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ORIGINAL ARTICLE

Clinical Laboratory Validation and Implementation of Quantitative, Real-Time PCR-based Detection of NPM1 Type A Mutation

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SUMMARY

***Background:* *NPM1* mutations have prognostic significance in acute myeloid leukemia (AML) and monitoring mutant *NPM1* levels during and after therapy has been described to predict relapse and survival. Despite the published significance of this molecular biomarker, routine monitoring for mutant *NPM1* levels has not been widely adopted in academic clinical laboratories. Therefore, our objective was to validate a quantitative, reverse transcription-PCR assay for the detection of NPM1 Type A mutant transcripts for use in the clinical laboratory.**

***Methods:* A quantitative, real-time, reverse-transcription PCR-based method for the detection of *NPM1* Type A mutant transcripts was validated for use in routine clinical practice. Results from this assay were compared to results from orthogonal methods, including next generation sequencing and digital droplet PCR.**

***Results:* This real-time, reverse-transcription PCR-based method is sensitive (limit of detection: 0.0150% NCN and reproducible (≤ 0.5 $\log_{10}$-fold variation). We summarize the rigorous validation results and share observations that will help other clinical laboratories that may wish to implement this testing. We show the superior sensitivity of this assay compared to other assays (e.g., 45 gene Myeloid Next Generation Sequencing panel) and present a representative case which highlights the assay's utility in the pathologic assessment of cases with borderline morphologic or flow cytometric findings.**

***Conclusions:* As molecular testing for residual disease in AML continues to expand, this sensitive and reproducible method will be an appropriate testing option for the detection of *NPM1* Type A mutant transcripts in clinical practice.**

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INTRODUCTION

Nucleophosmin (NPM1), a nucleolar protein expressed at high levels in many cell types, regulates several cellular functions including cell growth [1]. NPM1 is involved in tumorigenesis of different cell types through either gene rearrangement or mutation [1,2]. Importantly, approximately 50% of acute myeloid leukemia (AML) cases with a normal karyotype (overall, approximately one third of all adult AML patients) have been shown to have mutations in *NPM1* [2,3]. A variety of mutations have been reported, which consist of small

insertion/deletion (in/del) frameshift variants in exon 11, frequently involving codons Trp288 or Trp290 [3]. Type A mutations (c.860_863dupTCTG; p.W288Cfs* 12) are the most frequent, accounting for approximately 80% of all *NPM1* mutations. These mutations alter the NPM1 protein C-terminus, and functionally, they lead to aberrant cytoplasmic localization of NPM1 in leukemic cells [3].

NPM1 has been shown to have prognostic significance in AML that varies with co-existence of mutations in *FLT3* (internal tandem duplications, ITD) and *DNMT-3A*. In general, mutation of *NPM1* in AML is associated with a favorable impact on prognosis [2]. Patients with *NPM1* mutations without *FLT3* ITD or *DNMT3A* mutations have a better prognosis than those with co-existing *FLT3* ITD or *DNMT3A* mutations [2,4-7]. These prognostic implications can influence the management of patients, including decisions relating to bone marrow transplantation [2,8]. Importantly, *NPM1* has been reported to be a stable molecular marker that is detectable at the time of relapse in > 90% of patients who had *NPM1*-mutant AML at diagnosis [8-10].

Technically, the assessment of *NPM1* mutations has been reported using both RNA-based and DNA-based methods [10,11]. RNA-based methods have been reported to be more sensitive than DNA-based approaches and, therefore, have been used in key published monitoring studies [8,11]. The most widely used RNA-based approach is a quantitative, real-time, reverse transcription PCR (RT-PCR) method using mutant-*NPM1* specific primers where the *NPM1*-mutant transcript levels are normalized by ABL1 transcript expression (% Normalized Copy Number: (mutant *NPM1* copies/ABL copies) x 100).

Despite the biologic and prognostic significance of *NPM1* mutations in AML, routine testing for the quantitative monitoring of *NPM1* has not been widely implemented in academic clinical laboratories. Recently, there has been renewed interest and increasing clinical demand for monitoring mutant *NPM1* levels. Ivey et al. have recently demonstrated that AML patients with persistence of mutated *NPM1* transcripts in the blood after the second cycle of chemotherapy had a higher risk of relapse and a lower rate of survival than patients who did not have detectable mutated *NPM1* transcripts [8]. In addition, another recent study has suggested that *NPM1*-mutant allele burden at diagnosis may impact prognosis in *de novo* AML [12]. The ability to detect and monitor mutant *NPM1* is part of a renewed, broad-based interest to employ molecular and immunophenotypic markers to detect residual/recurrent disease in acute myeloid leukemia [13,14].

