## Afirma Genomic Sequencing Classifier (GSC) and Xpression Atlas Molecular Findings in Bethesda III Thyroid Nodules:

# Comparative Analysis of Nuclear Versus Other Atypia and Correlation with Histology

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## BACKGROUND

- The 2023 Bethesda System for Reporting Thyroid Cytopathology (TBSRTC) divides atypia of undetermined significance (AUS) nodules into two major subcategories: nuclear atypia (AUS-N) and other (AUS-O).
- While studies have reported the malignant risk of AUS thyroid nodules differs according to the nature of atypia, little is known about the difference of molecular profiles between AUS-N and AUS-O thyroid nodules.
- This study aims to investigate the molecular findings between AUS-N and AUS-O thyroid nodules across a large, real-world cohort of fine needle aspiration (FNA) samples and to correlate these findings with histological outcomes.

## DESIGN

- A retrospective study of consecutive thyroid FNA samples diagnosed as AUS and sent for Afirma Genomic Sequencing Classifier (GSC) testing.
- Afirma GSC classifier results, Xpression Atlas (expressed variants and fusions) results in GSC-suspicious samples, and tumor specific molecular signatures were analyzed and compared between AUS-N and AUS-O samples.
- A single center, retrospective analysis of histopathology specimens with AUS-N or AUS-O cytology were analyzed as well.

## RESULTS

- Between April 2024 – December 2024, there were 5,345 paired cytology and Afirma GSC samples sent through Thyroid Cytopathology Partners (TCP), read as AUS, and stratified as AUS-N or AUS-O (Table 1).
  - AUS-N = 920
  - AUS-O = 4,425
- The Afirma GSC ensemble classifier reported GSC-(S)uspicious results in 58.2% of AUS-N, compared to 28.1% of AUS-O (p<0.01) (Table 2).
- Overall, there were more molecular variants in the AUS-N Afirma GSC-S nodules than in AUS –O (34.6% vs. 27.8%), mainly driven by the proportion of *BRAF*V600E. There was no difference in the overall proportion of fusions between the two groups though higher risk fusions (*RET, NTRK3, ALK*) were more common in the AUS-N group (Table 3).
- AUS-N were significantly more *BRAF*-like on *BRAF-RAS* score (BRS) and had higher ERK and follicular-mesenchymal transition (FMT) signature expression than AUS-O nodules (Figure 1).
- There were 201 surgically resected AUS, Afirma GSC-S nodules analyzed from the University of Michigan (Table 4).
  - There were significantly more malignancies in the AUS-N (28/82=34.1%) vs AUS-O (18/119=15.1%).
  - There were significantly more Papillary Thyroid Carcinomas (PTC) in the AUS-N (25/27=92.6%) compared to AUS-O (12/18=66.7%).

**TABLE 1.**
### Demographics of the AUS cohort

| Demographics | | AUS-Nuclear (N=920) | AUS-Other (N=4,425) | P-value |
|---|---|---|---|---|
| **Age** | Mean [Q1, Q3] | 57.02 [45.27, 69.27] | 59.75 [49.32, 71.35] | <0.01 |
| **Nodule Size** | Mean [Q1, Q3] | 2.55 [1.55, 3.20] | 2.67 [1.70, 3.30] | <0.01 |
| **Sex** | Female | 717 (77.9%) | 3,429 (77.5%) | 0.803 |
| | Male | 203 (22.1%) | 996 (22.5%) | |
| **Nodule Location** | Isthmus | 53 (5.8%) | 222 (5.0%) | 0.297 |
| | Left | 413 (44.9%) | 1,901 (43.0%) | |
| | Right | 454 (49.3%) | 2,296 (51.9%) | |
| | Missing | 0 (0.0%) | 6 (0.1%) | |

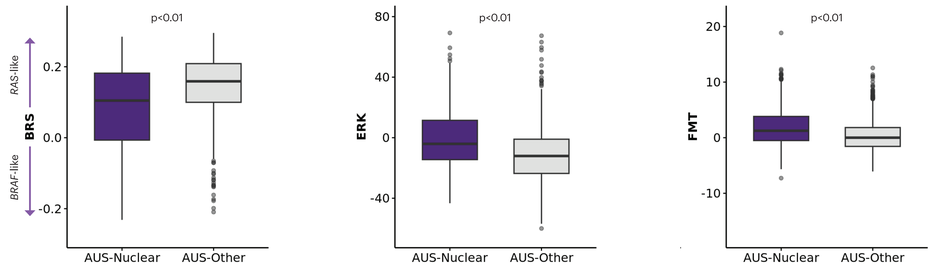
**TABLE 2.**
### Afirma GSC ensemble classifier results by AUS category

| Atypia | Total | GSC-B | GSC-S |
|---|---|---|---|
| **Nuclear** | 920 | 385 (41.8%) | **535 (58.2%)** |
| **Other** | 4,425 | 3,183 (71.9%) | **1,242 (28.1%)** |

