A Panel of Four miRNAs Accurately Differentiates Malignant from Benign Indeterminate Thyroid Lesions on Fine Needle Aspiration

Xavier M. Keutgen¹, Filippo Filicori¹, Michael J. Crowley¹, Yongchun Wang‡, Theresa Scognamiglio², Rana Hoda², Daniel Buitrago¹, David Cooper⁵, Martha A. Zeiger⁴, Rasa Zamegar¹, Olivier Elemento³, and Thomas J. Fahey III¹

Abstract

Purpose: Indeterminate thyroid lesions on fine needle aspiration (FNA) harbor malignancy in about 25% of cases. Hemi- or total thyroidectomy has, therefore, been routinely advocated for definitive diagnosis. In this study, we analyzed miRNA expression in indeterminate FNA samples and determined its prognostic effects on final pathologic diagnosis.

Experimental Design: A predictive model was derived using 29 ex vivo indeterminate thyroid lesions on FNA to differentiate malignant from benign tumors at a tertiary referral center and validated on an independent set of 72 prospectively collected in vivo FNA samples. Expression levels of miR-222, miR-328, miR-197, miR-21, miR-181a, and miR-146b were determined using reverse transcriptase PCR. A statistical model was developed using the support vector machine (SVM) approach.

Results: A SVM model with four miRNAs (miR-222, miR-328, miR-197, and miR-21) was initially estimated to have 86% predictive accuracy using cross-validation. When applied to the 72 independent in vivo validation samples, performance was actually better than predicted with a sensitivity of 100% and specificity of 86%, for a predictive accuracy of 90% in differentiating malignant from benign indeterminate lesions. When Hurthle cell lesions were excluded, overall accuracy improved to 97% with 100% sensitivity and 95% specificity.

Conclusions: This study shows that the expression of miR-222, miR-328, miR-197, and miR-21 combined in a predictive model is accurate at differentiating malignant from benign indeterminate thyroid lesions on FNA. These findings suggest that FNA miRNA analysis could be a useful adjunct in the management algorithm of patients with thyroid nodules. Clin Cancer Res; 18(7); 1–7. ©2012 AACR.

Introduction

Thyroid cancer is the most common endocrine neoplasm. Its incidence is increasing and females are affected nearly twice as often as males (1). Thyroid cancer typically presents as a thyroid nodule and 5% to 15% of all thyroid nodules will prove to be malignant. Currently, the gold standard for evaluating thyroid nodules is fine needle aspiration (FNA). FNA results in a definitive classification in approximately 70% to 80% of cases, whereas the remaining 20% to 30% of samples are characterized as indeterminate (2, 3). These lesions show a follicular growth pattern and FNA is not sufficient to distinguish between benign and malignant lesions. Indeterminate FNA lesions present a problem for both the patient and the clinician as at least 20% harbor a malignant diagnosis and require at least a hemithyroidectomy for determining final diagnosis (4). When the definitive diagnosis is consistent with malignancy, patients typically undergo a second surgical procedure in the form of a completion thyroidectomy. Furthermore, because 50% to 80% of indeterminate lesions are benign on final pathology, a significant number of patients undergo potentially avoidable surgery with its inherent risks and costs (2, 5, 6). Although many immunohistochemical and molecular markers have been investigated in recent years in efforts to improve sensitivity and specificity of FNA
Translational Relevance

Indeterminate thyroid lesions on fine needle aspiration (FNA) harbor malignancy in about 25% of cases. Hemi- or total thyroidectomy has, therefore, been routinely advocated for definitive diagnosis. Although many molecular markers have been investigated in recent years in an attempt to improve the sensitivity and specificity of indeterminate FNA cytology, none has been considered accurate enough to become an integral part of the diagnostic armamentarium for clinicians and cytopathologists. In this study, we were able to develop a statistical model that accurately differentiates malignant from benign indeterminate lesions on thyroid FNAs using a panel of 4 miRNAs (miR-222, miR-328, miR-197, and miR-21). Our model was 100% sensitive and 86% specific for differentiating malignant from benign indeterminate thyroid FNA lesions. When Hurthle cell lesions were excluded, specificity increased to 95%. On the basis of these results it would be reasonable to recommend a total thyroidectomy if malignancy is predicted using our model. In addition, a diagnostic hemithyroidectomy might be avoided in patients with benign lesions as predicted by this panel.

