Review

Current State and Future Perspective of Molecular Diagnosis of Fine-Needle Aspiration Biopsy of Thyroid Nodules

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Context: Fine-needle aspiration biopsy (FNAB) is the most sensitive and specific tool for the differential diagnosis of thyroid malignancy. Some limitations of FNAB can be overcome by the molecular analysis of FNAB. This review analyzes the current state and problems of the molecular analysis of FNAB as well as possible goals for increasing the diagnostic rate, especially in the indeterminate/follicular lesion cytological group.

Evidence Acquisition: Twenty publications were evaluated for the diagnostic material and assay systems used, the type, and the number of mutations screened. Sensitivity, specificity, and false-negative and false-positive rates were calculated for all publications.

Evidence Synthesis: Testing for a panel of somatic mutations is most promising to reduce the number of indeterminate FNAB. A mean sensitivity of 63.7% was achieved for indeterminate lesions. However, there is a broad sensitivity range for the investigation of mutations in the indeterminate lesions. Therefore, additional molecular markers should be defined by mRNA and microRNA expression studies and evaluated in FNAB samples of thyroid carcinomas without known somatic mutations, and especially for the many benign nodules in the indeterminate/follicular lesion fine-needle aspiration cytology category. This approach should improve the differential diagnosis of indeterminate/follicular lesion FNAB samples.

Conclusion: Testing for a panel of somatic mutations has led to an improvement of sensitivity/specificity for indeterminate/follicular proliferation FNAB samples. Further methodological improvements, standardizations, and further molecular markers should soon lead to a broader application of molecular FNAB cytology for the differential diagnosis of thyroid nodules and to a substantial reduction of diagnostic surgeries. **(*J Clin Endocrinol Metab* 96: 2016–2026, 2011)**

Thyroid nodules are frequent clinical findings. Their reported prevalence varies from 3–76%, depending on the screening method and the population evaluated (1). However, the incidence of thyroid cancer is low. The annual incidence in areas not affected by nuclear fallout has been reported to range between 1.2 and 2.6 cases per 100,000 in men and between 2.0 and 3.8 cases per 100,000 in women, with higher incidences in countries like Sweden, France, Japan, and the United States (2). Therefore, patients with thyroid nodules require evidence-based strategies for the differential diagnosis and risk stratification for malignancy. The revised ETA/AME/AACE guideline (1) together with other guidelines (3–5) recommends that after stratification for malignancy risk by history, clinical assessment, high-resolution ultrasonography, and sensitive TSH assay, fine-needle aspiration biopsy (FNAB) is the basis for the management of thyroid nodules.

The preoperative FNAB can reduce the number of surgeries for thyroid nodules to 10% compared with a strategy without FNAB use, with a concomitant increase of thyroid malignancies from 3.1 (without FNAB) to 34%

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Abbreviations: FA, Follicular adenoma; FN, false-negative; FNA, fine-needle aspiration; FNAB, FNA biopsy; FP, false-positive; FTC, follicular thyroid carcinoma; miRNA, microRNA; PTC, papillary thyroid carcinoma; TN, true negative.

(with FNAB) (6). Under optimal conditions, 60–80% of the biopsied nodules can be classified as benign by cytology, and 3.5–5% are classified as malignant (1). However, the ratio of malignant:benign results for patients undergoing resection of thyroid nodules is still 1:15 in Germany (7) or 1:7 in Italy (8), resulting in a high number of "diagnostic" thyroid surgeries (among the 110,000 annual thyroid surgeries in Germany). Besides other reasons like a lack of stringent selection of suspicious nodules for FNAB by ultrasound malignancy criteria, this unsatisfactory situation is mainly due to some limitations of this FNAB-focused strategy. It is difficult to determine the rate of false-negative (FN) cytologies, which can range from 6–17% (9) because a nodule diagnosed as benign by FNAB is usually managed conservatively. Most important, 10–20% of the FNAB samples are classified as follicular proliferation/indeterminate, which cannot distinguish between follicular adenoma (FA), adenomatoid hyperplasia, follicular thyroid carcinoma (FTC), and follicular variant of papillary thyroid carcinoma (PTC) (1, 10). Therefore, patients with this cytological finding currently have to undergo (diagnostic) surgery, which will detect thyroid malignancy in about 20% of these patients (11). This means that 80% of the thyroid FNAB samples that were classified as follicular proliferation/indeterminate lesion by cytology will undergo diagnostic (unnecessary) thyroidectomy. Thus, this category is the most problematic FNAB category.

