Molecular Profiling of 50,734 Bethesda III-VI Thyroid Nodules by ThyroSeq v3: Implications for Personalized Management

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Disclosure summary

B.R. Haugen serves on Advisory Board, Eisai; S.P. Hodak serves as a consultant for Sonic Healthcare USA; Lija Joseph is a consultant for Leica Biosystems; B. McIver serves on Advisory Boards for Eisai, Blueprint and Beyer, and has received speaker honoraria from Eisai, Eli Lilly, and Sonic Healthcare USA; M.N. Nikiforova and Y.E. Nikiforov own intellectual property related to ThyroSeq and receive royalties through the University of Pittsburgh; they also serve as consultants for Sonic Healthcare USA. All other authors have no conflicts of interests or disclosures to report.

Abstract

Context: Comprehensive genomic analysis of thyroid nodules for multiple classes of molecular alterations detected in a large series of fine-needle aspiration (FNA) samples has not been reported.

Objective: To determine the prevalence of clinically relevant molecular alterations in Bethesda categories III-VI (BCIII-VI) thyroid nodules.

Design: Retrospective analysis of FNA samples tested by ThyroSeq v3 using Genomic Classifier and Cancer Risk Classifier.

Setting: UPMC MGP laboratory.

Participants: A total of 50,734 BCIII-VI nodules from 48,225 patients.

Intervention: None

Main Outcome Measures: Prevalence of diagnostic, prognostic, and targetable genetic alterations.

Results: Among 50,734 informative FNA samples, 65.3% were test-negative, 33.9% positive, 0.2% positive for medullary carcinoma, and 0.6% positive for parathyroid. The benign call rate in BCIII-IV nodules was 68%. Among test-positive samples, 73.3% had mutations, 11.3% gene fusions, and 10.8% isolated copy number alteration. Comparing BCIII-IV nodules with BCV-VI nodules revealed a shift from predominantly RAS-like alterations to *BRAF* V600E-like alterations and fusions involving receptor tyrosine kinases (RTK). Using ThyroSeq Cancer Risk Classifier, a high-risk profile, which typically included *TERT* or *TP53* mutations, was found in 6% of samples, more frequently BCV-VI. RNA-Seq confirmed ThyroSeq detection of novel RTK fusions in 98.2% of cases.

Conclusions: In this series, 68% of BCIII-IV nodules were classified as negative by ThyroSeq, potentially preventing diagnostic surgery in this subset of patients. Specific genetic alterations were detected in most BCV-VI nodules, with a higher prevalence of *BRAF* and *TERT* mutations and targetable gene fusions compared to BCIII-IV nodules, offering prognostic and therapeutic information for patient management.

Introduction

Molecular testing is a well-established tool for the optimal management of patients with thyroid nodules classified on fine-needle aspiration (FNA) as indeterminate Bethesda Category (BC) III and IV (1-4). Because of the high negative predictive value of well-validated molecular tests in this setting, most diagnostic surgeries can be avoided for patients with negative molecular testing and likely benign nodules, reducing healthcare costs and improving patients' quality of life (5). An added benefit is that positive molecular test results can predict the type of malignancy in many cases, which may inform the extent of surgery and reduce the number of completion thyroidectomies that might otherwise be required following diagnostic lobectomy. Finally, when molecular testing is instituted, the wait for surgery may be shortened by decreasing the total number of patients requiring surgery and prioritizing patients with higher-risk cancer (2,5,6).

Molecular testing has been primarily used for diagnostic purposes. However, recent advances in our understanding of the association between genetic alterations and cancer phenotypes, patterns of spread, and risk of aggressive behavior have prompted the consideration for use of molecular testing for patients with the FNA diagnosis of Malignancy (BCVI) or Suspicious for Malignancy (BCV). Specifically, the seminal TCGA study and subsequent reports established the molecular classification of papillary thyroid carcinoma (PTC) and follicular thyroid carcinomas (FTC) in two main groups, RAS-like and BRAF V600E-like tumors (7,8). The former are follicular-patterned encapsulated neoplasms such as encapsulated follicular variant of PTC or FTC, which typically spread hematogenously rather than to regional lymph nodes, retaining the expression of genes associated with thyroid differentiation. The BRAF V600E-like tumors are classic type or

tall cell PTC; they frequently involve regional lymph nodes before spreading to distant sites and are prone to losing the expression of sodium iodide symporter (NIS) and other thyroid differentiation genes early in cancer progression. In addition, tumors with RAS-like alterations as the only genetic event have a low-risk of aggressive behavior, whereas BRAF V600E-like tumors are of intermediate risk for cancer recurrence (9,10). Furthermore, *TERT*, *TP53*, and several other genes have emerged as molecular markers of high-risk thyroid cancer associated with an increased risk of recurrence and mortality (11,12). In fact, molecular risk stratification is likely to predict the probability of aggressive cancer behavior, including distant metastasis, comparable to the ATA categories of low, intermediate, and high risk of structural disease recurrence (13). Growing evidence suggests that together with clinical and radiological findings, the availability of genetic profiling preoperatively may help to inform the optimal surgical management of patients with thyroid neoplasms (14). Molecular findings can support the current trend for de-escalation of treatment and initial conservative surgery for patients with differentiated thyroid cancer (DTC), consistent with management guidelines (2,3).

In addition to their diagnostic and prognostic utility, molecular markers may assist in therapeutic decisions for patients with radioactive iodine (RAI) resistant or refractory thyroid cancer. FDA approved therapeutic options exist for the treatment of *BRAF* V600E-mutated thyroid carcinoma (ATC, unresectable or metastatic poorly differentiated thyroid carcinoma [PDTC] and differentiated thyroid carcinoma [DTC]), *RET*-mutated medullary thyroid carcinoma (MTC) and *RET*-fusion positive PTC, and for metastatic and unresectable thyroid carcinomas carrying *NTRK* fusions (15,16). Additional FDA-approved off-label targeted therapies are available for patients with thyroid carcinomas carrying *ALK* or *ROS* fusions (15,17,18).

