Molecular markers in thyroid cancers

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ABSTRACT

Thyroid cancer is the most common of all endocrine tumors. In most countries, a steady increase in the incidence of thyroid cancer (mainly papillary carcinomas) was observed in both sexes, whereas mortality has declined. Thyroid nodule is the most common presentation of thyroid cancer and according to Bethesda reporting system is categorized into six different disease categories based on histopathological pattern. The indeterminate categories require further investigation which is generally in the form of a surgical or invasive procedure for confirmation of nature of disease. Our article is describing the various molecular markers that are linked with different subtypes of thyroid cancer and their usage in guiding diagnosis, investigative approach and prognostication in thyroid cancer especially the indeterminate categories. We discuss the various tests available for us currently for detecting the genetic alterations and thus help us frame further approach algorithms for detection of thyroid cancers.

Key words: Afirma, Molecular markers, Thyroid cancer, ThyroSeq

Introduction

Thyroid cancer is the most common of all endocrine tumors. In most countries, a steady increase in the incidence of thyroid cancer (mainly papillary carcinomas) was observed in both sexes, whereas mortality has declined. The declines in thyroid cancer mortality reflect both variations in risk factor exposure and changes in the diagnosis and treatment of the disease, while the increases in the incidence are likely due to the increase in the detection of this neoplasm by imaging modalities over the past few decades.[1]

Thyroid nodule is the typical presentation of thyroid cancer; they can be detected incidentally and may be present in up to 50% of people older than 60 years of age.[2] According to the recent data, overall prevalence of malignancy in thyroid nodule is 11-14%.[3] The challenge is to detect cancers without unnecessary procedure or surgery. Thus, now ultrasonography (USG) and fine needle aspiration (FNA) are standard of care for thyroid nodules.[2] Thyroid FNA was introduced 40 years back with the introduction of "The Bethesda System for Reporting Thyroid Cytopathology (TBSRTC)" for classifying thyroid pathology in 2007 by the National Cancer Institute. The advantage of TBSRTC is the standardization of the reporting of thyroid cytology, which before 2007, consisted of nonreproducible classification schemes that in some cases included either too few or too many disease categories. The six disease categories of TBSRTC arose from a probabilistic approach: The probability that a thyroid lesion placed into a specific category would show histological evidence of malignancy.[4] However, though having high positive predictive value (PPV) and negative predictive value (NPV), FNA is limited by inter-observer variation (high-volume vs. low-volume pathologist) and significant overlap between benign and malignant nodules resulting in abnormal but not conclusive reports.[3]

According to the tiered classification scheme, thyroid nodules can be broadly categorized as nondiagnostic (Type 1), benign (Type 2), indeterminate (Types 3 and 4), and suspicious for malignancy/malignant (Types 5 and 6).

1/3rd of all FNA cytology specimens are classified as indeterminate. Indeterminate categories include:

Bethesda Type 3

Atypia of undetermined significance OR follicular lesion of undetermined significance (AUS/FLUS); risk of malignancy is 5–15% and recommended management is repeat FNA. The FLUS/AUS category is the most heterogeneous and represents those cytology specimens that are neither benign nor malignant but may have a degree of cellular and/or architectural atypia that does not meet morphologic criteria for being follicular neoplasm (FN) or suspicious FN (SFN). A repeat FNA may lead to a benign cytology result in up to 40% of nodules;[5] however, the malignancy risk is as high as 16% and diagnostic surgery may still be necessary.[6] This category remains most controversial due to heterogenicity in its use among institutions and follow-up, probably as it impossible to establish distinct morphological criteria for diagnosing atypia among cytopathologist.[6]

Bethesda Type 4

FN/SFN (FN: Follicular Neoplasm, SFN: Suspicious for follicular neoplasm); risk of malignancy is 15–30% and the recommended management is surgical lobectomy.

Because FNA is unable to provide a definitive diagnosis for the nodules which are classified having indeterminate pathology most of them have to undergo diagnostic surgery to establish a histopathological diagnosis. However, only 10–40% of such surgically resected thyroid nodules will prove to be malignant. These unneeded operations lead to expenditure and associated risks of surgery and could be avoided if the FNA procedure could reliably establish the presurgical diagnosis of a benign nodule. In addition, because the standard of care is to offer a second surgery to complete the thyroidectomy, once diagnostic lobectomy confirms cancer, a more optimal surgical management would be a single up-front total thyroidectomy that can be offered if the diagnosis of cancer is established preoperatively. Thus, additional diagnostic markers are needed to diagnose nodules, especially in indeterminate category to reduce unnecessary investigations and two step thyroidectomies.