Given the increasing clinical demand for detecting and monitoring mutant *NPM1* levels, we report herein how our clinical laboratory has validated a quantitative, real-time, RT-PCR-based method for clinical detection of the *NPM1* Type A mutation in patients with acute myeloid leukemia. Of note, this assay is distinct from the PCR/capillary electrophoresis or Next Generation Sequencing (NGS)-based methods that may be used to test samples for *NPM1* mutations. Since the validation of this quantitative, real time, RT-PCR-based method was compliant with rigorous New York State Clinical Laboratory Evaluation Program (NY CLEP) standards, we believe this will be a helpful resource for other clinical laboratories that may decide to implement similar clinical, quantitative testing for *NPM1*.

MATERIALS AND METHODS

A quantitative, real-time, reverse transcription PCR (RT-PCR) assay for the detection and monitoring of *NPM1* Type A mutant transcripts in patient samples using RNA from peripheral blood and/or bone marrow aspirate specimens was validated for use in our clinical laboratory. A standard one-way work flow was used between separate work areas (e.g., pre-PCR area for specimen preparation, pre-PCR hood for master mix preparation, amplification area for thermocycling, and post-PCR area for detection).

Patient samples (EDTA tubes) may be collected and transported briefly at room temperature, but, upon receipt, should be stored at 2°C to 6°C in order to prevent RNA degradation. Samples should contain several mL of fresh peripheral blood or bone marrow aspirates (or fresh cell preparations with > 5 million cells). Red blood cell lysis and RNA extraction is performed according to standard protocol (QIAamp RNA Blood Mini Kit (Qiagen 52304)). The samples should provide at least 1 µg (1,000 ng) of total RNA upon RNA extraction with A260/A280 ratios of 1.7 to 2.0.

The RNA is reverse-transcribed into cDNA according to standard protocol with > 1µg (1,000 ng) of total RNA input per sample in 20 µL final volume (SuperScript III RT (Life Technologies, #18080085); RNaseOUT (Life Technologies, #10777019); Random Primers (Life Technologies, #48190011); dNTP mix, 10 mM (Promega, #U151B); First Strand Buffer, 5x and DTT, 0.1 M). Samples are incubated at 65°C for 5 minutes, then placed on ice or left at 4°C for at least 1 minute. Cycling condition are: 25°C (10 minutes), 50°C (50 minutes), 85°C (5 minutes), 4°C (hold), using an Applied Biosystems GeneAmp 9700 thermocycler or ProFlex PCR systems. cDNA samples may be kept at 4°C before immediate use or stored at -20°C for up to 1 week. The cDNA from the patient samples and positive and negative controls is diluted by adding 30 µL of nuclease-free water (50 µL total volume). Then, 5 µL of the diluted cDNA (corresponding to one tenth (> 100 ng RNA equivalent)) is then used as input into the quantitative, real-time PCR.

Quantitative, real-time PCR is conducted using the *NPM1* mutA MutaQuant primers and probes (Ipsogen, Qiagen, #677513) that includes primers and probes for *NPM1* Type A mutation and ABL, as well as *NPM1* Type A and ABL cDNA samples for standard curve generation. Quantitative PCR is performed in an ABI

7500 Fast Real-Time PCR system (Applied Biosystems) with TaqMan Universal PCR Master Mix (Life Technologies, #4304437) using the following cycling conditions: 50°C (2 minutes); 95°C (10 minutes); [95°C (15 seconds), 60°C (60 seconds)] x 50 cycles. For on-board amplification plot analysis, a threshold of approximately 0.1 is set for both *NPM1* and *ABL*. The results are reported as % Normalized Copy Number (%NCN) calculated as follows: (*NPM1* Mutant A Copy Number/ ABL Copy Number) x 100. The MV-4-11 cell line (negative for *NPM1* Type A mutation; ATCC, CRL-9591) and the OCI-AML3 cell line (positive for *NPM1* Type A mutation; DSMZ, ACC 582) may be used as control samples. An *NPM1* Type A mutation low positive (LP) control near the limit of detection of the assay as well as a negative control (e.g., MV-4-11 cell line) and a No Template Control (NTC) are included in every run. All samples and controls are run in duplicate reactions.

