Analytical and Clinical Validation of Pairwise MicroRNA Expression Analysis to Identify Medullary Thyroid Cancer in Thyroid Fine-Needle Aspiration Samples

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BACKGROUND: Medullary thyroid carcinoma (MTC) is an aggressive malignancy originating from the parafollicular C cells. Preoperatively, thyroid nodule fine-needle aspiration cytology (FNAC) and pathogenic gene mutations are definitive in approximately one-half of cases. MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded RNAs that regulate gene expression, a characteristic that confers the potential for identifying malignancy. In the current study, the authors hypothesized that differential pairwise (diff-pair) analysis of miRNA expression levels would reliably identify MTC in FNA samples. **METHODS:** The relative abundance of 10 different miRNAs in total nucleic acids was obtained from ThyraMIR test results. Diff-pair analysis was performed by subtracting the critical threshold value of one miRNA from the critical threshold values of other miRNAs. Next-generation sequencing with the ThyGeNEXT panel identified oncogenic gene alterations. The discovery cohort consisted of 30 formalin-fixed, paraffin-embedded benign and malignant thyroid neoplasms, including 4 cases of MTC. After analytical validation, clinical validation was performed using 3 distinct cohorts (total of 7557 specimens). **RESULTS:** In the discovery cohort, 9 diff-pairs were identified as having significant power using the Kruskal-Wallis test ($P < .0001$) to distinguish MTC samples from non-MTC samples. The assay correctly classified all MTC and non-MTC samples in the analytical validation study and in the 3 clinical validation cohorts. The overall test accuracy was 100% (95% confidence interval, 99%-100%). In indeterminate FNAC samples, the sensitivity of the diff-pair analysis was greater than that of the MTC-specific mutation analysis (100% vs 25%; $P = .03$). **CONCLUSIONS:** Pairwise miRNA expression analysis of ThyraMIR results were found to accurately predict MTC in thyroid FNA samples, including those with indeterminate FNAC findings. ***Cancer Cytopathol* 2021;129:239-249**.

KEY WORDS: fine-needle aspiration (FNA); medullary thyroid cancer (MTC); microRNA (miRNA); ThyraMIR; thyroid nodule.

INTRODUCTION

Medullary thyroid carcinoma (MTC) accounts for approximately 2% of all thyroid malignancies, originates from the parafollicular C cells, and is more aggressive than the more common well-differentiated thyroid carcinoma of follicular cell origin.[1,2] As with all forms of thyroid carcinoma, fine-needle aspiration cytology (FNAC) is

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the primary method of diagnosis for patients with MTC.[3] The typical cytological features of MTC include salt-and-pepper chromatin and dyshesive, single, or multinucleated cells with plasmacytoid morphology. However, the diagnosis of MTC on FNAC can prove challenging for cytopathologists because cytology samples may have inadequate cellularity or the features can be subtle or absent.[4-6] A meta-analysis performed by Trimboli et al in 2015 found that a correct diagnosis of MTC may be missed by conventional cytology approximately 50% of the time.[7]

Although ultrasonography often can provide valuable information in the risk assessment of well-differentiated cancers, it lacks specificity for the diagnosis of MTC. Furthermore, although serum calcitonin or the measurement of calcitonin in washout fluids can be useful tools, these typically are ordered when there is a diagnosis of or suspicion for MTC. Calcitonin measurement is not performed routinely in thyroid nodules with an indeterminate cytological diagnosis.[7]

DNA and messenger RNA (mRNA) sequence analysis for driver mutations has the potential to identify MTC, but to our knowledge is not completely sensitive or specific because not all driver mutations are known and there is overlap between MTC driver gene mutations and those of other thyroid neoplasms. Well-differentiated thyroid carcinomas arise from the thyroid epithelial cell, and the somatic driver mutations often are within genes of the mitogen-activated protein kinase (MAPK) pathway. These include mutations in *BRAF* and *RAS* genes and fusions involving the RET or NTRK1 tyrosine kinases.[8-12] It should be noted that the identification of a *RAS* mutation is associated with lower tumor staging and high disease-specific survival in MTC compared with those cases in which a RET mutation is identified. MTC is familial in approximately 25% of cases.[2,13] Activating mutations in the RET proto-oncogene cause nearly all familial MTC cases[14] and approximately 43% of sporadic MTC cases.[15] There is a genotype-phenotype correlation between the location of the mutation and the aggressiveness of the MTC.[3] The other genes of sporadic MTC have to our knowledge been incompletely characterized but include activating BRAF and RAS gene mutations and several ALK gene–related fusion transcripts that may be found in other thyroid malignancies.[16-19] RAS gene mutations also may occur in benign epithelial thyroid neoplasms.[9] Therefore, DNA driver mutation analysis cannot reliably distinguish MTC from other thyroid neoplasms.

