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Review

Molecular fine-needle aspiration biopsy diagnosis of thyroid nodules by tumor specific mutations and gene expression patterns

Markus Eszlinger*, Ralf Paschke

3rd Medical Department, University of Leipzig, Ph.-Rosenthal-Str. 27, D-04103 Leipzig, Germany

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ABSTRACT

Fine-needle aspiration biopsy (FNAB) is currently the most sensitive and specific tool for the presurgical differential diagnosis of thyroid malignancy, but has also substantial limitations. While approximately 75% of FNAB reveal benign lesions and 5% already cytologically prove malignancy, up to 20% of FNAB show follicular proliferation for which follicular adenoma, follicular carcinoma, and follicular variant of papillary carcinoma can only be distinguished histologically, thus requiring thyroid surgery. However, new biomarkers that might improve the accuracy of FNAB come along with the discovery of more and more details of the molecular etiology of thyroid tumors. So far molecular testing for somatic mutations is most promising (e.g., *BRAF*), since the proposed biomarkers from mRNA- and miRNA-expression studies need further evaluation, especially in FNAB samples. Nevertheless, the application of molecular markers will significantly improve thyroid tumor diagnosis and thus it will help to prevent unnecessary surgeries and it will also help to guide mutation-specific targeted therapies.

Contents

1. Epidemiology of thyroid nodules and cancer

Thyroid nodules constitute a very common clinical finding. Their prevalence varies between 4 and 76% depending on the screening method used and the population evaluated (AACE/AME Task Force on Thyroid Nodules, 2006). While on the basis of the least sensitive detection method (palpation) the prevalence of thyroid nodules has been estimated to be about 4% in iodine replete regions (Vander et al., 1968), the prevalence increases up to 67% using high-resolution ultrasound (Ezzat et al., 1994). Moreover, autopsy data indicate a prevalence of thyroid nodules between 8 and 65% (Wang and Crapo, 1997). Epidemiological and genetic risk factors for thyroid nodules are iodine deficiency, smoking, a history of head and neck radiation, as well as female sex, familial predisposition and increasing age. By far the best studied risk factor for thyroid nodules is iodine deficiency, e.g., it has been shown that the prevalence of thyroid nodules is inversely correlated with the population's iodine intake (Delange, 1994; Delange et al., 2001).

Although the majority of thyroid nodules are benign and asymptomatic, thyroid carcinoma account for about 5% of nodules (Hegedus, 2004). Even if thyroid carcinoma is rare among human malignancies (<1%) it is the most frequent endocrine cancer and its incidence is increasing (Hodgson et al., 2004). Especially in females there is a striking increase in incidence rates, which are nearly twice

* Corresponding author. Tel.: +49 341 9713312; fax: +49 341 9713239.
E-mail address: markus.eszlinger@medizin.uni-leipzig.de (M. Eszlinger).

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Table 1
Tumors of the thyroid according to DeLellis et al. (2004).

Papillary carcinoma
Histopathological variants:
Follicular variant
Macrofollicular variant
Oncocytic variant
Clear cell variant
Diffuse sclerosing variant
Solid variant
Cribriform carcinoma
Papillary carcinoma with focal insular component
Papillary carcinoma with squamous cell or mucoepidermoid carcinoma
Papillary carcinoma with spindle and giant cell carcinoma
Combined papillary and medullary carcinoma
Papillary microcarcinomas
Follicular carcinoma
Categories according to degree of invasiveness:
Minimally invasive
Widely invasive
Cytological variants:
Oncocytic variant
Clear cell variant
Minimally invasive
Widely invasive
Poorly differentiated carcinoma
Undifferentiated (anaplastic) carcinoma
Medullary thyroid carcinoma
Follicular adenoma

