

Recent Concepts in Thyroid Cytology with Emphasis on Ancillary Techniques

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Thyroid Fine needle aspiration cytology (FNAC) has gone through a paradigm shift in approach towards thyroid lesions. The 2017 Bethesda classification has helped to redefine the various conditions of the thyroid. An attempt has been made to utilize the classification with an example in each category. A total of 274 cases were studied over a period of one year (2018) (from January to December) at a tertiary referral hospital. The majority of cases were non neoplastic accounting to 246 cases with neoplastic cases accounting for 28 cases. The other ancillary techniques like use of Agnors, Morphometric, Pattern analysis, Cell block, ICC (immunocytochemistry) with LBC (Liquid based cytology) have been highlighted. Brief mentions of molecular techniques and recent advances, newer concepts like NIFTP have been discussed to highlight their utility in surgical management of Thyroid.

Keywords: FNAC, Thyroid, Bethesda system NIFTP.

Fine Needle Aspiration Cytology (FNAC) of Thyroid is a useful, cost effective rapid painless outpatient accurate method for patient with thyroid conditions. Significant numbers of thyroid nodules, almost quarter of the cases are classified in the indeterminate category which includes Bethesda categories 3rd, 4th and 5th according to the 2017 Bethesda system of reporting thyroid cytopathology. This makes it difficult for management of the cases¹.

Many clinicians and pathologists have come up with various methods to improve and subcategorize the various cytological subgroups which have been possible by the latest Bethesda system 2017².

In South East Asian countries where thyroid lesions are common, many of the

endocrinologist and pathologist have established their own system of clinical practice, reporting and their own methodology of management of thyroid cases which follow the *American Thyroid Association (ATA)* guidelines and the Bethesda system³.

There are new concepts like UMP (Uncertain Malignant Potential) and NIFTP in the thyroid tumor classification. There are recent studies which put NIFTP (Non-Invasive Follicular Neoplasm with Papillary –like nuclear features) as a newly designated category for 10%-30% of thyroid carcinomas.⁴

The ancillary studies which can be done on thyroid FNA material and liquid –FNA helps in molecular testing of thyroid tumors with the introduction of Next Generation Sequence (NGS)

for DNA and RNA. It helps in reducing ambiguity and sub categorizes the cases on cytology to improve the management of thyroid cases.⁵

The present article will deal with the latest Bethesda classification 2017, its use by presenting cases in each category and discuss the recent concepts of thyroid cytology:

The 2017 Bethesda System for Reporting Thyroid Cytopathology: includes

- Category-1 Nondiagnostic Or Unsatisfactory,
- Category-2 Benign,
- Category-3 Atypia Of Undetermined Significance Or Follicular Lesion Of Undetermined Significance,
- Category-4 follicular neoplasm or suspicious for a follicular neoplasm
- Category -5 Suspicious For Malignancy
- Category-6 Malignancy-⁽⁶⁾.

MATERIAL AND METHODS

Total number of thyroid cases sent to the Department which underwent FNAC in the year 2018 was 274 in number of these 44 cases were under ultrasound guidance. The rest 230 cases were done by the conventional method. Non neoplastic cases made up for benign category totaling to 246 cases while 28 cases were neoplastic. (Table-1)

The non-neoplastic cases included Colloid Goiter, Nodular colloid Goiter, Hashimotos and lymphocytic thyroiditis. The neoplastic conditions of thyroid included cases belonging to various Bethesda categories. We have presented with example for each category

Approach to Thyroid FNAC

Approach to thyroid swelling includes a combination of clinical findings, radiological

imaging with fine needle aspiration findings. The position of patient during the procedure of FNAC should be *in supine* position. Increased exposure of gland is done by keeping pillow under neck with instructions to patient, to keep still and refrain from swallowing. The numbers of passes required for an adequate thyroid aspirate are 2-4 passes. Depending on *the* size of the lesion, it can increase to 6-8 passes so as to represent the whole thyroid. The needle should have different angles or points of entry.⁷

The needle gauge used and technique of making smears are 24-27 gauge needle. For a hemorrhagic aspirate two step techniques should be used. If colloid material is aspirated, the aspirate should be subjected to centrifugation & deposit should be made into 1-2 smears and stained by Haematoxylin and Eosin (H&E), May-Grünwald Giemsa (MGG) and Papanicalaou (PAP) stain⁷