Molecular Pathogenesis of Thyroid Malignancies

Remarkable progress in understanding the molecular pathogenesis of thyroid cancer has been made in recent years, especially the past decade for signaling pathways, such as the mitogen-activated protein kinase (MAPK) and PI3K–AKT pathways. Activation of these pathways, by various genetic mutations and rearrangement, constitute the primary oncogenic mechanism that promotes the development and progression of thyroid cancer. This knowledge of oncogenetics can be used for developing molecular markers for detection or ruling out and planning novel treatment strategies for thyroid cancers. Furthermore, identification of markers that herald the development of aggressive biological behavior contributes to improved pre-operative risk classification.

The most common genetic alterations seen in thyroid cancer are BRAF (B-raf gene) and RAS-oncogene point mutations or gene rearrangements such as RET/PTC (RET:Rearranged during transfection,PTC:papillary thyroid carcinoma) and PAX8/PPARG (peroxisome proliferator-activated receptor gamma) rearrangements.

Other uncommon mutations observed are somatic or germline mutations of PTEN, TP53 mutations, and PIK3CA mutations. Mutations in thyroid-stimulating hormone receptor (TSHR) are also linked with thyroid cancer pathogenesis.

These genetic alterations generally cause dysregulation in one of these two pathways, the MAPK pathway and the (PI3K)/AKT signaling pathway. The MAPK pathway is frequently activated in thyroid cancer through point mutations of the BRAF and RAS genes and RET/PTC and TRK rearrangements.[7]

BRAF mutations

BRAF is a serine-threonine kinase belonging to family of RAF proteins which is mainly responsible for transmitting signals from the extracellular space into the nucleus, thus regulating cell proliferation and differentiation through the MAPK pathway.

BRAF mutations are present in 40–45% of papillary thyroid cancers. The majority of the cases with BRAF activating point mutations involve codon 600 leading to V600E mutation (up to 98–99% of cases), whereas other BRAF mutations can occur in 1–2% of cases.[7]

BRAF is not been found in follicular carcinomas and hence is quite a specific marker of papillary carcinoma and related tumor types. Despite being highly specific for papillary thyroid cancer, up to 55% thyroid cancer do not have BRAF mutation, thus making it less sensitive and use as isolated marker is of limited value.[8]

RAS mutations

Second in prevalence to BRAF mutation in thyroid cancer are RAS mutations. The RAS family of human genes includes the NRAS, HRAS, and KRAS. These membrane-associated proteins play a main role in the transduction of signals from tyrosine kinase and G protein-coupled receptors to effectors of the MAPK and PI3K-AKT signaling pathways, which mediate cell differentiation, proliferation, and survival. In thyroid tumors, most commonly found are NRAS codon 61 followed by HRAS codon 61, and KRAS codon 12 mutations.[8]

RAS is predominantly found in both benign and malignant follicular thyroid neoplasm. They are the most frequent mutation detected in cytologically indeterminate FNA results because of its association with follicular adenoma (FA), follicular thyroid carcinoma (FTC), and follicular variant of PTC.[5] RAS mutations have predominant distribution of 40–50% in follicular thyroid cancers, 10–20% in FvPTC, 35% in poorly differentiated carcinoma, and 55% in anaplastic carcinoma.[8]

In cytologically indeterminate nodules, RAS is associated with an 85–88% risk of thyroid cancer. Identification of RAS mutation is also helpful as a marker for follicular variant of papillary carcinoma and follicular carcinoma which are difficult to diagnose by cytology. RAS-positive benign FA (15% false positive) has potential for malignant transformation.[8]

RAS mutation and its value as marker for prognostication are still debatable as it is seen in encapsulated follicular variant of papillary carcinoma which mostly has an indolent course as well in follicular carcinomas with metastatic behavior and tumor dedifferentiation.

Although RAS positivity is not 100% predictive of malignancy, detection increases the risk to 80–85% which is often high enough to alter initial surgical management to total thyroidectomy for patients who may otherwise require 2 stage thyroidectomy based on indeterminant pathology.[7] Even if histologically benign, there is the potential for malignant

transformation associated with a RAS-positive FA and surgical resection may be a reasonable option.

