Journal of
Molecular Pathology

MDPI

Review

Molecular Testing of Thyroid Fine-Needle Aspiration: Local Issues and Solutions. An Interventional Cytopathologist Perspective

Claudio Bellevicine, Roberta Sgariglia, Mariantonia Nacchio, Caterina De Luca, Pasquale Pisapia, Francesco Pepe and Giancarlo Troncone *

Department of Public Health, University of Naples Federico II, 80131 Naples, Italy; claudio.bellevicine@unina.it (C.B.); roberta.sgariglia@unina.it (R.S.); mariantonia.nacchio@unina.it (M.N.); caterina.deluca@unina.it (C.D.L.); pasquale.pisapia@unina.it (P.P.); francesco.pepe4@unina.it (F.P.)
* Correspondence: giancarlo.troncone@unina.it; Fax: +39-081-7463-679

Abstract: Molecular testing has acquired a relevant role for diagnostic and prognostic stratification of indeterminate thyroid nodules. Besides the available commercial solutions marketed in the United States, various local testing strategies have been developed in the last decade. In this setting, the modern *interventional cytopathologist*, the physician who performs the both aspirate and the morphologic interpretation plays a key role in the correct handling of fine-needle aspiration (FNA) samples not only for microscopy but also for molecular techniques. This review summarizes experiences with local approaches to the molecular testing of thyroid FNA, highlighting the role of the modern interventional cytopathologist.

Keywords: interventional cytopathologist; FNA; thyroid; molecular techniques; molecular pathology; NGS; mRNA; microRNA; indeterminate

Citation: Bellevicine, C.; Sgariglia, R.; Nacchio, M.; De Luca, C.; Pisapia, P.; Pepe, F.; Troncone, G. Molecular Testing of Thyroid Fine-Needle Aspiration: Local Issues and Solutions. An Interventional Cytopathologist Perspective. *J. Mol. Pathol.* **2021**, *2*, 233–240. https://doi.org/10.3390/jmp2030020

Academic Editor: Paul A. VanderLaan

Received: 11 June 2021
Accepted: 9 July 2021
Published: 13 July 2021

1. Introduction

The updated Bethesda classification system for thyroid cytopathology (TBSRTC) includes molecular testing to refine fine-needle aspiration (FNA) indeterminate diagnoses [1]. Indeed, the American and European Thyroid Associations highlighted the importance of thyroid FNA molecular tests, although no consensus has yet been reached regarding the best molecular strategy [2–4]. In fact, advanced thyroid-specific molecular testing strategies are available, showing differences in their negative (NPV) and positive predictive values (PPV). The two most widely adopted commercially available thyroid FNA testing approaches are based on modern and comprehensive technologies either on *classifiers*, such as the RNA expression signatures to discriminate between benign and malignant nodules, or on the massive parallel sequencing for the detection of a very large number of genomic alterations, such as point mutations, gene fusions and copy number variations. The former includes tests with high NPV and moderate PPV (rule-out tests), whereas the latter may show high PPV (rule-in tests) or both high NPV and PPV [5]. The choice of the "ideal" molecular test is also influenced by the local prevalence of thyroid neoplasms and the resulting difference in malignancy rates in each TBSRTC category [3,6]. These advances and comprehensive thyroid-specific commercially available tests are centralized in North America private laboratories; in countries with universal healthcare system coverage like in Europe, the access to such advanced molecular tests can be prohibitive because of its very high cost and lack of reimbursement policies [7] As an alternative to thyroid-specific comprehensive testing, several centers have introduced sustainable solutions based on either the adoption of commercially available generic cancer panels not specifically designed for thyroid samples or the design and validation of narrow custom panels targeting the most relevant genomic alterations involved in thyroid carcinogenesis.

Comprehensive molecular testing may increase FNA accuracy, but only if special care is taken to ensure high quality in all the various steps inherent to the cytological workflow. Indeed, the relevance of proper nodule sampling under ultrasound (US) guidance, the correct handling of the aspirated material, the rapid on-site evaluation (ROSE), the careful cyto-preparations, the correct microscopic interpretation and standardized and meaningful reporting cannot be overemphasized. In this context, cytopathologists need to acquire increasingly complex skills beyond the traditional ones to ensure the full comprehension of the cytological material not only for the microscopic assessment but also for the advanced molecular technologies. Thus, cytopathologists should learn to interact as a bridge between the referring clinicians and the molecular laboratory staff, configuring a new multidisciplinary hybrid: the interventional molecular cytopathologist. This review summarizes experiences with local approaches to the molecular testing of thyroid FNAs, highlighting the role of the modern cytopathologist.

