Thyroid Cancer

36

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Abstract

Thyroid cancer is the most common type of endocrine malignancy. Initiation and progression of thyroid cancer involves multiple genetic and epigenetic alterations, of which mutations leading to the activation of the MAPK and PI3K/PTEN/AKT signaling pathways are crucial for tumor initiation and progression. Common mutations found in thyroid cancer are point mutations of the *BRAF* and *RAS* genes, as well as *RET/PTC* and *PAX8/PPARG* chromosomal rearrangements. More recently, a number of other mutations have been characterized, which occur in this cancer type with significantly lower frequency, but are associated with specific phenotypic and biological properties. These somatic mutations are useful diagnostic and prognostic markers for thyroid cancer and are being incorporated into clinical practice, offering a valuable tool for management of patients with thyroid nodules and cancer.

Keywords

Thyroid cancer • Thyroid nodules • FNA cytology • Molecular diagnostics • BRAF • RAS • RET/PTC • ALK

Introduction

Thyroid cancer is the most common endocrine malignancy, with a steadily growing incidence in the USA and other industrialized countries [1, 2]. The vast majority of thyroid tumors originate from follicular epithelial cells. The follicular cell-derived cancers are further subdivided into well-differentiated papillary carcinoma and follicular carcinoma, poorly differentiated carcinoma, and anaplastic (undifferentiated) carcinoma (Fig. 36.1) [3, 4]. Papillary carcinoma is the most common thyroid malignancy (80–85 %). In addition to classic-type papillary carcinoma, common histopathologic variants are microcarcinoma, follicular variant, and tall cell variant. Follicular carcinomas account for approximately 15 % of thyroid cancers and are subdivided into conventional type and oncocytic (Hürthle) type. Follicular adenoma is a benign tumor that is considered a precursor for follicular carcinomas (Fig. 36.1). Less differentiated thyroid cancers, i.e., poorly differentiated carcinoma and anaplastic carcinoma, can develop de novo, although many arise through the process of stepwise dedifferentiation of papillary and follicular carcinomas (Fig. 36.1). Medullary thyroid carcinoma originates from thyroid parafollicular or C cells, accounts for 3–5 % of thyroid cancers, can be a manifestation of an inherited genetic disease, and, therefore, is not discussed in this chapter.

Thyroid cancer occurs in thyroid nodules. Thyroid nodules are common in adults, particularly with increased age, and are typically detected by palpation or imaging [5–7]. However, most nodules are benign, and the rate of cancer in medically evaluated thyroid nodules ranges from 5–15 % [7–9]. A clinical challenge is to accurately diagnose cancer in these nodules and to avoid unnecessary thyroid surgery for benign disease. Sampling of thyroid nodules using fine needle aspiration (FNA) under ultrasound guidance followed by subsequent cytologic examination is the most accurate

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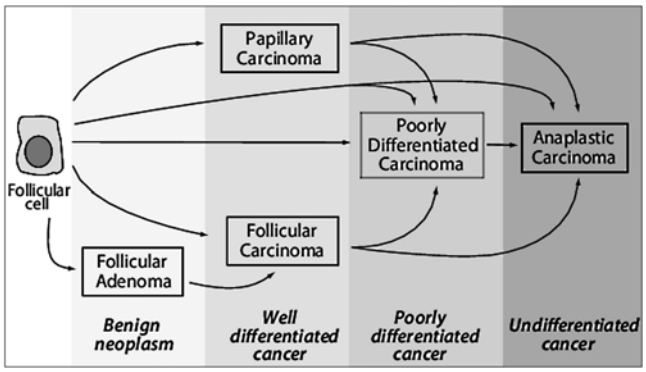


Figure 36.1 Pathways for thyroid cancer development from thyroid follicular cells

and widely used diagnostic tool at this time. FNA provides a definitive diagnosis of a malignant or benign nodule in the majority of cases. However, in about 25 % of nodules, FNA cytology cannot reliably exclude cancer, and such cases are placed in one of the indeterminate categories, hampering clinical management of these patients [6, 8, 10, 11]. By the current Bethesda System for Reporting Thyroid Cytopathology, the indeterminate categories include three specific cytologic diagnoses: atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS); follicular or oncocytic (Hürthle cell) neoplasm/suspicious for follicular or oncocytic (Hürthle cell) neoplasm (FN/SFN); and suspicious for malignant cells (SMC), with a predicted probability of cancer of 5–15 %, 15–30 %, and 60–75 %, respectively [12, 13]. Because FNA is unable to provide a definitive diagnosis for these indeterminate nodules, most patients with indeterminate cytology undergo diagnostic surgery to establish a histopathologic diagnosis. However, only 10–40 % of surgically resected indeterminate thyroid nodules are malignant [12]. The unneeded operations, with their attendant expenses and risks, may be avoided if the FNA procedure could reliably establish the presurgical diagnosis of a benign nodule. Additionally, since the standard of care is to offer a second surgery for total thyroidectomy if the diagnostic lobectomy confirms a cancer, a more optimal surgical management would be a single total thyroidectomy procedure that is planned when the diagnosis of cancer is established preoperatively.

Although well-differentiated thyroid cancer is a disease with an overall favorable outcome, some tumors entail a substantially worse prognosis and have to be treated more aggressively [14–16]. Multiple prognostic systems for differentiated thyroid cancer based on demographic and pathologic factors exist [17], but none of the systems accurately stratify thyroid tumors into appropriate risk categories. The rapidly expanding knowledge of the molecular genetics of thyroid cancer is being translated into clinical practice, offering significant improvement in the accuracy of the preoperative diagnosis of thyroid cancer and better tumor prognosis.

