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Detection of minimal residual disease in *NPM1*-mutated acute myeloid leukemia by next-generation sequencing

Stephen J. Salipante[1,2], **Jonathan R. Fromm**[1], **Jay Shendure**[2], **Brent L. Wood**[1], and **David Wu**[1]

[1]Departments of Laboratory Medicine, University of Washington, Seattle, WA

[2]Genome Sciences, University of Washington, Seattle, WA

Abstract

Detection of minimal residual disease predicts adverse outcome in patients with acute myeloid leukemia. Currently, minimal residual disease may be detected by RQ-PCR or flow cytometry, both of which have practical and diagnostic limitations. Here, we describe a next-generation sequencing assay for minimal residual disease detection in *NPM1*-mutated acute myeloid leukemia, which encompasses ~60% of patients with normal karyotype acute myeloid leukemia. Exon 12 of *NPM1* was PCR amplified using sequencing adaptor-linked primers and deep sequenced to enable detection of low-prevalence, acute myeloid leukemia-specific activating mutations. We benchmarked our results against flow cytometry, the standard-of-care for acute myeloid leukemia minimal residual disease diagnosis at our institution. The performance of both approaches was evaluated using defined dilutions of an *NPM1*-mutation positive cell line and longitudinal clinical samples from acute myeloid leukemia patients. Using defined control material, we found this assay sensitive to approximately 0.001% mutant cells, outperforming flow cytometry by an order-of-magnitude. Next-generation sequencing was precise and semi-quantitative over 4 orders-of-magnitude. In 22 longitudinal samples from 6 acute myeloid leukemia patients, next-generation sequencing detected minimal residual disease in all samples deemed negative by flow cytometry. Further, in one-third of patients, sequencing detected alternate *NPM1*-mutations in addition to the patient's index mutation, consistent with tumor heterogeneity. Next-generation sequencing provides information without prior knowledge of *NPM1* mutation subtype or validation of allele-specific probes as required for RQ-PCR assays, and without generation and interpretation of complex multi-dimensional flow cytometry data. This approach may complement current technologies to enhance patient-specific clinical decision-making.

Page proofs, correspondence and requests for reprints: David Wu, MD, PhD, Dept. of Laboratory Medicine, UW Hematopathology Laboratory at SCCA #G-7800, 825 Eastlake Ave East, Seattle WA, 98109, Phone: (206) 288-7662, Fax: (206) 288-7127, dwu2@uw.edu.

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Keywords

next-generation sequencing; minimal residual disease; acute myeloid leukemia; *NPM1*; prognosis; tumor heterogeneity; relapse

Introduction

Acute myeloid leukemia is an aggressive hematopoietic neoplasm associated with significant mortality and morbidity. The presence of minimal residual disease, that is, small numbers of neoplastic cells which persist after cancer therapy, has been shown in independent cohorts to correlate with disease relapse, even in patients with very limited disease. Minimal residual disease detection is particularly important in individuals treated with myeloablative hematopoietic stem cell transplantation, as often, these patients are younger (1-4).

In the United States, multi-parametric flow cytometric identification of minimal residual disease is the clinical standard of care for minimal residual disease detection in acute lymphoblastic leukemia and is increasingly used at many institutions for post-treatment monitoring of acute myeloid leukemia. Flow cytometry requires detection of leukemia-associated immunophenotypes in the context of regenerative hematopoiesis, as well as complex instrumentation and analysis of high-dimensional data, making it challenging to perform routinely or on a standardized basis (5, 6). Further, as no universal immunophenotype for relapsed blasts is observed in acute myeloid leukemia, it is necessary to employ a relatively large antibody panel in order to identify specific markers that can be used to monitor individual patients. Additionally, post-therapy immunophenotypic drift of leukemic blasts may confound consistent detection of minimal residual disease by flow cytometry, particularly in the context of marrow regeneration (7).