Patients tested by this assay should be previously known to have tested positive for the *NPM1* Type A mutation (*NPM1*, NM_002520, Exon 11, c.860_863dup TCTG, p.W288Cfs*) (HG19/GRCH37) through alternate genotyping methods. Samples from patients with *NPM1* mutations that are not Type A mutations are not appropriate for testing by this assay and require a separate mutation specific assay.

Myeloid NGS Panel: Targeted enrichment of 45 genes recurrently mutated in myeloid malignancies was performed using the Thunderstorm system (RainDance Technologies, Billerica, MA, USA) using a custom primer panel followed by sequencing using the Illumina MiSeq (v3 chemistry) yielding 260-bp paired-end reads. The Myeloid NGS Panel interrogates entire coding exons or select exons, as well as flanking intron sequence for single nucleotide variants (SNV) and insertion/deletions (INDEL). Each sample is run in duplicate. The detection sensitivity of the assay for SNV and INDEL, as determined by a separate comprehensive validation, is approximately 2% for SNV and 1% for INDEL.

Digital Droplet PCR: Digital droplet PCR was performed with the BioRad (Hercules, CA, USA) QX200 system (Automated Droplet Generator, Droplet Reader, C1000 Touch Thermocycler and PX1 PCR Plate Sealer). Primers included: NPM1-Common-Forward:
GAAGAATTGCTTCCGGATGACT,
NPM1-Type-A-Reverse:
CTTCCTCCACTGCCAGACAGA,
ABL1-Forward:
TGGAGATAACACTCTAAGCATAACTAAAGGT,
ABL1-Reverse:
GATGTAGTTGCTTGGGACCCA.
Probes included: NPM1 (6FAM/MGB):
ACCAAGAGGCTATTCAA,
ABL1 (VIC/MGB):
CATTTTTGGTTTGGGCTTC.
Final concentration was 250 nM and 500 nM for probe and primers; 100 ng cDNA was input per reaction. Droplet generation was performed according to manufacturer instructions and subsequent thermocycling conditions were: 95°C for 10 minutes, 40 cycles (94°C x 30 seconds and 60°C x 1 minute) (ramp rate 2°C/seconds), followed by 98°C for 10 minutes. Results were analyzed with the Quantasoft software (BioRad).

This work is covered under the IRB Protocol #: 1007 011151.

RESULTS

The accuracy of this quantitative, real-time, RT-PCR assay was assessed by comparing the real time RT-PCR results to orthogonal methods, including next generation sequencing and digital droplet PCR (ddPCR); the accuracy was 100% for 10 positive samples (see Table 1) and 100% for 10 negative samples (data not shown). The inter-run reproducibility (see Table 2) of the assay showed minimal variation (i.e., < 0.5 $\log_{10}$) for 10 different samples, including both high level and low level positive samples, near the lower limit of detection. In addition, the *NPM1* Type A Mutation and ABL1 standard curves also showed excellent reproducibility between runs with average slopes of -3.45, -3.47 and R^2 values of 1.00 (Supplemental Figures 1 and 2). In terms of sensitivity, the maximal reproducible analytic sensitivity of this assay was approximately 0.01% NCN (*NPM1* MutA/ABL) (0.006% - 0.013% NCN) demonstrated by patient sample and cell line serial dilutions (see Table 3 and Supplemental Table 1). To further assess analytic sensitivity, Probit analysis for 10 replicates across a range of dilution levels revealed a 95% probability (i.e., confidence) limit of detection (LOD) of 0.0150% NCN (Supplemental Table 2, Supplemental Figure 3). Lastly, this RT-PCR assay showed excellent concordance of the % NCN values with a separate digital droplet PCR (ddPCR) assay across the range of values in a dilution series and for several samples tested near the lower limit of detection; as seen in Supplemental Table 3 and Supplemental Figure 4. The results of the two assays showed excellent correlation ($R^2 = 1$). The power of NPM1 MRD surveillance by qRT-PCR has been documented in the literature by studies [8,11, 13,14] with large cohorts and longer follow up than we currently have available; therefore, we present a case which highlights another very important application of this assay, which is its ability to help resolve cases with borderline morphologic and flow cytometric findings by providing definitive, objective, molecular data that establishes the presence of the neoplastic clone in a more timely and more sensitivity manner than typical NGS testing. The patient is a 39-year-old woman who was recently diagnosed with acute myeloid leukemia, cytogenetic analysis of which revealed a normal female karyotype. Molecular testing by NGS at the time of diagnosis was positive for a *FLT3* internal tandem duplication (39 bp insert) and was also positive for the *NPM1* Type A mutation (c.860_863dupTCTG; p.W288Cfs*12, 42% VAF), as well as variants in *DNMT3A* (c.2171A>G;

Table 1. Accuracy for positive samples. Real Time PCR compared to reference method(s).

