Journal of the American Society of Cytopathology (2019) 8, 157–164

Available online at www.sciencedirect.com

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journal homepage: www.jascyto.org/

ORIGINAL ARTICLE

Diagnostic utility of fluorescence in situ hybridization testing on cytology cell blocks for the definitive classification of salivary gland neoplasms

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Received 10 December 2018; received in revised form 15 January 2019; accepted 16 January 2019

KEYWORDS
Salivary gland;
Fine needle aspiration;
Fluorescence in situ hybridization;
FISH;
Cytology cell block

Introduction Fine needle aspiration biopsy (FNAB) is a minimally invasive modality to evaluate salivary gland neoplasms and help guide clinical management. However, significant overlap in the cytomorphology findings among salivary gland neoplasms often renders the definitive diagnosis challenging. Recently, a number of benign and malignant salivary gland tumors have been characterized by specific chromosomal aberrations detectable using fluorescence in situ hybridization (FISH) testing. In the present study, we evaluated the role of FISH testing performed on cytology cell blocks in the diagnosis of salivary gland neoplasms by FNAB.

Materials and methods The data from 57 cases of primary salivary gland tumors diagnosed using FNAB at our institution and sent for ancillary FISH testing between 2012 and 2017 were retrospectively reviewed. The FISH studies were performed on cytology cell blocks, and break-apart probes were used to detect characteristic gene rearrangements for *PLAG1*, *MYB*, *MAML2*, and *ETV6* for pleomorphic adenoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, and secretory carcinoma (mammary analogue secretory carcinoma), respectively. Of the 57 cases sent for FISH testing, 6 were excluded because of FISH analysis failure (insufficient cell block cellularity).

Results Of the 51 cases included in the analysis, 15 samples were successfully subclassified after FISH testing, and 10 of these 15 FISH-positive cases were diagnostically confirmed by the surgical pathology review of excision material. Forty cases overall had undergone subsequent excision with the histopathologic follow-up diagnosis available, and all subclassified cases had concordant FNAB, FISH, and excision diagnoses.

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https://doi.org/10.1016/j.jasc.2019.01.006

Conclusions FISH testing performed on cytology cell blocks is a useful adjunct in establishing the diagnosis of salivary gland neoplasms by FNAB.

Introduction

Fine needle aspiration biopsy (FNAB) has emerged as a first-line, minimally invasive sampling modality to evaluate salivary gland neoplasms. FNAB is a relatively safe and reliable diagnostic technique with excellent sensitivity (range, 86%-100%) and specificity (range, 90%-100%) for the diagnosis of salivary gland lesions.[1-11] One of the significant advantages of FNAB is its ability to differentiate benign from malignant salivary gland tumors with high accuracy (range, 81%-100%), helping to guide the treatment of these patients.[1-11] Despite these advantages, FNAB is known to have highly variable accuracy (range, 48%-94%) for the definitive classification of salivary gland tumors.[1-11]

Moreover, FNAB of the salivary gland is considered to be one of the most diagnostically challenging areas of cytopathology. Primary salivary gland neoplasms represent a variety of distinct entities, which demonstrate both considerable intratumoral heterogeneity and cytomorphologic overlap.[1] The application of immunohistochemistry to the diagnosis of salivary gland tumors also has its disadvantages, including nonspecific and overlapping immunohistologic profiles.[12] In the absence of ancillary testing, descriptive diagnoses have been frequently rendered and have often encompassed a broad differential diagnosis of reactive, benign, and malignant processes.[1]

Recent molecular advances have revolutionized our ability to overcome these inherent limitations to diagnose salivary gland tumors with precision.[11] The discovery of characteristic translocations and resulting fusion oncogenes in a number of benign and malignant salivary gland tumors have allowed us to classify these tumors cytogenetically using fluorescence in situ hybridization (FISH).[11] FISH analysis performed on FNAB samples has emerged as a cornerstone in ancillary diagnostic testing. Major molecular alterations routinely detected by FISH testing include gene rearrangements for *PLAG1*,[13-16] *MYB*,[17-20] *MAML2*,[21-25] and *ETV6*,[26-28] which serve as diagnostic markers for pleomorphic adenoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, and secretory carcinoma (mammary analogue secretory carcinoma), respectively. Although a subset of these genetic aberrations has been described for tumors of other organ systems, they appear to be specific within the setting of salivary gland tumors and, thus, provide important diagnostic value.[17,29]

