Cytopathology in focus: Reflections on use of Milan System, edition 1: Areas to be explored for edition 2

Dana Razzano, MD
He Wang, MD, PhD

August 2021—Salivary gland neoplasms (SGN) are a special group of tumors due to the high variation in histologic subtypes that are further complicated by frequent overlapping morphological features. Fine-needle aspiration is a safe, cost-effective, first-line modality for diagnosing SGNs, an integral part of SGN preoperational workup. In 2018, Faquin and Rossi led the effort to standardize the reporting system of salivary gland lesions.[1] Their final product, Milan System for Reporting Salivary Gland Cytopathology (MSRSGC), has had a huge impact on salivary gland FNA practice in the United States and worldwide. The system has promoted improved communication between clinical practices by fostering consistency and transferability of diagnoses, resulting in improved patient care.

The system put forth a uniform tiered framework for classifying and reporting SGNs, an evidence-based scale for estimating the risk of malignancy (ROM), and clear-cut management recommendations. The MSRSGC also provides metrics for ongoing quality assurance and improvement. The six tiers of the MSRSGC and respective ROMs are as follows: 1) nondiagnostic (ND), 25 percent; 2) non-neoplastic, 10 percent; 3) atypia of undetermined significance (AUS), 20 percent; 4a) neoplasm, benign, less than five percent; 4b) neoplasm, salivary gland of uncertain malignant potential, 36 percent; 5) suspicious for malignancy, 60 percent; 6) malignant, 90 percent.

As of now, more than 100 articles have been published related to the MSRSGC, many of which are international collaborations. These studies have examined the applicability of the system in various clinical scenarios: retrospective versus prospective; interobserver reproducibility[2]; salivary gland cystic lesions[3]; submandibular lesions[4]; and lesions in pediatric patients.[5] Some studies even retrospectively evaluated the FNA diagnosis of resected specific entities such as pleomorphic adenoma or Warthin tumor.[6] The studies confirm that FNA has excellent diagnostic performance in differentiating between benign and malignant salivary gland lesions and effectively distinguishes low- from high-grade neoplasms. The MSRSGC is a valuable tool for preoperative risk stratification.

Like all good classification systems, widespread application has prompted new questions and suggestions for improvement and opportunities for clarification. A few recent advancements in salivary gland cytopathology deserve particular attention and could potentially be included in future discussion of updated guidelines:

● Questions related to the nondiagnostic category. Although the cytologic criteria of the ND category are tentatively defined by the MSRSGC as "<60 lesional cells or normal salivary gland tissue only within the clinical setting of an evident mass," the criteria have not been validated or established in the literature. Not everyone agrees that the criteria are adequate to address all potentially nondiagnostic scenarios. For instance, aspirates that consist of abundant matrix material without a cellular component should not be classified as nondiagnostic according to the current MSRSGC. This has impelled debate among pathologists. Further study is necessary to address the questions that have arisen surrounding this category.

A related question in the ND category of the MSRSGC is the relatively high risk of malignancy. Results from meta-analyses have demonstrated variation: Hollyfield, et al., reported a ROM of 38 percent, and Wei, et al., reported a ROM of 25 percent.[7] In more recent studies, the ROMs for the ND category are much lower than the 25 percent reported in the MSRSGC. As some authors have pointed out, the variability between ROM in different studies may be due to multiple factors such as sample size variation, nonrepresentative sampling, and/or the low surgical resection rates of ND specimens.[7] Ultimately, accumulated literature after the widespread application of the MSRSGC is likely to modify the ROM for the ND category.

● Subclassification of current MSRSGC categories. Suggestions have been proposed in the literature to subclassify certain categories of the MSRSGC. Because of differences in clinical management, it has been proposed that category six be divided into two subdivisions and one additional unique category (creation of category seven), as follows[8]: 6a) low-grade malignancy that requires complete surgical excision without concurrent neck dissection; 6b) high-grade malignant neoplasms that require more radical surgical excision with concurrent neck dissection. High-grade primary salivary gland neoplasms and metastatic lesions to the parotid gland lymph nodes (squamous cell carcinoma, metastatic melanoma, and Merkel cell carcinoma) were grouped together because both require treatment of draining the lymph node basin; 7) hematological malignancies, to ensure the clinician obtains appropriate hematological consultation and the specimen is sent fresh for flow cytometry.

Another study analyzed the risk stratification and clinical outcome of lesions in MSRSGC category three (AUS) when they were further subdivided. The risk of malignancy was found to be highest in the category of specimens with obscuring preparation artifacts and lowest in the cases categorized as indefinite for neoplasm with reactive and reparative atypia present. The authors therefore suggest it is important to subgroup AUS.[9]

● Molecular markers and antibody detection of gene rearrangements. The molecular features of SGNs are a rapidly evolving field, holding promise not only for specific diagnostic markers but also as potential targets for therapeutic precision medicine. **Table 1** illustrates the most up to date molecular features of SGNs.[10] These molecular alterations can be detected by using fluorescence in situ hybridization and greatly enhance diagnostic specificity and accuracy.