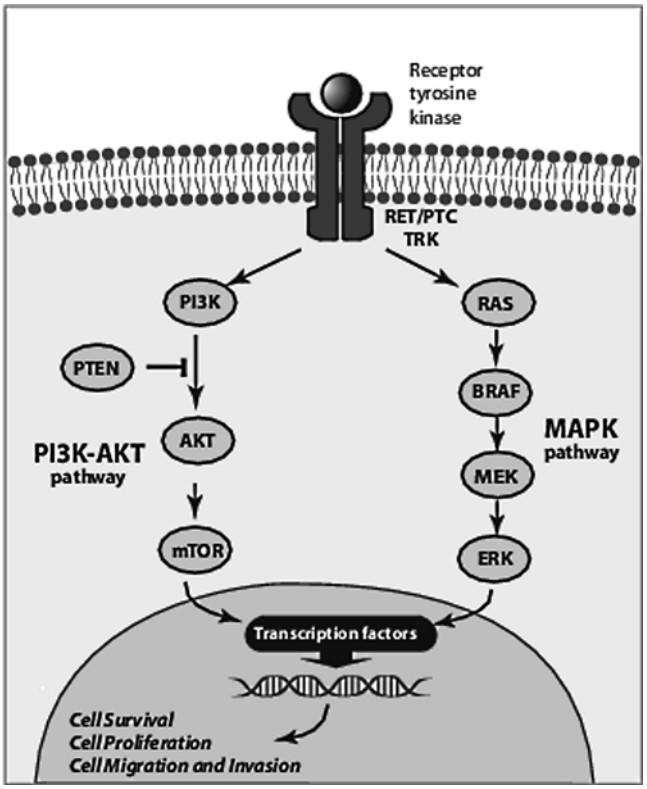


Figure 36.2 Thyroid cancer development and progression typically involves the activation of the mitogen-activated protein kinase (MAPK) and PI3K/PTEN/AKT signaling pathways. In thyroid cancer, the MAPK pathway is activated via point mutations of the *BRAF* or *RAS* gene, or chromosomal rearrangements involving the *RET* gene (known as RET/PTC rearrangement) or the *NTRK1* gene (TRK rearrangement). These non-overlapping genetic events are commonly found in well-differentiated papillary carcinomas and in some follicular carcinomas. Mutations in the genes coding for the effectors of the PI3K/PTEN/AKT pathway, such as *PIK3CA* (encoding a subunit of PI3K), *AKT1*, and *PTEN*, are found more frequently in follicular carcinomas and in more advanced and less-well-differentiated cancers

Molecular Basis of Disease

Thyroid cancer initiation and progression occurs through gradual accumulation of genetic and epigenetic alterations, including activating and inactivating somatic mutations, alteration in gene expression patterns, and miRNA dysregulation. Most mutations in thyroid cancer involve the effectors of the mitogen-activated protein kinase (MAPK) signaling pathway and the PI3K/PTEN/AKT signaling pathway (Fig. 36.2). Critical genes are frequently mutated in thyroid cancer via two distinct molecular mechanisms, point mutation or chromosomal rearrangement. MAPK activation frequently occurs via mutations in the cell membrane receptor tyrosine kinases RET and NTRK1, which are involved in chromosomal rearrangements, or through intracellular signal transducers BRAF and RAS, which are typically activated as a result of a point mutation. These mutually exclusive mutations occur in approximately 70 % of papillary thyroid carcinomas

(Table 36.1) [18–21]. In follicular carcinomas, mutations in the *RAS* genes are the most common, followed by *PAX8/PPARG* rearrangement. Thyroid cancer progression and dedifferentiation involves a number of additional mutations that affect the PI3K/PTEN/AKT signaling pathway and other cell signaling pathways (Table 36.1).

BRAF Mutations

BRAF is a serine-threonine kinase that is activated by RAS binding and protein recruitment to the cell membrane. BRAF phosphorylation leads to activation of MEK and other downstream targets along the MAPK signaling pathway (Fig. 36.2). In thyroid cancer, BRAF can be activated by point mutations, small in-frame deletions or insertions, or chromosomal rearrangement. The most common mechanism of activation is a point mutation of a thymine to adenine substitution at nucleotide 1799 (c.T1799A), resulting in a substitution of valine to glutamate at residue 600 (p.V600E) [19, 22]. The *BRAF* V600E mutation constitutes 95–99 % of all *BRAF* mutations found in thyroid cancer. Other alterations are *BRAF* p.K601E point mutation and small in-frame insertions or deletions surrounding codon 600 [23–26], as well as *AKAP9/BRAF* rearrangement [27]. The *BRAF* rearrangement seen in thyroid carcinomas is a paracentric inversion of chromosome 7q leading to the fusion of 3′ portion of the *BRAF* gene to the 5′ portion of the *AKAP9* gene and is more common in papillary carcinomas associated with radiation exposure [27]. More recently, several other fusion partners of *BRAF* have been identified in papillary carcinomas [28].

BRAF V600E is the most common genetic alteration in papillary thyroid carcinoma and is found in approximately 45 % of papillary thyroid tumors (Table 36.1) [29]. *BRAF* V600E also occurs in 10–20 % of poorly differentiated carcinomas and 30–40 % of anaplastic carcinomas arising from papillary carcinoma [30–33]. This mutation is typically found in papillary carcinomas with classic papillary histology and in the tall cell variant and is rare in the follicular variant of papillary carcinoma [18, 29]. In contrast, tumors with the *BRAF* K601E mutation are typically the follicular variant of papillary carcinoma [26]. *BRAF* V600E is not found in follicular carcinomas and benign thyroid nodules and therefore, among primary thyroid lesions, represents a specific marker of papillary carcinoma and related tumor types.

RAS Mutations

Point mutations of *RAS* are found in follicular carcinomas, papillary carcinomas, and follicular adenomas. Human *HRAS*, *KRAS*, and *NRAS* genes encode highly related G proteins that reside at the inner surface of the cell membrane and propagate signals arising from cell membrane receptors and G-protein-coupled receptors along the MAPK, PI3K/AKT, and other signaling pathways. Activating point mutations typically affect codons 12, 13, and 61 of the *RAS* genes. In thyroid cancer, *NRAS* codon 61 and *HRAS* codon 61 mutations are most common, followed by *KRAS* codon 12 and 13, although mutations have been found in different hot spots of all three genes. *RAS* mutations are present in 10–20 % of papillary carcinomas, 40–50 % of follicular carcinomas, and 20–40 % of poorly differentiated and anaplastic carcinomas [34–40]. Among papillary carcinomas, virtually all tumors with a *RAS* mutation belong to the follicular variant [18, 31]. *RAS* mutations also occur in 20–40 % of benign follicular adenomas [35, 36]. The finding of *RAS* mutations in benign adenomas as well as in follicular-patterned carcinomas suggests that *RAS* mutation positive follicular adenomas may serve as a precursor for *RAS* mutation positive follicular carcinoma and follicular variant of papillary carcinomas. Furthermore, *RAS* mutation may predispose well-differentiated cancers to dedifferentiation and anaplastic transformation [41–44]. Therefore, detection of this mutation at early stages may guide the therapy to prevent tumor progression.

RET/PTC Rearrangements

The *RET/PTC* chromosomal rearrangement is a characteristic of papillary thyroid cancer [45]. The rearrangement forms a fusion between the 3′ portion of the *RET* receptor tyrosine kinase gene and the 5′ portion of different partner genes. All chimeric genes contain the intact tyrosine kinase domain of *RET* fused to an active promoter of another gene that drives the expression and ligand-independent dimerization of the RET/PTC protein, leading to chronic stimulation of MAPK signaling (Fig. 36.2) [46–48]. The two most common fusions, *RET/PTC1* and *RET/PTC3*, are paracentric inversions since both *RET* and its respective fusion partners, *CCDC6* (*H4*) and *NCOA4* (*ELE1*), reside on the long arm of chromosome 10. In contrast, *RET/PTC2* and nine more recently discovered types of *RET/PTC* fusions are all interchromosomal rearrangements formed by *RET* fusion to genes located on different chromosomes (Table 36.2) [49].

