The Afirma Xpression Atlas for Thyroid Nodules and Thyroid Cancer Metastases: Insights to Inform Clinical Decision-Making From a Fine-Needle Aspiration Sample

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INTRODUCTION

The Afirma Xpression Atlas (XA) (Veracyte, South San Francisco, California), which uses RNA sequencing to detect expressed variants and fusions, was launched in May 2018, and its analytical and clinical validation data for thyroid nodules were published subsequently.[1] In November 2019, the Afirma patient report was expanded to enumerate the molecular insights of the Afirma Genomic Sequencing Classifier (GSC) and XA so that they could be incorporated into clinical decision making. In March 2020, the Afirma XA panel was expanded to include 593 genes informing on 905 variants and 235 fusions. Currently, the Afirma XA is available internationally and throughout the United States, except in New York, where approval is pending. This commentary reviews these developments and their potential impact on thyroid fine needle aspiration (FNA) utilization.

BACKGROUND

The Afirma GSC is a cancer rule-out test with a high negative predictive value so that cytologically indeterminate (Bethesda III/IV)[2] thyroid nodules with an Afirma GSC benign result can be considered for clinical observation in lieu of diagnostic surgical resection (Fig. 1). In blinded validation using a prospectively collected, multicenter cohort of Bethesda III/IV nodules with a cancer prevalence of 24%, its sensitivity (91%), specificity (68%), positive predictive value (47%), and negative predictive value (96%) were established.[3] Six consecutive independent clinical experience publications have subsequently supported its improved benign call rate (including significant improvement among Hürthle-dominant cytology specimens), high negative predictive value, and ability to safely reduce diagnostic surgery.[4-9]

The Afirma XA was introduced for thyroid nodules that are cytologically indeterminate and Afirma GSC suspicious, and those diagnosed as Bethesda V/VI (suspicious for malignancy or malignant) (Fig. 1). Afirma XA uses RNA sequencing of material collected via FNA to report expressed genomic variants and gene fusions that have been associated with thyroid cancer. These findings may refine the risk of cancer, especially among cytologically indeterminate nodules, depending on the specific alteration identified. However, Afirma XA is not a cancer rule-out test. Insight from these alterations can contribute to personalized care for biopsied lesions that

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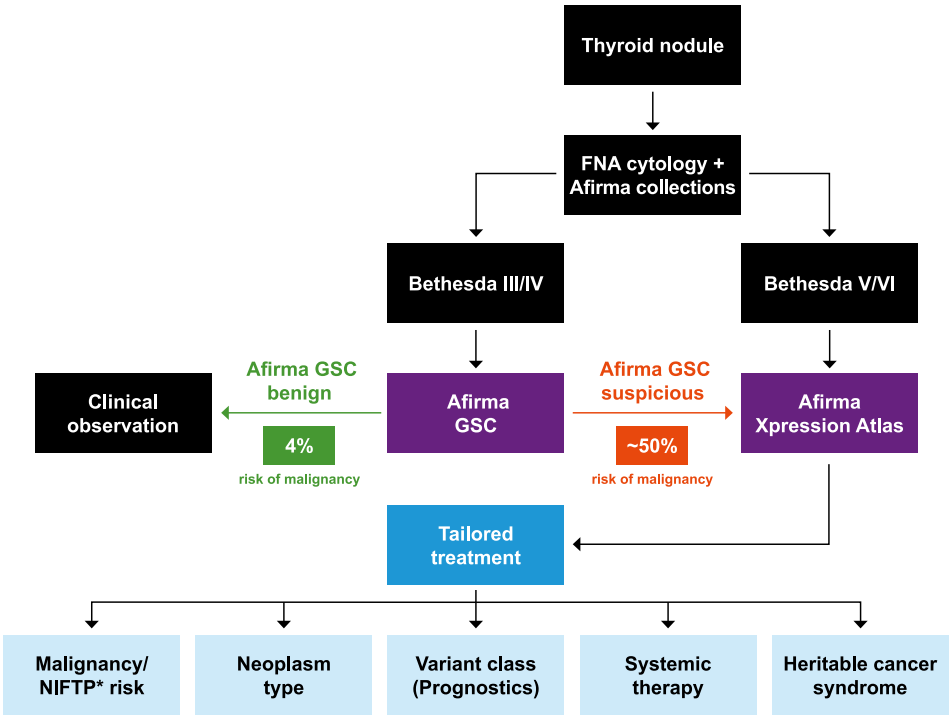