Previous studies have established the suitability and accuracy of ancillary molecular testing on FNAB specimens, including smears and cell block preparations.[30-32] However, to the best of our knowledge, the diagnostic utility of ancillary FISH testing to subclassify salivary gland neoplasms by FNAB has not been examined. The aim of the present study was to determine whether FISH testing performed on cytology cell blocks improves our ability to definitively diagnose salivary gland neoplasms by FNAB.

Materials and methods

During a 5-year period from January 2012 to December 2017, 57 cases of primary salivary gland tumors of the major salivary glands (parotid, submandibular, and sublingual glands) were diagnosed by FNAB at Stanford Hospital and sent for ancillary FISH testing. The indication for FISH analysis was the subclassification of cases with an indeterminate cytologic diagnosis. The cases were identified by querying the PowerPath computer database for major salivary gland cytology cases with FISH orders. The following search terms were used: "parotid," "submandibular," "sublingual," and "salivary." Cases were excluded if the final cytology report had rendered a diagnosis of metastatic malignancy, hematolymphoid malignancy, or a non-neoplastic or reactive process or if the FISH analysis had failed. The clinical information, cytologic diagnoses, and FISH results were retrospectively reviewed. The available histopathologic classification was reviewed and compared with the final cytologic diagnosis for subsequently resected tumors.

Cytologic evaluation

FNAB procedures were performed by either a cytopathologist using palpation or ultrasound guidance or a radiologist using computed tomography or ultrasound guidance. Each case had had a rapid on-site adequacy evaluation performed by an experienced cytotechnologist or board-certified cytopathologist to ensure appropriate lesional tissue sampling for diagnosis and to triage material for ancillary testing. The number of FNAB passes varied per procedure and was dependent on patient safety and tolerance and the ability to obtain appropriate material. In general, 3 to 5 FNAB passes were performed. The adequacy assessment involved examination of Diff-Quik (Protocol Hema 3; Fisher Scientific, Kalamazoo, MI)—stained smears from the FNAB passes. In most cases, 95% alcohol-fixed smears were prepared and formalin-fixed material was collected for cell block preparation for ancillary studies, including potential FISH testing.

A board-certified cytopathologist reviewed all available diagnostic material, including Diff-Quik—stained smears, Papanicolaou-stained smears, and hematoxylin and eosin—stained sections of the formalin-fixed cell block. The cytomorphologic features and any additional

immunohistochemical findings were interpreted to render a cytologic diagnosis before the results of the FISH studies. Immunohistochemical testing was used in conjunction with cytomorphology to aid in the diagnostic workup but was not used to triage cases sent for FISH testing. Immunohistochemical stains specific to the FISH probe targets, including *PLAG1* and *MYB*, were not available at our institution during the study period and could not be used diagnose or triage cases sent for FISH analysis. Before the FISH studies, immunohistochemical testing for *HER2* and androgen receptor was routinely performed to evaluate cases of suspected salivary duct carcinoma. In addition, an immunohistochemical panel of S100, mammaglobin, and DOG1 was performed to evaluate cases of suspected secretory carcinoma (mammary analogue secretory carcinoma) and acinic cell carcinoma. The results of the FISH studies were reported by the cytogenetics laboratory within 1 to 3 weeks, and the cytopathologist correlated the FISH results with the cytomorphologic and immunophenotypic findings. In some cases, a new diagnosis was rendered. Any reclassification of the final diagnosis owing to the FISH results was issued in an addendum or amended report.

