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ORIGINAL ARTICLE

Utility of microdissected cytology smears for molecular analysis of thyroid malignancy

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Abstract

Background: Molecular testing of thyroid fine-needle aspirates has demonstrated value in cases of indeterminate cytology (Bethesda categories III, IV, and V) enabling optimized individual patient management leading to better outcomes with health economic benefits. For most molecular testing modalities, including mutational panels and classifier analyses, part or all of a dedicated needle aspiration pass is required to obtain an adequate sample for testing. Our analysis, which is based on a combination approach (mutation detection and microRNA classifier status), has documented clinical validity and utility when performed on thyroid fine-needle aspirates placed directly into RNA preservative fluid. Here we show that the combination approach can be extended to microdissected stained cytology slides provides the physician greater opportunity to resolve cytological indeterminacy.

Methods: Extracted nucleic acid from needle aspirate and corresponding cytology preparations of 47 thyroid nodules were analyzed using identical methodology and results were compared.

Results: Of 94 molecular analyses (47 mutational analyses, 47 microRNA classifier assessments based on a validated 10 marker panel) only 5 samples showed discordant results.

Conclusion: These findings, together with supplementary work using archival specimens shows that the combination approach can be effectively applied to both direct aspirated thyroid nodule aspirates or to nucleic acid extracted from macrodissected and microdissected cytology slide smears, with the expectation of equivalent results. The advantages of both specimen sources, direct aspirate, and cytology slide smears are discussed.

KEYWORDS

cytology slide, microRNA classifier, NGS, personalized medicine, thyroid cancer diagnostic test

1 | INTRODUCTION

While thyroid nodules are quite common, only about 8%-15% are found to be histologically malignant.[1,2] Regardless, thyroid carcinoma is one of the most common endocrine malignancies.[3–5] Thyroid biopsies are performed using fine-needle aspiration (FNA), typically in concert with ultrasound guidance. These biopsies then undergo microscopic cytological evaluation for classification according to the Bethesda System for Reporting Thyroid Cytology. Diagnostic categories include (B-I) nondiagnostic; (B-II) benign; (B-III) atypia of undetermined significance or follicular lesion of undetermined significance (AUS/FLUS) or (B-IV) follicular neoplasm or suspicious for follicular neoplasm (FN/SFN) or (B-V) suspicious for malignancy or (B-VI) malignant.[6] According to NCCN guidelines, repeat biopsy is recommended for the solid lesions with nondiagnostic cytology, observation is recommended for the nodules of stable size with benign cytology, molecular diagnostic analysis is recommended for consideration in the indeterminate nodules (AUS/FLUS, FN/SFN), and

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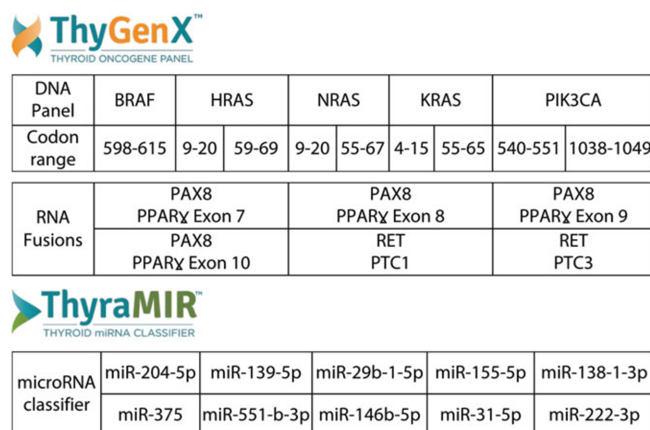
surgery is recommended for aspirates that are diagnosed as carcinoma or suspicious for carcinoma.[7]

Commercially available testing options for molecular analysis of nodules with the indeterminate cytology include a messenger-RNA gene expression classifier designed to define benign status of the nodule,[1] a microRNA expression classifier,[8] a Next Generation Sequencing (NGS) based oncogene panel also incorporating relevant RNA fusions and translocation,[9,10] and a combination test based upon NGS-based mutational analysis and a microRNA expression based classifier (Figure 1).[11,12]

Many current molecular tests that amplify nucleic acid can now be performed on extremely small amounts of tumor specimen including cytology smears prepared from fine-needle aspirates. Molecular testing of cytology smears can also be focused to specific areas of morphologic concern through targeted microdissection which can enrich the desired cell population for molecular analysis.[13–15] Based on well-known sample adequacy variation between fine needle passes, this technique of targeted microdissection can ensure molecular analysis of the actual diagnostic cells of concern.