the rates in males and which are possibly associated with reproductive factors and hormones (Hodgson et al., 2004). In addition, an elevated body mass index has been directly related to thyroid cancer risk in females but not in males (Del Maso et al., 2000). Besides these epidemiologic factors new diagnostic technologies (e.g., increased use of ultrasonography to detect nonpalpable cancers) are thought to affect thyroid cancer incidence, which has been recently shown by a French study (Leenhardt et al., 2004). Nevertheless, if this change in diagnostic practices alone would be the cause of increased thyroid cancer incidence one would expect an increased incidence in all the types of thyroid cancer (Table 1, DeLellis et al., 2004) (Hodgson et al., 2004). However, studies from different countries showing an increased thyroid cancer incidence attribute this to an increase in papillary thyroid carcinoma (PTC) (Hodgson et al., 2004). The most dramatic annual increase has been shown for the follicular variant of PTC (fvPTC) with an increase of 12.4%, while in contrast follicular thyroid carcinoma (FTC) appears to decline with a 0.7% increase (Hodgson et al., 2004). PTC comprise approximately 85–90% of all thyroid cancers with a female to male ratio of 4–5:1 whereas FTC account for about 10–15% of clinically evident thyroid malignancies (female:male ratio = 3–4:1) (DeLellis, 2006). In contrast to the well-differentiated PTC and FTC the poorly differentiated and (undifferentiated) anaplastic carcinoma are characterized by a rather low prevalence (approximately 2%). While the former are believed to develop from benign thyroid adenomas (or directly from follicular cells), the latter can arise from FTC and PTC (or come up de novo) (Fig. 1). Furthermore, while PTC and FTC have an excellent prognosis (10-year survival rate >90%), anaplastic carcinoma is highly aggressive and lethal (5-year survival rate 0–14%) (DeLellis et al., 2004). Poorly differentiated carcinoma form an intermediate entity between well- and undifferentiated carcinomas with a 5-year survival rate of about 50% (DeLellis et al., 2004).

2. Molecular etiology of thyroid nodules

2.1. Papillary thyroid carcinoma

The vast majority of PTC is characterized by mutations or rearrangements along the MAPK signaling cascade. The main targets of these genetic events are *RET*, *NTRK1*, *BRAF*, and *RAS*. So far more than 15 types of *RET* rearrangements, which primarily occur in radiation-induced tumors but also in variable proportions in sporadic (i.e., non-radiation associated) PTC, have been described (Tallini and Asa, 2001). The most common forms are *RET*/*PTC1* and *RET*/*PTC3*,

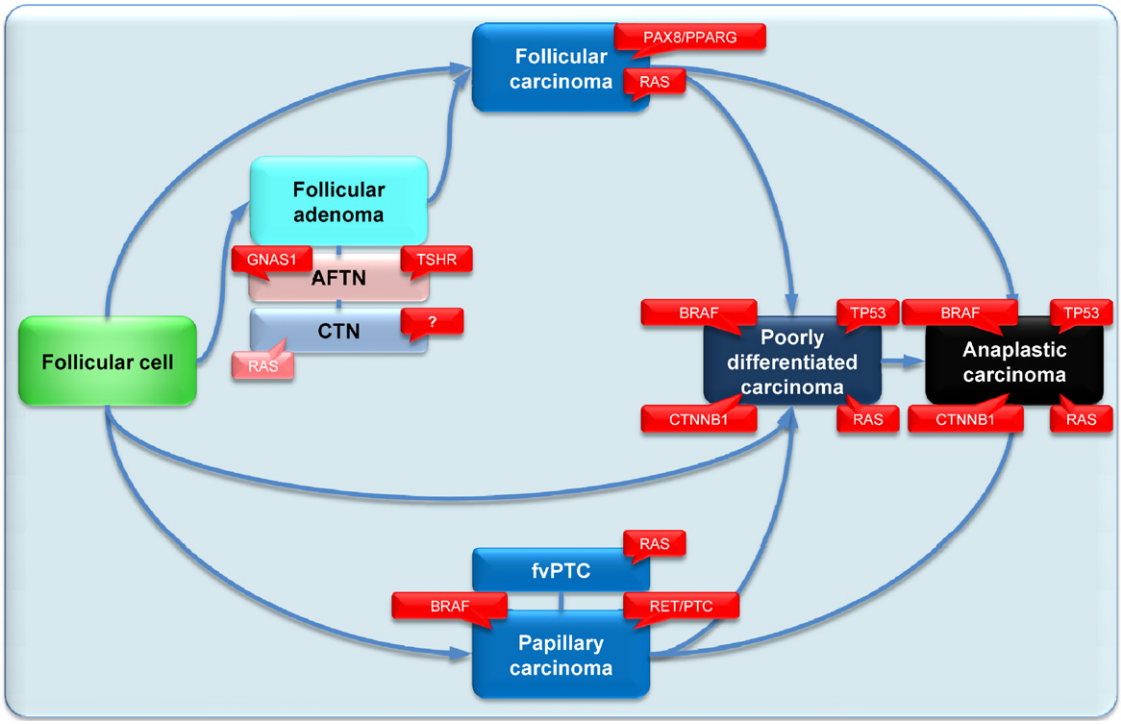

Fig. 1. Multi-step model of thyroid cancer origin and progression.

which are paracentric inversions resulting from the fusion of the 3′-terminal region of the *RET* receptor tyrosine kinase and the 5′-terminal region of *H4* and *NCOA4*, respectively, located on the long arm of chromosome 10 (Tallini and Asa, 2001). *RET*/*PTC1* is more common in classic PTC and the diffuse sclerosing variant of PTC, whereas *RET*/*PTC3* occurs mainly in the solid variant of PTC. The prevalence of *RET*/*PTC* rearrangements varies widely from 3 to 85%, which depends on detection methods used and the geographical location of patients (Kondo et al., 2006). The range for the prevalence of *RET*/*PTC* rearrangements is 13–43% (Kondo et al., 2006).