MicroRNAs (miRNAs) are endogenous, noncoding, single-stranded RNAs that regulate gene expression and subsequently differentiation, proliferation, and survival. These characteristics confer the potential for identifying malignancy. Early reports identified a role for individual miRNAs in thyroid cancer.[20] The use of miRNA-based molecular diagnostic testing to assess thyroid FNA samples was introduced after the development of machine learning–based classifier algorithms.[21-26] Many studies have identified a role for miRNAs in MTC.[27-35] Although miRNA expression classifiers have been developed to differentiate between benign and malignant thyroid nodules, to the best of our knowledge the ability of these classifiers to identify MTC has not been demonstrated previously.[21,22] Others have shown that pairwise analysis of differentially expressed miRNAs can identify distinct types of aggressive lesions.[36] In the current study, we determined whether pairwise comparisons of differentially expressed miRNAs detected using a commercially available diagnostic test, the ThyraMIR Thyroid miRNA Classifier, could reliably identify MTC in FNA samples.

MATERIALS AND METHODS

Study Design and Specimen Information

In the discovery phase, miRNA expression analysis of total nucleic acid (TNA) isolated from 30 formalin-fixed, paraffin-embedded (FFPE) tissue blocks of known histology was performed using the ThyraMIR Thyroid miRNA Classifier (ThyraMIR), which measures the relative expression of 10 miRNAs, including miR-29b-1-5p, miR-31-5p, miR-138-1-3p, miR-139-5p, miR-146b-5p, miR-155, miR-204-5p, miR-222-3p, miR-375, and miR-551b-3p. FFPE samples included 8 classic papillary thyroid carcinoma (cPTC) samples, 4 MTC samples, 12 poorly differentiated thyroid carcinoma (PDTC) samples, and 6 benign samples. For analytical validation of the test, archived TNAs from 18 FNA samples with known histology were evaluated. The precision and reproducibility were determined using TNA isolated from 3 MTC samples, 3 PTC samples, and 3 benign FFPE thyroid tissues tested using ThyraMIR by 3 operators on 2 independent runs. Intrarun variability was evaluated by testing 1 MTC sample, 1 PTC sample, and 1 benign sample in quadruplicate. To determine the lower limit of detection, serial dilutions ranging from 100 ng to 2 ng from 1 MTC sample and 1 PTC sample were assayed by 2 operators. Clinical

validation was performed on 3 different cohorts. The first cohort consisted of 130 FNA specimens with 1 MTC and 129 non-MTC surgical outcomes, which were collected as part of a prospective study approved by the Chesapeake institutional review board (approval # 00009811). The second validation cohort consisted of 7113 clinical FNA samples with known ThyGeNEXT and ThyraMIR data. The third validation cohort consisted of 314 clinical FNA samples from many participating sites across the United States that were submitted to Interpace Diagnostics Inc for molecular testing using ThyGeNEXT and ThyraMIR, and for which the surgical outcomes were made available. The institutional review board of record for protocol TT10 (cohorts 2 and 3) determined that these samples were exempt from human subject oversight.

Molecular Testing

miRNA classification was based on a clinically validated panel of 10 specific miRNAs performed by quantitative real-time polymerase chain reaction, the ThyraMIR test. The classifier yields a numerical value lying across a continuum from 0 to 1 that is used to identify those samples most likely to be associated with thyroid malignancy, as previously described.[37] Targeted next-generation sequencing mutation analyses to detect oncogenic RNA fusion transcripts and DNA mutation variants were performed using ThyGeNEXT as described previously.[38] All tests prescribed by physicians as part of the standard of care were performed at Interpace Diagnostics clinical laboratories (Pittsburgh, Pennsylvania and New Haven, Connecticut) according to standard clinical practices. Clinical FNA samples included cytology slides or dedicated needle passes placed directly into RNARetain preservative solution.

Statistical Analysis

All analyses were performed using R programming for statistical computing. Principal component analysis was performed to determine whether MTC samples could be differentiated from non-MTC samples using the raw critical threshold (Ct) value data obtained using ThyraMIR. Differentially expressed pairs of miRNAs (diff-pairs) then were used to evaluate potential biomarkers capable of differentiating MTC samples from non-MTC samples based on the Ct values from ThyraMIR. Diff-pairs were generated by subtracting the raw Ct values of one miRNA from the Ct values of other miRNAs. The Kruskal-Wallis test was performed to determine statistically significant differences between the MTC and non-MTC groups. Pairwise comparisons using Wilcoxon rank sum tests with a Benjamini-Hochberg P value adjustment were used to determine which groups were statistically significantly different. The McNemar test for paired proportions was used to compare the sensitivity of MTC-specific mutational analysis with that of the diff-pair analysis.