**TABLE 3.**
### Expressed variants and fusions in Afirma GSC-S nodules by AUS categories

| Total | Nuclear 535 | Other 1,242 | P-value |
|---|---|---|---|
| **FUSIONS** | **32 (5.9%)** | **55 (4.6%)** | **0.26** |
| *PAX8::PAARG* | 9 (1.7%) | 38 (3.1%) | 0.11 |
| ***RET*** | **5 (0.9%)** | **2 (0.2%)** | **0.029** |
| ***NTRK3*** | **7 (1.3%)** | **3 (0.2%)** | **0.011** |
| ***ALK*** | **4 (0.8%)** | **1 (<0.1%)** | **0.031** |
| *BRAF* | 2 (0.4%) | 2 (0.2%) | 0.59 |
| **VARIANTS** | **185 (34.6%)** | **345 (27.8%)** | **<0.01** |
| ***BRAFV600E*** | **65 (12.1%)** | **15 (1.2%)** | **<0.01** |
| *BRAFK601* | 3 (0.6%) | 19 (1.5%) | 0.10 |
| *NRAS* | 51 (9.5%) | 134 (10.8%) | 0.45 |
| *HRAS* | 36 (6.7%) | 98 (7.9%) | 0.43 |
| *KRAS* | 7 (1.3%) | 17 (1.4%) | 1.0 |
| *DICER* | 3 (0.6%) | 13 (1.1%) | 0.42 |
| *TSHR* | 11 (2.1%) | 15 (1.2%) | 0.20 |
| *EIF1AX* | 1 (0.2%) | 2 (0.2%) | 1.0 |
| *OBSCN* | 0 (0.0%) | 6 (0.5%) | 0.19 |

**FIGURE 1.**
### Prognostic molecular signatures by AUS category




BRS = *BRAF-RAS* score
FMT = follicular-mesenchymal transition

**TABLE 4.**
### Histopathology Outcomes

**A. Histopathology outcomes of AUS, GSC-S nodules**

| | AUS-N | AUS-O | P-value |
|---|---|---|---|
| **Total** | 82 | 119 | |
| **Malignant Histopathology** | 28 (34%) | 18 (15%) | |
| PTC | 18 (22%) | 10 (8.4%) | |
| PTC (follicular variant) | 7 (8.5%) | 2 (1.7%) | |
| Follicular carcinoma | 1 (1.2%) | 4 (3.4%) | |
| Oncocytic carcinoma | 0 | 2 (1.7%) | |
| Medullary carcinoma | 1 (1.2%) | 0 | |
| Lymphoma | 1 (1.2%) | 0 | |
| **Benign Histopathology** | 54 (66%) | 101 (85%) | |
| Follicular nodular disease | 30 (36.6%) | 61 (51.2%) | |
| Follicular adenoma | 16 (19.5%) | 28 (23.5%) | |
| Oncocytic adenoma | 2 (2.4%) | 7 (6%) | |
| Lymphocytic thyroiditis | 3 (3.7%) | 2 (1.7%) | |
| NIFTP* | 3 (3.7%) | 3 (2.5%) | |
| **Rate of Malignancy** | 28/82=34.1% | 18/119=15.1% | **p=0.002** |

*Noninvasive follicular thyroid neoplasm with papillary-like nuclear features

**B. Comparison of Histopathology Outcomes Between AUS-N and AUS-O Thyroid Carcinomas**

| | AUS-N (n=27) | AUS-O (n=18) | P-value |
|---|---|---|---|
| **Distribution of Carcinoma** | | | |
| PTC | 25 (92.6%) | 12 (66.7%) | **p=0.02** |
| Non-PTC | 2 (7.4%) | 6 (33.3%) | |
| **T Stage. No. (%)** | | | |
| T1 | 17 (63%) | 13 (72.2%) | p=0.42 |
| T2 | 8 (29.6%) | 3 (16.7%) | |
| T3 | 1 (3.7%) | 2 (11.1%) | |
| **Extrathyroidal Extension** | | | |
| Absent | 27 (100%) | 18 (100%) | |
| Present | 0 | 0 | |
| **Margin Status** | | | |
| Positive | 0 | 1 (5.6%) | p=0.4 |
| Negative | 27 (100%) | 17 (94.4%) | |
| **N Stage. No. (%)** | | | |
| Nx, N0 | 25 (92.6%) | 16 (88.9%) | p=0.08 |
| N1a | 0 | 2 (11.1%) | |
| N1b | 2 (7.4%) | 0 | |
| **M Stage. No. (%)** | | | |
| Mx, M0 | 27 (100%) | 18 (100%) | |
| M1 | 0 | 0 | |
| **Locoregional Recurrence** | | | |
| Yes | 0 | 0 | |
| No | 27 (100%) | 18 (100%) | |
| **Distant Metastasis** | | | |
| Yes | 0 | 1 (5.6%) | p=0.4 |
| No | 27 (100%) | 17 (94.4%) | |

## CONCLUSIONS

- AUS-N have a higher frequency of Afirma GSC-S results than AUS-O thyroid nodules.
- AUS-N have a higher frequency of *BRAF*p.V600E mutations and higher risk fusions than AUS-O thyroid nodules.
- Molecular signature expression of ERK and FMT are higher in AUS-N than AUS-O thyroid nodules, suggesting the potential for more aggressive tumor behavior.
- Consistent with the molecular findings, amongst Afirma GSC-S samples, AUS-N thyroid nodules are more likely to represent malignancy, specifically PTC, than AUS-O thyroid nodules.
- These findings support AUS subclassification and molecular testing to enhance risk stratification and potentially guide personalized management of AUS thyroid nodules.

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