# MOLECULAR ANALYSIS OF THYROID MALIGNANCY USING FNA CYTOLOGY SMEARS BY THE COMBINED THYGENX NGS AND THYRAMIR™ MIRNA CLASSIFIER TEST: A PROSPECTIVE STUDY

Gyanendra Kumar[1], Venkata Arun Timmaraju[1], Joanna Wanmin Song-Yang[1], Samantha Taylor[1], Sarada Gandhi Kolli[1], Seiyu Hosono[1], Alidad Mireskandari[1], and Sydney Finkelstein[1,2]

[1]Interpace Diagnostics Lab, New Haven, CT; [2]Interpace Diagnostics, Pittsburgh, PA

September 21-25, 2016
86th Annual Meeting of the American Thyroid Association
DENVER, COLORADO
www.thyroid.org

## BACKGROUND

Molecular diagnostic "rule-in/rule-out" tests to detect malignancy in thyroid nodules are either based upon the detection of oncogenic mutations and gene translocations or based upon mRNA and micro RNA expression classifiers (1, 2)

We have earlier reported a combined test (Figure 1), which incorporates the advantages of "rule-in and rule-out" tests. In the combined test, specimens are initially tested using NGS-based ThyGenX test to detect oncogenic driver mutations and gene fusions. If a mutations or fusion is detected, the specimen is called positive (malignant) and is not tested further. Otherwise it is further tested by ThyraMIR miRNA classifier to call samples malignant or benign. It is called malignant if either ThyGenX or ThyraMIR tests indicate malignancy, and is called benign if both ThyGenX and ThyraMIR test indicate lack of malignancy (3, 4). Using retrospectively collected FNA specimens, the combined ThyGenX/ThyraMIR test showed synergistic performance improvement over each test and resulted in 89% sensitivity and 85% specificity (3).

Here, we demonstrate the equivalency between FNA smears and FNA in protective buffer as sample types for molecular testing. We also report on the clinical performance of ThyGenX and ThyraMIR molecular testing using cytology smear slides from a multi-center double blinded prospective study.

## METHODS

**Equivalency study:** Matching clinical FNA samples (n=47) from the same patient, either stored in a nucleic acid stabilization solution or as air-dried smears on the cytology slides (both stained and unstained) were used to evaluate concordance between molecular results from the combined ThyGenX + ThyraMIR test, to validate routine molecular testing of thyroid nodule aspirates stored as smears on routinely used cytology slides.

**Prospective study:** The multi-center study was designed to evaluate the diagnostic performance of combined ThyGenX + ThyraMIR test. Ultrasound guided FNA biopsy, cytological and histological diagnosis was performed at the study sites. FNA biopsies were stored either as air-dried smears on cytological slides or a FNA pass in nucleic acid stabilization solution. From 1282 collected FNAs, correlation between post surgical histopathology calls and the molecular analysis from the combined ThyGenX + ThyraMIR test were made for 107 specimens to determine the diagnostic performance of the combined test.

**Molecular testing:** Total nucleic acids containing gDNA, mRNA and miRNA were isolated from FNA biopsies stored either in nucleic acid stabilization solution or as air-dried smears on cytology slides. ThyGenX and ThyraMIR assays were performed using validated clinical procedures under CAP/CLIA guidelines as described previously (3-5).

## REFERENCES

1. Nikiforova MN and Nikiforov YE. (2009) Molecular diagnostics and predictors in thyroid cancer. *Thyroid*. **19,** 12: 1351-61.
2. Nishino M. (2016) Molecular cytopathology for thyroid nodules: A review of methodology and test performance. *Cancer Cytopathol.* **124,** 14–27.
3. Labourier E. *et al.* (2015) "Accurate detection of oncogenic mutations in thyroid nodule aspirates with the next generation sequencing ThyGenX TEST" *Endocrine Practice.* **21**, S2: 1-257.
4. Kumar G. *et al.* (2015) "Thyroid miRNA Classifier (ThyraMIR) Complements Mutation Detection (ThyGenX) NGS Tests for Improved Molecular Diagnosis of Indeterminate Thyroid Nodule Needle Aspirates" *Thyroid*. **25,** S1: 1-337.
5. Wylie D. *et al*. (2016) Molecular classification of thyroid lesions by combined testing for miRNA gene expression and somatic gene alterations. *J. Pathol. Clin. Res.* **2,** 93–103.