Currently, however, sophisticated molecular techniques like FISH and next-generation sequencing are not widely available outside of major academic medical centers. More importantly, the low cellularity common in FNA specimens often makes molecular analysis ineffective. A practical solution would be the development of immunohistochemical surrogates for the diagnostic genetic tests. Earlier attempts at using such immunostains in SGN cytopathology yielded disappointing results. MYB protein is consistently expressed in adenoid cystic carcinoma, but it is also commonly detected in diagnostic mimickers. PLAG1 protein is usually seen in pleomorphic adenomas, but it is also expressed in various carcinomas such as ex-pleomorphic adenoma.

But a promising finding was reported recently. In 2021, Skaugen and colleagues[11] demonstrated that NR4A3 immunostaining is highly successful in diagnosing salivary gland acinic cell carcinoma on cell block material retrieved from FNA, outperforming not only DOG1 immunostaining but also *NR4A3* FISH. The diagnosis of acinic cell carcinoma is often challenging in FNAs because the routinely used acinar markers DOG1 and SOX10 do not help with the differential diagnosis between tumor and normal salivary acini. The authors demonstrate that because normal acini are negative, NR4A3 immunostaining solves this classic diagnostic dilemma with ease. NR4A3 immunostaining has also shown to be effective in samples with low cellularity that are insufficient for molecular analysis.[12] Thus, this immunohistochemical marker appears to make the FNA diagnosis of acinic cell carcinoma straightforward in the cases with adequate material for cell block.

Another exciting immunostain showing potential is the immunostain for Amphiregulin (AREG), an epidermal growth factor receptor ligand. AREG has been shown to be a downstream target of CRTC1-MAML2 fusion. Detection of AREG expression using immunohistochemistry helps identify fusion-positive MECs.[13] Ideally, additional immunohistochemical surrogates of genetic signatures could be developed and applied to salivary gland cytopathology to aid in difficult cases.

● In view of recently approved immune checkpoint inhibitors (e.g. nivolumab, atezolizumab, and pembrolizumab), testing for PD-L1 expression on tumor cells at the time of diagnosis has been required in pulmonary, gastric, urothelial, and head and neck squamous cell carcinoma. Guidelines for SGNs have not been established, but clinical studies are ongoing with positive results after PD-L1 inhibitor treatment.[14] PD-L1 expression is traditionally determined by IHC testing in histologic samples. Given that FNA is usually the first-line diagnostic modality for SGN, it is surprising that reports evaluating salivary gland FNA as an adequate substrate for PD-L1 expression

measurement are not found in the literature. Ongoing research studies by the authors will soon aid in providing answers to this important question.

Table 1 Characteristic Genetic Changes in Salivary Gland Neoplasms

Salivary Gland Neoplasms	Frequent Molecular Alterations	Frequencies (%)	Rare Molecular Alterations
Pleomorphic adenoma	*PLAG1* alterations *HMGA2* alterations	>50 10–20	
Mucoepidermoid carcinoma	*CRTC1-MAML2* and *CRTC3-MAML2* fusions	55–88 5	*EWSR1-POU5F1* fusion
Adenoid cystic carcinoma	*MYB-NFIB* and *MYBL1-NFIB* fusions	29–86 9–14	*MYB-PDCD1LG2, MYB-EFR3A, MYBL1-RAD51B, MYBL1-YTHDF3, NFIB-AIG1* fusions
Acinic cell carcinoma	*SCPP* gene cluster*—*NR4A3* fusions	84	*HTN3-MSANTD3* fusion
Secretory carcinoma	*ETV6-NTRK3* fusion	>95	*ETV6-RET, ETV6-MAML3, ETV6-MET* fusions
Polymorphous adenocarcinoma			
Classic type	*PRKD1* somatic mutations	70	
Cribriform adenocarcinoma of minor salivary glands type	*ARID1A-PRKD1, ARID1A-DD3* fusion and variant *PRKD1, PRKD2,* and *PRKD3* fusions	80	
Clear cell carcinoma	*EWSR1-ATF1* fusion	80–90	*EWSR1-CREM* fusion
Intraductal carcinoma			*TUT1-ETV5, KIAA1217-RET,* and *STRN-ALK* fusions
Intercalated duct type	*NCOA4-RET* fusion	47	*BRAF V600E* mutations
Apocrine or hybrid type	*TRIM27-RET* fusion		
Salivary duct carcinoma	*AR* gene alterations *ERBB2* amplification *TP53, PIK3CA, H-RAS, KIT, EGFR, BRAF, N-RAS, AKT1, FBXW7, ATM, NF1* mutations loss of heterozygosity of *CDKN2A/p16* and *PTEN*	40–70 29–35†	*NCOA4-RET ETV6-NTRK3 BCL6-TRADD HNRNPH3-ALK EML4-ALK ABL1-PPP2R2C* fusions
Myoepithelial carcinoma	*EWSR1* rearrangements	35	*PIK3CA* and *HRAS* mutations
Epithelial-myoepithelial carcinoma	*HRAS* mutations	33–83	*PLAG1, TP53, FBXW7, PIK3CA, CTNNB1, AKT1* mutations

Italic value indicates ETV6-NTRK3 fusion is detected in more than 95% of secretory carcinoma.
*Secretory Ca-binding phosphoprotein gene cluster, including the *NR4A3, STATH,* and *HTN3* genes.
†Frequencies of other molecular alterations in salivary duct carcinoma are described in Table 4.

