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Thyroid Nodules with Multiple Genetic Alterations on Fine Needle Aspiration Biopsy: a Case Series

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Abstract

Multiple genetic alterations in a thyroid nodule may predict the aggressive behavior of the tumor. A ThyroSeq molecular test was obtained from 557 nodules during thyroid biopsies. Eleven (2.0%) of 557 nodules had multiple genetic alterations. Eight patients had surgery showing 6 malignant and 2 benign nodules. Two of the 3 patients without surgery had metastatic disease (*BRAF V600E*, *TERT* plus *TP53,* and *NRAS* plus *TERT)* and were unstable for surgery. The 8 malignancies included 1 poorly differentiated cancer (PDTC)/anaplastic cancer harboring mutations in *BRAF V600E*, *TERT,* and *TP53* with metastatic disease causing death, 1 PDTC with a progressive disease with mutations *NRAS*, *TERT*, plus *EIF1AX,* and 3 classic PTC with mutations, *RET/PTC1* plus *TERT, BRAF V600E* plus *TERT and NRAS* plus *TERT* with a structural incomplete response, and 3 follicular variant PTC with mutations *KRAS* plus *TERT*, *NRAS* plus *TERT*, and *NRAS* plus *PTEN* with an acceptable response. Two histologically benign nodules contained *EIF1AX* plus *GNAS* and *NRAS* plus *EIF1AX* mutation. One additional patient without surgery had mutations, *NRAS* plus *TP53*. Thyroid nodules with multiple molecular alterations are uncommon and may have an aggressive malignant potential depending on the specific mutation.

Keywords: Thyroid cancer; Thyroid nodule; Molecular markers; Genetic alterations

Abbreviations: ATA: American Thyroid Association; ATC: Anaplastic Thyroid Cancer; AUS: Atypia of Undetermined Significance; FLUS: Follicular Lesion of Undetermined Significance; FN: Follicular Neoplasm; FNB; Fine Needle Biopsy; FTC: Follicular Thyroid Cancer; FV-PTC: Follicular Variant of Papillary Thyroid Cancer; PDTC: Poorly Differentiated Thyroid Cancer; PTC: Papillary Thyroid Cancer; RAI: Radioactive Iodine; SFN: Suspicious for a Follicular Neoplasm; SUSP: Suspicious for malignancy; Tg: Thyroglobulin; TgAb: Thyroglobulin antibody; TR: TI-RADS/Thyroid Imaging Reporting and Data System; VAF: Variant allelic frequency

Introduction

Thyroid nodules are common. A sizeable 15-year observation study revealed a prevalence of non-toxic thyroid nodules, approximately 6.5% in females and 1.5% in males in an iodine-sufficient area [1]. The prevalence of thyroid nodules is increased with age and more than 50% of the general population after age 65 years discovered thyroid nodules [2,3]. Once the nodule is detected, the main concern is that whether such nodule is benign or malignant. The management is straightforward for thyroid nodules, of which fine needle biopsy (FNB) reveals either benign (Bethesda II) or malignant (Bethesda VI) according to the Bethesda System for Reporting Thyroid Cytopathology [4]. Up to 25% of samples were reported as one of three indeterminate categories: atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS, Bethesda III), follicular neoplasm or suspicious for a follicular neoplasm (FN/SFN, Bethesda IV) and suspicious for malignancy (SUSP, Bethesda V) [5]. Rates of malignancy in these indeterminate categories are 6-18%, 10-40%, and 45-60%, respectively [4]. Due to the variable risk of malignancy, molecular markers analysis has been developed to facilitate a diagnosis of thyroid cancer, improve risk stratification, and tailor individual management.

Molecular markers in thyroid cancer have been considerably developed in the last several decades. ThyroSeq is a commercial assay that uses next-generation sequencing of RNA and DNA to detect genetic alterations, including gene mutations, translocations, and gene overexpression. An early ThyroSeq version using mutational analysis detected common gene mutations found in thyroid cancer, including *BRAF*, *RAS*, *RET/PTC,* and *PAX8-PPARG*. The recent version of ThyroSeq V.3 has expanded, covering more insertions/deletions and fusion of genes and abnormal expression levels of 112 genes associated with thyroid cancer with good positive predictive value (66-83%) and excellent negative predictive value (96-97%) [6,7]. Several genetic alterations have been well established correlating with thyroid cancer. *BRAFV600E* mutations and rearrangements of *RET* and *NTRK1* associate with papillary thyroid cancer (PTC), while *RAS* mutations were commonly found in follicular thyroid cancer (FTC) and follicular variant of PTC (FV-PTC). *PAX8/PPARG* fusions are also common in FTC. *TERT* promoter mutations are associated with both FTC and PTC, in which they are associated with aggressive tumors especially combined with a *BRAF V600E* mutation. Several genetic markers are associated with poorly differentiated thyroid cancer (PDTC) and anaplastic thyroid cancer (ATC), such as *TP53* and *TERT* mutations [8].