Sample #	Specimen	NPM1 Type A mutation RT-PCR Result	NPM1 Type A mutation RT-PCR (%, NCN [1])	Next Gen. Seq. reference result	ddPCR result (if available)	Path. diagnosis
P1	PB	positive	216.28	positive, NPM1 Type A[2], 56% VAF	positive	AML
P2	PB	positive	250.59	positive, NPM1 Type A[2], 40% VAF	positive	AML
P3	BM	positive	179.06	positive, NPM1 Type A[2], 28% VAF	positive	AML
P9	PB	positive	263.29	positive, NPM1 Type A[2], 51% VAF	positive	AML
P11	PB	positive	620.63	positive, NPM1 Type A[2], 47 % VAF	positive	AML
P13	PB	positive	405.03	positive, NPM1 Type A[2], 50% VAF	N/A	AML
P14	PB	positive	82.44	positive, NPM1 Type A[2], 43% VAF	positive	AML
P16	PB	positive	577.4	prior AML sample: NPM1 Type A[2] , 46% VAF. current sample: AML (87% Blasts)	positive	AML
P18	PB	positive	83.94	history of NPM1 Type A[2] ,48% VAF. Flow Cytometry (within 9 d of current sample) showed Residual AML: 1% Blasts	positive	residual AML
P19	PB	positive	398.09	history of NPM1 Type A[2]. NGS & Pathology (within 12 d of current sample) showed AML with NPM1 Type A[2], 18% VAF	positive	residual AML

NOTES:
1: %NCN = % Normalized Copy Number = (NPM1 Mutant Type A Copies/ABL Copies)*100.
2: NPM1 Type A mutation: c.860_863dupTCTG; p.W288Cfs*12.

Table 2. Inter-Run Reproducibility.

Sample	RUN1: % NCN	RUN2: % NCN	RUN3: % NCN	% NCN mean	% NCN SD	Max log fold change
N16	ND	ND	ND	0	0	/
N21	ND	ND	ND	0	0	/
N22	ND	ND	ND	0	0	/
P1, high+	216.28	482.78	520.04	406.37	165.67	0.38
P11, high+	620.63	554.7	625.07	600.13	39.41	0.05
P13, high+	407.97	624.36	626.5	552.94	125.56	0.19
P1, low+	0.28	0.28	0.33	0.30	0.03	0.07
P11, low+	0.096	0.106	0.116	0.11	0.01	0.08
P16, low+	0.065	0.088	0.117	0.09	0.03	0.26
Pos cell line, low+	0.045	0.042	0.084	0.06	0.02	0.30

NOTES: % NCN = % Normalized Copy Number = (NPM1 Mutant Type A Copies/ABL Copies)*100.

p.Y724C, 51% VAF and c.932_945delTGTCTTGGTG GATG; p.V311Dfs*8, 51% VAF) and NF1 (c.7789 T>C; p.S2597P, 52% VAF). The patient was treated with 7 + 3 induction chemotherapy, including midostaurin, and a bone marrow biopsy was performed on day 24. The bone marrow biopsy (Figure 1) revealed a hypercellular marrow with myeloid hyperplasia and left shifted myeloid maturation with 6% blasts as well as megakaryocytic hyperplasia. No circulating blasts were observed in the peripheral blood. The patient had re-

Table 3. Analytic sensitivity. Patient sample.

Patient sample dilution (% pos. control RNA)	NPM Type A mutant, number of copies	ABL, number of copies	% NCN, NPM1 Type A Mutant/ABL	Mean, % NCN	SD
P11_100%	121,808.750	35,411.234	343.983	355.525	16.323
P11_100%	131,026.953	35,695.621	367.067		
P11_10% dilution	27,605.953	50,782.969	54.361	51.901	3.479
P11_10% dilution	27,324.826	55,267.434	49.441		
P11_1% dilution	2,317.520	43,984.738	5.269	5.610	0.483
P11_1% dilution	2,429.558	40,819.695	5.952		
P11_10-1% dilution	342.882	40,465.391	0.847	0.822	0.036
P11_10-1% dilution	322.556	40,501.297	0.796		
P11_10-2% dilution	58.689	56,396.590	0.104	0.100	0.006
P11_10-2% dilution	54.337	57,233.090	0.095		
P11_10-3% dilution	13.156	94,619.969	0.014	0.013	0.002
P11_10-3% dilution	11.611	98,536.828	0.012		
P11_10-4% dilution	1.466	88,979.555	0.002	0.001	0.001
P11_10-4% dilution	0.110	90,285.188	0.000		
P11_10-5% dilution	0.000	96,103.969	0.000	0.000	0.000
P11_10-5% dilution	0.004	87,504.039	0.000		
N14	0.000	84,404.031	0.000	0.000	0.000
N14	0.000	75,076.672	0.000		

Table 4. Real Time PCR (RNA input) vs. 45 Gene-Myeloid NGS panel (Genomic DNA input).

