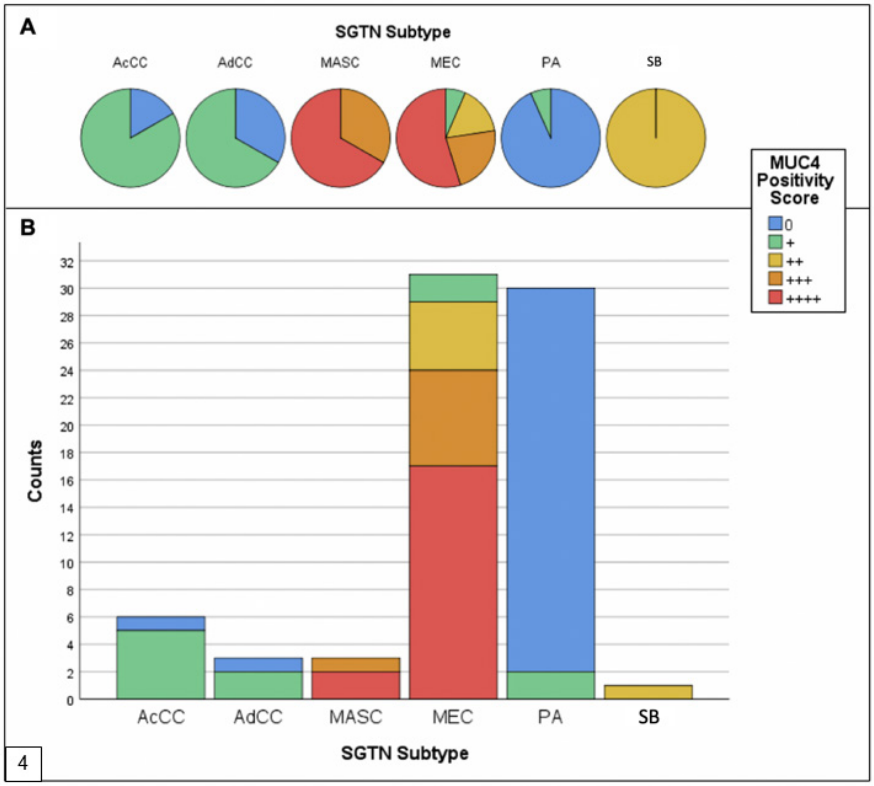

Figure 4: MUC4 Expression Profile for SGTN Subtypes. Score criteria was: 0% MUC4-positive cells observed = 0, 1-10% MUC4-positive cells observed = +, 11-50% MUC4-positive cell observed = ++, 51-90% MUC4-positive cell observed = +++, and >90% MUC4-positive cell observed = ++++. Colors represent MUC4-positivity score categories (see legend within figure). (**A**): Proportions of MUC4-positivity score categories for each SGTN subtype. (**B**): Counts of MUC4-positivity score categories for each SGTN subtype. Figures were created using IBM SPSS Statistics 26. *MUC4 = Mucin-4*, *SGTN = salivary gland-type neoplasm*, *AcCC = acinic cell carcinoma*, *AdCC = adenoid cystic carcinoma*, *MASC = mammary analogue secretory carcinoma*, *MEC = mucoepidermoid carcinoma*, *PA = pleomorphic adenoma*, *SB = sialoblastoma*.