Procedure of FNAC

The procedure for FNAC thyroid is by two techniques, the routine technique is by aspiration using 10ml syringe and needle (24-27g). In cases with haemorrhage, a non-aspiration sampling technique using only needle can be done. Both the techniques can be done under ultrasound guidance for non-diagnostic or unsatisfactory cases⁷

The Stains Used are PAP – for malignancy; MGG for background, colloid, fibroblasts; H&E for routine⁷

Category -1 Non diagnostic or unsatisfactory

Every thyroid FNA for diagnosis on cytology should be adequate or have a satisfactory smear. For an adequate or satisfactory smear for evaluation Bethesda criteria has been put forth. The criteria includes at least six groups of benign

Table 1. Case Distribution Of Thyroid Lesions On Cytology

Case	Conventional FNAC	USG Guided FNAC	Total
Colloid Goitre	96	21	117
Nodular Goitre	10	2	12
Autoimmune Thyroiditis	1		1
Hashimoto's	47	6	53
Lymphocytic	58	5	63
Follicular Neoplasm	13	4	17
Papillary Carcinoma	5	5	10
Medullary Carcinoma		1	1
Total	230	44	274

follicular cells with each group having at least 10 thyroid follicular cells.

The 2017 Bethesda BSRTC reinforces that if there is an abundant colloid or a specific diagnosis can be rendered or whenever there is significant atypia, the specimen is by definition adequate for evaluation.

Conditions where a definite diagnosis cannot be rendered due to increase in blood in smear, improper cell preservation and decrease in thyroid follicular cells, the diagnosis of unsatisfactory (UNS) or non-diagnostic (ND) ND/UNS is given⁸

This can be reduced by advising sonographic guidance, which is diagnostic in most cases. Surgery is advised if clinical and sonographic suspicion of malignancy is present.

Category -2 Benign

A case of a 50 year old Female with Clinical findings of neck swelling from midline to right side of neck. The swelling moves with deglutition. It is 3x3 cms in size with soft consistency. Clinical diagnosis is colloid goiter. Ultrasound finding shows a multi-nodular goiter with colloid degeneration. Biochemical findings are T3-1.62, T4- 12.11, TSH-0.005

Microscopy

Aspirate is satisfactory with smears which are moderately cellular. Benign thyroid follicular cells are seen and arranged in sheets. Background shows thick and thin colloid, with cyst macrophages.(Fig-1)

The diagnosis was confirmed by Histopathology as Nodular Colloid Goiter. The various differentials for multinodular goiter should be taken into consideration whenever the cellularity is more. The nuclear features mainly

help to distinguish between nodular colloid goiter, follicular neoplasm with micro follicles and cystic papillary carcinoma thyroid. The presence of abundant colloid in the background also helps in making a more definitive diagnosis^{9,10}

Category -3 AUS/FLUS

A 32 year old female with midline swelling since one year which is 3x2cms in size, oval, firm in consistency presented with Clinical diagnosis of Nodular colloid goiter. Ultra sound findings are: Nodular colloid goiter, showing heteroechoic lesion with hypo echoic halo, increased peripheral and internal vascularity.

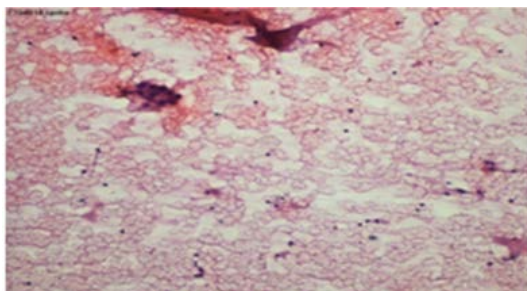
Microscopy

Aspirate is satisfactory. Smears studied shows thyroid follicular cells arranged in micro follicles. Background shows blood.