Ancillary FISH studies

FISH studies were performed on paraffin-embedded, formalin-fixed thin-tissue cell block sections pretreated by a standard protocol using the VP2000 slide pretreatment instrument (Abbott Molecular). In brief, the slides were deparaffinized with CitroSolv (Fisher Scientific), digested with CytoZyme Stabilized Pepsin (SciGene), pretreated with a sodium thiocyanate solution at 80°C (VP2000 Pretreatment Solution; Abbott Molecular), refixed in 10% buffered formalin, and dehydrated in an ethanol series. Dried dehydrated slides were denatured using a Vysis HYBrite instrument at 80°C for 6 minutes and hybridized for 48 hours at 37°C with the PLAG1, MAML2, or ETV6 break-apart probes (Empire Genomics) or the MYB break-apart probe (ZytoVision). The slides were washed with 2 × SSC/0.3% NP-40 at 73°C for 2 minutes, counterstained with DAPI and analyzed with an Olympus BX51 microscope equipped with an 100× oil immersion objective, appropriate fluorescent filters, and CytoVision imaging software (Leica Biosystems). Two hundred interphase nuclei were analyzed for each specimen. Nuclei demonstrating 5′/3′ signal separation >1 to 2 signal widths or extra-individual 5′ or 3′ signals without a colocalized partner signal consistent with variant rearrangement were considered to have a positive signal pattern. The positive signal pattern frequencies were compared to empirically established detection thresholds to determine positive and negative results (Fig. 1).

Results

In our cohort, the average patient age was 63.2 years (range, 11-87 years), with 29 men (56.9%) and 22 women (43.1%). The most common tumor site was the parotid gland, which constituted 46 cases (90.2%), followed by 3 submandibular gland cases (5.9%) and 2 sublingual gland cases (3.9%). The final diagnoses included pleomorphic adenoma (7 cases; 13.7%), adenoid cystic carcinoma (5 cases; 9.8%), mucoepidermoid carcinoma (4 cases; 7.8%), and secretory carcinoma (mammary analogue secretory carcinoma; 4 cases; 7.8%), which are known to harbor characteristic gene rearrangements detectable by FISH. Other tumor types included basal cell adenoma, Warthin tumor, lymphoepithelial cyst/cystic lymphadenoma, acinic cell carcinoma, epithelial myoepithelial carcinoma, carcinoma ex pleomorphic adenoma, and poorly differentiated or undifferentiated carcinoma (Table 1). Confirmatory histopathologic follow-up data were available for 40 cases (78.4%).

Of the 57 primary major salivary gland neoplasm cases diagnosed by FNAB with FISH studies performed, 6 (10.5%) were excluded because of a failed FISH analysis secondary to insufficient cell block cellularity (Fig. 2). Of the remaining 51 cases, 15 (26.3%) were positive by FISH

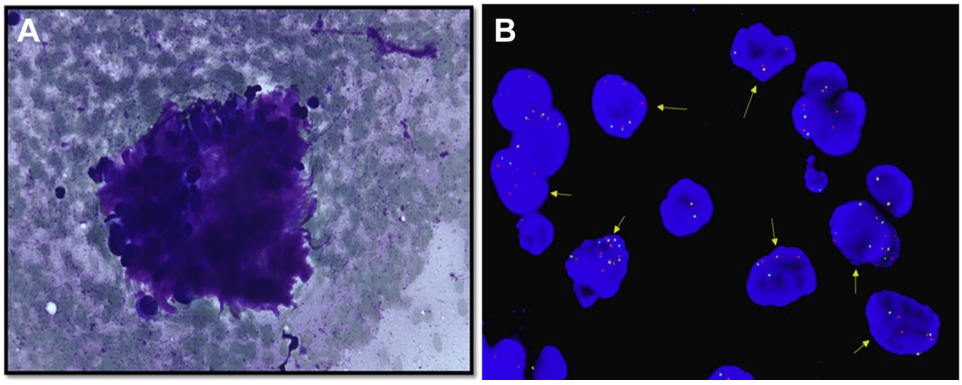

Figure 1 Cytology and fluorescence in situ hybridization (FISH) analysis in a representative patient with a pleomorphic adenoma. (A) Examination of the cytologic smear demonstrated the characteristic bland epithelial cells embedded in a fibrillary chondromyxoid stroma (Diff-Quik stain, original magnification ×400). (B) FISH analysis with *PLAG1* break-apart probe showed the presence of a *PLAG1* gene rearrangement. Nuclei (arrows) demonstrating 5′/3′ signal separation, consistent with a positive signal pattern.