To date, only limited information is available on the molecular profiles of thyroid nodules detected preoperatively in a large series of consecutive FNA samples with detection of all major classes of molecular alterations. In this study, we report the analysis of a 50,734 thyroid nodules classified as BC III-VI on preoperative FNA cytology for multiple classes of molecular alterations including point mutations, gene fusions, chromosomal copy number alterations (CNA) and gene expression alterations (GEA) with the aim to determine (i) the diagnostic utility of ThyroSeq v3 testing, (ii) the prevalence and distribution of molecularly-defined risk groups of thyroid cancer, and (iii) the frequency of therapeutically targetable genetic alterations detected in these nodules.

Methods

Samples

Retrospective analysis of consecutive FNA samples from primary thyroid nodules clinically tested by ThyroSeq v3 from January 2018 to May 2021 was performed following the approved University of Pittsburgh Institutional Review Board protocol. Samples were submitted as part of routine clinical care from 1,102 practice sites. Among the study patients, the median patient age was 58 years (range 5-91 years); 75.3% were female. The median nodule size was 2.1 cm (range, 0.5-9.4 cm). Cytologic diagnoses were extracted from the cytology reports generated at each clinical site and included, using The Bethesda System for Reporting Thyroid Cytopathology (19), 40,622 AUS/FLUS (BCIII), 7,725 of FN/SFN (BCIV), 2,028 of Suspicious for Malignancy (BCV), and 359 of Positive for Malignancy (BCVI) samples. Twenty-two FNA samples with

Non-diagnostic (BCI) and 97 with Benign (BCII) cytology tested during the same time period were excluded from this analysis. BCIII and IV nodules represent primary sample types that require diagnostic molecular testing and these samples are likely to represent consecutive samples from these BC submitted from participating institutions. Other samples were submitted for molecular testing for a variety of clinical reasons typically not stated in the requisition forms. These included BCV-VI nodules many of which were likely submitted for cancer risk stratification and therefore these cytology groups could be potentially enriched with higher-risk cancers.

Molecular Analysis

Molecular testing was performed using ThyroSeq v3 assay as described previously (20). The assay is based on targeted next-generation sequencing of DNA and RNA and evaluates 112 genes for point mutations, insertions, deletions, gene fusions, CNA, and GEA. Analysis of the genetic findings using the ThyroSeq v3 GC diagnostic algorithm classified results as Negative including Currently Negative (low probability of cancer/NIFTP) or Positive (high probability of cancer/NIFTP (1).

In addition, all test-positive samples were re-analyzed using ThyroSeq Cancer Risk Classifier (CRC) to establish a risk of aggressive behavior. The CRC algorithm categorized all samples into three molecular risk groups (MRG): MRG-low, MRG-intermediate, and MRG-high (13). Specifically, the low-risk group included *RAS, RAS*-like mutations and fusions (e.g., *BRAF* K601E mutation, *THADA/IGF2BP3* fusion) and/or focal chromosomal type (FC-type) CNA or RAS-like GEA. The intermediate risk group included *BRAF* V600E, BRAF-like alterations (e.g.,

RET fusions), and genome haploidization type (GH-type) CNAs characteristic of Hurthle cell neoplasms. The high-risk group was defined by the combination of an "early" driver alteration such as *BRAF* or *RAS* mutation or a fusion, with a "late-hit" mutation in the *TERT, TP53, AKT1*, and/or *PIK3CA* genes. This group also included *TERT* mutations at >10% allele frequency even if found as an isolated event. Additional rare combinations of genetic alterations also included in a high-risk group were (i) *DICER1* mutation found in combination with CNA characteristic of poorly differentiated thyroid carcinoma of children and young adults (21) and (ii) GH-type CNA with *MET* CNA (22,23).

Detection of Novel Fusions

ThyroSeq v3 test detects most clinically relevant gene fusions by direct amplification of the known fusion points. In addition, it is designed to detect all novel functional fusions in the *ALK, RET*, *NTRK1*, *NTRK3*, and *ROS1* genes by testing mRNA and calculating differential expression between the tyrosine kinase (TK) domain and the extracellular (EC) domain of each gene as previously described (20). Samples with disproportionally high expression of the TK over EC domain are suspicious for gene fusion and were subjected to whole transcriptome analysis (RNA-Seq) performed as reported previously (24,25).

Statistical Analysis

A two-sided t-test was used to compare continuous variables, and the chi-square test or Fischer Exact test was used for categorical variables. A p-value of less than 0.05 was considered statistically significant.

Results

Overall, 50,734 consecutive FNA samples from thyroid nodules classified as BCIII-VI on cytology from 48,225 patients with informative ThyroSeq v3 testing during the study period were identified. Of those, 17,205 (33.9%) revealed molecular alterations associated with follicular cell-derived thyroid cancer, 281 (0.6%) were positive for parathyroid cells, and 124 (0.2%) were positive for MTC. The remaining 33,124 (65.3%) samples had the molecular profile of follicular cell-derived nodules but were negative for cancer-related alterations.

Molecular Landscape of Follicular Cell-Derived Nodules

Among all follicular cell-derived nodules with a positive test result, 12,604 (73.3%) harbored one or more point mutations (total of 14,055 mutations), 1,945 (11.3%) had gene fusions either as a single event or in combination with other molecular alterations, 1,865 (10.8%) nodules had isolated CNA, and 791 (4.6%) showed GEA as the only detected abnormality.

Point mutations

The most common mutations in the follicular cell-derived nodules involved one of the three *RAS* genes (*NRAS, HRAS, KRAS*), which were found in 42.2% of test-positive cases, followed by *BRAF* V600E mutation found in 15.4% (Table 1). While *RAS* mutations were more frequent among BC III-IV as compared to BC V-VI nodules (46.5% vs. 8.8%, $p<0.001$), *BRAF* V600E was seen more often in BCV-VI as compared to BCIII-IV (65.8% vs. 8.8%, $p<0.001$) nodules.