RESULTS

Development of ThyraMIR Diff-Pair Analysis to Detect MTC

Principal component analysis of miRNA expression data (raw Ct values) was performed using a discovery cohort of 30 FFPE samples with known pathology (8 cPTC samples, 4 MTC samples, 12 PDTC samples, and 6 benign samples). As shown in Figure 1, MTC samples were found to cluster separately from cPTC samples, PDTC samples, and benign thyroid samples mainly based on the expression of miR-375 (principal component 2 on the y-axis). Quantitative analyses also demonstrated that miR-375 expression is markedly higher (lower Ct value) in MTC samples compared with cPTC, PDTC, and benign nodules (Fig. 2A). Differential miR-375 expression relative to the other 9 miRNAs included in ThyraMIR (miR-375 Ct minus miRNA Ct) yielded negative values only for the MTC samples (Fig. 2B). All 9 diff-pairs were identified as having significant power using the Kruskal-Wallis test ($P < .0001$) and subsequent pairwise comparisons using Wilcoxon rank sum tests ($P < .05$) to distinguish MTC samples from non-MTC samples (MTC vs benign: $P < .0001$; MTC vs cPTC: $P < .0001$; and MTC vs PDTC: $P < .0001$). These data demonstrated that pairwise analysis of miR-375 expression using ThyraMIR results can effectively differentiate MTC from non-MTC FFPE tissues.

Analytical Validation of ThyraMIR Diff-Pair Analysis to Detect MTC

The reproducibility and robustness of each diff-pair were evaluated by repeatedly testing TNA isolated from 3 MTC, 3 PTC, and 3 benign FFPE thyroid tissues (Table 1). Variability between operators and between runs was low as indicated by low standard deviation and coefficient of variation values. Similarly low coefficient of variation values for various diff-pairs also were obtained for intrarun variability (data not shown). The lower limit of detection of the test was determined by testing a serial dilution

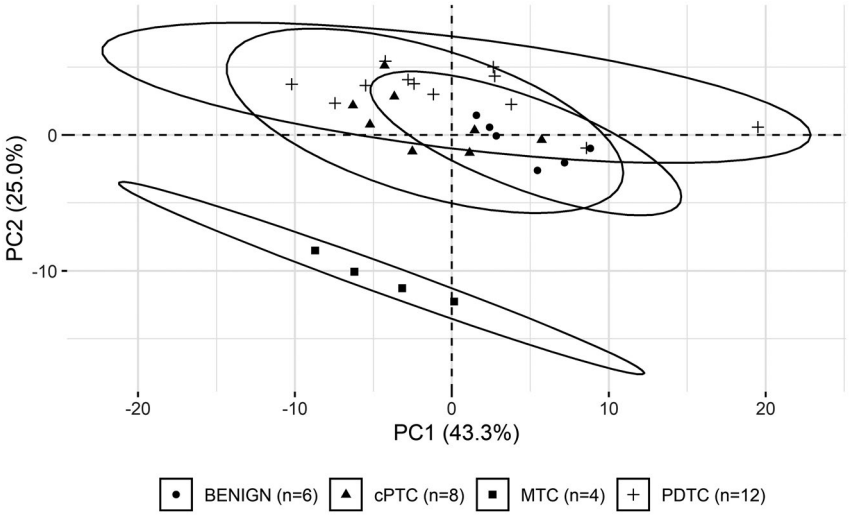

Figure 1. Principal component (PC) analysis for the discovery cohort using raw critical threshold (Ct) values for 10 microRNAs (miRNAs) from the ThyraMIR Thyroid miRNA Classifier. The 95% concentration ellipses are shown for each surgical outcome group. cPTC indicates classic papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma.

of MTC and PTC samples. As shown in Table 2, the ThyraMIR diff-pair analysis was able to successfully differentiate MTC from non-MTC samples at all inputs, from 100 ng to 2 ng of TNA. In addition to these experiments, 18 TNA samples isolated from thyroid FNA samples of known histology also were evaluated: 9 MTC samples, 5 cPTC samples, 2 nodular hyperplasia (NH) samples, and 2 follicular adenoma (FA) samples. Of a total of 108 tests performed during these analytical studies, 43 samples were identified as true-positive results (MTC samples called as MTC) and 65 samples were identified as true-negative results (non-MTC samples called as non-MTC), with no false-positive or false-negative results, suggesting a sensitivity of 100% (95% confidence interval [95% CI], 92%-100%) and a specificity of 100% (95% CI, 94%-100%) for ThyraMIR in the identification of MTC.

