Molecular approaches to thyroid cancer diagnosis

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Abstract

Thyroid nodules are common, and the accurate diagnosis of cancer or benign disease is important for the effective clinical management of patients. Molecular markers are a helpful diagnostic tool, particularly for cytologically indeterminate thyroid nodules. In the past few years, significant progress has been made in developing molecular markers for clinical use in fine-needle aspiration specimens, including gene mutation panels and gene expression classifiers. With the availability of next generation sequencing technology, gene mutation panels can be expanded to interrogate multiple genes simultaneously and to provide yet more accurate diagnostic information. In addition, recently several new molecular markers of thyroid cancer have been identified that offer diagnostic, prognostic, and therapeutic information that might be of value in guiding individualized management of patients with thyroid nodules.

Key Words
- thyroid cancer
- thyroid nodule
- molecular marker
- prognostic marker

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Introduction

Among endocrine tumors, thyroid cancer is the most common, with an estimated incidence of 12.2/100 000 per year in the USA (Howlader *et al.* 2013). The incidence of thyroid cancer, both in the USA and worldwide, has been increasing over the last four decades (Burgess & Tucker 2006, Davies & Welch 2006, Albores-Saavedra *et al.* 2007). The increased incidence of thyroid cancer diagnoses has been attributed, in part, to improved detection of small or subclinical thyroid nodules by thyroid ultrasonography and by other imaging techniques; however, increased incidence of thyroid tumors of all sizes has also been reported (Albores-Saavedra *et al.* 2007, Jung *et al.* 2014). The increased number of cases of papillary thyroid cancer is predominantly of follicular variant, *RAS* mutation-positive tumors, indicating a potential role for environmental (chemical/dietary) factors (Jung *et al.* 2014).

In addition to environmental factors, genetic factors are involved in thyroid cancer predisposition. Aside from the well-characterized familial forms of medullary thyroid cancer, non-medullary thyroid cancer in a first-degree relative increases the risk fourfold to tenfold higher than in the general population (Frich *et al.* 2001, Hemminki *et al.* 2005). Familial non-medullary thyroid cancer is characterized by autosomal-dominant inheritance with reduced penetrance, and has been estimated to account for approximately 5–10% of all thyroid cancers (Charkes 2006, Malchoff & Malchoff 2006, Moses *et al.* 2011, Mazeh & Sippel 2013). Genetic linkage studies have mapped susceptibility loci to several regions including 1q21, 2q21, 8p23, 8q24, 9q22, 14q31, and 19p13 (Bignell *et al.* 1997, Canzian *et al.* 1998, Malchoff *et al.* 2000, McKay *et al.* 2001, Cavaco *et al.* 2008, He *et al.* 2009, Tomaz *et al.* 2012). Definitive germline genetic mutations

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underlying thyroid cancer predisposition remain to be identified within candidate genes in these regions. Thyroid tumor development probably involves a complex interplay between genetic predisposition and environmental risk factors.

Thyroid cancer typically presents as a thyroid nodule. However, thyroid nodules are commonly found incidentally and may be seen in up to 50% of patients older than 60 years of age (Mazzaferri 1992, 1993, Guth *et al*. 2009). Only 5% of thyroid nodules are malignant (Brito *et al*. 2013). Most thyroid cancers are well-differentiated papillary carcinomas or follicular carcinomas and are associated with a low mortality rate, particularly in patients with stage I or II disease (survival rate >98%). However, a subset of these patients will have recurrent disease (Mazzaferri & Jhiang 1994). In addition, patients who present with higher-stage disease or distant metastases and patients with poorly differentiated or anaplastic thyroid cancer have higher mortality rates (Volante *et al*. 2004, Tanaka *et al*. 2011). Accurate identification of subsets of patients with risk factors for aggressive disease and higher mortality rates can help to guide treatment and management and as well as prevent overtreatment of patients with low-risk disease.

Thyroid cancer diagnosis using fine-needle aspiration and the need for molecular markers

The diagnosis of thyroid cancer is typically obtained through ultrasound examination and fine-needle aspiration (FNA) biopsy of suspicious nodules. Cytological examination of cells collected by FNA biopsy is the most reliable diagnostic method for evaluating thyroid nodules, and is able to definitely classify thyroid nodules as benign or malignant in the majority of cases (Cooper *et al*. 2009, Gharib *et al*. 2010). The Bethesda reporting system for classifying thyroid cytology was proposed in 2007 by the National Cancer Institute and provides diagnostic categories with accompanying risk stratification and recommended clinical management (Baloch *et al*. 2008, Ali & Cibas 2010). Thyroid FNA specimens in the benign category have a low risk of malignancy (approximately 0–3%), and thyroid FNAs in the malignant category have a 97–99% risk of malignancy (Ali & Cibas 2010). However, 20–30% of thyroid FNA specimens are indeterminate and fall into one of the following categories: atypia of undetermined significance/follicular lesion of undetermined significance (AUS/FLUS), follicular or oncocytic (Hürthle cell) neoplasm/suspicious for a follicular or oncocytic (Hürthle cell) neoplasm (FN/SFN), and suspicious for malignant cells (SUSP) (Baloch *et al*. 2008, Ohori & Schoedel 2011). The AUS/FLUS diagnostic category is associated with a 5–15% risk of malignancy and the recommended management is repeat FNA; for the FN/SFN category, the risk of malignancy is 15–30% and the recommended management is surgical lobectomy; and for the SUSP category, the risk of malignancy is 60–75% and the recommended management is near-total thyroidectomy or surgical lobectomy (Ali & Cibas 2010).

