Chapter

Recent Advances and Researches in the Field of Fine Needle Aspiration Cytopathology

Anjali Goyal

Abstract

Fine needle aspiration cytology/biopsy (FNAB) is quite often one of the first tests for the initial evaluation of lesions/swellings which are accessible to the needle tracts. The technique has its limitations in certain cases owing to the non-representative or inadequate material aspirated or due to the confusion arising from the lack of histologic pattern as observed on a biopsy. An immediate rapid on-site evaluation (ROSE) is valuable in minimizing the limitations arising from the non-representative/inadequate material. The introduction and application of several ancillary modalities, like immunocytochemistry, molecular tests and the advancements in interventional radiology, has further revolutionized the diagnostic scope of FNA biopsy. Molecular tests on the FNAC samples can aid in the distinction of benign from malignant lesions, in determining the genetic abnormalities and genetic makeup of tumors that can be useful not only for making a more specific diagnosis but also for determining prognosis, response to therapy and for the selection of patients for targeted therapy. FNAB biopsies have an added advantage in comparison with the core needle biopsies for molecular analysis since they have a much lower contamination of stroma. The chapter will be discussing the advancements and the uses of these ancillary techniques in the field of FNAC.

Keywords: FNAB, advances, ROSE, TME, cellular multiplexing, cytogenetics, ABCD, SCANT, FAST – PDL1, FAST cold/hot score, FISH, PCR, gene microarrays, scRNA Seq

1. Introduction

FNAB is the most frequently the first ordered investigation for palpable or non-palpable deep-seated lesions in the body, along with the advantage of acquiring tissues from small lesions and multiple sites. FNAB biopsies have an added advantage in comparison with the core needle biopsies for molecular analysis since they have a much lower contamination of stroma.

An advancement in the skill and the application of the various ancillary techniques has significantly reduced the need for an open biopsy.

Immunocytochemistry performed on direct smears, monolayered preparations and cell block sections of FNAB is the most commonly utilized ancillary technique,

helping in knowing the histogenesis of the tumor and its origin in cases of metastatic tumors, typing of tumors and determining prognostic and predictive markers [1].

Significant advancements have been made in the fields of pulmonary/bronchial cytology, breast FNAB, thyroid and the head and neck cancers along with the identification and advancements in the field of tumor microenvironment (TME) [2, 3].

The advent of the potential use of cytogenetics would aid as an adjunct to the sensitivity and specificity for the FNAB samples, thereby minimizing the indications of an open biopsy. The molecular tests are being studied to be carried out on FNAB samples from any site as an adjunct to the cytomorphologic diagnosis. These can be used for cancer detection, distinguishing benign from malignant tumors, targeted therapy and risk assessment for tumors, detection of clonality in hemopoietic neoplasms, typing of soft tissue sarcomas and the genetic make-up of tumors.

2. Newer advancements and procedures

2.1 The importance of tumor microenvironment

The tumor microenvironment includes the immune cells, fibroblasts, blood vessels and the stomal elements which play a role in the cancer progression as well as the treatment efficacy. A mapping of the same at the cellular and molecular levels will help in the decision making and treatment. The tumor microenvironment is reshaped dynamically as the cancer evolves, and hence, a repeated sampling of the tumor tissue to track the TME changes under treatment pressure is required. A FNAB biopsy hence becomes an important marker for the management of the cancer patients. [iii] Moreover, studying the tumor microenvironment at protein level is more important than the RNA studies due to the discrepancy between the protein and the RNA expression [4].

2.2 Cellular multiplexing technologies

Single-cell analytic technologies help in the further understanding of the TME. These involve the broad multiplexing and the cycling methods. Sample multiplexing/multiplex sequencing allows a large number of libraries pooled and sequenced simultaneously during a single run, which exponentially increases the number of samples analyzed without increasing the cost and time drastically. It is useful when targeting specific genomic regions [5, 6].

The ability to multiplex FNA samples opens new venues for a deeper and more informative analyses of TME, which in turn defines the tumor/immune biomarkers to predict the treatment options and outcomes [3].

2.3 Rapid on-site adequacy evaluation (rose)

The major drawback in FNAB procedures can be a sampling error, giving a high non-diagnostic (ND) rate, which can be minimized by an immediate on-site assessment. This could also provide a real-time communication of information including tissue triage recommendations for other ancillary tests including flow cytometry, cytogenetics and molecular testing [1, 3].

Principle: The principle of the ROSE remains the same, although the technique of specimen preparation may vary with the institutions and personnel. The most common practice is to deposit the sample on a slide and smear it by the second. The

various stains used can vary between laboratories and include Diff-Quik, toluidine blue, rapid papanicolaou or hematoxylin and eosin stain. One slide is stained with a fast stain for an immediate review, while the other is fixed in alcohol. The needle is rinsed in a collecting media and later processed along with the fixed slide.