The most common genetic change in sporadic PTC is the $BRAF^{V600E}$ mutation, which is present in about 29–83% of cases (Xing, 2005). Due to its high prevalence and its specificity for PTC (especially conventional PTC, oncocytic PTC and microcarcinomas) and PTC-derived anaplastic carcinomas (Xing, 2005), the *BRAF* mutation has become a target of intensive investigations (for review see Xing, 2007). There are controversial results regarding the association of the *BRAF* mutation with clinicopathological characteristics of PTC. Most of these studies demonstrate a significant association of the *BRAF* mutation with extrathyroidal invasion, lymph node metastasis, and advanced disease stage (Adeniran et al., 2006; Lee et al., 2007; Lupi et al., 2007; Namba et al., 2003; Nikiforova et al., 2003a; Puxeddu et al., 2008; Wang et al., 2008; Xing, 2007; Xing et al., 2005), whereas others do not (Costa et al., 2008; Fugazzola et al., 2004; Kim et al., 2005; Liu et al., 2005; Puxeddu et al., 2004). Moreover, the *BRAF* mutation occurs most commonly in PTC subtypes that are known to be more aggressive (Xing et al., 2005). In support for this finding a correlation of the *BRAF* mutation with a lower expression of both the sodium–iodide-symporter and the thyroperoxidase has been shown suggesting an early dedifferentiation which might be the basis for a poor prognosis (Romei et al., 2008).

RAS mutations occur in about 0–21% of PTC (Kondo et al., 2006). Nevertheless, they are rather uncommon in conventional PTC and they are almost always found in follicular variant PTC (and also in follicular adenoma/carcinoma and poorly differentiated and anaplastic carcinoma). Therefore, *RAS* mutations are believed to be an early event in thyroid tumor formation (Lemoine et al., 1988; Namba et al., 1990).

2.2. Follicular thyroid carcinoma

RAS mutations are found in 40–53% of FTC (Kondo et al., 2006). However, as mentioned above, *RAS* mutations are also found in follicular adenomas, fvPTC and poorly and undifferentiated carcinomas.

Kroll et al. (2000) identified a fusion gene between the thyroid specific transcription factor *PAX8* and *PPARG*, a ubiquitously expressed transcription factor playing an important role in the regulation of genes involved in lipid metabolism. Initially, the *PAX8*/*PPARγ* rearrangement was exclusively detected in FTC and therefore considered as a sign of malignancy (Kroll et al., 2000). The prevalence of *PAX8*/*PPARγ* in FTC varies between 25 and 63% (Kondo et al., 2006). Later the rearrangement was also shown in follicular adenoma (4–33%), and fvPTC (37.5%) (Castro et al., 2006; French et al., 2003; Kroll et al., 2000; Marques et al., 2002; Nikiforova et al., 2002), but not in poorly differentiated and undifferentiated carcinoma. The knowledge about the detailed molecular events by which *PAX8/PPARγ* contributes to the tumor development is so far limited. Kroll et al. (2000) showed that the fusion protein does not activate PPAR-responsive promoters, but functions as a dominant-negative inhibitor of *PPARγ*-induced reporter gene activation. Therefore, the inhibition of endogenous *PPARγ* was said to be an important mechanism by which the *PAX8/PPARγ* fusion protein causes FTC. However, Giordano et al. (2006) could show that the *PAX8/PPARγ* fusion protein can function in a *PPARγ*-like manner (although it also has transcriptional properties distinct from either *PAX8* or *PPARγ*), the original concept that the *PAX8/PPARγ* fusion protein contributes to follicular carcinoma by antagonizing endogenous PPARγ needs reevaluation.

