A Panel of four MicroRNAs accurately differentiates Malignant from Benign Indeterminate Thyroid Lesions on Fine Needle Aspiration

Xavier M. Keutgen*, Filippo Filicori†, Michael J. Crowley*, Yongchun Wang§, Theresa Scognamiglio†, Rana Hoda†, Daniel Buitrago*, David Cooper**, Martha A. Zeiger§, Rasa Zarnegar*, Olivier Elemento§, Thomas J. Fahey III†

*Division of Endocrine Surgery, Department of Surgery,
†Department of Pathology,
‡Institute for Computational Biomedicine,
New York Presbyterian Hospital – Weill Cornell Medical Center, 525 E 68th Street, Room A-1027, New York, NY, 10021

§Section of Endocrine Surgery, Department of Surgery,
**Division of Endocrinology, Department of Medicine,
The Johns Hopkins University School of Medicine, 600 N. Wolfe St, Baltimore, MD, 21287

Running Title: MicroRNAs and Fine Needle Aspiration

Keywords: MicroRNA, Indeterminate Lesion, Fine Needle Aspiration, Thyroid Cancer, Support Vector Machine

Accepted for presentation at the 97th Annual Meeting of the American College of Surgeons in San Francisco (CA), October 2011 and at the New York Surgical Society Annual Meeting, New York (NY), November 2011

Word Count – 3030, 4 Tables, 1 Figure

Conflict of Interest: The authors have no financial or personal relationships with other people or organizations that could inappropriately influence their actions

Role of the funding source: Dancer’s Care Foundation

Corresponding Author: Thomas J Fahey III MD, Dept. of Surgery, Weill Cornell Medical Center, 525 E 68th Street, Room A-1027, New York, NY, 10021, Email: tfahey@med.cornell.edu, Tel: 212-746-5187
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 pathological diagnosis.

Experimental Design: A predictive model was derived using 29 ex-vivo indeterminate thyroid lesions on FNA to differentiate benign from malignant 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 RT-PCR. A statistical model was developed using the Support Vector Machine (SVM) approach.

Results: A SVM model with 4 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 benign from malignant 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.
STATEMENT OF 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 four MicroRNAs (miR-222, 328, 197 and 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%. Based on these results it would be reasonable to recommend a total thyroidectomy if malignancy is predicted using our model. Additionally, a diagnostic hemithyroidectomy might be avoided in patients with benign lesions as predicted by this panel.
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 70-80% of cases, while the remaining 20-30% 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 since 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, since 50-80% 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 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.

MicroRNAs (miRNA) are single stranded non-coding small RNA segments, 19-23 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). MicroRNAs have been reported to be dysregulated in virtually all human cancer types, including all variants of thyroid cancer (9-11). MicroRNAs 222, -181a and -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 miRNAs 328, -197 and -21 are differentially expressed when comparing Follicular Thyroid Cancer (FTC) and Follicular Variant of Papillary Thyroid Cancer (FVPTC) to normal tissue and follicular adenomas (FA) (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 benign from malignant 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 (IRB) of Weill Cornell Medical College was obtained, written informed consent was collected from patients for the use of clinical specimens for research. All hemi and total thyroidectomies performed at our institution for indeterminate lesions were reviewed from 2005 to 2010. 181 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 in order 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 performed (Table 1) (Supplemental Table 1).

FNA Sampling and Data Collection

After thyroidectomy was performed, 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., Valencia, CA, USA) or Trizol (Invitrogen, Carlsbad, CA), 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 cytological cases were included in this study. Final diagnosis, FNA diagnosis, age, sex, tumor size and location, FNA location, surgical procedure, extrathyroidal extension, angiolympathic invasion and lymph node metastasis were entered for each patient sample into the tumor bank data sheet.

MicroRNA Selection, Extraction, Reverse Transcription and Real-Time-PCR

A systematic search of the databases PubMed, Ovidsp, and Google Scholar was performed 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 FA and CPTC, FVPTC and FTC and ultimately selected 6 miRNAs for further analysis (11-19).