2. The 7-Gene Test

One of the first genomic biomarkers introduced in thyroid cytopathology routine practice has been the *BRAF* exon 15 p.V600E point mutation. Despite its absolute specificity, high cost-effectiveness and simple detection, *BRAF* exon 15 p.V600E as a single marker has demonstrated low sensitivity, in particular in the atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) and follicular neoplasm/suspicious for follicular neoplasm (FN/SFN) categories [8]. A broader strategy is represented by the 7-gene test, which covers the genomic alterations most frequently occurring in thyroid neoplasms, i.e., *BRAF* exon 15 p.V600E, *HRAS, NRAS, KRAS, RET/PTC1, RET/PTC3, PAX8/PPARg*. Unfortunately, this assay also exhibits limited sensitivity and specificity ranging from 18–100% and 82–100%, respectively [9]. These discrepant results obtained from different series may be owed to local differences in cancer prevalence and cytopathology practice patterns. However, a consistent association between the genomic alterations detected by the 7-gene test and diagnostic categories identified by TBSRTC has been observed. In particular, *BRAF* exon 15 p.V600E and RET/PTC (grouped together as *BRAF*-like mutations), highly specific for malignant outcomes, are most frequently detected in both suspicious-for-malignancy and malignant thyroid FNAs, whereas *RAS* and *RAS*-like mutations (*PAX8/PPARg* and *BRAF* mutations other than p.V600E alterations), which are usually observed in benign or low-risk follicular patterned neoplasms, are more frequently retrieved in samples diagnosed as AUS/FLUS and FN/SFN [10]. Additionally, when limited molecular testing approaches are employed, gene alteration associated with adverse outcomes, such as *TERT* promoter mutations, are retrieved in FNAs classified as malignant and featuring "aggressive" cytomorphology (e.g., *tall cells*) [11]. This close correlation between molecular biomarkers and microscopic features is also observed in the AUS/FLUS sub-categories identified by the morphological qualifiers of atypia. In fact, the sub-categorization of AUS/FLUS can assist in the identification of the indeterminate nodules that require surgical management, that is, AUS/FLUS featuring rare thyrocytes with slight nuclear atypia or extensive but mild alterations [12]. In fact, these cases have shown a higher risk of malignancy (ROM) than the other AUS/FLUS qualifiers (Hurthle cells, architectural atypia) and, in turn, are more frequently associated with the occurrence of *BRAF* exon 15 p.V600E alteration, supporting from a molecular standpoint the sub-classification of the AUS/FLUS category [13–15].

3. From Single Gene Testing to Parallel and Massive Sequencing Analysis

After the introduction of the next-generation sequencing (NGS) platforms, testing strategies have moved away from the traditional method, which allows the sequencing of a limited number of genes, in favor of massive parallel sequencing. However, the custom NGS panels specifically developed to comprehensively cover thyroid-specific genomic alterations by centralized laboratories are proprietary and not commercially available or locally employable (e.g., *Thyroseq*) [7]. Conversely, cancer-generic NGS panels, featuring the

most common genomic alterations found in solid tumors, represent a possible solution for the processing of cytological samples. These panels proved able to generate NGS libraries even when the DNA input obtained from thyroid FNA was suboptimal [16–18]. However, their design is not specifically targeted to analyze thyroid FNAs; thus, the presence of non-informative genes undermined their analytical performance, owing to less than optimal thyroid-cancer-specific amplicon coverage [18].

In particular, Le Mercier et al. assessed the performance of the Ion AmpliSeq Cancer Hotspot Panel, a commercial panel not specifically designed for the thyroid, on a small retrospective series of 34 FNAs with available histological follow-up [16]. The authors extracted the DNA from either cell blocks or direct smears, obtaining sufficient material in the majority of cases (29/34, 85.2%). The FNAs were microscopically classified with their own classification system. In particular, these cases were classified as "follicular proliferations", a heterogeneous category that includes all the indeterminate cases including the AUS/FLUS. The differences between this classification system and TBSRTC hamper a comparison of the results obtained by Le Mercier et al. with other series, though they reached a sensitivity of 71%, specificity of 89%, NPV of 63% and PPV of 92%.

Although a fully comprehensive genomic profile of thyroid FNAs is still only provided by centralized North American laboratories, recent efforts have been made to develop custom NGS panels. These latter have specifically been designed to detect genomic alterations in thyroid nodules with indeterminate cytology. The results have been promising, since these panels have demonstrated a diagnostic efficiency higher than that yielded by the 7-gene testing solutions and by the commercially available cancer-generic NGS panels [19–22].