Other RAS-like mutations (such as *PTEN*, *DICER1*, BRAF K601E) were found in 12.2% (n=1,864) of BCIII-IV as compared to 1.1% (n=21) of BCV-VI nodules (p<0.001). The most common high-risk molecular alterations, *TERT* and *TP53*, were detected in 5.1% and 1.8% of all nodules, respectively, and were seen in combination with other molecular alterations in 81.4% of cases. *TERT* promoter mutations were more prevalent in BCV-VI than in BCIII-IV nodules (9.1% vs. 4.6%, $p<0.001$). Sixty-nine percent of *TERT* promoter mutations (602/873) co-occurred with another mutation or fusion, with the most common combinations being *RAS* + *TERT* (n=299), *BRAF* + *TERT* (n=249), and *EIF1AX* + *TERT* (n=30).

While most thyroid nodules harbored a single gene mutation, 8.8% of the mutation-positive nodules had multiple mutations. Among them, the most common combination of mutations was *RAS* + *EIF1AX* (33.7%), followed by *TERT* and/or *TP53* in combination with *RAS* (32.5%) or *BRAF* (25.0%).

Gene fusions

Overall, 1,945 (11.3%) test-positive FNA samples harbored a gene fusion (Table 1). The two most common fusions involved the *IGF2BP3* and *PPARG* oncogenes, followed by *NTRK3*, *RET*, *ALK*, *BRAF*, and *NTRK1* fusions. Other, less frequent fusions involved *GLIS3*, *FGFR2*, and *ROS1* genes. Single cases of *LTK*, *MET*, *SLC26A11*, and *ERBB4* fusions were also detected. Nodules carrying gene fusions were found in younger patients as compared to nodules harboring point mutations (mean age 44.1 vs 52.7 years, p<0.001).

When combined, fusions involving *IGF2BP3* and *PPARG*, both *RAS*-like alterations (26,27), were more prevalent in BCIII-IV than in BCV-VI nodules (6.6% vs. 1.5%, $p<0.001$). In contrast, receptor tyrosine kinase (RTK) fusions, including *RET, ALK, NTRK1, NTRK3, ROS1*, and *FGFR2*, which are typically BRAF-like, were more common in BCV-VI than in BCIII-IV nodules (8.4% vs. 4.1%, $p<0.001$).

Among the fusion-positive nodules, 27 (1.4%) harbored a co-existing high-risk mutation, either *TERT* (n=24) or *TP53* (n=3).

Gene expression alterations

Among 791 nodules positive for GEA alone, BRAF-like GEA were found in 340 (42.9%) nodules and RAS-like GEA in 451 (57.1%) nodules (Table 1). BRAF-like GEA were found in 1.7% of BCIII-IV and 3.7% of BCV-VI nodules ($p<0.001$).

RAS-like and BRAF-like alteration-positive nodules

Overall, among test-positive nodules, RAS-like mutations, fusions, and GEA were found more frequently in BCIII-IV (68.2%) compared to BCV-VI nodules (12.7%) ($p<0.001$) (Fig. 1). BRAF-like mutations, fusions, and GEA were found less frequently in BC III-IV (14.9%) compared to BC V-VI nodules (79.2%) ($p< 0.0001$) (Fig. 1), demonstrating a reversed ratio of RAS-like and BRAF-like alterations in BCIII-IV and BCV-VI nodules.

Chromosomal copy number alterations

Among 1,865 nodules with CNA as the only genetic finding, 1,179 (63.2%) of cases had a genome haploidization type (GH-type) CNA characteristic of Hürthle cell (oncocytic) tumors, whereas 36.8% had a focal chromosomal type (FC-type) CNA typically seen in various follicular-patterned thyroid tumors (Table 1). CNA were more common in BCIII-IV nodules compared to BCV-VI nodules (11.5% vs. 5.5%, $p < 0.001$) (Fig. 1). The difference was particularly prominent for the GH-type CNA, which were found almost exclusively (97.5%) in BCIII-IV nodules.

Molecular alterations in FNAs positive for parathyroid

In this series of FNA samples, 281 nodules were diagnosed as parathyroid lesions based on the high level of expression of parathyroid hormone (PTH) and other neuroendocrine gene mRNAs and low level of expression of thyroid follicular cell lineage genes. Among these samples, 16% harbored additional molecular alterations, providing evidence for a clonal parathyroid neoplasm (adenoma or carcinoma) rather than parathyroid hyperplasia (Table 2). *MEN1* mutations and CNA, either alone or in combination with mutations, represented the two most common genetic alterations identified in these nodules.

Molecular alterations in FNAs positive for medullary thyroid carcinoma

One hundred and twenty-four nodules showed high expression of Calcitonin and other neuroendocrine genes and low levels of thyroid follicular cell lineage genes, consistent with MTC. Although more than half (74/124, 59.7%) of the nodules diagnosed as MTC had BCIII-IV cytology, the molecular profile of MTC was more common in BCV-VI nodules (2.1% vs. 0.15%, $p<0.001$) (Fig. 2). Most (82%) of these cases showed mutations in *RET* or other genes including *HRAS*, *KRAS*, and *BRAF*, with all *BRAF* mutations being non-V600E (Table 3). In addition, in 16 cases, all with mutations, FC-type CNA were identified.

Diagnostic aspects of ThyroSeq testing

In BCIII-IV nodules, which are the primary target of diagnostic molecular tests, the benign (negative) call rate of ThyroSeq v3 Genomic Classifier was 68% (71% in BCIII and 52% in BCIV nodules), allowing potential avoidance of diagnostic surgery in the majority of these patients (Fig. 2). The proportion of nodules with negative test results decreased to 17% in BCV and 5% in BCVI nodules (Fig. 2), consistent with the expected high probability of cancer or NIFTP in these diagnostic categories. Among the 32% of test-positive BCIII-IV nodules, the vast majority had molecular profiles of follicular cell-derived neoplasms, whereas 1.76% were positive for parathyroid cells and 0.48% for MTC.