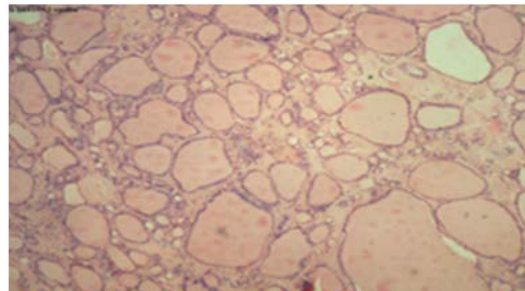
The diagnosis observed was Follicular Neoplasm/Follicular lesion of undetermined significance (FLUS) /Atypia of undetermined significance (AUS) Advised-Histopathological Examination, Immune markers on cell block. Histopathology gave a diagnosis of Follicular adenoma. Conditions associated with AUS and their importance is 3-6% of thyroid FNAC report, presence of architectural atypia and cellular atypia. The atypia is not sufficient to call follicular neoplasm or suspicious for malignancy, nor can be dismissed as benign/follicular lesion. Impression is it has a small but definite risk of malignancy which lies between that of benign and follicular neoplasm (5-10%).

The specific scenarios for AUS/FLUS (follicular lesions of undetermined significance) are,

- Nuclear features like ground glass appearance, nuclear grooving in an otherwise predominantly benign appearing sample.



a) Colloid goiter on cytology (H&E, 20x)



b) Colloid goitre HPE, Benign (H&E, 10X)

Fig. 1. Category-2 Benign

- Predominance of micro follicles in a sparsely cellular aspirate with scant colloid. Follicular cell atypia hindered by a sample preparation artifact (air- drying artifact or clotting artifact).
- Presence of hurtle cells in a clinical setting suggesting chronic lymphocytic thyroiditis or multinodular goiter^{11,12}

Approach to cellular thyroid aspirates in follicular patterned lesions

Whenever majority of the thyroid aspirate shows uniformly arranged follicles of thyroid cells with lumen filled with colloid on microscopic examination a diagnosis of follicular neoplasm is offered. The follicles can be based on their size either micro follicles or macro follicles; some lesions have both but a pattern dominates this follicular neoplasm on cytology can be further diagnosed as benign or malignant by the help of IHC markers or by BRAF molecular testing¹³

Category -4 Follicular Neoplasm/ Suspicious of Follicular Neoplasm-FN/SFN

The history is of a 30years old, Female with swelling in front of the neck from 6months 2x3 cms solitary nodule. Clinical diagnosis Colloid goiter. Ultrasonography showed features of thyroid lobe which is bulky and shows an isoechoic solid lesion, few hypo echoic areas noted within it. It shows both internal and peripheral vascularity. Tiradsva, T3-0.898 T4-7.18, TSH-1.84.

Microscopy

Aspirate shows moderately cellular smears which thyroid follicular cells arranged in micro follicles with mild nuclear atypia. Background shows blood. (Fig-2)

Diagnosis

Follicular Neoplasm or suspicious for

a Follicular Neoplasm. Differential of (follicular variant of papillary carcinoma thyroid FVPTC).and NIFTP(noninvasive follicular thyroid neoplasm with papillary-like nuclear features).

Advised Molecular testing, Surgery after risk Assessments definitive distinction between FVPTC and NIFTP is not possible on cytology alone. The diagnosis given on histopathology was Follicular Variant of Papillary Carcinoma of Thyroid.

Follicular-patterned cases with mild nuclear changes (increased nuclear size, nuclear contour irregularity, and/or chromatin clearing) can be classified as FN/SFN.⁶There should not be even a single papillae or a pseudo inclusion favouring a papillary carcinoma. A note of caution regarding follicular variant of papillary carcinoma or NIFTP should be mentioned with advice for molecular testing or IHC.

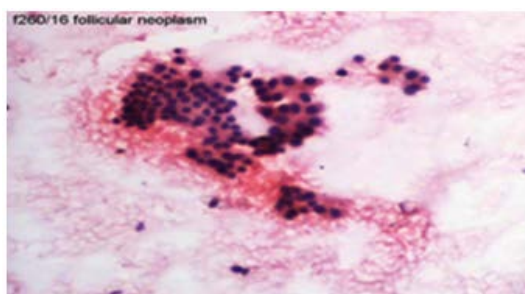
Category -5 Suspicious of malignancy

The clinical history is of a case of a 58 years old female with swelling in front of neck, 4x3 cm, globular and firm. Clinical Diagnosis was Colloid goiter. Ultrasound findings: Colloid nodules in both lobes of thyroid, cervical level II and III lymph nodes noted reactive. Right lobe swelling 3.7x7.5mm and left lobe 5x3 mm.