cytology, none has yet been accepted as integral to the diagnostic armamentarium by clinicians and cytopathologists. This is mainly due to the considerable overlap between follicular adenomas and differentiated thyroid carcinomas and the low prevalence of known mutations diagnostic for cancer in indeterminate FNA lesions.

miRNA are single-stranded noncoding small RNA segments, 19 to 23 nucleotides in length. Mature miRNAs operate via sequence-specific interaction with the 3′ untranslated region of mRNA targets and thereby cause suppression of translation and mRNA decay (7). In cancer, miRNAs have been shown to function as both tumor suppressors or oncogenes and have been useful for cancer classification and prognostication (8). miRNAs have been reported to be dysregulated in virtually all human cancer types, including all variants of thyroid cancer (9–11). miR-222, miR-181a, and miR-146b have been shown to be upregulated at least 10-fold in several studies when comparing classic variants of papillary thyroid carcinoma (CPTC) to normal thyroid tissue (12, 13). Furthermore, miR-328, miR-197, and miR-21 are differentially expressed when comparing follicular thyroid cancer (FTC) and follicular variant of papillary thyroid cancer (FVPTC) to normal tissue and follicular adenomas (14). In this study, we aimed to elucidate the expression patterns of these 6 miRNAs in indeterminate FNA lesions and to determine their potential for differentiating malignant from benign indeterminate FNA lesions.

Materials and Methods

Differential expression of 6 miRNAs (miR-222, miR-181, miR-146b, miR-328, miR-197, and miR-21) were initially measured in 29 ex vivo indeterminate FNA samples and used to develop a predictive model (derivation group). This model was then validated on an independent set of 72 prospectively collected indeterminate FNA samples (validation group).

Derivation group

After approval from the Institutional Review Board of Weill Cornell Medical Center (New York, NY) was obtained, written informed consent was collected from patients for the use of clinical specimens for research. All hemi- and total thyroidectomies carried out at our institution for indeterminate lesions were reviewed from 2005 to 2010. One hundred and eighty-one patients with indeterminate lesions that underwent surgery were identified during this time period. Of those, 14 indeterminate FNA lesions with malignant final histopathology could be found in our tumor bank. An additional 15 indeterminate lesions with benign final histopathology were randomly selected to match the malignant group. We purposely chose to keep an almost equal number of benign (52%) and malignant (48%) lesions in the derivation set to train the statistical model in recognizing benign and malignant lesions equally. All FNA specimens used for the derivation set were taken from ex vivo thyroid tissue samples after thyroidectomy was carried out (Table 1, Supplementary Table S1).

FNA sampling and data collection

After thyroidectomy was carried out, a 25-gauge needle was inserted in the thyroid nodule and 2 to 3 passages were used to collect the cytology specimen, which was then suspended in RLT lysis buffer, RNA later solution (Qiagen Inc.), or TRIzol (Invitrogen), snap frozen in liquid nitrogen and stored at −80°C. A cytopathologist reviewed all in-house and outside FNAs before surgery and an endocrine pathologist reviewed all surgery specimens. Only unequivocal cytologic cases were included in this study. Final diagnosis, FNA diagnosis, age, sex, tumor size and location, FNA location, surgical procedure, extrathyroidal extension, angiolymphatic invasion, and lymph node metastasis were entered for each patient sample into the tumor bank data sheet.