Molecular detection of minimal residual disease by RQ-PCR has been developed recently (1, 4), and takes advantage of common mutations in the gene nucleophosmin (*NPM1)* in acute myeloid leukemia. Activating insertion mutations in exon 12 of *NPM1* are frequent and recurrently observed in nearly one-third of all acute myeloid leukemia patients and in approximately 60% of patients with normal karyotype (8, 9). The most common mutation subtype, Type A, is a 4-base pair insertion of TCTG (p.Trp288Cysfs*10, NM_002520.4:c.956_959dup). Type A mutations are frequently seen in adults, representing approximately 75 to 80% of *NPM1* mutations in acute myeloid leukemia. However, at least 50 types of mutant *NPM1* have been described (8), and some patients may have private mutant alleles. Thus, the clinical laboratory use of RQ-PCR for acute myeloid leukemia minimal residual disease diagnosis requires that the patient's *NPM1* mutation be defined in advance and that probes targeting the specific mutation be available and validated. The ability to detect unexpected *NPM1* mutations may not be possible by RQ-PCR alone. Additionally, each probe differs in sensitivity and specificity, and for some mutation types probe cross-reactivity with the non-mutated allele becomes limiting (4).

Next-generation DNA sequencing represents an alternative molecular testing approach to the detection of minimal residual disease in acute myeloid leukemia. Next-generation

sequencing offers significant advantages in enabling the sensitive detection of low-prevalence mutations, as well as an unprecedented economy of scale (10, 11). We recently demonstrated the first application of next-generation sequencing for minimal residual disease detection in T-lineage acute lymphoblastic leukemia (12), and with others showed comparable potential for minimal residual detection in B-lineage acute lymphoblastic leukemia (13, 14). Here, we describe a next-generation sequencing assay for the detection of minimal residual disease in *NPM1*-mutated acute myeloid leukemia.

Materials and Methods

Samples

Residual, clinical samples were obtained and de-identified according to University of Washington Institutional Review Board guidelines. A total of 22 samples from 6 patients were used in this pilot study (Table 1). All patients had a confirmed histologic or flow cytometry diagnosis of acute myeloid leukemia and were selected for further study if sufficient residual DNA (at least 200 ng) was available for analysis. DNA extraction was performed using a QiAgen X-tractor with Reagent Pack DX (Germantown, MD).

Flow cytometry

Multi-parametric flow cytometry was performed at the University of Washington as part of the routine evaluation for minimal residual disease as described (6, 15). All samples were labeled with the antibody combinations in three tubes as follows: Tube A = HLA-DR PB, CD15 FITC, CD33 PE, CD19 ECD, CD117 PE-Cy5, CD13 PE-Cy7, CD38 A594, CD34 APC, CD71 APC-A700, and CD45 APC-H7; Tube B = HLA-DR PB, CD64 FITC, CD123 PE, CD4 ECD, CD14 PE-Cy55, CD13 PE-Cy7, CD38 A594, CD34 APC, CD16 APC-A700, CD45 APC-H7; Tube C = CD56 A488, CD7 PE, CD5 PE-Cy55, CD33 PE-Cy7, CD38 A594, CD34 APC, CD45 APC-H7. Reagents were purchased from either Becton-Dickinson (Franklin Lakes, NJ) or Beckman-Coulter (Pasadena, CA). Samples were processed using standard methods (red cell lysis using NH_4Cl+0.25% formaldehyde), and >400,000 events were acquired on a 4-laser, 10-color Becton-Dickinson LSRII flow cytometer. Abnormal clusters of events that differed from regenerative blasts were reported relative to total leukocytes following erythroid lysis. Groups of events differing from regenerative blasts by visual inspection were identified using in-house software and considered minimal residual disease.

Next-generation sequencing

Sequencing library production methods were modified from Caporaso, *et al.* (16). 200 ng DNA was amplified using KAPA HiFi HotStart ReadyMix (Kapa Biosystems, Woburn, MA) with primers specific to NPM1 (Reverse primer: 5′-AATGATACGGCGACCACCGAGATCTACACTATGGTGCCTGTAAACACGGTAGGGAAAGTTCTC-3′, Forward primer: 5′-CAAGCAGAAGACGGCATACGAGATNNNNNNNNAGTCAGTCAGTCTGTCTATGAAGTGTTGTGGTTCC -3′, where N's indicates the position of an 8 base pair, sample-specific index sequence). Amplicons from different specimens were pooled in equimolar amounts and sequenced on an Illumina MiSeq (San Diego, CA) using 150 paired-end

chemistries. Custom sequencing primers were used (Read 1: 5′-TATGGTGCCTGTAAACACGGTAGGGAAAGTTCTCA-3′, Read 2: 5′-AGTCAGTCAGTCTGTCTATGAAGTGTTGTGGTTCC-3′, Index Read: 5′-GGAACCACAACACTTCATAGACAGACTGACTGACT-3′). Oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, Iowa).