Preoperative Cancer Risk Assessment

For nodules with a positive test result, the availability of information on specific genetic alterations can be used to predict the risk of cancer recurrence using the ThyroSeq CRC (13,28,29). Overall, among 17,205 nodules positive for follicular cell-derived neoplasms, the majority (70%) were carrying low-risk molecular alterations (*RAS* mutations and other RAS-like mutations, gene fusions, and GEA) and were classified by ThyroSeq CRC as low risk (Table 4). Intermediate-risk molecular alterations (*BRAF* V600E; BRAF-like mutations, gene fusions and GEA; GH-type CNA) were detected in 24% of the nodules. High-risk molecular alterations (such as *TERT*, or *TP53*, typically present in combination with other mutations or gene fusions) were found in 6% of FNA samples. Molecular risk groups (MRG) distribution varied in different Bethesda cytology categories. Whereas the majority (76.3%) of BCIII-IV nodules had MRG-low profiles, BCV-VI nodules were more often MRG-intermediate 69.2% ($p<0.0001$). Furthermore, MRG-high profiles were significantly more common in BCV-VI nodules as compared to BC II-IV (10.2% vs 5.1%, $p < 0.0001$).

Potential therapeutic targets

Among nodules with MTC profiles, 66% were found to carry *RET* mutations, a target for FDA-approved RET inhibitors (30,31). *BRAF* V600E was found in 15% of nodules positive for alterations associated with follicular cell-derived tumors. However, only a small proportion of those cancers would be expected to represent ATC or RAI-resistant DTC, in which *BRAF* V600E is a therapeutic target. Among 17,205 of test-positive nodules composed of thyroid

follicular cells, fusions involving the RTK genes were detected in 784 (5%) cases (Table 1). These include targets for the FDA-approved therapies in thyroid cancer, such as *RET, NTRK1*, and *NTRK3* genes, as well as genes targetable in other cancer types, such as *ALK*, *ROS1*, and *FGFR2* (17,32-35). The RTK gene fusions were more prevalent in BCV-VI than in BCIII-IV nodules (8% vs. 4%, $p<0.001$).

Detection of new RTK gene fusion partners

A significant majority of the detected RTK gene fusions (86.1%) involved known fusion partners. Those were identified by amplification using primers flanking the known fusion point, a design that provides high sensitivity of fusion detection (20). The remaining 13.9% of RTK fusions detected by ThyroSeq involved a new partner fused with *RET*, *NTRK1*, *NTRK3*, *ALK*, or *ROS1* oncogenes, or a new fusion point between known partners. Those were detected by identifying a disproportionally high expression of the TK domain of these oncogenes compared with the EC domain (Fig. 3) (25,36). Such an approach should allow the detection of any fusions involving these genes and it also eliminates non-functional fusions, i.e., those that do not lead to the expression of the functional kinase domain of the RTK gene. However, this approach does not provide information regarding the fusion partner. In order to confirm the accuracy in the detection of novel RTK gene fusion types by this approach, 88 consecutive FNA samples with suspected fusions based on the preferential expression of the TK domain of these genes and with a sufficient amount of RNA remaining underwent whole transcriptome (RNA-Seq) analysis. It confirmed 87 (98.9%) of the suspected fusions, whereas one *NTRK1* fusion predicted by ThyroSeq testing was not found on the RNA-Seq analysis (Table 5). Most of these cases

contained fusions with previously unknown partner genes including *DLG5-RET*, *CTSB-ALK*, and *RUFY3-NTRK1*, whereas other fusions involved known partner genes but demonstrated novel fusion points.

Discussion

In this study, the availability of detailed genetic information on a large cohort of thyroid nodules across the range of FNA cytology diagnoses allowed us to establish the benign call rate of the test during routine clinical use across a variety of practice sites and to determine the molecular landscape of BC III-VI nodules with emphasis on genetic markers with diagnostic, prognostic, and therapeutic significance.

The benign call rate is an important performance characteristic of a diagnostic test as it reflects the proportion of patients with indeterminate FNA cytology that can potentially avoid diagnostic surgery for thyroid nodules. In the initial multicenter validation study, the benign call rate of ThyroSeq v3 GC in BCIII-IV nodules was 61% (37), and it has ranged from 58% to 71% in subsequent independent studies (6,37-39). In this study, which evaluated over 48,000 BCIII-IV nodules from more than one thousand clinical sites, the benign call rate was 65%, providing confirmation that this important test performance characteristic was in the range defined by the previous reports. In specific cytology groups, the benign call rate was 71% in BCIII and 52% in BCIV nodules, which is expected because of the higher cancer/NIFTP prevalence in BCIV nodules (19). The primary benefit of a Negative molecular test result is the reduction in diagnostic thyroid surgeries for benign nodules with associated complications and cost (2,5,6).

Indeed, based on the initial prospective validation study of ThyroSeq v3 GC that enrolled 247 patients with BCIII-IV nodules, all with surgical outcomes, the residual cancer probability in the test-negative nodules was 3% and all missed cancers were low-risk DTC, suggesting that non-surgical management is acceptable for these patients (37). Subsequent studies based on the real-world experience have shown that 89-100% of patients with ThyroSeq v3 Negative BCIII-IV nodules avoided immediate surgery, and on short follow-up most of the nodules remained stable and if excised, revealed intrathyroidal cancer/NIFTP only in a small proportion of cases (6,38,39).