Case	Real Time, RT-PCR NPM1 MutA/ABL, % NCN	Myeloid NGS panel, % VAF	Myeloid NGS panel, (mutant reads/total reads)
1	0.025	0	0/1,357, 0/1,972
2	0.049	0	0/2,462, 0/2,001
3	57.21	0.9	20/1,694, 12/1,810
4	0	0	0/1,297, 0/1,189
5	0.015	0	0/2,020, 0/1,935
6	65.98	0.8	2/102, 0/145
7	63.26	0.4	12/2,451, 6/2,226
8	0.007	0	0/1,572, 0/1,308
9	0	0	0/2,903, 0/2,672
10	0.02	0	0/2,258, 0/1,725
11	0	0	0/2,729, 0/2,869
12	0.82	0	0/3,403, 0/3,103
13	0	0	0/3,404, 0/3,017
14	0	0	0/4,544, 0/2,462
15	0	0	0/2,680, 0/2,556
16	0	0	0/1,712, 0/1,384
17	0	0	0/1,768, 0/1,659
18	0	0	0/1,888, 0/1,958
19	0.017	0	0/3,294, 0/3,784
20	0	0	0/3,036, 0/2,279
21	0.14	0	0/5,052, 0/4,566

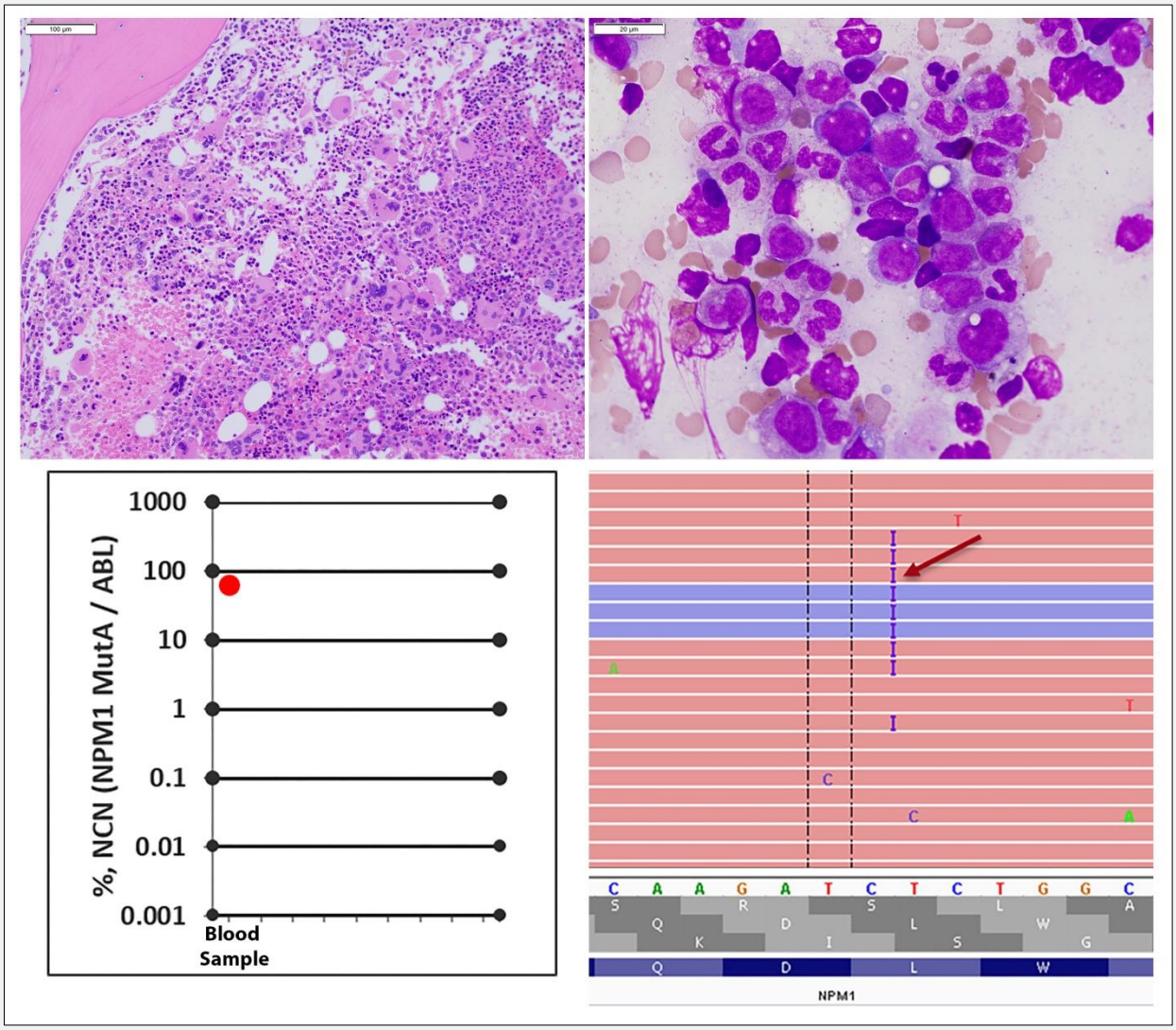

Figure 1. Data from a clinical patient.

An image of the H&E stained core biopsy (upper left) shows overall hypercellularity and relative myeloid hyperplasia. An image of the Wright-Giemsa stained bone marrow aspirate smear (upper right) shows maturing myeloid elements with full maturation, but with a slight left shift in maturation. Graph of the *NPM1* Type A mutation Real Time, RT-PCR results (lower left) showing the presence of the *NPM1* Type A mutation (63.3% NCN, Normalized Copy Number: *NPM1* Type A Mutation/ABL). Image of the sequencing reads (lower right) in Integrative Genomics Viewer (IGV©, Broad Institute) from genomic DNA-based sequencing also reveals the *NPM1* Type A mutation (0.4% variant allele frequency).

cently received growth factors, so it was not clear if the morphologic findings were a result of the recent growth factor administration, or whether they represented impending recurrence of the acute myeloid leukemia. Likewise, flow cytometry was not definitive for residual disease. Since the patient was known to have an *NPM1* Type A mutation, a peripheral blood sample was tested by the *NPM1* Type A mutation RT-PCR assay described above, and the patient was found to have a high level of *NPM1* Type A mutation (63.3% NCN, *NPM1* Type A Mutation/ABL; Figure 1 and Table 4 (case 7)). Subsequent next generation sequencing data with the Myeloid NGS Panel was performed on the genomic DNA from the peripheral blood sample and also revealed the Type A *NPM1* mutation (0.4% variant allele frequency (VAF); Figure 1 and Table 4 (case 7)). The prior *FLT3*

ITD (39bp) was also noted on NGS (0.4% VAF) and the prior *DNMT3A* variants were also seen at low VAF (*DNMT3A* c.2171A>G; p.Y724C, 1.5% VAF and c.932 _945delTGTCTTGGTGGATG; p.V311Dfs*8, 3.1% VAF). Given that the definitively positive RT-PCR results were available in a timely manner, the patient was closely followed over the following days and 14 days after the *NPM1* Type A mutation RT-PCR result, a follow up peripheral blood specimen was sent for flow cytometry and revealed that the patient had 25% circulating abnormal blasts, consistent with residual/recurrent acute myeloid leukemia. In this case, the high level of NPM1 Type A mutant transcripts appeared to more closely reflect the impending relapse, whereas the NGS findings revealed only a very low value, below the usual limit of detection for the NGS assay. Overall, the results from this case help demonstrate an important application of this assay (beyond routine serial MRD surveillance) which is to facilitate the interpretation of cases with borderline/unusual morphologic or immunophenotypic findings by providing objective molecular data which can identify the presence of a neoplastic clone in a timely manner, with greater sensitivity and more robust signal compared to other methods (e.g., routine NGS) and, thereby, this RT-PCR assay can help guide appropriate clinical follow up.