Below, two of these latter studies that focused on the clinical output of the custom panels along with their analytical performance are discussed in detail [19,23]. Song et al. designed a custom NGS panel, the *FSZ-Thyroid NGS* panel, covering at least 1000 hotspots in 16 thyroid-cancer-related genes, including the *AKT1, BRAF, CTNNB1, EIF1AX, EZH1, GNAS, HRAS, KRAS, NRAS, PIK3CA, RET, SPOP, TERT, TP53, TSHR* and *ZNF148* genes. This panel was adopted to process a prospective series of 196 indeterminate FNAs (153 AUS/FLUS and 43 FN/SFN) [23]. High-quality nucleic acids were extracted from cells obtained by a dedicated pass. Cells were collected in a preservative solution and stored at 4 °C, obtaining low-quality material in only eight cases (4.1%). As far as clinical validation is concerned, the interpretation of the molecular results was based on the association of a given mutation with a malignant histology. In particular, the authors distinguished between high-risk (e.g., *BRAF*-like and *RAS*-like mutations with allelic frequency $\geq$ 30%), low-risk (e.g., *EIF1AX* and *RAS*-like mutations with low allelic frequency) and benign-like genomic alterations. The authors obtained, among the indeterminate Bethesda FNAs, a sensitivity of 73%, specificity of 80%, PPV of 71% and NPV of 82%, by considering the low-risk neoplasms, such as non-invasive follicular thyroid neoplasms with papillary-like nuclear features (NIFT-P) and follicular and well-differentiated tumors of uncertain malignant potential (FT-UMP) as benign nodules. Although one of the strengths of this series is its prospective approach, the indications for surgery were given not only on the basis of cytological and molecular features of the nodules but also taking into account their clinical and US appearances [23].

Sponziello et al. developed a broad NGS custom panel, designed to cover 19 thyroid-cancer-related genes. Remarkably, the design included the detection of a very large number of gene fusions [19]. In addition to DNA and RNA targets, the panel also interrogates the expression levels of miR-146b-5p to increase assay sensitivity. As a matter of the fact, the highest NPV (100%) was obtained by this combined approach on a retrospective series of 40 FNA samples classified as indeterminate with the Italian Reporting System [19]. This custom NGS panel also included quality-control expression genes to provide estimates of each sample's cellular composition (e.g., follicular thyroid cells, parafollicular cells or parathyroid cells). However, one of the FNAs in their series classified as suspicious by microscopy, which harbored a *BRAF* exon 15 p.V600E mutation, was a false-positive

because the subsequent histology revealed a follicular adenoma. Since the allelic frequency of this mutation was 6%, the authors argued about the presence of a cellular subclone within the nodule [19]. Thus, as shown by the above-reported experience, the implementation of custom NGS panels, specifically designed to include the most relevant thyroid-cancer genes, may represent the first move to offer affordable access to NGS at the local level for patients with undetermined thyroid FNA.

4. Beyond Mutational Panels: Expression Analysis of mRNA and microRNA

Unlike the genomic tests based on the recognition of point mutations in DNA and fusions in RNA, the approach based on expression classifiers is designed to differentiate between benign and malignant nodules through mRNA expression patterns. This approach has the advantage, over mutation analysis, to identify gene signatures that reflect pathway activation generated not only by genomic alterations but also by epigenetic modulation. MicroRNAs (miRNAs), which are small single-stranded noncoding RNA sequences (19–25 nucleotides), whose function is to regulate gene expression, also act as tumor suppressors. In fact, miRNAs deregulation is a common finding in malignant neoplasms, supporting a role for these molecules in carcinogenesis. The assessment of miRNAs has several advantages over that of mRNA, including the high stability during the fixation and processing of cyto-histologic specimens [24].

Preclinical experiences based on RNA extracted from benchtop FNAs confirmed the feasibility of RNA analysis on cytological material. In particular Mazeh et al. evaluated a narrow panel of miRNAs by real-time polymerase chain reaction (RT-PCR), demonstrating a significantly increased expression of this set of miRNAs in tumor samples [25]. Moreover, the same authors tested a larger number of 279 miRNAs on an NGS platform (HiSeq 2500) and could identify a set of 19 miRNAs showing the most significant differences in their expression levels between malignant and benign nodules. This set of miRNA was further assessed on a group of 35 patients with indeterminate cytological diagnosis, showing 91% sensitivity, 100% specificity, NPV 87% and PPV 100% [26]. Conversely, Giannini et al. adopted a high-throughput nucleic acid hybridization technology (nCounter) to train a set of 34 genes on ex vivo FNAs. The unsupervised hierarchical clustering of this set of genes revealed a significant difference in the expression signatures of FNAs obtained from benign and malignant thyroid nodules [27].

The clinical relevance of different miRNAs was investigated in two studies focusing on follicular-patterned lesions. miRNAs were assessed by RT-PCR on retrospective and prospective FNAs [28,29]. Rossi et al. showed that, among five potential miRNAs candidates, only miR-375 was overexpressed in the majority of malignant lesions [29]. Conversely, Stokowy et al. adopted a minimal classifier that included only two miRNAs, miR-484 and miR-148b-3p. The latter yielded promising results when differentiating follicular adenomas from follicular carcinomas, reaching 89% sensitivity and 87% specificity [28]. Panebianco et al. used a combination of mRNAs and miRNAs to develop a Bayesian-neural-network-based classifier. The authors trained their method on a series of 118 histologically proven benign and malignant FNAs. These latter also included samples carrying a *BRAF* exon 15 p.V600E mutation as positive control. Their method showed high predictive power, correctly classifying 100% of the malignant FNAs and 69.23% of the benign samples [30].

