Molecular classification of thyroid lesions by combined testing for miRNA gene expression and somatic gene alterations

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Abstract

Multiple molecular markers contribute to the pathogenesis of thyroid cancer and can provide valuable information to improve disease diagnosis and patient management. We performed a comprehensive evaluation of miRNA gene expression in diverse thyroid lesions (n = 534) and developed predictive models for the classification of thyroid nodules, alone or in combination with genotyping. Expression profiling by reverse transcription-quantitative polymerase chain reaction in surgical specimens (n = 257) identified specific miRNAs differentially expressed in 17 histopathological categories. Eight supervised machine learning algorithms were trained to discriminate benign from malignant lesions and evaluated for accuracy and robustness. The selected models showed invariant area under the receiver operating characteristic curve (AUC) in cross-validation (0.89), optimal AUC (0.94) in an independent set of preoperative thyroid nodule aspirates (n = 235), and classified 92% of benign lesions as low risk/negative and 92% of malignant lesions as high risk/positive. Surgical and preoperative specimens were further tested for the presence of 17 validated oncogenic gene alterations in the BRAF, RAS, RET or PAX8 genes. The miRNA-based classifiers complemented and significantly improved the diagnostic performance of the 17-mutation panel (p < 0.001 for McNemar’s tests). In a subset of resected tissues (n = 54) and in an independent set of thyroid nodules with indeterminate cytology (n = 42), the optimized ThyraMIR Thyroid miRNA Classifier increased diagnostic sensitivity by 30–39% and correctly classified 100% of benign nodules negative by the 17-mutation panel. In contrast, testing with broad targeted next-generation sequencing panels decreased diagnostic specificity by detecting additional mutations of unknown clinical significance in 19–39% of benign lesions. Our results demonstrate that, independent of mutational status, miRNA expression profiles are strongly associated with altered molecular pathways underlying thyroid tumorigenesis. Combined testing for miRNA gene expression and well-established somatic gene alterations is a novel diagnostic strategy that can improve the preoperative diagnosis and surgical management of patients with indeterminate thyroid nodules.

Keywords: thyroid cancer; thyroid nodule; diagnosis; miRNA; gene mutation; gene rearrangement

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Introduction

Much progress has been made over the past 30 years in understanding the molecular pathogenesis of thyroid cancer. The majority of thyroid carcinomas involve activating somatic mutations in genes encoding effectors of the MAPK and PI3K/AKT signaling pathways [1,2]. Point mutations in the BRAF gene are relatively frequent in papillary thyroid carcinomas (PTC) while RAS mutations are typically found in follicular variants of PTC (FvPTC), follicular thyroid carcinomas (FTC) and follicular adenomas (FA). Chromosomal rearrangements resulting in oncogenic gene fusions, such as the RET/CCDC6 inversion in PTC or the PAX8/PPARG translocation in FvPTC and follicular neoplasms, are another hallmark of thyroid cancer [1,2]. Gene mutations occurring at lower frequency in the PI3K/AKT pathway (eg AKT1, PIK3CA, PTEN) and other signaling pathways (eg CTNNB1, TP53, TSHR) as well as accumulation of alterations with synergistic oncogenic effects leading to cancer progression have also been reported [1,2].
Recent advances further suggest that reactivation of telomerase activity through TERT promoter mutations is another cooperative driver of progression and aggressiveness in thyroid cancer [3,4].

Molecular testing for somatic gene alterations involved in tumorigenesis has considerably improved the preoperative diagnosis and surgical management of thyroid nodules [5–8]. Although nodules are frequent in adults, in particular in patients older than 60 years of age, only 5% of cases are ultimately diagnosed as malignant after surgical resection [9]. Approximately 50% of thyroid cancers are initially identified by imaging and cytological evaluation of fine-needle aspiration (FNA) biopsies [10]. Nodules with an indeterminate cytological diagnosis have a highly variable residual risk of cancer and require additional risk stratification to rule in or rule out cancer [10,11]. Because oncogenic gene alterations are in most cases highly specific for thyroid cancer, a nodule with a positive molecular result is associated with an increased risk of malignancy [5,6]. Such tests with high specificity and positive predictive value (PPV) are referred to as ‘rule in’ tests.