Data analysis

Sequencing runs were de-multiplexed using on-instrument software, allowing only perfectly matched index sequences to be assigned to their specimen of origin. Overlapping, paired-end reads were self-assembled using PANDAseq (17). The length of the DNA fragment sequenced (162 bp in a non-mutated gene) enables error correction of overlapping, independently sequenced paired-end reads (17) to be performed over the majority of the sequence fragment, including the documented sites of *NPM1* insertion mutations. Self-assembled reads were mapped to the human genome (hg19/Ghr37) using the bwasw alignment mode of the aligner bwa (0.6.2) (18) with non-default parameter “-r 1” as described elsewhere (19). Variants were called using VarScan (v2.3.6) (20) using parameters set for a minimum variant frequency 1×10^{-10}, a minimum of 1 variant read, and minimum average base quality of 5.

Results

Performance characteristics of next-generation sequencing and flow cytometry on a defined NPM1-positive sample

To explore the potential of next-generation sequencing for detecting *NPM1*-mutant minimal residual disease, we diluted *NPM1*-mutated cell line OCI-AML3 (21) into normal patient marrow (a staging specimen from a patient with classical Hodgkin lymphoma, which was negative by flow cytometry for abnormal B and T cell populations, myeloid blasts, and maturing myelomonocytic cells). Four separate technical replicates of each dilution were prepared for sequencing on each of three independent sequencing runs, yielding a total of 12 independent assessments per dilution. We found that semi-quantitative measurement of spiked cells could be achieved over 4 orders-of-magnitude (10% to 0.01%, Figure 1), with minimal intra- and inter-run variability (Table 2). At a level of 0.001% *NPM1*-mutated cells, flow cytometry did not detect any abnormal cells. At that dilution, next-generation sequencing replicates inconsistently recovered and detected *NPM1*-mutation positive reads: all four replicates from the first run were positive, whereas mutated sequence reads were detectable from only 1 and 2 replicates in the second and third sequencing runs, respectively. Thus, approximately 0.001% abnormal cells represents the limit-of-detection for conditions tested, comparable to the performance of RQ-PCR (4). Given an input of 200 ng DNA (corresponding to approximately 33,333 diploid cells), the assay has a theoretical detection limit of 1 cell in 33,333 (0.003%), consistent with our empirical observations when Poisson sampling from a population of low-prevalence molecules is considered. In this controlled experiment, next-generation sequencing and flow cytometry provide comparable detection of spiked OCI-AML3 cells to the level of 0.01%. Nevertheless, it should be noted that the flow cytometry diagnosis of minimal residual disease in clinical practice is typically

more challenging than this contrived scenario (5-7), which used an abnormal cell line with a distinct immunophenotype (data not shown), which was simple to identify.

Specificity of next-generation sequencing for detection of NPM1 mutations

We next evaluated the specificity of next-generation sequencing for detecting *NPM1*-mutated minimal residual disease by examining peripheral blood samples from 20 anonymous, normal blood donors. A total of 8,861,993 sequence reads were generated from that cohort, corresponding to an average of 443,100 ± 41,438 (mean ± standard deviation) reads per individual. On analysis, no sequence reads matched any of the 17 most common *NPM1* mutations (4), corresponding to a sensitivity of <1 in 221,550 cells (0.00045%) for detecting a false positive mutant allele. We conclude that identification of *NPM1* insertion mutations by next-generation sequencing is specific for minimal residual disease detection.

Comparison of next-generation sequencing to flow cytometry in longitudinal clinical samples

We investigated next-generation sequencing performance using 22 longitudinal clinical samples derived from 6 acute myeloid leukemia patients. In all samples tested, the patient's original *NPM1* mutation identified at diagnosis, which we term the “index clone”, could be convincingly detected. Of note, we could detect the index clone in six post-treatment samples that were interpreted as negative by clinical flow cytometry (Table 1). This finding, coupled with the high specificity of the assay, suggests that next-generation sequencing may enhance the sensitivity for minimal residual disease detection as compared to flow cytometry in clinical samples.

Detection of NPM1-mutation clonal heterogeneity

In samples with detectable *NPM1* mutations, it was possible to identify rare artifacts resulting from single-base substitution errors introduced through library preparation and sequencing as mutant alleles (19). In our cell line dilution studies, for which the specific *NPM1* mutation is known and defined, this empiric level of error was experimentally quantified as 0.02 ± 0.027% (mean ± standard deviation) of the true mutant alleles sequenced. Secondary mutations occurring significantly (several standard deviations) above that threshold, however, likely indicate the presence of other clones.