RET/PTC is found in approximately 10–20 % of adult sporadic papillary carcinomas [18, 49] but occurs with higher incidence in patients with a history of radiation exposure (50–80 %) and in papillary carcinomas from children and young adults (40–70 %) [50–52]. The distribution of *RET/PTC* rearrangement within the tumor may be quite heterogeneous, varying from involving almost all neoplastic cells (clonal *RET/PTC*) to being detected only in a small fraction of tumor cells (non-clonal *RET/PTC*) [53, 54]. Although a low level of *RET/PTC* rearrangement has been

Table 36.1 Average prevalence of mutations in thyroid cancer

Tumor type	Genes	Prevalence of common mutations (%)
Papillary carcinoma		
	BRAF	40–45
	RET/PTC	10–20
	RAS	10–20
	NTRK1	<5
	NTRK3	<5
	ALK	<5
Follicular carcinoma		
	RAS	40–50
	PAX8-PPARG	30–35
	PIK3CA	<10
	PTEN	<10
Poorly differentiated carcinoma		
	RAS	20–40
	TP53	20–30
	CTNNB1	<10
	BRAF	10–20
	AKT1	5–10
	PIK3CA	5–10
Anaplastic (undifferentiated) carcinoma		
	TP53	50–80
	CTNNB1	50–60
	RAS	20–40
	BRAF	20–40
	PIK3CA	10–20
	PTEN	5–15
	AKT1	5–10

reported in adenomas and other benign thyroid lesions in studies that used ultrasensitive detection techniques, the clonal *RET/PTC* (i.e., rearrangement that is found in most cells within the tumor) is specific for papillary thyroid carcinoma [49, 53]. Proper techniques for the clinically relevant detection of *RET/PTC* are discussed later in the chapter. Among different rearrangement types, *RET/PTC1* is typically the most common, followed by *RET/PTC3*, whereas *RET/PTC2* and other novel rearrangement types are rare (Table 36.2) [49].

NTRK1 and *NTRK3* Rearrangements

Chromosomal rearrangements involving the *NTRK1* and *NTRK3* receptor tyrosine kinase genes also occur in papillary thyroid carcinomas, although with a significantly lower prevalence. The *NTRK1* gene resides on chromosome 1q22 and can be fused to at least three different partner genes located on the same or different chromosomes, leading to the *TRK* rearrangement (Table 36.2) [55–57]. *NTRK* rearrangements occur in less than 5 % of papillary thyroid carcinomas [28]. Fusions involving another *NTRK* family gene, *NTRK3*, also occur in papillary thyroid cancer. *ETV6-NTRK3* fusions occur in approximately 2 % of sporadic papillary thyroid cancers and with a significantly higher prevalence (approximately 15 %) in tumors associated with exposure to ionizing radiation [28, 58].

ALK Rearrangements

Recently, rearrangements involving the *ALK* gene were identified in thyroid cancer. The most common fusion partner of *ALK* is the striatin (*STRN*) gene [59]. *ALK* fusions are found in 1–2 % of papillary carcinomas and with higher frequency (5–10 %) in poorly differentiated and anaplastic thyroid cancers [28, 59].

PPARG Rearrangements

PAX8/PPARG rearrangement is a t(2;3)(q13;p25) translocation that leads to fusion between a portion of the *PAX8* gene, which encodes a paired domain transcription factor, and the peroxisome proliferator-activated receptor (*PPARG*) gene [60]. *PAX8/PPARG* rearrangement leads to strong overexpression of the PPARG protein, although the mechanisms of cell transformation induced by this genetic event are not understood. Several types of *PAX8/PPARG* rearrangement occur, formed by the fusion of four *PAX8* gene regions (exons 1–7, 1–8, 1–9, or 1–7 plus 9) to *PPARG* exons 1–6. These different *PAX8* gene region fusions are apparently a result of the alternate splicing involving exons 8 and 9 known to affect the wild-type *PAX8*. The most commonly expressed *PAX8/PPARG* transcripts in follicular thyroid carcinomas contain exons 1–9 and 1–7 plus 9 of *PAX8*. In addition to *PAX8/PPARG*, the *PPARG* gene can fuse with the *CREB3L2* gene; however, this type of fusion is rare (Table 36.2) [61].

PAX8/PPARG is characteristically found in 30–35 % of follicular thyroid carcinoma [62–64]. This rearrangement also occurs in a small proportion (1–5 %) of the follicular variant of papillary carcinomas and in some (2–13 %) follicular adenomas [62–66]. Follicular adenomas with a *PAX8/PPARG* rearrangement typically have a thick capsule and show the immunohistochemical profile characteristic of thyroid cancer, suggesting preinvasive (in situ) follicular carcinomas or malignant tumors where invasion was overlooked during histological examination [63]. *PAX8/PPARG* rearrangements and *RAS* point mutations rarely occur in the same tumor, suggesting that they represent distinct oncogenic pathways in the development of follicular thyroid carcinoma [63].

Table 36.2 Common chromosomal alterations in thyroid cancer

	Tumor type	Fusion name	Fusion genes	Chromosomal alterations	Prevalence of subtypes
RET Fusions	PTC	*RET/PTC1*	*RET/CCDC6(H4)*	inv(10)(q11.2;q21)	60–70 %
		RET/PTC2	*RET/RIα*	t(10;17)(q11.2;q23)	<5 %
		RET/PTC3	*RET/NCOA4(ELE1)*	inv(10)(q11.2)	20–30 %
		RET/PTC4	*RET/NCOA4 (ELE1)*	inv(10)(q11.2), v.2	<1 %
		RET/PTC5	*RET/GOLGA5*	t(10;14) (q11.2;q?)	<1 %
		RET/PTC6	*RET/HTIF1*	t(7;10)(q32;q11.2)	<1 %
		RET/PTC7	*RET/RFG7*	t(1;10)(p13;q11.2)	<1 %
			RET/ELKS	t(10;12)(q11.2;p13)	<1 %
			RET/KTN1	t(10;14)(q11.2;q22.1)	<1 %
			RET/RFG9	t(10;18)(q11.2;q21-22)	<1 %
			RET/PCM1	t(8;10)(p21-22;q11.2)	<1 %
			RET/RFP	t(6;10)(p21;q11.2)	<1 %
			RET/HOOK3	t(8;10)(p11.21;q11.2)	<1 %
NTRK Fusions	PTC		*NTRK1/TPM3*	inv(1)(q23.1;q21.3)	Equally prevalent
			NTRK1/TPR	inv(1)(q23.1;q25)	
			NTRK1/TFG	t(1;3)(q21;q11)	
			ETV6-NTRK3	t(12;15)(p13;q25)	
ALK Fusions	PTC		*STRN/ALK*	inv(2)(p22;p23)	70 %
			EML4/ALK	inv(2)(p21;p23)	30 %
PPARG Fusions	FTC, PTC, FV		*PAX8/PPARG*	t(2;3)(q13;p25.2)	98 %
			CREB3L2/PPARG	t(7;3)(q33;p25.2)	2 %

FTC follicular thyroid carcinoma, *FV* follicular variant, *PTC* papillary thyroid carcinoma

TERT Mutations

Mutations of the telomerase reverse transcriptase (*TERT*) gene promoter were first described in melanoma at two specific hot spots (chr5:1295228C→T, termed C228T, and chr5:1295250C→T, termed C250T) and lead to increased transcriptional activity and expression of the gene [67, 68]. The C228T and C250T *TERT* promoter mutations occur with variable prevalence in different types of thyroid cancer and have strong association with tumor recurrence, distant metastasis, and tumor-related mortality [69–72]. *TERT* mutations are not found in benign thyroid nodules and therefore are useful diagnostically; they can also play a role in prognostication of thyroid cancer.