Altered signaling pathways ultimately lead to changes in the expression of genes regulating thyroid cell growth, proliferation and survival, as well as angiogenesis, invasion and metastasis [2]. These changes may be used as surrogate signatures to identify tumors with activated pathways [12]. Based on microarray expression data in 220 surgical specimens and 234 preoperative nodule aspirates, a proprietary diagnostic algorithm involving 167 genes and seven classification signatures has been developed for the preoperative diagnosis of indeterminate thyroid nodules [13,14]. This gene expression classifier was optimized for high sensitivity at the detriment of specificity and therefore cannot be used to rule in cancer. It can, however, identify and rule out approximately 50% of benign nodules with high negative predictive value (NPV) [13].

Another class of gene expression marker with diagnostic potential is microRNA (miRNA). miRNAs are small, non-coding RNAs that regulate fundamental cellular processes underlying cancer development and progression [15]. A large number of studies have shown that the expression of specific oncomiRs is deregulated in the most common types of differentiated thyroid cancer such as PTC [16–19], FvPTC [20–23] and FTC [22–26]. Similar to mRNA signatures, changes in miRNA expression can be assessed in thyroid nodule aspirates [27–30] and may be associated with the presence of activating gene mutations [18,20,22,30]. Although a systematic large-scale evaluation has not been performed to date, the bulk of these data suggest that classification algorithms based on miRNA expression may have clinical utility to rule in or rule out cancer in preoperative thyroid nodules with indeterminate cytology.

Recent integrated genomic studies have shown that thyroid cancer is more genetically diverse than previously recognized, with multiple layers of biomarkers contributing to the pathological genotypes and phenotypes [31]. The logical extension of this recognition is that a molecular test assessing this complexity should improve current tests based on a single molecular dimension. In this study, we performed a comprehensive evaluation of the diagnostic potential of known miRNA biomarker candidates, developed supervised machine learning classification algorithms and assessed their performance, alone or in combination with genotyping for known gene alterations.

Materials and methods

Thyroid specimens

Surgical specimens were obtained from the University of Michigan pathology archives under a research protocol approved by the university’s institutional review board. Thyroid tissues were histologically reviewed by a single expert pathologist (TJG) and classified according to current World Health Organization schemes. Remnant nucleic acids samples from preoperative thyroid nodule FNAs from 14 endocrinology centres across the United States were archived by Asuragen’s clinical laboratory under a research protocol approved by a central investigational review board. The initial set of 235 aspirates consisted of 118 nodules with benign cytology, including 13 with surgical outcome (12 benign, 1 malignant), 73 with malignant cytology, including 51 with surgical outcome (1 benign, 50 malignant), and 44 with indeterminate cytology, all with available surgical outcome. The second set of aspirates consisted of 42 distinct nodules with indeterminate cytology and surgical outcome.

Molecular assays

Total nucleic acids were extracted and tested for 17 gene alterations using the Luminex 200 System (Luminex, Austin, TX) and for miRNA expression using custom Pick-&-Mix microRNA PCR Panels (Exiqon, Vedbaek, Denmark) as previously described [5,32,33]. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for each target miRNA...
were performed in duplicate. The 31 miRNA candidates were selected based on literature review and on differential expression analyses performed using GeneChip miRNA Arrays v2 (Affymetrix, Santa Clara, CA), 122 benign or malignant resected thyroid lesions (Asterand, Detroit, MI) and blood samples from healthy donors. Genotyping of over 1500 unique gene alterations was performed using a custom sequencing assay [34,35].

Data analyses

Graphic and statistical analyses were performed in Excel (Microsoft Corp, Redmond, WA) or R (http://

Figure 1. miRNA expression profiling in resected thyroid tissues. (A) Unsupervised hierarchical clustering for histopathological categories represented by at least five independent specimens (n = 248 specimens total). The expression levels of 18 miRNAs as determined by RT-qPCR are colour-coded from low expression (high Ct, red) to high expression (low Ct, blue) after mean-centering normalization. The presence of gene alterations in the BRAF, RAS, PAX8 or RET genes detected with the Luminex platform is colour-coded at the top of the figure. (B) Principal component (PC) analysis using the same miRNAs and sample set. For clarity, the colour-coded markers corresponding to oFA, FTC, oFTC and PDC specimens are not displayed in the left panel. Abbreviations: HN, hyperplastic nodule (includes diffuse hyperplasia); CLT, chronic lymphocytic thyroiditis; FA, follicular adenoma; oFA, oncocytic FA; FTC, follicular thyroid carcinoma; oFTC, oncocytic FTC; PTC, papillary thyroid carcinoma (includes conventional and tall cell variants); FvPTC, follicular variant of PTC; PDC, poorly differentiated carcinoma; ATC, anaplastic thyroid carcinoma.