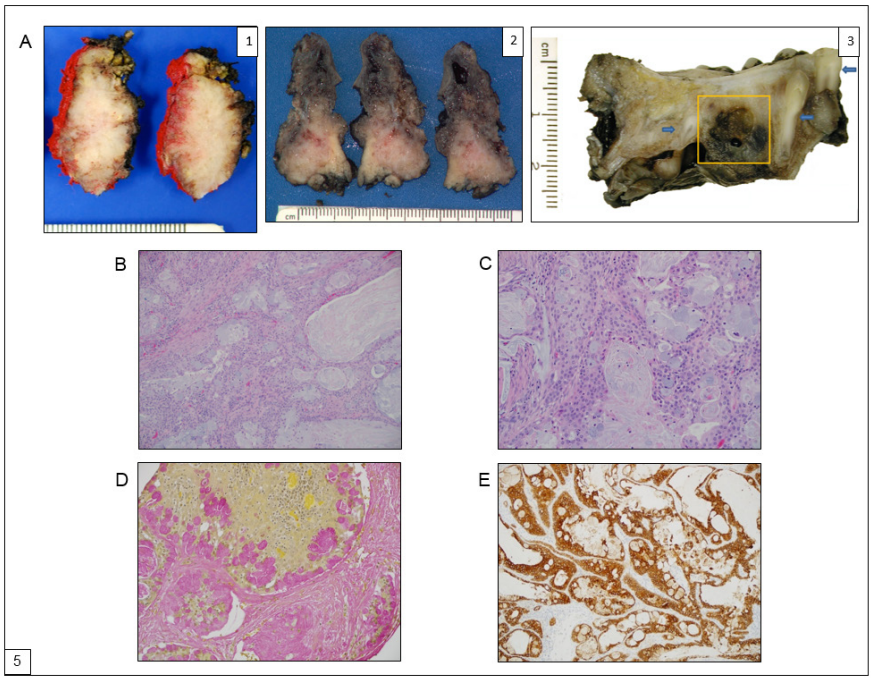

Figure 5: MEC gross and histopathology. (**A**): Representative gross images of MECs. (1) A MEC that presented as a left neck mass in a 10 y/o boy. MECs are typically 1-6cm in size, well-circumscribed, and un-encapsulated. They have a tan-white to grey to pink cut surface, as shown here. Higher grade MECs may have ill-defined margins and be fixed to skin or soft tissue. (2) MEC of the parotid gland. As shown here, MECs may have small cystic spaces and areas of hemorrhage. (3) A MEC that presented as a maxillary bone tumor in a 10 y/o girl. As shown here, MECs can be centered in bone and appear as ill-defined cavitary lesions. Here we see hemorrhage (see yellow box), and roots of teeth (see blue arrows) embedded in the maxilla. (B): H&E stained MEC at low magnification showing abundant mucin production (40X magnification). (C): Higher magnification shows the typical cell types

seen in MEC (squamoid, intermediate, and mucus cells; 200X). (D): Mucicarmine stain highlights abundant mucin production and mucus cells (200X). (E): The tumor shows strong and diffuse cytoplasmic MUC4-positivity by immunohistochemistry (100X). *MEC = mucoepidermoid carcinoma*, y/o = *year-old*, *H&E = hematoxylin and eosin*, *MUC4 = Mucin-4*.