Application of Tumor-Specific Mutation Detection in FNAB Diagnosis

In recent years, immunohistological markers like galectin-3 (12–16), HBME-1 (13, 14, 16), fibronectin-1, CITED-1, and cytokeratin-19 (13, 14) have been investigated to improve the differential diagnosis between benign and malignant thyroid nodules. However, they have barely been adopted in daily routine diagnostics, mainly because of different methods used and because these markers show prominent overlap between FA and differentiated thyroid carcinomas (17, 18). Currently, no single cytochemical (or genetic) marker is specific and sensitive enough to reliably further specify or replace the morphological diagnosis of follicular lesion or suspicious for neoplasm.

With the discovery of somatic mutations for 42% of PTC and 65% of FTC, new perspectives for the classification and diagnosis of thyroid tumors in addition to histology have emerged (19). Molecular testing for somatic mutation has become an immediate approach and is currently the most promising future approach for molecular FNAB diagnosis (20–22). It will allow a further discrimination of the follicular proliferation/indeterminate and suspicious FNAB categories and a reduction in the number of diagnostic thyroid surgeries and the rate of FN. In recent years, nearly all of the somatic mutations have been tested for their applicability in FNAB diagnosis in different settings. To date, 16 studies have analyzed one mutation [*e.g. BRAF* or *RET/PTC* (23–38)], and four studies have analyzed several mutations (*BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARg*) (20, 39–41).

We analyzed these studies and calculated the false-positive (FP), FN, sensitivity, and specificity rates of those studies that used indeterminate/follicular lesion, follicular lesion of indeterminate significance/atypia of indeterminate significance FNAB category samples by comparing the result of the FNAB mutation detection with the final histology. Details of these studies are summarized in Table 1 and in Supplemental Tables 1 and 2 (published on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org). We excluded those studies that did not use FNAB for mutation analysis (28), those without an "indeterminate/follicular lesion" category for FNAB analysis (38), or those that do not describe the correlation between cytology and histology (27). Two further studies (33, 37) were excluded from the analyses because they analyzed only PTC samples with final histology. Because only one study (39) has thus far also detected the FNAB mutations in the histology of the tumors, we considered the detection of a mutation in the FNAB of a histologically malignant sample as true positive. The detection of a mutation in a histologically benign thyroid lesion was categorized as a FP case. Detecting no mutation in an FNAB sample from a histologically benign surgical sample was considered a true negative (TN), and finding no mutation in a histologically malignant lesion was categorized as FN. The means for FP, FN, sensitivity, and specificity are given in Tables 3 and 4. To account for the major differences, the studies are classified according to the type and numbers of mutations investigated.

Some authors used a part of the same FNAB material for cytology and molecular analysis (20, 23, 30, 31, 39–41), such as leftover cells in the needle bevel plus the needle washing (40), or a third (39), or part of the total FNAB material obtained (20, 23, 31, 32). Others do not use the same FNAB sample for both morphological and molecular analyses but performed an additional FNAB (34), which can increase the likelihood of generating contradictory results even more. All four studies that investigated a panel of mutations and all studies that investigated *RET/PTC* rearrangements or *PAX8/PPARg* rearrangements in indeterminate FNAB samples used fresh FNAB material stored at −80 C, and only five studies that investigated only *BRAF* with DNA extraction used the FNAB material from

TABLE 1. Summary of four studies with a panel of mutation analyses

	First author, year (Ref.)			
	Nikiforov *et al.*, 2009 (40)	**Moses *et al.*, 2010 (20)**	**Ohori *et al.*, 2010 (41)**	**Cantara *et al.*, 2010 (39)**
Cytology categories investigated: sample number	Indeterminate: 52; cancer positive: 22; cancer negative: 12	Indeterminate: 110; malignant: 57; suspicious: 27; nondiagnostic: 2; benign: 257	Follicular lesion of indeterminate significance/atypia of indeterminate significance: 117	Indeterminate: 41; suspicious: 54; inadequate: 53; benign: 87
Material	FNAB (4 needle passes)—a small portion-Roche nucleic acids preservative solution for cytology and remaining material from needle washing for molecular analysis	FNAB—cytological analysis and remaining material for molecular analysis	FNAB (3 or 4 needle passes)—direct smear and monolayered slides by ThinPrep processing. Residual FNA material—nucleic acid preservative solution for molecular analysis	FNAB (1 or 2 needle passes) 2/3 of the material for cytology and 1/3 for molecular analysis. Tissue collected at surgery with 1.5 ml microcentrifuge tube containing Allprotect tissue reagent
Mutation identified (indeterminate samples)	Indeterminate: *N-, H-, KRAS*: 5; *BRAF*: 7; *RET/PTC1*-3: 2; *PAX8/PPARγ*: 1	Indeterminate: *BRAF*: 3; *RET/PTC1,3*: 1; *NRAS*: 8; *KRAS*: 0	Follicular lesion of indeterminate significance/atypia of indeterminate significance: *BRAF*: 3; *NRAS*: 7; *HRAS*: 1; *PAX8/PPARγ*: 1	Indeterminate: *N-, H-, KRAS*: 3; *BRAF*: 2; *RET/PTC1,3*: 2; *PAX8/PPARγ*: 0
Histology for indeterminate samples	PTC: 17; FTC: 4; FA: 4; HN: 27	PTC: 19; FA: 29; FTC: 8; Hurthle cell adenoma: 6; hyperplastic nodule: 40; Hurthle cell carcinoma: 2; lymphocytic thyroiditis: 6	PTC: 20; nonneoplastic: 79; adenoma: 18	PTC: 7; FA: 26; HN: 8
	Indeterminate	Indeterminate	Follicular lesion of indeterminate significance	Indeterminate; suspicious
FP/FN (n)	0/6	4/21[a]	0/8	1/1; 0/9
Sensitivity/specificity (%)	71/100	38/95[a]	60/100	85.7/97; 80.4/100

