Research Article

Next-Generation Sequencing Identifies a Highly Accurate miRNA Panel That Distinguishes Well-Differentiated Thyroid Cancer from Benign Thyroid Nodules

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Abstract

Background: Fine needle aspiration biopsy (FNAB) is the gold-standard procedure for diagnosing malignant thyroid nodules. Indeterminate cytology is identified in 10% to 40% of cases, and molecular testing may guide management in this setting. Current commercial options are expensive, and are either sensitive or specific. The aim of this study was to utilize next-generation sequencing (NGS) technology to identify informative diversities in the miRNA expression profile of benign versus malignant thyroid nodules.

Methods: *Ex vivo* FNAB samples were obtained from thyroid specimens of patients who underwent thyroidectomy at a referral center. miRNA levels were determined using NGS and multiplexing technologies. Statistical analyses identified differences between normal and malignant samples and miRNA expression profiles that associate with malignancy were established. The accuracy of the miRNA signature in predicting

histologic malignancy was validated using a group of patient specimens with indeterminate cytology results.

Results: A total of 274 samples were obtained from 102 patients undergoing thyroidectomy. Of these samples, 71% were benign and 29% were malignant. Nineteen miRNAs were identified as statistically different between benign and malignant samples and were used to classify 35 additional nodules with indeterminate cytology (validation). The miRNA panel's sensitivity, specificity, negative and positive predictive values, and overall accuracy were 91%, 100%, 87%, 100%, and 94%, respectively.

Conclusions: Using NGS technology, we identified a panel of 19 miRNAs that may be utilized to distinguish benign from malignant thyroid nodules with indeterminate cytology.

Impact: Our panel may classify indeterminate thyroid nodules at higher accuracy than commercially available molecular tests. *Cancer Epidemiol Biomarkers Prev; 27(8); 858–63. ©2018 AACR.*

Introduction

Thyroid nodules are very common and may be identified in as many as 50% of the population when neck ultrasound is performed (1). Fortunately, most thyroid nodules are benign, and only about 7% harbor malignancy. Well-differentiated thyroid cancer comprises over 90% of thyroid malignancies and includes papillary thyroid cancer (PTC) and follicular thyroid cancer (FTC). These cancers are the most common endocrine malignancies, with more than 64,000 new cases diagnosed in the United States every year (2). Diagnostic neck ultrasound should be performed in all patients with suspicious thyroid nodules. Thyroid ultrasound evaluates nodule characteristics, and certain sonographic features may raise the suspicion of malignancy. Nevertheless, these features lack accuracy to reliably distinguish benign from malignant nodules, and current guidelines continue to rely on ultrasound-guided fine needle aspiration biopsy (FNAB) as the procedure of choice in the evaluation of thyroid nodules (3).

The unmet clinical need

Although FNAB yields a benign diagnosis in 60% to 70% of the cases and malignant diagnosis in about 5%, indeterminate results are reported in 10% to 40% of the cases. The Bethesda system for reporting thyroid cytopathology provides an estimated cancer risk within each indeterminate category (4). The risk for malignancy ranges between 5% and 15% for follicular lesions of unknown significance (Bethesda category III), 15% and 30% for follicular lesions (Bethesda category IV), and 60% and 75% for suspicious for malignancy (Bethesda category V). Patients with thyroid nodules and indeterminate FNAB result (Bethesda category III or IV) are offered management options that include observation, repeat biopsy, surgery, or molecular testing (3, 4). Available commercial molecular tests are expensive and are either sensitive or specific (5–7). Highly sensitive tests may be used to rule out malignancy and consequently avoid surgery, whereas highly specific tests are used to rule in malignancy and indicate the need for a surgical procedure. At present, there is no ultimate molecular test with both sensitivity and specificity above 90%.

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miRNAs are endogenous short (21–23 nucleotides) noncoding RNAs that regulate cell function by silencing genes and hence also play a role in carcinogenesis. The miRNA expression profile changes during the course of the malignant transformation, and previous studies identified its diagnostic utility in differentiating between benign and malignant thyroid nodules (8). These studies were limited to a relatively small number of miRNAs that were investigated, and to date, no study evaluated the entire miRNome. Next-generation RNA sequencing (NGS) techniques enable to comprehensively and efficiently study the expression profile of miRNAs. The aims of this study were: (i) to identify differences in miRNA expression between biopsies taken from the same nodule; (ii) to utilize NGS technology to identify informative diversities in the miRNA expression profile of benign versus malignant thyroid nodules based on needle biopsies taken after surgery; and (iii) to validate the diagnostic role of miRNA in patients with indeterminate nodules.