The taking of a separate dedicated needle aspiration pass solely for molecular testing runs the risk that without microscopic confirmation, thyroid follicular cell adequacy cannot be assured. Molecular testing of cytology smears can therefore increase the diagnostic yield from FNA procedures, potentially eliminating the need for repeat biopsy in certain cases. Furthermore, slides offer the opportunity to perform clinical retrospective studies using archived materials. Comparative molecular analysis of archival slide format testing also affords the chance to understand the temporal sequence of molecular change over time.

The purpose of this study was to demonstrate the accuracy and potential utility of combined molecular analysis (mutational profiling and microRNA classifier status using ThyGenX and ThyraMIR tests) of FNA cytology smears. We compared molecular test results of cytology smears to that of preserved RNA from an FNA sample collected from the same patient, same nodule, during the same FNA procedure.



ThyGenX™
THYROID ONCOGENE PANEL

DNA Panel	BRAF	HRAS		NRAS		KRAS		PIK3CA	
Codon range	598-615	9-20	59-69	9-20	55-67	4-15	55-65	540-551	1038-1049

RNA Fusions			
	PAX8 PPARγ Exon 7	PAX8 PPARγ Exon 8	PAX8 PPARγ Exon 9
	PAX8 PPARγ Exon 10	RET PTC1	RET PTC3

ThyraMIR™
THYROID miRNA CLASSIFIER

microRNA classifier					
	miR-204-5p	miR-139-5p	miR-29b-1-5p	miR-155-5p	miR-138-1-3p
	miR-375	miR-551-b-3p	miR-146b-5p	miR-31-5p	miR-222-3p

FIGURE 1 ThyGenX and ThyraMIR panel designs. ThyGenX oncogene panel interrogates 9 hotspot regions in the *BRAF*, *HRAS*, *KRAS*, *NRAS*, and *PIK3CA* genes, 6 fusion transcripts, which play a role in thyroid tumorigenesis. ThyraMIR assay is a proprietary microRNA expression-based algorithm to classify the risk of malignancy in thyroid nodules

2 | MATERIALS AND METHODS

2.1 | Specimen collection

Two sets of samples were used in this study. The first set of samples was analyzed to determine whether molecular testing can be carried out from FNA smears. For this, total cytology specimen was scrapped (macrodissection) for the isolation of total nucleic acid (TNA) as explained below. The second set of samples was retrospectively evaluated to test our ability to apply the approach of targeted microdissection for molecular testing.

The first set of samples was collected as part of a prospective study approved by the Chesapeake Institutional Review Board (Approval # 00009811) and informed consent was obtained from study participants. A random subset of 47 patient specimens collected during the first study that included matched FNA samples of cytology slide smears (air-dried Diff-Quik or alcohol-fixed Pap) and RNA preserved (RNARetain, Asuragen) aspirates were evaluated. For these samples, the first drop of the FNA biopsy material was placed onto a slide, smeared and air-dried, and the remaining material was rinsed into RNA preservative solution. The second set of samples was comprised of 22 retrospective, de-identified cytology smears, with histological diagnosis of dissected tumors, which were also evaluated to determine the suitability of cytology smears for diagnostic molecular analysis of thyroid FNA.

2.2 | TNA isolation from RNA preserved FNA

TNA was isolated from RNA preserved FNA using the phenol-chloroform method.[16]

2.3 | TNA isolation from macrodissected FNA smears

All microscopic slides underwent whole slide imaging (Leica). Coverslips were removed by immersing in xylene (Fisher) for 48-72 hours.[13] Once the coverslips were off, slides were washed in xylene followed by a wash with 80% ethanol and air-dried.[17] Slides were then moistened using a drop of Tris-Tween buffer (10 mM Tris pH 8.0, 0.05% Tween 20) and all FNA smear material was scraped into RNA Retain (Asuragen), followed by TNA isolation using the phenol-chloroform method.[16]

2.4 | Targeted microdissection from cytology smears and TNA isolation

All microscopic slides underwent whole slide imaging (Leica). Diamond knife markings were placed on the slide underside representing cellular targets more representative for integrated molecular analysis. The coverslips were removed by immersion in xylene (Fisher) for 48-72 hours. Microdissection was performed on stained cytology smear slides (Pap stain, or Diff-Quik). Cell clusters were quickly microdissected using a simple manual technique approach under stereomicroscopic visualization. Accuracy was confirmed by comparison of premicrodissection and postmicrodissection stained slides providing detailed evidence regarding targeting accuracy. Microdissected cells were placed into digestion buffer from the Recoverall kit buffer and TNA was extracted using the Recoverall kit (Life Technologies) according to manufacturer's instructions.[13]