FIGURE 1. Clinical application of the Afirma Genomic Sequencing Classifier (GSC) and/or Xpression Atlas and the insights provided by these genomic analyses among thyroid nodules from fine needle aspiration (FNA) specimens. The patient report includes genomically derived understandings (bottom row) that may facilitate personalized treatment. *NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features. Adapted from Ali et al.[35]

are known or suspected to be malignant. Similarly, identification of genomic alterations from an FNA sample of known or suspected thyroid cancer metastases may characterize the oncogenic drivers of the tumor and inform decisions regarding systemic targeted therapies (Fig. 2).

AFIRMA XA ANALYTICAL AND CLINICAL VALIDATION

Clinical validity, analytical validity, and clinical utility are key components considered in an evidence-based assessment.[10] Analytical validation investigated Afirma XA across laboratories and reagent lots.[1] Using 69 variant-positive FNA samples, there was high accuracy between 2 different laboratories with different personnel for detecting variants (90%) and fusions (94%).

The Afirma XA clinical validation[1] demonstrated test sensitivity and accuracy to identify gene alterations associated with thyroid cancer using the same patient sample for both Afirma GSC and XA molecular testing. The molecular sample is collected into a Veracyte-provided tube containing FNAprotect (QIAGEN, Valencia, California) and shipped to Veracyte with frozen bricks (2 dedicated FNA passes per biopsied target are recommended). The test's ability to identify genomic variants in the sample's transcriptome was compared with currently accepted methods of targeted DNA and RNA sequencing.[1] Using 943 blinded FNA samples, the Afirma XA had high positive predictive agreement with targeted DNA sequencing (74% and 88% at 5% and 20% variant allele frequency, respectively) and targeted RNA sequencing (89%). Similarly, using 695 blinded FNA samples to look for RNA fusions, Afirma XA had an 82% positive predictive agreement with targeted RNA sequencing. Conversely, 95% or more of variants and fusions identified by Afirma XA were also identified by the reference DNA and RNA method. Others have shown similar RNA sequencing performance data among other tissue types.[11] Interestingly, some variants identified in DNA were not expressed—or were poorly expressed—in RNA. The contribution of these nonexpressed DNA variants to clinical pathology, neoplasm behavior, or response to targeted therapies is currently unknown. One may speculate that differences may be present between expressed and nonexpressed protein