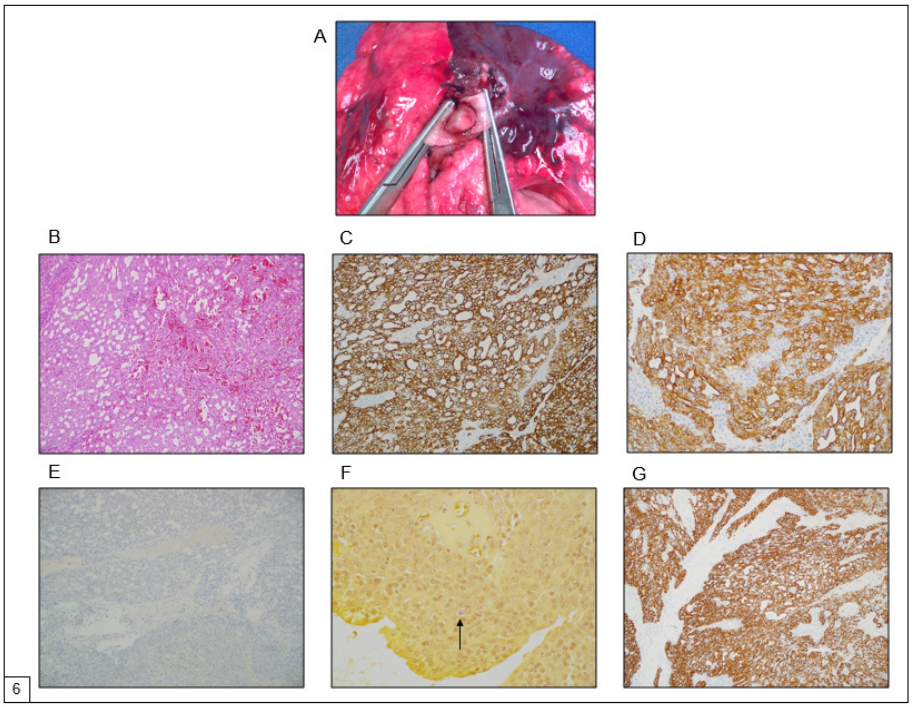

Figure 6: Non-salivary gland (bronchial) MEC gross and histopathology. (***A***): Gross image of a bronchial MEC in situ. (B): A microcystic growth pattern is seen on H&E stain (100X). (***C&D***): Strong and diffuse cytoplasmic staining for AE1/AE3 and CK7, respectively (both 100X). (***E***): Tumor is entirely DOG1-negative, making the diagnosis of AcCC unlikely (100X). (F): Mucicarmine highlights a rare focus of mucin production indicated by the **black arrow** (100X). (***G***): MUC4 immunohistochemical staining is strong and diffuse (100X). *MEC = mucoepidermoid carcinoma*, *AcCC = acinic cell carcinoma*, *MUC4 = Mucin-4*.

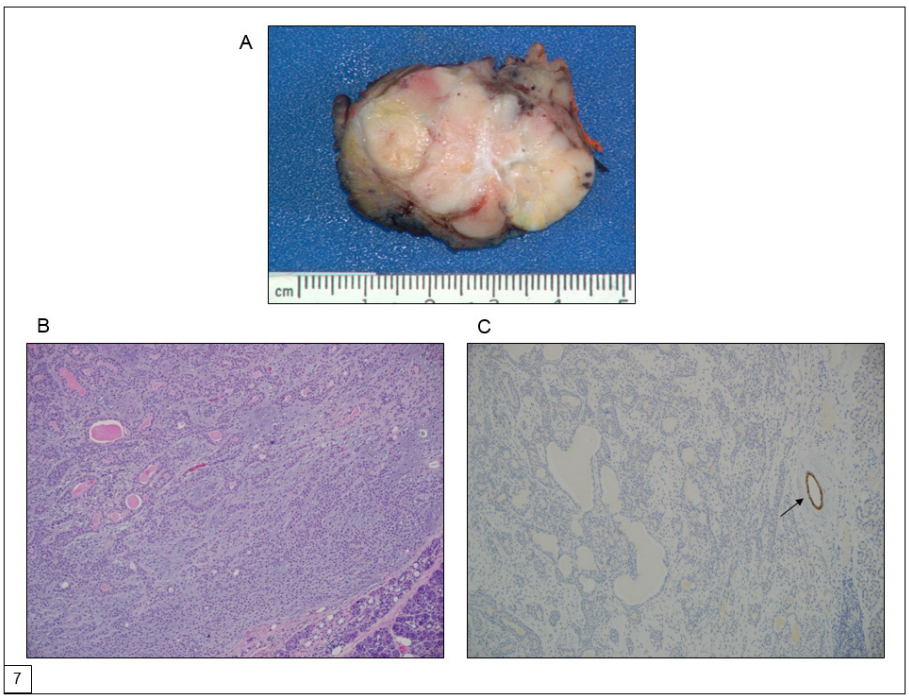

Figure 7: PA gross and histopathology. (***A***): Grossly, a PA often appears as a well-circumscribed mass with a bosselated surface. It has a rubbery, resilient consistency. Small extensions of the tumor often protrude into adjacent tissue. Islands of cartilage or bone may be present. (***B***): PA showing the classic biphasic histology with nests, cords, and glandular arrangements of neoplastic epithelial cells in the background of a chondromyxoid stroma. Residual uninvolved salivary gland parenchyma is noted in the bottom right corner (100X). (***C***): Tumor cells are entirely MUC4-negative; however, a residual salivary duct is highlighted by the immunostain (indicated by black arrow; 100X). *PA = pleomorphic adenoma*, *MUC4 = Mucin-4*.