2.5 | Next generation mutational sequencing (ThyGenX) test

Detailed procedures for the NGS-based mutational sequencing have been previously described.[12,18] Briefly, sample quality and copy number of extracted TNA were analyzed by qPCR analysis of *LINE1* retrotransposons. The *LINE1* copy number was used to determine optimal template inputs for multiplex Polymerase Chain Reaction (PCR).[12,18] RNA component of TNA was reverse transcribed, and the sequencing libraries were prepared using a multiplex gene-specific PCR with the DNA and the cDNA components of the TNA. Amplified gene products were then bar-coded using primers containing custom molecular barcodes. Libraries were purified using AxyGen mag prep kit (Corning) and quantitated using Illumina Library Quant Kit (KAPA). Finally, an 18 p-molar NGS library was sequenced on the Illumina MiSeq using custom primers and data was processed using a proprietary bioinformatics pipeline. MiSeq output files were de-multiplexed into sample and panel specific fastq files. Fastq files were preprocessed by removing low quality bases of ends of the reads (reads ≥Q20 only) followed by trimming of sequencing adapters. For the DNA panel, reads were initially aligned to a custom genome index of the panel amplicon sequences followed by an alignment to human refseq genome hg19 (Ensembl build GRCh37.74) and variant calling was performed a custom GATK based pipeline. For the RNA panel, fastq files aligned to a sequence database including hg19, the human transcriptome reference sequence (Ensembl annotation v74) and the fusion cDNA breakpoint sequences. Fusion variants were called according to a Poisson model that identifies fusions expressed at a level significantly greater than 10% of Tata Box Binding Protein(TBP) expression.

2.6 | MicroRNA expression classifier (ThyraMIR) test

The ThyraMIR test was used to provide the microRNA classifier status.[12] The microRNA component of TNA was converted to cDNA using miRCURY LNA Universal RT microRNA PCR kit (Exiqon) using 5 μL of the isolated TNA. cDNA products were diluted 1:100 and plated onto custom designed 384-well plates precoated with microRNA specific primers (Exiqon) along with PCR amplification reagents, and the qPCR was performed on an AB Quant Studio 6 (Applied Biosystems) using Exilent SYBR Green kit (Exiqon). TNA isolated from thyroid FNA or Formalin-Fixed Paraffin-Embedded tissue (FFPE) tissues, with known benign and malignant calls were used as plate controls. A Diagonal Linear Discriminant Analysis (DLDA) classifier based on the relative expression of 10 microRNAs, as described previously,[12] was utilized to classify FNA samples as positive or negative.

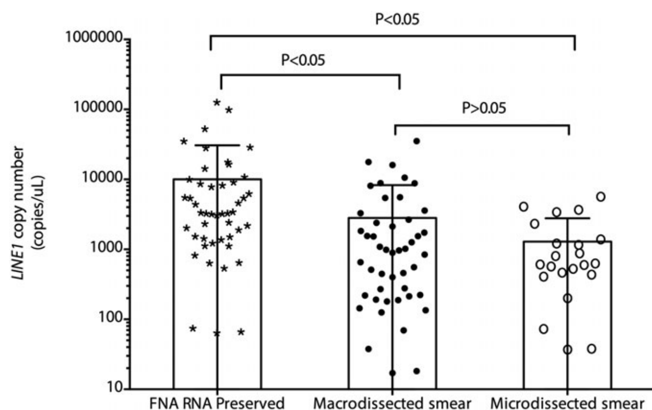


FIGURE 2 Analysis of sample quality and copy number. Graph shows successful total nucleic acid isolation from fine-needle aspiration (FNA) cytology smears as indicated by the amount amplifiable copies present in RNA preserved FNA, macrodissected cytology smears and microdissected cytology smears based on the of *LINE1* qPCR assay

2.7 | Data analysis

All the samples in this study were evaluated using ThyGenX and ThyraMIR tests as described above. A positive call was assigned to samples positive for the presence of mutations by ThyGenX or positive based upon the established classifier threshold for ThyraMIR. In samples that were scored as negative on both the tests, a negative diagnostic call was made.