Lastly, two papers explored on indeterminate thyroid FNAs the usefulness of a diagnostic algorithm that included additional markers besides the miRNA and mRNA expression assessment, in particular *BRAF* exon 15 p.V600E mutation [31,32], galectin-3 immunocytochemistry [31] and the ratio of mitochondrial to nuclear DNA [32]. In particular, Paskaš et al. demonstrated that a decision-tree model based on the assessment of *BRAF* mutations, galectin-3 immunostaining and miR-221/miR-222 expression levels provided a sensitivity of 73.5%, a specificity of 89.8%, a PPV of 80.6% and an NPV of 85.5%, reaching an accuracy of 75.7% [31]. Conversely, Titov et al. assessed *HMGA2* gene expression, miRNA- 146b, -221 and -375 levels, along with the ratio of mitochondrial to nuclear DNA

and *BRAF* mutational status, in 122 FNAs diagnosed as indeterminate (Bethesda III and IV), showing 89.2% sensitivity, 92.9% specificity, 84.6% PPV and 95.2% NPV, results that were comparable to those of commercial, centralized tests, such as Afirma and Thyroseq [32].

Taken together, these experiences showed the feasibility of miRNA and mRNA evaluation on thyroid FNAs. However, these results were too heterogeneous to be directly comparable, because each study adopted different sets of miRNAs and mRNAs whose expression cut-offs were established on training sample series with different compositions in terms of benign and malignant histological entities.

5. Interventional Molecular Cytopathology

Besides molecular testing, optimal sample collection, cytopreparation, interpretation and reporting are key. It is widely held that when FNA is performed directly by cytopathologists the percentage of non-diagnostic specimens is limited. Similarly, when the FNA is taken by a surgeon or by a radiologist, proper smear preparation and adequacy assessment ensured on-site by a cytopathologist or cytotechnologist also limit the number of inadequate specimens. The *interventional cytopathologist* is the physician who performs at the same time the aspirate and the morphologic interpretation. In a commentary published in 1988, Grohs described the interventional cytopathologist as the "single operator-interpreter system; the practitioner obtains the history and performs the physical examination, aspirates the specimen, prepares the smears, and interprets the aspirate in light of all the information obtained" [33]. Indeed, the role of the interventional cytopathologist had already been shaped in the 1950s–1960s of the past century at the Karolinska Institute in Sweden, where physicians like Frazen, Zajiceck, Lowagen and Pier Esposti advocated that the entire process of aspiration and cytological interpretation should be managed by the same person, because this approach results in higher diagnostic accuracy [34]. Recently, the palpation approach has been largely substituted by US-guidance to improve the aspiration of subclinical thyroid nodules [35–37]. In this regard, is still debated if the interpretation of US features by the interventional cytopathologist should be integrated in the FNA reporting system to "refine" the microscopic interpretation [38]. Furthermore, the incorporation of US teaching in the training of cytotechnology students has also been proposed [39].

In the molecular cytopathology era, cytopathologists must embrace the new challenges of the next-generation pathology, in order to integrate the results obtained from the new deep-sequencing technologies with the clinical vocation of cytopathology, thus interfacing with both the molecular laboratory and referring clinicians. Cytopathologists can achieve this goal by performing FNAs by themselves or by assisting other physicians on-site, ensuring diagnostic material, not only for microscopy and traditional ancillary techniques such as immunocytochemistry, but also for the molecular characterization of FNAs [40,41].

In fact, cytopathologists' contributions are fundamental to obtain not only fewer inadequate specimens for microscopy but also to obtain more adequate samples for molecular tests. In Table 1, a preliminary set of unpublished data from thyroid FNAs tested with the 7-gene assay at the University of Naples Federico II [10] demonstrated that the number of FNAs inadequate for molecular testing was significantly higher among the samples taken by non-cytopathologists.

Table 1. Comparison of inadequate FNAs for molecular test between different proceduralists (interventional-cytopathologist and non-cytopathologist) at our Institution [10].

Proceduralist	Inadequate/Total FNAs	%	*p*-Value
Interventional-cytopathologist	129/1278	10%	0.0037
Non-cytopathologist	171/1186	14.4%	

Interestingly, a recent study showed that sample adequacy for microscopy and molecular tests might be influenced by different factors. In fact, although microscopic adequacy seems to benefit from ROSE performed either by a cytotechnologist or cytopathologist, the

adequacy of FNAs for molecular testing could also be related to the adoption of longer, thin needles by which a higher number of cells are entrapped and subsequently rinsed in the nucleic acid preservative solution [42]. When the on-site presence of the cytopathologist is not warranted, telepathology tools allow for an adequacy check of the specimens, increasing the success rate of molecular testing performed on cytopathology samples [43].