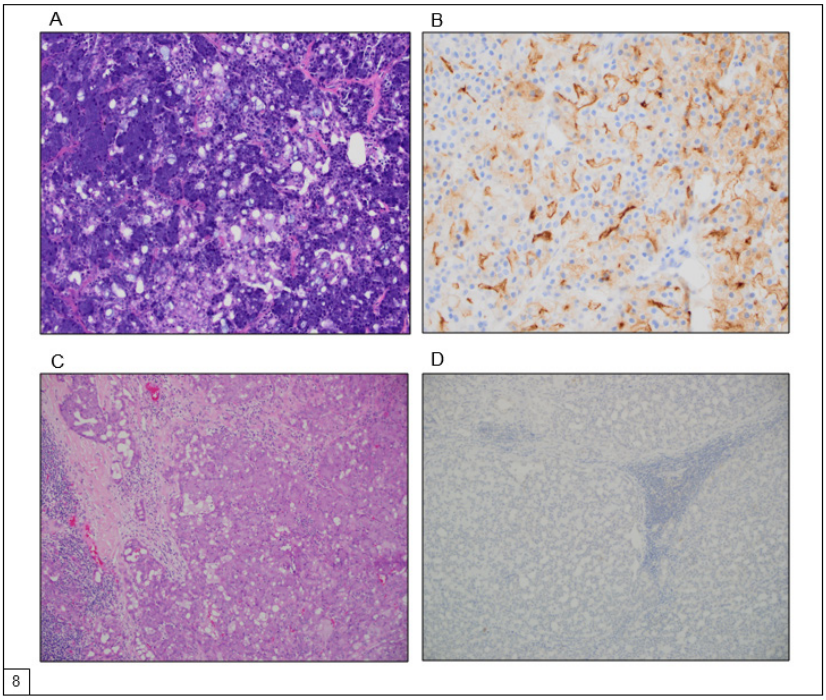

Figure 8: AcCC Histopathology. (***A***): Acinic cell carcinoma (AcCC) characterized by large neoplastic cells with abundant basophilic cytoplasm infiltrating the parotid gland. Non-neoplastic salivary ducts can be seen between nests of malignant cells (200X). (***B***): The tumor is focally positive for DOG-1, a marker that is usually positive in AcCC (***C***): Another AcCC composed of solid nodules of tumor cells separated by dense, fibrotic bands of connective tissue. The neoplastic cells are large with small nuclei and voluminous, basophilic cytoplasm. (***D***): Neoplastic cells are Mucin-4-negative (100X).