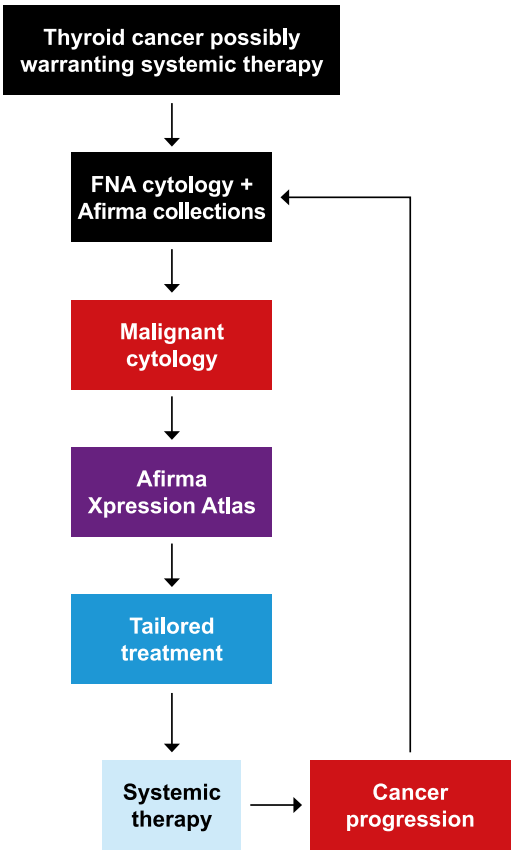

FIGURE 2. Clinical application of the Afirma Xpression Atlas (XA) for known or suspected thyroid cancer metastases from fine needle aspiration (FNA) specimens. Collection of FNA cytology confirms adequate tissue sampling and confirmation of the biopsied target. XA findings may characterize the oncogenic drivers of the tumor and inform personalized decisions regarding systemic targeted therapies. Adapted from Ali et al.[35]

coding variants that are thought to be drivers of oncogenesis. Promoter variants, like those of *TERT*, are not identified by Afirma XA.

AFIRMA XPRESSION ATLAS EXPANSION

Our understanding of the molecular landscape of thyroid neoplasia continues to expand, including that of alterations associated with thyroid cancer and resistance to targeted therapies.[12,13] Genomic variants and fusions identified from the literature and via ongoing discovery, with analysis of more than 37,000 Bethesda III/VI samples for novel and rare *NTRK1*, *NTRK3*, *RET*, *ALK*, and *BRAF* fusions, formed the basis for the first update to the Afirma XA panel in March 2020. An additional 104 novel or rare gene fusions were identified, all of which may be targeted with specific kinase inhibitors.[14] The panel now informs on 905 gene variants and 235 fusions from 593 genes.

THE NEW AFIRMA GSC AND XPRESSION ATLAS REPORT

Positive Predictive Value

The risk of malignancy or noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) among Bethesda III/IV nodules is an important factor for both patients and providers in the decision to surgically remove a thyroid nodule. This risk, or positive predictive value, is provided in Afirma test reports based on GSC and/or XA findings with the supporting evidence referenced (Figs. 3 and 4). For Afirma XA, PPV is reported either for the specific alteration identified or for alterations of that gene. These values were derived from validation data,[3,15,16] a systematic review of the published literature[17] and its periodic update, or real-world experience among consecutively identified patients with available surgical histopathology.[18] These PPVs are reported to their nearest quartile (eg, PPV 50% or 75%), except for those >95% (*BRAF*V600E, *NTRK* fusions, and *RET* fusions) and >99% (medullary thyroid cancer classifier positive).[15] NIFTP are considered true positive findings given the current recommendation of surgical resection of NIFTP for both diagnostic and therapeutic purposes. For Bethesda V/VI nodules, the PPV of genomic alterations is reported based on Bethesda III/IV knowledge and qualified by a statement that a similar or higher risk of malignancy is expected among Bethesda V/VI nodules (given their higher pretest risk of malignancy).

FDA-Approved Alteration-Specific Therapy

US Food and Drug Administration (FDA)-approved therapies are available for advanced thyroid carcinomas that are locally recurrent or metastatic, progressive or symptomatic, and refractory to standard treatment (surgery and radioactive iodine for differentiated thyroid cancer and surgery for medullary thyroid cancer), regardless of their underlying oncogenic drivers.[19] These therapies include oral multi-kinase inhibitors, often targeting the vascular endothelial growth factor receptor, that have demonstrated improved progression-free survival with better responses than have been seen with cytotoxic