The availability of a large series of samples classified as Positive on ThyroSeq allowed us to provide a detailed molecular characterization of thyroid nodules that underwent routine FNA testing yielding cytological diagnoses ranging from BCIII to BCVI. The modern molecular classification of follicular cell-derived thyroid tumors recognizes RAS-like, BRAF-like, and CNA-positive neoplasms, each with characteristic histological correlates, modes of spread, and expression of thyroid follicular differentiation markers (7,12,23,40). The results of this study demonstrate that the molecular landscape of test-positive BCIII-IV nodules is dominated by *RAS* mutations and RAS-like alterations found in close to three-fourths of cases whereas BRAF-like alterations occurred in only 15% of these nodules. This finding was expected because RAS-like tumors are known to have follicular or microfollicular architectures and typically show subtle or absent nuclear features of papillary carcinoma, meeting cytologic criteria for BCIII and BCIV categories (19). Histologically, RAS-like tumors typically fall into the spectrum of encapsulated follicular-patterned tumors including NIFTP, encapsulated follicular variant of papillary carcinoma, follicular adenoma, and follicular carcinoma (1,7,8,41). Identifying a smaller

subgroup of BCIII-IV nodules carrying *BRAF* V600E and BRAF-like alterations is clinically important because those are associated with a near 100% probability of cancer, typically classic or tall cell variant of papillary carcinoma, and a higher likelihood of regional lymph node metastasis (1,42).

We observed that the transition from the BCIII-IV category to the BCV-VI category resulted in a reversal of the proportions of these two main classes of genetic alterations, with *BRAF* V600E mutations and BRAF-like alterations becoming dominant in the BCV-VI category (79%). At the same time, the rate of RAS-like alterations decreased to 15%. This distribution of molecular profiles correlates with the cytologic criteria for BCV and BCVI categories that rely on the identification of nuclear features of papillary carcinoma, which are most prominent in classic and tall cell variants of papillary carcinoma. Virtually all these nodules are expected to be papillary cancers on histologic evaluation, with a propensity to spread to regional lymph nodes. This information and the presence or absence of additional high-risk alterations may help to guide optimal surgical management of these patients (39,43).

Another molecular class of thyroid tumors includes neoplasms driven by chromosomal CNA found in isolation, without other driver mutations or gene fusions. Two types of CNA are recognized, GH-type characteristic of Hürthle cell tumors (12,23,44,45) and FC-type CNA common in a subgroup of follicular variant papillary carcinomas and follicular carcinomas (7,8). In this study, 11% of the test-positive nodules belonged to this molecular group, of which two-thirds had GH-type CNA and one-third FC-type CNA, both found predominantly in nodules with BCIII-IV cytology. Upon excision, most of these nodules are found on histology to be Hurthle

cell tumors, follicular variants of papillary carcinoma, or NIFTP, with a small proportion of other variants of papillary carcinoma (1,45,46).

The Cancer Risk Classifier (CRC) algorithm is a more recently introduced step in the analysis of the genetic profiles obtained by ThyroSeq, stratifying thyroid tumors into molecular groups with low-, intermediate-, and high-risk, with expected 5-year risk of distant metastasis of <1%, 5-10%, and 20-35%, respectively (13). The MRG-high group, defined primarily by the presence of multiple mutations including high-risk mutations such as *TERT* and *TP53*, included several less common molecular profiles such as an isolated *TERT* mutation, known for its association with distant metastasis and tumor recurrence (11), which in our validation analysis was associated with high-risk thyroid cancer only when found at >10% allele frequency. Such CRC-based molecular risk profiles detected in FNA samples can predict the risk of structural disease recurrence (28,29). In this study, 70% of the test-positive follicular cell-derived nodules had a low-risk molecular profile. Optimal management may include therapeutic thyroid lobectomy or active surveillance for most of these patients without suspicious ultrasonographic or clinical findings. In contrast, the 6% of patients with nodules with high-risk molecular alterations should be prioritized for surgery. Because of the significant risk of distant spread, pre- or post-operative work-up for distant metastatic disease and strong consideration for total thyroidectomy may be of benefit for many of these patients. Notably, the proportion of thyroid nodules with high-risk molecular profiles in this study was small, particularly in BCIII-IV categories (4-8% of test-positive nodules), which is consistent with the indolent clinical behavior of most well-differentiated thyroid cancers (47). The higher rate of high-risk molecular profiles in BCVI nodules (21%) probably reflects a selection bias as many of those samples were likely submitted

for molecular analysis to aid with cancer risk stratification, enriching this group with higher-risk cancers. Tumors in the intermediate-risk profile have a predicted risk of cancer recurrence similar to the ATA intermediate-risk for recurrence (3). Clinical management of these patients, including the extent of surgery, would need to include clinical factors and imaging characteristics, such as thyroid nodule size and sonographic appearance. However, it is important to acknowledge that not all nodules with the CRC-defined risk determined in the FNA samples are malignant on surgical pathology and some of those should be expected to be histologically benign or NIFTP, as it was seen in the previous studies among RAS-like tumors (1), most of which fall into the MRG-low group.

The prevalence of incidentally identified parathyroid lesions in this series was 0.6%, comparable to that in a smaller prior study that also described the clinical impact of the incidental discovery of a parathyroid lesion while working up a presumed thyroid nodule (48). The prevalence of incidental parathyroid lesions did not significantly vary between Bethesda Categories in this study. Identification of likely somatic genetic alterations in a subset of parathyroid lesions indicates that up to 16% of incidentally identified parathyroid lesions are neoplastic. The genetic profile of these lesions (i.e., *MEN1* mutations, CNA) may assist in distinguishing parathyroid hyperplasia from clonal parathyroid adenomas or carcinomas.

A molecular diagnosis of MTC was rendered in 0.2% of thyroid nodules overall and in 2.1% of BCV-VI nodules. This finding corroborates a recent observation highlighting that ~30% of patients with MTC fail to receive an accurate diagnosis on FNA, precluding optimal surgical management of these patients (49). For patients whose MTC harbored a *RET* mutation, germline

genetic testing of the specific *RET* exon harboring this mutation could be considered, rather than sequencing the entire *RET* gene. In contrast, the 28% of patients with MTC harboring *RAS* or *BRAF* mutation can be reassured of the sporadic nature of their disease with no need for additional germline testing, or workup for pheochromocytoma preoperatively (50).