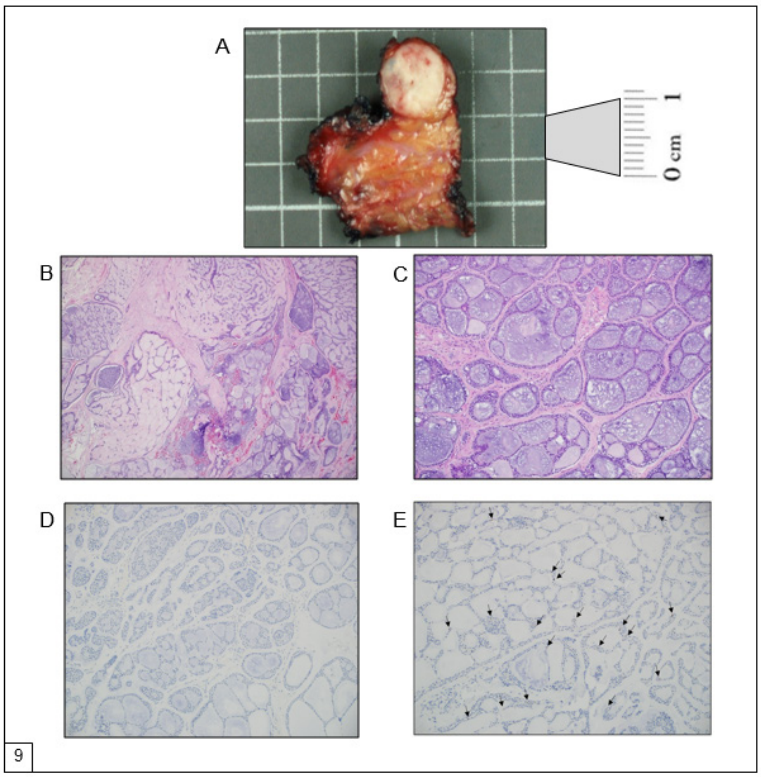

Figure 9: AdCC gross and histopathology. (***A***): Gross appearance of AdCC. This type of tumor has tendency for perineural spread. (***B***): A mixture of histologic patterns may be seen in AdCCs (e.g. solid, tubular, cribriform). Adenoid cystic carcinoma showing cords of tumor cells in a somewhat nodular, fibrotic background, and associated with abundant mucin production (40X). (***C***): Higher magnification showing a microcystic pattern with a rim of malignant cells surrounding abundant basophilic mucin (100X). (***D***): The tumor is MUC4-negative (100X). (***E***): A second AdCC with scattered, single MUC4-positive neoplastic cells (indicated by black arrows; 100X). *AdCC = adenoid cystic carcinoma, MUC4 = Mucin-4. Ruler image: https://images-na.ssl-images-amazon.com/images/I/51mdrK0XkQL._SY355_.png*.