Materials and Methods

Patients and biopsies

The study protocol was reviewed and approved by the Independent Ethics Committee, Hadassah-Hebrew University Medical Center (Jerusalem, Israel, Helsinki committee, protocol #HMO-0375). Adult patients who were referred to elective thyroid surgery between January and December 2015 were offered to participate in the study. The investigators obtained written informed consent from patients with a preoperative diagnosis of benign goiter, PTC, or indeterminate cytology. Once the thyroid gland was surgically removed, an *ex vivo* FNAB was performed as described previously (9). In brief, in patients with thyroid cancer, the nodule was palpated and 2 to 3 passes were performed using a 19-guage needle. In patients with benign thyroid disease, the biopsy was performed from normal appearing thyroid tissue. All preoperative diagnoses were confirmed by standard histopathology. The "validation group" consisted of patients with preoperative indeterminate cytology report (Bethesda III or IV). In these patients, the ultrasound report was carefully reviewed to confirm the exact location of the thyroid nodule that was biopsied before surgery. *Ex vivo* biopsies were performed from the very same nodule as described above. Samples were coded without identification of the group classification (benign, malignant, validation). Data were collected on each patient including age, gender, indications for surgery, detailed preoperative ultrasound report, preoperative cytology diagnosis, and detailed final histopathology diagnosis. All data were recorded on a Case Report Form designed for this study.

RNA extraction and small RNA cDNA library preparation and sequencing

All cell samples were collected from the aspiration biopsy needle by air injection and irrigation into a collecting tube containing 1 mL of RNA*later* (ThermoFisher Scientific). Tubes were stored at 4°C until further processing. Total RNA was extracted with TRIzol, following Polytron homogenization using disposable tips. Concentration was measured with Qubit 2.0 Fluorometer and RNA BR assay reagents (Thermo Fisher Scientific), and RNA was stored at −80°C.

Total RNA was subjected to in-house multiplexed small RNA cDNA library preparation, which entailed ligation of barcoded 3′ adapters to 24 different samples, pooling of samples, ligation of a 5′ adapter, reverse transcription, and PCR, as described

previously (10), with modifications allowing multiplexing of several 24-sample libraries on a single HiSeq lane, namely, 48 to 120 small RNA samples per lane (11), using TruSeq-like PCR primers. Libraries were sequenced on an Illumina HiSeq 2500 sequencer, and the information obtained was analyzed by an automated computer pipeline to decode and annotate small RNA reads (12).

Statistical analysis

Statistical procedures on count data were based on the "DESeq2" R/Bioconductor package for analysis of differential expression in RNA sequencing experiments (13), as described previously (14), and complemented by calculations with "edgeR" (15) and "samr" (16). Differential expression analyses were performed upon a matrix of read counts with samples as columns and miRNA as rows, after filtering out low-coverage miRNA (retaining miRNA with at least 3 reads in at least 1/3 of study samples). To overcome the difficulty of multiple testing, the DESeq2 package uses an adjusted P value for which we determined that less than 0.05 would be regarded as significant. The samr package utilizes a Q-value to address multiple testing, which we also determined to be significant if <0.05. Prior to any analysis based on miRNA read counts, we applied the "sva" package (17) to remove batch biases from the data. In addition, prior to analyses other than differential expression, miRNA read counts were transformed using DESeq2's variance stabilizing transformation, to generate normally distributed data. To describe the extent of technical variability (namely differences between miRNA profiles generated from repeated aspiration biopsies from the same nodule or normal tissue), we calculated correlation distances between samples based on miRNA abundance profiles, and compared the distribution of distances derived from all possible pairwise tests to the distribution of distances from technical repeat pairwise tests, using empirical cumulative distribution function plots and a Mann–Whitney U test. When testing for differential expression, we first aggregated miRNA counts from all technical repetitions of each specimen (separately for tumor and adjacent normal tissue), such that each biological sample was represented once, by the sum of counts of its technical repetitions. In the differential expression models, patient was included as a blocking factor, being that often malignant and benign tissues were obtained from the same specimen. The ability of differentially expressed miRNA to discriminate malignant from benign/normal tissue was evaluated in the discovery set of samples by calculating the c-statistic, equivalent to the area under the ROC curve. To maximize the discrimination ability of miRNA, we devised a score for each sample ("miRNA score"), assembled by summing the standardized levels (z-values) of all significantly upregulated miRNA, and the negative of the z-values of all significantly downregulated miRNA. In the validation set of samples, z-values were calculated by relating to the normal mean and SD values from the discovery set. ROC curve was used to test the discriminatory characteristics of the miRNA score in the validation set. Sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy were calculated at an optimal cutoff point. Plots were generated with "ggplot2."