3 | RESULTS

3.1 | TNA isolation from cytology smear specimens

TNAs were isolated from FNA specimens in RNA preservative, macrodissected FNA cytology smears, and microdissected FNA cytology smears. The samples were analyzed by *LINE1* qPCR to determine sample quality and to estimate number of amplifiable copies present in the sample. The *LINE1* copy number ranged from 55 to 108 359 copies/μL in RNA preserved FNA specimens, from 15 to 30 898 copies/μL in macrodissected cytology smears and 36 to 5510 copies/μL in microdissected cytology smears. These data indicate that TNA was successfully isolated from all 3 specimen types. A one-way analysis of variance was performed to compare the differences in *LINE1* copy number between the 3 specimen types. The *LINE1* copy number observed was significantly higher for RNA preserved FNA compared to macrodissected FNA smears ($P = .0302$) and microdissected smears ($P = .0377$). There were no differences in the in the *LINE1* copy numbers observed between macrodissected FNA smears and microdissected FNA smears ($P = .902$). Also, there was no impact of staining on the extraction of TNA from samples (Figure 2).

3.2 | Equivalency of molecular results from FNA specimens in RNA preservative and macrodissected FNA cytology smears

We compared the molecular results of FNA specimens in RNA preservative (RNA*Retain*) and matched FNA cytology smears ($n = 47$). The TNA, isolated from these samples, were first tested for somatic mutations. Mutations were detected in 17/47 cytology smear and 16/47 RNA preservative samples (Figure 3B) demonstrating 98% concordance of mutation testing between specimen types.

Samples were subsequently tested using microRNA expression levels to examine their relative abundance in macrodissected cytology smears and RNA preservative samples. A summary of the microRNA expression data is shown in Figure 3C. In total, 8/47 macrodissected cytology smear samples had positive and 39/47 had negative microRNA classifier calls. Comparatively, 9/47 RNA preservative samples

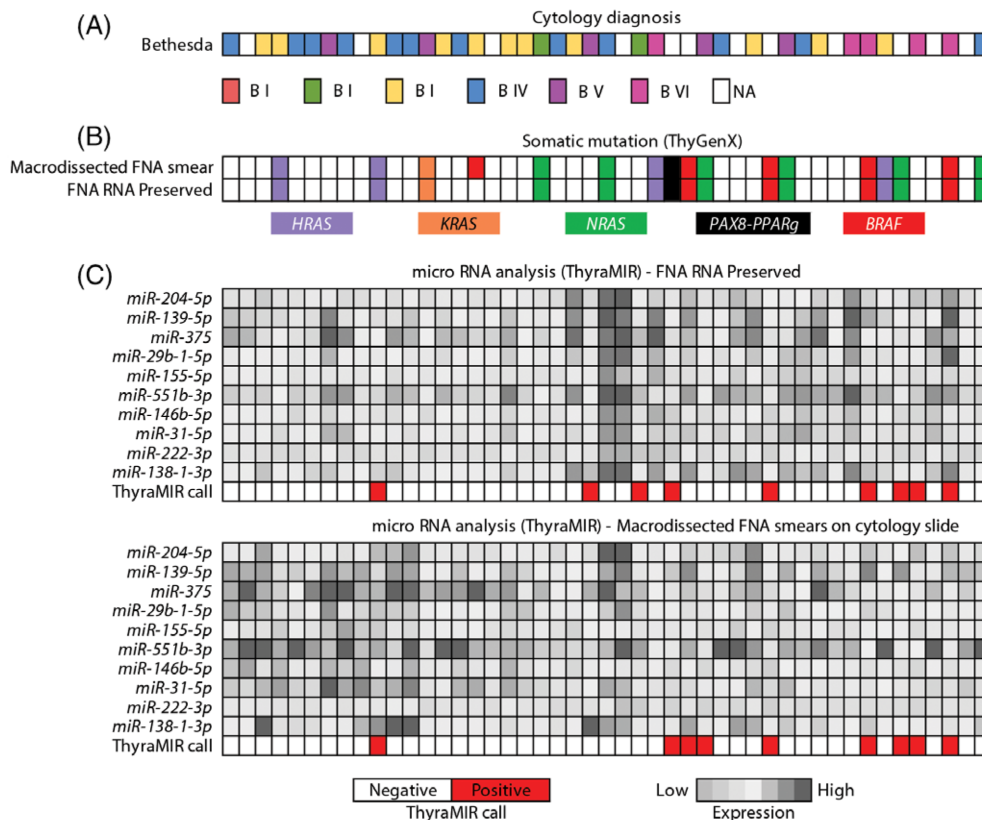


FIGURE 3 A, Comparison of molecular testing results between RNA preserved fine-needle aspiration (FNA) and macrodissected FNA smears. A, Cytology diagnosis (Bethesda); B, mutation testing results (ThyGenX calls); and C, raw microRNA expression data and ThyraMIR calls (highlighted red) for the 47 FNA samples 47 prospectively collected FNA samples stored either as a smear or in RNA preservative

had positive and 38/47 had negative microRNA classifier calls. Discordant results were observed in only 5 cases.

