Diagnostic challenges in the gray-zone lesions of fine-needle aspiration cytology

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ABSTRACT

Fine-needle aspiration cytology (FNAC) is an excellent technique for rapid diagnosis due to its speed, accuracy, and cost-effectiveness. However, there are many gray-zone areas in cytology that needs attention. These lesions in the aspiration cytology can be overcome by applying the selective use of the series of tests. This review discusses the diagnostic challenges in the gray-zone areas in FNAC. It emphasizes the use of selective ancillary techniques to solve the problems in this area.

Keywords: Fine-needle aspiration cytology, Ancillary techniques, Gray-zone, cytology

INTRODUCTION

Cytopathology, with its relatively painless, safe, and straightforward procedures for sample acquisition, has emerged as the first-line investigation for rapid diagnosis as well as follow-up. For several decades, the cytopathology has been considered as a screening tool, and inferior to biopsy, regarding the specific diagnosis. At present, the cytopathologists are armed with several ancillary techniques to resolve the issue of the various gray-zone areas. It is now possible to diagnose the different tumors and their subsets with considerable certainty. The morphological evaluation is indeed the cornerstone and the guiding light for these ancillary techniques. In the present review, we have discussed the various ancillary methods that may help to solve the problems of the gray-zone areas of fine-needle aspiration cytology (FNAC).

Ancillary techniques: The weapons to conquer the gray-zone areas

The foremost goal of the cytopathologist is to provide an optimum diagnosis. With the help of ancillary techniques, the limitations of the cytopathologists are shrinking day by day. Table 1 enumerates the various ancillary techniques available. It ranges from basic techniques for the identification of specific microorganisms to cytogenerics and assessment for mutations to liquid biopsy (LB). Cytology provides the best alternative source of readily extractable, reasonably stable well-preserved DNA, using all the different cytopreparatory methods including freshly prepared, unstained direct smears. For instance, the usage of intact cell preparation methods as smears, cytospins, and LB cytology (LBC) for fluorescent in situ hybridization (FISH) is advantageous as compared with formalin-fixed paraffin-embedded tissue (FFPE) as no sectioning artifacts are present. Figure 1 depicts the approach to sample collection for ancillary techniques in cytdiagnosis.
THYROID CYTOLOGY

FNAC is a useful diagnostic technique for the diagnosis of thyroid lesions due to high sensitivity, specificity, and low cost. However, 15–30% of thyroid FNACs are categorized as indeterminate and put in the category 3/4 in “The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC).” It is challenging to categorize “Atypia of undetermined significance/follicular lesions of undetermined significance (AUS/FLUS)” and “follicular neoplasm/suspicious of follicular neoplasm (FN/SFN)” from “suspicious for malignancy (SFM).” The non-neoplastic nodules may have overlapping cytological features with neoplastic lesions. Moreover, the inability to demonstrate the capsular and vascular invasion on cytology, the subcategorization of follicular neoplasm has been a big diagnostic challenge.

The diagnosis of non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFTP) on cytological smears is not feasible and forms a large proportion of the “Indeterminate” TBSRTC categories. Major discordances are seen in the diagnosis of follicular patterned lesions – follicular variant of papillary thyroid carcinoma (FV-PTC), follicular carcinoma, and the new entity NIFTP. The Bethesda system discusses the risk of malignancy after inclusion of NIFTP, which is 6–18% in AUS category, 10–40% in FN/SFN category, and 45–60% in SOM category.

The diagnosis of gray-zone lesions of the thyroid is one of the biggest challenges in cytopathology practice. Various ancillary modalities have been introduced for categorization of thyroid cytology aspirates classified into one of the indeterminate categories. Immunohistochemistry (IHC) has been tried with limited success as no single marker is reliable. A variety of immunomarkers has been described in the literature including “Hector Battifora mesothelial-1” (HBME-1), galectin-3 (GAL3), cytokeratin 19 (CK19), CD56, trophoblast cell surface antigen 2 (TROP2), “Cbp/p300-interacting transactivator with Glu/Asp-rich carboxyterminal domain 1”, and TPO. The most commonly proposed panel is GAL-3, HBME-1, and CK19. HBME-1 is often strongly expressed in PTC.

To differentiate follicular patterned lesions, a panel of a combination of TROP-2 and HBME-1 has been used.