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miRNA expression data (cycle threshold or Ct) were normalized by mean-centering. For m miRNA evaluated in a given sample, the mean miRNA expression level \(1/m \times \sum_{i=1}^{m} C_{ti}\) was subtracted from each individual raw Ct. For hypothesis testing, p values were calculated using Welch’s t-test and adjusted using the Benjamini-Hochberg procedure. The tests were considered significant when the adjusted p values were smaller than a false discovery rate set at 5% \(Q\) value < 0.05. The predictive performance of the algorithms trained by supervised machine learning was assessed using the area under the receiver operating characteristic curve (AUC). The probability scores generated by classifiers A and B were calculated using raw Ct results. Given an intercept \(c_0\), coefficients \(c_i\) and raw Ct values \(x_i\), the score of each classifier with \(m\) miRNA interrogated was calculated as \(1/(1+e^{c_0+\sum_{i=1}^{m} c_i x_i})\). Diagnostic sensitivity and specificity were calculated by comparing qualitative binary molecular test results (positive/high risk or negative/low risk) to the reference pathology diagnoses (benign or malignant). PPV and NPV were estimated using sensitivity, specificity, thyroid cancer prevalence and the Bayes theorem. Additional information is available in the Supplemental Materials and Methods.

**Results**

miRNA expression profiling in thyroid tissues

The expression levels of 31 target miRNAs were determined by RT-qPCR in 257 unique surgical specimens representing 17 histopathological diagnostic categories. Unsupervised hierarchical clustering and principal component analyses confirmed highly variable miRNA expression profiles with distinct expression patterns in various benign or malignant lesions (Figure 1A and 1B). Almost all PTC separated from the other samples along the first principal component with FvPTC clustering between the PTC and follicular carcinomas (ATC) clearly separated from the other samples along the second component, consistent with their highly distinct histology. The chronic lymphocytic thyroiditis (CLT) cases formed a very tight cluster between the follicular cell-derived tumours and the ATC.

### Differential miRNA expression in thyroid tissues

Statistical hypothesis testing identified specific miRNAs differentially expressed in malignant versus benign samples (Table 1). There were 13 miRNAs overexpressed in thyroid carcinoma by more than one Ct on average (effect size >1) and with a significant adjusted p value \(Q\) value < 0.05), mainly miR-31-5p, -146b-3p/-5p, -221-3p, -222-3p and -551-3p. Conversely, there were only three miRNAs significantly over expressed in benign thyroid lesions, miR-96-5p, -19b and -204-5p. Similar analyses using various combinations of histopathological categories showed that specific biomarker candidates such as miR-221-3p/-5p, -222-3p and -551-3p were differentially expressed in both PTC and FTC relative to benign lesions (Supplemental Table 1). Other, such as miR-31-5p, -146b-3p/-5p and -375, were statistically significant only in PTC, including FvPTC.