In few of the studies, the average reported ND rate was improved by 12% when ROSE was implemented, in addition to the overall cost effectiveness, although an increase in the number of passes was recorded. The drawbacks included an increased cost of the procedure, prolonged procedure time and increased burden on the laboratories [7, 8].

2.4 ABCD and SCANT

Most of the cycling methods developed earlier were meant for paraffin-embedded sections and were not compatible with the FNAB samples. Hence, gentler cycling methods were developed including DNA-barcoded antibody technologies such as antibody barcoding with cleavable DNA (ABCD) and single-cell analysis for tumor phenotyping (SCANT).

DNA barcode-modified antibodies are used in both the methods. After staining, each barcode type is read out by beads, hybridization probes or sequencing [9].

The SCANT method has been used to interrogate drug relevant pathways in FNAB samples from breast cancer tissues. One of the obstacles with SCANT is its long destaining times between cycles (0.5–1 hour). Hence, additional methods with a faster turnaround time were developed in the form of fast analytical staining technique (FAST-FNA) [10].

2.5 Fast-FNA technology

The technology involves ultrafast destaining chemistry and automated image cytometry readers for a rapid analysis. The method bypasses the shortcoming of other cellular cycling technologies and allows a fast cycling while maintaining the integrity of the dispersed cells. The studies have been performed using both mouse and human FNAB samples of head and neck squamous cell carcinoma (HNSCC) [3, 6].

Two newly developed scores include FAST PD-L1 (programmed cell death-Ligand1) and FAST cold/hot scores on the FNAB samples. The PD-L1 score is used to define PD-L1 expression in various cells like the tumor cells, macrophages and the other immune cell in reference to a minimum of Viable Cells analogous to the combined positive scoring (CPS) score on histologic sections. Although false positive scores were not recorded in the cell blocks and aspiration specimen, an excisional biopsy may be recommended for the negative results [11]. The FAST PD-L1 score is more quicker (<2 hours from cell harvesting to report), serially deployable and more cost effective [10].

The FAST cold/hot score is based on the observation that the efficacy of immunotherapies depends on the presence of a baseline immune response, or a pre-existing immunity, and quantifies relevant immune components to define whether a given tumor is hot, altered or cold [1].

2.6 Molecular techniques in FNAC

Several molecular tests including in-situ hybridization, polymerase chain reaction (PCR), Southern blotting and gene microarrays have been described using the FNA specimen, indicating the excellent potential of molecular tests in FNAB acquired material. The need for a molecular testing arises where a core needle biopsy is not

available, in cases with an indeterminate or suspicious cytology. Multitargeted FISH assays can be used to distinguish benign from malignant lesions, in addition to their role for targeted therapy, e.g., epithelial growth factor receptor (EGFR) inhibitors for NSCLC. The FNAB biopsies have an additional advantage in comparison with the core needle biopsies in having a much lower contamination of stroma [1].

2.7 Emerging advances

A few other scores identified by small conditional RNA sequencing (scRNA Seq) mapping are emerging involving the FNA mapping of the other tumor microenvironment (TME) cell types. Immuno-FNA grams can rapidly convey the TME landscape and its changes during treatment.

An additional aspect to automating the FNAB staining interpretation is the development of automated image cytometers which incorporate the advances in bioengineering and artificial intelligence (AI) for a rapid analysis. The use of automated systems incorporates quality measures of control and lowers the variation in interpretation [3].

3. FNA advances in the specific fields

3.1 Advances in the diagnosis of pulmonary carcinoma

Pulmonary nodules are frequently encountered in routine imaging which can range from benign nodules to cancerous nodules. An early, accurate diagnosis is of paramount importance for initiating therapy in malignant lesions and avoiding unnecessary investigations for the benign ones. Hence, a direct tissue sampling is essential which can be accomplished by non-invasive techniques like FNAB.

The FNAB sampling can be performed via airways (endobronchial/transbronchial) or the chest wall (CT-guided percutaneous FNAB.), which can be used for molecular studies and therapeutic decision making.

With the newer advances in the field of pulmonary pathology, an accurate classification of the lung tumors is mandatory along with the need for a characterization of the correct molecular alterations which predict the response to certain drugs.

Because of the availability of a limited material, an efficient panel of immunohistochemical (IHC) markers is required for the cytology samples, to largely differentiate the poorly differentiated squamous cell carcinoma (SQC) from small cell lung carcinoma (SCLC) and adenocarcinomas from metastasis [12].