Salvatore *et al.* (37) study was not included in this table because they used just PTC as final histology. This would lead to a false specificity of 0% by the absence of TN samples. HN, Hyperplastic nodule.

[a] It was not possible to calculate the suspicious samples because the data correlating histology and mutation were not given.

the routine air-dried fine-needle aspiration (FNA) smear (25, 26, 29, 30, 36). Only this latter approach has major advantages, including:

1) Exactly the same routine air-dried smear that was analyzed by the cytopathologist is used for the molecular analysis. Therefore, possible differences between molecular and morphological results cannot be due to different samples.
2) Integrated and focused molecular diagnostics are performed only for those (same) FNAB samples that the cytopathologist cannot decide based on cytology criteria.
3) There is no need to prepare part of or further FNAB material for RNA preservation.
4) There is no need to store part of the FNA material or additional FNAB material at −80 C until completion of cytological diagnosis to then select those stored samples with indeterminate cytology reports for further molecular analysis.
5) There is no second FNAB for molecular diagnostics and thus less burden for the patient.
6) There is lower total diagnostic cost due to spared unnecessary parallel morphological and molecular diagnostics (and lower total cost due to spared surgeries).

Major differences between previous studies result from the different cytological classifications (Table 2) of the FNAB sample results. The "indeterminate" category described by the American Thyroid Association (ATA) in 2006 (42) and used in many studies up to 2010 comprised both indeterminate and suspicious samples thus further complicating the interpretation of the data. Suspicious and indeterminate samples are often placed in the same category (25, 26, 31, 32, 36, 40), which is likely to result in higher malignancy rates compared with indeterminate/follicular proliferation and also in completely different results for sensitivity and specificity.

The individual evaluation of the studies showed for the prospective study of Nikiforov *et al.* (40) that testing for a panel of mutations significantly improves the accuracy of the cytological diagnosis (Table 1). They reported a gain in sensitivity (from 44 to 80%) and in accuracy (from 93.3 to 97.4%). Especially in samples with an indeterminate cytology, the detection of any mutation is highly predictive for malignancy and therefore provides a strong indication for surgery (40). Moreover, similar to Xing *et al.* (43), Nikiforov *et al.* (40) suggest that patients with nodules showing indeterminate (and suspicious) cytology, but which are positive for a mutation (especially *BRAF* and *RET/PTC*), would be strong candidates for total thyroidectomy. Furthermore, the preoperative knowledge of a *BRAF* mutation may be useful for guiding the decision for prophylactic central compartment neck dissection (43). Although the results for *BRAF* and *RET/PTC* mutations

TABLE 2. Current classification of thyroid FNAB by different organizations and the respective goals for molecular FNAB

AACE/AME/ ETA, 2010 (1)	BTA, 2007 (3)	ATA, 2009 (5)	NCI, 2008 (83)	Molecular FNAB goals
Nondiagnostic	Nondiagnostic	Nondiagnostic/ inadequate	Nondiagnostic/unsatisfactory	26% Mutation positive (39). Reduce rate of a second FNAB
Benign	Benign	Nonneoplastic	Benign	Reduce the FN rate in settings with high FN between 6 and 17% (9)
Follicular lesion	Follicular lesion	Indeterminate	Follicular lesion of undetermined significance/ atypia of undetermined significance	Improve the differential diagnosis between benign and malignant. Sensitivity 85.7/97%, specificity 97/ 100% (39, 40). Reduce the rate of diagnostic surgery. Increase the rate of total thyroidectomy as first surgery
Suspicious	Suspicious	Suspicious (for PTC)	Follicular-neoplasm/suspicious for follicular neoplasm Hurthle cell neoplasm. Suspicious for malignancy	Improve the differential diagnosis between benign and malignant. Increase the rate of total thyroidectomy as first surgery (53)
Malignant	Malignant	Malignant	Malignant	Increase the rate of total thyroidectomy as first surgery and define the extension of the surgery

are very promising, the detection of a *RAS* mutation might be more problematic. Although in the study of Nikiforov *et al.* (40) the finding of a *RAS* mutation conferred an 87.5% probability of malignancy for an indeterminate FNAB, the diagnostic potential of *RAS* and *PAX8/PPARg* mutations is lower because *RAS* (44–47) and *PAX8/ PPARg* (44, 48, 49) mutations were detected in both malignant and histologically benign tumors.