2.3. Follicular adenoma

In many studies the differentiation between follicular adenoma (FA) and hyperplastic nodules appears rather arbitrary. Even if many do not diagnose an adenoma but regard all the lesions as nodules as long as there is no evidence of malignancy, FA is usually solitary and surrounded by a thin capsule, and adenomatoid nodules are typically multiple and lack a well-defined fibrous capsule and are composed of follicles morphologically similar to those in the surrounding thyroid tissue. However, in the frequent background of nodular hyperplasia or thyroiditis in the surrounding tissue it is hard to distinguish hyperplastic nodules and adenomas. Therefore, the current WHO classification recommends that the biological basis for separating hyperplastic nodules from follicular adenomas (true tumors) should depend on their clonality (Chan et al., 2004). However, the clonality approach is not always decisive because of a technical failure rate of about 10% and other discrepancies (Trulzsch et al., 2001). With respect to their function, benign thyroid nodules can be subdivided into autonomously functioning thyroid nodules (AFTN), cold thyroid nodules (CTN), or less often as so-called warm nodules. A further differentiation between "hot" and "cold" nodules within the entity of benign nodules appears important for appropriate phenotype correlations as the histologic distinction between adenomas and adenomatoid nodules is not related to thyroid function or specific entities defined by specific molecular defects. The molecular defects in AFTN are *TSHR* mutations with a reported prevalence varying between 38 and 82% and $G_s\alpha$ mutations varying between 8 and 75%, respectively (Führer et al., 1997; Georgopoulos et al., 2003; Gozu et al., 2006; Holzapfel et al., 1997; Lyons et al., 1990; O'Sullivan et al., 1991; Parma et al., 1997; Tonacchera et al., 2000; Trulzsch et al., 2001; Vanvooren et al., 2002), The variable mutation frequencies can most likely be explained by different sensitivities of the mutation screening methods and the different qualities of the tissue samples studied (Krohn et al., 2005). Up to date no obvious relationship between the activating mutations *in vitro* activity and the patient's phenotype could be shown (for review see Arturi et al., 1998; Corvilain et al., 2001; Krohn and Paschke, 2002). Therefore, currently a *TSHR* or $G_s\alpha$ mutation screening does not seem helpful with regard to diagnosis, prognosis or the treatment of hot nodules. In contrast to AFTN, benign CTN do not contain somatic *TSHR* or $G_s\alpha$ mutations but a defective targeting of the sodium–iodide-symporter to the cell membrane, leading to a decreased iodide uptake (Dohan et al., 2001, 2003; Neumann et al., 2004). Therefore, a distinction between hot and cold nodules will more likely reflect different pathopysiologies than the histologic distinction between FA and adenomatoid nodules because the molecular pathophysiology of CTN is very different from AFTN and because CTN and AFTN exhibit marked differences in gene expression which further explain these functional differences (Eszlinger et al., 2004, 2005, 2006a,b; Lazar et al., 1999; Neumann et al., 2004; Paschke and Neumann, 2001).

3. Current standard of distinguishing benign and malignant tumors

Currently benign and malignant thyroid tumors are distinguished and assigned into specific subtypes based on a histologic classification (Table 1). Additional thyroid epithelial tumor subtypes have been suggested to accommodate borderline cases (Williams, 2000).