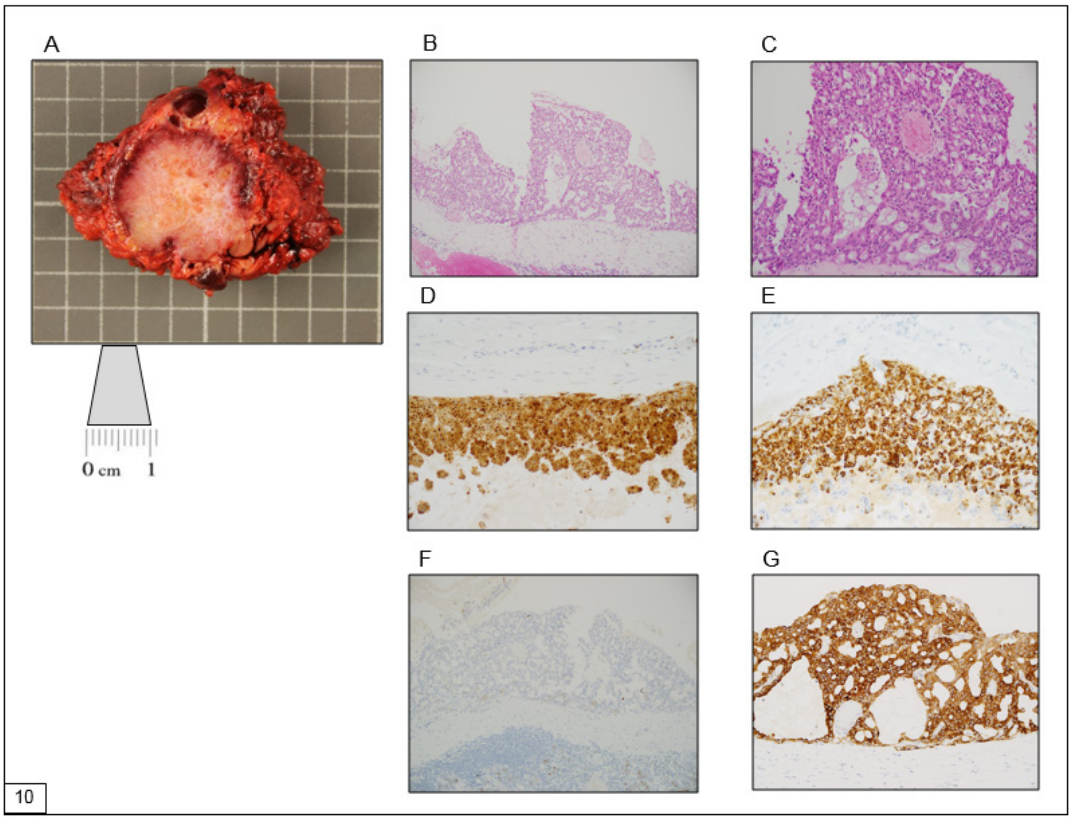

Figure 10: MASC gross and histopathology. (***A***): Gross photograph of a MASC showing a well-circumscribed, un-encapsulated, solitary, solid mass with attached soft tissue, including fat. Grossly, MASCs often display a brown or grey cut surface, and have a rubbery consistency. MASCs are variable in terms of size (0.2-5.5cm typically). (***B***): Low magnification of a cystic tumor shows a cellular neoplasm with a microcystic arrangement of tumor cells (100X). (***C***): At higher magnification, the tumor is seen to be composed of cells with abundant eosinophilic cytoplasm. Pink secretory material is present within the microcysts (200X). (***D&E***): The tumor is strongly and diffusely positive for S100 and mammaglobin, respectively (200X). (***F***): DOG-1 is negative. (***G***): MUC4 is strongly positive. *MASC = mammary analogue secretory carcinoma, MUC4 = Mucin-4. Ruler image: https://images-na.ssl-images-amazon.com/images/I/51mdrK0XkQL._SY355_.png*.

As a marker for diagnosing pediatric MEC, MUC4-positivity has a sensitivity of 100%, a specificity of 79.41%, a positive predictive value (PPV) of 75.86%, and a negative predictive value (NPV) of 100% (Table 2). As a marker for pediatric PA, MUC4-negativity has a sensitivity and PPV of 93.33%, and a specificity and NPV of 95.45%. A Freeman-Halton extension of Fisher's Exact Test comparing SGTN subtype to MUC4 expression score yielded a $\chi 2$ statistic of 85.283, indicating that the SGTN subtypes are statistically significantly different in their expression of MUC4 ($p < 0.001$). When MUC4-positivity score was converted from categorical to numeric data, and a Kruskal-Wallis test was performed (which is a non-parametric test and, therefore, makes no assumptions regarding normality of data), SGTN subtypes were found to be statistically significantly different within each expression score category except for +++ (in other words, significant differences were seen for categories: 0, +, ++, and ++++; $p < 0.005$). When pairwise comparisons (15 for each expression score category) were evaluated (with a Bonferroni-adjusted $\alpha A = 0.0033$), the following pairs were found to be statistically significantly different: MASC vs. PA ($p = 0.002$), MEC vs. PA ($p < 0.001$), and AcCC vs. PA ($p = 0.001$) for the "0" MUC4-expression category; MASC vs. AcCC ($p = 0.001$), MEC vs. AcCC ($p < 0.001$), and PA vs. AcCC ($p < 0.001$) for the "+" MUC4-expression category; AcCC vs. SB ($p = 0.001$), AdCC vs. SB ($p = 0.002$), MASC vs. SB ($p = 0.002$), PA vs. SB ($p < 0.001$), and MEC vs. SB ($p = 0.003$) for the "++" MUC4-expression category; and PA vs. MEC ($p < 0.001$) for the "++++" MUC4-expression category.

Table 2: MUC4 as a Diagnostic Marker

	Sensitivity	Specificity	PPV	NPV
$MUC4^+$ for MEC:	100%	79.41%	75.86%	100%
$MUC4^-$ for PA:	93.33%	95.45%	93.33%	95.45%

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for: *MUC4 = Mucin 4, MEC = mucoepidermoid carcinoma, PA = pleomorphic adenoma*.

Discussion

In terms of comparing the results of this study to what has been previously reported in the literature, we, too, observed that the parotid gland was the most common site from which SGTNs were excised (45.95%) [6]. Other studies have shown that poorer outcomes are seen in SGTNs that are high grade, have perineural invasion, and/or have extracapsular spread [8]. Most of our study's cases were low-grade, and had neither perineural invasion or metastases.