The same panel of mutations was analyzed by Cantara *et al.* (39) in samples of 174 patients undergoing thyroid surgery for indeterminate/suspicious/inadequate/benign FNAB results (Table 1). The mutation analysis was positive in 28.5% of all samples. The most prevalent was *BRAF* (49.3% of the positive samples), followed by *RAS* (34.3%) and *RET/PTC* (16.4%). The diagnostic performance of molecular analyses was superior to that of traditional cytology in diagnosing malignancy, and the combination of the two techniques could improve the accuracy for diagnosing cancer from 83 to 93.2% when compared with cytological analysis alone. Molecular analyses detected eight thyroid cancers that cytology missed from a total of 32 cancers by diagnosing them as FNAB indeterminate/inadequate/benign. Moreover, Cantara *et al.* (39) also compared the FNAB mutation analysis with a mutation analysis of the corresponding histological material from the surgical sample. In 88.2% of the cases, the mutation found in the FNAB material was also detected in the histological samples. The 11.8% discrepant results were due to the presence of a mutation in the tissue sample that was not found in the cytology sample.

Similarly, Ohori *et al.* (41) performed a mutation screening in 117 FNAB cytology samples classified as follicular lesion of indeterminate significance/atypia of indeterminate significance (Table 1). *BRAF*, *RAS*, *RET/PTC*, or *PAX8/PPARg* were detected in 10% of this FNAB category. They demonstrated that the probability of having cancer in this cytology category together with detection of one of the somatic mutations investigated is 100%, whereas the probability of having cancer without mutation detection decreases to 7.6%. Again, a positive molecular test was very helpful to further refine this FNAB category into high-risk and low-risk categories (41).

Moses *et al.* (20) prospectively tested 110 indeterminate FNAB samples for *BRAF*, *NRAS*, *KRAS*, and *RET/PTC* 1 and 3 and *TRK1* mutations (Table 1). In contrast to Nikiforov *et al.* (40), Moses *et al.* (20) reported a lower sensitivity for the detection of malignancy for the analysis of a panel of mutations and explained this lower detection rate by the use of remnant material from the FNAB for the molecular analysis and the lack of *HRAS* and *PAX8/ PPARg* rearrangement analysis. However, even when testing only two mutations, Salvatore *et al.* (37) reported an increase in the diagnosis of malignancy from 27% by cytology alone to 34% for the combination of cytology and mutation detection.

The most extensively studied mutation in this diagnostic context is definitely $BRAF^{V600E}$ (24–33, 35, 36).

The nine presurgical FNAB studies with *BRAF* mutation detection and their correlation with histological results are summarized in Supplemental Table 1 (24–26, 29–32, 35, 36). Many *BRAF* FNAB studies have been conducted in Korea (24, 29, 31), a country with a high iodine intake. The prevalence of *BRAF* mutation in this country is much higher than in Western countries, reach-

TABLE 3. Means from studies classified according to the type and number of mutations investigated and FNAB category

	Indeterminate	
	Several mutations analysis[a]	**RET/PTC rearrangements analysis[b]**
FP	1.25 (0–4)	0
FN	9 (1–21)	3.5 (1–6)
Sensitivity	63.7% (38–85.7%)	55% (50–60%)
Specificity	98% (95–100%)	100%

Data are expressed as means (range). The means for suspicious samples were not calculated because only data from Cantara *et al.* (39) were available. The suspicious samples from Moses *et al.* (20) were not calculated because data for a correlation of histology with mutation status were not given.

[a] Means from four studies (20, 39–41) that analyzed a panel of mutations of indeterminate samples.

[b] RET/PTC rearrangements analysis (23, 34) of indeterminate samples.

ing 83% for histologically verified PTC (28). All of these *BRAF* studies concluded that the *BRAF* mutation is a specific marker for PTC and showed a high specificity and reliability for searching for this mutation in FNAB. However, there are distinct cytological hallmarks for PTC.

Comparing the data between the studies and also the means, for the group of indeterminate samples the mean number of FP in the four studies analyzing a panel of mutations was higher than when just one mutation was analyzed (Tables 3 and 4). One *RAS* mutation was detected in a FA (39), and four *NRAS* mutations were identified in four FA (20), resulting in a mean of 1.25 FP samples. The mean of 0.5 FP samples in indeterminate FNAB (Table 4) is due to the identification of a *BRAF* mutation in a sample with the final histology of nodular hyperplasia by Chung *et al.* (24). The explanation given is that it might be a precursor for PTC in a background of Hashimoto's thyroiditis. Kim *et al.* (29) also found a FP *BRAF* sample. This was explained by a very low cutoff for the dual-priming oligonucleotide-based PCR. Currently, the low FP rates for investigating a panel of mutations (Table 3) are very likely also due to the low number of (suspicious) FNAB samples analyzed to date. The possible significance of *RAS* mutations in histologically benign samples is discussed below.