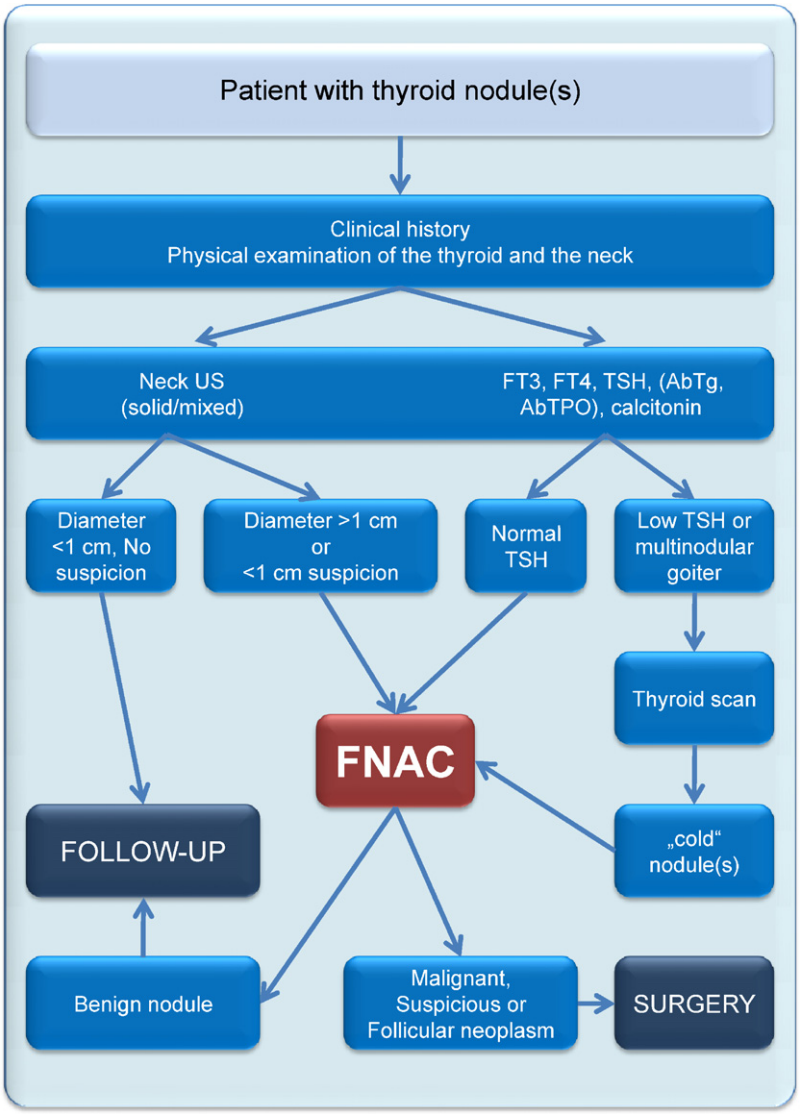

Fig. 2. Flow chart of the diagnostic evaluation of thyroid nodules according to the ETA/ETA-CRN (Pacini et al., 2006).

However, the prognostic significance of these histologic classifications is limited (Verburg et al., 2009). Moreover, the application of the current WHO classification in clinical routine is hampered by considerable interobserver variability that is most pronounced for follicular-patterned tumors (Fassina et al., 1993; Franc et al., 2003; Saxen et al., 1978) and the probability to miss a focus of capsular invasion is rather high especially if less than the total circumference of the nodule is investigated. As the morphology of FA and FTC is very similar and since the diagnostic hallmarks of FTC e.g., vascular and capsular invasions cannot be detected by cytology it is impossible to make the differential diagnosis in FNA smears. Nevertheless, FNAB is currently the best method to select suspicious thyroid nodules for surgery. Therefore FNAB is a central element in the diagnostic evaluation of thyroid nodules according to the ETA and the ETA cancer research network guidelines (Fig. 2) (Pacini et al., 2006), the ATA (Cooper et al., 2009) and AACE/AME/ETA guidelines (Gharib et al., 2010). However, about 20% of the FNAB samples show follicular proliferation which cannot distinguish between FA, FTC, and fvPTC (Gharib, 1997). This is mainly due to the fact that the diagnostic hallmarks of FTC (e.g., vascular and capsular invasions) cannot be detected by cytology. Therefore, patients with this cytologic finding currently have to undergo (diagnostic) surgery, which will detect thyroid malignancy in about 20% of these patients. Although many immunohistological markers have been investigated in recent years in order to improve the differential diagnosis between benign and malignant thyroid tumors little has entered daily routine work as many of the immunohistological markers show prominent overlap between FA and differentiated thyroid carcinomas (Faggiano et al., 2007).

4. Application of tumor specific mutation detection in FNAB diagnosis

Due to the discovery of somatic mutations for about two-thirds of PTC (*BRAF* mutations and *RET*/*PTC* rearrangements) and FTC (*RAS* mutations and *PAX8*/*PPARγ* rearrangements) there are new perspectives for the classification and diagnosis of thyroid tumors. Nearly all of these mutations have been tested for their applicability in FNAB diagnosis in different settings in the recent years (Table 2) (Bentz et al., 2009; Cheung et al., 2001; Chung et al., 2006; Cohen et al., 2004; French et al., 2008; Jin et al., 2006; Jo et al., 2009; Nikiforov et al., 2009; Pizzolanti et al., 2007; Rowe et al., 2006; Sapio et al., 2007a,b; Xing et al., 2004, 2009). The most extensively studied mutation in this diagnostic context is definitely $BRAF^{V600E}$. However, there is hardly any diagnostic potential of *BRAF* mutation detection for the most frequent FNAB problem,

Table 2
Settings of studies testing mutation detection for its applicability in FNAB diagnosis.