RET/PTC

The RET proto-oncogene codes for a receptor tyrosine kinase protein, which is involved in intracellular signal transduction. Somatic RET rearrangements have been identified in papillary thyroid cancer, thus named RET/PTC. The two most common RET/PTC rearrangements associated with PTC are RET/PTC1 and RET/PTC3. They have been reported with increased incidence in patients with a history of radiation exposure and in younger patients. Its prevalence is reported in up to 45% of PTCs among the age group of 6–21 years. RET/PTC rearrangements occur in approximately 10–20% of PTCs.

Although testing for RET/PTC is extremely specific for thyroid cancer, the diagnostic utility of this test as an isolated molecular marker is limited because of its low prevalence.[8] Apart from PTC, RET gene is commonly found to be mutated in medullary thyroid carcinoma, in both familial and sporadic cases.[7]

PAX8/PPARG

The PAX8/PPARG fusion protein is a somatic tumor genetic rearrangement resulting from fusion between a paired domain transcription factor and the peroxisome proliferator-activator receptor. It is known to occur in about 36% of follicular thyroid cancers, 13% of follicular variant PTCs, 2% of oncocytic (Hurthle cell) carcinomas and anaplastic thyroid cancers. However, when only nodules with indeterminate cytology are considered, PAX8/PPARG has been shown to have a specificity of 100% for thyroid cancer. Although PAX8/PPAR,G occur with variable prevalence in FA, positive FAs may be premalignant lesions that eventually may develop into cancer.[8]

Other important molecular markers

The PI3K/AKT pathway is being unrecognized now more than ever before for thyroid cancer development. It can be activated by activating mutations in PIK3CA and AKT1 as well as by inactivation of PTEN, which negatively regulates this pathway. 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. Activating mutations in PIK3CA have been found in FTC, poorly differentiated thyroid carcinomas and anaplastic thyroid carcinomas. AKT1 mutations have been reported in metastatic thyroid cancer.

Additional genes mutated in thyroid cancer include TP53 and CTNNB1 (b-catenin). These genes tend to be mutated in more aggressive and advanced thyroid tumors. In addition, mutations in TSHR and guanine nucleotide binding protein, alpha stimulating gene have also been shown to play a role in thyroid tumorigenesis.[7]

Methods used for Molecular Diagnosis

The recent advancements in identifying the genes involved in the pathogenesis and development of tests to identify them have helped us to assist the cytopathological report in determining whether a lesion is likely to be benign or malignant. It can complement clinical, USG and cytopathology reports to avoid unnecessary surgical interventions.

Although many markers are in development and have been studied in a research setting, following principal tests are currently marketed for use to improve the malignancy risk assessment of "indeterminate" thyroid nodules. "Rule in" and "Rule out" tests that attempt to confirm or exclude, respectively, the presence of cancer within a thyroid nodule. The rule in tests assess for the presence of single gene point mutations (such as BRAF or RAS) or gene rearrangements (such as RET/PTC and PAX8/PPARG) which have been shown to increase the ability to predict cancer, while the rule out test (Afirma Gene Expression Classifier [GEC]) utilizes a proprietary GEC (RNA expression) specifically designed to maximize the ability to define a process as benign. Tests with high PPV can be used as rule in tests, whereas tests with high NPV are used as rule out tests. The PPV and NPV of a test are different from variables such as sensitivity and specificity of a test. They depend on:

a. The category of cytologically "indeterminate" nodule (AUS, FLUS vs. FN, SFN)
b. Prevalence of the malignancy within the population being tested
c. The patterns of cytopathology practice.

Rule Out Test

Gene Expression Classifier

In 2010, Chudova *et al*. analyzed the amplified transcription profile from mRNA of FNA biopsy specimen from a patient undergoing thyroidectomy and developed a gene expression test to predict lesion with low risk of malignancy.[9] From this further mathematical analysis led to the development of the Afirma GEC which is a custom thyroid microarray developed by Veracyte Inc., in San Fransisco, California. It is used to analyze mRNA expression of 167 different genes and is designed to identify nodules with benign histology. Two dedicated samples of FNA taken from each thyroid nodule are immediately stored in nucleic acid preservative solution. It is used in nodules 1 cm or larger. The test is not recommended for indeterminate nodules with suspicious of malignancy (Category V) histology. The results help classify the tumor into benign and suspicious.