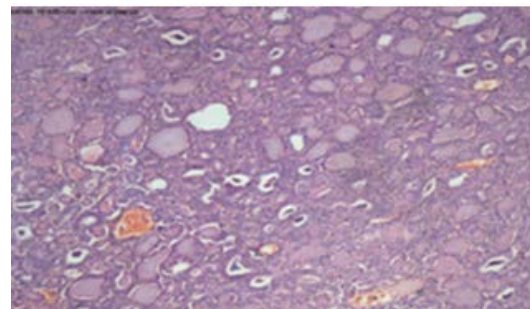
Microscopy

Smears are cellular and show pleomorphic cells showing abundant cytoplasm with hyper chromatic nucleus. Single tumorcells seen in the background. Amorphous amyloidseen which stained with Congo red. Cell Block shows Calcitonin positive tumor cells. (Fig-3)

A diagnosis of malignant thyroid neoplasm was done on cytology with suspicion of



a) Thyroid Follicular Neoplasm on Cytology(PAP,20X)



b) Follicular variant of Papillary carcinoma Thyroid HPE (H&E,20X)

Fig. 2. Category-4 Follicular neoplasm

medullary carcinoma. Confirmation was done by using special stain Congo Red on cytology slide and on histopathology section. IHC Calcitonin was done on cell block which showed positivity. Serum Calcitonin level assay showed raised levels. Diagnosis of Medullary carcinoma thyroid was confirmed both by histopathology and IHC on cell block.

Medullary thyroid carcinoma (MTC) is a rare, well differentiated neuroendocrine carcinoma that arises from parafollicular thyroid cell. It accounts for approximately 1-2% of thyroid malignancies¹⁴. As medullary carcinoma presents with a wide variety of histologic patterns, it is important to perform immuno-histochemistry in any thyroid tumour that lacks definite colloid or is simply not typical of another type of thyroid carcinoma.

Amyloid is a characteristic histologic feature in Medullary thyroid carcinomas (MTC). and present in approximately 80% of the tumors and is composed of Calcitonin secreted by the tumour cells. Amyloid can be readily detected by histochemical stains such as Congo red¹⁵. Calcitonin is the most useful marker for the

diagnosis of MTC, with a good sensitivity ranging from 74% to 100% on cytology¹⁶.

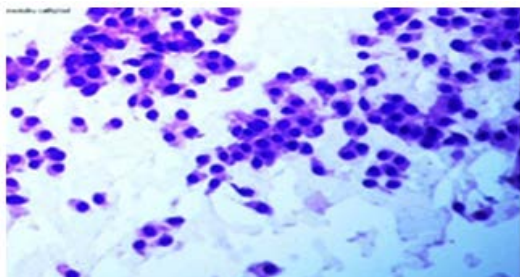
Calcitonin has been identified in the amyloid stroma by immuno- histochemistry and immunofluorescence. Congo red and Calcitonin were used on cytology & cell block respectively with confirmation of the case by histopathology.

Category-6 Malignancy

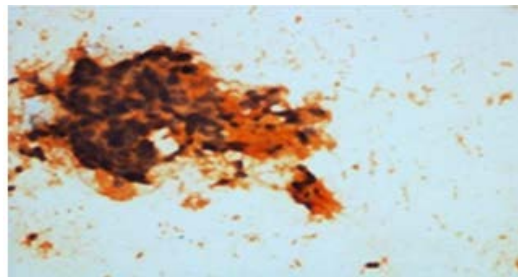
History of a 43year old female, with clinical features of neck swelling, measuring 3x3cms firm solitary nodule. Clinical diagnosis: was Colloid goiter, Ultrasound showed well defined solid hyper echoic nodule in right lobe of thyroid with peripheral and central vascularity and multiple calcifications TIRADS IVA, T3-1.37, T4-8.12, and TSH 4.42.