Mutations Associated with Tumor Dedifferentiation

Thyroid cancer progression and dedifferentiation is more frequent in tumors with *BRAF* and *RAS* mutations and typically involves the accumulation of additional genetic alterations (Table 36.1). Point mutations affecting the *TP53* gene are very common in anaplastic carcinomas (50–80 % of cases) [73–76] but less frequent in poorly differentiated carcinomas and extremely rare in well-differentiated thyroid cancer. *TP53* mutations are most common in exons 5–8 and lead to loss of function of this important cell cycle regulator. The *CTNNB1* gene frequently mutates in anaplastic carcinoma and encodes β-catenin that is involved in cell adhesion and the wingless (Wnt) signaling. Point mutations in exon 3 of *CTNNB1* occur in up to 60 % of anaplastic carcinomas and with lower prevalence in poorly differentiated thyroid carcinomas [77, 78]. In addition, mutations in *PIK3CA*, *PTEN*, and *AKT1* genes are found in anaplastic and poorly differentiated carcinomas, but they are less common [33, 79–82].

Gene Expression and miRNA Expression

Thyroid papillary carcinomas and other types of thyroid cancer have distinct alterations in gene expression [21, 83–86]. Gene expression changes include downregulation of genes responsible for specialized thyroid function such as thyroid hormone synthesis, upregulation of many genes involved in cell adhesion, motility, cell-cell interaction, and different patterns of deregulation of genes coding for cytokines and other proteins involved in inflammation and immune response. Among papillary carcinomas, different mRNA expression profiles correlate with classic papillary histology,

follicular variant, and tall cell variant [83, 87]. Moreover, presence of *BRAF*, *RAS, RET/PTC*, and *NTRK1* mutations correlates with different patterns of gene expression, providing a molecular basis for distinct phenotypic and biologic features associated with each mutation type [21, 83].

Many miRNAs are deregulated in thyroid cancer [88–91]. Generally, miRNA expression profiles are different between papillary carcinoma, follicular carcinoma, and other types of thyroid tumors [92]. Several specific miRNAs, such as miR-146b, miR-221, and miR-222, have increased expression in papillary carcinomas and may play a role in the development of these tumors [89, 91, 92]. Possible target genes for these miRNAs are the regulator of the cell cycle *p27(Kip1)* gene and the thyroid hormone receptor (*THRB*) gene [93, 94]. Several abnormally expressed miRNAs were found in follicular carcinomas (miR-197, miR-346, miR-155, miR-224) [90, 95] and anaplastic carcinomas (miR-30d, miR-125b, miR-26a, and miR-30a-5p) [96].

Clinical Utility of Testing

Preoperative Diagnosis of Thyroid Cancer

Molecular markers are helpful in improving the preoperative diagnosis of cancer in thyroid nodules. Despite the high diagnostic value of FNA cytology, it cannot reliably diagnose cancer in 20–30 % of nodules, and such cases are considered as indeterminate for malignancy. The inability to rule out cancer in these nodules leads to diagnostic lobectomy for most of these patients, although most surgically removed thyroid nodules are benign [12]. Additionally, those patients that are found to have cancer on surgery have to undergo a second surgery to complete thyroidectomy. Both the unnecessary surgeries and two-step surgical management can be avoided with more accurate preoperative diagnosis of cancer. The current American Thyroid Association's management guidelines recommend testing for mutational markers for nodules with indeterminate FNA cytology to help guide clinical management [97].

Mutational markers that have been most extensively validated and clinically used for preoperative diagnosis of thyroid cancer in FNA samples include *BRAF* and *RAS* point mutations and *RET/PTC*, *PAX8/PPARG*, and *NTRK1* rearrangements [98–101]. Finding of any of these mutations in thyroid FNA samples is a strong predictor of malignancy in thyroid nodules irrespective of the cytological diagnosis [98–100]. *BRAF* mutation has been studied most extensively, and in a meta-analysis of 22 studies of thyroid FNA samples, *BRAF* mutation correlated with malignant outcome in 99.3 % of cases [102]. The presence of a *RET/PTC* or *PAX8/PPARG* rearrangement also correlates with malignancy in close to 100 % of cases. Therefore, patients with these mutations would be candidates for total thyroidectomy irrespective of the cytologic diagnosis (Fig. 36.3). This would eliminate the need for intraoperative pathology consultation and subsequent second surgery for complete thyroidectomy, reducing costs and additional morbidity. Detection of a *RAS* mutation, which is the second most common mutation after *BRAF*, conferred a 74–87 % probability of malignancy [98, 99, 103]. Importantly, *RAS* mutations are found in tumors which are difficult to diagnose by cytology alone, i.e., follicular variant of papillary carcinoma and follicular carcinoma [104]. The remaining *RAS*-positive nodules are diagnosed as a benign follicular adenoma, which is most likely a precursor lesion for follicular carcinoma [63]. Therefore, surgical removal of follicular adenomas that carry this oncogenic mutation by lobectomy may be considered as justifiable to prevent tumor progression.

Testing for a seven-gene panel (*BRAF*, *NRAS*, *HRAS*, *KRAS*, *RET/PTC1*, *RET/PTC3*, and *PAX8/PPARG*) is particularly helpful in nodules with indeterminate cytology. In a prospective study of 1,056 consecutive thyroid FNA samples with indeterminate cytology, detection of any mutation in specific categories of indeterminate cytology, i.e., AUS/FLUS, FN/SFN, and SMC, conferred a risk of histologic malignancy in 88 %, 87 %, and 95 % of nodules, respectively [103]. The risk of cancer in mutation-negative nodules was 6 % in the AUS/FLUS group, 14 % in FN/SFN, and 28 % in SMC [103]. The clinical algorithm outlined in Fig. 36.3 recommends that any positive result in the mutational panel is an indication for total thyroidectomy in all categories of indeterminate cytology as the initial surgical approach [103]. This avoids a repeat of FNA and proceeds with optimal surgical management without delay. This clinical approach also eliminates the need for the current two-step surgery, i.e., diagnostic lobectomy followed by completion thyroidectomy for most patients with malignant nodules. In a series of 471 patients with thyroid nodules that had indeterminate cytology (AUS/FLUS or FN/SFN), patients with no access to mutation testing were 2.5-fold more likely to require a two-stage surgery [105]. A mutation-negative result of the seven-gene panel does not eliminate the risk of cancer, as expected based on its sensitivity of approximately 70 %. Therefore, diagnostic lobectomy is justified as the initial surgical intervention for mutation-negative nodules with FN/SFN and SMC cytology, whereas conservative management with ultrasound follow-up and repeat FNA can be considered for nodules with AUS/FLUS cytology (Fig. 36.3). Although larger gene panels are available, even the seven-gene panel, when applied routinely to thyroid FNA samples with indeterminate cytology, leads to an overall cost saving for patients with thyroid nodules due to the up-front offering of optimal surgical management [106].