Table 1: Various available ancillary techniques in cytopathology.

| Cytopreparatory methods: CB and LBC | Special stains and microbiological cultures |
| ICC | FCM |
| Fluorescence in situ hybridization | Reverse transcriptase PCR |
| NGS | Digital imaging and ANN |
| Electron microscopy (EM) |

CB: Cell block, LBC: Liquid biopsy cytology, ICC: Immunocytochemistry, FCM: Flow cytometry, NGS: Next-generation sequencing, PCR: Polymerase chain reaction, ANN: Artificial neural network

Figure 1: Approach to sample collection for ancillary techniques in fine-needle aspiration cytology diagnosis.
by Zargari and Mokhtari[5] to diagnose carcinoma with equivocal morphology. In his study, carcinomas show diffuse strong membranous TROP-2 immunoreactivity and weak focal reactivity in the benign lesion. However, the oncocytic variant of follicular neoplasms (“Hurthle cell neoplasm”) is strongly positive for TROP-2 and thus cannot be used for differentiating indeterminate oncocytic neoplasms. Zargari and Mokhtari used HBME-1 to differentiate malignant and benign lesions and showed a higher rate of membranous staining in malignancy and cytoplasmic staining in benign lesions.[5] Furthermore, BRAF mutation-specific antibody helps in the detection of the BRAF V600 mutation, with high accuracy.[4]

The American Thyroid Association (ATA) has recommended either a second FNAC or molecular testing in AUS/FLUS category.[6] The molecular testing in thyroid cytology can increase the predictive power of cytopathology of indeterminate lesions.

The currently available molecular testing for cytologic specimens is based on next-generation sequencing (NGS) and polymerase chain reaction (PCR) that give information on gene fusion, m-RNA expression profile, and other chromosomal changes.[7] A combination testing based on NGS-based mutational analysis and a microRNA expression-based classifier is also available. Nikiforov has discussed the various tests, namely, Afirma, ThyroSeq, RosettaGX Reveal, ThyroGenX, and ThyraMIR [Table 2].[8]

The artificial neural network (ANN) is a relatively new concept to distinguish between benign and malignant follicular neoplasm. Fractal dimension, chromatin textural analysis, and an ANN can be useful in indeterminate thyroid nodules.[9,10]

**SALIVARY GLAND CYTOLOGY**

FNAC is an essential diagnostic tool of the salivary gland neoplasms due to its high sensitivity (82–92%) and specificity (93–100%). The Milan system for reporting salivary gland cytopathology, published in 2018, comprises seven categories, of which AUS, salivary gland neoplasm of uncertain malignant potential (SUMP), and suspicious of malignancy do not provide a specific categorization of the lesion and have increased risk of malignancy.[11]

The management of neoplastic versus (vs.) non-neoplastic and low-grade versus high-grade malignancies are different, and thus, an accurate diagnosis is imperative for therapy. In the majority of cases, the cytomorphological diagnosis can be straightforward. The various factors may pose a diagnostic challenge to the cytopathologists. These include the heterogeneity of salivary gland tumors, evidence of cystic change, a variable degree of atypia, and overlapping cytomorphological features. For example, salivary glands aspirates with increased lymphoid population will have differential diagnosis ranging from a reactive process to lymphoproliferative disorder or cases of secretory carcinoma can be misinterpreted as acinic cell carcinoma. Ancillary studies are the mainstay of diagnosis in such cases.

Newly introduced chromosomal translocations and the fusion of oncogenes are instrumental in the diagnosis of a specific subset of tumors. The salivary gland neoplasms constitute a wide variety of benign and malignant tumors with cytomorphological overlap, and the usage of appropriate ancillary technique can help immensely in the formulation of a definitive diagnosis in AUS and SUMP categories. The characteristic immunophenotypic and cytogenetic features of various salivary gland tumors are described in the literature and now have significant diagnostic implications.[11] Figure 2 elaborates the basic difficulties and differential diagnoses in routine cytopathology practice.

In the salivary neoplasms, a large number of genetic changes can be assessed either by immunohistochemical surrogates or FISH.[12] The FNAC, along with FISH, provides high specificity in the diagnosis and even prognostic assessment such as in mucoepidermoid carcinoma.[13]

Flow cytometric immunophenotypic can be used to differentiate between reactive and lymphoproliferative disorder reliably. ANNs have been used to differentiate pleomorphic adenoma and adenoid cystic carcinoma on FNAC smears.[14] Further neural networks can be developed for other matrix rich tumors as well.