Availability of data

miRNA deep sequencing count data for all study samples, along with partial metadata, are available as a spreadsheet file online.

Table 1. Patient characteristics

Variable	Patients without cancer, $n = 64$	Patients with cancer, $n = 38$	P	Validation group, $n = 35$
Female gender	80%	76%	0.687	81%
Mean age, median (IQR)	43 (32–59)	47 (37–57)	0.715	47 (32–57)
Thyroid function				
Euthyroidism	60.0%	91.6%	0.004	87.1%
Hyperthyroidism	31.7%	5.6%		9.7%
Hypothyroidism	8.3%	2.8%		3.2%
Preoperative TSH, median (IQR)	0.62 (0.30–1.23)	1.71 (1.24–2.66)	<0.001	1.75 (1.17–2.82)
Multinodular goiter	56.9%	26.3%	0.005	21.9%
Hashimoto thyroiditis	12.3%	39.5%	0.003	25.0%
Graves disease	18.5%	0%	0.012	0%
Family history of thyroid cancer	1.7%	5.6%	0.650	0%
Radiation history	1.7%	2.8%	1	0%

NOTE: P values were calculated with χ^2 tests for proportions and Mann–Whitney tests for continuous variables (age and TSH). IQR, 1st–3rd quartile range.

Demultiplexed sequencing read files were deposited at NCBI's Gene Expression Omnibus (GEO record GSE116196).

Results

During the study period, 340 biopsies were performed in 137 patients (Table 1). Age (mean ± SD) was 45.7 ± 16.4 years with a 3.9:1 female-to-male ratio. Of the entire sample set, 195 (57.4%) were classified as benign, 79 (23.2%) malignant, and 66 (19.4%) served as the validation group (Fig. 1). miRNAs were profiled using deep sequencing of multiplexed (120 samples/Illumina HiSeq 2500 lane) small RNA cDNA libraries prepared from each sample following total RNA extraction (Supplementary Figs. S1–S3; Supplementary Tables S1–S3). Correlation-based distance measurement between the technical repetitions demonstrated differences in miRNA expression that were smaller in magnitude compared with biological repetitions (Supplementary Fig. S4), thus supporting the soundness of our approach.
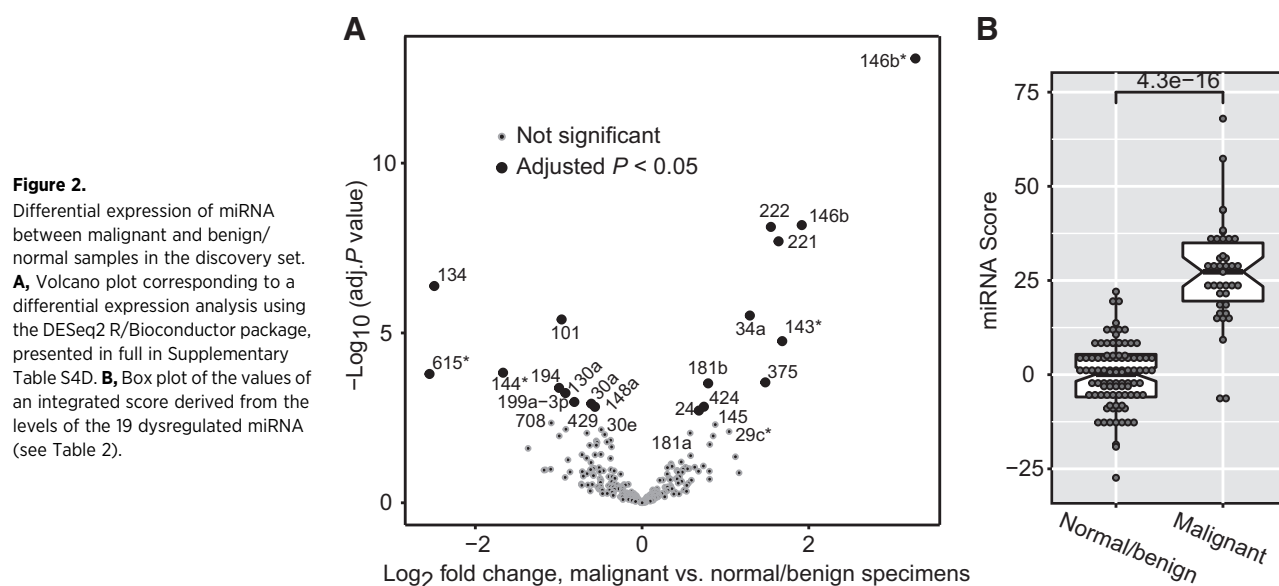
Biomarker discovery