3.3 | Suitability of microdissection based molecular testing using archived cytology smears

A retrospective study using archived, de-identified cytology smears from thyroid nodule FNAs with known, de-identified surgical pathology outcomes was performed to evaluate clinical suitability for microdissection based molecular testing. Twenty-two cases were included of which 13 had benign and 9 had malignant pathology. Of these, 6 cases were classified as B II, 7 cases as B III, 4 cases as B IV, 2 cases as B V, and 3 cases as B VI cytology based on the Bethesda cytology classification system. In total 8/22 microdissected cytology smears harbored mutations, including 4 *BRAF*, 3 *KRAS*, and 1 *HRAS* mutation (Figure 4A), whereas microRNA expression results were positive in 10/22 tested cases (Figure 4A,B). The combination of mutation and microRNA analysis correctly identified 7/9 samples as malignant and 10/13 samples as benign.

3.4 | Quality metrics of data from sequencing and microRNA expression for FNA samples stored using RNA preservative, whole slide cytology smear, and targeted microdissected cytology smears

In order to ensure that cytology smears are suitable for clinical testing we compared various QC metrics that are monitored during routine clinical testing of FNA samples in RNA preservative to that of cytology smears. As a quality measure, both DNA and RNA panel libraries were prepared as 2 independent replicates for sequencing.

For NGS-based testing, metrics for coverage depth and read alignment were used as indicators of both DNA and RNA-Seq quality. As shown in Figure 5A-D, the median coverage for the DNA panel was greater than 18 000 reads per sample in all the 4 sample sets tested. Similarly, for the RNA panel, median coverage was greater than 13 500 reads per sample for all the 4 sample sets tested. For the 4 sets of samples tested, all samples had an average of 97% of fraction of amplicons with in 5× of median coverage (indicator of PCR efficiency) and 88% fraction of target bases sequenced were covered at >1000 reads (indicator of NGS coverage depth). NGS QC metrics for microdissected cytology smear samples were comparable to the data from whole slide FNA smears and FNA in RNA preservative. This indicates that successful ThyGenX testing can be carried out from FNA material stored as cytology smears, or from microdissected cells from the cytology slides (Figure 5A-D). In addition to monitoring NGS QC metrics, lower limit of detected (LLD) studies were also carried out to demonstrate the quality of the library preparation process. LLD was tested by diluting a positive clinical sample into a negative clinical sample across a dilution series. DNA panel LLD was carried out diluting a *NRAS_61R positive* sample into a negative sample and RNA panel LLD was carried out by dilution a *NCOA4_RET4* fusion positive sample into a negative sample. Figure 5E shows the reliable detection of *NRAS_Q61R* across a range of variant allele frequency (%VAF) as low

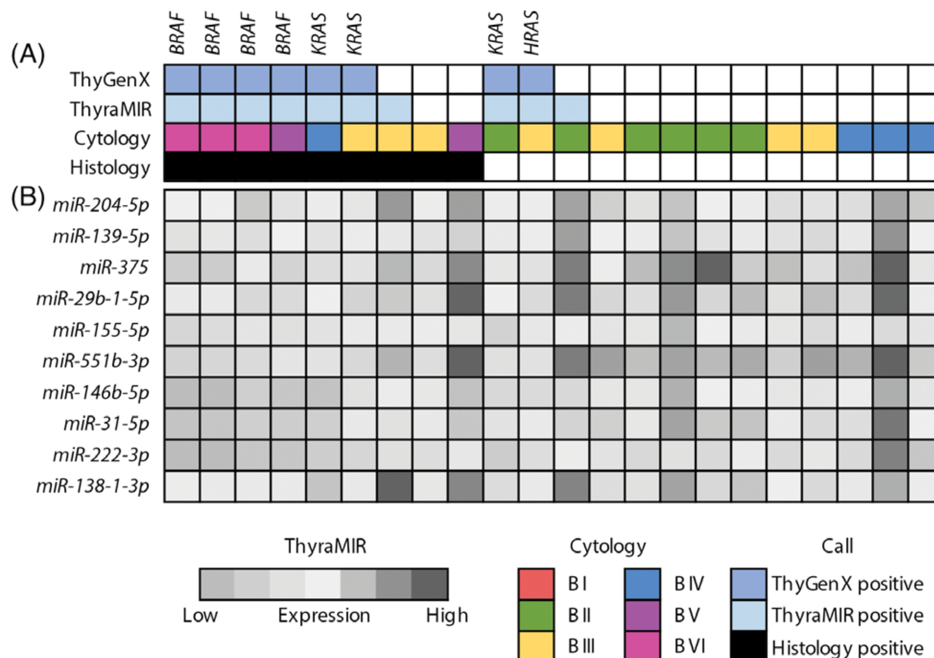


FIGURE 4 Clinical and genomic annotation of microdissected cytology smears. A, Correlation between molecular profiling by ThyGenX and ThyraMIR tests and the associated cytology and histopathology diagnoses (row) in each of the 22 microdissected fine-needle aspiration cytology smear samples (column) tested by both assays. B, Raw microRNA expression data from ThyraMIR testing of microdissected cytology smears