While the presence of a single molecular marker may be sufficient to stratify the risk of cancer and guidance for surgery presumptively, co-occurrence of multiple molecular markers is uncommon and may associate with aggressive cancer [9]. We now report a case series of thyroid nodules harboring more than 1 genetic alteration detected by the ThyroSeq next-generation sequencing molecular/genetic assay in 557 thyroid nodules. The ultrasound appearance, pathology, initial staging, risk stratification, and response to therapy are evaluated.

Materials and Methods

All patients, who underwent FNB of thyroid nodules from February 2015 to June 2018 at a single academic hospital endocrinology clinic, were reviewed. The thyroid nodules were diagnosed by ultrasound (US) using a high frequency 12 MHz linear probe and a Toshiba Xario 200 ultrasound system. During this period, molecular analysis was performed on all biopsies with indeterminate cytology. In addition, all specimens were sent to CBL Pathology (Rye Brook, NY) for a cytology second opinion. Our institutional practice was to send specimens with insufficient follicular content (Bethesda I) for molecular testing. An analysis of case records of thyroid nodules and molecular marker analysis with ThyroSeq V.2 or V.3® with more than 1 genetic alteration was performed. Cases were excluded if the genetic alteration was not associated with thyroid malignancy, including *TSHR* and *EZH1* mutations and *NIS* overexpression. The electronic medical records were retrospectively reviewed for the age of diagnosis, size of thyroid nodules, sonographic characteristics, Thyroid Imaging Reporting and Data System (TI-RADS) classification, cytopathology using the Bethesda System, molecular marker with allele frequency, pathology result, cancer staging, initial risk stratification, treatment modality, and dynamic risk stratification. The ages reported in clinical vignettes are the ages at diagnosis of thyroid nodules. We used the 8th (2017) tumor, node, metastasis classification system from the American Joint Committee on Cancer for thyroid cancer staging [10] and the 2015 ATA guidelines (5) for both initial and dynamic risk stratification and response to therapy. The US features for thyroid for malignancy were classified using TI-RADS (TR) level according to the 2017 American College of Radiology TIRADS committee [11]. In the absence of thyroglobulin antibody (TgAb), serum thyroglobulin (Tg) is measured by the Beckman Coulter immunometric assay with the lower limit of detection at 0.1 ng/mL. While TgAb is present, serum Tg is measured by a radioimmunoassay method (USC Endocrine Laboratory). All subjects gave their informed consent for inclusion before they participated in the study. The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee of Boston University School of Medicine (H-34976).

Results

Between February 2015 to June 2018, 1,596 sequential biopsies were performed on thyroid nodules >1 cm in greatest dimension according to the 2015 ATA Thyroid Nodule and Cancer Guideline (5) or per physician preference. 602 thyroid nodules (14.5% Bethesda I, 6.2% Bethesda II, 3.5% Bethesda VI, and 75.9% Bethesda III, IV, V) from 693 patients were evaluated by ThyroSeq due to indeterminate cytology (Bethesda III, IV, and V) or physician's preference. Several Bethesda II nodules had a molecular test performed because the second evaluation at CBL Pathology showed a higher Bethesda III classification. None of the Bethesda II nodules was reclassified as Bethesda IV, V, or VI from the second evaluation. 557 of 602 (92.5%) samples had adequate DNA/RNA for molecular analysis. 11/557 (2.0%) samples had multiple positive markers (Table 1). Eight of 11 had thyroid surgery with pathology showing 6 cancers and 2 benign thyroid nodules. Although there was no surgical pathology in 3 patients, one was diagnosed with a PDTC/ATC on cytology while the other 2 patients included one with a Bethesda V cytology with multiple suspicious lymph nodes and pulmonary nodules on CT scan but medically unstable for thyroid surgery, and 1 with a solitary nodule. The 8 malignancies included 1 PDTC/ATC, 1 PDTC, 3 classic PTC, and 3 FV-PTC. The details of each case are described below.

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Malignant Pathology Group

Table 1: The pathology, initial staging, risk stratification, and response to therapy of 11 patients with thyroid nodules with multiple genomic markers.