In Patient-1, an alternate clone was clearly identified. In the index sample, only one *NPM1* mutation (NM_002520.6:c.[864G>C;869delinsTCCTA]) was detected. However, in four subsequent samples spanning 38 months, next-generation sequencing detected increasing levels of the Type A allele that were quantified significantly above the empiric error frequency (>3.5 standard deviations), with a concurrent decrease of the index allele (Figure 2A, B). At days 1127 and 1162, the read counts of Type A allele surpassed those of the index allele. In Patient-2, we similarly detected heterogeneity (Figure 2A, C), harboring a Type B index mutation (NM_002520.6:c.863_864insCATG). A low-prevalence, but significant level (>900 standard deviations above the empiric error frequency) of Type R allele (NM_002520.6:c.863_864insTATG) was found in the index case, but became undetectable after initialization of treatment. By day 37, a low-prevalence Type A clone was identified at significant levels (>25 standard deviations above the empiric error frequency),

which became dominant at day 110. At ∼1 year, the amount of Type A allele was surpassed by the index clone, but nevertheless persisted.

In both of these patients, our findings are most consistent with the detection of genetically distinct *NPM1* clones. These alternate alleles are unlikely to be artifact due to the abundance of reads detected. Moreover, the Type A *NPM1* mutant allele differs from the index allele seen at diagnosis for Patient-1 by 10 nucleotides and from that of Patient-2 by 2 nucleotides (Figure 2A), a degree which is unlikely to result from simultaneous sequencing artifacts in multiple reads. The interpretation of alternative clones is further supported by flow cytometry in Patient-1 (Figure 2D). Not only did the relapse myeloid blast population differ from the index monoblast clone by immunophenotype, this relapse myeloid blast population continued to increased in proportion over time (Table 1), mirroring the results for the second *NPM1* clone as detected by sequencing. These results together provide evidence that next-generation sequencing can detect distinct tumor clones as defined by different *NPM1* mutations. Alternate *NPM1* mutations were not identified in other patients examined.

Discussion

Mutations in *NPM1* have been shown to represent an early genetic lesion in acute myeloid leukemia and are therefore thought to be one of several driver mutations in this disease (8). Several groups have demonstrated that mutant *NPM1* is generally stable through treatment, and typically, *NPM1* mutation can be detected in post-treatment relapse samples as a measure of recurrent disease (22). Some have suggested that mutant *NPM1* is not identified in relapse samples from a small subset of patients, implying that mutation in *NPM1* is not always an early lesion or that *NPM1*-mutated cells may respond to treatment (23, 24). Nevertheless, the prognostic value of monitoring *NPM1* mutations in minimal residual disease has been independently demonstrated (4, 25). Of particular note, *NPM1* mutation level has been confirmed as an independent clinical prognostic factor for relapse and survival (26). Thus, monitoring of acute myeloid leukemia through *NPM1* mutation is generally agreed to be an important prognostic marker.

The most widely used approaches for detecting minimal residual disease in *NPM1*-mutated acute myeloid leukemia, in general, are RQ-PCR (1, 4) and flow cytometry (2, 3). However, as each approach offers its own combination of benefits and drawbacks, the best method to assess minimal residual disease in a patient care context remains a matter of debate (27-29). Importantly, it should be noted that in limited studies in which molecular diagnosis of various mutated targets and flow cytometry were directly compared, results have demonstrated equivalency of these approaches (27), and it is generally believed that both RQ-PCR and flow cytometry are suitable for diagnosis of minimal residual disease in acute myeloid leukemia (28, 30). In this work we explored the utility of next-generation sequencing to identify minimal residual disease in *NPM1*-mutated acute myeloid leukemia and report several key findings.