Of the indeterminate thyroid nodules that are surgically resected, 10–40% are confirmed to be malignant (Mazzaferri 1993, Baloch *et al*. 2002, 2008). As a result, most diagnostic surgeries are performed for benign thyroid nodules. On the other hand, for those patients who have undergone a surgical lobectomy and been found to have a tumor larger than 1 cm, a second surgery is usually performed to remove the remaining thyroid lobe. Therefore, additional diagnostic markers are needed to guide the management of patients with indeterminate thyroid nodules in order to reduce the frequency of unnecessary diagnostic lobectomies and two-step surgeries.

Several types of ancillary approaches have been used to improve the diagnostic yield of FNA biopsies in indeterminate thyroid nodules. These include immunohistochemical stains, microRNAs, gene mutations/rearrangements, and gene expression panels (Bartolazzi *et al*. 2008, Nikiforov *et al*. 2011, Alexander *et al*. 2012, Keutgen *et al*. 2012). While many of these ancillary tools have not yet reached clinical practice, two such approaches, gene mutation/rearrangement panels and a gene expression classifier, are currently being used for clinical management (Nikiforov *et al*. 2011, Alexander *et al*. 2012). These panels are based on the wealth of knowledge on thyroid cancer genetics accumulated over the last three decades. In addition, gene mutation/rearrangement panels have the added benefit of providing information that could be useful in prognostication and targeted therapy.

The utility of molecular markers in indeterminate thyroid nodules can be evaluated based on the sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of each test. Sensitivity is a measure of the proportion of actual positives that are correctly identified as such, and specificity is the proportion of negatives that are correctly identified as such. NPV is the percentage of patients with a negative test result who do not have the disease, and PPV is the percentage of patients with a positive test result who have the disease. A negative result in a molecular test with high sensitivity

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and NPV, would indicate that the patient with an indeterminate thyroid nodule has a higher likelihood of having a benign nodule and could be managed with active surveillance, whereas a positive result in a molecular test with high specificity and PPV indicates that the patient with an indeterminate thyroid nodule probably has thyroid cancer and would be an indication for surgery. Importantly, although specificity and sensitivity depend only on test performance, NPV and PPV depend on the prevalence of disease in the tested population. Therefore, institutional differences in malignancy rates conferred by each cytologic diagnosis may result in significant variation of NPV and PPV, which should be taken into account when any molecular test is used clinically.

Molecular genetics of thyroid cancer

From the 1990s, when pathogenesis of only approximately 25% of thyroid cancers was understood, to the present, when genes involved in the pathogenesis of over 90% of thyroid cancers have been described, much progress has been made in elucidating the molecular mechanisms underlying thyroid cancer (Fig. 1). This progress provides the basis upon which new diagnostic and prognostic markers, as well as new targeted therapies, have been developed.

The molecular pathogenesis of the majority of thyroid cancer involves dysregulation of the MAPK and phosphatidylinositol-3 kinase (PI3K)/AKT signaling pathways (Fig. 2). The MAPK pathway is frequently activated in thyroid cancer through point mutations of the *BRAF* and *RAS* genes and *RET/PTC* and *TRK* rearrangements (Kimura *et al*. 2003, Soares *et al*. 2003, Frattini *et al*. 2004, Adeniran *et al*. 2006). Point mutations in *BRAF* are found in approximately 45% of papillary thyroid cancers (Cohen *et al*. 2003, Kimura *et al*. 2003). *BRAF* is a serine–threonine kinase which, upon activation by *RAS*, activates *MEK* and leads to activation of downstream effectors of the MAPK pathway. In nearly all cases (98–99% of cases) activating point mutations of *BRAF* involve codon 600 and result in the V600E mutation, and in 1–2% of cases other *BRAF* mutations such as the K601E mutation, small in-frame insertions or deletions, or *BRAF* rearrangement can occur (Soares *et al*. 2003, Ciampi & Nikiforov 2005, Ciampi *et al*. 2005, Hou *et al*. 2007*a*, Chiosea *et al*. 2009).

RAS genes (*HRAS, KRAS*, and *NRAS*) are G proteins that signal to both the MAPK and PI3K/AKT pathways. Point mutations in the *RAS* genes typically occur in codons 12, 13, and 61, and are found in 40–50% of follicular carcinomas and in 10–20% of papillary thyroid carcinomas (Lemoine *et al*. 1989, Namba *et al*. 1990, Suarez *et al*. 1990). *RAS*-mutated papillary thyroid carcinomas typically are of the follicular variant (Zhu *et al*. 2003, Adeniran *et al*. 2006). *RAS* mutations are also seen in 20–40% of follicular adenomas (Lemoine *et al*. 1989, Namba *et al*. 1990, Suarez *et al*. 1990, Motoi *et al*. 2000).