#### Table 1. Differential miRNA expression in benign versus malignant resected tissues

| miRNA     | Effect size | p value | Q value | AUC  |
|-----------|-------------|---------|---------|------|
| miR-138-1-3p | -0.77       | 1.1 E -03 | 1.7 E -03 | 0.64 |
| miR-139-5p  | -0.40       | 3.8 E -02 | 0.051   | 0.58 |
| miR-141-3p  | 0.04        | 0.904    | 0.904   | 0.53 |
| miR-144-5p  | 0.29        | 0.288    | 0.308   | 0.51 |
| miR-146b-3p | 3.75        | 5.4 E -28 | 1.7 E -26 | 0.83 |
| miR-146b-5p | 3.78        | 3.8 E -24 | 5.9 E -23 | 0.80 |
| miR-151-3p  | 0.51        | 6.5 E -03 | 9.6 E -03 | 0.57 |
| miR-155-5p  | 0.39        | 0.069    | 0.088   | 0.55 |
| miR-15a-5p  | 0.73        | 2.7 E -05 | 5.9 E -05 | 0.64 |
| miR-181a-2-3p| 1.14        | 4.3 E -07 | 1.1 E -06 | 0.68 |
| miR-187     | 1.73        | 2.8 E -08 | 7.9 E -08 | 0.72 |
| miR-197-3p  | 0.58        | 1.0 E -04 | 1.8 E -04 | 0.62 |
| miR-19b     | 0.47        | 8.3 E -03 | 1.2 E -02 | 0.57 |
| miR-204-5p  | -1.14       | 9.6 E -05 | 1.8 E -04 | 0.65 |
| miR-21-5p   | 1.22        | 2.6 E -09 | 1.0 E -08 | 0.72 |
| miR-221-3p  | 1.90        | 8.8 E -13 | 4.5 E -12 | 0.75 |
| miR-221-5p  | 1.69        | 9.9 E -10 | 4.4 E -09 | 0.73 |
| miR-222-3p  | 1.98        | 2.4 E -16 | 1.8 E -15 | 0.78 |
| miR-222-5p  | 1.20        | 2.8 E -06 | 6.7 E -06 | 0.68 |
| miR-224-5p  | 1.12        | 1.2 E -08 | 3.8 E -08 | 0.70 |
| miR-29b-1-5p| 0.17        | 0.300    | 0.309   | 0.51 |
| miR-29b-3p  | 0.55        | 4.9 E -03 | 7.6 E -03 | 0.61 |
| miR-31-5p   | 2.29        | 4.0 E -14 | 2.5 E -13 | 0.75 |
| miR-328     | 0.30        | 0.100    | 0.119   | 0.54 |
| miR-34a     | 0.69        | 7.0 E -05 | 1.5 E -04 | 0.64 |
| miR-375     | 2.34        | 1.0 E -08 | 3.4 E -08 | 0.70 |
| miR-424-5p  | 0.36        | 0.125    | 0.143   | 0.54 |
| miR-551b-3p | 3.32        | 8.8 E -24 | 9.1 E -23 | 0.82 |
| miR-7-5p    | -1.42       | 1.5 E -04 | 2.6 E -04 | 0.65 |
| miR-885-5p  | 0.45        | 0.071    | 0.088   | 0.57 |
| miR-96-5p   | 0.37        | 0.164    | 0.181   | 0.55 |

AUC, area under the receiver operating characteristic curve.
miR-21-5p was highly expressed in all thyroid specimens, in particular in cPTC/TcPTC and ATC (see also Figure 1A), but was not significant in FvPTC ($Q < 0.69$) and was barely significant in FTC ($Q < 0.025$) when compared to benign lesions. ATC samples had miRNA expression patterns mostly similar to other carcinomas but were characterized by high expression levels of miR-155-5p and low levels of miR-141-3p and -375 (see also Figure 1A). The five poorly differentiated carcinomas (PDC) were distributed along the first principal component (Figure 1B) and out of 11 miRNAs with an effect size >1 $Ct$, only miR-96-5p and miR-139-5p were statistically significant relative to benign lesions ($Q = 0.024$ and 0.0028, respectively). Interestingly, all miRNAs were expressed at lower levels in CLT samples relative to all other samples combined, with the exception of miR-155-5p that was over expressed by 1.7 $Ct$ on average ($Q = 3.1 \times 10^{-10}$).

miRNA classifiers development

Predictive models to discriminate benign from malignant thyroid lesions were trained on surgical specimens (Supplemental Figure 1) and further tested in an independent set of 235 representative thyroid nodule FNAs. Principal component analyses confirmed that miRNA expression profiles were similar in surgical and preoperative specimens for both benign and malignant lesions (Supplemental Figure 2). miR-187, -222-5p, -224-5p and -885-5p were excluded from model training because of the lack of reliable detection ($Ct > 35$) in various histopathology categories and nodule FNAs. In addition, eight miRNAs over expressed in blood relative to thyroid tissue, that is, for which potential blood contamination in a nodule aspirate could interfere with small but diagnostically relevant variations in miRNA expression, were excluded from model training (Figure 2; miR-7-5p, -15a-5p, -19b, -96-5p, -151-3p, -144-5p, -197-3p and -424-5p). Classification models were built using the 19 remaining miRNAs and eight supervised learning methodologies (Table 2). Diagonal linear discriminant analysis (DLDA) proved very robust with invariant AUC calculated on specimen scores predicted under leave-one-out cross-validation in the training set and with optimal performance in the nodule FNA set. In addition, DLDA resulted in the highest correlation between duplicates, indicating that this classification model was less sensitive to small-to-moderate variations in $Ct$ signal.
Classification of benign and malignant lesions