Surgery and, when indicated, RAI remain the standard therapy for follicular-cell derived thyroid cancer. A subset of metastatic and RAI-resistant DTCs and ATC may require systemic TKI therapy. Several drugs are currently approved by the FDA for thyroid carcinomas or may be used off-label based on FDA approval for non-thyroid cancers. Current FDA-approved therapeutic options for thyroid cancer include dabrafenib plus trametinib for *BRAF* V600E-positive ATC and unresectable or metastatic PDTC and DTC (51); selpercatinib or pralsetinib for *RET*-mutated medullary thyroid carcinoma (MTC) and *RET*-fusion positive PTC (15,52); and larotrectinib or entrectinib for metastatic and unresectable thyroid carcinomas carrying *NTRK* fusions (53,54). FDA-approved off-label therapeutic options targeting ALK or ROS fusions include crizotinib, brigatinib, or entrectinib (15,17,18). In this study, we observed that among BCV-VI nodules, up to 10% carried an RTK fusion, and close to 70% harbored the *BRAF* V600E mutation. Although only a small proportion of these patients have advanced tumors meeting indications for targeted therapies, the information on therapeutic targets may be valuable in the future management of many of these patients diagnosed with follicular cell-derived cancer or MTC.

Overall, NGS-based tests are favored for detecting multiple therapeutic targets in the same assay (55,56). There are two approaches for detecting gene fusions by NGS, hybrid capture-based and

amplification-based, each with its advantages and disadvantages. The hybrid capture-based approach is expected to identify all known and novel fusions involving RTK genes covered by the assay. However, accurate fusion detection requires a significant number of cells (at least ~40 ng of RNA or DNA). In contrast, a standard amplification-based approach can detect known fusions in low-cellularity samples, requiring only 5-10 ng of RNA, but will miss novel fusion types. To combine the advantages of both approaches, ThyroSeq v3 was designed to enable the detection of all known fusion types in low-cellularity FNA samples using the amplification-based approach, paired with the detection of novel RTK fusions by differential expression of the RTK gene regions located upstream and downstream of the fusion point (20). This study demonstrated that such an approach has high specificity, with 98% of fusions suspected based on the differential expression of RTK gene regions confirmed by RNA-Seq.

This study uniquely provides molecular profiles of a large series of thyroid nodules with various cytologic diagnoses, however, it has a number of limitations. First, whereas the BCIII and BCIV groups likely to represent a more consecutive series of thyroid nodules from various institutions submitted as part of routine care for patients with indeterminate cytology nodules, the BCV and BCVI groups likely represent only a proportion of all nodules assigned to these categories that underwent molecular testing for various clinical reasons. As such, they may not be representative of all BCV and BCVI nodules. Second, the cytologic diagnoses were rendered by local cytopathologists with likely different diagnostic thresholds, and the cytology was not centrally reviewed. Thus, this series inherits the local cytologic interpretation, and the results may not be fully extrapolated to every clinical practice. Finally, the study design did not include a

correlation with subsequent histologic diagnoses or clinical follow-up, collection of which would not be feasible for a large sample size of this study.

In summary, molecular profiling of over 50,000 thyroid FNA samples using a large thyroid-specific targeted NGS panel demonstrated that two-thirds of the nodules in the Bethesda III and IV categories lack genetic alterations and are most likely benign hyperplastic nodules. About one-third of these nodules carry one or more genetic alterations and are neoplasms. Most of these neoplasms are driven by RAS-like genetic alterations, in contrast to Bethesda V-VI nodules, the majority of which harbor BRAF-like alterations and are enriched in intermediate- and high-risk genetic alterations, including therapeutically relevant mutations and gene fusions. The availability of broad genetic information for thyroid nodules in the preoperative setting should not only enhance cancer diagnosis in nodules with indeterminate cytology but additionally provide valuable prognostic and therapeutic information informing for personalized management of patients with thyroid cancer.

Data Availability

Restrictions apply to the availability of some or all data generated or analyzed during this study to preserve patient confidentiality or because they were used under license. The corresponding author will on request detail the restrictions and any conditions under which access to some data may be provided.

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Tables and Figures Legends

Table 1. Main Types of Molecular Alterations Detected in Bethesda Category III-VI Thyroid Nodules.

Table 2. Molecular Alterations Detected in Thyroid Nodules with Parathyroid Profile

Table 3. Molecular Alterations Detected in Thyroid Nodules with Medullary Thyroid Carcinoma Profile.

Table 4. Molecular Prediction of Cancer Recurrence by ThyroSeq Cancer Risk Classifier.

Table 5. ThyroSeq v3 Detection of Novel RTK Fusions Using Differential Expression of Tyrosine Kinase (TK) Domain and Confirmation Rate by RNA-Seq.

Figure 1. Molecular Profiles of ThyroSeq v3-Positive Nodules with Different Bethesda Categories

Figure 2. Molecular Profiles of Test-Positive Nodules with Different Bethesda Categories

Figure 3. ThyroSeq v3 Detection of Novel RTK Fusions by Measuring Differential Expression of the Tyrosine Kinase (TK) and Extracellular Domains of the RTK Genes. **A**. Since wild-type

RTK genes are not expressed in thyroid follicular cells, no expression or very low expression of both parts of the gene is expected (no or few sequencing reads detected by ThyroSeq v3); **B**. As a result of gene fusion, the TK domain of the RTK gene is fused with an actively transcribed partner gene leading to an increased expression of the TK domain (high number of sequencing reads), whereas the extracellular domain remains undetectable (no or few sequencing reads).