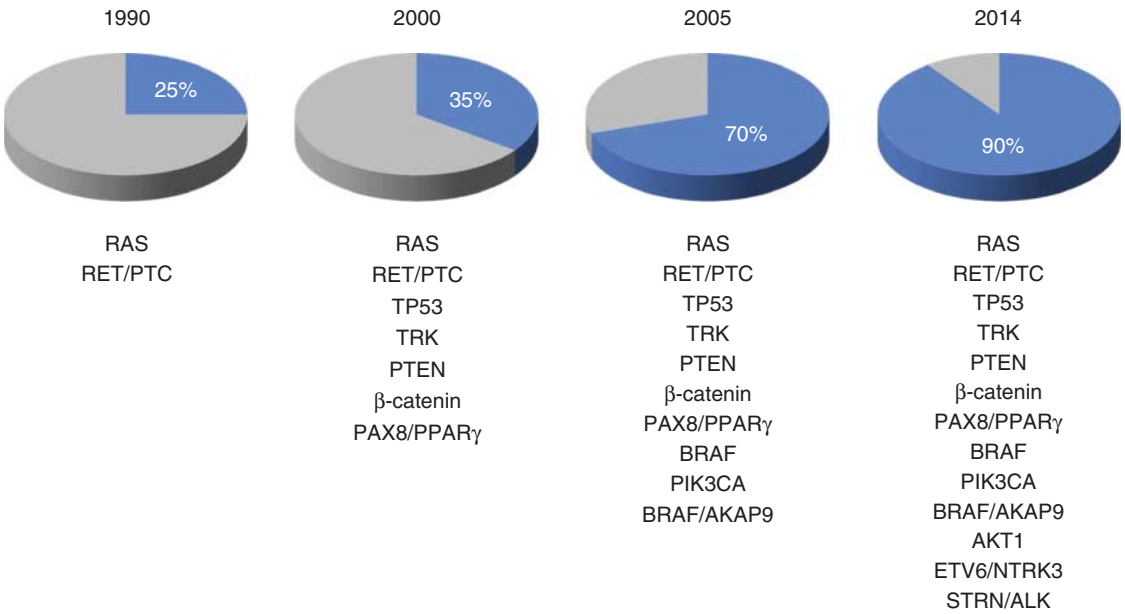

Figure 1
Progress in identifying mutational markers in thyroid cancer.

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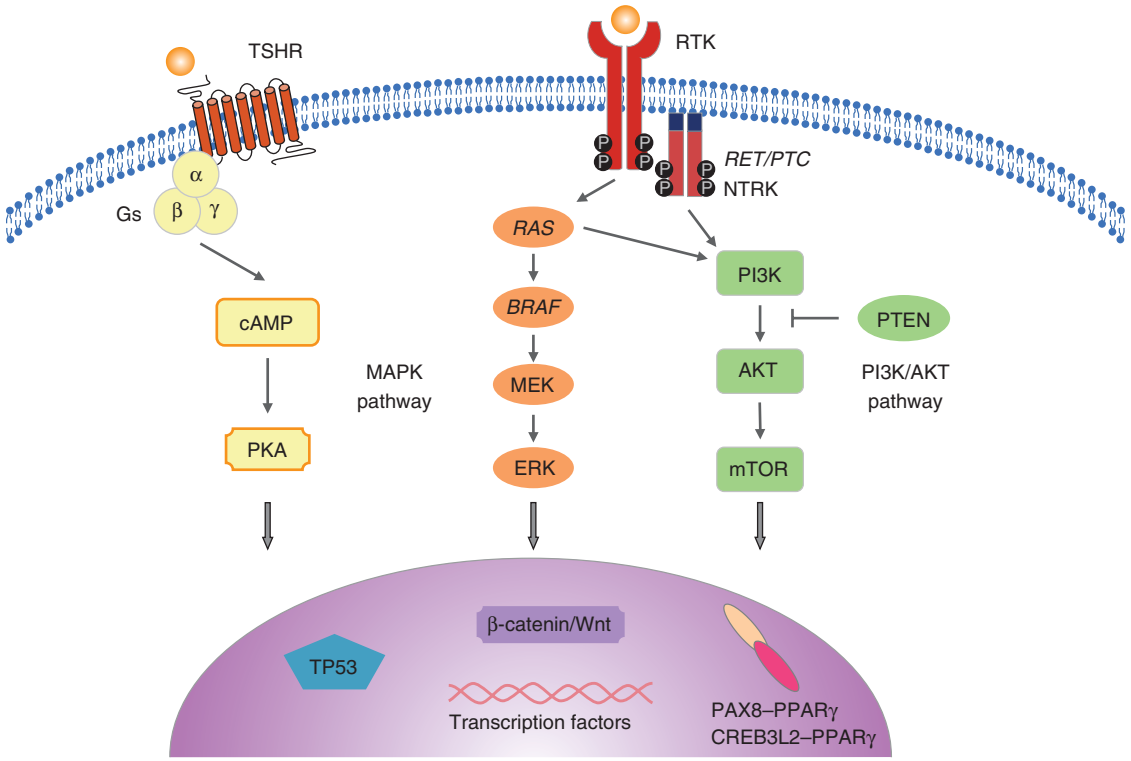

Figure 2

Molecular pathways in thyroid cancer. The molecular pathogenesis of thyroid cancer involves dysregulation of the MAPK, phosphatidylinositol-3 kinase (PI3K)/AKT, and the TSHR cAMP signaling pathways. The MAPK pathway is frequently activated in thyroid cancer through point mutations of the *BRAF* and *RAS* genes and *RET/PTC* and *TRK* rearrangements, and the PI3K pathway is frequently activated through point mutations of *PIK3CA* and mutation/deletion of *PTEN*.