Case Number	Age, Gender	Bethesda class	Molecular Markers, VAF	Size (Long x AP x TR)	Ultrasound characteristics	TI-RADS	Pathology	AJCC 8th staging	ATA initial risk stratification	RAI Rx (mCi)	Length of follow up (months)	ATA response to therapy
1†	58M	I	•RET/PTC1, high level •TERT (c.1-124C>T), 21%	Thyroid bed mass, 0.7 x 1.6 cm (AP x TR)	Thyroid bed mass with several abnormal LN after surgery for PTC	N/A	Moderately differentiated PTC in lateral LN, ENE	IVB -lung, bone metastasis	High	144.5	60	Structural incomplete
2†	59M	VI-PTC	•NRAS (c.182A>G), 37% •TERT (c.1-124C>T), 35% •EIF1AX (c.371G>C), 78%	>6.5 x 4.0 x 5.5 cm	Isoechoic, well-defined margin	TR3	PDTC, >4 vascular inv.	II	High	110.2	31	Structural incomplete
3†	66F	V	•BRAF V600E (c.1799T>A),10% •TERT (c.1-124C>T),10%	1.7 x 1.2 x 2 cm	Hypoechoic, lobulated margin with several abnormal LN.	TR4	Classic PTC, microscopic ETE, PTC in 9 of 16 lateral LN.	II	Intermediate	103.2	19	Structural incomplete
4†	55F	V	•KRAS (c.182A>G), 38% •TERT (c.1-124C>T), 45%	1.9 x 1.7 x 1.4 cm	Isoechoic, well-defined margin, T>W	TR4	FV-PTC, ETE, capsular inv, >4 vascular inv.	I	Intermediate	No	30	Excellent
5†	81M	V	•NRAS (c.182A>G), 41% •TERT (c.1-146C>T), 40%	2.2 x 3.6 x 4.6 cm	Isoechoic, well-defined margin, T>W	TR4	FV-PTC (incidental 1.4 cm PTC, +BRAF V600E)	II	Intermediate	107.5	36	Excellent
6†	38F	VI-PTC	•NRAS (c.182A>G), 10.4% •PTEN (c.696delA), 10.2%	3.8 x 1.8 x 3.3 cm	Isoechoic, well-defined margin	TR3	FV-PTC, focal capsular inv.	I	Low	No	52	Excellent
7‡	76M	VI-PDTC/ Anaplastic	•BRAF V600E (c.1799T>A), 12.9% •TERT (c.1-124C>T),62.6% •TP53 (c.842A>T), 15.5%	4.8 x 3.1 x 2.5 cm	Hypoechoic, irregular margin, T>W, microcalcification	TR5	No surgery, passed away from invasive disease	Likely IVB with distant metastasis	N/A	No	3	Death from tumor
8§	66F	III-AUS	•NRAS (c.182A>G), 22% •EIF1AX (c.3382A>T), 23%	1.6 x 1.2 x 1.3 cm	Isoechoic, well-defined margin	TR3	Benign	N/A	N/A	No	25	No change in size, no adenopathy by US at 25 months
9§	40M	III-AUS	•EIF1AX (c.38G>C),55% •GNAS (c.681G>T),28%	2.7 x 2.3 x 2.4 cm	Hypoechoic, well-defined margin	TR4	Benign	N/A	N/A	No	23	No change in size, no adenopathy by US at 23 months
10‡	77F	V	•NRAS (c.182A>G), 21% •TERT (c.1-124C>T), 11%	Complex nodule, >3.7 cm in largest dimension	Hypoechoic, well-defined margin, T>W	TR5	No surgery medically unstable	N/A	N/A	No	41	CT neck/chest show stable thyroid mass and enlarged mediastinal nodes after 41 months
11¶	49F	III-AUS	•NRAS (c182A>G), 6% •TP53 (c.509C>T), 52%	1.6 x 0.8 x 1.4 cm	Hypoechoic, well-defined margin in adenomatous goiter	TR4	Surgery declined	N/A	N/A	No	19	No change in size, no adenopathy by US at 19 months

† Surgical pathology confirmed malignancy; ‡ Likely malignant by cytology and cross-sectional imaging; § Surgical pathology confirmed benign lesion; ¶ Likely benign by cytology and clinical course

M: male, F: female, VAF: variant allele Frequency, RAI: radioactive iodine, LN: lymph node, PTC: papillary thyroid cancer, AUS: atypia of undetermined significance, PDTC: poorly differentiated thyroid cancer, FV-PTC: follicular variant of PTC, ENE: extranodal extension, ETE: extrathyroidal extension, Inv: invasion, T>W: taller than wide, N/A: Non-applicable

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Case 1

A 58-year-old male presented with metastatic PTC. He had total thyroidectomy followed by 144.5 mCi radioactive iodine (RAI) ablation for a 6 cm PTC (stage III: pT4a, N1b, Mx and ATA high risk) with a biochemical incomplete response at 1 year after treatment (non-TSH stimulated Tg 4.8 ng/mL and negative TgAb with no evidence of radioiodine avid tissue). Two years after surgery, a 1.6 cm mass was found in the right thyroid bed (Figure 1A), which FNB was non-diagnostic and molecular analysis was positive for *RET/PTC1* (high level expression) and *TERT* mutation (c.1-124C>T). The *TERT* mutation had a variant allelic frequency (VAF) of 21%. In addition, multiple bilateral abnormal lymph nodes were found in the neck, and the FNB of the left level IV lymph node (Figure 1B) revealed metastatic PTC. The patient underwent lymph node dissection, which confirmed metastatic PTC with the maximum node size of 2.5 cm. Subsequently, after a 60-month follow-up, he has had an increasing number and size of lung nodules and lytic bone lesions consistent with progression of non-iodine avid distant metastatic disease. He is currently ATA high risk with a progressive structural incomplete response to therapy.