Author	Mutations analyzed	Material studied	Methodologies used
Bentz et al. (2009)	BRAF	DNA from cytologic slides	LightCycler PCR/fluorescent probe melting curve analysis
Cheung et al. (2001)	RET/PTC1, -2, -3	RNA from FNAB	RT-PCR/Southern hybridization
Chung et al. (2006)	BRAF	DNA from post-surgical aspitared nodules	PCR/sequencing, PCR-RFLP
Cohen et al. (2004)	BRAF	DNA from cytologic slides	PCR/sequencing, Mutector assay
French et al. (2008)	PAX8/PPARγ	Cells from FNAB	FISH
Jin et al. (2006)	BRAF	DNA from cytologic slides	PCR/sequencing, Mutector assay, LightCycler PCR with FRET probes, allele-specific LightCycler PCR with CYBR Green
Jo et al. (2009)	BRAF	DNA from FNAB	PCR/pyrosequencing, PCR/dideoxy sequencing
Nikiforov et al. (2009)	BRAF, RAS, RET/PTC, PAX8/PPARγ	DNA/RNA from FNAB	LightCycler PCR/fluorescent probe melting curve analysis, RT-PCR for rearrangements, PCR/sequencing
Pizzolanti et al. (2007)	BRAF, RET/PTC	DNA from FNAB, RNA from ex vivo nodules	Allele-specific LightCycler PCR with CYBR Green, Mutector assay, RT-PCR for rearrangements
Rowe et al. (2006)	BRAF	DNA from cytologic slides	LightCycler PCR/fluorescent probe melting curve analysis, PCR/sequencing
Sapio et al. (2007a)	BRAF (+galectin-3 expression)	DNA from FNAB	Mutant allele-specific PCR amplification (MASA), (ICC)
Sapio et al. (2007b)	RET/PTC, TRK, BRAF	DNA/RNA from FNAB	Mutant allele-specific PCR amplification (MASA), RT-PCR
Xing et al. (2004)	BRAF	DNA from FNAB	PCR/sequencing, Mutector assay
Xing et al. (2009)	BRAF	DNA from FNAB	Mutector assay

the "indeterminate follicular neoplasm", which comprises and is unable to distinguish FA, FTC and fvPTC. Moreover, the prevalence of *BRAF* mutations is associated with a high geographical variability (the prevalence of *BRAF* mutations is considerably higher in countries with a high iodine uptake). Nonetheless, preoperative *BRAF* mutation testing of FNAB specimens can be an informative preoperative risk stratification strategy by more reliably defining the optimal scope of initial surgery and also the medical management of PTC: Xing et al. (2009) examined the *BRAF* mutation status on preoperative FNAB specimens from 190 PTC patients and analyzed its relation to clinicopathologic characteristics of the patients. They found that the occurrence of the *BRAF* mutation in the FNABs strongly predicted extrathyroidal extension, capsular invasion, and lymph node metastasis. Moreover, the BRAF mutation was strongly associated with PTC persistence and recurrence (Xing et al., 2009). More important, Marchetti et al. (2009) reported an increase in the diagnostic accuracy for PTC of 20% by searching for $BRAF^{V600E}$ mutations in routine FNABs of patients with histologically proven PTC. However, much smaller increases in diagnostic accuracy for PTC were reported by Nikiforov et al. (2009) and Zatelli et al. (2009) investigating smaller numbers of PTC. This would be in line with the clinical finding that the cytologic diagnosis of PTC is mostly feasible by routine cytology. Therefore, the preoperative knowledge of a *BRAF* mutation may help to define the optimal extent of initial thyroidectomy (total versus lobectomy), may help deciding about prophylactic central neck dissection and may help to plan more appropriately the postsurgical therapy (e.g., ^{131}I therapy, TSH suppression and follow-up) (Xing et al., 2009).

Recently Nikiforov and coworkers published a large prospective study including 470 FNAB samples of thyroid nodules from 328 patients, which were tested for *BRAF*, *RAS*, *RET*/*PTC* and *PAX8*/*PPARγ* mutations (Nikiforov et al., 2009). They could show that testing for a panel of mutations significantly improved the accuracy of the cytological diagnosis. Especially in samples presenting an indeterminate cytology the detection of any mutation is highly predictive for malignancy and therefore provides a strong indication for surgery (Nikiforov et al., 2009). Moreover, similar to Xing et al. (2009), Nikiforov and colleagues suggest that patients with nodules showing indeterminate cytology but which are positive for a mutation (especially *BRAF* and *RET*/*PTC*) would be strong candidates for total thyroidectomy, whereas for nodules with indeterminate cytology and a negative mutation screening a reduced risk of malignancy can be anticipated and hence lobectomy might be an appropriate initial surgical approach (Nikiforov et al., 2009). Interestingly, in this study of Nikiforov et al. (2009) a decision according to this algorithm would have decreased the need for a second surgery in 30% of patients with an indeterminate cytology and no inappropriate total thyroidectomy would have been performed. While the results for *BRAF* and *RET*/*PTC* mutations are very promising, the detection of a *RAS* mutation might be more problematic. Although in the study of Nikiforov et al. the finding of a *RAS* mutation conferred an 87.5% probability of malignancy (62.5% of PTC, 25% of FTC), the diagnostic potential of *RAS* mutations is lower as *RAS* mutations were detected in both, malignant and benign tumors (Esapa et al., 1999; Ezzat et al., 1996; Lemoine et al., 1989; Namba et al., 1990; Nikiforova et al., 2003b). However, since *RAS* positive FA may be precursors for *RAS* positive FTC and since *RAS* mutations are associated with a worse prognosis, Nikiforov et al. (2009) suggest surgical removal of *RAS* positive FA to prevent putative progression.