All SQC (irrespective of differentiation) showed expression of p63 and negative for TTF-1. Adenocarcinomas showed a positive staining for both the markers. SCLC has an expression similar to adenocarcinoma which can be differentiated by the morphologic criteria and the neuroendocrine markers. The following algorithm can be useful to classify the lung carcinomas.

Expression of three markers—TTF-1, p63 and HMW-Cytokeratin-can be used for a fair assessment of the histogenesis of the lung tumors (**Table 1**).

4. Biopsy procedures

1. Fiberoptic bronchoscopy (FBS) has a high diagnostic yield for endobronchial lesions, but a low diagnostic yield for the non-endobronchial and peripheral lesions.

Type of Lung Tumors	TTF 1	P63(Nuclear)	HMWCK	Napsin A cytoplasmic	Desmocolin 3
SQC	Negative	Positive	Positive	Negative	Positive
ADC	Positive	+/−	+/−	Positive 80%	+/−
SCC	Positive	+/−	Negative	+/−	+/−

SQC—squamous cell carcinoma, ADC—adenocarcinoma, SCC—small cell carcinoma.

Table 1.
IHC algorithm for lung carcinomas [12].

2. Endobronchial ultrasound-guided transbronchial needle aspiration (EBUS-TBNA) helps in the acquisition of real-time ultrasound images with an accessibility to the central lung lesions and surrounding lymph nodes (mediastinal, paratracheal, subcarinal, hilar and interlobar). The technique recently emerged as a minimally invasive method for mediastinal staging of lung cancers and diagnostic work up for centrally located mases, with a limitation for evaluating the peripheral lung lesions, other lymph nodes and diagnostic material for small lesions.

3. Endobronchial ultrasound using a guide (EBUS-GS) helps in the acquisition of real-time ultrasound images with an improved accessibility to more peripheral lesions. However, it is an expensive technique and requires professional training.

4. Navigation bronchoscopy helps in an improved accessibility to more peripheral lesions along with a virtual road map to target. The technique is expensive and requires a navigation programme.

5. Computerized tomography (CT)-guided percutaneous biopsy (CT-NAB) provides a high diagnostic yield for the peripheral lung lesions (diameter > 1 cm) with possible complications of hemorrhage and pneumothorax.

6. Gun biopsy, high diagnostic yield using core-biopsy needles. Patient cooperation is required along with the complications including pneumothorax and pulmonary hemorrhage.

7. Electromagnetic navigation bronchoscopy (ENB)—This is a recently emerging technology. It makes use of a localization device which assists in placing the forceps/brush in the desired foci. Low-frequency electromagnetic waves are used with a 3D reconstruction of the signals. The technique localizes and samples lesions in the lung parenchyma and mediastinum that are beyond the reach of a standard endoscopy [1, 12, 13].

The commonly used molecular markers for lung cancer include epithelial growth factor receptor (EGFR), receptor tyrosine kinase (ROS1) and gene encoding protein B-raf (BRAF) mutations for adenocarcinomas and anaplastic lymphoma kinase (ALK) mutations for non-small cell carcinoma (NSCLC), used as targets for lung carcinoma treatment. Other gene mutations including vascular endothelial growth factor (VEGF), KRAS oncogene, rearranged during transfection), and mesenchymal epithelial transition factor (MET) are also being used for cancer treatment in addition

to the immunologic markers like PD1 and PD-L1. Other molecular techniques like the next generation sequencing (NGS)-based tests and transcriptome analysis—involving the RNA sequencing and microarrays are being studied as the diagnostic and prognostic markers in lung cancer management [12].

4.1 Head and neck cancers

The success of FNAB in the initial evaluation of the head and neck masses has been established. It is especially useful for clinically and radiologically equivocal nodules, initial tumor staging and treatment planning and the surveillance of postoperative lymph nodes.

The newer advancements include the incorporation of ultrasound-guided FNA (UGFNA) into the existing practice of palpation-guided FNA (PGFNA) [14].

Additional studies employed on FNA material may be crucial for arriving at a diagnosis. Various antibody panels being explored for FAST-FNA analysis in human HNSCC [3, 6].

4.2 Breast pathology

The International Academy of Cytology (IAS) Yokohama system for breast carcinoma reporting for FNAB has been proposed by the NIH for a uniformity of the cytology reports. The system is providing an impetus for further research and a focus on contentious areas, in addition to providing the practice guidelines for indications, techniques of breast FNAB and smear making it provide the key diagnostic cytopathologic features of breast lesions.

The system defines the breast lesions into five reporting categories including insufficient/inadequate, benign, atypical, suspicious of malignancy and malignant—each stratified with a distinct risk of malignancy (ROM) and a direct link to management algorithm. It also reviews and recommends appropriate ancillary testing to improve the quality assurance (**Table 2**) [15, 16].