The expansion of knowledge on driver mutations in thyroid cancer and the availability of new high-throughput tech-

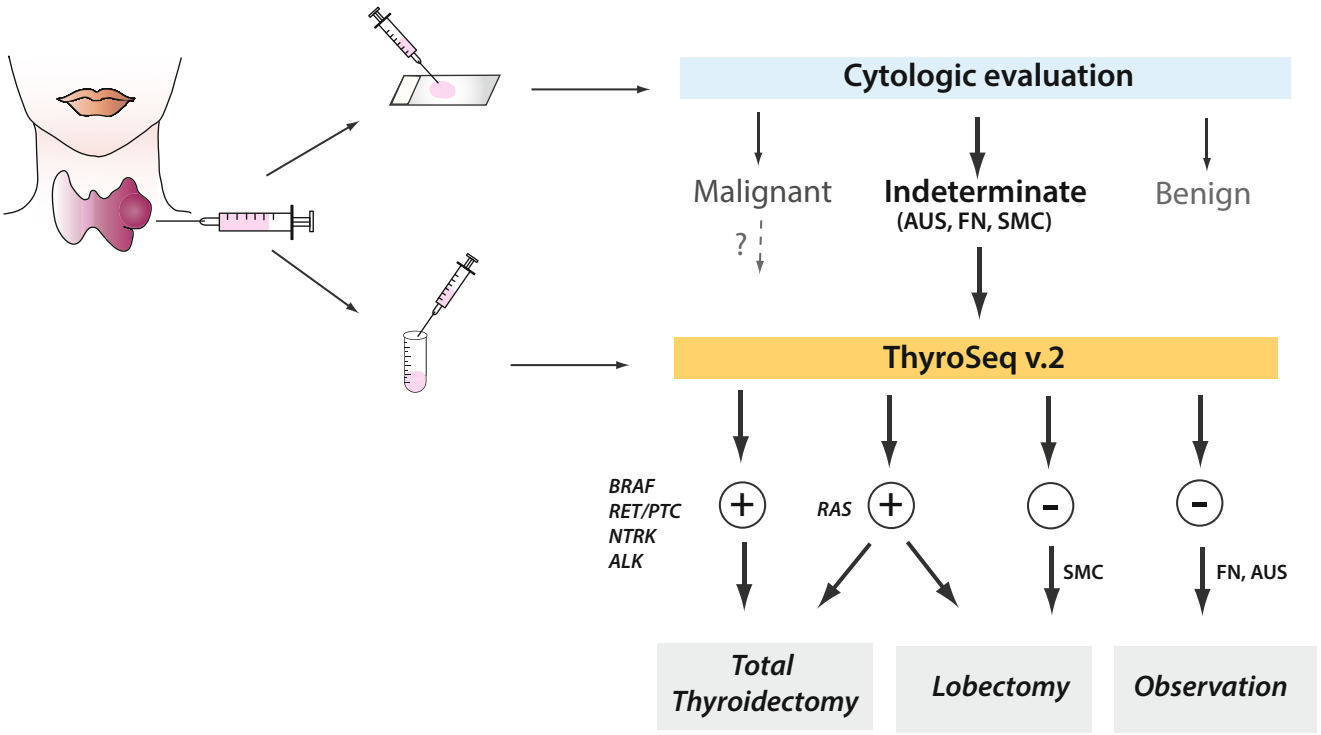


Figure 36.3 Clinical management of patients with thyroid nodules based on the combination of cytological examination and ThyroSeq v.2 mutational analysis of fine needle aspiration (FNA) samples. Molecular testing is particularly helpful for nodules with indeterminate cytology. Due to a high risk of cancer in nodules with *BRAF* mutations or *RET/PTC*, *PAX8/PPARG*, and *TRK* rearrangement, surgical treatment can proceed directly to total thyroidectomy. *RAS* mutations confer a 70–80 % risk of cancer, and these patients may benefit from either total thyroidectomy or lobectomy, depending on the additional clinical and imaging findings. Nodules without mutations found on ThyroSeq v.2 panel that have a cytologic diagnosis of follicular neoplasm/suspicious for follicular neoplasm (FN/SFN) or atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS) have a 4–5 % residual probability of cancer and may be followed conservatively. Nodules with suspicious for malignant cells (SMC) cytology and negative for mutations have approximately 20 % residual risk of cancer and should be managed by lobectomy.

nologies for simultaneous detection of multiple genetic mutations provided the basis for expanding gene panels for thyroid FNA samples. A 12-gene panel of selected gene regions from *AKT1*, *BRAF*, *CTNNB1*, *GNAS*, *PIK3CA*, *TP53*, *TSHR*, *PTEN*, *HRAS*, *KRAS*, *NRAS*, and *RET* (e.g., ThyroSeq [107]) utilizes next generation sequencing (NGS) to expand the original seven-gene panel and test for mutations in additional genes implicated in thyroid tumors. Detection of additional mutations together with higher sensitivity of detecting all mutations offered increased test performance. A study on 228 thyroid nodule samples including 51 FNA samples showed accurate detection of multiple mutations with a sensitivity of 3–5 % [107]. Common *BRAF* and *RAS* mutations were identified at low level in 27 tumors by NGS, which were not detected by the Sanger sequencing. This indicates that NGS-based panels not only can assess additional genes and hot spots mutations in a single test but also detect common driver mutations at a higher rate, thereby increasing the sensitivity and negative predictive value (NPV) of cancer detection in thyroid nodules [107]. Although most thyroid tumors had a single oncogenic mutation, this panel identified 2–3 mutations in each of the nine tumors, most of which presented at a higher stage, with dedifferentiation on histopathology [107]. As a result, identification of multiple mutations may be used to preoperatively identify those patients that need a significantly more aggressive treatment plan to maximize the chances for disease cure.

Commercially available NGS gene panels, such as AmpliSeq, contain many of the genes important to thyroid carcinogenesis. One study analyzed 34 indeterminate FNA samples using DNA obtained from cell blocks or from stained smears and tested them for mutations in 50 genes (AmpliSeq panel) [108]. Mutations in *BRAF*, *NRAS*, *KRAS*, and *PTEN* were detected in these samples, and the presence of a mutation in any of these genes was a strong indicator of cancer. In this study, the residual risk of cancer in nodules with indeterminate cytology and a negative molecular test result was 8 %.