Bethesda Category	BC III, n (%)	BC IV, n (%)	BC V, n (%)	BC VI, n (%)	Total, n (%)
ThyroSeq v3 Result	Positive (n=11565)	Positive (n=3665)	Positive (n=1643)	Positive (n=332)	Positive (n=17205)
Mutations (n=14,055)					
RAS	5615 (48.6%)	1474 (40.2%)	149 (9.1%)	25 (7.5%)	7263 (42.2%)
BRAF V600E	1168 (10.1%)	174 (4.7%)	1071 (65.2%)	229 (69.0%)	2642 (15.4%)
TERT	426 (3.7%)	267 (7.3%)	120 (7.3%)	60 (18.1%)	873 (5.1%)
TP53	155 (1.3%)	115 (3.1%)	19 (1.2%)	14 (4.2%)	303 (1.8%)
Gene Fusions (n=1,945)					
IGF2BP3	551 (4.8%)	54 (1.5%)	7 (0.4%)	0 (0%)	612 (3.6%)
PPARG	293 (2.5%)	114 (3.1%)	20 (1.2%)	3 (0.9%)	430 (2.5%)
NTRK3	214 (1.9%)	32 (0.9%)	50 (3.0%)	5 (1.5%)	301 (1.7%)
RET	156 (1.3%)	48 (1.3%)	57 (3.5%)	20 (6.0%)	281 (1.6%)
ALK	85 (0.7%)	13 (0.4%)	15 (4.5%)	3 (0.9%)	116 (0.7%)
BRAF	40 (0.3%)	9 (0.2%)	14 (0.9%)	11 (3.3%)	74 (0.4%)
NTRK1	32 (0.3%)	8 (0.2%)	10 (0.6%)	5 (1.5%)	55 (0.3%)
FGFR2	20 (0.2%)	7 (0.2%)	1 (0.1%)	0 (0%)	28 (0.2%)
ROS1	2 (0.02%)	1 (0.03%)	0 (0%)	0 (0%)	3 (0.02%)
Other (e.g., GLIS3)	29 (0.3%)	11 (0.3%)	5 (0.3%)	0 (0%)	45 (0.3%)
All Fusions	1422 (12.3%)	297 (8.1%)	179 (10.9%)	47 (14.2%)	1945 (11.3%)
RTK fusions	509 (4.4%)	109 (3.0%)	133 (8.1%)	33 (9.9%)	784 (4.6%)
Copy Number Alterations (n=1,865)					
CNA (all)	949 (8.2%)	808 (22.0%)	102 (6.2%)	6 (1.8%)	1865 (10.8%)
GH-type	585 (5.1%)	565 (15.4%)	24 (1.5%)	5 (1.5%)	1179 (6.9%)
FC-type	364 (3.1%)	243 (6.6%)	78 (4.7%)	1 (0.3%)	686 (3.9%)
Gene expression alterations (n=791)					
GEA (all)	518 (4.5%)	175 (4.8%)	87 (5.3%)	11 (3.3%)	791 (4.6%)

RAS-like	316 (2.7%)	110 (3.0%)	23 (1.4%)	2 (0.6%)	451 (2.6%)
BRAF-like	202 (1.7%)	65 (1.8%)	64 (3.9%)	9 (2.7%)	340 (2.0%)

Molecular Alterations	**Number of FNAs, n (%)**
MEN1	14 (5.0%)
HRAS	3 (1.1%)
*BRAF**	1 (0.4%)
CTNNB1	2 (0.7%)
TP53	3 (1.1%)
PIK3CA	3 (11.1%)
IDH1	2 (0.7%)
EIF1AX	1 (0.4%)
Copy number alterations alone	17 (6.0%)
Copy number alterations with other mutations	5 (1.8%)
Total FNAs with alterations	46 (16.4%)
Total FNAs with parathyroid profile, n	281 (100%)

* *BRAF* V600E

Molecular Alterations	**Number of FNAs, n (%)**
HRAS	26 (21.0%)
KRAS	6 (4.8%)
*BRAF**	3 (2.4%)
RET	67 (54.0%)
Copy number alterations alone	0 (0%)
Copy number alterations with other mutations	16 (12.9%)
Total FNAs with alterations	102 (82.3%)
Total FNAs with MTC profile, n	124 (100%)

**BRAF* K601E (n=1) and G469A (n=2) mutations

Molecular Risk Groups (MRG)*	BC III, n (%)	BC IV, n (%)	BC V, n (%)	BC VI, n (%)	Total, n (%)
Low*	8649 (74.8%)	2969 (81.0%)	371 (22.6%)	36 (10.8%)	12025 (69.9%)
Intermediate**	2431 (21.0%)	405 (11.1%)	1140 (69.4%)	227 (68.4%)	4203 (24.4%)
High***	485 (4.2%)	291 (7.9%)	132 (8.0%)	69 (20.8%)	977 (5.7%)

*MRG-Low includes *RAS* or *RAS*-like alterations and FC-type CNA
**MRG-Intermediate includes *BRAF* V600E or *BRAF*-like alterations and GH-type CNA
***MRG-high includes "early" driver alteration in combination with a "late-hit" mutation (*TERT*, *TP53*, *AKT1*, and/or *PIK3CA*), isolated *TERT* mutations at >10% allele frequency, *DICER1* mutation plus CNA characteristic of poorly differentiated thyroid carcinoma of children and young adults, and GH-type CNA in combination with *MET* CNA.

Fusion oncogene	Fusions suspected by ThyroSeq v3	Fusions Confirmed by RNA-Seq
RET	36	36 (100%)
ALK	25	25 (100%)
NTRK3	13	13 (100%)
NTRK1	12	11 (92.3%)
ROS1	2	2 (100%)
Total	88	87 (98.9%)