Whereas *NRAS, HRAS*, and *KRAS* mutations are found in follicular-cell-derived thyroid tumors, mutations in *HRAS* and *KRAS* also occur in medullary thyroid cancers (Agrawal *et al*. 2013).

The *RET* gene is a receptor tyrosine kinase that is expressed in thyroid C cells, but not in follicular cells. The *RET* gene can be activated by fusion with various partners that drive the expression of the 3′ portion of the *RET* gene coding for the tyrosine kinase domain of the receptor, and provide the dimerization motif to lead to the constitutive activation of RET kinase. The most common rearrangement types are *RET/PTC1* (formed by fusion of *RET* with the *CCDC6* gene) and *RET/PTC3* (formed by fusion of *RET* with the *NCOA4* gene) (Grieco *et al*. 1990, Santoro *et al*. 1994). The *RET/PTC1* and *RET/PTC3* rearrangements are found in 10–20% of papillary thyroid carcinomas (Nikiforov 2006, Zhu *et al*. 2006), and their incidence is progressively decreasing (Jung *et al*. 2014). These rearrangements are found at higher frequencies in children/young adults and in patients with a history of radiation exposure (Nikiforov *et al*. 1997, Fenton *et al*. 2000, Rabes *et al*. 2000). In addition to rearrangements involving *RET*, which are found in papillary thyroid tumors, the *RET* gene is commonly found to be mutated in medullary thyroid carcinomas, in both familial and sporadic cases (de Groot *et al*. 2006, Kloos *et al*. 2009).

The *PAX8/PPARγ* rearrangement, a fusion between a paired domain transcription factor and the peroxisome proliferator-activated receptor genes, is found in 30–40% of follicular carcinomas (Dwight *et al*. 2003, French *et al*. 2003, Nikiforova *et al*. 2003). The *PAX8/PPARγ* rearrangement can also be seen, at lower prevalence, in the follicular variant of papillary thyroid carcinoma and in follicular adenomas (Marques *et al*. 2002, Nikiforova *et al*. 2002, 2003, Dwight *et al*. 2003, French *et al*. 2003).

The importance of the PI3K/AKT pathway in thyroid tumorigenesis has been increasingly recognized in the last decade. The PI3K/AKT pathway can be activated by activating mutations in *PIK3CA* and *AKT1* as well as by

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inactivation of *PTEN*, which negatively regulates this pathway (Fig. 2). Somatic mutations of *PTEN* have been reported in follicular thyroid tumors and anaplastic thyroid carcinoma, and germline mutations of *PTEN* can result in follicular thyroid tumors arising in patients with Cowden syndrome (Dahia *et al*. 1997, Gustafson *et al*. 2007, Hou *et al*. 2007*b*, Nikiforova *et al*. 2013). Activating mutations in *PIK3CA* typically occur at hotspots within exons 9 and 20 and have been reported in follicular thyroid carcinomas, poorly differentiated thyroid carcinomas, and anaplastic thyroid carcinomas (Garcia-Rostan *et al*. 2005, Hou *et al*. 2007*b*, Ricarte-Filho *et al*. 2009). *AKT1* mutations have been reported in metastatic thyroid cancer (Ricarte-Filho *et al*. 2009).

Additional genes mutated in thyroid cancer include *TP53* and *CTNNB1* (β-catenin). *TP53* is a tumor suppressor that plays important roles in cell cycle regulation and DNA repair and *CTNNB1* is involved in Wnt signaling. These genes tend to be mutated in more aggressive and advanced thyroid tumors (Dobashi *et al*. 1994, Garcia-Rostan *et al*. 2001). In addition to these well-characterized mutations, mutations in thyroid-stimulating hormone receptor (*TSHR*) and *GNAS* have also been shown to play a role in thyroid tumorigenesis. Also, novel markers have been recently identified, including *ETV6/NTRK3*, *STRN/ALK*, and *TERT*. These markers will be discussed in further detail below.