## ThyGenX + ThyraMIR combined test

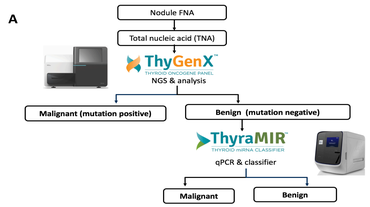


**Figure 1A** Total nucleic acids are isolated from FNA biopsies and tested by the ThyGenX oncogene panel to rule-in malignancy for the presence of Thyroid cancer driver mutations. Samples negative by the ThyGenX panel are reflexed to the ThyraMIR miRNA classifier to rule-in and/or rule-out malignancy.

B

| DNA Panel | BRAF | HRAS | | NRAS | | KRAS | | PIK3CA | |
|---|---|---|---|---|---|---|---|---|---|
| Codon range | 598-615 | 9-20 | 59-69 | 9-20 | 55-67 | 4-15 | 55-65 | 540-551 | 1038-1049 |

| RNA Fusions panel | | | |
|---|---|---|---|
| | PAX8 PPARγ Exon 7 | PAX8 PPARγ Exon 8 | PAX8 PPARγ Exon 9 |
| | PAX8 PPARγ Exon 10 | RET PTC1 | RET PTC3 |

| miRNA classifier panel | | | | | |
|---|---|---|---|---|---|
| | miR-204-5p | miR-139-5p | miR-29b-1-5p | miR-155-5p | miR-138-1-3p |
| | miR-375 | miR-551-b-3p | miR-146b-5p | miR-31-5p | miR-222-3p |

**Figure 1B** ThyGenX and ThyraMIR panel designs: ThyGenX oncogene panel interrogates 9 hotspot regions in thyroid cancer driver genes - BRAF, HRAS, KRAS, NRAS and PIK3CA genes and 6 oncogenic fusion transcripts. ThyraMIR assay is a proprietary miRNA expression classifier to diagnose thyroid malignancy.

## Validation of routinely used FNA cytology smears as a sample type for molecular testing

| ThyGenX test | FNA on cytology slide | FNA in RNARetain |
|---|---|---|
| ThyGenX + | 17 | 16 |
| ThyGenX - | 30 | 31 |
| Concordance = 46/47 = 97.8% | | |

**Table 1** Matched nodule FNA's from the same biopsy (n=47) stored in nucleic acid stabilization buffer and as air-dried smears on cytology slides were tested by the ThyGenX assay. Concordance between diagnostic calls between sample types was 98%.

| ThyraMIR test | FNA on cytology slide | FNA in RNARetain |
|---|---|---|
| ThyraMIR + | 8 | 9 |
| ThyraMIR - | 39 | 38 |
| Concordane = 42/47 = 89.4% | | |

**Table 2** Following ThyGenX testing, samples, were then tested by ThyraMIR assay. Concordance between diagnostic calls between sample types was 90%.

| ThyGenX+ ThyraMIR test | FNA on cytology slide | FNA in RNARetain |
|---|---|---|
| Benign | 26 | 23 |
| Malignant | 21 | 24 |
| Concordance = 42/47 = 89.4% | | |

**Table 3** The diagnostic call concordance for the combined ThyGenX + ThyraMIR test between both sample types was 90%. These results validated the utility of FNA biopsy material stored as air-dried smears on cytology slides as a suitable specimen type for carrying out routine molecular testing.

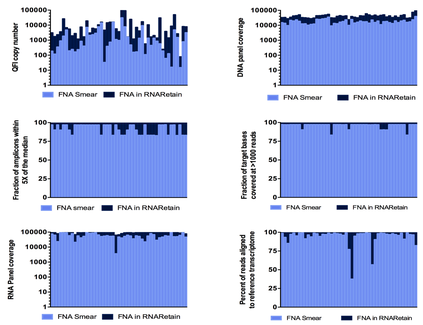


**Figure 2** Comparative plots show various QC metrics for NGS testing FNA stored as a smear and in preservation buffer. **A)** Summary of copy number/µl per sample measured by QFI assay. **B)** DNA panel amplicon coverage **C)** Targeted amplicon PCR efficiency **D)** Fraction of target bases coverage at >1000 reads **E)** RNA panel amplicon coverage **F)** % RNA panel sequencing reads aligning to reference transcript.