Nevertheless, further studies have to clarify if *BRAF* mutation positive patients would benefit from more extensive surgery and have to especially examine the usefulness of *RAS* mutation screening. Although the examples discussed here give a rather optimistic view, some drawbacks have to be mentioned. For example, the incidence of detected somatic mutations in FTC and PTC has been shown to vary between different studies (Kondo et al., 2006) and different methods of mutation screening (e.g., direct sequencing, denaturing gradient gel electrophoresis, LightCycler PCR, PCR-RFLP) are characterized by different sensitivities (Chung et al., 2006; Rowe et al., 2006; Trulzsch et al., 1999; Zhu et al., 2006). In fact, the use of very sensitive methods led to the detection of *RET*/*PTC* thought to be specific for PTC also in Hashimoto's disease (Rhoden et al., 2006) and *PAX8*/*PPARγ* thought to be specific for FTC also in some thyroid adenomas (Cheung et al., 2003; Marques et al., 2002; Nikiforova et al., 2002). Moreover, in spite of the above indications for an improved accuracy by the evaluation of the "problematic" thyroid FNABs with the diagnosis follicular lesion or suspicious (see Table 3, for the different FNAB classifications) still 37 of 52 indeterminate samples investigated for *BRAF*, *RAS*, *RET*/*PTC* and *PAX8*/*PPARγ* (Nikiforov et al., 2009); 88 of 89 follicular lesions investigated for $BRAF^{V600E}$ (Zatelli et al., 2009) and 19 of 21 indeterminate FNAB samples investigated for *BRAF*, *TRK*, and *RET*/*PTC* (Sapio et al., 2007b) could not be clarified by mutation testing. These limitations of the mutation search in FNABs with the cytologic diagnoses follicular lesion or suspicious is supported by the clinical finding, that

Table 3
Current classification of thyroid FNA by different organizations.

AACE/AME/ETA (2009); Gharib et al. (2010)	ATA (2009); Cooper et al. (2009)	BTA (2007) (British Thyroid Association and Royal College of Physicians, 2007)	NCI (2008); Baloch et al. (2008); Theoharis et al. (2009)
Nondiagnostic	Nondiagnostic/inadequate	Nondiagnostic	Unsatisfactory
Benign	Non-neoplastic	Benign	Benign
Follicular lesion	Indeterminate	Follicular lesion	Follicular lesion, follicular neoplasm
Suspicious		Suspicious	Suspicious
Malignant	Malignant	Malignant	Malignant

(diagnostic) surgery will detect thyroid malignancy in only 20% of these patients (Cooper et al., 2009; Gharib et al., 2010) and the fact described above that known mutations can only be found in about 2/3 of PTC and FTC. Therefore, as long as we do not know all the possible point mutations in the different thyroid cancer types and especially for the benign adenomas (to be also able to discriminate the benign samples) we will need additional molecular approaches (outlined below) for the diagnostic discrimination of problematic FNAB diagnoses.

5. Application of tumor specific mRNA/miRNA expression patterns in FNAB diagnosis

A further specification of FNAB diagnosis can be achieved by the use of gene expression signatures in addition to the screening for known thyroid cancer related mutations discussed above. In the thyroid field several studies have investigated the gene expression profiles of different thyroid cancers by the help of microarrays (for review see Eszlinger et al., 2007). However, despite the fact that microarray studies revealed very distinct changes in the expression of certain genes, none of the genes identified by array studies as differentially regulated was proven to be an ideal single marker of PTC (Aogi et al., 1998; Bernet et al., 2002; Casey et al., 2003; Haugen et al., 1997; Inohara et al., 1999; Ippolito et al., 2001; Mase et al., 2003; Raphael, 2002; Sack et al., 1997; Saggiorato et al., 2001). Therefore, the aim of current approaches, which is to identify the minimal number of discriminating genes, appears more promising for diagnostic purposes (Cerutti et al., 2004; Eszlinger et al., 2006b; Foukakis et al., 2007; Fujarewicz et al., 2007; Jarzab et al., 2005; Kebebew et al., 2006; Krause et al., 2008; Mazzanti et al., 2004; Weber et al., 2005). For example, Mazzanti et al. (2004) compared the gene expression patterns of PTC, fvPTC, FA, and adenomatoid nodules and identified two classifiers comprising 6 and 10 genes, respectively, which allowed a correct classification with regard to malignancy in all samples of the test set. A further possibility to improve the diagnosis in FNAB with cytological findings suspicious for thyroid cancer might be the combination of measuring differentially expressed genes and detecting cancer specific mutations (e.g., *BRAF*, *RET*/*PTC*). A very promising study, based on the combined analysis of galectin-3 and the $BRAF^{V600E}$ mutation, has recently been published (Sapio et al., 2007a). Even if most of these studies are very promising, almost all of them are based on the investigation of thyroid tissue samples. Therefore, the proposed markers need to be prospectively established in FNB samples.