The first major finding of this work is that although next-generation sequencing has proven only semi-quantitative, likely secondary to PCR bias during sequencing library construction, the use of next-generation sequencing overcomes several of the limitations presented by

both flow cytometry and RT-PCR for minimal residual disease detection. As compared to flow cytometry, we found that one advantage of minimal residual disease detection by next-generation sequencing is the potential for increased sensitivity for detecting abnormal blasts. In our cell dilution studies, next-generation sequencing was determined to have a limit of detection of approximately 0.001% minimal residual disease, an order of magnitude greater than observed in concurrent flow cytometry studies for the conditions tested (Figure 1). Even so, consistent clinical detection of abnormal myeloid blasts and particularly blast equivalents (promonocytes or monoblasts) by flow cytometry is often more challenging than this contrived experimental scenario, and the level of success depends greatly upon on the immunophenotype of the abnormal blasts and how distinct it is from background regenerative blasts (5-7). This was also observed in our analysis of clinical samples. Specifically, it is noteworthy that all clinical specimens examined in this study, even if diagnostically negative by flow cytometry, were found to harbor minimal residual disease by next-generation sequencing (Table 1). The clinical implication of these low frequency positive results though merits further study and consideration. Some investigators have postulated that the persistence of low-levels of minimal residual disease in some patients may indicate effective immune surveillance (27), whereas others have suggested that these results may alternatively represent an early harbinger of relapse (31). Irrespective, the consistent detection of minimal residual disease by next-generation sequencing after treatment in 6 of 6 randomly selected patients of our cohort suggests that low level clonal persistence may be a consistent biological feature of acute myeloid leukemia. Accordingly, monitoring of patients by next-generation sequencing may provide important clinical information regarding the kinetics of these populations and thus insight into the biologic and clinical behavior of a patient's disease.

Another advantage of next-generation sequencing as compared to flow cytometry is the potential for enhanced specificity. While in general the level of blasts estimated by next-generation sequencing was comparable to that of flow cytometry, in a subset of cases, the measured leukemic blast proportion by next-generation sequencing differed from flow cytometry based-estimates (Table 1). There are two important points to note regarding these apparent discordances. First, by flow cytometry, admixed regenerative myeloid blasts may be variably present in the post-treatment setting and consequently difficult to consistently distinguish from leukemic blasts due to an overlap of immunophenotypes between the two populations. This may therefore lead to either over- or underestimation of leukemic blasts by flow cytometry depending on how gating of the leukemic blast versus regenerative blast populations is performed. Second, as flow cytometry is typically performed on samples subjected to erythroid lysis protocols that also result in the removal of nucleated erythroid precursors, the blast estimate by flow cytometry is enumerated and reported with respect to total white cells. However, DNA from early erythroid precursors will contribute to the total templates examined by DNA sequencing. Thus, in cases with erythroid hyperplasia, flow cytometry estimates of blast percentage may be greater than those estimated by sequencing. As next-generation sequencing is not subject to either of these artifacts, it is likely that next-generation sequencing more accurately estimates the minimal residual disease burden, albeit in a semi-quantitative fashion. This conclusion is supported by our cell spike-in studies

(Figure 1), in which we observed strong concordance of next-generation sequencing results versus theoretical dilutions for *NPM1*-mutation detection.

As compared to RT-PCR, there are several potential advantages to the next-generation sequencing approach we propose herein. As the primers used in our assay are situated outside of *NPM1* exon 12, all reported *NPM1* mutations (9) should be detectable with comparable efficiency using this approach. Accordingly, this assay eliminates the need for mutation-specific probes as required for RQ-PCR and the related challenges in ensuring suitable performance characteristics for multiple probe sets (4, 30). As a case in point, Patient-1 had a novel mutation (NM_199185.3:c.777_782delinsCCAGTTCCTA), previously unreported in COSMIC (Figure 2A). This contrasts with RQ-PCR assays that require prior knowledge of the *NPM1* mutation subtype or use of multiple mutation-specific probes (4). In light of these considerations, next-generation sequencing for minimal residual disease could serve as a universal approach for clinical laboratory detection in *NPM1*-mutated acute myeloid leukemia, providing a technically defined analytic method that is equally applicable to all patients with *NPM1*-mutated blasts. Notably, both RQ-PCR and our proof-of-principle next-generation sequencing assay are still limited to use in cases in which *NPM1*-mutation positive minimal residual disease is present. However, because next-generation sequencing technologies permit high-throughput analysis with enhanced economy of scale, next-generation sequencing-based assays could be readily expanded to include additional potentially informative targets, such as *DNMT3A*, *KIT*, *IDH1*, *IDH2* or other driver mutations, for minimal residual disease detection (32-37).