miRNA selection, extraction, reverse transcription, and real-time PCR

A systematic search of the databases PubMed, Ovidsp, and Google Scholar was carried out to identify studies related to miRNA and thyroid lesions published in the English language through December 2010. We focused on miRNAs that were differentially expressed between normal thyroid tissue or follicular adenomas and CPTC, FVPTC, and FTC and ultimately selected 6 miRNAs for further analysis (11–19).

miRNA levels of miR-328, miR-222, miR-197, miR-181a, miR-146b, and miR-21 were determined in FNA specimens using real-time PCR. miRNAs were extracted and reverse transcribed to cDNA according to the standard protocol by the mirVana Kit (Ambion Inc.), the mirPremier Kit (Sigma
miRNAs and Fine Needle Aspiration

Table 1. Demographics and pathologic characteristics of derivation and validation groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Derivative group</th>
<th>Validation group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (mean ± SD)</td>
<td>50 ± 15</td>
<td>55 ± 16</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>22</td>
<td>59</td>
</tr>
<tr>
<td>Tumor size, cm (mean ± SD)</td>
<td>2.7 ± 1.7</td>
<td>2.8 ± 1.7</td>
</tr>
<tr>
<td>Range</td>
<td>0.8–6</td>
<td>0.7–7.4</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total thyroidectomy</td>
<td>12</td>
<td>35</td>
</tr>
<tr>
<td>Hemi thyroidectomy</td>
<td>17</td>
<td>37</td>
</tr>
<tr>
<td>Completion thyroidectomy</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>FNA pathology (^a)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Follicular lesion of undetermined significance</td>
<td>17</td>
<td>32</td>
</tr>
<tr>
<td>Follicular neoplasm</td>
<td>9</td>
<td>23</td>
</tr>
<tr>
<td>Hurthle cell neoplasm</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Suspicious for malignancy</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Final pathology</td>
<td></td>
<td></td>
</tr>
<tr>
<td>CPTC</td>
<td>4</td>
<td>8</td>
</tr>
<tr>
<td>Poorly differentiated PTC</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>FVPTC</td>
<td>8</td>
<td>8</td>
</tr>
<tr>
<td>FTC</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Hurtle cell cancer</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>FA</td>
<td>13</td>
<td>9</td>
</tr>
<tr>
<td>HPN</td>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>Thyroiditis</td>
<td>3</td>
<td>19</td>
</tr>
</tbody>
</table>

Abbreviation: FA, follicular adenoma.

\(^a\)Classification according to The NCI Thyroid Fine Needle Aspiration State of the Science Conference scheme.

Statistical analysis and derivation group model development

We evaluated several methodologies for building statistical models that can predict benign versus malignant status in indeterminate thyroid FNA lesions based on expression profiles of the 6 miRNAs. These methodologies were regression trees, logistic regression, linear discriminant analysis (LDA), quadratic discriminant analysis (QDA), and support vector machines (SVM) with different kernel functions (20, 21). By representing lesions as data points in a multidimensional space, and combining the expression levels of several miRNAs in a linear or nonlinear manner, these approaches find decision surfaces that best separate malignant and benign lesions.

SVMs identify the hyperplane such that the distance (also called margin) between the hyperplane and the closest data points is maximized. The data points that are exactly the margin distance away from the hyperplane are called support vectors. Because in many cases no separating hyperplane exists, some data points are allowed to be on the wrong side of their margin. The extent to which such flexibility is allowed is controlled by a cost parameter C > 0. Moreover, a set of variables describing lesions can be expanded by mapping the original variables to a higher dimensional space using a nonlinear function. Such mapping is obtained using kernel functions; a commonly used kernel function is the radial basis function kernel, $K(x_i, x_j) = \exp(-\gamma ||x_i - x_j||^2)$, with $\gamma > 0$ and where $x_i$ and $x_j$ are vectors containing expression values describing lesions. More extensive descriptions of SVMs and other approaches used here have been described previously (20, 21).