Among the new skills that a wannabe interventional molecular cytopathologist needs to acquire is the additional responsibility of effective communication with patients. In fact, although indeterminate thyroid FNA diagnoses carry a moderate to low ROM, patients may perceive these diagnoses as very worrisome, asking for a radical and often unnecessary surgical procedure [44]. In particular, it has been demonstrated that the anxiety of patients with a non-definitive FNA diagnosis persists long after the FNA procedure [45].

Besides the traditional skill of ROSE of the aspirated material to check adequacy by microscopy, a rapid on-site molecular evaluation (ROME) is also feasible, thanks to the availability of automated RT-PCR assays [46]. In fact, the interventional molecular cytopathologist can easily put the needle rinse material into a cartridge in which the RT-PCR process is miniaturized and fully automated, obtaining results in less than 2 h. With this fully automated approach, the interventional molecular cytopathologist could obtain a same-day cytological and molecular diagnosis that may reassure the patients, especially in peripheral FNA clinics where more limited financial resources are only sufficient to reimburse a preliminary screening based on less comprehensive and simpler assays [46,47].

Thus, thanks to the development of custom molecular panels together with the availability of automated assays, affordable testing solutions for thyroid FNAs can be introduced in local settings near patients' homes, moving the practice of interventional cytopathology into the modern era by integrating the traditional skills of FNA sampling, smearing and microscopic interpretation with expertise in molecular biology.

Author Contributions: Conceptualization, C.B., G.T.; Methodology, all authors; Software, all authors; Validation, all authors; Formal Analysis, all authors; Investigation, all authors; Resources, all authors; Data Curation, all authors; Writing—Original Draft Preparation, C.B., G.T.; Writing—Review and Editing, all authors; Visualization, all authors; Supervision, C.B., G.T.; Project Administration, C.B., G.T. All authors have read and agreed to the published version of the manuscript.

Funding: The authors have not declared a specific grant for this review from any funding agency in the public, commercial or not-for-profit sectors.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Data sharing not applicable.

Conflicts of Interest: Giancarlo Troncone reports personal fees (as speaker bureau or advisor) from Roche, MSD, Pfizer, Boehringer Ingelheim, Eli Lilly, BMS, GSK, Menarini, AstraZeneca, Amgen and Bayer, unrelated to the current work. The other authors have nothing to disclose.

References

1. Cibas, E.S.; Ali, S.Z. The 2017 Bethesda System for Reporting Thyroid Cytopathology. *Thyroid* **2017**, *27*, 1341–1346. [CrossRef]
2. Haugen, B.R.; Alexander, E.K.; Bible, K.C.; Doherty, G.M.; Mandel, S.J.; Nikiforov, Y.E.; Pacini, F.; Randolph, G.W.; Sawka, A.M.; Schlumberger, M.; et al. 2015 American Thyroid Association Management Guidelines for Adult Patients with Thyroid Nodules and Differentiated Thyroid Cancer: The American Thyroid Association Guidelines Task Force on Thyroid Nodules and Differentiated Thyroid Cancer. *Thyroid* **2016**, *26*, 1–133. [CrossRef]
3. Ferris, R.L.; Baloch, Z.; Bernet, V.; Chen, A.; Fahey, T.J.; Ganly, I.; Hodak, S.P.; Kebebew, E.; Patel, K.; Shaha, A.; et al. American Thyroid Association Statement on Surgical Application of Molecular Profiling for Thyroid Nodules: Current Impact on Perioperative Decision Making. *Thyroid* **2015**, *25*, 760–768. [CrossRef] [PubMed]
4. Paschke, R.; Cantara, S.; Crescenzi, A.; Jarzab, B.; Musholt, T.J.; Simoes, M.S. European Thyroid Association Guidelines regarding Thyroid Nodule Molecular Fine-Needle Aspiration Cytology Diagnostics. *Eur. Thyroid. J.* **2017**, *6*, 115–129. [CrossRef]
5. Nishino, M.; Krane, J.F. Role of Ancillary Techniques in Thyroid Cytology Specimens. *Acta Cytol.* **2020**, *64*, 40–51. [CrossRef] [PubMed]