A total of 279 miRNAs were detected (with sequencing depths of at least 3 reads in at least 1/3 of study samples). Although the majority of miRNAs did not show a significantly different expression between normal (or benign) and malignant samples, several miRNAs were either over- or underexpressed in malignant samples as compared with normal (Fig. 2A). Nineteen miRNAs, with the highest dysregulation significance, were selected to be included in a diagnostic panel (Table 2). A score derived from the panel of diagnostic miRNAs was most accurate in distinguishing benign from malignant samples (Fig. 2B). Further analyses of the discovery set of samples are provided in Supplementary Figs. S5–S7 and Supplementary Tables S4 and S5. Many of these miRNAs have
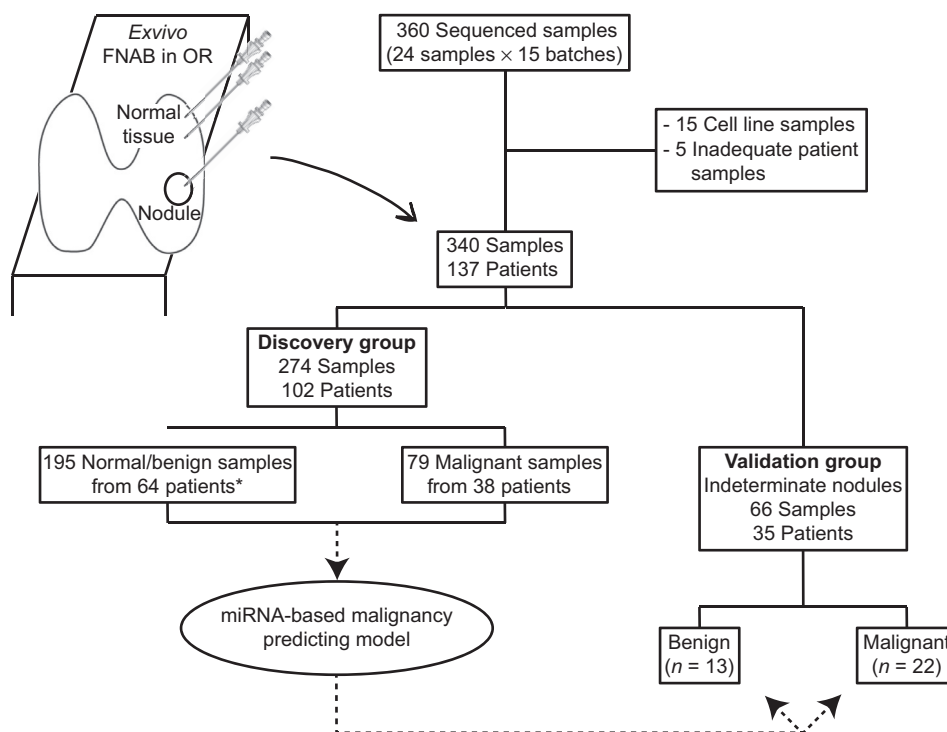
Figure 1.
Overview of the study design. 360 small RNA cDNA libraries were sequenced on 5 Illumina HiSeq 2,500 lanes (24 libraries × 15 batches, with 5 batches/lane). Of those, 15 libraries represented cell line RNA (not included in analyses), and 5 libraries were inadequate for various reasons. The remaining 340 libraries represented valid FNABs taken *ex vivo* in the operating room (OR) from 137 patients. Of these patients, 102 had cytological evidence of malignancy or were operated for nononcological indications (discovery group), while 35 had indeterminate preoperative cytology (validation group). A miRNA deep sequencing–based discriminator was developed using discovery set data and was tested on the validation set sequencing data. *, Note that in the discovery group, patients with tumors also contributed benign samples, in addition to the 64 patients without cancer.