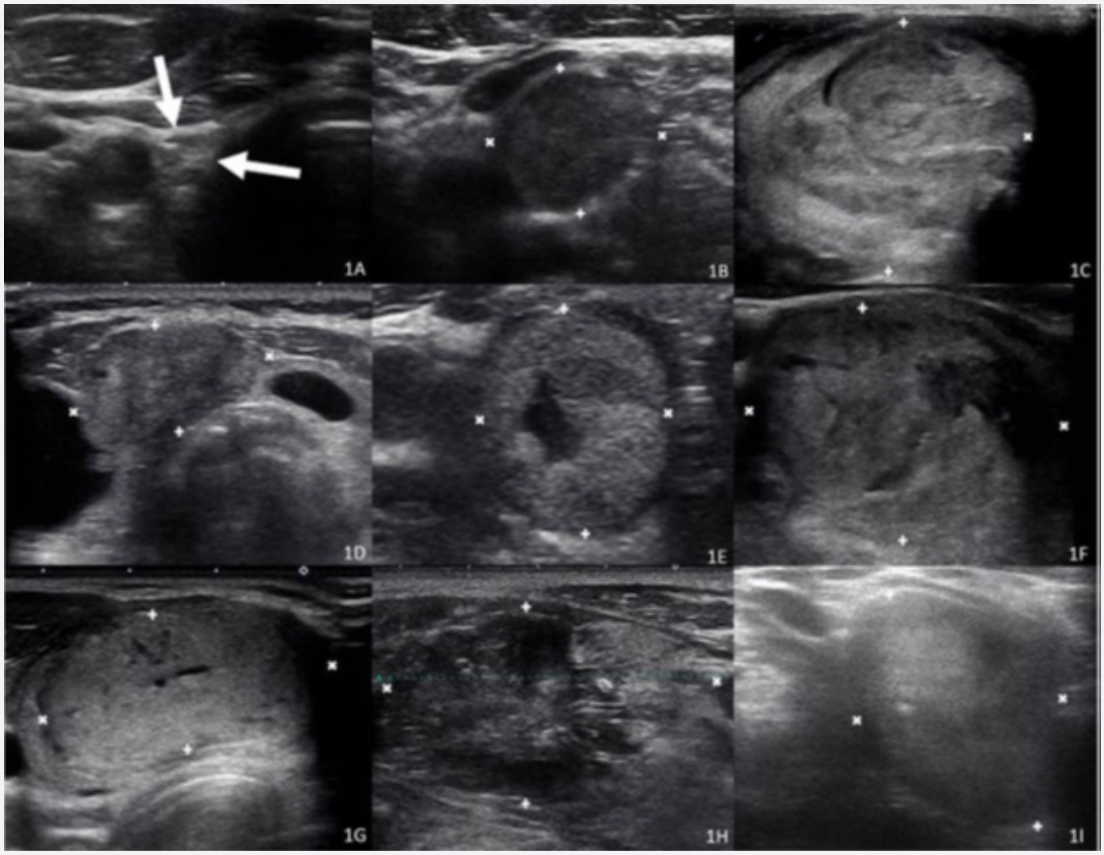

Figure 1: Thyroid ultrasound of cases with pathology confirmed malignancy or likely malignant by cytology and clinical course. Surgical pathology confirmed malignancy in case 1 (1A: right thyroid bed mass (arrows), 1B: left level IV 1.5 cm lymph node), case 2 (1C: 6.5 cm TR3 nodule), case 3 (1D: 1.7 cm TR4 nodule), case 4 (1E: 1.9 cm TR4 nodule), case 5 (1F: 4.6 cm TR4 nodule) and case 6 (1G: 3.8 cm TR3 nodule). Case 7 (1H: 4.8 cm TR5 nodule) had Bethesda VI from FNA. Case 10 (1I: >3.7 cm TR5 nodule) was likely malignant by clinical course and Bethesda V from FNA. All images are transverse views except for case 7 (1H), which is a longitudinal view.

Case 2

A 59-year-old male presented with a 6.5 cm isoechoic thyroid nodule (TR3) in the right thyroid (Figure 1C). There was evidence of mass effect but no invasion on the trachea, esophagus, right common carotid artery, and jugular vein. FNB showed Bethesda VI, positive for PTC. Three molecular markers were detected, *NRAS* mutation (c.182A>G; VAF 37%), *TERT* promoter mutation (c.1-124C>T; VAF 35%) and *EIF1AX* mutation (c.371G>C; VAF 78%). He underwent total thyroidectomy followed by 110.2 mCi RAI ablation with remnants observed on the post-therapy scan. Pathology revealed 8 cm PDTC with no extrathyroidal extension or node metastasis. The cancer was stage II: pT3a, Nx, Mx, ATA high initial risk with structurally incomplete response to therapy at 31 months with a non-stimulated Tg rising after surgery from 0.93 to 24.80 ng/mL with an undetectable TgAb and a non-localizing 18F-fluorodeoxyglucose-positron emission tomography scan (FDG-PET scan) but with small 3-4 mm pulmonary nodules on CT scan.