Toper MH, Sarioglu S. Molecular pathology of salivary gland neoplasms: diagnostic, prognostic, and predictive perspective. *Adv Anat Pathol.* 2021;28(2):81–93. Reprinted with permission.

Significant progress has been made in the diagnosis and characterization of salivary gland lesions after the widespread application of the MSRSGC in 2018, coupled with several important clinical factors that are pertinent to patient management. It is exciting to learn that the second edition of the MSRSGC reporting guidelines is expected to be published in the later part of 2022 and will feature updated risks of malignancy based on new evidence in the literature and other significant advances in salivary gland cytopathology.

1. Rossi ED, Faquin WC. The Milan System for Reporting Salivary Gland Cytopathology (MSRSGC): an international effort toward improved patient care—when the roots might be inspired by Leonardo da Vinci. *Cancer Cytopathol.* 2018;126(9):756–766.
2. Layfield LJ, Esebua M, Yang Z, Vatzmitsel M, Giorgadze T, Schmidt R. The Milan system for reporting salivary gland cytopathology: a study of inter-observer reproducibility. *Diagn Cytopathol.* 2019;47(8):765–768.
3. Maleki Z, Allison DB, Butcher M, Kawamoto S, Eisele DW, Pantanowitz L. Application of the Milan System for Reporting Salivary Gland

Cytopathology to cystic salivary gland lesions. *Cancer Cytopathol.* 2021;129(3):214–225.
4. Maleki Z, Baloch Z, Lu R, et al. Application of the Milan System for Reporting Submandibular Gland Cytopathology: an international, multi-institutional study. *Cancer Cytopathol.* 2019;127(5):306–315.
5. Wang H, Weiss VL, Borinstein SC, et al. Application of the Milan System for Reporting Pediatric Salivary Gland Cytopathology: analysis of histologic follow-up, risk of malignancy, and diagnostic accuracy. *Cancer Cytopathol.* 2021;129(7):555–565.
6. Allison DB, Smith AP, An D, et al. Assessing the diagnostic accuracy for pleomorphic adenoma and Warthin tumor by employing the Milan System for Reporting Salivary Gland Cytopathology: an international, multi-institutional study. *Cancer Cytopathol.* 2021;129(1):43–52.
7. Castrodad-Rodríguez CA, Lajara S, Khader SN, et al. Application of the Milan System for Reporting Salivary Gland Cytopathology: experience of an academic institution in a tertiary academic medical center. *Cancer Cytopathol.* 2021;129(3):204–213.
8. Mazzola F, Gupta R, Luk PP, Palme C, Clark JR, Low TH. The Milan System for Reporting Salivary Gland Cytopathology—proposed modifications to improve clinical utility. *Head Neck.* 2019;41(8):2566–2573.
9. Wangsiricharoen S, Maleki Z. Risk stratification and clinical outcome in the atypia of undetermined significance category in the Milan System for Reporting Salivary Gland Cytopathology. *Cancer Cytopathol.* 2021;129(2):132–139.
10. Toper MH, Sarioglu S. Molecular pathology of salivary gland neoplasms: diagnostic, prognostic, and predictive perspective. *Adv Anat Pathol.* 2021;28(2):81–93.
11. Skaugen JM, Seethala RR, Chiosea SI, Landau MS. Evaluation of NR4A3 immunohistochemistry (IHC) and fluorescence in situ hybridization and comparison with DOG1 IHC for FNA diagnosis of acinic cell carcinoma. *Cancer Cytopathol.* 2021;129(2):104–113.
12. Bishop JA. Immunohistochemistry surrogates for molecular alterations: a new paradigm in salivary gland tumor cytopathology? *Cancer Cytopathol.* 2021;129(2):102–103.
13. Shinomiya H, Ito Y, Kubo M, et al. Expression of amphiregulin in mucoepidermoid carcinoma of the major salivary glands: a molecular and

clinicopathological study. *Hum Pathol.* 2016;57:37–44.
14. Cohen RB, Delord JP, Doi T, et al. Pembrolizumab for the treatment of advanced salivary gland carcinoma: findings of the phase 1b KEYNOTE-028 study. *Am J Clin Oncol.* 2018;41(11):1083–1088.

Dr. Razzano is a cytopathology fellow and Dr. Wang is associate professor of pathology—both at Yale University School of Medicine. Dr. Wang is a member of the CAP Cytopathology Committee.