Using these methodologies and specific parameter choices (e.g., for SVMs), models were trained using all 29 samples and variable subsets of the 6 miRNAs were used. The predictive accuracy of each of these models was estimated using leave-one-out cross-validation (20, 21). Feature selection was carried out using both backward elimination and forward addition procedures using the 6 miRNAs. SVM kernel functions (linear, radial basis) and parameter values were also explored and selected using cross-validation. All statistical procedures were implemented using the R statistical analysis language and software. The e1071, MASS, and rpart R third-party libraries were used for SVM, LDA, and regression trees, respectively.

Validation group

After model selection, 72 consecutive specimens were collected prospectively in an in vivo fashion using standard ultrasound-guided transcutaneous FNA technique. Briefly, 1 to 2 passes were obtained with a 25-gauge needle and cytologic smears made. Samples from both the clinic and presurgical FNAs were then obtained from the residual material in the needle after the cytologic smears were made. All nodules were indeterminate lesions and no other selection criteria were applied except that the patient had to be going to surgery. All patients were euthyroid and none required preoperative treatment.
with thyroxine. The validation group consisted of 22 indeterminate lesions with malignant final pathology (30.5%) and 50 indeterminate lesions (69.5%) with benign final pathology (Table 1, Supplementary Table S2). This benign to malignant sample ratio is consistent with the ratio reported in the literature for indeterminate FNA lesions, namely 20% to 30% for malignant and 70% to 80% for benign pathology (22–26). Sixteen indeterminate FNA samples were obtained from Johns Hopkins Hospital (Baltimore, MD), of which 3 were malignant and 13 were benign on final pathology. Thirty-three samples were obtained in surgery clinic and 39 FNA samples were obtained in the operating room before the surgical incision. All samples were obtained under identical conditions using ultrasound guidance. Cytologic smears were prepared for all presurgical samples at the time of biopsy to ensure an adequate sample. All presurgical cytology samples were reviewed by a cytopathologist in a blinded fashion and all proved to be indeterminate lesions with identical features to the prior outside office FNA. An endocrine pathologist reviewed the histopathology of all specimens. None of the 72 FNA samples collected in vivo for validation were used for model selection or training. Results

A total of 101 indeterminate thyroid FNA samples were included in this study, 29 ex vivo samples in the derivation group and 72 in vivo samples in the validation group (Table 1).

Table 2. Mean \( \Delta C_T \) values in derivation group with 2-tailed t test \( P \) values

<table>
<thead>
<tr>
<th>miRNA</th>
<th>Benign ( \Delta C_T )</th>
<th>Malignant ( \Delta C_T )</th>
<th>( t ) test, ( P ) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>miR328</td>
<td>1.75</td>
<td>2.35</td>
<td>0.38</td>
</tr>
<tr>
<td>miR222</td>
<td>-3.45</td>
<td>-7.73</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>miR21</td>
<td>-6.07</td>
<td>-8.72</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>miR197</td>
<td>-0.99</td>
<td>-2.11</td>
<td>0.11</td>
</tr>
<tr>
<td>miR181a</td>
<td>-4.00</td>
<td>-5.71</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>miR146b</td>
<td>-3.50</td>
<td>-6.95</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Statistical models accurately predict benign versus malignant FNA status within the derivation group

Expression levels of 6 miRNAs with known differential expression in thyroid cancer were measured on 29 samples from the derivation group using RT-PCR (see Methods). \( \Delta C_T \) values were used as expression measures, using RNU6B as housekeeping reference gene. Normalized \( \Delta C_T \) values are shown [Fig. 1A; normalized \( \Delta C_T \) values for the derivation group (A) for each miRNA using a heatmap representation]. In addition, mean \( \Delta C_T \) values for each miRNA for samples in the derivation group are shown in Table 2.