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Case 3

A 66-year-old female presented with incidental thyroid nodules from a CT scan. The US of the thyroid confirmed a 1.7 cm hypoechoic isthmus nodule with lobulated margin suggesting extrathyroidal invasion (TR 4) (Figure 1D). FNB revealed Bethesda V with *BRAF V600E* mutation (c.1799T>A, VAF 10%) and *TERT* mutation (c.1-124C>T, VAF 10%). There were abnormal lymph nodes in the right neck level III and VA. The largest lymph node was 2 cm in size. She underwent total thyroidectomy with right modified radical neck dissection. Pathology revealed a 1.7 cm classic PTC with extrathyroidal extension. Nine of 15 lymph nodes were positive for metastatic disease, with the most prominent metastatic nodes measuring 1.1 cm without extranodal extension. Her cancer was stage II: pT1b, N1b, M0, and ATA intermediate initial risk. She received 103.2 mCi RAI ablation with no metastatic disease on the post-therapy scan. US 6 months after RAI ablation demonstrated a new metastatic 1.1 cm lymph node in the right level IIA with a rise in her non-stimulated Tg to 1.7 ng/mL with an undetectable TgAb. At 19 months of follow-up, she has a slowly progressive locally structural disease.

Case 4

A 55-year-old female with Hashimoto's thyroiditis presented with a 1.9 cm isoechoic, taller than wide thyroid nodule (TR 4) (Figure 1E). FNB showed Bethesda V, suspicious for malignancy, with *KRAS* mutation (c.182A>G, VAF 38%) and *TERT* mutation (c.1-124C>T, VAF 45%). The patient had a total thyroidectomy, and pathology confirmed 1.8 cm FV-PTC with capsular invasion, microscopic extrathyroidal extension, and extensive angioinvasion. Her cancer was stage I: pT1b, Nx, Mx, ATA intermediate initial risk. She did not receive RAI ablation. After 30 months follow-up, she has an unremarkable neck US and a non-stimulated Tg < 0.1 ng/mL with negative TgAb, an excellent response to therapy.

Case 5

An 81-year-old male presented with a multinodular goiter. The largest nodule was 4.6 cm, isoechoic, and taller than wide (TR 4) in the right lobe (Figure 1F). FNB revealed Bethesda V with *NRAS* mutation (c182A>G; VAF 41%) and *TERT* mutation (c.1-146C>T; VAF 40%). He had a total thyroidectomy, and pathology showed 5.2 cm FV-PTC without capsular or vascular invasion. His initial staging was stage II: pT3a, N0, M0, and ATA low initial risk. However, 1.4 cm classic PTC was incidentally found in the left lobe nodule, which was not biopsied, and was positive for *BRAF V600E* mutation (c.1799T>A). He received 107.5 mCi RAI ablation with no metastatic disease on the post-therapy whole-body scan. He has an excellent response to therapy at 36 months of follow-up with a negative neck US and a non-stimulated Tg <0.1 ng/mL with negative TgAb.

Case 6

A 38-year-old female presented with a neck mass and compressive symptoms. US showed a 3.8 cm isoechoic nodule in the right lobe (TR 3) (Figure 1G). FNB revealed Bethesda VI consistent with PTC with *NRAS* mutation (c.182A>G; VAF 10.4%) and *PTEN* mutation (c.696delA; VAF 10.2%). She had a total thyroidectomy, and pathology confirmed 3.3 cm encapsulated FV-PTC with focal capsular invasion. There was neither angioinvasion nor extrathyroidal extension. Her initial staging was stage I: pT2, N0, Mx, and ATA low initial risk. She has an excellent response to treatment at 52 months after surgery without RAI with a negative neck US, a non-stimulated Tg 0.12 ng/mL, and an undetectable TgAb.

Case 7

A 76-year-old male presented with a rapidly enlarged neck mass in 3 months along with progressive dysphagia. CT scan revealed a 5.4 cm poorly defined heterogeneous mass in the right thyroid. The mass encased the right common carotid artery and right internal jugular vein and invaded the prevertebral space. US confirmed the hypoechoic 4.8 cm mass with infiltrative borders and microcalcifications (TR 5) (Figure 1H). There were several abnormal lymph nodes in the bilateral lateral neck. FNB of the mass showed Bethesda VI consistent with PDTC/ATC with triple molecular markers, including *BRAF V600E* mutation (c.1799T>A; VAF 12.9%), *TERT* mutation (c.1-124C>T; VAF 62.6%), and *TP53* mutation (c.842A>T; VAF 15.5%). CT scan of the head revealed a 1.2 cm temporal lobe lesion suggesting metastatic disease. The patient died due to respiratory failure from aerodigestive invasion by the tumor 3 months after diagnosis.

Benign Pathology Group

Case 8

A 66-year-old female with a 30-year history of recurrent Graves' disease and a prior history of RAI treatment had several sub centimeter thyroid nodules which had never been biopsied. Surveillance ultrasound showed a 1.6 cm isoechoic nodule in the right lobe (TR 3) (Figure 2A). This nodule was increased in size from 1.0 cm on the last US exam performed 5 years before. FNB revealed Bethesda III with *NRAS* mutation (c.182A>G, VAF 22%) and *EIF1AX* mutation (c.338-2A>T, VAF 23%). She had total thyroidectomy, and pathology showed no evidence of malignancy and adenomatous nodules.