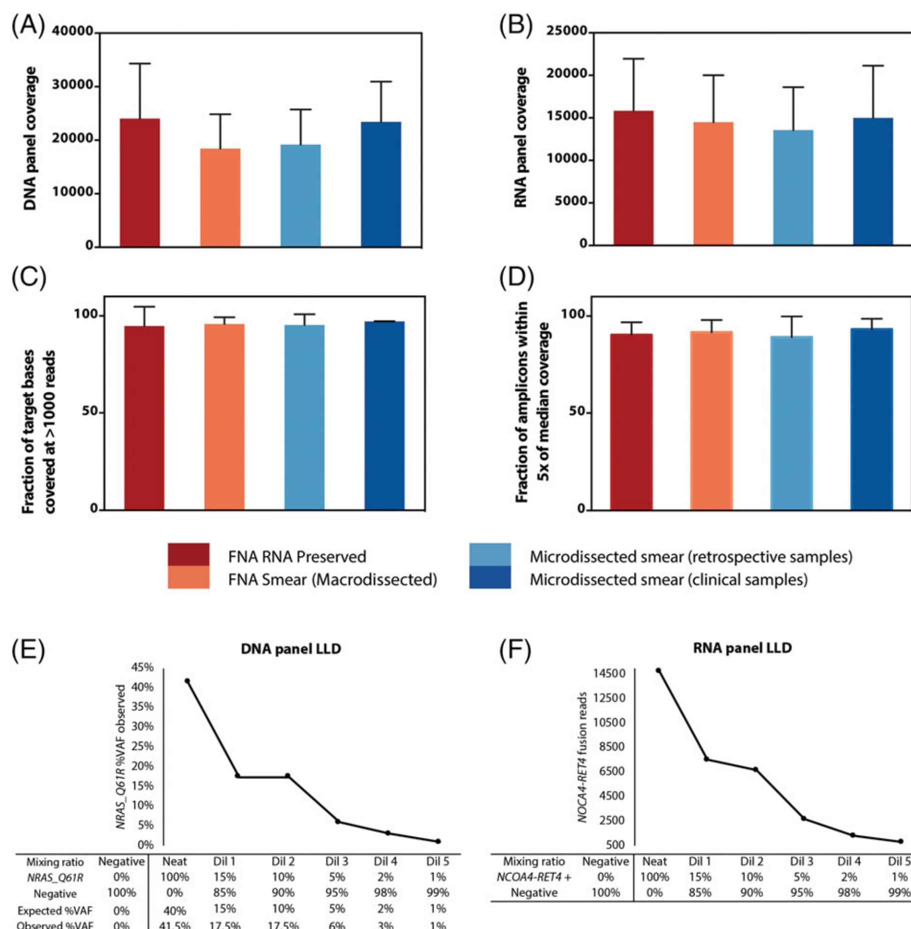


FIGURE 5 Quality metrics for Next Generation Sequencing (NGS) testing from cytology smears. Comparative plots show various QC metrics for NGS testing fine-needle aspiration stored as a smear and in preservation buffer. A, DNA panel amplicon. B, RNA panel amplicon coverage. C, Fraction of target bases coverage at >1000 reads coverage. D, Fraction of amplicon within 5× of median coverage (targeted amplicon PCR efficiency). E, DNA panel lower limit of detected (LLD) and F, RNA panel LLD

as 1%VAF. Similarly, Figure 5F shows the detection the *NCOA4_RET4* fusion reads across a range of dilutions.

For microRNA expression profiling, testing was also carried out as 2 technical replicates. Figure 6 below shows that correlation between replicate qPCR Ct values for the microRNA expression was greater than 0.95 for all 4 sample types. Correlation between replicates for FNA in RNA preservative was 0.99 (*n* = 47), for macrodissected FNA smear specimens 0.97 (*n* = 47), for retrospectively collected microdissected smear specimens was 0.98 (*n* = 22) and for clinical microdissected smear specimens was 0.99 (*n* = 14). These data indicate the robustness of the microRNA assay (Figure 6).