For the FN samples, it is important to note that although many tumors are histologically malignant, no mutation was identified in FNAB material. Even for the four studies evaluating several mutations (Table 1), a mean of nine of 80 tumors with indeterminate cytology and no mutation identified had a malignant histology (11.25% of the samples).

The highest sensitivity for the indeterminate/follicular proliferation category (63.7%) was achieved by analyzing a panel of mutations (Table 1 compared with Supplemental Tables 1 and 2) as demonstrated by Nikiforov *et al.* (40), by Ohori *et al.* (41), and especially by Cantara *et al.* (39) with the highest sensitivity of 85.7%. However, because one group identified only a small number of mutations in the indeterminate samples (20), there is a broad sensitivity range of 38–85.7% for the investigation of the mutation panel in the indeterminate/follicular proliferation lesions (Table 3). Moreover, a detailed comparison of the results is difficult because the different studies adhered to different methodologies and classification schemes. The detailed data in the study of Nikiforov *et al.* (40) especially illustrate this variation of classifications. This study's indeterminate group [according to the 2006 ATA classification (42)] comprises suspicious and indeterminate lesion samples. In comparison to other studies analyzing follicular lesion of indeterminate significance/atypia of indeterminate significance, the malignant samples are overrepresented in the 2006 ATA (four cytology class classifications) indeterminate group. Moreover, in the Nikiforov study (40), the treating physicians were aware of the results of the molecular testing. These two characteristics are likely to explain the higher prevalence of malignant tumors diagnosed by molecular FNAB analysis of the indeterminate FNAB in the Nikiforov study (40) compared with other mutation panel studies that analyzed "suspicious" samples (20, 39) or follicular lesions of undetermined significance/atypia of undetermined significance (41).

Nikiforov *et al.* (40) and Ohori *et al.* (41) used melting curve analysis for point mutation analysis, and Nikifo-

TABLE 4. Means from studies that analyzed BRAF mutations

	Indeterminate (24, 29, 30, 35)	**Suspicious, follicular-neoplasm/suspicious for follicular neoplasm, Hurthle cell neoplasm, suspicious for malignancy (26, 29, 30)**
FP	0.5 (0–1)	0
FN	6 (3–12)	13.3 (8–12)
Sensitivity	12.95% (0–37.5%)	62% (55–71%)
Specificity	92.3% (75–100%)	100%

Data are expressed as means (range). The studies from Pizzolanti *et al.* (32), Nam *et al.* (31), Cohen *et al.* (25), and Zatelli *et al.* (36) did not enter in the calculi of the mean because of the different classification used.

rov verified it with Sanger sequencing, whereas Cantara *et al.* (39) and Moses *et al.* (20) used standard PCR and verified the PCR-positive samples with direct sequencing. However, it is unlikely that the differences in mutation detection are due to the different methods because the sensitivities of these are very similar. Also, different rearrangement analyses were used. Nikiforov *et al.* (40) and Ohori *et al.* (41) used standard RT-PCR, and Cantara *et al.* (39) and Moses *et al.* (20) nested PCR. However, many further variables such as number of samples, method of extraction, and conservation of the material are also of importance. Therefore, more studies evaluating and comparing different methodologies are needed. Jin *et al.* (50) compared the detection of *BRAF* by comparing four different methods, including direct sequencing, Colorimetric Assay Mutector, real-time LightCycler PCR with fluorescence resonance energy transfer probes, and an allele-specific PCR with LightCycler SYBR Green. They demonstrated a similar sensitivity for the four detection methods. However, the allele-specific PCR with LightCycler SYBR Green was the most rapid, easiest to perform, and least expensive technique, and was classified as readily performed in most molecular diagnostic laboratories.

The limitations of the mutation search in FNAB with the cytological diagnosis of follicular lesion/indeterminate and its improvement by molecular diagnostics are illustrated in Fig. 1. (Diagnostic) Surgery will detect thyroid malignancy in 20% (1, 11) of the follicular lesion samples. Based on the frequency of the mutations/rearrangements described above, somatic mutation detection will only be able to diagnose about half of these 20% malignant lesions for the FNAB diagnosis follicular lesion. Therefore, somatic mutation detection in indeterminate/follicular lesion samples is unable to identify the 50–70% (50% in Fig. 1) of the malignant cases in this FNAB category. This is due to the fact that there are currently no molecular diagnostic markers for the further 50–70% (50% in Fig. 1) malignant cases without known somatic mutations, and especially for the 80% benign nodules in the follicular proliferation group.