Based on the above results, two DLDA classifiers were selected for further evaluation. Each classifier generated a probability score between 0 and 1 that could be further interpreted as high risk for malignancy (score ≥ 0.5) or low risk for malignancy (score < 0.5). There was 100% qualitative agreement between the resubstituted and cross-validated scores in the training set with an overall score variability of ±1.37% (Supplemental Figure 3). The classifier A, consisting of miR-138-1-3p, -146b-5p, -155, -204-5p and -375, correctly identified 81 of 130 malignant cases (62%) as high risk and 103 of 110 benign cases (94%) as low risk (Table 3). The classifier B interrogated the same miRNAs plus miR-29b-1-5p, -31-5p, -139-5p, -222-3p, and -551b-3p. It classified only 64 benign cases (58%) in the low risk category but correctly identified 118 malignant cases (91%) as high risk. Similar distribution of molecular results was observed in the independent set of thyroid nodule FNAs (Table 3). Overall, the classifier A efficiently identified 92% (233/252) of benign lesions as low risk while the classifier B efficiently identified 92% (205/223) of malignant lesions as high risk.

Combination with mutation testing

All specimens were also tested for the presence of 17 well-characterized oncogenic gene alterations in the BRAF, RAS, RET or PAX8 genes using the Luminex platform [5,33]. A correlation between genotype, miRNA expression and histology was observed in the resected specimen set (Figure 1A). Gene alterations were distributed as expected, with BRAF and RET-PTC detected mainly in carcinomas with true papillary architecture (cPTC and TcPTC) and RAS and PAX8-PPARG detected mainly in FvPTC and follicular neoplasms. In the training set, 55% (71/130) of the malignant cases were mutation-positive and 86% (95/110) of the benign cases were negative (Table 3). Mutation and miRNA testing identified overlapping but distinct subsets of thyroid lesions. Among the 59 mutation-negative malignant specimens, classifier A identified 26 cases as high risk (44%) and classifier B identified 51 cases as high risk (86%). The cancer detection rate was significantly increased from 55% for mutation testing alone to 75% for mutation testing combined with classifier A (97/130; \( p = 9.4E-07 \) for McNemar test) or 94% for mutation testing combined with classifier B (122/130; \( p = 2.5E-12 \)). Similar results were obtained with preoperative thyroid nodule FNAs (Table 3). Mutation testing correctly identified 70% (65/93) of malignant nodules and the detection rate was increased to 95% (88/93, \( p = 4.5E -06 \)) for mutation testing combined with classifier B. In both the resected and FNA sample sets, the majority of mutation-negative benign specimens were correctly identified as low risk by classifier A (219/
227 or 96%), that is, the benign detection rate for combined testing was only slightly reduced compared to that of mutation testing alone (87% [219/252] versus 90% [227/252], \( p = 0.013 \)).

Comparison with high-throughput targeted sequencing

An optimized miRNA classification algorithm, commercially available as the ThyraMIR Thyroid miRNA Classifier (Interpace Diagnostics Group Inc., Parsippany, NJ) [32], was next evaluated in parallel to a targeted next-generation sequencing (NGS) assay interrogating mutation hotspots in 20 genes and 46 aberrant transcripts (Supporting Information Table 2). In a subset of resected thyroid tissues negative by the 17-mutation panel \( (n = 54) \), additional gene alterations were detected by NGS in 13 out of 36 malignant cases, corresponding to a potential increase in diagnostic sensitivity of 36% relative to the 17-mutation panel (Figure 3A). With ThyraMIR, 14 of the malignant cases were classified as high risk/positive (39% increase in sensitivity relative to the 17-mutation panel), including five specimens negative by NGS (Supplemental Figure 4). None of the 18 benign lesions was high risk by miRNA testing (100% specificity), while seven cases were positive by NGS, corresponding to a potential decrease in diagnostic specificity of 39%. The distribution of molecular results was similar in a set of 42 thyroid nodule FNAs with indeterminate cytology (Bethesda categories III or IV) that had not been used for classifier development (Supplemental Figure 4). Both NGS and miRNA testing improved the diagnostic sensitivity of the 17-mutation panel but NGS decreased its specificity by detecting additional gene alterations in 19% of benign cases (Figure 3B). Among 30 nodules negative by the 17-mutation panel, ThyraMIR correctly classified 67% of malignant cases as high risk/positive and 100% of benign cases as low risk/negative. Testing for miRNA expression combined with genotyping for 17 well-characterized gene alterations resulted in optimal diagnostic sensitivity (85%) and specificity (95%).