In addition to mRNA expression patterns the quantification of specific miRNAs in FNAB appears very promising. Investigations of the miRNA expression patterns of FTC and PTC compared to benign thyroid tissues have been done and identified several differentially expressed miRNAs (He et al., 2005; Pallante et al., 2006; Weber et al., 2006). Pallante et al. (2006) showed strong differences in the

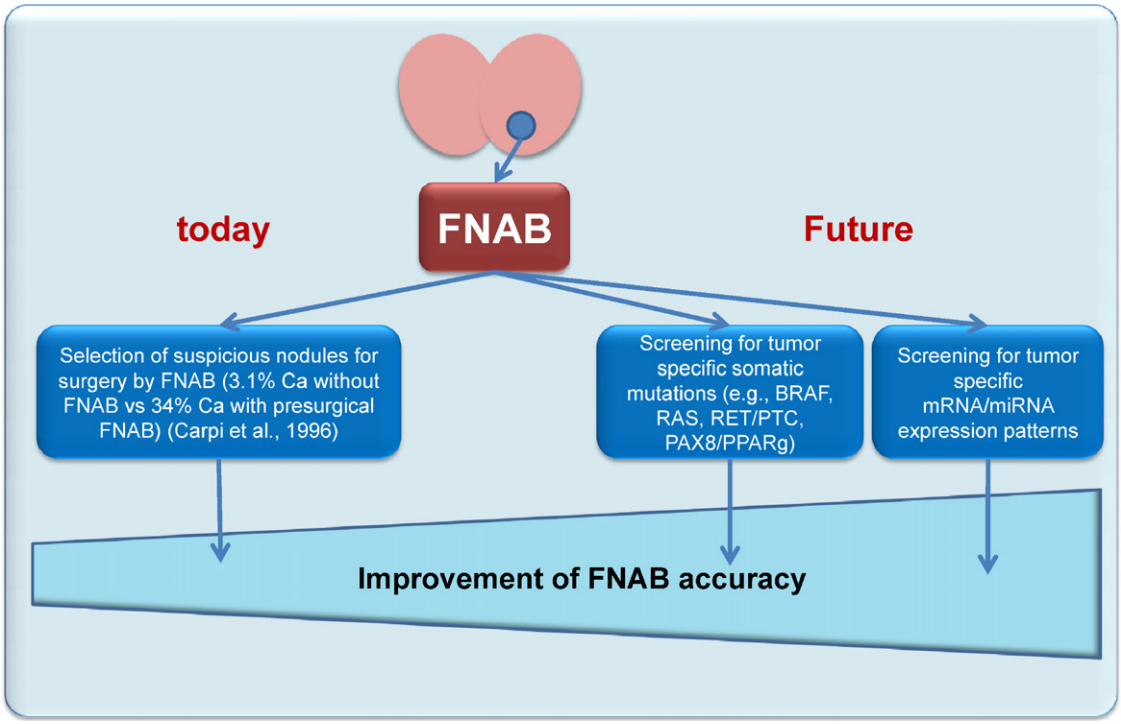

Fig. 3. FNAB diagnosis of thyroid nodules today and in the future.

expression patterns of miR-221, -222, and -181b between FNAB of PTC and benign thyroid nodules with 5–35fold differences. Furthermore, a recent study using a large series of well-characterized FNA samples also demonstrated the high diagnostic potential of miRNA testing in FNAB samples (Nikiforova et al., 2008).

6. Conclusions

While the selection of suspicious nodules for surgery was substantially enhanced by the introduction of FNAB (3.1% carcinomas in nodules resected without FNAB versus 34% carcinomas in nodules with preoperative FNAB selection (Carpi et al., 1996)), the discovery of the molecular etiology of thyroid nodules (e.g., identification of mutations, differentially expressed genes and miRNAs) provides the basis for further improvements (Fig. 3). So far molecular testing for common somatic mutations is most promising (e.g., *BRAF*), since the proposed biomarkers from mRNA- and miRNA-expression studies need further evaluation, especially in FNAB samples. The application of both, histologic and molecular markers will refine the definition of thyroid tumors. It will help to better characterize tumors with histologicaly uncertain biological behavior and it will help to guide mutation-specific targeted therapies.

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