Lastly, given the difference in the *NPM1* Type A mutation levels observed by this real time, RT-PCR method (using RNA input) and the next generation sequencing (NGS) method (using genomic DNA input) for the case described above, we compared the results of the *NPM1* Type A mutation levels as measured by the RT-PCR method to the mutation levels determined by NGS (using genomic DNA-based input) for samples which have data available from both assays. The results are summarized in Table 4 and demonstrate the superior sensitivity of the RNA-based RT-PCR method for *NPM1* Type A mutation detection, compared to a genomic DNA-based Myeloid NGS panel with abundant read depths (e.g., 1,500 - 2,000 x) at this *NPM1* genomic location. This greater sensitivity of the RNA-based RT-PCR method is consistent with prior reports indicating a greater sensitivity of RNA-based methods compared to genomic DNA-based input [11,14] and is also consistent with the known high expression of NPM1 [15-17].

Taken together, our validation studies demonstrate that this quantitative, real-time, RT-PCR method using RNA input is a sensitive and reproducible method for the detection of NPM1 Type A mutant transcripts in clinical samples. In addition, the clinical case results demonstrate how this assay can provide important findings when used in the routine clinicopathological workup of patients being monitored for residual acute myeloid leukemia. Lastly, we have found that this assay format provides greater sensitivity than a typical genomic DNA-based NGS approach (e.g., 45 gene Myeloid NGS panel).

DISCUSSION

Residual disease monitoring is becoming increasingly adopted in routine clinical care for acute myeloid leukemia (AML); flow cytometric methods focus on detecting abnormal myeloid cells with altered immunophenotypic profiles, but this approach has some limitations due to the heterogeneity of immunophenotypic abnormalities in AML, as well as technical challenges [14]. Molecular methods, which can complement flow cytometric-based studies, are increasingly being implemented as an approach for monitoring residual disease in AML. Although AML cases frequently harbor at least 1 or 2 driver mutations [18], not all of these mutations are suitable for residual disease monitoring [14,19, 20].

NPM1 mutations in AML were described approximately 15 years ago [3] and the mechanism of pathogenesis continues to be studied [21]. Meanwhile, the utility of tracking mutant *NPM1* as a reliable marker of disease status in AML continues to expand [8,10,13,14]. Herein, we report the validation and clinical implementation of a quantitative, real-time, RT-PCR assay for the detection and monitoring of the most common *NPM1* mutation (i.e., Type A *NPM1* mutation) in AML or other myeloid disorders. We have found that this assay is sensitive (i.e., maximal reproducible sensitivity approximately 0.01% NCN) and reproducible (i.e., 100% concordance with known positive and known negative samples and < 0.5 $\log_{10}$-fold variation between replicates/ batches) for the detection of *NPM1* Type A mutant transcripts.

Based on our experience, the following observations may be helpful to other laboratories who wish to implement this assay. In terms of sample requirements, we have found that it is important to receive 1 or 2 EDTA tubes each containing several milliliters of peripheral blood or bone marrow aspirate, and that RNA extraction and elution should be optimized to permit appropriate RNA concentrations, so that the requisite RNA amount can be put into the cDNA synthesis reaction. This is important since it will influence the overall sensitivity of the assay, by impacting the number of ABL copies obtained (ideally, at least 10,000 ABL copies obtained per replicate reaction, *see below for additional details*). In terms of results interpretation for assay controls: the NTC (No Template Control) sample should be negative for both *NPM1* Type A and ABL; the negative control sample (e.g., MV411 cell line or other known negative sample) should be negative for *NPM1* Type A mutant transcripts but positive for ABL; the low positive control sample should be positive for NPM Type A mutant transcripts (e.g., near the lower limit of the reportable range of the assay, 0.01 - 0.1% NCN) and should also be positive for ABL. The standard curves (*NPM1* Mut A and ABL) should each show slopes of -3.3 to -3.6 and R^2 values of > 0.95. The data obtained should be consistent between duplicates such that the CT (cycle threshold) value in between duplicates is +/- 1 to 2 cycles. For

patient samples, *NPM1* Type A may be considered negative if it is not detected in both duplicate reactions and the replicates show at least 10,000 ABL copies. *NPM1* Type A may be considered positive if it is detectable in both duplicate reactions with at least 10 *NPM1* mutA copies in at least 10,000 ABL Copies (i.e., > 0.1% NCN (*NPM1* mutA/ABL). If one of the duplicates has a positive value for *NPM1* mutA and the other replicate is not detected, or if both duplicates show a value of < 0.1% NCN (*NPM1* mutA/ABL) or < 10 *NPM1* mutA copies, then new cDNA is prepared and the assay may be repeated. If the repeat reactions yield a positive result in both of the new replicates, then the result may be considered positive. Similarly, if the repeat reactions yield a negative result in both of the new replicates, then the result may be considered negative. Lastly, if one of the new replicates is low positive and then the other new replicate is negative, then the average of the positive replicates from the first and second runs may be taken, and the result may be considered positive. Lastly, if a patient sample is negative for *NPM1* Type A, but the ABL copy number is less than 10,000, then the sample may be considered suboptimal for minimal residual disease, and a note can be added to the report to suggest testing a new sample. As is true for all molecular testing, interpretation of the results in the context of the clinical and pathologic findings (e.g., flow cytometry residual disease testing, etc.) is helpful.