Using the 2-tailed \( t \) test, we found that 4 miRNAs were differentially expressed between malignant and benign lesions; these included miR-222, miR-21, miR-181a, and miR-146b. MiRNA expression of miR-197 and miR-328 was not statistically significantly different (Table 2).

To investigate how to use miRNA expression values to achieve maximum accuracy in predicting benign versus malignant status of indeterminate thyroid lesions on FNA, we developed predictive models using several statistical methodologies, ranging from simple linear approaches (logistic regression) to more complicated nonlinear ones (SVMs with nonlinear kernel functions). Predictive performances were assessed using cross-validation. For each statistical methodology used, we determined whether using subsets of miRNAs instead of all 6 miRNAs improved predictive performances.

The results of these analyses are shown in Table 3. The regression tree methodology provided easily interpretable results as it identifies the combination of \( \Delta C_T \) thresholds that...
best discriminate between malignant and benign lesions. To determine whether methods that use nonlinear combination of miRNA expression values would improve predictive performances, we evaluated QDA and SVMs with a radial basis kernel (SVM-RBF). We found that both approaches selected the same subset of miRNAs (miR-328, miR-222, miR-197, and miR-21) and both approaches had similar and improved performances: 85% to 86% accuracy, 86% to 87% sensitivity, and 85% to 86% specificity. However, SVM-RBF had better performance on the training set (100% accuracy vs. 93% for QDA); therefore, we selected SVM-RBF as the best predictive model.

**Model validation using an independent, in vivo FNA sample set**

We then sought to validate prospectively the performance of our best predictive model (SVM-RBF) using the 4 miRNAs on an independent set of 72 in vivo indeterminate thyroid lesions. Twenty-two lesions were malignant and 50 were benign on final histopathology. Normalized $-\Delta C_t$ values are shown [Fig. 1B; normalized $-\Delta C_t$ values for the validation group (B) for each miRNA using a heatmap representation]. In addition, mean $-\Delta C_t$ values for all samples and each miRNA for the validation group are shown in Table 4. When applied to the $-\Delta C_t$ for all 4 miRNAs the pretrained SVM-RBF model correctly classified 65 of 72 in vivo FNA samples, with 100% sensitivity and 86% specificity for a diagnosis of cancer, for an overall accuracy of 90%. Five of the 7 lesions that our model predicted incorrectly had a diagnosis of Hurthle cell neoplasm on FNA. Three were hyperplastic nodules with oncocytic features and 2 were follicular adenomas with oncocytic features on final patholog-

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**Table 3. Statistical models and their predictive performances in the ex vivo FNA samples**

<table>
<thead>
<tr>
<th>Methodology</th>
<th>miRs selected</th>
<th>% Accuracy training set</th>
<th>% Accuracy cross-validation</th>
<th>% Sensitivity cross-validation</th>
<th>% Specificity cross-validation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Regression tree</td>
<td>miR-21</td>
<td>78</td>
<td>78</td>
<td>69</td>
<td>86</td>
</tr>
<tr>
<td>Logistic regression</td>
<td>miR-21</td>
<td>78</td>
<td>78</td>
<td>69</td>
<td>86</td>
</tr>
<tr>
<td>LDA</td>
<td>miR-21</td>
<td>78</td>
<td>78</td>
<td>69</td>
<td>86</td>
</tr>
<tr>
<td>SVM with linear kernel, cost = 1</td>
<td>miR-21, miR-328</td>
<td>78</td>
<td>78</td>
<td>71</td>
<td>85</td>
</tr>
<tr>
<td>QDA</td>
<td>miR-328, miR-222, miR-21, miR-197</td>
<td>93</td>
<td>85</td>
<td>86</td>
<td>85</td>
</tr>
<tr>
<td>SVM with radial basis kernel, $\gamma = 0.5$, cost = 64</td>
<td>miR-328, miR-222, miR-21, miR-197</td>
<td>100</td>
<td>86</td>
<td>86</td>
<td>87</td>
</tr>
</tbody>
</table>