Microscopy

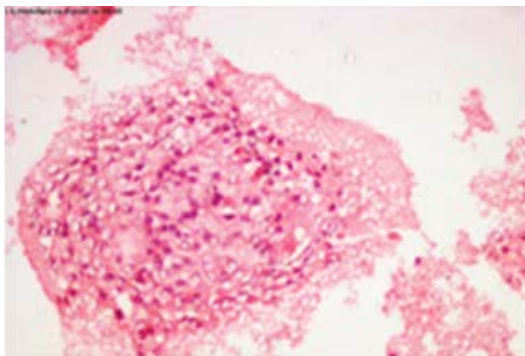
Aspirate is satisfactory. Smears are highly cellular. Smears studied show tumor cells arranged in papillary fronds, sheets and clusters. Individual tumor cells are arranged in palisading pattern. Nuclear grooves and intranuclear inclusions seen. Background shows colloid and blood. The diagnosis offered is Papillary Carcinoma Thyroid which was confirmed on Histopathology. (Fig-4)



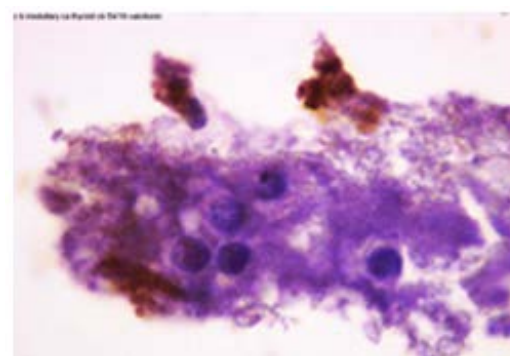
a. FNAC Medullary carcinoma(MGG,20X)



b. Congo Red positive (20x)



c. Cell Block of thyroid (H&E,20x)



d. Calcitonin positive on cell block(40x)

Fig. 3. Category- 5 Medullary carcinoma Thyroid

Cytologic Major & Minor criteria's for diagnosis of Papillary Carcinoma

Major criteria

Enlarged oval and irregular nuclei, fine chromatin, multiple eccentric micronucleoli. Longitudinal chromatin ridge or nuclear groove, intranuclear cytoplasmic inclusions. All criterias are essentially nuclear. A constellation of features are necessary for diagnosis.

Minor criteria

The minor criteria are the following. Papillary cyto-architecture with syncytial monolayers, thick colloid, psammoma bodies with calcific debris, occasional giant cells, and histiocytoid cells⁽¹⁷⁾.

Ancillary Techniques

Use of AgNORS in Thyroid Lesions

Nucleolar organizer regions (NORS) can be located by using silver nitrate on cytopreparations. It is a simple, easily manageable, reproducible and cost-effective method for differentiating benign from malignant lesions of thyroid. Colloid Goitre is taken as the control for studying any condition of thyroid⁽¹⁸⁾.

Role of morphometry in diagnosis of Follicular Neoplasms

Four nuclear parameters are selected and compared with a standard the factors of the nucleus assessed are Mean nuclear area, Mean nuclear perimeter, largest to smallest diameter ratio of the nuclei, and coefficient of variation of the nuclear area it helps to differentiate between benign and malignant follicular neoplasm⁽¹⁹⁾

Uses of Liquid Based Cytology in Thyroid FNAC

Artifacts caused by conventional sample method account for significant proportion of inconclusive or AUS/FLUS results in thyroid

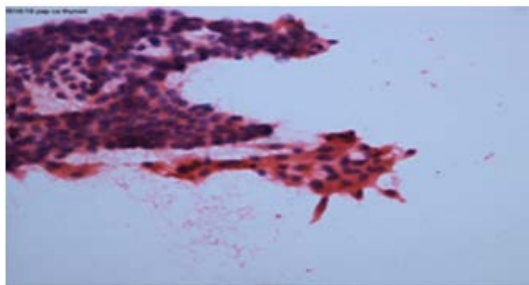
FNA. Liquid based cytology helps to improve the diagnosis and reduce the number of AUS/FLUS. It is done by improving the quality of cytomorphology with absence of obscuring elements and preservation of cells adequately. It helps in using the residual material obtained by LBC for ancillary studies. Eg. like molecular testing for BRAF/RAS in Papillary carcinomas.⁽²⁰⁾