Evaluation of EGFR (HER2/neu) gene amplification is an important application of FISH using FNAB material, particularly important in inoperable cases which is valuable in targeted therapy with Trastuzumab. FNAB smears or cell block sections from metastatic tumors can be used for evaluating the same [1, 17].

Several molecular tests like FISH can be applied for chromosomal aneusomy, PCR for allelotyping and clonality assays. The results have been reported to give reproducible results in conjunction with cytomorphology for diagnosis, risk

	Diagnostic category	Risk of malignancy
1	Insufficient	30.3
2	Benign	4.7
3	Atypical	51.5
4	Suspicious of malignancy	85.4
5	Malignant	98.7

IAC—International Academy of Cytology; ROM—Risk of Malignancy.

Table 2.
Yokohama system for classification of breast lesions [15].

assessments, clinical progression and therapy. Numerous studies and researches on the transcriptional profiling on the FNAB specimen are under research and need to be validated [1].

4.2.1 Fractal analysis of Kirsh edge images for tissue (FKT) fragment innerstructure

Recent advances in high-precision mammography and ultrasound screening have led to an increase in the detection of early lesions, appearing as micro-calcified or microcystic images. These need to be an improvement in the accuracy of FNAB in assessing these lesions. Fractal analysis of Kirsh edge images for the tissue fragment inner structure is useful in breast FNAB. FKT measures tissue fragment chromasia of hyperchromatic crowed tissue fragments (HCG), tissue fragment shape unevenness and the inner structure complexity. This might serve as a useful system assisting in the cytopathological assessment of the breast FNAB.

The cluster gray image-fractal analysis evaluating the darkness of clusters, cluster unevenness and complexity of the hyperchromicity/cluster density of the deep-stained clusters known as hyperchromatic crowed cell groups (HCG) on breast FNAB is a useful cytology assistance system for breast FNA [18].

1. Cluster size classification: clusters were classified into small, middle and large clusters (small cluster: $<40 \times 102\ \mu m^2$; large cluster $>100 \times 102\ \mu m^2$ or larger. Fibroadenomas showed the presence of small, middle and large clusters at a similar frequency with a higher frequency of large clusters, in contrast to infiltrating breast carcinoma where the small clusters were more frequent and a lower frequency of large clusters was seen.

2. Cluster gray image-fractal analysis: (a) the darkness of clusters (luminance), (b) cluster unevenness (complexity) and (c) complexity of cluster density (roundness-corrected fractal value) were assessed.

The FNA from the infiltrating breast carcinoma (NST), the luminance of the small clusters was low (dark), with a high unevenness and a higher complexity of the cluster density. The luminance of the large clusters was high(bright), with a high unevenness and complexity as compared to that in fibroadenomas [18].

4.3 Soft tissue neoplasms

FNAB specimen can be used for the karyotypic analysis of soft tissue sarcomas. Cytospin and monolayer preparations of the FNAB samples of the primary or recurrent sarcomas are excellent specimen for FISH testing because of the availability of single cells for analysis.

Molecular analysis to demonstrate c-kit mutations can also be performed on the FNAB material making a diagnosis of primary and recurrent gastrointestinal stromal tumors (GIST) [1, 3].

4.4 Hemapoetic neoplasms

FNAB can performed on the lymphoid lesions. Cytomorphology between the reactive and the low-grade lymphomas can be confusing and has a limited value in the classification and the typing of the various lymphoma subtypes.

Ancillary techniques like immunophenotyping and molecular analysis are necessary for establishing monoclonality (differentiate benign from malignant) and the typing of malignant lymphomas. The analyses can be done on FNAB samples with a good cellularity. However, the inability to procure a satisfactory aspirate because of fibrosis or necrosis can lead to failure in rendering a specific diagnosis. This can be overcome by practising ROSE, hence reducing the ND rate in these tumors.

Whereas the cytomorphologic features coupled with IHC can provide a diagnosis in most of the cases, additional molecular testing might be required for confounding cases, wherein molecular testing using FISH or PCR may be asked for. The specific molecular tests can be used to detect gene rearrangements and chromosomal abnormalities useful for targeted therapy and prognosis [1, 3].

4.5 Thyroid neoplasms

FNAB is routinely used for screening and preoperative evaluation of the thyroid nodules. The most prestigious of the thyroid associations like American Association of Clinical Endocrinologists (AACE), American Thyroid Association (ATA) and the European Thyroid Association (ETA) provide detailed guidelines on which the nodules should be biopsied, which was revised in 2017 and named as the Bethesda system. It divided the FNA categories into six groups.