Expansion of the gene panels to include additional and more recently discovered point mutations and gene fusions further increases the sensitivity and overall performance of gene tests for cancer diagnosis in thyroid nodules. An NGS gene panel that includes 56 genes and gene fusions (ThyroSeq®

v.2, UPMC/CBLPath, Rye Brook, NY) was validated with 143 thyroid nodules with FN/SFN cytology and known surgical outcome and showed both high sensitivity (90 %) and specificity (93 %) for cancer detection, with the NPV of 96 % [109]. Based on the high sensitivity and specificity, the NPV and positive predictive value (PPV) are expected to remain high in a broad range of cancer prevalence in the tested population.

Another approach to cancer detection in thyroid nodules is through the analysis of gene expression changes associated with cancer development. A commercial test, known as Afirma Thyroid FNA Analysis (Veracyte, South San Francisco, CA), utilizes the mRNA expression profiles of 142 genes to classify indeterminate thyroid nodules into a benign or suspicious category using a proprietary algorithm [110, 111]. This test was validated in a multi-institutional prospective double-blind study that included 265 nodules with indeterminate cytology [111]. The study showed high NPV in nodules with AUS/FLUS (95 %) and FN/SFN (94 %) cytology, whereas the PPV remained low (38 % for AUS/FLUS and 37 % for FN/SFN) [111]. High NPV suggests that this test is particularly helpful as a "rule-out" test, thereby helping to avoid unnecessary surgeries [112, 113].

Prognostic and Treatment Implications

Among prognostic markers, one of the best studied is the *BRAF* V600E mutation. *BRAF* V600E is associated with poor prognostic factors in papillary thyroid cancer such as extrathyroidal invasion, lymph node metastases, and tumor recurrence (reviewed in Ref. 114). In thyroid FNA specimens, preoperative testing that identifies a *BRAF* V600E mutation may be associated with disease persistence and recurrence [14], although some studies did not find such association [115–117]. A meta-analysis of multiple studies encompassing almost 2,500 patients demonstrated that a *BRAF* V600E mutation was significantly associated with tumor recurrence or persistent disease, which was found in 25 % of tumors with a *BRAF* V600E mutation compared to 13 % of *BRAF* mutation-negative tumors [118]. In addition, a large, multicenter study of 1,849 patients found the presence of the *BRAF* V600E mutation to be significantly associated with increased mortality from papillary thyroid cancer [119]. The overall mortality was 5 % in patients with a *BRAF* V600E mutation and 1 % in patients with a *BRAF* mutation-negative tumor. The results of these studies indicate that *BRAF* V600E is overall a sensitive but not specific marker of unfavorable outcome.

The presence of multiple driver mutations in thyroid cancer is associated with more aggressive tumor behavior. Coexisting mutations in the early driver genes, such as *BRAF* or *RAS*, with mutations in *PIK3CA*, *AKT1*, or *TP53* in the same tumor occur in poorly differentiated and anaplastic tumors [79, 81, 120]. More recently, an NGS-based mutation analysis demonstrated that approximately 4 % of well-differentiated papillary cancers have more than one mutation, and these tumors are aggressive and typically present with distant metastases [107].

TP53 mutation is a well-characterized genetic event governing thyroid tumor dedifferentiation and is found with high frequency in poorly differentiated and anaplastic thyroid cancer [73, 74]. However, *TP53* mutation also occurs in some well-differentiated cancers such as papillary thyroid carcinoma and oncocytic follicular carcinoma [107]. Well-differentiated cancers carrying a *TP53* mutation may have greater tumor dedifferentiation and a more aggressive clinical course.

Another prognostic molecular marker for thyroid cancer is a *TERT* mutation. The C228T and C250T mutations have a significantly higher prevalence in aggressive thyroid tumors including widely invasive oncocytic (Hürthle cell) carcinoma and anaplastic thyroid carcinoma [69–72]. In the largest study of thyroid cancer reported to date, *TERT* promoter mutations were an independent risk factor for persistent disease, distant metastases, and disease-specific mortality for well-differentiated thyroid cancer and separately for papillary carcinoma and follicular carcinoma [72]. Overall, testing for specific mutations and their combinations may provide important prognostic information and accurately identify patients who may benefit from more extensive initial thyroid surgery to prevent tumor recurrence and from more frequent monitoring of disease recurrence.

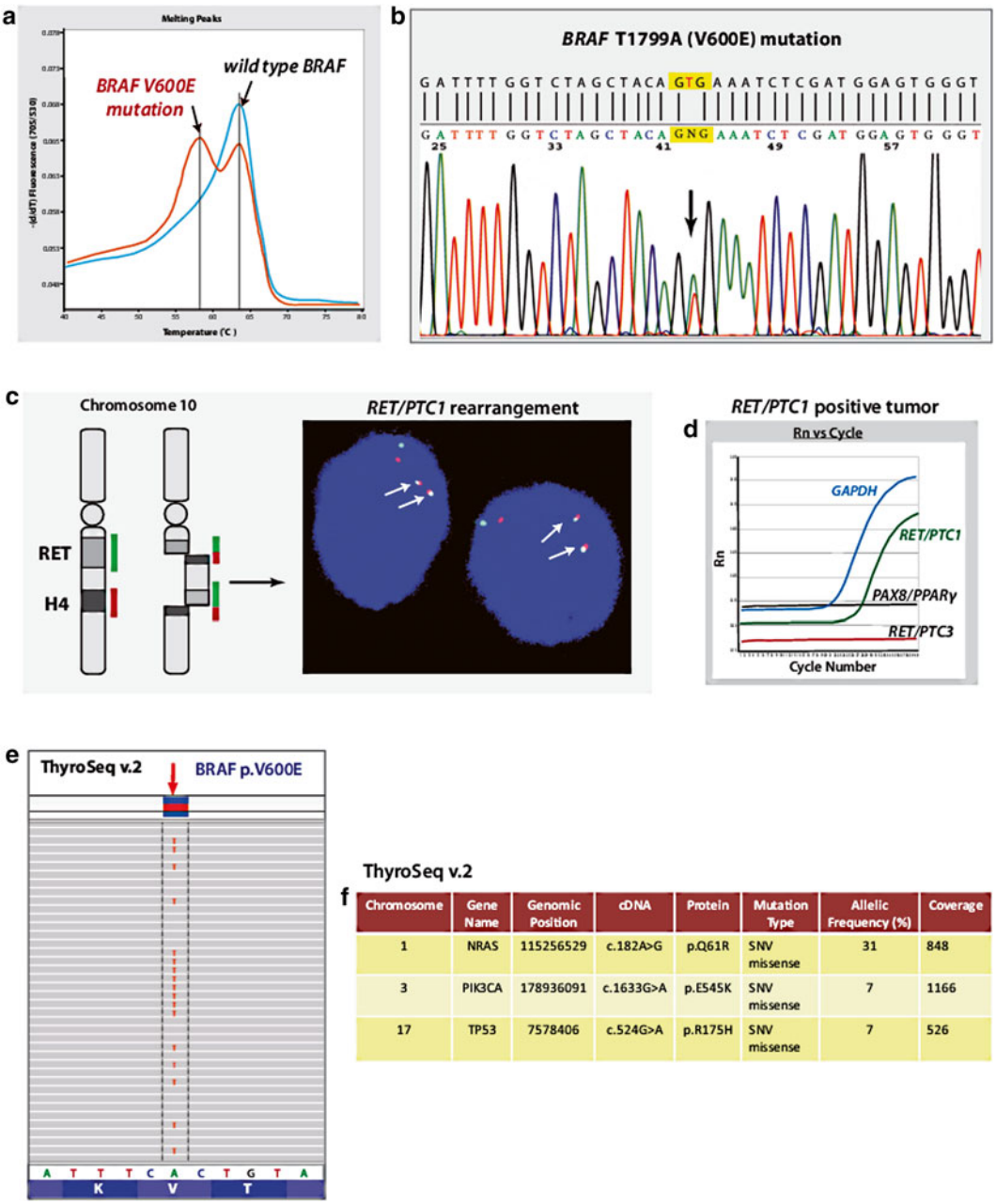
Patients with advanced thyroid cancer carrying activating mutations in the MAPK and PI3K pathways may benefit from treatment with tyrosine kinase inhibitors (sorafenib, vandetanib, axitinib, sunitinib) [121]. Also, selective inhibitors of the V600E mutant BRAF kinase (vemurafenib, PLX4032) showed promising early results in clinical trials, as well as inhibitors of ALK and NTRK kinases.