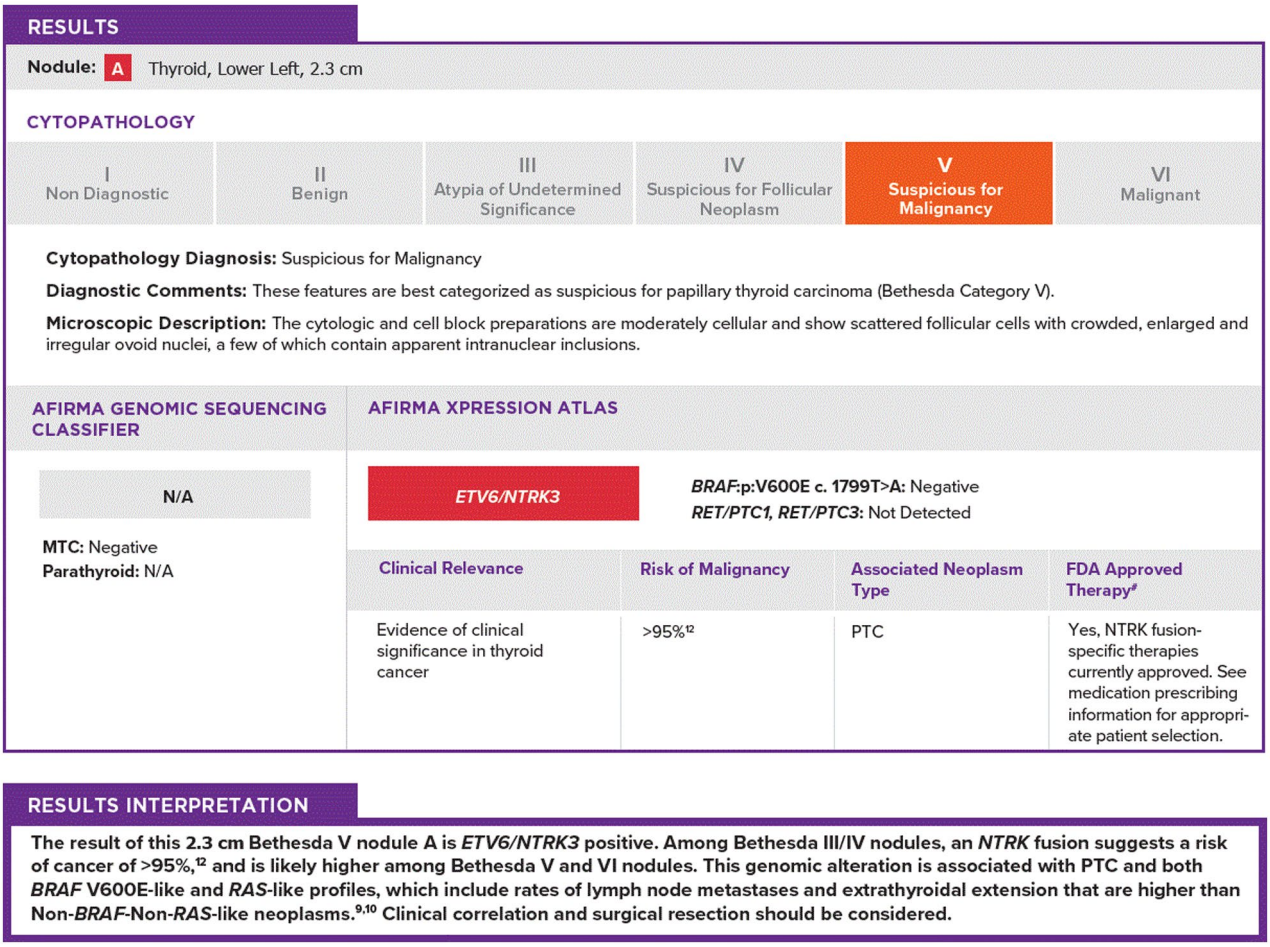

FIGURE 3. Example of an Afirma patient report of a hypothetical 2.3-cm thyroid nodule from the left lower lobe that was classified as Bethesda V ("suspicious for malignancy") via cytopathology. The Afirma Genomic Sequencing Classifier is a thyroid cancer rule-out test for Bethesda III/IV nodules, thus it is not applicable (N/A) in this setting. The Afirma Xpression Atlas (XA) detected an *ETV6/NTRK3* fusion, reported in red because of its high risk of malignancy. This molecular alteration is associated with papillary thyroid cancer (PTC), and alteration-specific US Food and Drug Administration (FDA) therapies exist for this alteration, which may be appropriate in the right clinical context. Additional information and context are provided in the "Results Interpretation" section. Image superscripts and their references are 9,[30] 10,[31] and 12.[18] #FDA-approved therapies for thyroid cancer, both specific for genomic alterations and nonspecific, may be found at https://www.cancer.gov/about-cancer/treatment/drugs/thyroid and https://www.cancer.gov/about-cancer/treatment/drugs/solid-tumors. See https://clinicaltrials.gov for potentially relevant clinical trials. Afirma XA is not a companion diagnostic and is not conclusive for any therapy.

chemotherapy. Nonetheless, the clinical impact of these drugs has been limited by modest tumor burden reductions and adverse effects that can impact quality of life. Recently, more alteration-specific targeted therapies have entered clinical trials or have received FDA approval. These agents have shown potentially greater tumor responses, fewer side effects, and efficacy across multiple cancers harboring the targeted genomic alteration. This has led to approved usage or clinical trials for refractory cancers harboring the appropriate genomic alteration, and in some cases regardless of the cancer type (ie, they are "tissue agnostic").[12,20-23]

The Afirma patient report now states if an FDA-approved alteration-specific targeted therapy exists for the identified oncogenic driver (Figs. 1-4). Internet addresses (associated with the superscripted number sign in the "FDA-Approved Therapy" column of the patient report [Figs. 3 and 4]) are provided for both alteration-specific and alteration-nonspecific, FDA-approved therapies and clinical trials (which change frequently).

Currently approved alteration-specific targeted therapies include 2 oral *NTRK* fusion inhibitors for solid tumors (entrectinib[20] and larotrectinib[12]) and for *BRAF*V600E-positive anaplastic thyroid cancer: the