Another important aspect is the fact that the detection of *RET/PTC* and *PAX8/PPARg* rearrangements that requires RNA is methodologically more demanding than DNA-based search for the point mutations. Therefore, the two studies that investigated *RET/PTC* and *PAX8/PPARg* have used RNA extraction from FNAB material transferred into RNA preserving solutions and stored at −80 C. The reported PCR failure rates for *RET/PTC* alone or *RET/PTC* and *PAX8/PPARg* PCR in FNA material were 50 and 25.3% in the two studies (39, 40). Despite these technical difficulties, the added diagnostic value of the *RET/PTC* and *PAX8/PPARg* detections was considerable because nearly all *RET/PTC* or *PAX8/PPARg*-positive FNAB samples were malignant in these two studies (39, 40).

These data suggest that *PAX8/PPARg* detection can apparently contribute to the molecular FNA diagnostics, despite its detection in histologically benign adenomas (48, 49, 51). Also, *RAS* mutations have been reported in FA (20–40%) (44–47, 52). However, in analogy to *PAX8/PPARg* according to Nikiforov *et al.* (40), the presence of *RAS* mutation in a FNAB sample confers a probability of malignancy in 87.5% of the *RAS*-positive cases. Moreover, most important *RAS* (40, 44) and also *PAX8/PPARg*-positive FA (49) may very likely be precursors for *RAS* or *PAX8/PPARg*-positive FTC, as also suggested by further studies in transgenic models (52).

As long as we do not know all the genetic alterations in the different thyroid cancer types, and especially in the benign nodules, we will need additional molecular approaches (outlined below) for the diagnostic identification of the follicular lesion/indeterminate FNAB that are mutation negative but show a malignant histology (Fig. 1) and also for the molecular diagnosis of benign nodules. However, as summarized in Table 2, already now with the current possibilities (of detecting the known mutations in FNAB) there are additional benefits due to molecular diagnosis not only for the follicular proliferation/indeterminate but also for the suspicious, malignant, and even nondiagnostic FNAB results by reducing the number of completion surgeries for suspicious (and malignant) samples (53) or the number of nondiagnostic/inadequate (39) FNAB results, respectively (Table 2). A widespread clinical implementation will require a simple test that is compatible with clinical practice and robust and reproducible in multiple laboratories with routinely obtained air-

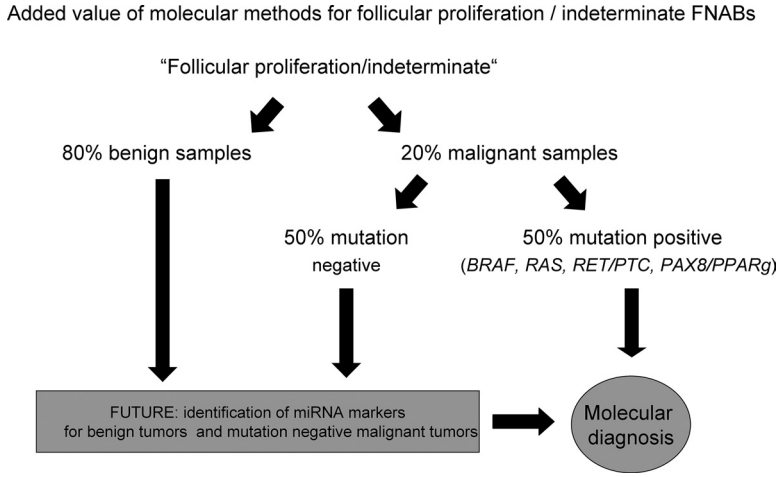

FIG. 1. Added value of molecular methods for follicular proliferation/indeterminate FNAB based on published malignancy rates for follicular proliferation/indeterminate FNAB of 20% (11).

dried material (21, 54). Moreover, reliable and competent FNAB diagnostics with a well-defined classification of diagnostic FNAB cytology categories will remain a prerequisite for molecular FNAB diagnosis. Because of the heterogeneity of thyroid tumors and the resultant difficulty to obtain repeated identical FNAB cytology results and to ensure a clinically feasible integrated morphological and molecular FNAB diagnosis, the molecular analysis should be done with the same sample that has been analyzed by the cytopathologist.

Which Additional Approaches Can Improve the Molecular FNA Diagnosis?

The current level of molecular analysis in FNAB is still restricted to a few specialized laboratories. There is a need for certified laboratories, adequate material, sensitive and standardized methods for extracting DNA and especially mRNA and microRNA (miRNA) from routine air-dried smear samples, and also a need for sensitive methods for mutation detection before it is possible to introduce molecular FNAB cytology diagnostics in the daily routine thyroid nodule workup. As outlined above, the search for the known somatic mutations will only allow us to diagnose a part of the follicular proliferation/indeterminate FNAB samples (Fig. 1). Therefore, to reduce the high number of diagnostic thyroid surgeries, there is an urgent need for further markers that can reliably identify about 50% of the malignant but mutation-negative lesions and especially 80% of benign nodules in this follicular proliferation/indeterminate cytology category.