As we have previously noted, the sensitivity of our assay was similar to published values for RQ-PCR assays (4). Nevertheless, minor modifications could increase this value even further. The sensitivity with which next-generation DNA sequencing is able to detect low-prevalence mutations is proportional to the number of DNA molecules examined, the effective number of sequence reads generated for each template molecule, and the rate with which sequencing or library preparation errors result in false-positive mutation calls (19, 38). Here, the specificity of the assay for *NPM1* insertion mutations has proven non-limiting, with no false positive *NPM1* mutation reads detected in normal blood donor samples: this specificity is due to the fact that it is quite unlikely by PCR or sequencing artifact to replicate 4-base pair or larger *NPM1* insertion mutations. Accordingly, it should theoretically be possible to further increase sensitivity by proportionally scaling the amount of template DNA and the read depth allocated per sample, assuming that sufficient quantities of sample are available. We also note that, while others previously explored the use of next-generation sequencing for detecting *NPM1* mutations, the reported limit-of-detection for those studies was 0.02% (11), 20-fold higher than reported here. Aside from technical considerations including differences in sequencing platforms used, this difference likely resulted in part from under-sampling of template alleles, given the limited read-depth (~7,758 reads). Thus, some reported negative results in the previous study may represent false negatives (11).

The second major finding of this study is the presence of alternate, genetically distinct *NPM1*-positive sub-clones that were identified in a surprisingly high proportion (2 of 6) of the randomly selected acute myeloid leukemia patients we studied. In Patient-1 (Figure 2B),

the original, index clone was seen to decrease after initiation of therapy, but a secondary population bearing a different *NPM1* mutation gradually increased in proportion, coinciding with the detection of an immunophenotypically distinct population of abnormal blast by flow cytometry (Figure 2D). In the other, Patient-2 (Figure 2C), an alternate sub-clone was detected at the time of diagnosis, but was not identified in other specimens from the same patient, suggesting its elimination during treatment. Nevertheless, a separate, genetically distinct clone was detected just over one month after the index sample and persisted through the remaining 2 time points. Intriguingly, it was the patient's originally index clone, and not either sub-clone, that returned during the patient's apparent relapse a year after diagnosis. Findings from this unselected, albeit limited cohort suggest both that the dynamics of minimal residual disease and acute myeloid leukemia relapse may be more complex than previously appreciated and that sub-clones may occur in an apparently significant proportion of acute myeloid leukemia patients. The identification of such sub-clones has not previously been possible by existing clinical technologies, although recent work has suggested that acute myeloid leukemia clonal heterogeneity can represent an adverse prognostic marker in some patients (39). Longitudinal studies will be required to determine the clinical significance of clonal heterogeneity detected by next-generation sequencing in *NPM1*-mutated acute myeloid leukemia.

Lastly, the clinical detection of uncommon (non-Type A) alleles of *NPM1* may be clinically important and is efficiently achieved by next-generation sequencing. Alpermann and colleagues recently reported that rare *NPM1* mutation types had a trend toward favorable event-free and overall survival as compared to patients with the common Type-A mutations (40). Moreover, in patients with concurrent *FLT3*-ITD mutation, which is typically regarded as a poor prognostic genetic marker that overrides the favorable prognostic effect of *NPM1* mutation, only patients with Type A *NPM1* mutations showed poor overall survival, with uncommon *NPM1* mutations conferring significantly better event-free and overall survival (40). Next-generation sequencing offers a robust approach to ascertain the specific *NPM1* mutation present and the presence of subclones or relapse clones bearing prognostically different *NPM1* mutations simultaneously.