**Figure 3.** Comprehensive molecular characterization of thyroid lesions by miRNA expression and mutation testing. (A) Resected thyroid tissues. The graph shows the diagnostic specificity (Sp) and sensitivity (Se) for the panel of 17 gene alterations detected with the Luminex platform (17-panel), next-generation sequencing at 5% allele variant threshold (NGS 5%), the ThyraMIR test (miRNA), and for combination testing with the 17-mutation panel and ThyraMIR in 54 resected tissues all negative with the 17-mutation panel (100% specificity, 0% sensitivity). (B) Thyroid nodule aspirates. Same analysis for molecular testing performed in 42 aspirates from nodules with an indeterminate cytology diagnosis of atypia of undetermined significance/follicular lesion of undetermined significance (Bethesda category III) or follicular neoplasm/suspicious for a follicular neoplasm (Bethesda category IV).

**Discussion**

In the present study, we interrogated miRNA expression in a large and histologically diverse set of thyroid lesions and applied rigorous statistical analyses to the development of miRNA classification algorithms that discriminate between benign and malignant lesions. We show that multidimensional molecular analyses for miRNA expression, mRNA fusions and DNA mutations improve the predictive classification of thyroid lesions. Our data underline the relevance of miRNAs in the pathology of thyroid cancer and the value of combination testing in the preoperative risk-based assessment of thyroid nodules with indeterminate cytology.

Ultrasound-guided FNAs are the sample of choice for the diagnostic evaluation of thyroid nodules but present specific limitations for the development of a gene expression classifier. Those limitations include the imperfect traceability to the reference diagnosis,
the unknown proportion of representative follicular cells in specimens dedicated to molecular testing and classifier training, and the potential contamination by other cellular components that may bias the expression levels of the biomarkers of interest. The latter is particularly significant for blood-containing thyroid biopsies. For example, our data indicate that miR-7-5p and miR-144-5p, two miRNAs differentially expressed in FTC [22–24,26], would not be dependable in a diagnostic FNA contaminated with a few percent of blood. Based on strict design requirements for test robustness and reliability, we elected to train our miRNA classification models using exclusively well-characterized surgical specimens after exclusion of all the miRNAs that could interfere with the diagnostic signature ($n = 8$) or generate high signal variability or test failure ($n = 4$).

Following cross-validation and evaluation of performance in an independent set of preoperative FNAs, this approach enabled us to select the most robust learning algorithm. Interestingly, algorithms with the highest initial AUC in the training set (random forest and support vector machine) showed the strongest drop in predictive performance after migration to FNA, suggesting that these statistical models overfitted the training data set. Overfitting is a well-known problem in machine learning where overtraining complex algorithms on noisy features or training on a sample set too small or not diverse enough can lead to high training set performance that will not generalize well to a new data set [36]. Significant decreases in predictive accuracy have previously been reported for miRNA classifiers that had optimal performance during training [27,30]. In contrast, we chose our DLDA classification model not based on the highest AUC during initial training but based on steady performance under leave-one-out cross-validation and after testing in an independent set of nodule FNAs.

DLDA is a relatively simple linear classification model that assumes a common diagonal feature covariance matrix for the different classes to be predicted and is known to yield low error rates in situations where more complex predictors may suffer from overfitting [37]. The parameters of the statistical model, estimated using the maximum likelihood method, can be easily constrained so that the sum of the model coefficients attributed to each miRNA predictor is equal to zero (built-in normalization). The resulting classifier A contained two miRNAs down regulated in PTC, miR-138-1-3p and miR-204-5p, and two miRNAs highly over expressed in PTC, miR-146b-5p and miR-375 [17–21]. It also included the only miRNA overexpressed in CLT, miR-155, which is known to control immune response and inflammation [38]. The classifier B further interrogated two miRNAs with modest differential expression (miR-139-5p and miR-29b-1-3p) and three miRNAs highly expressed in PTC (miR-31-5p) or in both PTC and FTC (miR-222-3p and miR-551b-3p) [16,18–22,25].