Available Assays

Testing for Mutations

For preoperative diagnosis of thyroid cancer, FNA samples can be tested for a shorter or broader panel of mutations. Those should contain most frequently occurring alterations including point mutations (*BRAF*, *HRAS*, *NRAS*, *KRAS*) and chromosomal rearrangements (*RET/PTC1*, *RET/PTC3*, *PAX8/PPARG*).

Detection of point mutations can be performed using many different methods, including Sanger sequencing, real-time PCR, pyrosequencing, allele-specific PCR, snapshot array, or restriction fragment polymorphism analysis



Chromosome	Gene Name	Genomic Position	cDNA	Protein	Mutation Type	Allelic Frequency (%)	Coverage
1	NRAS	115256529	c.182A>G	p.Q61R	SNV missense	31	848
3	PIK3CA	178936091	c.1633G>A	p.E545K	SNV missense	7	1166
17	TP53	7578406	c.524G>A	p.R175H	SNV missense	7	526

Figure 36.4 Laboratory techniques for detection of mutations in thyroid cancer. (**a**) Real-time PCR with post-PCR fluorescence melting curve analysis showing two melting peaks, one corresponding to a wild-type allele and the other to a mutant *BRAF* c.T1799A (p.V600E) allele. (**b**) Sanger sequencing detection of a *BRAF* c.T1799A (p.V600E) mutation, with vertical arrow indicating the heterozygous T and A nucleotides of the heterozygous wild-type and V600E alleles. (**c**) FISH detection of *RET/PTC1* rearrangement (arrows) using the fusion probe design. (**d**) Real-time RT-PCR analysis showing *RET/PTC1* rearrangement and no *RET/PTC3* and *PAX8/PPARG* rearrangements. (**e**) Detection of *BRAF* c.T1799A (p.V600E) mutation using targeted next generation sequencing gene panel. (**f**) Results of testing of a thyroid FNA sample using a 56-gene mutation panel showing the presence of three mutations involving the *NRAS*, *PIK3CA*, and *TP53* genes, which indicates a high risk of cancer in this nodule and suggests that the cancer may be prone to dedifferentiation and more aggressive biological behavior

(Fig. 36.4a, b) [19, 31, 122–126]. Other methods can be used for more sensitive detection of point mutations, e.g., coamplification at lower denaturation polymerase chain reaction (COLD-PCR), locked nucleic acids (LNA)-PCR, and others [103, 127]. In one study, detection of *BRAF* mutations was compared using probe-specific real-time PCR, real-time allele-specific PCR, direct sequencing, and a colorimetric assay and showed similar sensitivity in *BRAF* detection in archival FNA samples [125].

Real-time PCR methods are rapid, easy to perform, cost-efficient, and run in a closed PCR system that reduces the risk of PCR amplicon contamination. Real-time PCR followed by

fluorescence melting curve analysis is frequently used for detection of *BRAF* and *RAS* mutations [63, 128]. Two probes complementary to wild-type sequences are designed to span the mutation site for each mutational hot spot, including codons 12, 13, and 61 of the *RAS* genes and codons 600 and 601 of the *BRAF* gene. If no mutation is present, probes will bind perfectly to the sample DNA and melt at a higher temperature, showing a single peak on post-PCR melting curve analysis (Fig. 36.4a). In contrast, if a heterozygous mutation is present, probes will bind to mutant DNA imperfectly, i.e., with one nucleotide mismatch, and will melt (dissociate) earlier, producing two melting peaks (Fig. 36.4a). Each nucleotide substitution produces a melting peak at a specific melting temperature (T_m). This method detects all possible mutation variants at the interrogated hot spot using a minimal amount of DNA.

The two most common approaches for detection of chromosomal rearrangements (*RET/PTC*, *PAX8/PPARG*, and *TRK*) are reverse transcription PCR (RT-PCR) and fluorescent in situ hybridization (FISH). RT-PCR is a reliable and sensitive technique for detection of fusion transcripts in fresh FNA samples and frozen tissue specimens. Assays frequently use real-time RT-PCR with fluorescently labeled probes, which increase the specificity of transcript detection and allow quantification of the amplified product (Fig. 36.4c) [98]. Amplification of a housekeeping gene in each RT-PCR reaction monitors RNA quality and quantity. When RT-PCR is used for detection of rearrangements from formalin-fixed paraffin-embedded (FFPE) tissue samples, amplification of short PCR products can overcome poor-quality RNA and avoid false-negative results.

Highly sensitivity techniques (such as nested PCR amplification or blotting of PCR products with specific probes) are not optimal for detection of rearrangements due to an increased risk of false-positive results due to RT-PCR contamination or amplification of nonspecific sequences and require rigorous use of negative controls. In addition, ultrasensitive techniques may result in the detection of rearrangements that are present in a small fraction of the tumor. This is particularly problematic for the detection of the *RET/PTC* rearrangement, which can vary from involving almost all neoplastic cells (clonal *RET/PTC*) to involving only a small fraction of tumor cells (non-clonal *RET/PTC*) [53, 54]. Since only clonal *RET/PTC* rearrangement is specific for papillary carcinoma [45, 53], the sensitivity of detection will not be greater than 1 % of tumor cells (i.e., detection of 1 % or more tumors cells in the background of normal cells) to avoid detecting non-clonal rearrangements, which have no clinical implications at this time.