In a prospective multicenter study involving 265 nodule with indeterminant cytology and histology follow up, this test was validated. It has a high NPV for Bethesda Category III and IV which is 95% and 94%, respectively. Hence, it can be used as a "rule out" test for these diagnostic categories. But in Bethesda

Category V, it has a NPV of only 85% hence leading to a 15% risk of malignancy thus less helpful as a rule out test in this category.[10] A multicenter cross-sectional study showed that a negative Afirma GEC result has led to a decrease of histologic thyroid surgical resection rate from 74% to –7.6% which is both dramatic and significant.[11]

However, in an independent study, the NPV for the Afirma GEC was 89.6% (lower than expected), in a practice with a high incidence of thyroid cancer in patients with indeterminate FNAs (33% incidence for local practice).[12] Another independent study has demonstrated a lower than expected rate of benign Afirma GEC reports in AUS/FLUS and FN/HCN, increasing the number of tests needed to avoid one surgery from two to four and raising questions about the costs of widespread application of this assay. In addition, it was found the PPV of a suspicious classifier result to be lower than previously reported (16 vs. 38%), so that more than 80% of GEC suspicious nodules proved to be benign at surgery.[13] This disappointing result, however, is consistent with the performance of the classifier when applied to a group of patients at low risk for malignancy.[10]

The PPV in different studies have shown to vary from 14% to 57% hence it cannot be reliably used as a rule in test and limits its clinical utility to predict the risk of malignancy. Therefore, the role of a "suspicious" result is less well defined. If diagnosis is benign in indeterminant category, the patient can be followed up clinically with no need for surgery, but suspicious diagnosis needs a surgical consultation. There is also a tendency for Afirma GEC test to report benign Hurthle cell nodules as suspicious frequently.[14]

The Afirma GEC test is expected to provide most useful information in a practice setting with a prevalence of malignancy in indeterminate thyroid nodule of 12–25%. Outside this range, this test is unlikely to provide information that would alter the management.[14] Because the diagnostic performance of the GEC in heterogeneous clinical populations is limited, and classifier NPV heavily depends on disease prevalence rates that are often completely unknown or not well characterized among cases submitted for evaluation, clinicians are cautioned to consider how the GEC will perform in their unique patient populations. Despite these limitations, the Afirma GEC remains an intriguing molecular diagnostic tool for evaluation of cytologically indeterminate thyroid nodules.

In 2014, Veracyte has introduced a test known as Afirma malignancy classifiers to enhance the Afirma GEC test results to assess the risk of malignancy. This test is performed on FNA samples which are reported as suspicious GEC result. It tests for gene mutations and mRNA profile which are involved with different types of thyroid carcinoma and enhances both the NPV and PPV of Afirma GEC tests. At present studies are ongoing to verify for the validation of this added on test.

Rule In Tests

ThyGenX test

This test identifies mutations and translocation fusions of different types of thyroid carcinomas. It uses a next-generation-sequencing platform to identify more than 100 genetic alterations across eight genes associated with thyroid malignancy. It requires only one dedicated FNA sample (minimum 50 ng of cellular material preserved) and uses only cases identified as Bethesda Class III or Class IV. This test is currently being offered by Interpace Diagnostics (New Jersey).

ThyraMIR

This is a new molecular test introduced to be used in conjunction with ThyGenX test. It is based on analysis of 10 different microRNAs (miRNAs). The miRNA molecules are involved in cell-cycle progression, differentiation and proliferation in thyroid tissue thus can be of potential diagnostic value in indeterminate thyroid nodules. Combination of both above tests demonstrated sensitivity and specificity of 89% and 85%. While NPV and PPV were reported as 94% and 74%, respectively. When both tests were a negative residual risk of cancer was low 6%. Used together, it has been reported to have NPV similar to that of Afirma Gene classifier but with much higher PPV.[14]

ThyroSeq test

This test is a next-generation sequencing based test which tests for gene mutation and fusion panel initially designed to target 12 cancer genes with 284 mutational hot spots. It is cost effective as it tests allows detection of an expanded set of informative point mutations, gene rearrangements, and small insertion/deletion mutations. This test has proven to have PPV of 88% and 87% for AUS/FLUS and FN/SFN, respectively.[15] This high PPV indicates that ThyroSeq test can be used as "rule in" test. This has been updated to an enhanced version ThyroSeq v2 in 2014 which has a more extensive panel detecting DNA (14 genes, including 1400 mutations) and RNA alteration (42 fusion 16 genes for expression).[14] A study using this test showed increased accuracy with PPV of 88% and NPV of 96% with 92% accuracy rates.[16] Therefore, this test may potentially function as both rule in or rule out tests. However, in settings with low pretest probability of malignancy according to bayesian modeling the NPV would remain high but PPV could drop significantly thus, limiting utilization as only a rule out test. Furthermore, considering the wide and expanded mutational profile being screened by this test, the chances of detecting a 'false positive" also remains.[14]

Although, further studies are needed to validate ThyroSeq V2 test in clinical setting, present data are encouraging.