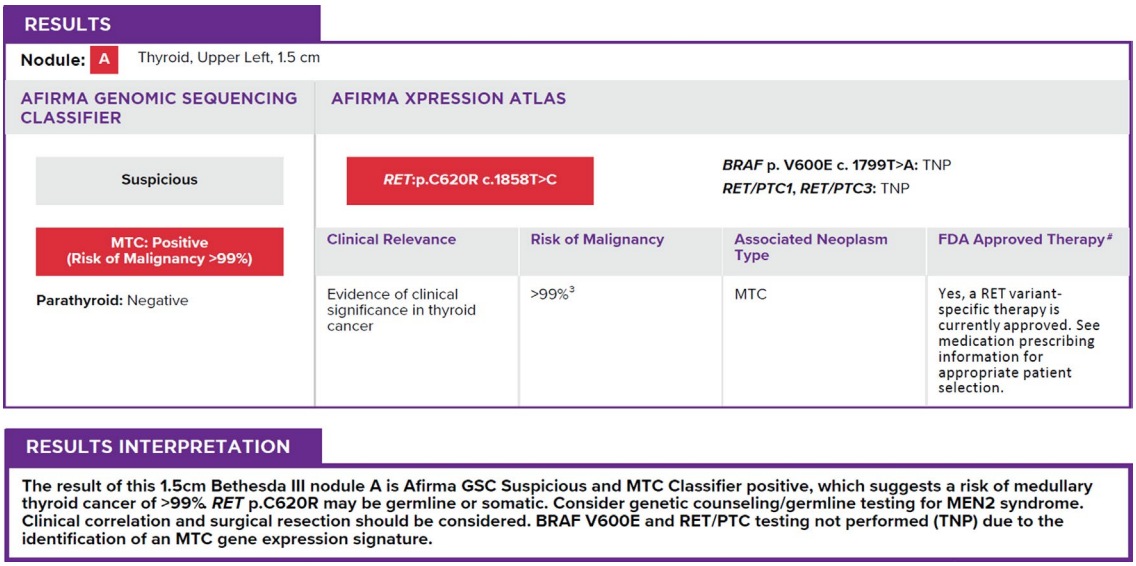

FIGURE 4. Example of an Afirma patient report of a hypothetical 1.5 cm thyroid nodule from the left upper lobe that was classified as Bethesda III by cytopathology. The Afirma Genomic Sequencing Classifier (GSC) result was "Suspicious," but the usual orange color (representing ~50% risk of malignancy) of this result is replaced with gray, foreshadowing that the risk of malignancy is altered by an additional finding. The "MTC Classifier" box reports a positive result and is colored red, indicating a high risk of malignancy. The Afirma Xpression Atlas reports the detection of a *RET*:p.C620R c.1858T>C variant. It is also reported in red, denoting the high risk of malignancy of this variant in the setting of an "MTC Positive" result and clinical relevance. These molecular findings are associated with medullary thyroid cancer. An alteration-specific FDA-approved therapy emerged with the FDA approval of selpercatinib on May 8, 2020. Additional information and context are provided in the "Results Interpretation" section, including the recommendation to consider genetic counseling/germline testing for multiple endocrine neoplasia type 2 (MEN2) syndrome. The superscript number 3 indicates a reference to Randolph et al.[15] #FDA-approved therapies for thyroid cancer, both specific for genomic alterations and nonspecific, may be found at https://www.cancer.gov/about-cancer/treatment/drugs/thyroid and https://www.cancer.gov/about-cancer/treatment/drugs/solid-tumors. See https://clinicaltrials.gov for potentially relevant clinical trials. Afirma XA is not a companion diagnostic and is not conclusive for any therapy.

BRAF inhibitor dabrafenib plus the MEK inhibitor trametinib.[21] FDA approvals of additional selective inhibitors are expected soon. Promising responses to selective *RET* inhibitors (selpercatinib [LOXO-292], pralsetinib [BLU-667]) have been reported among thyroid carcinomas harboring *RET* fusions and point mutations.[22-24] Selpercatinib received FDA approval on May 8, 2020, for patients ≥12 years of age with advanced or metastatic *RET* mutant medullary thyroid cancer (MTC) who require systemic therapy, or with advanced or metastatic *RET* fusion-positive thyroid cancer who require systemic therapy and who are radioactive iodine refractory (if radioactive iodine is appropriate).[25]

Calling out alterations for which alteration-specific targeted therapy exists may benefit patients and educate clinicians about this rapidly changing field, but there are several potential pitfalls—including a misunderstanding that when no alteration-specific FDA-approved therapy exists, no FDA-approved therapy exists. This is not true given the existence of FDA-approved therapies that are not alteration specific. Furthermore, when an FDA-approved therapy exists, it may be misconstrued to mean that this therapy should be administered regardless of other considerations. Systemic therapy is reserved for malignancies in patients that have failed, or are poor candidates, for standard treatments such as surgical resection, radioiodine, focal therapies (including radiation, thermal ablation), or clinical observation. This potential for misunderstanding is mitigated by instructing readers on the patient report to "see medication prescribing information for appropriate patient selection" (Figs. 3 and 4).

Clinical Relevance

Information regarding FDA-approved alteration-specific inhibitors and evidence-based PPVs are summarized in the new Afirma report in a section called "Clinical Relevance" (Figs. 3 and 4). The FDA fact sheet[26] lists 3 levels of clinical evidence for companion diagnostics. Although Afirma XA is not a companion diagnostic, similar language was adopted to conform with FDA recommendations. Identified alterations are considered to have "evidence of clinical significance"

when there exists an FDA-approved alteration-specific therapy or an established PPV >95% among Bethesda III/IV nodules. Alterations are considered to have "potential clinical significance" when their PPV is <95% as established from at least 10 representative cytologically indeterminate nodules. Finally, alterations associated with thyroid cancer from published literature, but without an established PPV, are reported as "unknown clinical significance."