Validation of ThyraMIR Diff-Pair Analysis to Detect MTC in Clinical FNA Samples

Three validation cohorts were used to assess the performance of the ThyraMIR diff-pair analysis in FNA samples. The first cohort verified the test specificity in 130 FNA samples with known surgical outcomes representing a variety of non-MTC diagnoses: 47 hyperplastic nodule samples, 8 FA samples, 6 follicular carcinoma samples, 21 follicular variant of PTC samples, 47 cPTC samples, and 1 MTC sample (see Supporting Fig. 1). This MTC case had a cytology diagnosis of MTC, molecular findings included a KRAS_Q61R mutation, and ThyraMIR findings were strongly positive. The corresponding histology revealed an MTC diagnosis with positive lymph nodes. All miR-375 diff-pair values were negative for the single specimen with an MTC outcome whereas the 129 non-MTC samples demonstrated both positive and negative diff-pair values, corresponding to a specificity of 100% (95% CI, 97%-100%). The second cohort consisted of 7113 clinical FNA specimens without a known surgical outcome but with known ThyGeNEXT mutation status: 8 FNA samples with mutations characteristic of MTC (4 RET C634R and 4 RET M918T mutations); 302 FNA samples with gene alterations frequently found in PTC (287 BRAF V600E, 9 CCDC6-RET, and 6 NCOA4-RET gene alterations); and 6803 FNA samples with no mutations found on the oncogene panel and negative miRNA classifier results, which therefore had a very high likelihood of being benign (see Supporting Fig. 2). The 9 miR-375 diff-pair values were systematically negative only for the 8 FNA samples carrying MTC-specific RET mutations, further indicating that diff-pair analysis of ThyraMIR data can distinguish MTC from non-MTC nodules in FNA samples (see Supporting Table 1).

The third cohort consisted of 314 FNA samples with mostly Bethesda category III or IV cytopathology (224 of 314 samples; 71%), a known surgical pathology outcome, ThyGeNEXT test results, and ThyraMIR test results. This cohort included 11 MTC samples and 303 non-MTC samples: 58 FA samples, 16 follicular thyroid carcinoma samples, 30 follicular variant of PTC samples,