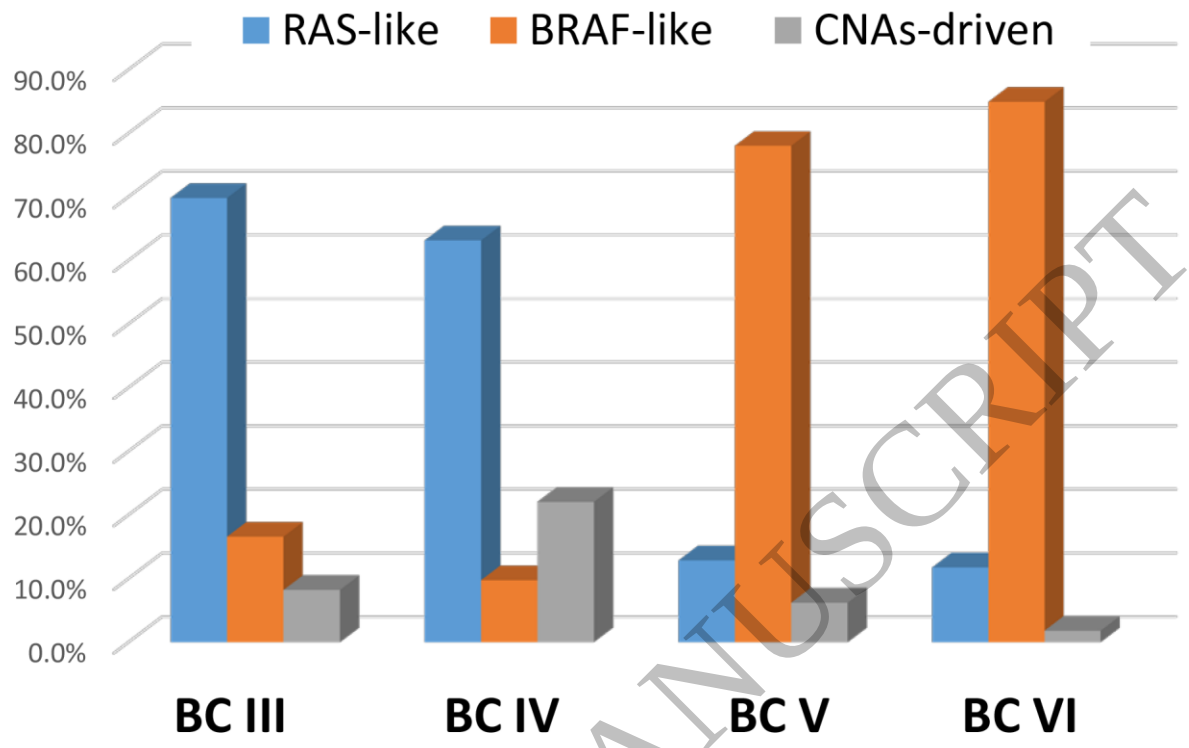

Figure 1
158x100 mm (x DPI)

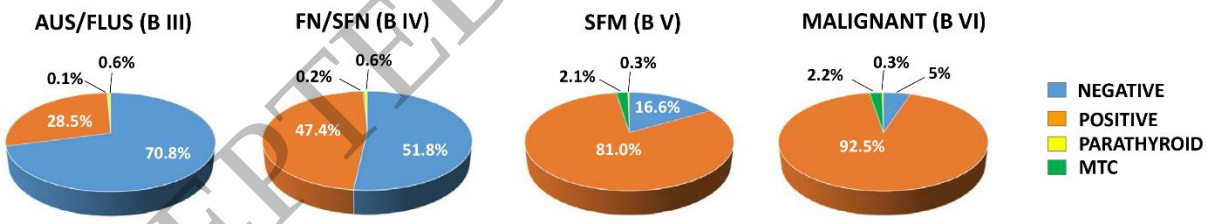

Figure 2
165x30 mm (x DPI)

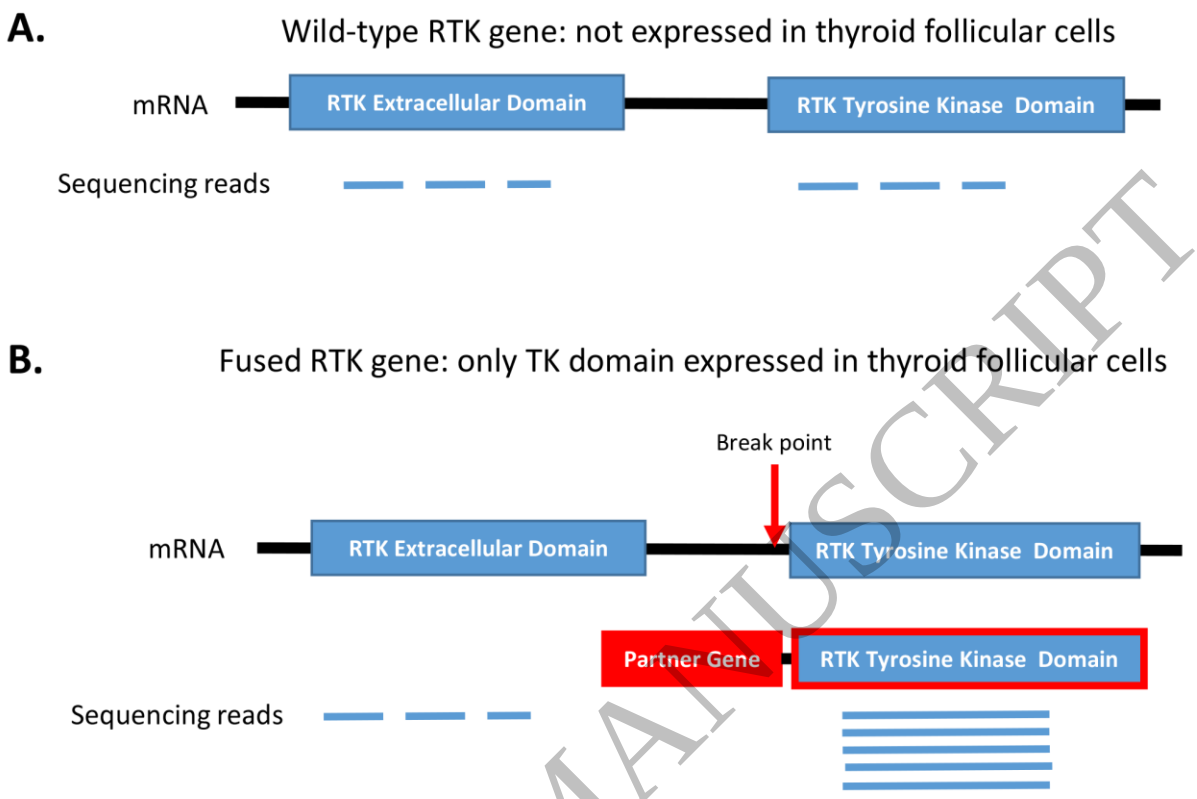

Figure 3
165x107 mm (x DPI)