Utility of mutational molecular markers in preoperative FNA samples

BRAF and *RAS* point mutations and *RET/PTC* and *PAX8/PPARγ* rearrangements are the most common genetic alterations found in thyroid cancer and have been used for cancer detection in thyroid nodules with indeterminate FNA cytology. A seven (or eight) gene mutation panel which includes *BRAF, KRAS, HRAS, NRAS*, and *RET/PTC1*, *RET/PTC3*, *PAX8/PPARγ* (and *TRK*) rearrangements is the best characterized molecular panel. The presence of any mutation has high specificity and high PPV for malignancy, as demonstrated in three prospective studies, one of which involved two institutions and the rest of which were based on a single institution (Nikiforov *et al*. 2009, 2011, Cantara *et al*. 2010). All three studies have demonstrated a high specificity and PPV of the positive test for cancer detection in all categories of indeterminate cytology (Nikiforov *et al*. 2009, 2011, Cantara *et al*. 2010). These results provide strong evidence that the presence of any of these mutations, with the possible exception of *RAS* (discussed below), is an indication for surgery, and specifically for total thyroidectomy. The strategy of using mutational markers combined with cytological diagnosis to guide the extent of surgery was recently shown to be effective in reducing the need for two-step surgery, i.e. initial lobectomy followed by completion thyroidectomy (Yip *et al*. 2014). In a series of 471 FNA biopsies that had indeterminate cytology (AUS/FLUS or FN/SFN), patients who did not have mutational marker testing were 2.5-fold more likely to require two-stage surgery (Yip *et al*. 2014).

RAS mutations, compared with *BRAF* mutation or *RET/PTC* and *PAX8/PPARγ* rearrangements, have a lower PPV for cancer of 74–87% (Nikiforov *et al*. 2009, 2011, Cantara *et al*. 2010). Benign nodules positive for *RAS* mutation were found, on surgical resection, to be follicular adenomas. Although follicular adenomas are benign, evidence exists that follicular adenomas represent a pre-cancerous change that can progress to malignancy (Burns *et al*. 1992, Fagin 2002, Zhu *et al*. 2003, Nikiforov & Ohori 2012). Thus such patients would benefit from removal of these nodules before possible progression.

NPV of mutational panels has been best characterized for the seven-gene panel. In mutation-negative nodules with AUS/FLUS cytology, the residual risk of malignancy in a large series of cases (with a disease prevalence of 14%) was 6%, with a 2.3% risk of invasive cancer (Nikiforov *et al*. 2011). In these AUS/FLUS nodules, the NPV is high at 94% (Nikiforov *et al*. 2011). As the residual risk of cancer approaches that of the risk in nodules with benign cytology, and as most missed cancers are intra-thyroidal (0.5% risk of extra-thyroidal spread), low-grade tumors, this test can be used to eliminate the need for surgery in AUS/FLUS nodules, as long as the disease prevalence in the AUS/FLUS category does not exceed 14–15%.

In mutation-negative nodules with FN/SFN cytology, the seven-gene panel decreases the risk of malignancy from 27 to 14% and in mutation-negative nodules with SUSP cytology from 54 to 28% (Nikiforov *et al*. 2011). As the risk of malignancy is significantly decreased, these patients could be offered a diagnostic lobectomy rather than a total thyroidectomy. However, an improved NPV would still be desired to reduce the risk further.

This molecular mutational panel may also play a valuable role in pediatric patients with thyroid nodules, in which thyroid FNA biopsy may result in an indeterminate diagnosis in as many as 38% of cases (Monaco *et al*. 2012). In a small series of cases, preoperative molecular testing was able to guide surgical management and prevent the need for a second surgery in 60% of cases (Buryk *et al*. 2013).

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Gene expression markers for FNA diagnosis of indeterminate nodules

A panel of gene expression markers was identified by examining mRNA expression profiles in thyroid nodules and utilizing that data to train a molecular classifier (Chudova *et al*. 2010). This gene expression classifier uses the expression of 142 genes to classify thyroid nodules into a benign or suspicious category (Chudova *et al*. 2010). The reported genes used in the gene expression panel are involved in many processes, including energy metabolism and cell differentiation/development (Alexander *et al*. 2012).

The molecular test based on the gene expression classifier is commercially offered as Afirma. The test was validated in one study, which was a multi-institutional prospective double-blind study which included 265 nodules with indeterminate cytology from 49 clinical sites (Alexander *et al*. 2012). The study limitations include a relatively small sample size (129 AUS/FLUS, 81 FN/SFN, and 55 SUSP samples) and a high rate of post-unblind exclusion of samples. The study demonstrated that, with a disease prevalence of 24, 25, and 62% in the AUS/FLUS, FN/SFN, and SUSP cytology groups, respectively, the NPV was 95% in AUS/FLUS, 94% in FN/SFN, and 85% in SUSP nodules. The PPV was significantly lower and varied by cytologic category (AUS/FLUS, 38%; FN/SFN, 37%; and SUSP, 76%; Alexander *et al*. 2012). Therefore, this test is marketed as a 'rule-out' test, i.e. a test to identify nodules likely to be benign and thus avoid unnecessary surgery (Duick *et al*. 2012, Alexander *et al*. 2014). Importantly, the NPV for this test also depends on the disease prevalence in each category of indeterminate cytology. A recent study has shown a lower NPV for the Afirma gene expression classifier test, which was found to be 89.6% for AUS/FLUS and FN/SFN cytology nodules (Harrell & Bimston 2014). This is probably due to a higher disease prevalence in patients with nodules of indeterminate cytology, which was 33% in this study. Therefore, additional independent, not industry-supported studies are required to establish the performance of this test in different patient populations.