Case 9

A 40-year-old male was referred for subclinical hyperthyroidism. US revealed a solitary 2.7 cm hypoechoic, taller-than-wide nodule with possible microcalcification and minimal cystic degeneration (TR 4) (Figure 2B). Because of the high-risk sonographic appearance with microcalcification, the nodule was immediately biopsied. FNB showed Bethesda III with positive *EIF1AX* mutation (c.38G>C, VAF 55%) and *GNAS* mutation (c.681G>T, VAF 28%). Additionally, the high expression of the *NIS* (*SLC5A5*) gene was reported. A subsequent thyroid scan identified this nodule as a hot nodule. He underwent a right thyroid lobectomy to treat his subclinical hyperthyroidism, and pathology revealed no evidence of malignancy.

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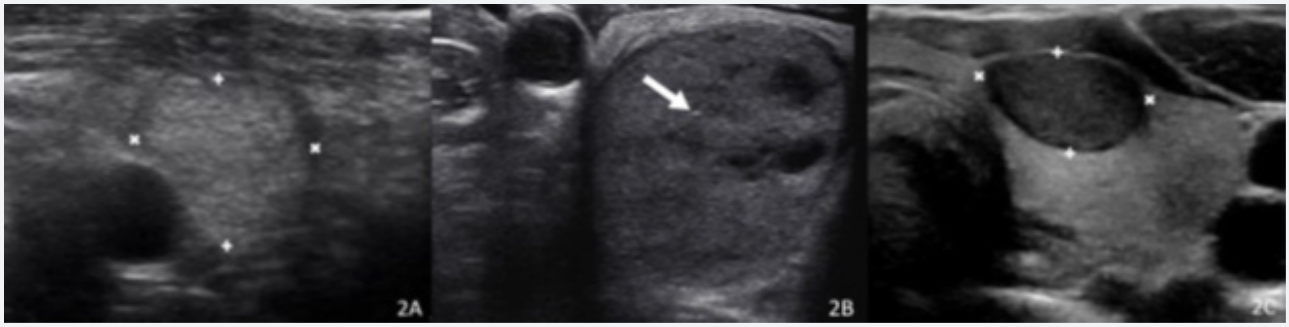

Figure 2: Thyroid ultrasound of cases with benign pathology or likely benign by cytology and clinical course. Surgical pathology confirmed benign in case 8 (2A: 1.6 cm TR3 nodule) and case 9 (2B: 2.7 cm TR4 nodule) with punctate, non-shadowing hyperechoic focus, possible microcalcification (arrow). Case 11 (2C: 1.6 cm TR4 nodule) was Bethesda III and was clinically stable after follow-up for 2 years. All images are transverse views.

No Surgical Pathology Group

Case 10

A 77-year-old female with a history of COPD was incidentally found an enlarged substernal goiter with a 3.1 cm nodule in the right lobe from a CT scan, causing a mild narrowing of a trachea. US confirmed a hypoechoic nodule, taller than wide (TR 5), although the entire nodule was not well visualized given substernal position (Figure 1I). FNB revealed Bethesda V, suspicious for PTC, and molecular markers were positive for *NRAS* mutation (c.182A>G; VAF 21%) and *TERT* mutation (c.1-124C>T; VAF 11%). Multiple enlarged mediastinal and hilar lymph nodes and small pulmonary nodules on the CT scan suggested metastatic disease. Her pulmonary status prevented thyroidectomy. The tumor and metastases are stable in size on CT scan after 41 months of follow-up.

Case 11

A 49-year-old female presented with a non-toxic multinodular goiter. The US of the thyroid revealed multiple nodules. The largest was a 1.6 cm very hypoechoic nodule (TR 4) in the left lobe (Figure 2C). The cytology showed Bethesda III, AUS with *NRAS* mutation (c.182A>G, VAF 6%) and *TP53* mutation (c.509C>T, VAF 52%). The patient declined surgery, and with US monitoring, the nodule was stable in size for 19 months. Unfortunately, the patient eventually was lost to follow up.

Discussion

Thyroid nodules with more than one genetic alteration were uncommon. They were found in 2.0% of 557 nodules derived from a cohort of 1,596 sequential thyroid nodule biopsy at a single academic endocrine clinic over a 28-month period. The previous studies reported approximately 4% prevalence of the simultaneous presence of 2-3 genetic alterations [9,12]. Nikiforova et al. reported that 9 of 228 samples (3.9%) with 2-3 positive markers were 6 ATC, 1 PDTC, and 2 PTC [9]. A similar result was earlier reported by Hou et al. as 13 cases among 303 patients (4.3%) were 8 ATC, 2 FTC, 1 PTC, and 1 benign [12]. Compared to the previous studies, the present cohort demonstrates a slightly lower prevalence of nodules with multiple markers possibly due to our genetically and racially diverse population and because our cohort includes a broader range of cytology classification: Bethesda I plus indeterminate cytology. The few analyses of Bethesda I and VI cytology were performed based on physician request. The Bethesda II cases were performed when a second review performed on every thyroid biopsy by CBL Pathology showed a higher Bethesda classification (only Bethesda III). At the time of this study, all consecutive indeterminate cytology results (Bethesda III, IV, and V) and not just the current recommendation of Bethesda III and IV were referred for molecular testing regardless of insurance or socioeconomic status of our racial and ethnically diverse patient population. The lack of selection bias is a strength of this study.