Table 1 Distribution of fluorescence in situ hybridization results for specific histopathologic diagnoses.

Final diagnosis (n)	FISH performed (n)	FISH specimen tested (FNA; excision)	FISH gene rearrangement or amplification[a] (detected; not detected)	Utility of FISH performed on FNA specimen for salivary gland diagnosis: brief comments based on practice patterns
Pleomorphic adenoma (7)	*PLAG1* (4)	3; 1	3; 1	*PLAG1* FISH performed on FNA specimen to rule in pleomorphic adenoma (3/7 cases; 43%); other FISH tests ordered to exclude other neoplasms (6/10 cases; 60%)
	MYB (2)	2; 0	0; 2	
	MAML2 (3)	3; 0	0; 3	
	ETV6 (1)	1; 0	0; 1	
Adenoid cystic carcinoma (5)	*MYB* (5)	5; 0	5; 0	*MYB* FISH performed on FNA specimen to rule in suspected adenoid cystic carcinoma (5/5 cases; 100%)
	HER2 (1)	0; 1	0; 1	
Mucoepidermoid carcinoma (4)	*MAML2* (5)	4; 1	3; 2	*MAML2* FISH performed on FNA specimen to rule in suspected mucoepidermoid carcinoma (4/4 cases; 100%)
	MYB (2)	1; 1	0; 2	
Mammary analogue secretory carcinoma (4)	*ETV6* (4)	3; 1	4; 0	*ETV6* FISH performed on FNA specimen to rule in mammary analogue secretory carcinoma (3/4 cases; 75%)
Salivary duct carcinoma (6) and Carcinoma ex pleomorphic adenoma (3)	*HER2* (8)	1; 7	5; 1	*HER2* FISH performed on excision specimens to support a diagnosis of salivary duct carcinoma (9/9 cases; 100%)
	MAML2 (1)	1; 0	0; 1	
Basaloid salivary gland lesion (5)	*MYB* (5)	5; 0	0; 5	In most cases (4/5; 80%), excision was not performed because a benign diagnosis was rendered by FNA when *MYB* FISH results provided no support for adenoid cystic carcinoma
	PLAG1 (3)	3; 0	0; 3	
Acinic cell carcinoma (4)	*ETV6* (5)	2; 3	0; 5	*ETV6* and *MAML2* performed on FNA and excision cases to exclude other neoplasms (4/4 cases; 100%)
	MAML2 (2)	1; 1	0; 5	
Other diagnoses[b] (13)	*MAML2* (5)	2; 3	0; 5	FISH performed frequently on FNA to exclude certain diagnoses and support the final diagnosis[b]
	MYB (4)	4; 0	0; 4	
	ETV6 (2)	2; 0	0; 5	
	PLAG1 (1)	1; 0	0; 5	
	EWSR1 (1)	1; 0	0; 5	

Abbreviations: FISH, fluorescence in situ hybridization; FNA, fine needle aspiration.
Sums might not equal totals because multiple FISH tests could have been ordered for 1 case or FISH could have been ordered for both FNA biopsy and excision specimens.
[a]Gene rearrangement detection for *ETV6*, *MAML2*, *MYB*, *EWSR1*, and *PLAG1* or gene amplification detection for *HER2*.
[b]Basal cell adenoma (n = 2), basal cell carcinoma (n = 1), epithelial myoepithelial carcinoma (n = 2), low grade salivary gland carcinoma (n = 1), high-grade carcinoma (n = 2), cystic lymphadenoma (n = 1), myoepithelial carcinoma (n = 1), undifferentiated pleomorphic sarcoma (n = 1), lymphoepithelial cyst (n = 1), and Warthin tumor (n = 1).