In summary, the favorable performance characteristics and expanded diagnostic capabilities of next-generation sequencing suggest that this method may enhance minimal residual disease detection in *NPM1*-mutated acute myeloid leukemia, augmenting technologies for minimal residual disease detection currently in clinical use. In this regard, next-generation sequencing may permit opportunities for earlier clinical intervention, either through detecting very low levels of minimal residual disease, the detection of acute myeloid leukemia heterogeneity, or by enabling monitoring of the rate of change in minimal residual disease abundance (31). Defining the clinical significance of low-level minimal residual disease by next-generation sequencing, however, remains an important task.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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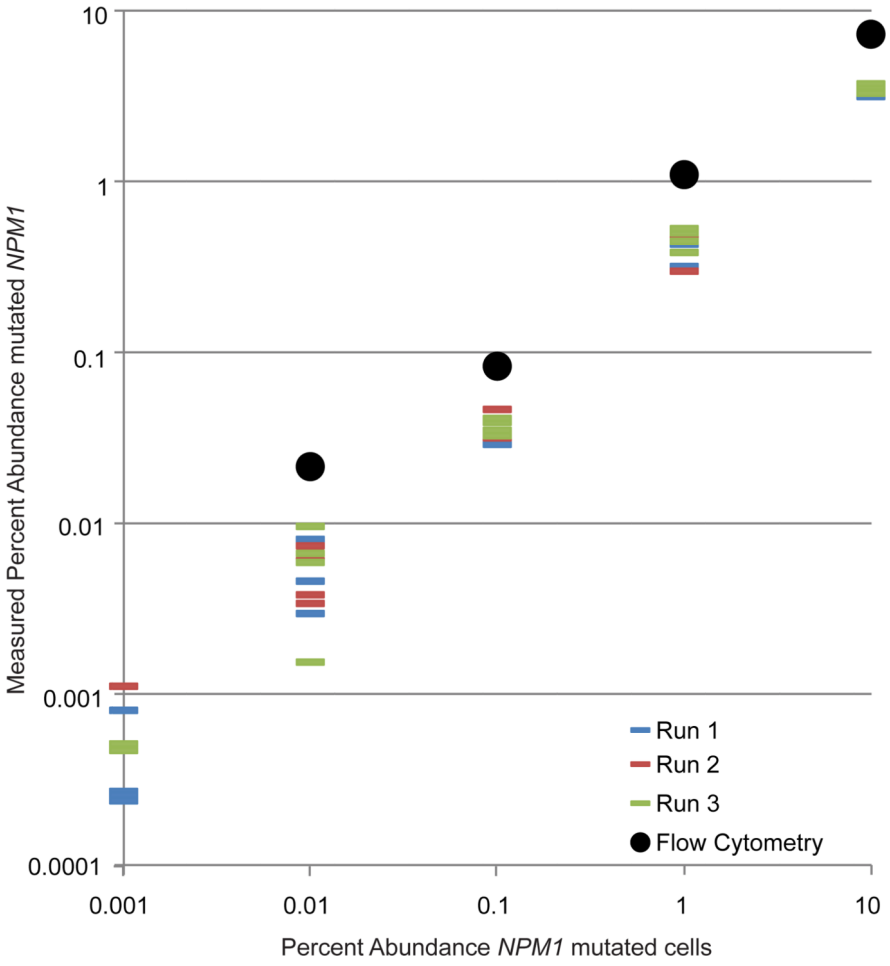

Figure 1. Next-generation sequencing for minimal residual disease detection of *NPM1* mutations

Comparison of next-generation sequencing (dash) versus flow cytometry (circle) results using cell mixtures of the *NPM1*-mutation positive OCI-AML3 cell line diluted into normal marrow. Graph depicts four technical replicates for each next-generation sequencing sample, with each set of technical replicates sequenced on three separate sequencing runs (red, green, or blue). Intra- and inter-run reproducibility data are shown in Table 2.

