Do Molecular Mutations and Alterations Explain the Differences in Clinical Behaviour in Indeterminate Bethesda III and IV Thyroid Nodules?

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1 Background

Thyroid Cancer

- Thyroid cancer is an ever-present disease whose prevalence is **highest** among all other **endocrine** malignancies.
- Approximately **5%** of the population has a **thyroid nodule** that can be palpated, yet the likelihood of the nodule being **malignant** ranges from **10-15%** [1].

Management

- Prior to surgical intervention, clinicians utilize **ultrasound** to image the thyroid nodule followed by a fine needle aspiration (**FNA**) [3].
- Cytopathological results of the FNA are scored in accordance with the **Bethesda system** outline by the American Thyroid Association 2015 guidelines which ranges from a score of **I** (non-diagnostic) to **VI** (malignant) [4].

Molecular Testing

- The majority of thyroid cancer driver alterations lead to dysregulated mitogen-activated protein kinase (**MAPK**) and phosphatidylinositol-3 kinase (PI3K)-AKT pathways [6]. As a result, **genetic analysis** of thyroid cancers has become increasingly salient.
- **ThyroSeq v3** is a molecular test that identifies a broad spectrum of molecular alterations such as gene fusions, copy number alterations (CNAs), gene expression profiles (GEPs), and point mutations [7].
- Thyroid nodules with **RAS-like** mutations in the MAPK pathway tend to be **less proliferative** as they respond to negative feedback inhibition. **BRAF-like** mutations, however, tend to have much **higher** levels of cellular **proliferation** that lead to **aggressive** cancer since they do not respond to negative feedback [6].
- The literature has begun to associate **Bethesda score** to respective **molecular alterations**.
- Although **Bethesda III** nodules are more likely to be **benign** than **Bethesda IV**, when **malignant,** they are more likely to have **aggressive** histopathological features [10].

2 Objective

This study aims to assess whether thyroid nodules classified as **Bethesda III** exhibit different **molecular alterations**, **frequencies**, and **aggressiveness** levels compared to **Bethesda IV** nodules.

3 Methods

Study Design

- A multicenter retrospective chart review of over 1000 patient files was conducted at two McGill University teaching hospitals from January 2016 – April 2022 in Montreal, Quebec, Canada.

Inclusion Criteria

- Above 18 years of age.
- Had thyroid nodules categorized as atypia of undetermined significance (AUS/FLUS; Bethesda III) or follicular neoplasm/suspicious of follicular neoplasm (FN/SFN; Bethesda IV) on FNA.
- Had ThyroSeq v3 molecular testing performed on the targeted nodule.
- Had diagnostic surgery.

Data Collected

- Patient characteristics (sex and age)
- Tumor characteristics findings on ultrasound, FNA results, ThyroSeq v3 results, and histopathological findings after diagnostic surgery)
- surgical management decisions.
- Aggressive malignancies: lymph node metastasis, poorly differentiated carcinoma, aggressive variants of papillary thyroid cancer (hobnail, tall cell, solid/trabecular and columnar) and extrathyroidal extensions (ETE).

Statistical Plan

- Descriptive analysis was performed.
- Associations between Bethesda category, ThyroSeq v3 molecular testing results, age, sex, and histopathology were examined.
- Statistical significance between groups was defined as p-value < 0.05
- Statistical analyses of associations between the categorical variables were performed by the chi-square test or two-sided Fisher's exact test. All analyses were performed with STATA-13 (STATA Corporation, College Station, TX, USA).