3.5 | Mutation and microRNA testing of cytology smears in cases where FNA in RNA preservative provided insufficient quantity of TNA for molecular analysis

During routine mutation and microRNA testing of FNA specimens, we occasionally found that total amount of nucleic acid (TNA) in the FNA in RNA preservative specimen was insufficient for molecular analysis. We hypothesized that molecular testing of cytology smears could increase the diagnostic yield in such cases without requiring an additional FNA procedure. In total, 14 clinical cases that had limited nucleic acid in the RNA preserved FNA specimens were examined. There was insufficient TNA present in 4/14 cases for mutation analysis and 14/14 cases for microRNA analysis (Table 1). All cases that initially had insufficient TNA in the original FNA specimen in RNA preservative had corresponding cytology smears that provided sufficient TNA for molecular analysis. In cases in which mutation analysis was originally assessable, mutation results of cytology smears were 100% concordant with those of FNAs in RNA preservative. Importantly, one case that otherwise would have been nonassessable by molecular because of insufficient TNA was deemed positive when cytology smears were used. These results demonstrate the successful application of cytology smear as a valuable tool in analyzing clinical patient samples.

4 | DISCUSSION

This study was designed to determine if routinely prepared thyroid FNA cytology smears can be used for molecular profiling by NGS for oncogene mutations and oncogenic fusion transcripts and by a microRNA expression classifier analysis. Two studies were initiated as follows: (1) an equivalency study to determine the utility of FNA clinical cytology smears as a sample type for molecular testing by analyzing whole slide macrodissected FNA smears in comparison to the matched aspirate material stored in RNA preservative buffer and (2) a retrospective study from archival cytology slides to show the utility of molecular testing microdissected regions from cytology slides. Our results clearly demonstrate that both the FNA in RNA stabilizing solution and the FNA smears on the cytology slides can be successfully used in the dual platform thyroid molecular diagnostic test for mutational change (ie, ThyGenX) and microRNA expression-classification (ie, ThyraMIR).

Comparison of test results obtained using the 2 specimen types from the same patients demonstrated 98% concordance between the results of NGS-based mutation sequencing tests and 90%

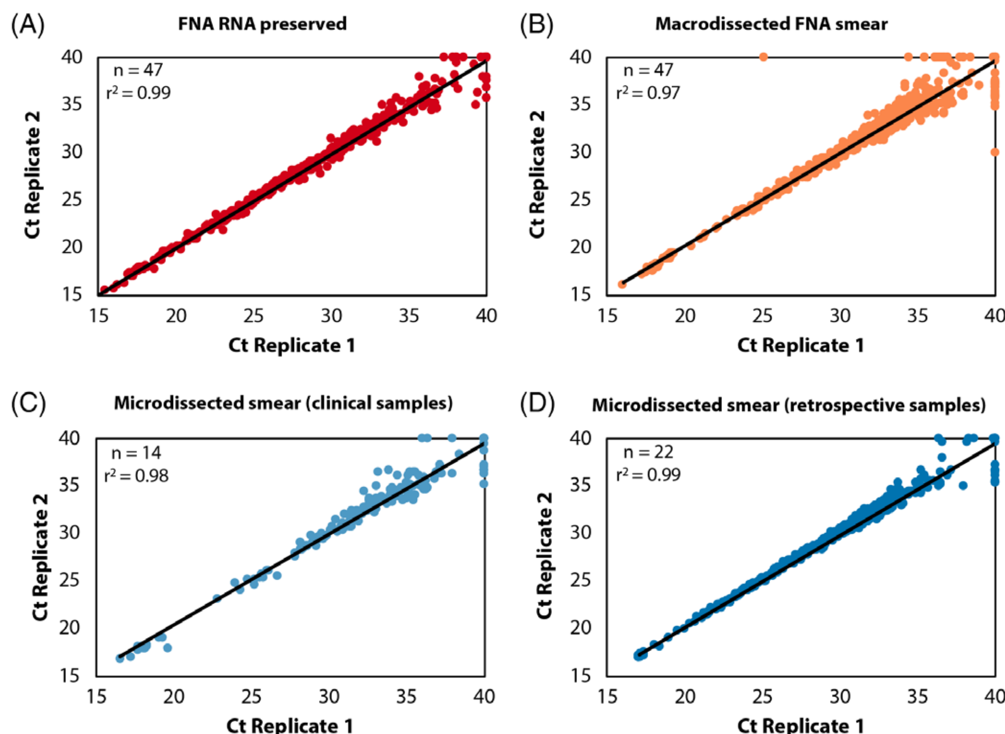


FIGURE 6 Quality metrics from microRNA expression profiling from cytology smears. Plots show correlation between replicates from qPCR for A, fine-needle aspiration (FNA) in RNA preservative; B, macrodissected FNA smears; C, microdissected smears (clinical samples); and D, microdissected smears (retrospective samples)