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Figure 2.
Differential expression of miRNA between malignant and benign/normal samples in the discovery set. **A,** Volcano plot corresponding to a differential expression analysis using the DESeq2 R/Bioconductor package, presented in full in Supplementary Table S4D. **B,** Box plot of the values of an integrated score derived from the levels of the 19 dysregulated miRNA (see Table 2).

been previously reported to be dysregulated in thyroid cancer (Supplementary Tables S6 and S7).

Biomarker validation

Of the 35 specimens that were included in the validation group (after merging technical replicates), 22 were identified on final histopathology as malignant and 13 as benign (Supplementary Fig. S8). Of the 22 malignant specimens, 15 (68%) were identified as PTC and 7 (32%) were identified as FTC. Panel miRNA-based diagnostic scores according to histopathology are presented in Fig. 3A. ROC curve analysis showed an AUC of 0.95 and identified a score of 9.27 as the optimal cutoff between benign and malignant samples (Fig. 3B and C). Of the validation cohort,

only 2 malignant samples had a score that was lower than 9.27, yielding values for sensitivity, specificity, NPV, PPV, and overall accuracy of 91%, 100%, 87%, 100%, and 94%, respectively (also see Supplementary Figs. S9 and S10).

Discussion

This study demonstrates that 19 miRNAs can serve as a highly accurate diagnostic tool and differentiate between benign and malignant thyroid nodules. Although several of the miRNAs identified in this study have been described previously (Supplementary Table S6), the strength of this study is the utilization of NGS technique that enabled the evaluation of the complete

Table 2. Significantly dysregulated miRNA in the comparison of malignant versus benign thyroid tissues[a] within discovery specimens

| miRNA | Model 1 | | | | Model 2 | Model 3 |
	Log_2FC	P	P_{adj}	C-statistic[b]	P	P
hsa-miR-146b*	3.3	8.3E−14	2.2E−11	0.898	3.5E−11	1.9E−11
hsa-miR-146b	1.9	6.7E−09	6.6E−07	0.887	2.7E−07	1.3E−07
hsa-miR-222	1.5	7.5E−09	6.6E−07	0.897	2.8E−07	8.9E−09
hsa-miR-221	1.6	2.0E−08	1.3E−06	0.873	9.3E−07	9.4E−08
hsa-miR-134	−2.5	4.1E−07	2.2E−05	0.201	4.8E−06	1.4E−04
hsa-miR-34a	1.3	3.1E−06	1.3E−04	0.848	7.1E−05	3.3E−06
hsa-miR-101	−1.0	4.0E−06	1.5E−04	0.120	9.1E−06	9.1E−06
hsa-miR-143*	1.7	1.7E−05	5.7E−04	0.782	1.9E−04	1.2E−04
hsa-miR-144*	−1.7	1.5E−04	4.2E−03	0.210	3.6E−04	1.3E−04
hsa-miR-615*	−2.5	1.6E−04	4.2E−03	0.240	4.9E−04	1.2E−02
hsa-miR-375	1.5	2.8E−04	6.7E−03	0.750	3.5E−03	5.5E−03
hsa-miR-181b	0.8	3.0E−04	6.7E−03	0.696	7.2E−04	8.8E−06
hsa-miR-194	−1.0	4.1E−04	8.4E−03	0.222	1.7E−04	3.0E−03
hsa-miR-130a	−0.9	5.9E−04	1.1E−02	0.264	4.4E−03	5.2E−04
hsa-miR-199a-3p	−0.8	1.1E−03	1.9E−02	0.244	7.4E−03	3.7E−03
hsa-miR-30a	−0.6	1.2E−03	2.0E−02	0.217	4.6E−03	7.9E−03
hsa-miR-424	0.7	1.5E−03	2.2E−02	0.781	1.4E−03	4.9E−03
hsa-miR-148a	−0.6	1.5E−03	2.2E−02	0.221	5.6E−03	9.5E−03
hsa-miR-24	0.7	1.9E−03	2.7E−02	0.816	3.0E−03	1.7E−02