Several reports revealed that thyroid cancers carrying multiple oncogenic markers tend to have more aggressive behavior since they usually occur in ATC or PDTC [9,11-13]. ATC was the most common cancer type harboring multiple oncogenic markers with a prevalence of up to 17% (9, 12). However, a report by Liu et al. revealed the prevalence of the coexistence of genetic alterations as high as 72.5% among ATC and 64.1% among FTC [13]. There was no pure ATC in the present cohort. The biopsy of case 7 was Bethesda VI suspicious PDTC/ATC, died of rapidly progressive invasive and metastatic disease with triple mutations (*BRAF V600E* (VAF 12.9%), *TERT* (VAF 63.6%), and *TP53* (VAF 15.5%). The only other cancer with a triple mutation (*NRAS* (VAF 37%), *TERT* (VAF 35%), *EIF1AX* (VAF 78%) was a poorly differentiated tumor with an incomplete structural response and a rapidly rising Tg level. If co-occurrence of markers happens in well differentiated thyroid cancers, they typically carry poor prognostic features such as distant metastasis or local tumor recurrence, especially if associated with *BRAF V600E* and *TERT* [9,14].

In the present case series, 8 of 12 patients (case 1-7 and 10) clinically or pathologically had thyroid cancer, and 6 of them had

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aggressive features including local or distant metastases, death from thyroid cancer, poorly differentiated tumor, or extensive vascular invasion. Of note, the patients with the aggressive or structurally persistent disease were PDTC/ATC or classical PTC. In contrast, the patients with FV-PTC with *RAS* mutations had an excellent response to therapy despite the multiple positive markers. On the other hand, 3 of 11 patients (cases 8, 9, and 11) behaved as benign nodules despite multiple genetic alterations with 2 nodules confirmed pathologically benign. In summary, nodules with multiple genetic markers likely have more aggressive features, but they are not always cancer or carry a poor prognosis. Further, this study demonstrates that genomic and gene expression alternations have different risks of malignancy corresponding with the published data, which showed *BRAF V600E,* especially in combination with *TERT* has a high risk for an aggressive malignancy [14]. *NRAS* is associated with lower risk PTC (28.6%), noninvasive follicular thyroid neoplasm with papillary-like nuclear features (NIFTP) (12%), and benign nodules (58.9%) [15] while *TSHR* with a low VAF (3%- 22%) and *EZH1* mutations *and NIS* overexpression have an extremely low risk of malignancy [16,17].

TERT promoter mutations were the most common mutations found among 8 patients who were clinically or pathologically had a thyroid malignancy. Furthermore, they were detected in all 6 cases with aggressive disease. Although *TERT* promoter mutations were reported as an independent risk factor for poor prognostic features, including distant metastasis, higher stage, persistent disease, and mortality in well-differentiated thyroid cancer [18], it was clinically relevant in the setting of co-occurrence with other mutations such as *BRAF V600E* and *RAS* [14,19]. Therefore, the effect of isolated *TERT* mutations remains unclear regarding risk stratification. The present case series reproduces the idea of multiple oncogenic markers, including *TERT* mutations yielding aggressive cancer with poor prognosis and response to therapy. We found *TERT* mutations, without a specific combination pattern, in all 6 cases with aggressive disease (case 1-4, 7 and 10) combining with *KRAS* mutation, *NRAS* mutation, *RET/PC1* fusion. Two cases (case 2 and 7) harbored triple mutations, *TERT* plus *NRAS* plus *EIF1AX,* and *TERT* plus *BRAF V600E* plus *TP53,* respectively. Both had overly aggressive and progressive disease with the death of one patient due to distant metastasis and iodine avid progressive residual disease in the other patient.

Among 3 patients with clinically or pathologically benign disease (case 8, 9, and 11), markers found in this group included mutations with lower or no risk of malignancy of *NRAS*, *EIF1AX*, *GNAS*, and *TP53*. Our prior study demonstrated that *RAS* mutation, when occurring alone, was associated with malignancy in only 28.6% [15]. This study suggests that thyroid cancer with a single *RAS* mutation is associated with a non-aggressive form of FV-PTC or classic PTC with an absence of recurrence up to 26 months follow-up. The current case study expands this observation and suggests multiple genetic alterations in addition to *RAS* are not always associated with aggressive disease (case 2, 4, 5, 6, 8, 10, and 11). Two of the cases clinically or pathologically benign (case 8 and 11). Four of the 5 patients with malignancy demonstrated clinically or pathologically had the nonprogressive disease (case 10) or FV-PTC (case 4, 5, and 6) with an excellent response to therapy. Case 10 had a local and distant metastatic disease but has been untreated because of other medical problems without progression for 41 months after diagnosis. Case 4 and 5 with *RAS* plus *TERT* mutation and case 6 with *RAS* plus *PTEN* had an excellent response to therapy after 30, 36, and 52 months, respectively, of follow-up. The only patient with a *RAS* mutation with a structurally incomplete disease with a high risk of recurrence had a triple mutation (case 2), *NRAS* (VAF 37%), *TERT* (VAF 35%), and *EIF1AX* (VAF 78%). Additional investigations are needed to determine which thyroid cancers with *RAS* mutations are associated with local and distant disease and poor response to therapy.

