A first-of-its-kind, microRNA-based Diagnostic Assay for Accurate Thyroid Nodule Classification

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Introduction

- The accurate diagnosis of thyroid nodules can be challenging, with 15-30% of Fine Needle Aspiration (FNA) samples classified as indeterminate. Patients with indeterminate nodules are often referred to surgery, though most of these nodules prove to be benign.

- microRNAs are an important class of non-coding RNAs that play key roles in gene expression regulation. microRNA expression has been shown to be a reliable method for cancer subtype classification.

- We describe here RosettaGX Revealm™, a clinically validated microRNA-based assay that stratifies thyroid lesions as benign or suspicious for malignancy.

- This is the first thyroid diagnostic test that works on stained FNA smears.

- Assay development involved more than 400 samples and three profiling platforms used in discovery and training. Blinded validation was conducted on a separate set of approximately 200 samples using qPCR.

Methodology

- Samples: For discovery studies, cytology samples (smears and cellblocks) from archived materials as well as Formalin Fixed Paraffin Embedded (FFPE) resections were used. For training studies, FNA samples (all Bethesda classes) were used. For independent validation, indeterminate stained smears were used. Samples were collected from seven medical centers. Thyroid lesions were ultimately classified as malignant or benign based on histological diagnosis of the resected tumors. Each of the validation samples were reviewed by the institute’s pathologist as well as two additional expert pathologists.

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RNA extraction and profiling: Highly purified RNA, including the well-preserved microRNA fraction, was extracted from samples using proprietary protocols. In the discovery stage, cytology and FFPE samples were profiled using three platforms: (1) custom printed microarrays measuring over 2000 microRNAs, (2) proprietary qPCR and (3) Next Generation Sequencing (NGS). In training and validation, FNA smears were profiled for the expression of a set of selected biomarkers using qPCR.

Assay Development Scheme

- Discovery: Aim: to identify microRNAs that distinguish malignant from benign thyroid lesions

  - Over 150 samples profiled by microarray for the expression of over 2000 microRNAs

  - Eleven FFPE samples from resections were profiled by NGS.

  - A set of 96 microRNAs was selected based on microarray and NGS studies and confirmed in over 200 FFPEs using qPCR

  - A set of 24 microRNAs including malignant, benign, epithelial and blood markers was selected for subsequent training studies

- Training:

  - Over 350 stained FNA smears (MGD, Diff-Quik, Papacriocath) were profiled through qPCR for the expression of the 24 microRNA set

  - A microRNA-based classifier combining discriminant analysis and K-nearest neighbors (KNN) was developed

  - Assay protocol was established, including RNA extraction and qPCR protocols, positive and negative controls, QA measures

- Analytical Validation:

  - Over 350 samples profiled by the R&D lab & Rosetta’s CLIA certified lab in parallel and lab-to-lab concordance was established

Results

RosettaGX Revealm™ Works on Stained Smears Regardless of the Stain Type Being Used

Objective: As various stains are commonly used in clinical practice, we examined the expression of our malignant markers in pairs of samples originating from the same nodule and stained with different dyes (May Grunwald Giemsa (MGG), Diff-Quik and Papacriocath).

Results: The expression levels of mir-148b-5p, one of our malignant markers is similar for different stains of the same nodule; left panel, 52 MGG-Diff-Quik pairs; right panel, 15 Diff-Quik-Papacriocath pairs.

hsa-miR-375 is a Significant Marker for Medullary Thyroid Carcinoma in FNA Smears

Expression levels of hsa-miR-375 were compared between medullary thyroid carcinoma samples (n=23) and samples from other malignant (n=165) and benign thyroid nodules (n=184).

Assay Does Not Incorrectly Diagnose Smears That Contain Only Blood

72 blood smears (whole blood and various blood fractions) were profiled for the expression of the assay microRNAs in 71 of 72 blood samples failed QA and only 1 sample was classified as benign (lymphocytes)

Figure shows expression of epithelial and blood markers in malignant and benign thyroid nodules as well as blood smears. As seen, the expression of blood markers is extremely high in blood smears while the expression of epithelial markers is extremely low in these samples

Analytical Validation

Concordance between the R&D lab and Rosetta’s CLIA certified lab (Philadelphia, PA) is demonstrated

- 173 RNA samples from smears and FFPEs were profiled by the two laboratories

- Pearson correlation coefficient was calculated on microRNA expression levels found in the two laboratories

- Correlation was higher than 0.9 in 100% of the samples

- The agreement between labs is 94.1%

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- This demonstrates the precision and robustness of the test.

Blinded Validation Studies

- The independent, multi-center blinded validation study consisted of 203 FNA samples collected from centers in the USA, Europe and Israel

- Each of the validation samples were reviewed by the institute’s pathologist and two additional expert pathologists. Samples for which an additional pathologist was in agreement with the institutional pathologist was included in the study

- The samples were processed according to the Standard Operating Procedures (SOPs) by personnel blinded to the reference diagnosis

- Classification was automatically generated by a dedicated software

- 191 (94%) samples passed QA

Performance (10 fold cross validation)

<table>
<thead>
<tr>
<th>Sensitivity (%)</th>
<th>Specificity (%)</th>
<th>NPV (%)</th>
<th>PPV (%)</th>
<th>#Benign</th>
<th>#Malignant</th>
<th>RoM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bethesda II, III</td>
<td>84</td>
<td>66.1</td>
<td>60.5</td>
<td>85</td>
<td>39</td>
<td>71</td>
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<tr>
<td>Bethesda IV, V</td>
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<td>80.1</td>
<td>66.1</td>
<td>12</td>
<td>0</td>
<td>100</td>
</tr>
</tbody>
</table>

NPV- Negative Predictive Value; PPV- Positive Predictive Value; RoM- Rate of Malignancy; Failure rate- 7.8%

- Calculated post-validation, based on validation fold

Summary

- A first-of-its-kind assay utilizing microRNA expression in FNA smears for distinguishing benign from malignant thyroid nodules was developed

- Assay validation results:

  - Where final diagnosis confirmed by three pathologists - Sensitivity- 95%; Specificity- 79%; Negative Predictive Value- 98%; Positive Predictive Value- 63%

  - Overall sample set- Sensitivity- 84%; Specificity- 72%; Negative Predictive Value-90%; Positive Predictive Value- 60%

- RosettaGX Revealm™ is a simple and reliable diagnostic assay, which offers a valuable tool for the classification of pre-operative thyroid samples, including those that are presently indeterminate according to cytological evaluation and is the only assay that can run off of cytology smears.