Molecular Markers for Prognostication

The diagnosis of genes associated with thyroid cancer using the diagnostic tests will help us prognosticate also and may guide

the surgical approach undertaken, and bad prognostic markers will guide us to adopting a more extensive initial surgery.

BRAF V600E mutation

It is associated with poor prognostic factors such as extrathyroidal invasion, lymph node metastases, advanced tumor stage at presentation and recurrence. BRAF V600E and poor prognosis could be liked to dedifferentiation and this gene altering the function of sodium iodide symporter, thus decreasing the ability of tumor to trap radioiodine and leading to treatment failure and thus recurrence.

Thus, patients with BRAF V700E positive status detected in FNA nodules sampled preoperatively we should choose more extensive initial surgery, higher dose of post-surgical radioiodine, lower suppression of thyroid stimulating hormone, and closer follow-up.

TERT promoter

This a novel prognostic marker has been shown to be prevalent in more aggressive and advanced thyroid cancers. The mutations in TERT promoter cause increase in telomerase activity thus protecting telomere repeats and promoting tumorigenesis. It is known to coexist with other tumorigenic alterations such as BRAF and RAS mutations. The coexistence of BRAF and TERT promoter association is an indicator of the worst prognosis.

p53 mutations

They are rarely seen in thyroid cancer but are generally believed to point to a poorly differentiated and aggressive phenotype and associated with anaplastic carcinomas.

AACE/ACE/AME: Thyroid Nodule Guideline 2016 Update

Important points on molecular markers

- Molecular marker may be used to complement, not to replace usual management. Should be ordered when the results are expected to influence clinical management.
- Molecular testing is ordered for cytologically indeterminate nodules.
- Cytopathology expertise, patient characteristics, and prevalence of malignancy within the population being tested impact the NPVs and PPVs of molecular tests, which should be kept in mind.
- Consider the detection of BRAF and RET/PTC and, possibly, PAX8/PPARG and RAS mutations if such selection is available. Because of the insufficient evidence and the limited follow-up, there is no recommendation either in favor of or against the use of GECs for cytologically indeterminate nodules.
- At present, with the exception of mutations such as BRAFV600E that has a PPV approaching 100% for PTC, evidence is insufficient to recommend in favor of or

against the use of mutation testing as a guide for the extent of surgery.
- Since the false-negative rate for indeterminate nodules is 5–6% and the experience and follow-up for mutation-negative nodules or nodules classified as benign by a GEC are still insufficient, close follow-up is recommended.
- While molecular analysis of FNA genetic material from thyroid nodules shows great promise in refining the diagnosis, prognosis, and treatment of thyroid cancer, there are currently insufficient data to support a universal recommendation for molecular testing in the further categorization of "indeterminate" thyroid nodules.

Summary

Research in the past three decades in thyroid cancers has led to identification of most mutations and other genetic alteration in thyroid cancer. Significant advances have been made in understanding genetic mechanism for thyroid cancer and in the development of molecular test for cancer diagnosis in thyroid nodule.

With cost of molecular test going down and it becoming more efficient would make molecular test more feasible and cost effective for indeterminate thyroid nodules. However, the need for molecular diagnosis only occurs in the minority of case in which the cytology is indeterminate; therefore, one can consider sending samples for molecular diagnostics only in 10-25% of cases where it is pertinent.

In addition, utility of any molecular test is useful only when combined with clinical data, USG risk factors as well as understanding of the prevalence of malignancies for different cytological categories at the proposing institution. Rule out test such as GEC will perform better in low cancer frequency and in cytological category of AUS/FLUS or FN. A rule in test like Thyseq or Thygenx performs better in categories and settings of higher cancer prevalence.

Thus, molecular test cannot supplement clinical judgment and other well-proven modalities of investigations but it adds exciting new paradigm in uncertainties of indeterminant thyroid nodules. Future research may delineate these molecular test in detail to be included in routine decision making in approach to cytologically indeterminate thyroid nodule.

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