TABLE 1 ThyGenX and ThyraMIR testing from cytology slides in cases where FNA pass specimens were found insufficient for molecular testing

		FNA		Microdissected smear	
Case	Cytology	ThyGenX	ThyraMIR	ThyGenX	ThyraMIR
1	B III	Failed	Failed	No mutation	Negative
2	B III	No mutation	Failed	No mutation	Negative
3	B II	No mutation	Failed	No mutation	Negative
4	B III	*HRAS Q61R*	Failed	*HRAS Q61R*	Positive
5	B III	Failed	Failed	No mutation	Negative
6	B III	No mutation	Failed	No mutation	Negative
7	B IV	Failed	Failed	No mutation	Negative
8	B III	Failed	Failed	No mutation	Negative
9	NA	No mutation	Failed	No mutation	Negative
10	B II	No mutation	Failed	No mutation	Negative
11	B III	No mutation	Failed	No mutation	Negative
12	NA	No mutation	Failed	No mutation	Negative
13	B III	No mutation	Failed	No mutation	Negative
14	B III	No mutation	Failed	No mutation	Negative

Table above shows cytology as indicated by the Bethesda category. B II, Bethesda II; BIII, Bethesda III; B IV, Bethesda IV; and NA, cytology not available.

concordance between the results of microRNA expression-based tests. The higher level of concordance for the mutational sequencing test is most likely due the algorithm by which each test is considered positive. A positive result for the mutational sequencing test is based on the detection of oncogenic mutations and fusions, whereas a positive result for the microRNA expression test is based on the complex expression profile of 10 microRNAs within the specimen. This expression profile correlates to a microRNA expression coefficient that lies within a range of coefficients that are more or less indicative of malignancy.[11,12] Given the potential enrichment of malignant cells using microdissection, the microRNA coefficient is more likely to be at the extreme of this range, well above the threshold for a positive result. By contrast, FNA specimens in RNA preservative typically have mixed cell populations, including both benign and malignant cells, such that microRNA coefficients are nearer to the threshold for a positive result. Consistently, when discordant results were found for the microRNA expression test, the microRNA coefficient for the FNA specimen in RNA preservative was in proximity to the threshold for a positive microRNA result whereas the coefficient for the cytology smears was above the threshold.

Importantly, metrics of data quality from cytology specimens were as robust as those used clinically for FNA specimens in RNA preservative. However, when we tested instances where the quality and quantity of nucleic acid derived from FNA's in RNA preservative was insufficient for molecular analysis, microdissection of cytology slide smears provided sufficiently high quantity and quality of nucleic acid for analysis. In these cases, molecular testing of microdissected cytology slides provided clinically meaningful results.

The ability to perform molecular analysis of microdissected cytology smears could minimize concern for sampling variation between needle passes. In this alternative approach, molecular testing of the microdissected cytology slide material can ensure molecular analysis of the same diagnostic cellular material used to render the cytology diagnosis. The obvious disadvantage is that the archived cytology slide material may be consumed for molecular analysis. In another scenario, initial molecular testing could be done using FNA in an RNA stabilizing buffer and the FNA on cytology smears could be used for additional testing in cases where initial molecular results do not correlate with the cytological results and other clinical findings.

Noteworthy issues, when employing ancillary molecular analysis, are sampling variation when the specimen is divided for separate testing and competition for representative material for optimal correlative interpretation. Slide-format sample procurement using microdissection has the advantage of using cytologic features to ensure optimal specimen division for molecular testing after microscopic assessment is based on the entire sample. Even with direct analysis of the needle aspirate, the requirements for both NGS sequencing analysis and microRNA profiling as quite low enabling the bulk of the aspirate to be used for cytology interpretation. Careful attention is required however when multiple needle aspiration passes are employed since individual needle passes are more likely to lead to sampling variation. Individual passes should be managed in a fashion that enables each part of each pass to contribute to both microscopic and molecular analysis.

In summary, we provide a comprehensive approach to maximize the diagnostic material of each individual FNA pass, offering options to carry out mutational and microRNA testing using FNA specimens either in RNA preservative fluid or on cytology slides, depending upon limiting factors. Furthermore, when molecular results from an FNA pass are insufficient or where more clarity is desired, we show that combination mutation and microRNA testing of cytology slides can to provide supplemental results for each FNA pass.

CONFLICT OF INTEREST

The authors are employees of Interpace Diagnostics Corporation.

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