Several studies have investigated the gene expression profiles of different thyroid cancers by microarrays (55). However, despite the fact that microarray studies revealed distinct changes in the expression of certain genes, none of the genes identified as differentially regulated were proven to be an ideal single marker of PTC (56–65). Moreover, there is a distinct lack of reliable markers for FTC and especially for benign nodules. This topic has thus far only been addressed by one study (66). Therefore, the aim of several current approaches to identify the minimal number of discriminating genes appears promising to close this diagnostic gap (66–76). Moreover, no study to date has applied the combination of detecting cancer-specific mutations and evaluating molecular classifiers by measuring differentially expressed genes or miRNA to improve the diagnosis for FNAB cytology results with indeterminate/follicular proliferation findings. One study developed in this direction combined the analysis of galectin-3 and the *BRAF* mutation detection (76). Even if most of the classifier studies are very promising, almost all of them except the recent one by Chudova *et al.* (77), which used FNAB material obtained in addition to the routine cytology FNAB material, are based on the investigation of thyroid tissue samples. Therefore, the proposed markers need to be prospectively evaluated and established in routinely obtained FNAB samples as outlined above.

In addition to mRNA expression patterns, the differential quantification of specific miRNA in thyroid FNAB cytology appears very promising. Investigations of the miRNA expression patterns of FTC and PTC compared with benign thyroid tissues have identified several differentially expressed miRNA (78–80). Pallante *et al.* (79) showed strong differences in the expression patterns of miR-221, -222, and -181b between FNAB of PTC and benign thyroid nodules. Furthermore, a recent study using a large series of well-characterized FNAB samples demonstrated the high diagnostic potential of miRNA testing in FNAB samples (81). Most important, miRNA are less susceptible to degradation than mRNA and have been shown to allow better diagnostic classifications than mRNA classifiers (82). Recently, this has also been demonstrated for the differential classification of benign thyroid nodules (21).

However, both the mRNA and miRNA array gene expression investigations thus far lack the definition of specific markers for benign nodules and further comparisons of FTC and benign nodules to specifically identify markers for the FNAB follicular proliferation category. One way of searching for these markers is to analyze and compare the miRNA expression profiling of histologically benign thyroid nodules with normal surrounding tissues and PTC or especially FTC. To date this has been done in small numbers of samples (75) without verification in routine FNAB.

Conclusions and Future Perspectives

Although the selection of suspicious nodules for surgery was substantially enhanced by the introduction of FNAB (6), the stepwise unraveling of the molecular etiology of thyroid nodules (*e.g.* identification of mutations, differentially expressed mRNA, and miRNA) provides the basis for further improvements (Fig. 2). Because immunohistological markers did not show enough specificity and sensitivity, as a first step molecular testing for the common somatic mutations *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARg* in all indeterminate/follicular or suspicious cytologies appears to be the most promising approach (Fig. 2). This mutation detection should be performed with routine FNAB cytology material because this new molecular diagnostics approach needs close integration with FNAB cytology, which will remain the first approach able to clar-