and 36 (63.2%) were negative by FISH. Of the 51 cases included in the analysis, 15 (29.4%) were successfully subclassified after FISH testing, 10 (19.6%) of which were confirmed by histopathologic diagnosis on follow-up surgical pathology excision (Fig. 3). All definitively subclassified cases with available histopathologic follow-up data available had concordant cytologic diagnoses and FISH results. Of the 15 cases successfully subclassified by FISH analysis, 3 were pleomorphic adenomas demonstrating *PLAG1* gene rearrangements, 5 were adenoid cystic carcinomas demonstrating *MYB* gene rearrangements, 3 were mucoepidermoid carcinomas demonstrating *MAML2* gene rearrangements, and 4 were secretory carcinomas (mammary analogue secretory carcinomas) demonstrating *ETV6* gene rearrangements. One case of pleomorphic adenoma confirmed by histopathologic examination after excision was negative for a *PLAG1* gene rearrangement. Two cases of mucoepidermoid carcinoma confirmed by histopathologic examination showed no detectable *MAML2* gene rearrangements.

In the subset of cases with a histopathologic classification available, all subsequently resected tumors with no known defining chromosomal aberrations were also negative using FISH. These diagnoses included basal cell adenoma, Warthin tumor, lymphoepithelial cyst/cystic lymphadenoma, acinic cell carcinoma, epithelial myoepithelial carcinoma, carcinoma ex pleomorphic adenoma, and poorly differentiated or undifferentiated carcinoma. The cytohistologic

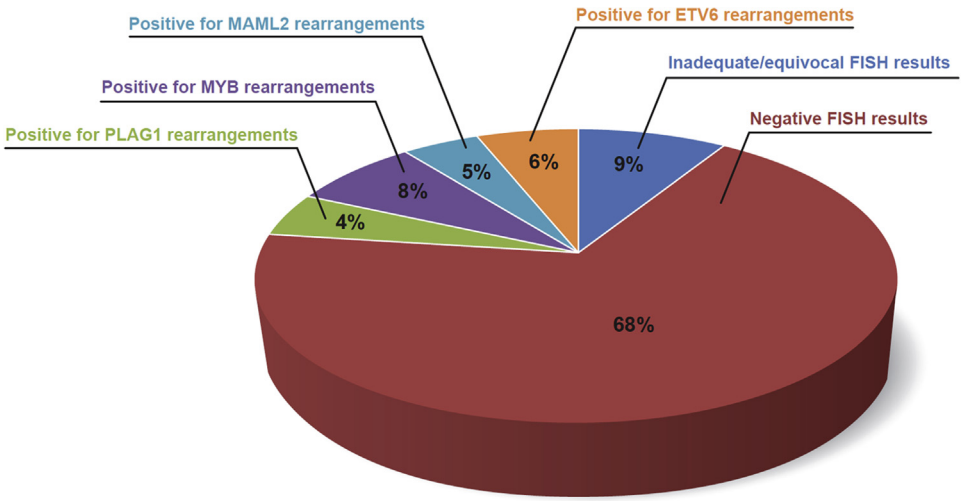

Figure 2 A significant proportion of cases included in the analysis were successfully subclassified after fluorescence in situ hybridization (FISH) testing.

correlation before FISH testing revealed that the cytologic diagnosis definitively subtyped the tumor in 8 cases (20%), favored the subtype in 10 (25%), provided the subtype in the differential diagnosis in 17 (42.5%), and was discordant in 5 cases (12.5%; Table 2).

Discussion

FNAB of salivary gland tumors is an effective and minimally invasive technique with excellent sensitivity and specificity.[1-11] FNAB can accurately distinguish between benign and malignant salivary gland neoplasms but is not well-suited for definitive subtyping of these tumors.[1-11] Moreover, the diagnosis of salivary gland tumors by cytology alone can be quite challenging, owing to the diversity of these tumors and their overlapping cytomorphologic and immunophenotypic features.[1-11] Recent molecular discoveries have characterized a number of specific translocations and fusion oncogenes in benign and malignant salivary gland tumors, allowing for ancillary FISH testing to optimize our ability to establish a diagnosis on FNAB specimens.[1-11] Cytology specimens are inherently enriched for neoplastic cells and are ideal for FISH applications.[30-32] FISH testing can also be performed on an array of cytologic preparations, including formalin-fixed paraffin-embedded cell block sections, direct unstained smears, destained Diff-Quik–stained slides, destained Papanicolaou-stained slides, and cytospin preparations, among others.[30-33] However, FISH testing on cytology specimens has certain preanalytic limitations, including low tumor cellularity, nonviable tumor sampling, contamination by non-neoplastic tissue (including inflammatory cells and peripheral blood), and sampling error, which can yield false-negative results.[30-32] Previous studies have reported on the important diagnostic