Role of Cell Block in Thyroid FNAC

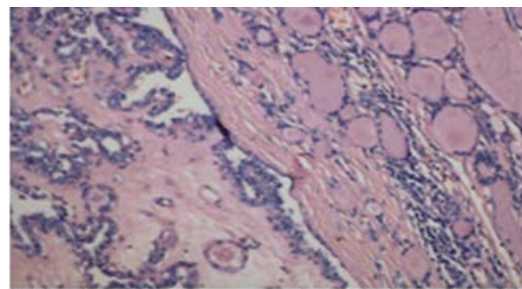
Advantages are that the cell block section helps in meeting the architectural details especially to differentiate papillary carcinoma from papillary hyperplasia. It helps in diagnosing papillary carcinoma of thyroid at metastatic sites. Marker studies, special stains can be done on cell block preparations for better diagnostic accuracy of thyroid nodules. Confirmation of malignant thyroid conditions, can be done by IHC studies on cell block²¹

Role of immunocytochemistry in Thyroid lesions

Immunocytochemistry (ICC) and cytoenzymology (CE) are widely available nowadays in the market and be used to diagnose intermediate categories of thyroid conditions. They can be performed on smears (cell blocks, or liquid-based preparation). Markers that are, most important for diagnosis on thyroid FNA are Hectforamesothelial antigen-1 (HBME-1), thyroid peroxidase (TPO), and dipeptidylaminopeptidase IV (DPP4) can be used directly on smears. TTF-1, Thyroglobulin & Calcitonin are primary thyroid markers to identify and distinguish the cell type in less well differentiated neoplasms.⁽²²⁾ Cytokeratin 19 and CD44 are helpful in distinguishing Papillary carcinoma from other thyroid neoplasms & metastatic papillary carcinoma to thyroid. Gelectin – 3 & CD44v6 are helpful in diagnosing follicular carcinoma.



a) Papillary CA thyroid on cytology (PAP, 20x)



b) Papillary ca thyroid (H&E, 10X)

Fig. 4. Category 6 Malignancy

CD markers are helpful to confirm lymphoma in thyroid. Immunohistochemical markers including galectin-3, Hectortin-1, and cytokeratin-19 can be used to differentiate between benign and malignant thyroid lesions, the latter, in which it can be diffusely expressed.⁽²³⁾

Approach to cellular thyroid aspirates in follicular patterned lesions

There can be a method of pattern analysis in diagnosis of solitary nodule thyroid. Diagnostic accuracy by pattern analysis is very high, with specificity and sensitivity. It reduces the number of surgeries. It is reproducible. Various studies by different authors have found it to be an efficient and clinically reliable method.⁽²⁴⁾

Role of Radiology

Advantages of ultrasonography in FNAC of thyroid

Ultrasonography (US) has several advantages over palpation. In approaching thyroid FNAC, Real-time US permits visualization of the needle within the lesion, this helps to reach and aspirate small nonpalpable thyroid nodules which are suspicious of cancer. Even in palpable thyroid nodules, US guidance is superior to palpation as it helps to obtain the correct and adequate material for diagnosis of the lesions. It thus improves diagnostic accuracy of thyroid lesions.⁽²⁵⁾

Recent Advances

FNAC of thyroid has undergone many changes with many advances in the diagnostic categories brought in by Bethesda classification 2017. There are many cases in the indeterminate category AUS/FLUS, (Low-risk) to Follicular neoplasm/ Suspicious for Follicular Neoplasm (FN/SFN) of intermediate risk or suspicious for malignancy [SFM]. They are high risk on cytology, which need to be confirmed by other methods. Also it is imperative for other ancillary methods to be added to decide the surgical and clinical management of thyroid nodules. The emphasis of the latest classification is not to over diagnose and over treat benign cases of thyroid which present as solitary nodule.^{26,27} The new classification of thyroid which comes under the Endocrine Tumour classification includes borderline tumours. The new terms included are UMP and NIFTP wherein. (UMP) means uncertain malignant potential, NIFTP (non-invasive follicular neoplasm with

papillary-like nuclear features) which comes under the 2A2 category of the latest classification of endocrine tumor.⁽²⁸⁾

NIFTP comes under category 3rd of Bethesda classification FN/SFN which accounts for (10% to 30%) of Follicular neoplasms and has an indolent course. Another aspect of follicular neoplasm FVPTC (follicular variant of papillary thyroid carcinoma) has also to be noted and diagnosed by ancillary techniques.⁽⁴⁾

There have been advances in techniques to differentiate the different low risk to high risk FN/SFN to AUS/FLUS from benign and malignant. These are molecular testing techniques to detect changes in DNA and RNA of thyroid follicular cells by using next generation sequencing (NGS).⁽⁵⁾