4 Results

Overview n=203 patients

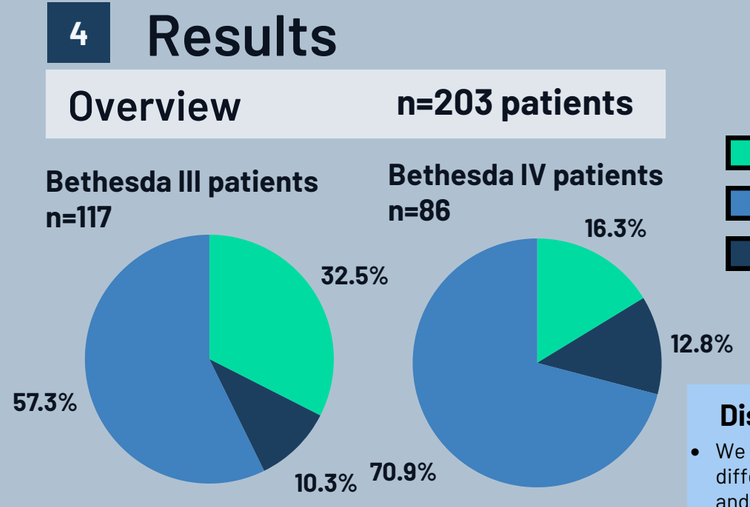

Table 1. Baseline Characteristics of Total Population

Variant	Bethesda III (%) (n = 117)	Bethesda IV (%) (n = 86)
Age (years)		
mean (min-max, ±SD)	**54.4 (22-88, ±12.3)**	**51.9 (16-78, ±15.0)**
Sex		
Female	90 (76.9)	73 (84.9)
Male	27 (23.1)	13 (15.1)
Nodule size (cm)		
mean (min-max, ±SD)	2.17 (0.1-7.5, ±1.4)	2.27 (0.1-6.7, ±1.1)
Pathology		
Benign	38 (32.5)	14 (16.3)
Malignant/NIFTP*	79 (67.5)	72 (83.7)
Aggressive features		
Present	12 (15.2)	11 (15.3)
Not present/NIFTP	67(84.8)	61 (84.7)

Discussion

- We observed a statistically significant difference in the presence of **CNAs** and **GEP** in mainly **malignant Bethesda IV** thyroid nodules (**Table 3**).
- Despite not meeting the threshold for statistical power defined by this study, we can appreciate the observable trend that only **malignant Bethesda III** nodules demonstrated **BRAFV600E** (four (5.1%)) mutations on molecular testing, three of which led to **aggressive cancer** (**Table 4**).
- We noted that **BRAFV600E** (25.0%), **TP53** (25.0%) and **ALK fusion**(8.3%) tend to associate with **aggressive Bethesda III** thyroid nodules (Table 4)
- In analyzing the aggressive and non-aggressive/NIFTP **Bethesda IV** thyroid nodules, we can appreciate the **absence** of both **KRAS** and **BRAFV600E** mutations (Table 5).
- We noted that all three **THADA fusions** (4.9%) were in **Bethesda IV** nodules **lacking aggressive** features.
- It was reported that of the nine malignant **Bethesda IV** nodules with **EIF1AX** mutations, five(55.6%) did not lead to aggressive thyroid cancer.

Table 2. Pathological and clinical characteristics of patients deemed malignant on final pathology.

Variant	Malignant/NIFTP Bethesda III (%) (n = 79)	Malignant/NIFTP Bethesda IV (%) (n = 72)
Sex		
Female	60 (76.0)	61 (84.7)
Male	19 (24.1)	11 (15.3)
Surgical intervention		
Hemithyroidectomy	65 (82.3)	65 (90.3)
Total Thyroidectomy	14 (17.7)	7 (9.7)
Type of Malignancy/NIFTP		
NIFTP	11 (13.9)	4 (5.6)
Papillary carcinoma	55 (69.6)	54 (75.0)
Follicular carcinoma	1 (1.3)	6 (8.3)
Hürthle cell carcinoma	5 (6.3)	3 (4.2)
Poorly differentiated thyroid cancer	1 (1.3)	6 (8.3)
Well differentiated neoplasm of undetermined malignant potential	4 (5.1)	3 (4.2)

Table 3. Association between molecular mutation and malignant/NIFTP Bethesda III/IV thyroid nodules.