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**Table 2: Currently available molecular tests in thyroid specimens.**

| Molecular tests | Methodology | Specimen                  | Results                  |
|-----------------|-------------|---------------------------|--------------------------|
| Afirma          | mRNA gene expression | FNAC material in nucleic acid preservative | Benign/suspicious |
| ThyroGenX       | Multiplex PCR by sequence-specific probes | FNAC material in nucleic acid preservative | Specific gene location/ translocation |
| ThyroMIR        | microRNA expression | FNAC material in nucleic acid preservative | Negative/positive |
| ThyroSeq        | NGS         | FNAC material in nucleic acid preservative | Specific gene location/ translocation |
| Rosetta GX reveal | miRNA classifier test | Direct smear, ThinPrep LBC | Negative/positive |

PCR: Polymerase chain reaction, ANN: Artificial neural network, CB: Cell block, FNAC: Fine-needle aspiration cytology, FFPE: Formalin-fixed paraffin-embedded tissue, LBC: Liquid biopsy cytology
LYMPH NODE CYTOLOGY

The broad range of differential diagnosis of lymphadenopathy and need for early management in lymphomas has made FNAC as the first-line diagnostic modality. The revised “World Health Organization classification of lymphoreticular neoplasms” (2016) has described many specific entities based on cytomorphology, immunophenotypic characteristics, genetic abnormalities, and clinical features. Due to subtle cytomorphologic differences in the cellular morphology in low-grade non-Hodgkin lymphoma (NHL), a “false-positive” diagnosis on cytology cannot be excluded. The question of florid reactive versus low-grade lymphoma is always difficult for even experienced cytopathologists. Furthermore, on FNAC, it is difficult to provide a definitive diagnosis for various lymphomas on morphology alone. Many of these lymphomas bear characteristic morphological, immunophenotypical, and molecular abnormalities that can aid in a definitive subtyping. FNAC, along with ancillary tests, helps in diagnosis of these subtypes.

Diffuse large B-cell lymphoma (DLBCL) is the most common type of NHL. An accurate FNAC diagnosis is essential, along with information on prognostic and predictive markers. Gene expression profiling has devised the classification of DLBCL into three different groups – germinal center B-cell-like DLBCL and non-germinal center B-cell-like DLBCL with approximately 80% concordance with gene expression profiling. Cytogenetics has an essential role in the prognostic assessment in lymphomas and for the selection of therapeutic strategies. Many lymphomas have typical chromosomal aberrations and translocations between the antigen receptor (Ig/TCR) genes and oncogenes. The translocations involving MYC in Burkitt lymphoma and BCL2 in the diagnosis of follicular lymphoma, cyclinD1 in Mantle cell lymphoma are crucial in difficult cases.

OVARIAN CYTOLOGY

FNAC is being increasingly used in primary diagnosis of patients with ovarian masses who can be potential candidates for neoadjuvant chemotherapy before surgical intervention and those patients which can be good candidates for fertility sparing surgeries. The subclassification of ovarian epithelial neoplasms as benign, borderline, and malignant,
on cytomorphology alone, considering factors as tumor heterogeneity, evidence of cystic, and mucinous change, poses a diagnostic challenge. The distinction between low-grade versus high-grade serous carcinoma is critical and has therapeutic significance. Another diagnostic challenge is evidence of mucinous neoplasm on cytology smears, where it becomes difficult to differentiate between primary versus metastatic mucinous carcinoma or a part of teratoma. The distinction between subtypes of germ cell tumors and sex cord tumors can be a diagnostic dilemma and often, an accurate cytological diagnosis cannot be rendered. However, a cell block (CB) and usage of further ancillary techniques can be instrumental in making a conclusive diagnosis.