---

**Table 4. Mean $-\Delta C_t$ values in validation group with 2-tailed $t$ test $P$ values**

<table>
<thead>
<tr>
<th></th>
<th>miR328</th>
<th>miR222</th>
<th>miR21</th>
<th>miR197</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>$-3.38$</td>
<td>$-3.51$</td>
<td>$-5.97$</td>
<td>$-1.27$</td>
</tr>
<tr>
<td>Malignant</td>
<td>$-3.12$</td>
<td>$-7.62$</td>
<td>$-8.61$</td>
<td>$-3.11$</td>
</tr>
<tr>
<td>$t$ test, $P$ value</td>
<td>$&lt;0.05$</td>
<td>$&lt;1e-7$</td>
<td>$&lt;0.001$</td>
<td>$&lt;0.05$</td>
</tr>
</tbody>
</table>

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**Discussion**

We have generated a predictive model to differentiate malignant from benign indeterminate thyroid lesions on 29 FNA samples using 4 miRNAs (miR-222, miR-328, miR-197, and miR-21). This was validated on an independent group of 72 indeterminate lesions, which accurately identified 100% of malignant and 86% of benign indeterminate FNA lesions. The use of molecular markers has recently been advocated to improve classification and diagnosis of indeterminate thyroid nodules on FNA. Several studies have examined the role of mutation analysis in FNA samples and have identified BRAF, RET/PTC, RAS, and PAX8/PPARγ in this context (25–27). Because mutation analysis has been shown to be highly specific for malignancy in indeterminate lesions, several authors have suggested to carry out total thyroidectomy in these lesions (26, 27). Although some studies suggest an improved accuracy for evaluation of indeterminate FNA lesions by mutation analysis, several reports have shown that a large number of these lesions carry no mutations and that many benign lesions also have mutations (25, 28–30). Therefore, mutation analysis alone is insufficient to classify most malignant indeterminate lesions on FNA. Several reports have identified gene panels ranging from 6 to 105 genes with high sensitivity and specificity at differentiating benign from malignant thyroid tissue samples (31–36). Although these findings are encouraging, the predictive accuracy of these gene panels in FNA samples in a real-world setting remains limited, as these were validated on small cohorts of patients and did not analyze samples with indeterminate cytology on FNA.

miRNAs have been identified as important prognostic and diagnostic markers in human cancers over the last decade. One report by Nikiforova and colleagues analyzed the utility of a panel of 7 miRNAs (miR-187, miR-222, miR-
221, miR-146b, miR-155, miR-224, and miR-197) to differentiate benign from malignant thyroid pathology in 62 FNA samples. They found that if one of these miRNAs was upregulated at least 2-fold, the sensitivity of their panel was 88% with a specificity of 94% and an accuracy of 95% (16). However, only 8 of 62 FNA samples had a diagnosis of an indeterminate lesion on cytology. Mazeh and colleagues found that miR-221 had a sensitivity of 95% and a specificity of 100% at differentiating between papillary thyroid carcinoma (PTC) and benign thyroid nodules in 27 FNA samples (37). Furthermore, Chen and colleagues found that miRNAs 222 and 146b were significantly differentially expressed between 20 benign and 20 malignant ex vivo FNA specimens (15). However, neither of these studies used indeterminate thyroid lesions on cytology for analysis.

Kitano and colleagues found that miRNAs 7 and 126 are significantly differentially expressed in thyroid tissue samples with indeterminate cytology, but no FNA samples were used to validate these findings (38). Although these 4 reports have studied and confirmed the utility of miRNA detection in thyroid FNA samples, only one used indeterminate FNA samples for analysis and none looked at the utility of miRNAs in a prospective way using a predictive statistical model. As noted previously, the samples analyzed in the current study were all indeterminate samples on FNA cytology.