Associated Neoplasm Type

The preoperative identification of the nodule's likely neoplasm type adds important clinical information for decision making. The Afirma XA report associates the neoplasm type expected in at least 80% of cases with the alteration identified (Figs. 1, 3, and 4).

Parathyroid classifier–positive samples predict parathyroid hyperplasia, adenoma, or parathyroid carcinoma[16] and should prompt an appropriate biochemical and clinical correlation that may be followed by parathyroid-specific treatment plans. The MTC classifier predicts MTC (Fig. 4),[15] which should be followed by specific clinical, biochemical, radiological, genetic, and treatment activities specific to MTC.[27] Among MTC classifier–positive samples, Afirma XA identifies a variant or fusion in approximately three quarters, most often *RET* or *RAS* variants.[28] Beyond MTC, *TSHR* variants may be associated with autonomously functioning neoplasms and should prompt correlation with thyroid function studies. The *PAX8/GLIS3* fusion is associated with hyalinizing trabecular tumor.[29] For other alterations, The Cancer Genome Atlas (TCGA)[30] and Yoo et al[31] published data sets that were used to associate the alteration with the neoplasm type.

Variant Class

Clinical decision making regarding the need and extent of surgery for nodular thyroid disease incorporates multiple pieces of data; these include clinical history, physical examination, and preoperative imaging. Molecular insights may add prognostic insights regarding the risk of lymph node metastases, N1b lymph node metastases, extrathyroidal extension, and gross-extrathyroidal extension.[30,31] While speculative, it is possible that the potential risk for these findings may be better informed by the results of molecular testing, even when these findings are not appreciated on preoperative imaging or surgical pathology.

TCGA investigated PTC (including the subtypes of classical, tall cell, and follicular variants) and developed a 71-gene signature to classify gene expression as either *BRAF*V600E-like or *RAS*-like.[30] Yoo et al[31] subsequently studied minimally invasive FTC, FA, classical PTC, and follicular variant PTC. Gene expression analysis demonstrated 3 molecular subtypes (regardless of histological features), including *BRAF*V600E-like, *RAS*-like, and non-*BRAF*–non-*RAS*. Importantly, these data can help to stratify the risk of lymph node metastases (and N1b lymph node metastases) and extrathyroidal extension (and gross extrathyroidal extension) in a highest to lowest order: *BRAF*V600E-like > *RAS*-like > NRNR.[30,31] Given the improved understanding of genotype–phenotype correlations in thyroid tumors, the Afirma XA patient report now incorporates this information. Each genomic alteration assigned a PPV is also assigned a signaling class based on the pooled TCGA and Yoo et al data sets (see "Results Interpretation" in Fig. 3).

Clinicians may find this additional "natural history" information helpful in their management deliberations and patient counseling. The intent, however, is not necessarily to drive more aggressive treatment in the *BRAF*V600E-like class, given its higher rate of lymph node metastases and extrathyroidal extension (as well as multifocality[30,31]). Instead, this information can empower treating physicians to perform a careful evaluation and have an informed conversation with the patient regarding appropriate risks and benefits. Despite the apparently increased risk of lymph node metastases and extrathyroidal extension among *BRAF*V600E-like neoplasms, patients without these features on preoperative imaging and intraoperative inspection may, in the right clinical context, be well-served by hemithyroidectomy. Ultimately, the role of known genetic alterations in contributing to initial clinical management should be determined in clinical trials.

Hereditary Syndromes

Hereditary syndromes associated with thyroid cancer are important to recognize for both the patient and at-risk affected family members. The identification of a potential germline genomic alteration via preoperative FNA testing is expected to heighten awareness and prompt appropriate further evaluation.