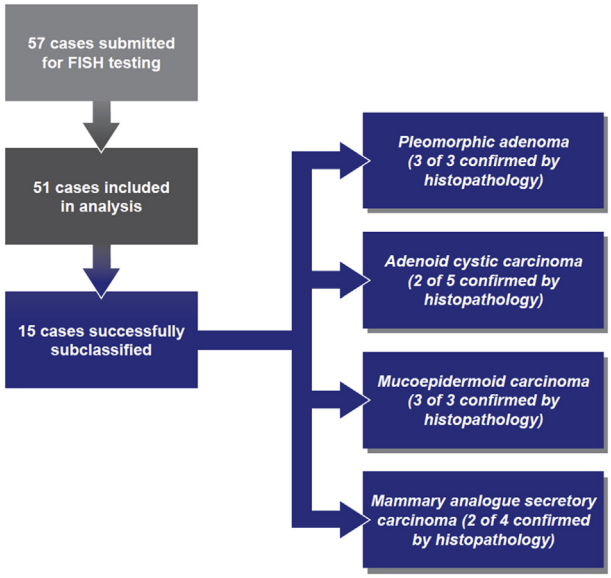

Figure 3 Of the 51 cases included in the analysis, 15 (29.4%) were successfully subclassified after fluorescence in situ hybridization (FISH) testing, 10 (19.6%) of which were confirmed by histopathologic diagnosis on follow-up surgical pathology excision. All definitively subclassified cases with available histopathologic follow-up data had concordant cytologic diagnoses and FISH results.

Table 2 Cytohistologic correlation of cytology cases sent for FISH testing.

Cytomorphologic diagnosis before subtyping by FISH result	Cases (n; % of total)
Definitive subtype	8 (20)
Favored subtype	10 (25)
Subtype in differential diagnosis	17 (43)
Subtype discordant	5 (13)

Abbreviation: FISH, fluorescence in situ hybridization.

role of ancillary molecular testing on FNAB specimens.[30-32] However, to the best of our knowledge, the utility of FISH testing performed on FNAB samples for the definitive classification of salivary gland neoplasms has not yet been investigated.

In our study, we retrospectively analyzed 57 cases of primary salivary gland neoplasms diagnosed by FNAB and sent for FISH testing from our institution from 2012 to 2017. FISH studies were performed on cytology cell blocks, and separation probes were used to detect the characteristic gene rearrangements for *PLAG1*, *MYB*, *MAML2*, and *ETV6* for pleomorphic adenoma, adenoid cystic carcinoma, mucoepidermoid carcinoma, and secretory carcinoma (mammary analogue secretory carcinoma), respectively. Of the 51 cases, 15 (26.3%) were positive by FISH and 36 (63.2%) were negative by FISH. Of the 51 cases included in the analysis, 15 (29.4%) were successfully subclassified after FISH testing. Moreover, all successfully subclassified cases with surgical pathology follow-up data available had concordant histopathologic and FISH results. Our study found that FISH testing performed on cytology cell blocks is a useful adjunct in establishing the diagnosis of salivary gland neoplasms by FNAB for cases with indeterminate cytologic findings.

Overall, we were able to subclassify nearly one third of our salivary gland cases with an indeterminate cytologic diagnosis, obviating the need for repeat sampling. Complete surgical resection is currently the mainstay treatment for most benign and malignant salivary gland tumors.[34-37] However, superficial or conservative parotidectomy could be indicated for patients with benign tumors or lower grade malignant tumors. Total parotidectomy with possible facial nerve and/or neck dissection and adjuvant radiation therapy should be performed for high-grade tumors or deep malignant tumors.[34-37] FISH studies might be especially crucial for preoperative planning in cases in which both benign and malignant entities cannot be entirely excluded.