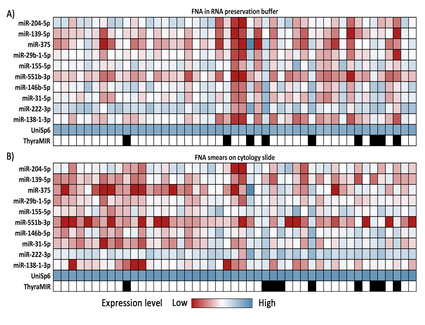


**Figure 3** miRNA expression heat maps and ThyraMIR classifier diagnostic calls for **A)** FNA stored in RNA preservation buffer **B)** FNA smears on cytology slides. miRNA expression was measured using pre-plated Exiqon assays. Ct measurements were fed to a DLDA classifier algorithm to distinguish samples as either benign or malignant

## Diagnostic Performance of ThyGenX + ThyraMIR tests

**ThyGenX calls All samples**

| ThyGenX | Histopathology: Benign (n=56) | Histopathology: Malignant (n=51) |
|---|---|---|
| Positive | 10 (FP) | 34 (TP) |
| Negative | 46 (TN) | 13 (FN) |

**Table 4** ThyGenX correctly rules-in malignancy in 34/51 samples. **ThyGenX false positives are driven by Ras mutations and PAX8-PPARg2 fusions.**

**ThyraMIR calls in ThyGenX negative samples**

| ThyraMIR | Histopathology: Benign (n=46) | Histopathology: Malignant (n=17) |
|---|---|---|
| Positive | 0 (FP) | 8 (TP) |
| Negative | 46 (TN) | 9 (FN) |

**Table 5 ThyraMIR correctly rules-out all ThyGenX negative benign samples (no false positive calls).** ThyraMIR rescues 8 ThyGenX mutation negative samples as malignant.

**Combined ThyGenX + ThyraMIR calls – all samples**

| ThyGenX + ThyraMIR | Histopathology: Benign (n=56) | Histopathology: Malignant (n=51) |
|---|---|---|
| Positive | 10 (FP) | 42 (TP) |
| Negative | 46 (TN) | 9 (FN) |

**Table 6** The 9 false negative calls by the combined testing strategy are likely due to low tumor cellularity of the specimen.

## Clinical and genomic annotation of prospective study samples

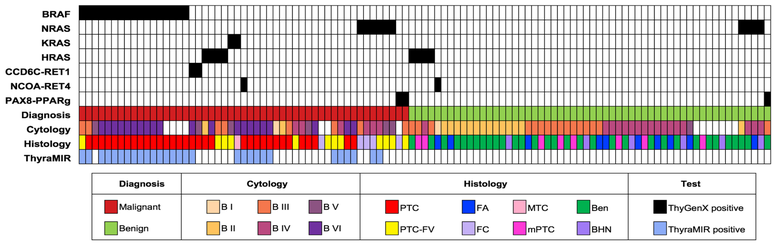


**Figure 4** Integrative plot shows correlation between molecular profiling, cytology and histology diagnoses (row) in each of the 107 samples (column) successfully tested by both assays. Cytology is scored based on the Bethesda category (BI-BVI). Histology categories PTC - Papillary thyroid carcinoma, mPTC - micro PTC, PTC-FV - Papillary thyroid carcinoma follicular variant, FA - Follicular adenoma, FC - Follicular carcinoma, MTC - Medullary thyroid carcinoma, Ben - Benign and BHN - Benign hurtle nodule.

## Clinical Performance of the combined ThyGenX + ThyraMIR test

| | Test performance | |
|---|---|---|
| Sensitivity | 82% | 95% CI, 70-92 |
| Specificity | 82% | 95% CI, 70-91 |
| PPV | 81% | 95% CI, 68-91 |
| NPV | 84% | 95% CI, 72-93 |

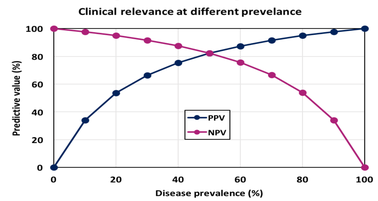


**Figure 5** Correlation of ThyGenX + ThyraMIR test diagnostic calls to post-surgical histopathologic diagnosis and analysis of test performance. The combined assay results in clinically actionable 91% NPV and 69% PPV at 32.5% cancer prevalence

## CONCLUSIONS

1. Demonstrated the equivalency and clinical utility of FNA specimens stored as cytology smear slides as a sample type for ThyGenX and ThyraMIR molecular testing compared to routinely used FNA stored in RNARetain. Between the two specimen types, 97.8% concordance was observed for ThyGenX calls and 89.4% concordance was observed for the combined ThyGenX/ThyraMIR test.

2. Demonstrated the clinical performance of the combined ThyGenX + ThyraMIR test by carrying out a multi-center, and double blinded prospective study using cytology smears as the specimen type. **Correlation of post surgical histopathology calls with molecular diagnostic calls indicated the sensitivity and specificity of the combined test to be 82% each, reflecting 69% PPV and 91% NPV at 32.5% of disease prevalence**.

3. ThyGenX + ThyraMIR can be performed from archived cytology slides for retrospective and/or longitudinal studies