Expanded mutational marker panels for FNA specimens

The continuing discovery of genes involved in thyroid carcinogenesis together with the availability of new high-throughput technologies has led to a rapid expansion of mutational panels that can be used in FNA samples. The expanded panels can detect more mutations and with higher sensitivity, which is expected to increase significantly the sensitivity and NPV of mutational panels. Next generation sequencing (NGS) technologies allow the high-throughput, massively parallel sequencing of nucleic acid sequences and offer a cost-effective way to analyze a large number of genetic alterations in small samples. NGS-based approaches generate an increased amount of complex information with specialized requirements for analysis and reporting that can be effectively managed with bioinformatics applications (e.g. SeqReporter; Roy *et al*. 2014). A 15-gene mutational panel expanded to include other clinically significant genes that have been found to be mutated in thyroid cancer, such as *PIK3CA, TP53, TSHR, PTEN, GNAS, CTNNB1, AKT1*, and *RET*, has recently been reported (Nikiforova *et al*. 2013).

Mutations in *PIK3CA* and *AKT1* have been reported in thyroid tumors, and occur more frequently in advanced and dedifferentiating tumors (Garcia-Rostan *et al*. 2005, Hou *et al*. 2007*b*, Ricarte-Filho *et al*. 2009). Mutations in *PIK3CA* were detected, in a limited series of this expanded mutational panel, in papillary thyroid carcinoma, poorly differentiated thyroid carcinoma, and anaplastic thyroid carcinoma, and were found both in the presence and absence of other mutations (Nikiforova *et al*. 2013). *PTEN* mutations, consistent with previous reports, were detected in follicular carcinoma, as well as in benign follicular adenomas (Dahia *et al*. 1997, Hou *et al*. 2007*b*, Nikiforova *et al*. 2013).

Additional mutations known to occur in more aggressive types of well-differentiated thyroid cancer as well as in poorly differentiated and anaplastic carcinomas involve *TP53* and *CTNNB1* (Dobashi *et al*. 1994, Garcia-Rostan *et al*. 2001). Interestingly, in one study, *TP53* mutations were found not only in anaplastic carcinomas, but also in 22% of oncocytic follicular carcinomas (Nikiforova *et al*. 2013).

Somatic mutations of the *TSHR* gene are known to frequently occur in autonomously functioning thyroid nodules (Garcia-Jimenez & Santisteban 2007, Nishihara *et al*. 2009). However, *TSHR* mutations at specific hotspots were also found in thyroid carcinomas (Nikiforova *et al*. 2013). Similar to *TSHR* mutations, mutations in *GNAS*, a gene which encodes an α subunit of heterotrimeric G protein complexes, occur predominantly in benign hyperfunctioning nodules. In a limited series, all nodules found to carry an isolated *GNAS* mutation were found to be benign after surgery (Nikiforova *et al*. 2013). Therefore, *GNAS* mutations may uniquely function as markers of benign nodules.

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The *RET* gene is commonly found to be mutated in medullary thyroid carcinomas, in both familial and sporadic cases. Among sporadic medullary carcinomas, the *RET* M918T mutation is the most common, accounting for more than 75% of all somatic *RET* mutations found in these tumors (de Groot *et al*. 2006, Kloos *et al*. 2009). The *RET* M918T mutation is also the most common mutation in multiple endocrine neoplasia type 2B (MEN2B), whereas in MEN2A and familial medullary thyroid carcinoma, mutations in *RET* typically occur in one of five cysteine codons within the cysteine-rich extracellular domain (Mulligan *et al*. 1995, Hansford & Mulligan 2000). Detection of specific mutations of *RET* in medullary thyroid carcinoma (or mutation of *KRAS* or *HRAS*, which can occur in sporadic cases), thus can not only inform diagnosis but can also facilitate genetic analysis of germline mutations (Agrawal *et al*. 2013).

An expanded 15-gene NGS-based panel has been validated in a study of 228 samples from thyroid nodules including 51 FNA samples, and showed accurate detection of multiple mutations with a sensitivity of 3–5% of mutant alleles (Nikiforova *et al*. 2013). This analysis was able to identify mutations in 27 tumors, which would not have been detected by mutational analysis of *BRAF* and *RAS* genes, indicating that it is expected to increase the sensitivity and NPV of cancer detection in thyroid nodules (Nikiforova *et al*. 2013). In addition, although most tumors in this study were positive for a single mutation, in nine tumors, two to three mutations were identified (Nikiforova *et al*. 2013). These results indicate that in clinical practice, a targeted NGS panel will further increase the accuracy of cancer risk assessment in thyroid nodules and will provide information about the presence of multiple mutations, which has prognostic implications.