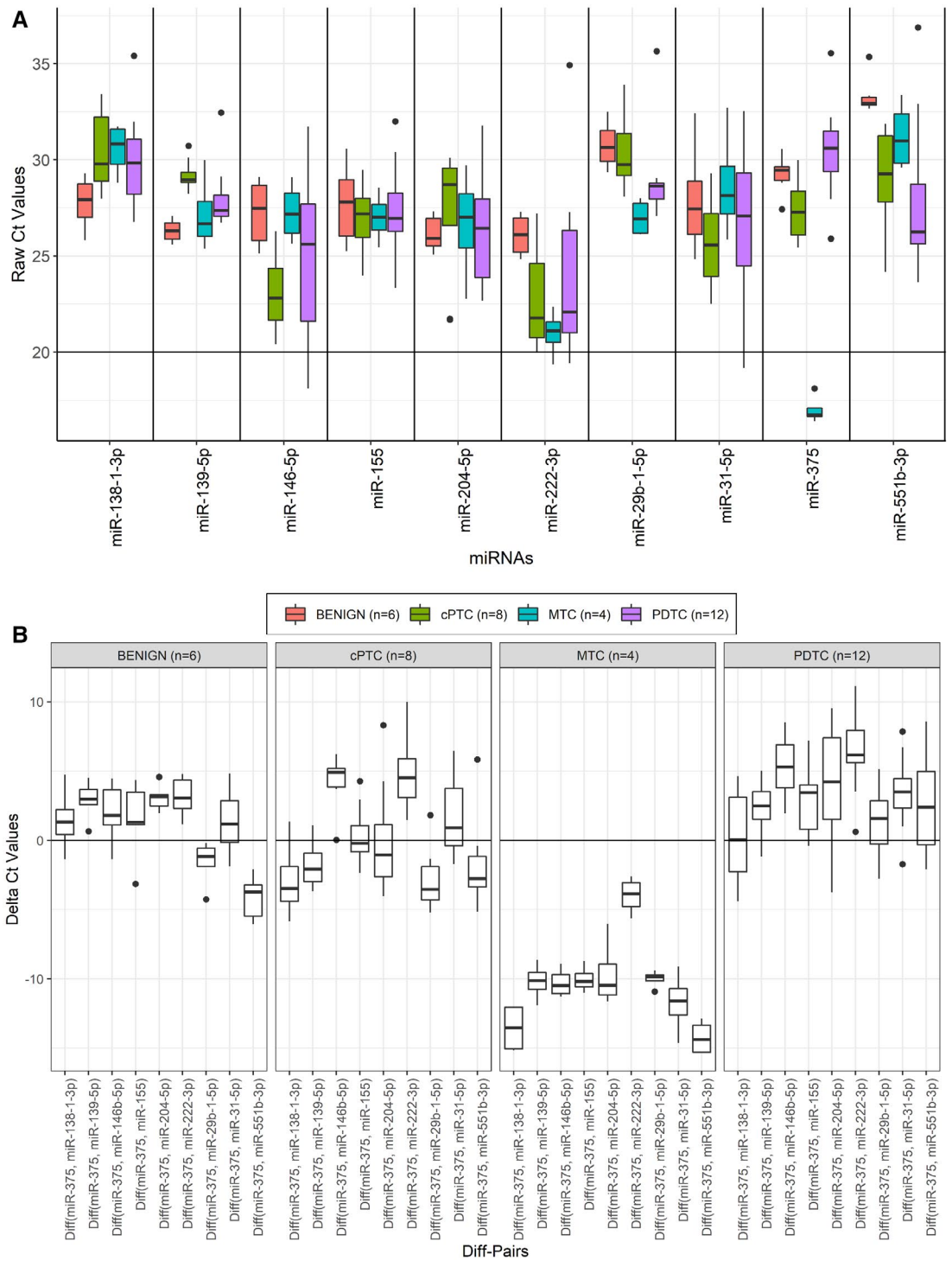

Figure 2. Quantitative analysis of microRNA (miRNA) expression in the discovery cohort. (A) Box plot representing the raw critical threshold (Ct) values generated with the ThyraMIR Thyroid miRNA Classifier in samples with the indicated surgical outcomes. (B) Box plot for the ThyraMIR differential pairwise (Diff) analysis using the same sample set. Boxes represent the 25th, 50th (median), and 75th percentiles of the Ct or delta Ct distributions; whiskers correspond to 1.5 times the interquartile ranges; and black circles indicate the individual Ct or delta Ct values outside theses distributions. cPTC indicates classic papillary thyroid carcinoma; MTC, medullary thyroid carcinoma; PDTC, poorly differentiated thyroid carcinoma.

TABLE 1. Reproducibility of the ThyraMIR Diff-Pair Analysis for the Detection of MTC

Sample	Metric	Diff (miR-375, miR-204-5p)	Diff (miR-375, miR-139-5p)	Diff (miR-375, miR-29b-1-5p)	Diff (miR-375, miR-155)	Diff (miR-375, miR-551b-3p)	Diff (miR-375, miR-146b-5p)	Diff (miR-375, miR-31-5p)	Diff (miR-375, miR-222-3p)	Diff (miR-375, miR-138-1-3p)	Diff-Pair Result
MTC	Mean	−8.43	−9.38	−9.90	−9.45	−13.72	−9.71	−10.25	−4.05	−13.28	MTC (18/18)
	SD	1.75	0.74	0.55	1.11	0.96	0.69	1.17	1.02	1.31	
	CV	21%	8%	6%	12%	7%	7%	11%	25%	10%	
PTC	Mean	−1.72	−1.48	−3.70	−0.34	−0.44	4.24	3.56	4.48	−3.97	Non-MTC (18/18)
	SD	1.53	0.61	0.33	0.26	0.47	0.82	0.20	0.29	0.36	
	CV	89%	41%	9%	78%	107%	19%	6%	7%	9%	
Benign	Mean	3.08	3.88	1.45	5.00	−1.62	3.93	4.04	5.24	1.28	Non-MTC (18/18)
	SD	1.05	0.91	0.42	1.19	0.66	0.95	0.96	0.36	0.90	
	CV	34%	24%	29%	24%	41%	24%	24%	7%	70%	

Abbreviations: CV, coefficient of variation; Diff, differential microRNA expression; miR, microRNA; MTC, medullary thyroid cancer; PTC, papillary thyroid cancer; SD, standard deviation.

TABLE 2. Lower Limit of Detection of the ThyraMIR Diff-Pair Analysis for the Detection of MTC

Sample	TNA Input, ng	ThyraMIR Result	Diff (miR-375, miR-204-5p)	Diff (miR-375, miR-139-5p)	Diff (miR-375, miR-29b-1-5p)	Diff (miR-375, miR-155)	Diff (miR-375, miR-551b-3p)	Diff (miR-375, miR-146b-5p)	Diff (miR-375, miR-31-5p)	Diff (miR-375, miR-222-3p)	Diff (miR-375, miR-138-1-3p)	Diff-Pair Result
MTC	100	Positive	−9.73	−9.84	−9.94	−11.01	−13.82	−10.04	−11.19	−4.23	−14.86	MTC
	50	Positive	−9.69	−9.74	−9.72	−11.00	−13.22	−9.94	−10.95	−4.26	−14.71	MTC
	25	Positive	−9.52	−9.54	−9.39	−10.77	−13.28	−9.96	−10.94	−3.95	−14.30	MTC
	10	Positive	−9.70	−9.85	−9.73	−10.98	−13.76	−9.85	−11.09	−4.24	−15.16	MTC
	5	Positive	−9.76	−9.99	−9.48	−10.97	−13.92	−10.03	−10.98	−4.04	−14.34	MTC
	2	Positive	−9.70	−10.01	−9.72	−11.26	−14.72	−10.59	−11.01	−4.13	−14.90	MTC
PTC	100	Positive	−3.13	−2.18	−3.75	−0.73	−0.44	5.08	3.50	4.67	−3.54	Non-MTC
	50	Positive	−2.91	−2.25	−3.75	−0.50	−0.45	5.26	3.53	5.11	−3.39	Non-MTC
	25	Positive	−3.13	−2.14	−3.77	−0.64	−0.60	5.21	3.62	4.76	−3.66	Non-MTC
	10	Positive	−3.47	−2.50	−3.89	−0.70	−0.54	5.11	3.35	4.78	−3.41	Non-MTC
	5	Positive	−2.95	−2.69	−3.73	−0.40	−0.32	5.09	3.68	4.85	−3.34	Non-MTC
	2	Positive	−3.00	−1.85	−2.95	−0.48	−0.12	5.12	3.50	4.93	−3.84	Non-MTC