The material for extracting DNA or RNA material can be done by using directly the FNA material or liquid based fine needle aspiration LB-FNA to improve mutation profiling. It thus improves diagnostic accuracy and thus avoids unwanted thyroidectomies. These help to decrease the number of indeterminate nodules in thyroid.⁽²⁹⁾

Molecular methods used are :

Affirm a gene expression classifier

This uses micro RNA profiles for the diagnosis of indeterminate nodules where surgery is not desirable. Material collected from two needle passes are preserved in RNA preservative. Good number of gene profiles from the common 142 genes to the high risk 25 gene profiles are studied. Any of the important 6 gene profiles, if they show changes then the nodule are considered suspicious.⁽³⁰⁾

BRAFV 600E

Gene mutation alone is an important marker to indicate malignancy, It is present in more than 99% of thyroid nodules and thus helps to differentiate the lesion from FN/SFN Bethesda 5th from malignancy Bethesda 6th which is important for surgical management.⁽³¹⁾

The seven gene test

A panel of combination of BRAF V600E, PAX8/PPARG, RET/PTC, HRAS, KRAS and NRAS is the seven gene panel. Another test panel which can be suggested wherever possible as it has been shown to have greater sensitivity and specificity.⁽³²⁾

Thyroseq v2 full ngs panel

The ThyroSeq v2 panel uses NGS for simultaneous analysis of multiple genes. ThyroSeq v2 tests for point mutations and small insertions/deletions in 14 genes, 42 types of gene fusions, and expression levels of 16 genes. It helps in detecting mutations or genetic changes in RET (medullary thyroid carcinoma) TERT promoter mutation, a mutation which is both a diagnostic and prognostic marker, and present at increased frequency in aggressive tumors such as poorly differentiated carcinoma and anaplastic thyroid carcinoma. It is also associated with poor result like increased risk of distant metastasis, persistent disease, and cancer specific mortality.⁽³³⁾ This can be detected by use of thyroseq v2⁽³³⁾

NIFTP (Noninvasive follicular variant of papillary thyroid carcinoma)

The 2017 revised thyroid cytopathology (TBSRTC) has introduced a new category wherein it has reclassified noninvasive follicular variant of papillary thyroid carcinoma to a new term which is noninvasive follicular thyroid neoplasm with papillary like nuclear features (NIFTP)^(6, 34) the subset which is included in the Bethesda category 3 of FN/SFN represents a benign indolent well circumscribed thyroid tumour with follicular architecture, absence of any papillary structures but with nuclear features which overlap with those of papillary carcinoma.

The cytological diagnosis of this subgroup is difficult as capsular or vascular invasion cannot be commented about on FNA. Also as there is subtle overlap of nuclear features between benign NIFTP and follicular variant of papillary thyroid carcinoma (FVPTC) there is some limitation to categorize this indeterminate condition.⁽³⁵⁾

Thus the factors which help to differentiate NIFTP from FVPTC rely on nuclear features which has an semiquantitative scoring system which are six parameters divided into three categories.

The three categories are,

1. Nuclear size, shape of nucleus whether overlapping or increased in size.
2. Nuclear membrane whether irregular contour or not.
3. Chromatin as to whether cleared or glassy appearance. A score of zero is given if each factor shows the features in less than 50% of the cells a score of one is given if more than 50% of the cells

show the features. A total score ranges from zero to three.⁽³⁶⁾

If the score is (0/1) it is benign, score (1/2) NIFTP score (2/3) FVPTC, with slight overlapping of the nuclear features⁽³⁷⁾.

Molecular analysis of NIFTP

NIFTP has lineage towards RAS mutations rather than BRAF mutations. Thus, showing that they are less prone for metastasis. This leads to a more conservative approach to these neoplasms with thyroid lobectomy alone being enough and obviates the need for complete thyroidectomy and radiation.⁽³⁸⁾

CONCLUSION

Thyroid FNAC has come a long way in diagnosis of thyroid conditions. The latest classification of thyroid. Bethesda 2017 helps to reduce the indeterminate cases of thyroid i.e. Bethesda 4th and 5th by the utility of IHC, Molecular testing and Liquid Based Cytology.

The new terms of NIFTP and FVPTC are given importance so as to reduce unwanted surgeries of indeterminate cases.

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