In the Afirma XA, variants associated with a thyroid cancer syndrome are identified and a recommendation to consider genetic counseling and confirmatory germline testing is included in the patient report (Fig. 4). These

genes include *RET* (multiple endocrine neoplasia type 2), *PTEN* (*PTEN* hamartoma tumor syndrome; Cowden syndrome), *APC* (*APC*-associated polyposis; familial adenomatous polyposis), and *DICER1* (*DICER1* syndrome). Similar comments are made for variants within Afirma XA genes that are associated with other syndromes on the American College of Medical Genetics and Genomics minimal reporting list.[32] These include *BRCA1*, *BRCA2*, *RB1*, *WT1*, *NF2*, *COL3A1*, *TGFBR2*, *TP53*, and *MSH2*. Afirma XA is not intended to serve as a germline test, and alterations identified may be somatic. Conversely, a negative result does not exclude a germline syndrome. Regardless of the Afirma XA result, patients suspected of harboring an inherited syndrome should be considered for genetic counseling and potential germline testing according to appropriate guidelines.

Results Interpretation

This section summarizes the insights gained for the patient sample in the context of their Bethesda category (Figs. 3 and 4) or metastatic presentation. For example, the risk of malignancy is not reported among specimens received from metastatic sites. Because no test is perfect, these results do not supersede clinical judgment. The molecular findings are expected to assist the overall patient management decision-making process.

IMPLICATIONS FOR CYTOPATHOLOGY

The increasing ability to leverage knowledge of molecular alterations in thyroid FNA specimens could further encourage the use of FNA, particularly for specimen acquisition from patients with locally advanced or metastatic disease. In such patients, the diagnosis may not be in question, but the opportunity to obtain clinically valuable molecular data in the least invasive manner possible is highly beneficial. Morphologic analysis in this setting can also serve to document disease progression to more aggressive tumors, such as poorly differentiated or anaplastic carcinomas. The opportunity to perform cytologic, histologic, and molecular correlation also has the potential to refine and further define cytologic criteria for malignancy on thyroid FNA specimens.

SUMMARY

Molecular testing of thyroid FNA specimens is widely practiced and adds considerable insight toward clinical decision making. It is exciting to witness genomic insights supplement the traditional factors used in patient management. Despite this excitement for "genomics," clinicians should not forget that the FDA has generally followed a hands-off policy of enforcement discretion among laboratory-developed tests.[33] Thus, clinicians have had thrust upon them heightened gatekeeper roles of evaluating test quality and safety. As patient advocates, they must demand validation data for all laboratory-developed tests and reject those that are unsubstantiated.

The Afirma GSC and XA have been validated both analytically and clinically.[1,3,34] The recent validation of the Afirma XA demonstrated test reliability and the identification of genomic alterations that may inform patient management. The updated Afirma GSC and XA reports aim to optimize the understanding of these contributions, including decisions about observation versus surgery, the need for disease-specific preoperative testing, associated neoplasm types, prognostics, identification of molecular targets for systemic therapy, and the recognition of potential hereditary syndromes. Patients and their doctors can now consider more personalized management options informed not only by the clinical, cytological, and radiological data, but also important insights from molecular analysis of the FNA material.

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CONFLICT OF INTEREST DISCLOSURES

Mimi I. Hu serves on the advisory board for Blueprint Medicines, Loxo Oncology, and Eli Lilly and is a consultant for Veracyte. Lori J. Wirth has received personal fees from Bayer, Blueprint Medicine, Cue Biopharma, Eisai, Exelexis, Genentech, Lilly, Loxo Oncology, Merck, and Rakuten Medical and has received nonfinancial support from Eisai, Lilly, Loxo Oncology, and Merck. Richard T. Kloos is an employee and equity owner of Veracyte. The other authors made no disclosures.

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