While selecting the most appropriate miRNAs for our analysis, we observed that miRNA 146b and miRNA 181a were not useful for our prediction algorithm, which enabled us to narrow our panel down to 4 miRNAs. With the proposed miRNA panel we covered most malignant pathologies present in indeterminate FNA lesions: miR-222 for CPTC and FVPTC, miR-328 and miR-197 for FTC and miR-21 for FVPTC and FTC (16, 19).

The 4 miRNAs that make up the panel seem to all be involved to some extent in cell-cycle control or cell proliferation. miR-222 has been shown to regulate p27kip1 (cyclin-dependant kinase inhibitor), which inhibits G1–S-phase cell-cycle progression and serves as a checkpoint for cell proliferation (18). miR-197 and miR-328 have been shown to target a variety of genes that are involved in cell proliferation and apoptosis (19). miR-21 has been shown to target mRNAs encoding important cell-cycle checkpoints regulators and also to be upregulated in thyroid and lung tumors harboring the RAS mutation (39). Furthermore, one study recently showed that miR-21 targets the thyroid hormone receptor (40), thus downregulating its expression in thyroid cancer cells (40).

In this study, we developed a model that is accurate for differentiating malignant from benign indeterminate lesions on FNA with a specificity of 86%. When Hurthle cell neoplasms were excluded the specificity improved to 95%. This difference in performance may be explained by the fact that the 4 miRNAs that were ultimately selected in the model are not significantly differentially expressed in Hurthle cell tumors. To our knowledge there is no study directly comparing miRNA expression between benign and malignant Hurthle cell tumors. One study previously found that oncogenic follicular adenomas and FTCs have different miRNA expression patterns than their conventional counterparts (16). In addition, our group has previously shown that gene expression profiling of Hurthle cell adenomas is similar to FTCs, suggesting that even benign Hurthle cell lesions seem to have molecular characteristics more closely related to carcinomas than adenomas (35). We believe that Hurthle cell lesions represent a separate entity with a different miRNA expression profile and, therefore, that a separate predictive model should be designed and applied to FNAs with a preoperative diagnosis of "Hurthle cell neoplasm." We are currently actively working on developing such a model.

Because our miRNA panel seems to be 100% sensitive for malignant pathology of indeterminate FNA lesions, it would be reasonable to recommend a total thyroidectomy if malignancy is predicted. Furthermore, our model was also 95% predictive for benign pathology of indeterminate lesions when excluding Hurthle cell lesions. Because the risk of a false negative result was only 5% in those lesions, a diagnostic hemithyroidectomy with its inherent risks and costs might be avoided in patients with benign lesions as predicted by this model.

The application of a panel of 4 miRNAs in daily clinical practice is realistic and feasible and can be carried out in an easy and rapid way using commercially available products and statistical software for classification. FNA specimens are usually low in total RNA and preamplification is often required for further analysis, but RT-PCR of miRNAs may be quicker and easier as it can be carried out with concentrations as low as 1 ng/μL and miRNAs can be extracted from indeterminate lesions with a very high success rate.

This study is the largest reported to date on miRNA analysis of indeterminate thyroid FNA lesions. A limitation of the study, however, is the small sample size of FTCs in the validation group. Although these tumors are uncommon and all FTCs were predicted correctly in our model, larger scale studies will be required to further validate the predictive role of the 4 miRNA panel in indeterminate thyroid lesions in general and more specifically in those harboring a diagnosis of FTC on final pathology.

In summary, we developed a predictive model using 4 miRNAs (miR-222, miR-328, miR-197, and miR-21) that is 100% sensitive and 86% specific for differentiating malignant from benign indeterminate FNA thyroid lesions. When Hurthle cell neoplasms were excluded from the analysis our model had an improved specificity of 95% and an overall accuracy of 97% whereas retaining a sensitivity of 100% for malignant lesions. With further confirmation, application of this model may permit more informed decisions by patients and clinicians when faced with an FNA diagnosis of an indeterminate lesion.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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