Abbreviations: Diff, differential microRNA expression; miR, microRNA; MTC, medullary thyroid cancer; PTC, papillary thyroid cancer; TNA, total nucleic acids.

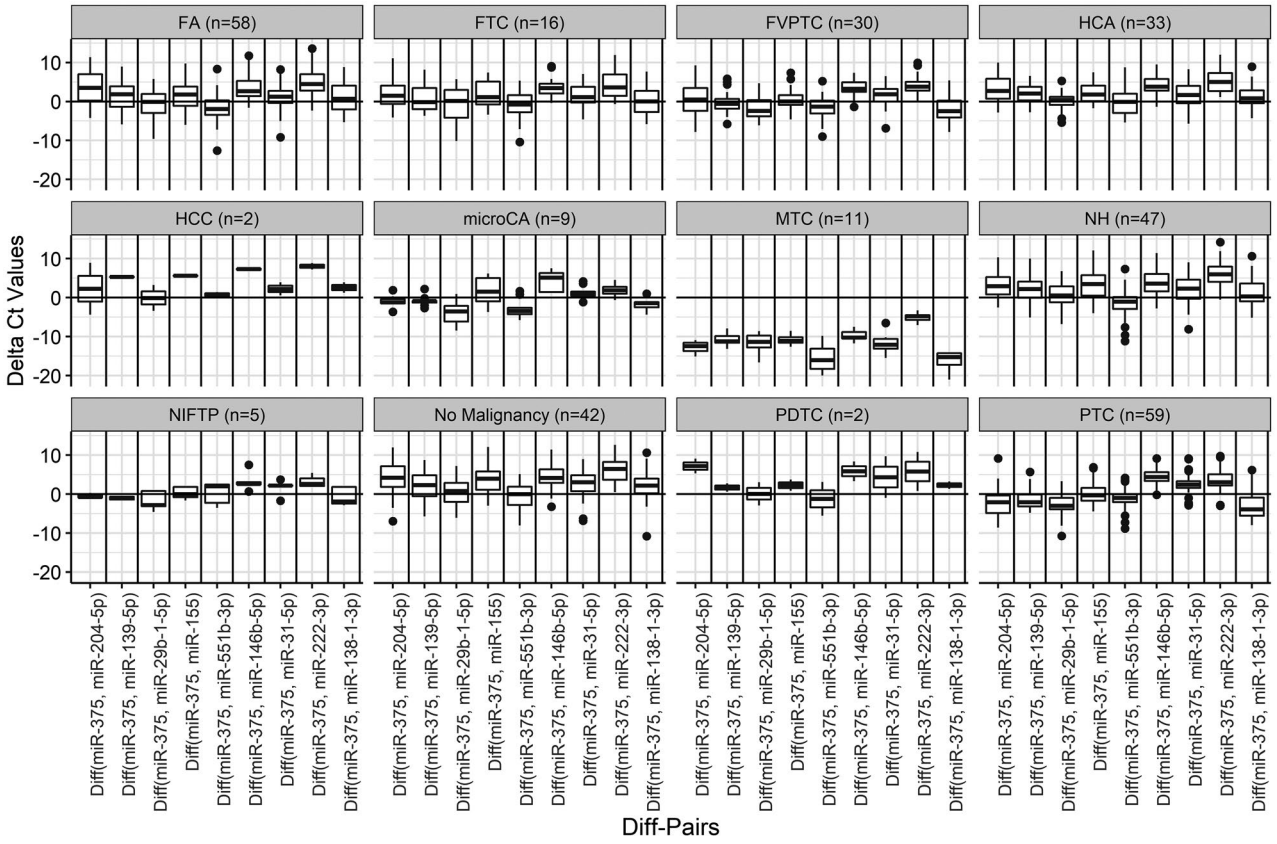

Figure 3. Box plot for the ThyraMIR differential pairwise (Diff) analysis in the third validation cohort consisting of 314 fine-needle aspiration samples with the indicated surgical outcomes. Boxes represent the 25th, 50th (median), and 75th percentiles of the delta critical threshold (Ct) distributions; whiskers correspond to 1.5 times the interquartile ranges; and black circles indicate the individual Ct or delta Ct values outside theses distribution. FA indicates follicular adenoma; FTC, follicular thyroid carcinoma; FVPTC, follicular variant of papillary thyroid carcinoma; HCA, Hurthle cell adenoma; HCC, Hurthle cell carcinoma; microCA, microcarcinoma; MTC, medullary thyroid carcinoma; NH, nodular hyperplasia; NIFTP, noninvasive follicular thyroid neoplasm with papillary-like nuclear features; PDTC, poorly differentiated thyroid carcinoma; PTC, classic papillary thyroid carcinoma.