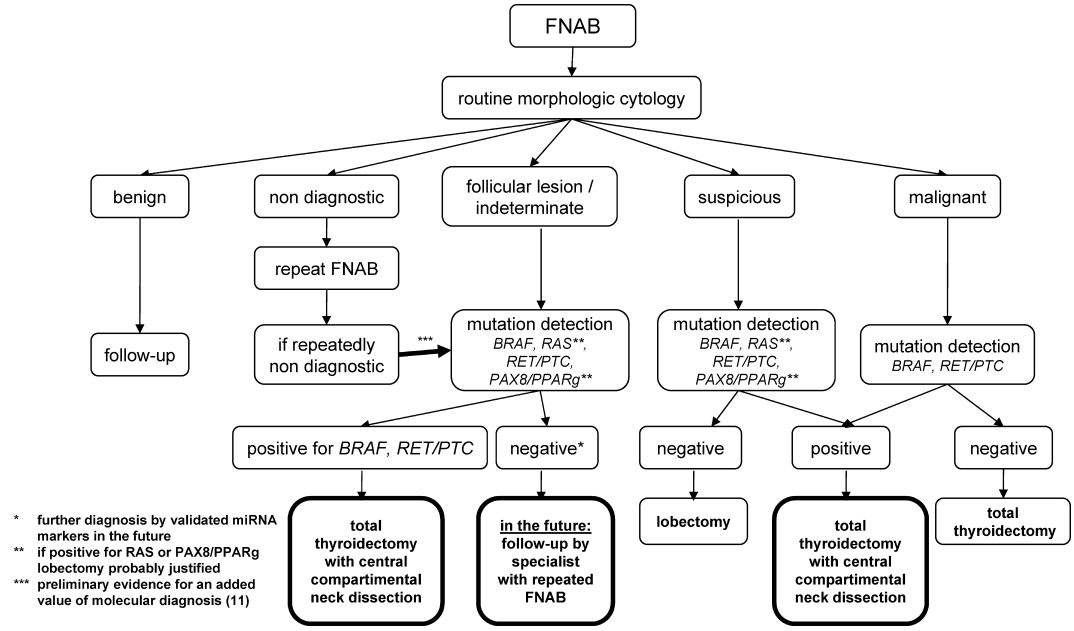

FIG. 2. Current and future possibilities to improve the current morphological cytological diagnosis of thyroid nodules by integrated (morphological and molecular) multilevel diagnostics of routine air-dried thyroid FNAB cytology specimens to reduce the rate of "diagnostic" surgeries and to increase the rate of primary thyroidectomies with primary central compartment neck dissection. The improvements by molecular diagnostics in comparison to conventional cytology are highlighted in *bold*. *, miRNA markers validated in future studies should allow further differentiation of *BRAF*, *RAS*, *RET/PTC*, and *PAX8/PPARG*-negative follicular lesion/indeterminate or suspicious cytologies into benign and malignant nodules and thus to further reduce the probability of having cancer without detected mutation and without miRNA malignancy markers, especially in the case of potential miRNA markers for benign nodules. **, *RAS* or *PAX8/PPARg*-positive follicular lesion/indeterminate or suspicious cytologies probably justify lobectomy even in the case of a benign histology because *RAS* mutations very likely identify a premalignant lesion (see text). ***, Preliminary evidence for an added value of molecular diagnostics according to Cantara *et al.* (39).

ify the diagnosis for many thyroid nodules. Moreover, basing the mutation detection on clearly defined cytological classification categories will also define and focus the different additional possibilities for molecular testing that are determined by and are dependent on the morphological FNAB result (Table 2). In addition to the specific malignancy risk, the FNAB diagnosis will also determine the probability for the most common and most likely type of differentiated thyroid carcinomas in a given FNA sample (11, 83), which can serve to focus the additional molecular testing (*e.g.* only *BRAF* and *RET/PTC* testing) in the malignant category (Fig. 2).

According to first studies, the application of molecular diagnostics in the indeterminate category will lead to definitive diagnoses in this cytological category resulting in a 50% decreased malignancy rate, *i.e.* from 20% (11) to 8% (39) or 10% (41). If the malignancy rate for the indeterminate category can be further reduced to 3%, *e.g.* by miRNA markers (see above), follow-up by a specialist with ultrasound and repeat FNA instead of diagnostic thyroidectomy might be possible in the future (84–86) as outlined in Fig. 2.

Performing integrated molecular FNAB diagnostics in routine FNAB material will require the development of methods that allow us to extract DNA, mRNA, and miRNA in parallel from those routine (air-dried or liquid-based) cytology samples that were used for the morphological FNAB diagnosis. Integrated molecular FNAB diagnostics in routine FNAB material have thus far only been performed for point mutations (20, 23–26, 29–32, 34–41) that can be done with DNA extraction. Extraction of mRNA for *RET/PTC* and *PAX8/PPARg* analysis has been judged as not feasible (22). However, preliminary data of our group show that both the detection of rearrangements and the quantification of miRNA in FNAB samples is possible by using improved extraction methods that allow the extraction of mRNA and miRNA. Moreover, amplifying very small PCR fragments can also increase the PCR success rate. Furthermore, it appears easier to quantify miRNA from these routine FNAB samples than mRNA or to detect rearrangements.

Because the known somatic mutations currently only allow us to diagnose a part of the indeterminate FNAB samples (Fig. 1), additional classifiers derived from mRNA and miRNA expression analysis will have to fill this diagnostic gap. To be able to quantify these future markers in routine FNAB samples, the DNA, mRNA, and miRNA extraction methods will have to be quantitatively reliable. This multilevel approach integrated into current diagnostic routine (Fig. 2) should soon considerably improve the differential diagnosis between benign and malignant samples by reducing the number of undefined

FNAB samples. These increased FNAB possibilities should soon lead to a broader application of FNAB cytology for the differential diagnosis of thyroid nodules, to better FNAB training of clinicians and cytopathologists, and thereby to a substantial reduction of diagnostic surgeries for thyroid nodules and an increase in the rate of primary total thyroidectomies with central compartment neck dissection instead of repeated thyroid surgeries for thyroid cancer. Moreover, the molecular profiling of thyroid tumors will in the future also help to guide mutation-specific targeted therapies.

Acknowledgments

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