MicroRNA levels of mir-328, mir-222, mir-197, mir-181a, mir-146b and mir-21 were determined in FNA specimens using Real-Time-PCR. MicroRNAs were extracted and reverse
transcribed to cDNA according to the standard protocol using the MirVana Kit (Ambion Inc.), the mirPremier Kit (Sigma Aldrich, St Louis MO) or the microRNA Purification Kit (Norgen Biotek Corp, Canada). Quantity and integrity of microRNA yield was assessed using the NanoDrop™ (NanoDrop Technologies, Willmington, DE) and Bioanalyzer 2100 and RNA 6000 Nano/Pico LabChip® (Agilent Technologies, Palo Alto, CA). This method yielded RNA concentrations from 2 to 184 ng/ul. Reverse transcription was performed for each microRNA (TaqMan MicroRNA gene-expression array kit, Applied Biosystems, Inc) using 10ng for 15μl of reverse transcription reaction. 1.33μl of cDNA was then used for each 20μl PCR reaction. A quantitative reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay was used to measure miRNA levels using the TaqMan MicroRNA gene-expression assay kit (Applied Biosystems, Foster City, CA, Part Number: 4427975; Assay IDs: 001093, 002276, 000543, 000543, 000543). RT-PCR was performed using an ABI PRISM 7000 Sequence Detection System. A total of 45 cycles of amplification were performed, each cycle consisting of 15s at 95°C and 1min at 60°C according to the standard protocol. Control amplification was performed in all samples in triplicate. RNU6B was used as a housekeeping gene and ΔCt values were used for data analysis.

**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
non-linear manner, these approaches find decision surfaces that best separate malignant and benign lesions.

Support Vector Machines 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. Since 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 non-linear 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 Support Vector Machines 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 performed 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) (Supplemental Table 2). This benign to malignant sample ratio is consistent with the ratio reported in the literature for indeterminate FNA lesions, namely 20-30% for malignant and 70-80% for benign pathology (22-26). Sixteen indeterminate FNA samples were obtained from Johns Hopkins Hospital, of which 3 were malignant and 13 were benign on final pathology. Thirty-three samples were obtained in surgery clinic and thirty-nine FNA samples were obtained in the operating room prior to the surgical incision. All samples were obtained under identical conditions using ultrasound guidance. Cytologic smears were prepared for all pre-surgical samples at the time of biopsy to assure an adequate sample. All pre-surgical 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).
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). \( \Delta \)CTs were used as expression measures, using RNU6B as housekeeping reference gene. Normalized \( -\Delta \)CTs are shown in Figure 1A (Normalized \( -\Delta \)CTs for the Derivation Group (A) and Validation Group (B) for each miRNA using a heatmap representation). Additionally, mean \( \Delta \)CT values for each miRNA for samples in the derivation group are shown in Table 2.

Using non-parametric Wilcoxon rank-sum tests, we found that four miRNAs were differentially expressed between malignant and benign lesions; these included miR-222 (\( p<0.005 \)), miR-21 (\( p<0.03 \)), miR-181a (\( p<0.04 \)) and miR-146b (\( p<0.03 \)). MiRNA expression of mir-197 (\( p=0.3 \)) and mir-328 (\( p=0.6 \)) was not statistically significantly different.

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 non-linear ones (Support Vector Machines with non-linear 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 since it identifies the combination of \( \Delta \)CT thresholds that best discriminate between malignant and benign lesions. To determine whether methods that use non-linear combination of miRNA expression values would improve predictive performances, we
evaluated quadratic discriminant analysis (QDA) and Support Vector Machines 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: 85-86% accuracy, 86-87% sensitivity and 85-86% specificity. However, SVM-RBF had better performance on the training set (100% accuracy versus 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 -ΔCTs are shown in Figure 1B (Normalized -ΔCTs for the Derivation Group (A) and Validation Group (B) for each miRNA using a heatmap representation). Additionally, mean ΔCT values for all samples and each miRNA for the validation group are shown in Table 4. When applied to the -ΔCT for all 4 miRNAs the pre-trained SVM-RBF model correctly classified 65 out 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 seven 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 pathology. When all 13 Hurthle cell neoplasms were excluded, the predictive performance of our model improved dramatically with 100% sensitivity, 95% specificity 97% overall accuracy in differentiating malignant from benign indeterminate lesions on FNA.

**DISCUSSION**
We have generated a predictive model to differentiate benign from malignant indeterminate thyroid lesions on 29 FNA samples using 4 miRNAs (miR-222, 328, 197, 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). Since mutation analysis has been shown to be highly specific for malignancy in indeterminate lesions, several authors have suggested performing 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, since these were validated on small cohorts of patients and did not analyze samples with indeterminate cytology on FNA.

MicroRNAs have been identified as important prognostic and diagnostic markers in human cancers over the last decade. One report by Nikiforova et al. analyzed the utility of a panel of 7 miRNAs (miRNAs 187, 222, 221, 146b, 155, 224 and 197) to differentiate benign from malignant thyroid pathology in 62 FNA samples. They found that if 1 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 the 62 FNA samples had a diagnosis of an indeterminate lesion on cytology. Mazeh et al. found that mir-221 had a sensitivity of 95% and a specificity of 100% at differentiating between PTC and benign thyroid nodules in 27 FNA samples (37). Furthermore, Chen et al. 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 et al. 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 four 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 FTC and mir-21 for FVPTC and FTC (16, 19).