33 Hurthle cell adenoma samples, 2 Hurthle cell carcinoma samples, 9 micro-PTC samples, 47 NH samples, 5 samples of noninvasive follicular thyroid neoplasm with papillary-like nuclear features, 2 PDTC samples, 59 cPTC samples, and 42 samples classified as benign or no malignancy. As shown in Figure 3, the 11 MTC samples demonstrated negative diff-pair values, whereas the 303 non-MTC samples demonstrated both positive and negative diff-pair values. With 11 true-positive results (MTC detected as MTC), 303 true-negative results (non-MTC identified as non-MTC), no false-negative results, and no false-positive results, the diagnostic test performance for the detection of MTC was 100% sensitivity (95% CI, 72%-100%), 100% specificity (95% CI, 99%-100%), and 100% accuracy (95% CI, 99%-100%). Study results for the 11 MTC samples are summarized in Table 3. Only 2 of the 11 samples had a MTC-specific mutation (RET M918T) and for the 8 FNA samples with indeterminate FNAC results (Bethesda category III or IV), the diagnostic sensitivity of mutation testing was found to be significantly lower than the sensitivity of pairwise miRNA analysis (25% vs 100%, respectively; $P = .03$).

DISCUSSION

The current retrospective study demonstrated the analytical and clinical validity of a pairwise miRNA analysis for the detection of MTC using ThyraMIR results in thyroid FNA samples. Principal component analysis was instrumental during the feasibility stage in demonstrating the possibility of differentiating MTC from non-MTC thyroid nodules solely based on raw miRNA expression data. Pairwise analysis further demonstrated that, when compared with the other 9 miRNAs of the ThyraMIR panel, miR-375 was highly expressed in

TABLE 3. Summary of Results for 11 MTC Samples From the Third Validation Cohort

Specimen	Sample Type	Bethesda Diagnosis	Suspicion for MTC Noted	ThyGeNEXT Result	ThyraMIR Result	Diff-Pair Result	Surgical Outcome
TT10-02	FNA in RNARetain	AUS (category III)	No	Negative	Positive	MTC	MTC
TT10-03	Diff-Quik stain	AUS (category III)	Yes	RET M918T	Positive	MTC	MTC
TT10-04	FNA in RNARetain	FN (category IV)	No	RET M918T	Positive	MTC	MTC
TT10-05	FNA in RNARetain	Malignant (category VI)	Yes	Negative	Positive	MTC	MTC
TT10-08	FNA in RNARetain	AUS (category III)	Yes	Negative	Positive	MTC	MTC
TT10-11	Diff-Quik stain	AUS (category III)	No	HRAS Q61R	Positive	MTC	MTC
TT10-24	FNA in RNARetain	SFM (category V)	No	Negative	Positive	MTC	MTC
TT10-27	FNA in RNARetain	MTC (category VI)	Yes	Negative	Positive	MTC	MTC
TT10-29	FNA in RNARetain	SFN (category IV)	Yes	Negative	Positive	MTC	MTC
TT10-31	FNA in RNARetain	FN (category IV)	No	Negative	Positive	MTC	MTC
TT10-33	FNA in RNARetain	AUS (category III)	No	KRAS G12R	Positive	MTC	MTC

Abbreviations: AUS, atypia of undetermined significance; Diff, differential microRNA expression; FN, follicular neoplasm; FNA, fine-needle aspiration; MTC, medullary thyroid cancer; SFM, suspicious for malignancy; SFN, suspicious for neoplasm.