Emerging novel molecular markers

In addition to the *RET/PTC* and *PAX8/PPARγ* rearrangements, which are found in approximately 15% of thyroid tumors, many other gene fusions have been described, such as rearrangements involving *NTRK* or *BRAF*. Fusion of *BRAF* with A kinase anchor protein 9 (*AKAP9*) is a rearrangement rarely found in sporadic thyroid cancer, although it also occurs at higher frequencies (up to 11%) in patients with a history of radiation exposure (Ciampi *et al*. 2005). *NTRK1* is a receptor tyrosine kinase and when rearranged with one of three potential fusion partners activates MAPK pathway signaling (Bongarzone *et al*. 1998, Musholt *et al*. 2000). *NTRK1* rearrangements are found in approximately 1–5% of papillary thyroid carcinomas and at higher frequencies in patients with radiation exposure (Ciampi *et al*. 2005, Leeman-Neill *et al*. 2013). More recently, whole-transcriptome (RNA-Seq) analyses have led to the discovery of novel gene fusions in thyroid cancer. RNA-Seq analysis of radiation-associated thyroid cancer identified a novel *ETV6–NTRK3* chromosomal rearrangement, which occurs in 2% of sporadic papillary thyroid cancers and 14.5% of radiation-associated tumors (Leeman-Neill *et al*. 2014). Another interesting gene fusion with therapeutic implications has been recently identified by RNA-Seq analysis of aggressive forms of thyroid cancer. The fusion of the striatin (*STRN*) gene and the anaplastic lymphoma kinase (*ALK*) gene was found in 9% of poorly differentiated thyroid cancers, 4% of anaplastic thyroid cancers, and 1.2% of well-differentiated papillary thyroid cancers (Kelly *et al*. 2014). Whereas in the past, testing for rare fusions and other mutational events was difficult to apply to clinical FNA samples due to the high cost and the need for large amounts of DNA and RNA for testing using individual gene assays, at the present time the availability of targeted NGS offers a convenient and cost-effective technique for detection of multiple point mutations and gene rearrangements in clinical FNA samples.

In addition to these markers, another recently discovered molecular marker is mutation of the telomerase reverse transcriptase (*TERT*) promoter (Landa *et al*. 2013, Liu *et al*. 2013*a*,*b*, Melo *et al*. 2014). TERT promoter mutations have not been found in benign thyroid nodules. Therefore, the presence of a TERT mutation is expected to not only be useful in the diagnosis of malignant nodules, but is also likely to play an important role in disease prognostication, and will be discussed in the next section. In our preliminary validation study, the use of an extended panel of mutational markers that includes point mutations and gene fusions in over 60 genes results in a NPV of over 95% for thyroid nodules with AUS/FLUS and FN/SFN cytology that were found to be negative for these mutations (Y Nikiforov, personal communication).

Molecular markers for cancer prognostication

The utility of molecular markers in FNA biopsies may extend beyond diagnostic information to have a role in preoperatively identifying the subsets of tumors with more aggressive biological behavior. Such patients may benefit from more extensive initial surgery to include central compartment lymph node dissection to prevent tumor recurrence. Among prognostic markers, one of the best studied is the *BRAF* V600E mutation. The presence of

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the *BRAF* V600E mutation in papillary thyroid cancer was found to be associated with poor prognostic factors such as extrathyroidal invasion, lymph node metastases, and recurrence (reviewed in Xing (2007)). In thyroid FNA, preoperative testing for *BRAF* V600E mutation was found to be useful in predicting disease persistence and recurrence (Xing *et al*. 2009). However, not all studies have shown such associations (Kim *et al*. 2005, Liu *et al*. 2005, Ito *et al*. 2009). A recent meta-analysis of 14 studies including a total of 2470 patients has revealed that the *BRAF* V600E mutation was significantly associated with tumor recurrence or persistent disease, which was found in 25% of BRAF-V600E-positive tumors versus 13% of *BRAF*-mutation-negative tumors (Tufano *et al*. 2012). In addition, a large, multicenter study of 1849 patients found the presence of the *BRAF* V600E mutation to be significantly associated with increased mortality from papillary thyroid cancer (Xing *et al*. 2013). The overall mortality was 5% in patients with *BRAF* V600E mutation and 1% in *BRAF*-mutation-negative patients. These results indicate that overall *BRAF* V600E-positive tumors have a higher chance of having more aggressive disease features at presentation and as a group have an increased risk of recurrence and overall mortality. However, it is important to note that the majority of patients with *BRAF* V600E mutation do not have recurrent disease and overall survival remains very high in both groups of patients (Fig. 3). This indicates that *BRAF* V600E taken in isolation is a relatively sensitive but not a specific marker of tumor recurrence and tumor-related mortality.

Recently, additional and more specific markers of more aggressive tumor behavior have emerged. One of these markers is the presence of multiple driver mutations in thyroid cancer. Co-existing mutations in the early driver genes such as *BRAF* or *RAS* with mutations in *PIK3CA*, *AKT1*, or *TP53* in the same tumor have been shown to occur in poorly differentiated and anaplastic tumors (Garcia-Rostan *et al*. 2005, Hou *et al*. 2007*b*, Liu *et al*. 2008). More recently, an NGS-based mutational analysis revealed that approximately 4% of well differentiated papillary cancers have more than one mutation, and these tumors are distinctively aggressive and typically present with distant metastases (Nikiforova *et al*. 2013).

TP53 mutations are known to occur at a high frequency in poorly differentiated thyroid cancers (25%) and anaplastic thyroid cancers (70–80%), and are a well-characterized genetic event governing thyroid tumor dedifferentiation (Donghi *et al*. 1993, Fagin *et al*. 1993). However, *TP53* mutation has also been found in some well-differentiated cancers such as papillary thyroid carcinoma and oncocytic follicular carcinoma (Nikiforova *et al*. 2013). It is likely that well-differentiated cancers carrying a *TP53* mutation have a potential for tumor dedifferentiation and more aggressive clinical course, which should be addressed in further studies.