The results of the cytologic examination on the FNAB material provide the basis for the further clinical assessment. However, the assessment of the cells can be challenging in some instances and assessed into the Bethesda Class III (indeterminate), or class IV (suspicious of follicular neoplasm), thereby resulting in a resection where more than 70% are benign nodules (**Table 3**) [19].

Hence, the use of molecular markers in routine assessment of such nodules would be helpful to assess the risk of malignancy, as recommended in the second edition of Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) and in the American Thyroid Association(ATA) guidelines. Additionally, the introduction of a new diagnosis by the WHO in 2017 is an important aspect in the diagnosis of thyroid cancer and included a new section termed "other encapsulated follicular patterned thyroid tumors" (**Figure 1**) [19, 20].

In lesions like the (NIFTP), the features of invasion are required to exclude the malignancy, and the cytomorphologic features alone are not helpful. Hence, the use

Category	Reporting	Management
1	Inadequate/non-diagnostic	Repeat FNA (with USG guidance)
11	Benign	Clinical follow up
111	Atypia of unknown significance (AUS)/ follicular lesion of unknown significance (FLUS)	Repeat FNA/molecular testing/lobectomy (diagnostic)
1 V	Follicular neoplasm/suspicious of follicular neoplasm	Molecular testing/lobectomy (diagnostic)
V	Suspicious for malignancy	Lobectomy/near total thyroidectomy
V1	Malignant	Lobectomy/near total thyroidectomy

Table 3.
Bethesda system for reporting thyroid cytopathology (TBSRTC) [18].

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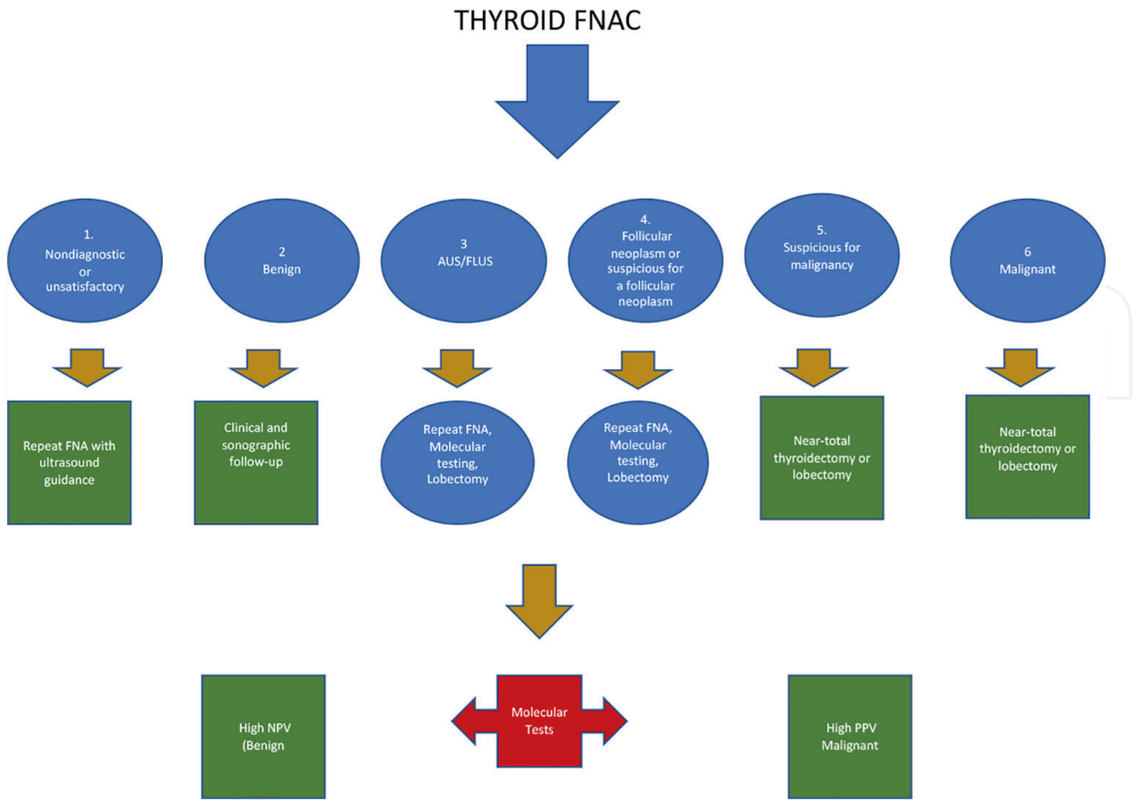

Figure 1.
Preoperative management of thyroid nodules [19, 20]. (AUS-Atypia of unknown significance, FLUS-follicular lesion of unknown significance).

of molecular markers is indicated to assess the malignancy in these lesions to avoid an unnecessary surgical treatment.