The fact that MUC4, as a marker for MEC, has a sensitivity (and NPV) of 100% indicates that it can effectively be used to rule in MEC in the differential diagnosis of pediatric SGTNs. It is worth noting that all MECs in our cohort were MUC4+, regardless of their tissue of origin (i.e. whether they were isolated from the parotid gland, bronchus, neck, palate/maxillary bone, etc.). The clinical utility of MUC4 as a diagnostic tool would be further enhanced when used in combination with other tests currently used to either diagnose MEC or rule out other tumor types (i.e. IHC for AE1/AE3, CK5/6, S100, mammaglobin, GCDFP-15 and p63, as well as FISH for the PLAG1/CTNNB1, MECT1/MAML2, and ETV6/NTRK3fusion genes) [21]. MUC4 may be especially useful in situations in which tumor samples are small (pediatric samples tend to be smaller than adult samples) or when samples are of insufficient quality for FISH/genetic analysis. It also appears that MUC4 expression may be a method to rule out PA, as 92.59% of PAs were MUC4-negative. Interestingly, MUC4 could serve as a tool to rule in/out PA, seeing as MUC4-negativity has a specificity and NPV of 95.45%. We have summarized the results of our study with those previously reported in the literature in a Supplementary Table [1, 21, 39].

Supplementary Table: Proposed IHC & FISH Profiles for Diagnosis of SGTNs

	AcCCs	AdCCs	MASCs	MECs	PAs	SBs
MUC4	--	--	Positive	Positive	--	Positive
Mucicarmine	Positive	--	Positive	Positive	--	--
Alcian Blue	Positive	--	--	Positive	--	--
DOG1	Positive	--	Positive	--	--	--
Mammaglobin	--	--	Positive	--	--	--
P63	Positive	Positive	--	Positive	Positive	Positive
S100	--	Positive	Positive	--	Positive	Positive
AE1/AE3	Positive	Positive	--	Positive	Positive	--
CK7	Positive	Positive	--	Positive	Positive	--
PLAG1, HMGA2	--	--	--	--	Positive	--
MECT1-MAML2	--	--	--	Positive	--	--
MYB-NFIB	Positive	--	--	--	--	--
ETV6-NTRK3	--	--	Positive	--	--	--

Though the SGTN subtypes were found to be statistically significantly different in their expression of MUC4 by $\chi 2$ analysis ($p < 0.001$), it must be noted that the sample sizes were not the same for each SGTN subtype (which would be ideal), and only 1 representative sample of SB was included. Because Kruskal-Wallis tests were statistically significant for MUC4-expression scores 0, +, ++, and ++++ ($p < 0.005$), it was appropriate to evaluate pairwise comparisons using a Bonferroni-adjusted αA. For the "0" MUC4-expression category, PA was found to be statistically significantly different from MASC, MEC, and AcCC ($p \leq 0.002$). For the "+" MUC4-expression category, AcCC was found to be statistically significantly different from MASC, MEC, and PA ($p \leq 0.001$). For the "++" MUC4-expression category, SB was found to be statistically significantly different from AcCC, AdCC, MASC, PA, and MEC ($p \leq 0.003$). For the "++++" MUC4-expression category, PA and MEC were found to be statistically significantly different ($p < 0.001$). Together, these data suggest that high MUC4-positivity is most significantly associated with MEC, and MUC4-negativity is most significantly associated with PA.

PAs and MECs together comprised 82.43% of our samples. This may simply be a reflection of the fact that PA and MEC are the most common benign and malignant SGTNs (respectively) seen in the pediatric population [7, 11]. Regardless, for the sake of statistical significance, in the future we hope to replicate these results with a larger total number of SGTN samples, and to equally represent each subtype in terms of sample size. This could be done using a large, publicly-available dataset like the National Cancer Institute's TCGA (The Cancer Genome Atlas Program).

Conclusions

Taken together, these results indicate that MUC4 can serve as an additional, powerful screening tool to diagnose the MEC subtype of pediatric SGTNs. To our knowledge, this is the first study to show the utility of MEC4 as a sensitive marker for pediatric MEC.

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