In terms of the performance characteristics, the limited sensitivity and specificity of these FISH assays for the diagnosis of salivary gland neoplasms has been well-established.[11] In our series, FISH was negative for gene rearrangements in 36 cases (63.2%), which represented most of our FISH results. However, in the 78% of cases with histopathologic follow-up data available, all tumors lacking defining gene rearrangements in *PLAG1*, *MYB*, *MAML2*, and *ETV6* were negative by FISH, suggesting 100% specificity for tumors, which are not known to harbor these translocations. In our histopathologically confirmed cases, 1 case of pleomorphic adenoma was negative for a *PLAG1* gene rearrangement, and 2 cases of mucoepidermoid carcinoma showed an absence of the *MAML2* gene rearrangement. *PLAG1* gene rearrangements in pleomorphic adenomas are mediated in some cases by small intrachromosomal rearrangements not easily detected by FISH analysis.[38] *MAML2* gene rearrangements will be observed in ~50% of mucoepidermoid carcinoma cases and are less likely to be observed in high-grade mucoepidermoid carcinoma cases.[39] Both cases of mucoepidermoid carcinoma with negative FISH for *MAML2* demonstrated high-grade cytologic features on histologic evaluation. Our analysis might have achieved greater sensitivity with our institutional FISH assays because break-apart probes were used without regard of the fusion gene partner and could detect a wider array of molecular aberrations.

In our series, 5 cases (12.5%) also showed discordant cytohistologic findings before FISH testing, highlighting the potential value of FISH testing for cases that are diagnostically challenging or had suboptimal sampling. The cases were defined as discordant if the cytologic differential diagnosis rendered excluded the final histopathologic diagnosis. Two of these difficult lesions were accurately characterized as basaloid neoplasms on cytologic assessment, raising a broad differential diagnosis that included both benign and malignant entities. However, the final histopathologic diagnosis of epithelial-myoepithelial carcinoma was excluded from the differential diagnosis. Epithelial-myoepithelial carcinoma is an unusual low-grade malignant neoplasm with biphasic luminal and abluminal differentiation and is a rare diagnostic consideration.[1] Both lesions showed an absence of the *MYB* gene rearrangement, supporting the final diagnosis. One challenging case demonstrated features suggestive of an atypical cystic basaloid salivary gland lesion, although the cytologic evaluation was limited by extensive necrotic debris. The histopathologic follow-up revealed a pleomorphic adenoma. The FISH testing for the *MAML2* gene rearrangement was negative, providing no support for a mucoepidermoid carcinoma. In 2 of these cases, the specimens were suboptimal owing to paucicellularity and a descriptive cytomorphologic diagnosis was rendered, raising a limited differential diagnosis that did not include the final histopathologic diagnosis. The FISH analysis was negative for the *MAML2* gene rearrangement in both cases, providing support for the uncommon diagnoses of salivary duct carcinoma and lymphoepithelial cyst found on the follow-up surgical pathology excisions. Even in the paucicellular samples, ancillary FISH testing could still play an important role in the workup of salivary gland lesions by helping to exclude certain diagnoses.

Our study has demonstrated the diagnostic utility of FISH testing performed on cytology cell blocks for the definitive classification of salivary gland neoplasms in the setting of indeterminate cytologic diagnoses. FISH analysis failed for only 6 cases (10.5%) owing to insufficient tumor cellularity, and these cases were excluded from our analysis. Although cell blocks can provide adequate material for molecular testing, formalin-fixed paraffin-embedded tissue sections are prone to DNA fragmentation and nuclear truncation artifacts.[31,40] Further investigation is warranted to determine which cytologic preparations will be the most optimal for ancillary FISH testing of salivary gland tumors.

Conclusion

FISH testing performed on cytology cell blocks is a useful adjunct to subclassify salivary gland neoplasms using FNAB. Ancillary FISH testing allows for definitive classification in cases with indeterminate cytology, helping to guide clinical management and surgical planning, and obviating the need for repeat sampling.

Funding sources

No specific funding was disclosed.

Conflict of interest disclosures

The authors made no disclosures.

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