TP53 mutations are associated with dedifferentiating thyroid cancer [20], especially in combination with *BRAF* or *NRAS* mutation [9], such as case 7 with *BRAF V600E*, *TERT,* and *TP53* who died within 3 months from aggressive local tumor growth and distant metastasis. In contrast, case 11 was positive for both *TP53* and *NRAS* with a sonographically low-risk nodule in a multinodular goiter. The nodule was stable in size without local adenopathy by the US after 19 months of follow-up. Case 8 carried *NRAS* and *EIF1AX* mutations. Although the pathology revealed a benign tumor, Karunamurthy et al. [21] reported that *EIF1AX* mutations had a 20% risk of cancer and even higher if it co-occurred with *RAS* mutations as 3 out of 4 cancers in their cohort harboring coexistence of *EIF1AX* and *NRAS* mutations [21].

One of the 3 benign nodules (case 9) was an autonomous nodule that was biopsied despite mild hyperthyroidism because a single microcalcification was suspected on US. The ATA or ACR malignancy risk assessment is not validated for hot nodules, and in retrospect, a proper clinical diagnosis with a nuclear thyroid scan would have identified the very low risk of malignancy and prevented the unnecessary biopsy and molecular test. Case 9 carried *EIF1AX* and *GNAS* mutations, both of which are low-risk markers. Additionally, a high expression of *NIS* (*SLC5A5*) was detected. Unlike *EIF1AX*, both *GNAS* mutation and high *NIS* expression occur in benign hyperfunctioning nodules [22,23], while their roles in cancer stratification are still unclear.

The molecular test was performed regardless of insurance status when it was acceptable to perform molecular testing on all indeterminate cytology classes, Bethesda III, IV, and V. A number of molecular tests were performed on select malignant Bethesda VI nodules when requested by a clinician. One of the essential elements of this study is that it will not be repeated in a clinical use study since every malignant nodule associated with multiple genetic alterations occurred in Bethesda I, V, and VI cytology. Since the current insurance payment for the molecular tests is approved for only Bethesda III and IV, all these patients with aggressive behavior would be denied payment, and testing would not have been performed. The importance of the pre-operative knowledge

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of multiple mutations allowed case 2 with a well-defined isoechoic nodule without extrathyroidal extension or abnormal nodes to have the correct initial surgery of total thyroidectomy and central neck dissection for a PDTC rather than a simple thyroidectomy. All three of the molecular tests with multiple genetic alterations and a Bethesda III, IV cytology that would currently be covered by insurance were benign on pathology or by clinical behavior after 19, 23, and 25 months of follow-up. This study utilized the next-generation sequencing of ThyroSeq V.2® and ThyroSeq V.3® that examines up to 112 genes panel. However, other molecular tests (Afirma GSC/Xpression Atlas® and ThyGenX/ThyraMir®) examine a smaller number of genetic alterations for thyroid nodules. These tests cannot be assessed in the context of this study.

Conclusion

Thyroid nodules with multiple markers which detect genomic alterations are uncommon, but they are likely to be thyroid cancer depending on the specific alternation seen. Specific molecular markers are associated with aggressive behavior include mutations of *BRAF V600E*, *NRAS,* or *RET* rearrangement in combination with *TERT* promoter mutations, while other combinations such as *GNAS* are found in tumors with low malignant potential. Recognizing the combination of high-risk markers, especially when more than 2 are detected, would be helpful in cancer risk stratification and decision for surgery. Interestingly 4 of the 6 nodules with multiple markers with surgical confirmation of malignancy were isoechoic and otherwise low risk on ultrasound. Without the multiplicity of molecular markers, they would have been handled as a low- risk tumor with lobectomy or observation. Multiple high-risk genomic and expression alterations detected preoperatively predict aggressive behavior. In this cohort, the three patients with thyroid cancer and structural incomplete response to therapy contained a *TERT* mutation with *RET/PTC1*, *BRAF V600E,* or *NRAS* plus *EIF1AX* mutations. At the same time, the only death occurred in a patient with a triple mutation of *BRAF V600E*, *TERT,* and *TP53*. This cohort study supports the consideration for future studies to determine if preoperative molecular markers assays should be performed on all cytology classes at risk for cancer (Bethesda III, IV, V, and VI) to detect high-risk alterations in genomic expression that would permit an initial more aggressive surgery and treatment necessary for tumors with a higher risk of local and distant metastases and poor response to therapies.

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