The use of immunohistochemical (IHC) markers that assess the malignancy in the lesions would increase the sensitivity of FNAC. The postulated markers included galectin 3, HBME, fibronectin 1 and cytokeratin 19, which proved insufficient for assessment of the biopsy material. Hence, the molecular markers become the next option for assessment of these lesions.

One of the first molecular markers to be used was the commonly observed molecular markers in papillary thyroid carcinoma (PTC) such as BRAF gene mutations, RET and NTRK rearrangements or PAX8/PPARγ fusion characteristic of follicular tumors.

Multi gene panels with mutations characteristic of follicular and papillary thyroid cancers like point mutations within BRAF, KRAS, HRAS and NRAS for follicular and RET/PTC1,RET/PTC3 and PAX8/PPARγ rearrangements for papillary carcinoma, respectively. The sensitivity of the techniques is further improved with the introduction of the oligonucleotide arrays and next-generation sequencing (NGS), which allows the assessment of many gene alterations in one analysis. The classifiers are grouped into two broad groups—rule out a malignant lesion "Rule out test" or to confirm a malignant lesion "Rule in test" determined by the negative predictive value (NPV) or the positive predictive value (PPV). The higher negative the NPV, the higher probability of benign lesion. Similarly, the higher positive the PPV, the higher probability of malignant lesion (**Table 2**, **Figure 1**) [21].

The use of ultrasound guided biopsies has become a common practice in all nodules measuring >10 mm [7].

Rapid on site evaluation (ROSE) is a useful adjunct to the thyroid FNAC for the specimen adequacy and diagnosis. A comparison of the ROSE and non-ROSE groups improves the specimen adequacy with a decrease in the number of needle passes [8].

Hurthle cell predominant nodules present with a confounding picture on FNAC in terms of their malignant potential and hence assigned a Bethesda Category III or IV. Thyroseq V3 molecular profiling can be used for tailoring the surgical management of the Hurthle cell neoplasms. A molecular profiling with ThyroSeq V3 helps in predicting the malignant potential of these nodules [22].

Mutation of BRAF gene occurs almost exclusively in papillary thyroid carcinoma. (PTC), which can be detected by the IHC marker BRAF(V600E) [23].

Low-cost qualifiers based on the PCR method which support FNAC are being developed which need to be validated.

Telomerase activity is shown to distinguish benign from malignant lesions in various studies. Telomerase activity with hTERT gene expression is seen in malignancy along with in few inflammatory conditions like lymphocytic thyroiditis and follicular adenomas in some cases.

Molecular testing is a useful adjunct to surgical decision making. Mutational profiling of thyroid nodule, and mainly the BRAF and RAS can be done using droplet digital PCR. Gene expression profiling by RT-PCR helps in the distinction of benign from malignant thyroid nodules. Upregulation of genes like (extracellular matrix protein (ECM1) and transmembrane protease serine 4 (TMPRS4) mRNA was determined to be an independent predictor of malignant thyroid neoplasm [24]. Classification of the thyroid neoplasms can be done on cell blocks using FISH [25].

Other molecular tests are being increasingly used which can help in differentiating benign from the malignant nodules more accurately. The Afirma gene sequencing classifier (Thryoseq v2) and other molecular tests are based on micro-RNA alterations [26, 27]. The tests can help in improving the diagnosis for appropriate therapy. ThyGEnX/ThyraMIR is another combination test waiting for validation.

TERT promoter mutations have a diagnostic and prognostic significance in the thyroid FNAC. TERT mutation positive nodules might be indeterminate on FNAC and present with aggressive clinicopathological behaviors like extra thyroid invasion, lymph node metastases, distant metastases, disease recurrence or patient death, underscoring the importance of the TERT analysis.

Telomerase activity is shown to distinguish benign from malignant lesions in various studies. Telomerase activity with hTERT gene expression is seen in malignancy along with in few inflammatory conditions like lymphocytic thyroiditis and follicular adenomas in some cases [28].

Use of analytical chemistry procedures allows for the potential recognition of cancer-based metabolites for the purposes of advancing the era of personalized medicine. Nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) coupled with separation techniques, e.g., gas chromatography (GC) and liquid chromatography (LC), are the main approaches for metabolic studies in cancers [29].

The immense metabolite profiling has provided a chance to discover novel biomarkers for early detection of thyroid cancer and reduce unnecessary aggressive surgery.

An assessment of the cyclooxygenase-1 and 2 gene expression levels in chronic autoimmune thyroiditis, papillary thyroid carcinoma and non-toxic nodular goiter

showed that an up-regulation of cyclooxygenase-2(COX-2) activity is associated with the papillary thyroid cancer (PTC) [30].