Mutation	Malignant/NIFTP Bethesda III (%) (n = 79)	Malignant/NIFTP Bethesda IV (%) (n = 72)
HRAS	8 (10.1)	13 (18.1)
KRAS	2 (2.5)	0 (0)
NRAS	20 (25.3)	12 (16.7)
EIF1AX	13 (16.5)	9 (12.5)
PTEN	3 (3.8)	3 (4.2)
DICER1	3 (3.8)	8 (11.1)
CNA	11 (13.9)	23 (31.9)
GEP	11 (13.9)	24 (33.3)
TP53	3 (3.8)	3 (4.2)
BRAFV600E	4 (5.1)	0 (0)
***THADA* fusion**	6 (7.6)	3 (4.2)
***ALK* fusion**	1 (1.3)	1 (1.4)

Table 4. Association between molecular mutation and aggressive features in Bethesda III thyroid nodules.

Mutation	Non-Aggressive/NIFTP Bethesda III (%) (n = 67)	Aggressive Bethesda III (%) (n = 12)
HRAS	7 (10.4)	1 (8.3)
KRAS	2 (3.0)	0 (0)
NRAS	19 (28.4)	1(8.3)
EIF1AX	11 (16.4)	2 (16.7)
PTEN	3 (4.5)	0 (0)
DICER1	3 (4.5)	0 (0)
CNA	9 (13.4)	2 (16.7)
GEP	10 (14.9)	1 (8.3)
TP53	0 (0)	3 (25.0)
BRAFV600E	1 (1.5)	3 (25.0)
***THADA* fusion**	6 (9.0)	0 (0)
***ALK* fusion**	0 (0)	1 (8.3)

Table 5. Association between molecular mutation and aggressive features in Bethesda IV thyroid nodules.

Mutation	Non-Aggressive/NIFTP Bethesda IV (%) (n = 61)	Aggressive Bethesda IV (%) (n = 11)
HRAS	11 (18.0)	2 (18.2)
KRAS	0 (0)	0 (0)
NRAS	11 (18.0)	1 (9.1)
EIF1AX	4 (6.6)	5 (45.5)
PTEN	2 (3.3)	1 (9.1)
DICER1	7 (11.5)	1 (9.1)
CNA	20 (32.8)	3 (27.3)
GEP	21 (34.4)	3 (27.3)
TP53	0 (0)	3 (27.3)
BRAFV600E	0 (0)	0 (0)
***THADA* fusion**	3 (4.9)	0 (0)
***ALK* fusion**	0 (0)	1 (9.1)

5 Conclusion

- Marked increase in copy number alterations and gene expression profiles in Bethesda IV nodules when compared to Bethesda III nodules.
- **All of the thyroid nodules in this study bearing a BRAF V600E mutation were from Bethesda III nodules.**
- EIF1AX mutations in malignant Bethesda IV thyroid nodules tended to be associated with more indolent thyroid cancers.
- As a result, when comparing malignant Bethesda III and IV tumors, Bethesda III cancers were more likely to have a BRAF V600E mutation whereas Bethesda IV were more likely to have more indolent RAS-like mutations (including EIF1AX) and molecular alterations (CNA and GEP).
- This may explain the phenomenon of more invasive malignancies in Bethesda III nodules from a molecular mutation/alteration perspective.
- **While Bethesda III nodules are more likely to be benign, when malignant, they tend to exhibit molecular mutations that lead to aggressive thyroid cancer. Bethesda IV nodules, however, are more likely to have molecular alterations that cause non-aggressive thyroid cancer.**

Study limitations

- The **retrospective nature** of the chart review is inherently limited when compared to a prospective study.
- The files admitted into the study were subject to **geographic selection bias** as they all originated from **McGill University** tertiary care centers in the metropolitan area of Montreal.
- Patients had to **pay** out-of-pocket for **molecular testing**, thereby imposing a subsequent selection bias in the study. Therefore, there may be an overrepresentation or underrepresentation of **aggressive** or **non-aggressive** molecular mutations or **malignancies** in this study due to the **costs** of molecular testing.

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