6. Valderrabano, P.; Leon, M.E.; Centeno, B.A.; Otto, K.J.; Khazai, L.; McCaffrey, J.C.; Russell, J.S.; McIver, B. Institutional prevalence of malignancy of indeterminate thyroid cytology is necessary but insufficient to accurately interpret molecular marker tests. *Eur. J. Endocrinol.* **2016**, *174*, 621–629. [CrossRef]
7. Nishino, M.; Bellevicine, C.; Baloch, Z. Molecular Tests for Risk-Stratifying Cytologically Indeterminate Thyroid Nodules: An Overview of Commercially Available Testing Platforms in the United States. *J. Mol. Pathol.* **2021**, *2*, 135–146. [CrossRef]
8. Trimboli, P.; Treglia, G.; Condorelli, E.; Romanelli, F.; Crescenzi, A.; Bongiovanni, M.; Giovanella, L. BRAF-mutated carcinomas among thyroid nodules with prior indeterminate FNA report: A systematic review and meta-analysis. *Clin. Endocrinol.* **2015**, *84*, 315–320. [CrossRef]
9. Nishino, M. Molecular cytopathology for thyroid nodules: A review of methodology and test performance. *Cancer Cytopathol.* **2016**, *124*, 14–27. [CrossRef]
10. Bellevicine, C.; Migliatico, I.; Sgariglia, R.; Bs, M.N.; Vigliar, E.; Pisapia, P.; Iaccarino, A.; Bruzzese, D.; Fonderico, F.; Salvatore, D.; et al. Evaluation of BRAF, RAS, RET/PTC, and PAX8/PPARg alterations in different Bethesda diagnostic categories: A multicentric prospective study on the validity of the 7-gene panel test in 1172 thyroid FNAs deriving from different hospitals in South Italy. *Cancer Cytopathol.* **2020**, *128*, 107–118. [CrossRef] [PubMed]
11. Dell'Aquila, M.; Fiorentino, V.; Martini, M.; Bd, S.C.; Cenci, T.; Lombardi, C.P.; Raffaelli, M.; Pontecorvi, A.; Fadda, G.; Pantanowitz, L.; et al. How limited molecular testing can also offer diagnostic and prognostic evaluation of thyroid nodules processed with liquid-based cytology: Role of TERT promoter and BRAF V600E mutation analysis. *Cancer Cytopathol.* **2021**. [CrossRef] [PubMed]
12. Nishino, M.; Wang, H.H. Should the thyroid AUS/FLUS category be further stratified by malignancy risk? *Cancer Cytopathol.* **2014**, *122*, 481–483. [CrossRef]
13. Bellevicine, C.; Sgariglia, R.; Migliatico, I.; Vigliar, E.; D'Anna, M.; Nacchio, M.A.; Serra, N.; Malapelle, U.; Bongiovanni, M.; Troncone, G. Different qualifiers of AUS/FLUS thyroid FNA have distinct BRAF, RAS, RET/PTC, and PAX8/PPARg alterations. *Cancer Cytopathol.* **2018**, *126*, 317–325. [CrossRef]
14. Park, H.J.; Moon, J.H.; Yom, C.K.; Kim, K.H.; Choi, J.Y.; Choi, S.I.; Ahn, S.-H.; Jeong, W.-J.; Lee, W.W.; Park, S.Y. Thyroid "Atypia of undetermined significance" with nuclear atypia has high rates of malignancy and BRAF mutation. *Cancer Cytopathol.* **2014**, *122*, 512–520. [CrossRef] [PubMed]
15. Hyeon, J.; Ahn, S.; Shin, J.H.; Oh, Y.L. The prediction of malignant risk in the category "atypia of undetermined significance/follicular lesion of undetermined significance" of the Bethesda System for Reporting Thyroid Cytopathology using subcategorization and BRAF mutation results. *Cancer Cytopathol.* **2014**, *122*, 368–376. [CrossRef] [PubMed]