Abbreviations: Log_2FC, log base 2 of the fold change; P_{adj}, P value adjusted for multiple testing (false detection rate).
[a]Model 1 refers to patient-adjusted analysis; Model 2 includes patient and thyroid function state (euthyroid, hypothyroid, or hyperthyroid); and Model 3 includes patient and presence of noncancer thyroid pathology (Hashimoto thyroiditis or Graves disease).
[b]The area under the ROC curve, which corresponds to the discrimination ability between malignant and benign samples (values closer to 0 or 1 represent higher discrimination ability, whereas values approaching 0.5 signify lack of discrimination).
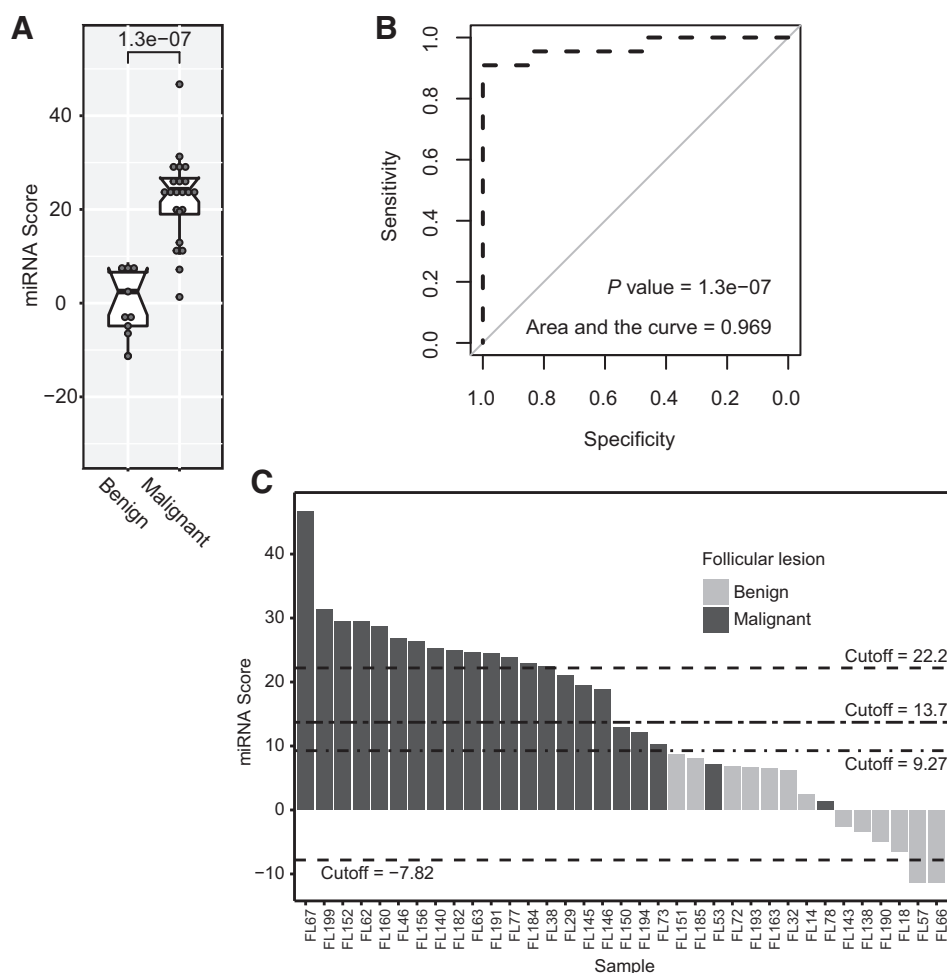
Figure 3.
Differential expression of miRNA between malignant and benign nodules that had indeterminate cytology and were defined as follicular lesions (validation set). A score summing the standardized levels of all 19 significant ("signature") miRNAs (**A**) (derived from the discovery set, see Fig. 2B) was considerably higher in follicular lesions found to be malignant on pathology compared with benign tissue (*P* value is derived from a Mann–Whitney test), and discriminated malignant specimens from benign lesions at high accuracy (**B**). **C,** The miRNA scores are shown as column plot with horizontal lines depicting four possible cutoff values for malignancy (determined by the "OptimalCutpoints" package in R). Cutoff I (−7.82, MaxSe) has 100% sensitivity and 15% specificity. Cutoff II (9.27, MinValueSe = 0.94) has 94.9% sensitivity and 86% specificity. Cutoff III (13.71, MaxSpSe) has 92.3% sensitivity and 95.7% specificity. Cutoff IV (22.2, MaxSp) has 71.8% sensitivity and 100% specificity.