The metabolic alterations in thyroid cancer can serve as potential therapeutic targets. Altering the balance between cancer and stromal cells might serve as a promising therapeutic strategy [31].

4.6 Lymph nodes

PCR for selected markers has been studied for their utility in increasing the sensitivity of cytomorphology for detecting the metastasis and their histogenesis, particularly pertaining to the endoscopic ultrasound (EUS)-guided FNAB, and detection of micro-metastasis.

Various studies involving the aberrations in genes like the Cyclin D1 by FISH for squamous cell carcinoma and FNA-PCR for tyrosinase to detect metastasis in melanoma [1, 3, 32].

The results could serve as an independent marker for prognosis, tumor aggressiveness and recurrence.

4.7 Pancreatic neoplasms

The EUS-FNA for the diagnosis of pancreatic neoplasms has the potential drawbacks of a paucity of diagnostic material along with the overlapping features of a low-grade tumor and reactive processes like chronic pancreatitis [33].

Franseen Needle used for EUS-guided FNAB has a role in tissue acquisition for study of microsatellite instability in unresectable pancreatic lesions, required for a treatment with the immune checkpoint inhibitors for the unresectable pancreatic lesions [34].

Mutations of the K-ras oncogene have been most frequently studied as an adjunct to conventional cytology to aid in the diagnosis of pancreatic adenocarcinoma. Other markers being studied include PLAT by RT-PCR and Lipocalin 2.

An expression of S100P, IMP3 and Maspin with a non-expression of von Hippel-Lindau gene product (pVHL) were significantly correlated with pancreatic ductal adenocarcinoma (PDAC) [35].

4.8 Renal neoplasms

Molecular tests have been studied as an ancillary adjunct for the diagnosis of malignancy in renal FNAB. However, a very few reports are available on the same [1, 3].

4.9 Infectious diseases

A PCR is one of the most popular diagnostic adjuncts in addition to the cytomorphology with microbiologic staining and cultures for the diagnosis of tuberculous lymphadenitis. Molecular tests can be used to increase the rate of detection and identify the resistant strains [1].

Various studies have advocated the use of RT-PCR to detect M. Tuberculosis with a higher sensitivity. Primers and Probes for the real-time PCR were designed on the basis of the internal transcribed spacer sequence, enabling the recognition of Mycobacterium Avium and Mycobacterium Tuberculosis. The tests showed no false

positive with other mycobacterial species and other pathogens causing lymphadenitis. The technique needs no hybridization or further processing time for analysis [36].

Even in the absence of epithelioid granulomas/necrosis, Avidor et al. developed a PCR method for amplification of Bartonella Henselae DNA for an accurate diagnosis of Cat Scratch disease, obviating the need for excisional biopsy [1].

Starac et al. reported the presence of human papilloma virus (HPV) DNA by PCR which can be successfully performed on FNA material. This can guide the clinicians in assigning the site of origin of metastatic squamous cell carcinoma, since the anogenital SCC has a high prevalence of high-risk HPV DNA particularly in the cervix [37].

4.10 Salivary glands

FNAB has a well-established role in the evaluation of salivary gland tumors. However, they have a significant morphologic diversity with a confounding presentation on FNAC which may not allow a specific diagnosis in many cases, making it a challenging area in cytopathology.

There has been an emergence of newer entities in the salivary gland tumors over the last decade, along with the characterization of specific translocations in a subset of these tumors.

Mammary analogue secretory carcinoma is a recently described entity characterized by a t(12;15)(q21;p13) translocation resulting in ETV6-NTRK3 fusion. Hyalinizing clear cell carcinoma is a low-grade tumor with infrequent nodal and distant metastasis, shown to harbor EWSR1-ATF1 gene fusion. The CRTC1-MAML2 fusion gene resulting from a t(11,19)(q21:13) translocation is now known to be a feature of mucoepidermoid carcinoma associated with an improved survival. A t(6,9)(q22–23:p23–24) translocation resulting in MYB-NFIB gene fusion has been identified in majority of adenoid cystic carcinomas.

Polymorphous low-grade adenocarcinoma is characterized by hot spot E710D mutations in PRKD1 gene, whereas the cribriform carcinomas are characterized by translocations in PRKD1–3 genes.

Notably, salivary duct carcinoma is a high-grade adenocarcinoma with the molecular and morphologic features similar to the invasive ductal carcinoma of breast, including the HER2 gene amplification, mutations of TP53, PIK3CA and HRAS and loss or mutation of PTEN. A subset of SDC with apocrine morphology is associated with overexpression of the androgen receptors [38].