16. Le Mercier, M.; D'Haene, N.; De Nève, N.; Blanchard, O.; Degand, C.; Rorive, S.; Salmon, I. Next-generation sequencing improves the diagnosis of thyroid FNA specimens with indeterminate cytology. *Histopathology* **2015**, *66*, 215–224. [CrossRef]
17. Roy-Chowdhuri, S.; Goswami, R.S.; Chen, H.; Patel, K.P.; Routbort, M.J.; Singh, R.R.; Broaddus, R.R.; Barkoh, B.A.; Manekia, J.; Yao, H.; et al. Factors affecting the success of next-generation sequencing in cytology specimens. *Cancer Cytopathol.* **2015**, *123*, 659–668. [CrossRef] [PubMed]
18. Bellevicine, C.; Sgariglia, R.; Malapelle, U.; Vigliar, E.; Nacchio, M.; Ciancia, G.; Eszlinger, M.; Paschke, R.; Troncone, G. Young investigator challenge: Can the Ion AmpliSeq Cancer Hotspot Panel v2 be used for next-generation sequencing of thyroid FNA samples? *Cancer Cytopathol.* **2016**, *124*, 776–784. [CrossRef]
19. Sponziello, M.; Verrienti, A.; Pecce, V.; Abballe, L.; Grani, G.; Ramundo, V.; Fadda, G.; Brunelli, C.; Durante, C.; Filetti, S. Performance of a dual-component molecular assay in cytologically indeterminate thyroid nodules. *Endocr. Abstr.* **2020**, *68*, 458–465. [CrossRef]
20. Verrienti, A.; Pecce, V.; Abballe, L.; Ramundo, V.; Falcone, R.; Jak, F.I.N.; Brunelli, C.; Fadda, G.; Bosco, D.; Ascoli, V.; et al. Analytical validation of a novel targeted next-generation sequencing assay for mutation detection in thyroid nodule aspirates and tissue. *Endocrinology* **2020**, *69*, 451–455.
21. De Biase, D.; Acquaviva, G.; Visani, M.; Sanza, V.; Argento, C.M.; De Leo, A.; Maloberti, T.; Pession, A.; Tallini, G. Molecular diagnostic of solid tumor using a next generation sequencing custom-designed multi-gene panel. *Diagnostics* **2020**, *23*, 250. [CrossRef]
22. Sgariglia, R.; Nacchio, M.; Migliatico, I.; Vigliar, E.; Malapelle, U.; Pisapia, P.; De Luca, C.; Iaccarino, A.; Salvatore, D.; Masone, S.; et al. Moving towards a local testing solution for undetermined thyroid fine-needle aspirates: Validation of a novel custom DNA-based NGS panel. *J. Clin. Pathol.* **2021**. [CrossRef]
23. Song, Y.; Xu, G.; Ma, T.; Zhu, Y.; Yu, H.; Yu, W.; Wei, W.; Wang, T.; Zhang, B. Utility of a multigene testing for preoperative evaluation of indeterminate thyroid nodules: A prospective blinded single center study in China. *Cancer Med.* **2020**, *9*, 8397–8405. [CrossRef]
24. Monroe, R.J.; Vora, A. Molecular Diagnostics in Thyroid Cytopathology. In *Molecular Cytopathology*; Springer: Berlin/Heidelberg, Germany, 2016; pp. 67–97.
25. Mazeh, H.; Mizrahi, I.; Halle, D.; Ilyayev, N.; Stojadinovic, A.; Trink, B.; Mitrani-Rosenbaum, S.; Roistacher, M.; Ariel, I.; Eid, A. Development of a microRNA-based molecular assay for the detection of papillary thyroid carcinoma in aspiration biopsy samples. *Thyroid* **2011**, *21*, 111–118. [CrossRef] [PubMed]