MTC but not in other thyroid histopathologic categories. These findings were highly reproducible when performed by multiple operators on different days. Verification studies demonstrated that pairwise analysis using the ThyraMIR results generated in FNA samples differentiated MTC from non-MTC thyroid lesions with high sensitivity and specificity. Pairwise analysis correctly identified all MTC cases with 100% sensitivity and 100% specificity. These results are consistent with the important role played by miR-375 in MTC. Prior work has demonstrated that overexpression of miR-183 and miR-375 in MTC correlates with lateral lymph node metastasis and mortality,[29] that miR-375 is involved in the downregulation of the growth inhibitor YAP1,[29] and that higher expression of miR-375 plays a pivotal role in MTC tumorigenesis.[27,28,33]

This pairwise miRNA expression analysis has several advantages over other strategies for the diagnosis of MTC on FNA samples. First, compared with a strategy that uses only specific MTC driver mutations, pairwise miRNA expression analysis is more sensitive for the determination of MTC. It can identify MTC without MTC-specific driver mutations, and it can identify MTC in samples that are positive for nonspecific mutations that also are found in non-MTC neoplasms, such as RAS. The finding of a RAS mutation in MTC conveys a lower tumor stage and much higher disease-specific survival compared with the finding of a RET mutation. Second, no additional experimentation or sample volume is required for the test. The raw data obtained from the combined ThyraMIR classifier and ThyGeNEXT oncogene panel, which are performed for the risk stratification of thyroid FNA samples,[22-24] simply need to be reanalyzed using diff-pair analysis. Third, pairwise analysis confers a robust functionality to the assay. Unlike raw Ct values, which are a function of both miRNA expression and the total amount of nucleic acids used in the assay, the pairwise analysis is self-normalizing and dependent only on miRNA expression. Therefore, the assay is accurate across a large range of TNA input in the assay.

The unique biology of miRNAs demonstrates further advantages of pairwise analysis for the detection of MTC in FNA samples. Because miRNAs are more stable than mRNAs, cytology slides can be used as the sample source, thereby precluding the need for repeat FNA or the collection of additional passes. In addition, miRNAs can migrate from one cell to another in the

vicinity as well as at a distance by means of packaging within membrane-bound exosomes and be transported through extracellular fluid and circulating blood.[39] This communication functionality enables mutation-bearing cells to recruit adjacent nonmutated cells as part of neoplastic progression.[40] From a diagnostic perspective, this is especially valuable with respect to sampling variations related to needle aspiration of discrete microscopic sites within a relatively large-sized nodule. Both MTC and non-MTC neoplasms manifest intratumoral heterogeneity, in which somatically acquired mutations are present in only a subset of tumor cells.[41,42] When the overall mutation variant percentage is relatively low, the FNA sample may capture a percentage of mutated cells that falls beneath the lower limit of mutation detection, leading to a false-negative mutation testing result. This false-negative result is less likely to occur with the use of miRNA analysis because the miRNAs can be present in mutation-negative cells.[43]

The major strength of the current study was the large number of non-MTC samples in the validation cohorts, providing considerable confidence in the specificity of the pairwise analysis. In the validation cohort with known final pathology, the 95% CI for specificity ranged from 99% to 100%. A potential weakness of the current study was the availability of a relatively small number of MTC samples. In the validation cohort with known final pathology, there were 11 MTC samples and the 95% CI for test sensitivity ranged from 72% to 100%. The relative rarity of MTC does limit our confidence regarding the clinical sensitivity of this assay, although it is likely superior to other approaches, as discussed above.

In the current study, we have reported on the development and validation of a molecular test performed in a College of American Pathologists (CAP)– and/or Clinical Laboratory Improvement Amendments (CLIA)–certified laboratory to differentiate MTC from non-MTC FNA samples. The approach used for the molecular diagnosis of MTC was based on the combination of a comprehensive next-generation sequencing–based oncogene panel (ThyGeNEXT) and a miRNA expression profiling panel (ThyraMIR) that was subjected to pairwise analysis. The ThyGeNEXT test detects the DNA mutations and RNA fusions commonly found in thyroid cancers and in MTC, and ThyraMIR further determines the risk of malignancy. By using pairwise analysis of the individual miRNA Ct values, MTC is differentiated from non-MTC samples with high sensitivity and specificity. The use of this unique combination testing approach provides particular value to samples with a Bethesda category III (atypia of undetermined significance/follicular lesion of undetermined significance) or category IV (follicular neoplasm/suspicious for neoplasm) cytology diagnosis, particularly in the absence of cytological features of MTC. Pairwise miRNA expression analysis of ThyraMIR results accurately identifies MTC in thyroid FNA samples, including those with indeterminate FNAC findings.

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CONFLICT OF INTEREST DISCLOSURES

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AUTHOR CONTRIBUTIONS

Andrea M. Ciarletto: Data curation, formal analysis, investigation, methodology, visualization, and writing–review and editing. **Christina Narick:** Conceptualization and writing–review and editing. **Carl D. Malchoff:** Resources and writing–original draft. **Nicole A. Massoll:** Resources and writing–review and editing. **Emmanuel Labourier:** Formal analysis, methodology, visualization, and writing–original draft. **Keith Haugh:** Data curation and software. **Alidad Mireskandari:** Resources, supervision, and project administration. **Sydney D. Finkelstein:** Conceptualization and writing–review and editing. **Gyanendra Kumar:** Data curation, formal analysis, methodology, project administration, supervision, and writing–original draft.

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