As these mutations are recurrent, they serve as powerful diagnostic tools in the salivary gland tumor diagnosis and refinement of salivary gland cancer classification, along with serving as promising prognostic biomarkers and targets of therapy.

Although the molecular consequences of these translocations and their potential prognostic and therapeutic values are not well characterized, the resulting fusion oncogenes and oncoproteins can be used as diagnostic clues in salivary gland FNAB material in order to overcome the limitations of the cytomorphological evaluation alone [33].

4.11 Upper gastrointestinal lesions

Automated multiband imaging system can be used for EUS-FNA biopsy specimen for the evaluation of upper gastrointestinal subepithelial lesions.

Sample isolation processing by spectromicroscopy (SIPS) has been introduced as an alternative to rapid on-site cytologic evaluation, which is a useful but complicated procedure.

Automated multiband imaging system (AMUS) helps in calculating the whitish core amounts in EUS-FNA samples in patients with subepithelial lesions, which is found to be more useful than SIPS for an on-site evaluation for the gastrointestinal SEL [39].

5. Conclusions

The effectiveness of FNAB for rendering a specific diagnosis can be improved tremendously by the application of several ancillary modalities. Although in many cases cytomorphologic features alone might be sufficient for making a diagnosis, the use of ancillary tests is often necessary not only for rendering a specific diagnosis but, where malignant lesions are involved, for determining prognostic and predictive factors from the procured aspirate. Most currently available ancillary techniques can be used on FNAB specimens. Immunocytochemistry performed on direct smears, monolayered preparation and cell block sections of FNAB is the most commonly utilized ancillary technique. This can be widely routinely used on FNAB specimens for determining the organ of origin of a metastatic tumor, for classification and typing of tumors and for determining prognostic and predictive markers.

There are, however, no immunostains that can help in the distinction of benign from malignant lesions on FNAB. In comparison with immunocytochemistry, molecular tests can aid in the distinction of benign from malignant lesions, in determining the genetic abnormalities and genetic makeup of tumors that can be useful not only for making a more specific diagnosis but also for determining prognosis, response to therapy, and determining the presence or absence of specific molecular targets for selection of patients for targeted therapy.

The emerging concepts and technologies have a potential to affect the field of cytopathology and clinical practice. Although many of the advances are in a trial phase, further researches in the fields like FAST-FNA, Kisher image analysis, along with a biomarker cocktail would be useful to predict treatment options, targeted drug therapy and outcomes.

Conflict of interest

The authors declare no conflict of interest.

Abbreviations

FNAC	fine needle aspiration cytology
FNAB	fine needle aspiration biopsy
IHC	immunohistochemistry
ROSE	rapid on-site evaluation
TME	Tumor microenvironment
ND	non-diagnostic
ABCD	antibody barcoding with cleavable DNA
SCANT	single-cell analysis for tumor phenotyping
FAST-FNA	fast analytic screening technique for fine needle aspirates
PD-L1	Programmed cell death ligand 1

FAST	cold/hot score
HNSCC	head and neck squamous cell carcinoma
PCR	polymerase chain reaction
FISH	fluorescent in situ hybridization
EGFR	epithelial growth factor receptor
scRNA Seq	small conditional RNA sequencing
AI	artificial intelligence
SQC	squamous cell carcinoma
SCLC	small cell lung carcinoma
NSCLC	non-small cell carcinoma
TTF-1	thyroid transcription factor-1
HMW	high molecular weight
FBS	fiberoptic bronchoscopy
EBUS-TBNA	endobronchial ultrasound guided transbronchial needle aspiration
EBUS-GS	endobronchial ultrasound using a guide
CT-NAB	Computerized Tomography (CT)-guided percutaneous biopsy
ENB	electromagnetic navigation bronchoscopy
ROS1	receptor tyrosine kinase
ALK	anaplastic lymphoma kinase
VEGF	vascular endothelial growth factor
RET	rearranged during transfection
MET	mesenchymal epithelial transition factor
NGS	next genomic sequencing
UGFNA	ultrasound-guided FNA
PGFNA	palpation-guided FNA
HER2	human epidermal growth factor receptor 2
TBSRTC	the Bethesda system of reporting thyroid cytopathology
NIFTP	non-invasive follicular thyroid neoplasm with papillary like nuclear features
TMPRS4	transmembrane protease serine 4
ECM1	extracellular matrix protein
PDAC	pancreatic ductal adenocarcinoma
SIPS	sample isolation processing by spectromicroscopy
AMIS	automated multiband imaging system

Author details

Anjali Goyal
Department of Pathology, Smt NHL MMC Ahmedabad, Gujarat, India

*Address all correspondence to: anjali@knee.in

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