Notably, miR-21 and miR-221, two well-known oncomiRs over expressed in many cancer types including thyroid cancer [15,16,19–21,29], were not selected by the learning algorithms. Those miRNAs were outperformed by other candidates with similar expression patterns in various histopathological categories but better predictive performance, in particular miR-146b-5p and miR-375 over miR-21-5p in PTC and miR-222-3p and miR-551b-3p over miR-221-5p/-5p in FTC. However, our data did confirm that miR-21 expression levels seem to correlate with aggressive histopathological features and may have prognostic value [31]. Finally, several miRNAs such as miR-7-5p, -15a-5p, -96-5p, -187 or -885-5p were valid candidates based on the literature and our data but were excluded based on our stringent design specifications. Previous work suggested that miR-885 might be a good marker to discriminate oFTC from FTC and FA [25]. Our data indicate that miR-885-5p is a general marker of oncogenicity over expressed in both oFTC and oFA, thus limiting its potential diagnostic utility at this time.

Importantly, predictive models interrogating the expression levels of a few miRNAs identified a subset of malignant lesions that lack oncogenic gene alterations. The sensitivity of mutation testing in thyroid tissues and nodule FNAs was 61% (136/223), in the range expected based from the literature [5,6], and increased to 78% (175/223) when combined with classifier A ($p = 1.2 \times 10^{-9}$ for McNemar’s test). Among the specimens positive by mutation and/or miRNA testing, the residual risk of cancer was high and unchanged relative to mutation testing alone (84%, $p = 1.0$). This performance would correspond to a calculated PPV of 60–80% in a given set of indeterminate thyroid nodules with a thyroid cancer prevalence of 20–40%. The sensitivity was further increased to 94% (210/223) when mutation testing was combined with classifier B ($p = 2.1 \times 10^{-17}$), leading to a low residual risk of cancer (8%) among specimens negative by mutation and miRNA testing. This performance would correspond to a calculated improvement from 78–90% NPV to 94–98% NPV at 20–40% cancer prevalence. These pivotal observations in our case-control study suggest that miRNA testing could complement mutation testing and potentially improve the preoperative clinical management.
of patients with indeterminate thyroid nodules. Further optimization of the classifiers A and B resulted in the commercial ThyraMIR test that was recently evaluated in a multicenter cross-sectional cohort study [32]. As expected based on our data, mutation testing combined with ThyraMIR correctly ruled in 89% of malignant nodules with a PPV of 74% and correctly ruled out 85% of benign nodules with a NPV of 94% at 32% cancer prevalence [32].

An alternative method to improve the diagnostic performance of mutation testing is to interrogate additional genetic alterations associated with thyroid cancer. Single institution studies with unblinded surgical pathology review have previously shown that comprehensive genotyping by NGS can classify indeterminate nodules with high sensitivity and specificity [8,39]. Our data confirmed that expansive targeted NGS panels can increase test sensitivity in both resected tissues and indeterminate nodules. This higher sensitivity would correspond to a decrease in the number of false negative malignant nodules that are incorrectly ruled out, that is, an increase in NPV. However, NGS also uncovered additional gene alterations in specimens negative by ThyraMIR and classified as benign by independent pathologists from diverse endocrinology centres. Those cases included four mutations in TP53 (6–20% allele variant), two in TSHR (>30% allele variant), and four in HRAS, NRAS, PTEN or RET (5–8% allele variant) (Supplementary Table 3). TSHR mutations have previously been reported in benign nodules and low-level mutations <10% of alleles may represent subclones within larger nodules that could be monitored for potential tumour growth by repeat FNA and molecular testing [8].