The four miRNAs that make up the panel appear to all be involved to some extent in cell cycle control or cell proliferation. MicroRNA-222 has been shown to regulate p27\textsuperscript{kip1} (cyclin-dependant kinase inhibitor), which inhibits G1-S phase cell-cycle progression and serves as a checkpoint for cell proliferation (18). MicroRNA 197 and 328 have been shown to target a
variety of genes that are involved in cell proliferation and apoptosis (19). MicroRNA 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 demonstrated 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 benign from malignant 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 oncocytic FAs and FTCs have different miRNA expression patterns than their conventional counterparts (16). In addition, our group has previously demonstrated that gene expression profiling of Hurthle Cell adenomas is similar to FTCs, suggesting that even benign Hurthle Cell lesions appear to have molecular characteristics more closely related to carcinomas than adenomas (34). 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.

Since our miRNA panel appears 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. Since 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 four miRNAs in daily clinical practice is realistic and feasible and can be performed in an easy and rapid way using commercially available products and statistical software for classification. FNA specimens are usually low in total RNA and pre-amplification is often required for further analysis, but RT-PCR of miRNAs may be quicker and easier since it can be performed with concentrations as low as 1ng/µl and miRNA is 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, 328, 197 and 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% while 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.
REFERENCES


Table 1. Demographics and Pathological 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><strong>Age (Mean±SD)</strong></td>
<td>50±15</td>
<td>55±16</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>3</td>
<td>13</td>
</tr>
<tr>
<td>Female</td>
<td>26</td>
<td>59</td>
</tr>
<tr>
<td><strong>Tumor Size in cm (Mean±SD)</strong></td>
<td>2.7±1.7</td>
<td>2.8±1.7</td>
</tr>
<tr>
<td><strong>Range</strong></td>
<td>0.8-6</td>
<td>0.7-7.4</td>
</tr>
<tr>
<td><strong>Surgery</strong></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><strong>FNA Pathology</strong></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><strong>Final Pathology</strong></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>Hurthle 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><strong>Thyroiditis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>19</td>
</tr>
</tbody>
</table>

*Classification according to The NCI Thyroid Fine-Needle-Aspiration State of the Science Conference Scheme; CPTC: Classic Variant of Papillary Thyroid Cancer, FVPTC: Follicular Variant of Papillary Thyroid Cancer, FTC: Follicular Thyroid Cancer, FA: Follicular Adenoma
Table 2: Mean ΔCt in Derivation group with two-tailed t-test p-values

<table>
<thead>
<tr>
<th></th>
<th>mir328</th>
<th>mir222</th>
<th>mir21</th>
<th>mir197</th>
<th>mir181a</th>
<th>mir146b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benign</td>
<td>-1.75</td>
<td>-3.45</td>
<td>-6.07</td>
<td>-0.99</td>
<td>-4.00</td>
<td>-3.50</td>
</tr>
<tr>
<td>Malignant</td>
<td>-2.35</td>
<td>-7.73</td>
<td>-8.72</td>
<td>-2.11</td>
<td>-5.71</td>
<td>-6.95</td>
</tr>
<tr>
<td>t-test (p-value)</td>
<td>0.38</td>
<td>&lt;0.001</td>
<td>&lt;0.01</td>
<td>0.11</td>
<td>&lt;0.05</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>
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>

* Linear Discriminant Analysis, ^ Support Vector Machines, † Quadratic Discriminant Analysis
Table 4: Mean ΔCt in Validation group with two-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>-1.38</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>
Figure 1. Normalized -ΔCTs for the Derivation Group (A) and Validation Group (B) for each miRNA using a heatmap representation.
Figure 1

A) Derivation cohort

Malignant (n=14)  Benign (n=15)

2.0 High

miRNA expression (normalized –ΔCT)

-2.0 Low

B) Validation cohort

Malignant (n=22)  Benign (n=50)

Pathology

miRNA expression

mir328

mir222

mir21

mir197

mir181a

mir146b
Clinical Cancer Research

A Panel of four MicroRNAs accurately differentiates Malignant from Benign Indeterminate Thyroid Lesions on Fine Needle Aspiration

Xavier M. Keutgen, Filippo Filicori, Michael J. Crowley, et al.

Clin Cancer Res  Published OnlineFirst February 20, 2012.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-11-2487

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2012/02/20/1078-0432.CCR-11-2487.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.