Another very promising prognostic molecular marker is mutation of the *TERT* promoter. Telomerase is a reverse transcriptase that utilizes an RNA template to add telomeric repeats to the ends of chromosomes. Telomerase is not expressed in most normal tissues, but is frequently activated in tumor cells (Shay & Bacchetti 1997). Maintenance of telomere length, either through telomerase activation or a recombination-based mechanism known as alternative lengthening of telomeres (ALT), is required for immortalization of cancer cells. Recently, two mutations in the promoter of *TERT* (chr5: 1295228C>T, termed C228T and chr5: 1295250C>T, termed C250T) were discovered in melanomas and were found to result in increased transcriptional activity of the promoter (Horn *et al*. 2013, Huang *et al*. 2013). The C228T and C250T *TERT* promoter mutations were also detected in follicular cell-derived thyroid cancers, but were absent in benign lesions and in medullary thyroid cancers (Landa *et al*. 2013, Liu *et al*. 2013*a*,*b*, Melo *et al*. 2014). The C228T and C250T mutations have a significantly higher prevalence in aggressive thyroid tumors including widely invasive oncocytic carcinoma and anaplastic thyroid carcinoma (Landa *et al*. 2013, Liu *et al*. 2013*a*,*b*, Melo *et al*. 2014). Interestingly, *TERT* mutations in some studies were found

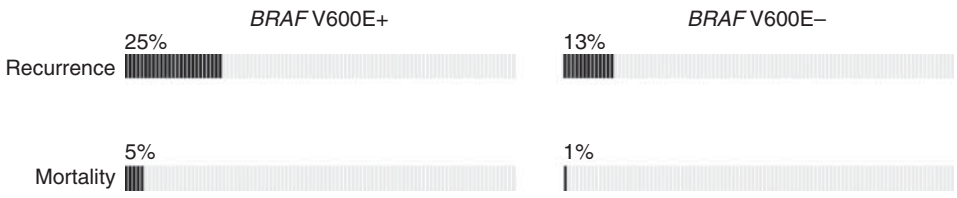

Figure 3
Associations between *BRAF* V600E and tumor recurrence and mortality. Based on the results reported by Tufano *et al*. (2012) and Xing *et al*. (2013).

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to be more common in tumors with the *BRAF* V600E mutation, which may indicate a possible synergistic interplay between MAPK pathway activation and telomerase activation to promote aggressive tumor behavior (Landa *et al*. 2013, Liu *et al*. 2013*b*). In a recent large study of 469 patients with a mean follow-up of 8 years, *TERT* promoter mutations have been found to be an independent risk factor for persistent disease, distant metastases, and disease-specific mortality for well-differentiated thyroid cancer (Melo *et al*. 2014).

Summary and future directions

Over the last several years, significant progress has been made in understanding the genetic mechanisms of thyroid cancer and in the development of molecular tests for cancer diagnosis in thyroid nodules. Work from multiple research labs as well as genomic sequencing data from papillary thyroid carcinomas from The Cancer Genome Atlas (TCGA) has led to the identification of mutations and other driver genetic alterations in over 90% of thyroid cancers, making it one of the best characterized human cancers from a genetic standpoint. Moreover, NGS technology offers the reliable detection of most of these genetic alterations in the limited cell samples obtained by FNA biopsy, offering significant improvement in the accuracy of cancer detection in thyroid nodules as compared with currently available clinical tests. As a result, it is likely that in the near future NGS-based molecular tests will be able to predict the risk of cancer in thyroid nodules with very high accuracy, which will eliminate the uncertainty of indeterminate FNA cytology. Furthermore, as the cost of NGS continues to plummet and analytical tools become more efficient, the cost of molecular testing will decrease, making routine use of NGS-based molecular tests even more feasible and cost-effective for management of patients with cytologically indeterminate thyroid nodules.

Moreover, molecular markers are expected to have a significant effect on cancer prognostication. While the *BRAF* V600E mutation can be considered a relatively sensitive prognostic marker for papillary cancer, it is not specific and cannot be used in isolation for tumor prognostication. Recent results obtained using broad tumor genotyping have shown that several specific molecular signatures (such as the presence of several driver mutations, *TP53* mutations, or *TERT* promoter mutations) are found in a small fraction of well-differentiated papillary and follicular cancers and are associated with more aggressive tumor behavior. It is expected that these molecular signatures will be confirmed and perhaps further improved in additional studies and will offer more specific detection of well-differentiated thyroid cancers that have higher risk for tumor recurrence and cancer-related mortality. Future studies will be needed to define the optimal surgical and post-surgical management of patients based on these molecular signatures. With these advances, determination of a personalized cancer genome will become feasible in thyroid nodules in the near future, and will offer truly individualized medicine for patients with thyroid nodules and cancer.

Declaration of interest

Y E Nikiforov serves as consultant for Quest Diagnostics. S J Hsiao has no potential conflicts of interest.

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