The ovarian masses can be most commonly accessed through ultrasound-guided FNAC. An adequate CB preparation, along with cytological smears, can facilitate a morphological diagnosis. Immunocytochemistry (ICC) on the CB can determine the exact nature of the neoplasm and aid in the differentiation of epithelial versus non-epithelial tumors and metastatic deposits. The specific tissue diagnosis of high-grade serous carcinoma (HGSC) of the ovary may be achieved in FNAC by a combination of morphology and immunocytochemistry. A minimal panel comprising CK7, PAX8, WT1, and p53 can be used to diagnose HGSC. A basic panel to differentiate primary ovarian and metastatic adenocarcinoma should include PAX8 (Mullerian marker), CK7, CK20, WT1, and p16. CK7 negative (−)/CK20 positive (+), CDX2+ indicates a colorectal primary while a CK7+, CK20−, PAX8+, indicates an ovarian primary. WT1 expression is seen in serous carcinomas, GCDFP is expressed by breast carcinomas, loss of DPC4 indicative of pancreatic origin. A strong diffuse p16 immunoreactivity supports a cervical origin.

Figure 4 highlights the differential diagnosis of the gray-zone areas of ovarian neoplasms.

SOFT-TISSUE TUMORS IN CYTOLOGY

The cytomorphological diagnosis of soft-tissue tumors on cytology is often difficult and is based largely on identification of patterns. The cytology smears have limited diagnostic accuracy as in some sarcomas, cells may appear bland, while in many benign mesenchymal lesions, cellular atypia can be seen. The reactive fibroblasts, myofibroblasts, regenerating, and degenerating muscle fibers can be misinterpreted in the cytology smears. Thus, additional material for ancillary techniques is a mandate for cytodiagnosis of soft-tissue tumors.

The utility of FNAC in soft-tissue tumors is mostly for triaging patients and providing broad differential diagnoses. ICC and molecular testing permit a more accurate and definitive diagnosis.

Many of the soft-tissue tumors are characterized by highly specific translocations and amplifications. Molecular advances helped in the development of useful surrogate IHC markers of molecular changes. ICC, FISH, and sequencing-based methods can be excellent ancillary diagnostic modalities. Table 3 enumerates the IHC and molecular
markers of soft-tissue tumors frequently subjected to FNAC.

**The future is more for less**

**NGS**

The introduction of NGS to the field of molecular diagnostics has broken barriers by utilizing restricted cytology samples for molecular analysis of multiple genes. To overcome the issue of heterogeneity of cytology samples, the appropriate use of the molecular test is recommended.[24] NGS technology can help to assess multiple genetic abnormalities from the minimum amount of tissue.[24] FNAC smears or even residual material in the FNAC syringe may be used for NGS when the CB is not available.[26,27] The full potential of limited volume cytology samples can be harnessed by recognizing the prospects and pitfalls of performing NGS testing.[28]

**LB**

LB is another upcoming minimally invasive, readily repeatable technique for detection and molecular profiling of malignancies. LB is most useful in detection of the inaccessible tumors. Cell-free DNA, circulating tumor cells, exosomal microRNA, and small RNA assessment in LB can give the information on the molecular genetic abnormalities of the tumor at the time of chemotherapy.[29] LB is a non-invasive technique and bears lot of potential to diagnose minimal residual disease, dynamic supervision of newer genetic changes of the tumor, and even early detection of the cancer.[2]

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**Table 3: Immunohistochemical and molecular characterization of soft-tissue tumors.**

| Soft-tissue tumors | IHC                        | Molecular pathology                        |
|--------------------|----------------------------|-------------------------------------------|
| Atypical lipomatous tumor | MDM2, CDK4                  | Chromosome 12q13-15 amplification through supernumerary ring or giant markers |
| Myxoid liposarcoma        | –                           | FUS-DDIT3                                 |
| Neurofibroma             | S100 (multifocal), SOX10, neurofilament (axons) | –                                   |
| Desmoid fibromatosis      | β-catenin, SMA              | CTNNB1 or ACP mutations                     |
| GIST                   | KIT, DOG1                   | KIT mutations; subset with PDGFRA mutations |
| IMT                    | SMA±, ALK (50%)             | ALK rearrangement                          |
| Solitary fibrous tumor   | CD34, STAT6                 | NAB2-STAT6                                |
| DFSP                   | CD34                       | COL1A1-PDGFβ                               |
| Synovial sarcoma SMA     | EMA, keratin, TLE1         | SS18-SSX1, SS18-SSX2                      |
| Leiomyosarcoma           | Desmin, caldesmon           | –                                         |
| MPNST                  | Focal S100, SOX10, GFAP, loss of H3K27me3 | NF1, CDKN2A, SUZ12, EED1 mutations       |
| Ewing sarcoma           | CD99 (diffuse membranous), NKK2, FLI-1 | EWSR1-FLI1, EWSR1 rearrangement         |
| Alveolar RMS            | Diffuse desmin and myogenin | PAX3-FOXO1                                |
| DSRCT                 | EMA, NSE, desmin, WT1 (C terminus) | EWSR1-WT1                                |

IHC: Immunohistochemistry
Whole slide imaging (WSI)

WSI represents the first but momentous step that has enabled the entry of wide range of digital tools which are revolutionary for upcoming practice of pathology.