According to European Leukemia Net MRD Working Party (ELN) consensus document [14], a sensitivity of 0.1% is suggested for residual disease platforms, and real-time qPCR platforms with cDNA-based inputs are recommended because of their sensitivity. Recommended sampling time points include at diagnosis, after 2 cycles of standard induction/consolidation chemotherapy and at the end of treatment, using peripheral blood and bone marrow samples. In addition, after the end of treatment, molecular MRD assessment is recommended every 3 months for 24 months. Upon serial sampling, molecular progression has been proposed to be defined as an increase in copy numbers of 1 $\log_{10}$ or more between any 2 positive samples.

In addition to its sensitivity, advantages of the quantitative RT-PCR assay include: *i)* its technical suitability for widespread implementation (e.g., similar to BCR-ABL for CML) due to standardized instrumentation and *ii)* its shorter turn-around time (e.g., 2 days) compared to NGS (e.g., 1 - 2 weeks). Nevertheless, one of the limitations of the current qRT-PCR assay is that it is mutation-specific. Therefore, for monitoring of *NPM1* Type A mutation levels with this assay, the patient should be known to have had the *NPM1* Type A mutation (c.860_863dupTCTG; p.W288Cfs*12) at initial diagnosis. Since the *NPM1* Type A mutation primers and probes may show unpredictable cross-reactivity against other types of *NPM1* mutations, patients with "Non-Type A" *NPM1* mutations require testing by separate mutation-specific primer sets for qRT-PCR. Recently, a multiplex digital droplet platform capable of detecting and quantitating several different *NPM1* mutation types in one assay has been described, although in that assay platform, the type of *NPM1* mutation detected in a sample is not specifically identified and validation of the performance (and potential cross reactivity) of the degenerate multiplex reverse primer mix is still required [22].

As technology evolves, NGS-based methods will likely be increasingly integrated into MRD testing algorithms, especially as the need for tracking several potential molecular biomarkers becomes increasingly desired. The Myeloid NGS assay used for comparison in this study, was a "typical" Myeloid NGS panel (i.e., 45 gene panel), similar to those often used in academic centers and commercial laboratories for testing at diagnosis, which are frequently amplification-based sequencing assays, often without unique molecular barcodes and consist of multigene panels (e.g., 40 - 50 genes). As technology advances, the use of smaller NGS panels that incorporate error reduction measures (e.g., unique molecular barcodes) and provide ultrahigh sequencing depths that permit very sensitive detection of a few, select consensus "molecular MRD biomarkers" in myeloid diseases will likely expand. Indeed, such "ultradeep" NGS sequencing approaches have recently been published for *NPM1* [23,24] with 300,000 or more sequencing depths using genomic DNA input. The advantages of DNA-based NGS methods include the stability of DNA as an input substrate and the ability to detect all NPM1 mutation types with one assay. However, the disadvantages that limit the widespread use of such biomarker-specific "ultradeep" NGS assays for routine clinical testing, at this point in time, include variability in institutional informatics support (i.e., "in-house scripts") required for such complex NGS assays, the high amount of input DNA required in some protocols, the expense of NGS (especially if separate "ultradeep" NGS panels are needed for different biomarkers), and the significant institutional investment in instrumentation and human resources required for developing and maintaining such NGS assays. As mentioned previously, for monitoring NPM1, the European Leukemia Net MRD Working Party (ELN) [14] has recommended real-time qPCR platforms with RNA/cDNA-based inputs.

In conclusion, an overall testing strategy for AML is evolving whereby several assays are performed at diagnosis (including NGS panel testing, cytogenetic analysis as well as rapid, focused assays interrogating potential therapeutic targets (e.g., *FLT3*)), which is followed (i.e., during and post-therapy) by sequential monitoring of mutations known to be reliable markers of residual disease (e.g., *NPM1*). In this respect, the assay described herein offers an effective option for the testing of patients with myeloid diseases with the *NPM1* Type A mutation.

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JR, WT, and MK declare that they have no conflict of interest.

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