In term of diagnostic performance, this predictable decrease in specificity with NGS would correspond to an increase in the number of false positive benign nodules that are incorrectly ruled in and that may be referred for unnecessary surgery, that is, a decrease in PPV. In contrast, combination testing for miRNAs and a few well-characterized oncogenic mutations provided a better balance between sensitivity and specificity. A key advantage of this strategy lies in its ability to rule in malignant nodules that lack oncogenic mutations and to rule out benign nodules with germline or low-level somatic mutations of unknown clinical significance. Predictive performance could be further improved in the future by interrogating additional genetic alterations, provided they are highly specific for thyroid cancer. High test specificity is not only a requirement to efficiently rule in cancer with high PPV, it is also beneficial to increase the benign diagnostic yield, that is, the rate of benign nodules correctly ruled out by a test. For that reason, combination testing for oncogenic mutations and miRNA expression also represents a significant improvement over current mRNA-based gene expression classification that can only rule out approximately 50% of benign nodules with indeterminate cytology [13,32].

In summary, we have shown that miRNAs are practical surrogate markers to identify subsets of thyroid tumours with altered oncogenic pathways. This observation is consistent with their documented function in almost all aspects of cancer development and progression, and their strong association with distinct signaling changes, differentiation states and progression risks in thyroid cancer [15,31]. A direct application of our findings is that a molecular test combining an optimized miRNA classification algorithm with a validated panel of somatic driver mutations displays high diagnostic sensitivity and specificity. This novel approach can rule in cancer with high PPV and rule out cancer with high NPV, thus increasing the diagnostic yield of molecular cytology relative to current methods and further improving the preoperative risk-based management of thyroid nodules with indeterminate cytology.

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Author Contributions

DW designed the miRNAs classifiers, performed statistical analyses and generated figures and tables. SBH conceived the miRNA experiments, managed all specimens and collected the associated data. BCH conceived the next-generation sequencing experiments and analyzed the results. TJG selected, reviewed and annotated the surgical specimens. EL designed the study, analyzed data and wrote the manuscript. All authors reviewed, revised and approved the manuscript before its submission.
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**SUPPLEMENTARY MATERIAL ON THE INTERNET**
The following supplementary material may be found in the online version of this article:

Supplemental Materials and Methods

**Supplemental Table 1.** Multiple hypothesis testing for differential miRNA expression in various histopathological categories

**Supplemental Table 2.** Genes targeted by next-generation sequencing and number of PCR amplicons

**Supplemental Table 3.** Benign specimens negative by the ThyraMIR test and by the 17-mutation panel but positive by targeted next-generation sequencing

**Supplemental Figure 1.** Expression of 31 miRNAs in 240 resected tissue specimens used for classifier training. The strip plots show the expression levels (Ct) of each miRNA in specimens grouped and colour-coded by histopathology category. Abbreviations: HN, hyperplastic nodule (includes diffuse hyperplasia); CLT, chronic lymphocytic thyroiditis; FA, follicular adenoma; oFA, oncocytic FA; FTC, follicular thyroid carcinoma; oFTC, oncocytic FTC; PTC, papillary thyroid carcinoma (includes conventional and tall cell variants); FvPTC, follicular variant of PTC.

**Supplemental Figure 2.** Comparison of miRNA expression profiles in resected thyroid tissues and preoperative thyroid nodule aspirates. Principal component (PC) analyses were performed for benign or malignant specimens using miRNA expression levels determined by RT-qPCR.

**Supplemental Figure 3.** Comparison between resubstituted and cross-validated scores in the 240 resected tissue specimens used for classifier training. The correlation plot (left) and the Bland and Altman bias plot (right) were built using n=960 scores generated with the classifiers A and B. The dashed lines in the bias plot represent the 95% limits of agreement or 1.96 times the standard deviation of the differences between resubstituted and cross-validated scores (±0.0137).

**Supplemental Figure 4.** Sunburst plot showing the distribution of molecular results in a subset of resected tissues negative by the 17-mutation panel (n = 54, left) and an independent set of nodules aspirates indeterminate by cytology (n = 42, right). Each sample is represented by one spoke and the total numbers of samples within each category are indicated. From the inner-most to the outer-most circle, the chart shows the hierarchical relationships between surgical pathology (benign or malignant) and the molecular results obtained for the panel of 17 gene alterations detected with the Luminex platform (17-panel), the ThyraMIR test (miRNA), and next-generation sequencing (NGS) at 5% allele variant threshold.