WSI generates digital illustrations of routine glass slides, ensuring preservation of digital representations with good image quality. This system will allow digital analysis anywhere and at any time. Not only can these images be used for remote consultations, telepathology, they are an ideal educational material. Whole slide images are storehouse of information regarding morphology and complex cell phenotypes. Digital images in cytology can be for developing educational modules, telecytology, external quality control, and assurance, along with applications as image analysis.

ANN

ANN is a new technology for formulating a diagnosis and predicting disease outcome. ANN is basically a software program with an algorithmic approach simulating the biological neuronal network of human brain. ANN is typically useful in the gray-zone lesions of cytology. In cytology, ANN has been used for classification of breast lesions, identification of malignancy in effusion, and in thyroid lesions, salivary gland tumors.

CONCLUSION

The usage of ancillary techniques has broadened the cytopathologist’s horizon, facilitating in the formulation of diagnosis, detection of prognostic and predictive markers, thereby ensuring rapid and effective treatment of patients. In the modern era of personalized and targeted therapy, cytology provides a suitable platform for biomarker testing. FNAC specimens are being increasingly used for immunocytochemistry, cytogenetics, molecular analysis, and image analysis, thereby breaking the limitations of morphology alone. Molecular cytopathology and ANNs based on image analysis are rapidly progressing fields in the age of whole slide scanners. The selection of the appropriate ancillary technique, along with the clinical and radiological features, is immensely valuable in the final diagnosis. The cytopathologists need to stay vigilant and also be aware of the usefulness of the various types of cytology samples for the diagnosis and management of the patient. The cytopathologists need to stay vigilant and also be aware of the usefulness of the various types of cytology samples for the diagnosis and management of the patient.

COMPETING INTEREST STATEMENT BY ALL AUTHORS

There is no competing interest in this papers as declared by both the authors.

AUTHORSHIP STATEMENT BY ALL AUTHORS

Shruti Gupta has collected data and drafted the manuscript. Pranab Dey has analyzed data and guided in writing the manuscript.

LIST OF ABBREVIATIONS (In alphabetic order)

ACC – Acinic cell carcinoma
AdCC – Adenoid cystic carcinoma
AFP – Alpha fetoprotein
ALCL – Anaplastic large cell lymphoma
AR – Androgen receptor
ANN – Artificial neural network
AUS – Atypia of undetermined significance
BCA – Basal cell adenoma
CDX2 – Caudal-type homeobox 2
CITED1 – Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain, 1
CB – Cell block
CLL – Chronic lymphocytic leukemia
DNA – Deoxyribonucleic acid
DLBCL – Diffuse large B-cell lymphoma
DOG1 – Discovered On GIST 1
ETV1 – ETS Variant Transcription Factor 1
FNAC – Fine needle aspiration cytology
FCM – Flow cytometry
FISH – Fluorescent in situ hybridization
FC – Follicular carcinoma
FLUS – Follicular lesions of undetermined significance
FL – Follicular lymphoma
FVPTC – Follicular variant of Papillary thyroid carcinoma
FFPE – Formalin-fixed paraffin-embedded tissue
GAL 3 – Galectin-3
HBME-1 – Hector Battifora mesothelial-1
HNF1 – hepatocyte nuclear factor 1
HMGA – High mobility group A
HGSC – High-grade serous carcinoma
HL – Hodgkin lymphoma
IHC – Immunohistochemistry
LB – Liquid biopsy
LBC – Liquid-based cytology
LBL – Lymphoblastic lymphoma
LPL – Lymphoplasmacytoid lymphoma
MCL – Mantle cell lymphoma
MSRS GC – Milan System for Reporting Salivary Gland Cytopathology
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