miRNome. Some of these miRNAs are not specific to thyroid cancer. miR-221/222 and miR-146b are overexpressed in colon, breast, ovarian, hepatic, prostate, and several other malignancies. The mechanisms of these miRNAs in thyroid carcinogenesis are not entirely understood; however, attempts to characterize miRNA–gene regulatory networks in thyroid cancer have been made (18). Specific thyroid target genes include *BRAF*, *RAS*, *PAX8*, *P51*, *PTEN*, *mTOR*, and *PPAR* among others, and several studies aim to identify the therapeutic role of these miRNA–gene interactions. Other investigators use miRNA expression profile as a prognostic tool to identify tumor aggressiveness as well as a marker for tumor recurrence when isolated in serum samples (19, 20).

Nevertheless, the focus of this translational study is on the diagnostic role of miRNA expression in thyroid nodules. At present, patients with indeterminate thyroid FNAB results are offered to either undergo diagnostic thyroidectomy (total or lobectomy) or molecular testing. Current commercial molecular tests include Afirma, ThyroSeqV2, and Rosetta (5–7). The former two include mRNAs and gene testing, whereas the latter is a 24 miRNA-based assay. The two major disadvantages of the commercial tests are their accuracy and costs. All commercial tests highlight their very high NPV, whereas their PPV is much lower. Thus, these tests are extremely useful as "rule out" tests while their ability to correctly identify cancer is between 50% and 70%. The other limitation of these tests is their cost, ranging

Table 3. Comparison with commercially available molecular assays

Molecular assay/report	Sensitivity	Specificity	NPV	PPV	Comments
Afirma GEC (5)	92%	52%	93%	47%	Multiple validation studies
Afirma GSC	91%	68%	96%%	47%	One meta-analysis
Other reported results	83%–100%	10%–73%	75%–100%	14%–57%	
ThyroSeq v2 (7)	90%	93%	96%	83%	Limited validation data
Other reported results	70%–95%	60%–77%	91%–98%	27%–66%	
Rosetta (6)	74%–100%	74%–80%	92%–100%	41%–43%	Difference between the entire and agreement validation sets
Current study	91%	100%	87%	100%	

between 2,000 and 3,000 USD, a price that many patients cannot afford. Our study implies that these two obstacles can be overcome. Notwithstanding the need for external validation, our overall accuracy is the highest reported to date with a NPV and PPV of 87% and 100%, respectively (Table 3).

Our study has several limitations. Samples were obtained *ex vivo*, and although we have previously demonstrated that similar results can be obtained from real FNAB samples, a future clinical trial needs to validate the results in patient management. All thyroid malignancies evaluated in the discovery set were PTCs. FTC has a somewhat similar miRNA profile; however, medullary thyroid cancer is a distinctly different malignancy and is not the focus of this study. Several specimens included in the discovery set were taken from patients with benign thyroid pathology—for example, Hashimoto thyroiditis or Graves disease—however, statistical adjustment did not alter the results. Finally, it has been shown that the prevalence of malignancy in indeterminate thyroid FNABs affects the accuracy of molecular testing. Our results need to be validated in other countries as well.

Conclusions

Using NGS technology, we identified a panel of 19 miRNAs that may be utilized to distinguish benign from malignant thyroid nodules with indeterminate cytology at higher accuracy than commercially available molecular tests and lower expected costs. A future multi-center clinical trial is needed to establish its utility in patients with indeterminate FNAB results.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: H. Mazeh, A. Karas, I. Mizrahi, I.Z. Ben-Dov
Development of methodology: H. Mazeh, T. Deutch, A. Karas, I. Mizrahi, D. Gur-Wahnon
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): A. Karas, K.A. Bogardus, I. Mizrahi
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Mazeh, T. Deutch, A. Karas, I. Mizrahi, I.Z. Ben-Dov
Writing, review, and/or revision of the manuscript: H. Mazeh, T. Deutch, A. Karas, I. Mizrahi, I.Z. Ben-Dov
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): T. Deutch, A. Karas, I. Mizrahi
Study supervision: H. Mazeh, T. Deutch, D. Gur-Wahnon, I.Z. Ben-Dov

Acknowledgments

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