For detection of gene rearrangements in FFPE samples, where RNA is degraded, FISH is a reliable method (Fig. 36.4d). FISH utilizes fluorescently labeled DNA probes for targeted detection of gene rearrangements in interphase or metaphase nuclei. The FISH probes are relatively large in size, ranging from 20 to 200 kb. Currently, probes for detection of *RET/PTC* or *PAX8/PPARG* rearrangements are not commercially available, but bacterial artificial chromosomes clones are available [53, 54, 60]. Several positive and negative controls are required to validate the scoring criteria for accurate FISH results. For *RET/PTC* rearrangement, the cutoff level for positive test results is 7–30 % positive cells, depending on the probe design [53, 129].

Introduction of NGS technology has enabled high-throughput detection of multiple genetic alterations in both constitutional and cancer genomes. NGS has clear advantages over conventional sequencing techniques, such as Sanger sequencing, by allowing sequencing of large regions of the genome at lower cost and with higher sensitivity. NGS can be used to sequence the genome, exome, transcriptome (mRNA), and targeted multigene panels. While genome or exome analyses are essential for discovery projects, targeted gene panels are advancing into routine clinical testing of thyroid cancer. Targeted NGS gene panels include testing for common mutations in thyroid cancer and for multiple genetic alterations known to occur in thyroid cancer with low prevalence, such as mutations in the *PIK3CA*, *AKT1*, *PTEN*, and *TP53* genes [102, 108] and chromosomal rearrangements of the *BRAF*, *ALK*, and *NTRK* genes. An NGS-based panel that includes 56 genes and gene fusions (ThyroSeq v.2) has been recently validated for preoperative diagnosis of thyroid nodules [109]. Commercially available targeted NGS gene panels that offer sequencing for mutations in cancer-related genes or custom NGS thyroid panels can be used.

Testing for Gene Expression and miRNA Expression

In addition to gene mutations, changes in mRNA and miRNA expression have been explored for diagnostic use in thyroid samples. Search for a limited number of differentially expressed genes that can be used diagnostically appears to be promising. Upregulation of the *HMGA2* gene in malignant thyroid tumors has been found in several studies and may be of diagnostic utility for thyroid nodule FNA samples [130, 131]. Aberrant expression of *MET*, *TPO*, *TIMP1*, *DPP*, and other genes was observed in several studies and explored for diagnostic use [83–85, 131, 132]. At least one company is exploring the use of gene expression profiling of thyroid FNA samples as a tool for determining the benign or malignant potential of thyroid nodules [110]. The possibility of applying a combination of cytological evaluation, mutational analysis, and gene expression markers to improve the FNA diagnosis of thyroid nodules may improve clinical care for these patients.

The diagnostic utility of miRNA expression in thyroid FNA samples has been also explored [88, 92, 133, 134]. In one study, preoperative assessment of several miRNAs (miR-221, miR-222, miR-146b, miR-224, miR-155, miR-197,

miR-187) in thyroid nodule FNA samples demonstrated that upregulation of three or more of these miRNAs can predict papillary or follicular thyroid cancer with 98 % accuracy [92]. This demonstrates the feasibility of miRNA detection in thyroid FNA samples and provides initial evidence for its possible diagnostic use pending further validation.

Laboratory Issues

Collection of FNA Sample

Freshly collected and fixed FNA and resection specimens can be used for clinical molecular testing. Collection of fresh FNA samples during routine FNA procedures is simple, does not prolong the FNA procedure, and yields DNA and RNA of excellent quality. Typically, the FNA procedure is conducted under ultrasound guidance to ensure sampling of the nodule, with thyroid cells collected using a 23, 25, or 27 gauge needle and sent for cytological evaluation. In most cases, 3–4 FNA needle passes are performed. To collect a sample for molecular testing during an FNA procedure, either one entire pass is taken for molecular testing or most of the aspirated sample from the first two passes (the most representative sample) is used for direct cytology smears for cytological evaluation, with the residual material in the needle and the needle wash from both passes placed into a tube containing nucleic acid preservative solution, e.g., RNAlater (Qiagen) or Trizol (Invitrogen) (Fig. 36.3). The latter approach allows successful sampling of the nodule in 90–98 % of cases [98, 103]. After collection, the FNA specimen for molecular testing can be stored at −20 or −80 °C until molecular testing is performed. If collection of fresh FNA material is not possible, fixed cytology FNA material, i.e., stained cytology smear or cytology cell block, can be used for molecular testing. Use of a fixed specimen provides reliable detection of point mutations but is not ideal for detection of chromosomal rearrangements due to the suboptimal quality of the RNA.

Quality Assurance

The quantity and quality of nucleic acids isolated from FNA specimens can be assessed either by spectrophotometric measurements or by PCR amplification. Real-time PCR can be used to assess the quantity and quality of nucleic acids in a simple and cost-efficient way via evaluating PCR amplification of the *RAS* or *BRAF* genes for DNA and amplification of the *GAPDH* housekeeping gene for RNA.

Fresh FNA samples should be evaluated for sample adequacy prior to molecular testing to assess the proportion of thyroid epithelial cells and tumor cells within the sample. Thyroid FNA samples may contain a number of "contaminant" cells, i.e., lymphocytes, other white blood cells, and stromal cells. An abundance of these non-epithelial cells may decrease sensitivity of detection and lead to a false-negative result. Assessment of the proportion of thyroid epithelial cells within an FNA sample can be performed by comparing the expression of the universal housekeeping gene (i.e., *GAPDH*), which is uniformly expressed in all cell types, with the expression of a gene that is expressed only in thyroid cells or in several types of epithelial cells including thyroid cells, such as the thyroid peroxidase (*TPO*) gene, the thyroglobulin (*TG*) gene, and cytokeratin genes (*KRT7* and *KRT19*) [103, 135].

For assurance of quality of molecular testing, a set of positive and negative controls at different levels of allelic frequencies has to be used during each analytical run. Some controls are available through the commercial sources, e.g., Horizon Diagnostics (Cambridge, UK). In addition, the College of American Pathologists offers proficiency testing for several of the most commonly mutated genes in thyroid cancer, including *BRAF* and *KRAS*.

Conclusions

Our understanding of the molecular changes in thyroid cancers is improving the clinical management of patients with thyroid nodules. Molecular testing can enhance the accuracy of cancer diagnosis in thyroid nodules, cancer prognosis, and will likely be important for selection of targeted therapies for thyroid cancer. The most significant impact of molecular testing is the improved diagnosis of cancer in nodules with indeterminate cytology results. Research discoveries using NGS technologies will lead to identification of novel mutations and other genetic and epigenetic events in thyroid cancer with the potential for further improvement in the care of patients with thyroid cancer.

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