26. Mazeh, H.; Deutch, T.; Karas, A.; Bogardus, K.A.; Mizrahi, I.; Gur-Wahnon, D.; Ben-Dov, I.Z. Next-Generation Sequencing Identifies a Highly Accurate miRNA Panel That Distinguishes Well-Differentiated Thyroid Cancer from Benign Thyroid Nodules. *Cancer Epidemiol. Biomark. Prev.* **2018**, *27*, 858–863. [CrossRef] [PubMed]
27. Giannini, R.; Torregrossa, L.; Gottardi, S.; Fregoli, L.; Borrelli, N.; Savino, M.; Macerola, E.; Vitti, P.; Miccoli, P.; Basolo, F. Digital gene expression profiling of a series of cytologically indeterminate thyroid nodules. *Cancer Cytopathol.* **2015**, *123*, 461–470. [CrossRef] [PubMed]
28. Stokowy, T.; Wojtas, B.; Jarzab, B.; Krohn, K.; Fredman, D.; Dralle, H.; Musholt, T.; Hauptmann, S.; Lange, D.; Hegedus, L. Two-miRNA classifiers differentiate mutation-negative follicular thyroid carcinomas and follicular thyroid adenomas in fine needle aspirations with high specificity. *Endocrine* **2016**, *54*, 440–447. [CrossRef] [PubMed]
29. Rossi, E.D.; Bizzarro, T.; Martini, M.; Capodimonti, S.; Sarti, D.; Cenci, T.; Bilotta, M.; Fadda, G.; Larocca, L.M. The evaluation of miRNAs on thyroid FNAC: The promising role of miR-375 in follicular neoplasms. *Endocrinology* **2016**, *54*, 723–732. [CrossRef]
30. Panebianco, F.; Mazzanti, C.; Tomei, S.; Aretini, P.; Franceschi, S.; Lessi, F.; Di Coscio, G.; Bevilacqua, G.; Marchetti, I. The combination of four molecular markers improves thyroid cancer cytologic diagnosis and patient management. *BMC Cancer* **2015**, *15*, 918. [CrossRef]
31. Paskas, S.; Jankovic, J.; Živaljević, V.; Tatic, S.; Bozic, V.; Nikolić, A.; Radojković, D.; Savin, S.; Cvejič, D. Malignant risk stratification of thyroid FNA specimens with indeterminate cytology based on molecular testing. *Cancer Cytopathol.* **2015**, *123*, 471–479. [CrossRef]
32. Titov, S.; Demenkov, P.S.; Lukyanov, S.A.; Sergiyko, S.V.; Katanyan, G.A.; Veryaskina, Y.A.; Ivanov, M.K. Preoperative detection of malignancy in fine-needle aspiration cytology (FNAC) smears with indeterminate cytology (Bethesda III, IV) by a combined molecular classifier. *J. Clin. Pathol.* **2020**, *73*, 722–727. [CrossRef] [PubMed]
33. Grohs, H.K. Starting Out: The Interventional Cytopathologist: A New Clinician/Pathologist Hybrid. *Am. J. Clin. Pathol.* **1988**, *90*, 351–354. [CrossRef]
34. Linsk, J.A. Aspiration cytology in sweden: The karolinska group. *Diagn. Cytopathol.* **1985**, *1*, 332–335. [CrossRef] [PubMed]
35. Bellevicine, C.; Vigliar, E.; Malapelle, U.; Pisapia, P.; Conzo, G.; Biondi, B.; Vetrani, A.; Troncone, G. Cytopathologists can reliably perform ultrasound-guided thyroid fine needle aspiration: A 1-year audit on 3715 consecutive cases. *Cytopathology* **2016**, *27*, 115–121. [CrossRef] [PubMed]
36. Wu, M.; Choi, Y.; Zhang, Z.; Si, Q.; Salem, F.; Szporn, A.; Zhang, D.; Burstein, D.; Teng, M.; Miles, B.A.; et al. Ultrasound guided FNA of thyroid performed by cytopathologists enhances Bethesda diagnostic value. *Diagn. Cytopathol.* **2016**, *44*, 787–791. [CrossRef] [PubMed]
37. Rollins, S.D. Teaching FNA techniques and ultrasound guided FNA. *Cancer Cytopathol.* **2019**, *127*, 7–8. [CrossRef] [PubMed]
38. Renshaw, A.A.; Gould, E.W.; Russ, G.; Poller, D.N. Thyroid FNA: Is cytopathologist review of ultrasound features useful? *Cancer Cytopathol.* **2020**, *128*, 523–527. [CrossRef]
39. Varley, K.E.; Monaco, S.E. Guiding cytotechnology students toward the future: Incorporating the use of ultrasound in FNA training. *Cancer Cytopathol.* **2020**, *128*, 685–687. [CrossRef]
40. Pagni, F.; Di Bella, C. The Interventional Pathologist. *Arch. Pathol. Lab. Med.* **2013**, *137*, 11–12. [CrossRef]
41. Fischer, A.H.; Benedict, C.C.; Amrikachi, M. Five Top Stories in Cytopathology. *Arch. Pathol. Lab. Med.* **2013**, *137*, 894–906. [CrossRef]
42. Landau, M.S.; Pearce, T.M.; Carty, S.E.; Wolfe, J.; Yip, L.; McCoy, K.L.; Lebeau, S.O.; Tublin, M.E.; Ohori, N.P. Comparison of the collection approaches of 2 large thyroid fine-needle aspiration practices reveals differing advantages for cytology and molecular testing adequacy rates. *J. Am. Soc. Cytopathol.* **2019**, *8*, 243–249. [CrossRef] [PubMed]
43. Al Zoughbi, W.; Kim, D.; Alperstein, S.A.; Ohara, K.; Manohar, J.; Greco, N.; Khani, F.; Robinson, B.D.; Rao, R.A.; Elemento, O. Incorporating cytologic adequacy assessment into precision oncology workflow using telepathology: An institutional experience. *Cancer Cytopathol.* **2021**. [CrossRef]
44. Pitt, S.C.; Saucke, M.M.C.; Wendt, M.E.M.; Schneider, D.F.; Orne, J.; Macdonald, C.L.; Connor, N.P.; Sippel, R.S. Patients' Reaction to Diagnosis with Thyroid Cancer or an Indeterminate Thyroid Nodule. *Thyroid* **2021**, *31*, 580–588. [CrossRef] [PubMed]
45. Lodewijk, L.; Vriens, M.R.; Vorselaars, W.M.; Van Der Meij, N.T.; Kist, J.W.; Barentsz, M.W.; Verkooijen, H.M.; Rinkes, I.H.B.; Valk, G.D. Same-Day Fine-Needle Aspiration Cytology Diagnosis for Thyroid Nodules Achieves Rapid Anxiety Decrease and High Diagnostic Accuracy. *Endocr. Pract.* **2016**, *22*, 561–566. [CrossRef] [PubMed]
46. De Luca, C.; Sgariglia, R.; Nacchio, M.; Pisapia, P.; Migliatico, I.; Clery, E.; Gragnano, G.; Campione, S.; Vigliar, E.; Malapelle, U. Rapid On-site Molecular Evaluation in thyroid cytopathology: A same-day cytological and molecular diagnosis. *Diagn. Cytopathol.* **2020**, *48*, 300–307. [CrossRef] [PubMed]
47. Hynes, S.O.; Pang, B.; James, J.A.; Maxwell, P.; Salto-Tellez, M. Tissue-based next generation sequencing: Application in a universal healthcare system. *Br. J. Cancer* **2017**, *116*, 553–556. [CrossRef] [PubMed]