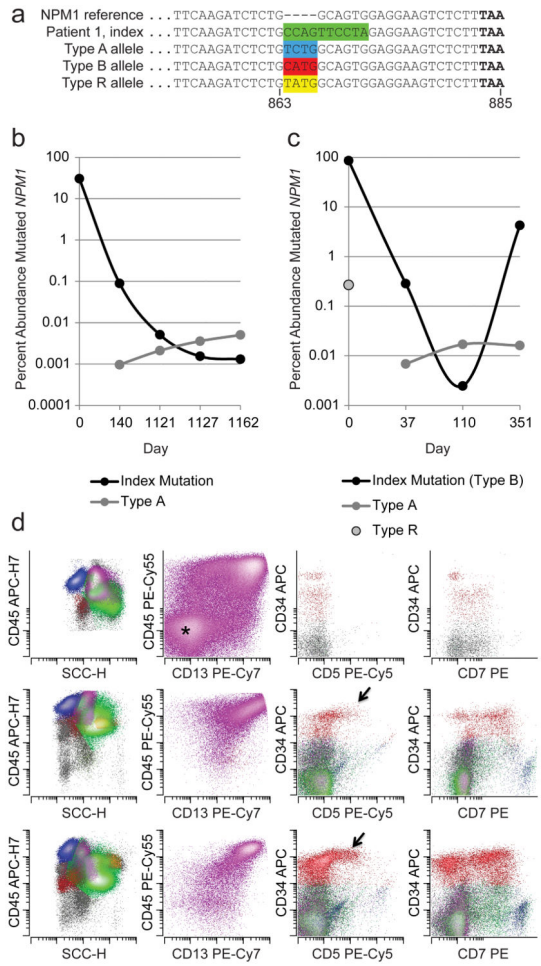

Figure 2. Evidence for *NPM1*-mutation heterogeneity in clinical samples

(**A**) Multiple alignment of presentation (index) and variant sub-clone *NPM1* mutations detected in Patients-1 and -2. Bold indicates *NPM1* stop codon. (**B**) Detection of sub-clones in Patient-1 showing decline of the index clone and subsequent rise of secondary clone (Type A mutation). (**C**) Detection of sub-clones in Patient-2, showing development and low-level persistence of secondary Type A mutation. Heterogeneity is also demonstrated in the index case by the presence of a Type R allele. (**D**). Flow cytometry immunophenotype for Patient-1 at time-points: 0 (index), top row, day 1162, middle row, and then one additional time-point, approximately 2.5 months after day 1162, bottom row. The original index clone was an expanded monoblast population, (top row, 2nd column, asterisk) without a substantial myeloid blast population component (top row, 3rd and 4th columns). The alternate clone in subsequent samples (bottom 2 rows) no longer had a monoblastic immunophenotype but rather had an abnormal myeloid blast population with aberrant lymphoid antigen expression and accounted for 0.3% and then 1.8% of total white cells, respectively (bottom two rows, 3rd and 4th columns, arrows)

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Table 1

Comparison of minimal residual disease in patient samples by next-generation sequencing versus flow cytometry

Patient	Time [days]	Flow cytometry (%WBCs)*	Mutation (allele)	Next-generation sequencing (% reads)*	Total Reads
1	0 (index)	25.20	864G>C;869delinsTCCTA (index)	30.5700	534,528
			860_863dup (A, relapse)	Not detected	
	140	0.00	864G>C;869delinsTCCTA (index)	0.0900	413,989
			860_863dup (A, relapse)	0.0034	413,989
	1,121	7.70	864G>C;869delinsTCCTA (index)	0.0100	569,095
			860_863dup (A, relapse)	0.0900	569,095
	1,127	not done	864G>C;869delinsTCCTA (index)	0.0015	586,102
			860_863dup (A, relapse)	0.0020	586,102
	1,162	0.30	864G>C;869delinsTCCTA (index)	0.0013	534,930
			860_863dup (A, relapse)	0.0050	534,930
	1,244	1.80	Not done		
2	0 (index)	68.00	863_864insCATG (B, index)	85.8476	176,098
			863_864insTATG (R)	0.2670	188,059
	37	Not done	863_864insCATG (B, index)	0.2844	204,901
			860_863dup (A)	0.0069	201,729
	110	0.90	863_864insCATG (B, index)	0.0025	201,729
			860_863dup (A)	0.0169	204,901
	351	5.50	863_864insCATG (B, index)	4.2404	188,059
			860_863dup (A)	0.0160	176,098
3	0 (index)	57.70	860_863dup(A)	70.3056	200,511
	390	67.00	"	41.8142	211,340
	405	44.10	"	24.6797	237,541
4	0 (index)	51.00	860_863dup(A)	39.1643	329,921
	291	0.00	"	0.0156	256,616
	622	0.00	"	0.0082	207,984
	914	51.40	"	31.1361	214,919

Patient	Time [days]	Flow cytometry (%WBCs)*	Mutation (allele)	Next-generation sequencing (% reads)*	Total Reads
	957	0.00	"	0.0271	221,846
5	0 (index)	15.00	860_863dup (A)	54.2468	154,307
	27	0.00	"	2.6826	983,942
	43	0.00	"	0.1397	258,701
6	0 (index)	84.40	860_863dup (A)	69.9585	490,422
	48	34.00	"	49.7879	374,717

*Note: Apparent discordances in blast estimates between flow cytometry and next-generation sequencing may be due in part to two reasons: 1) admixed regenerative myeloid blasts or neoplastic leukemic blasts may be inadvertently included or excluded, respectively, in the blast estimate by flow cytometry due to overlap of the immunophenotype of these populations; 2) estimates of blasts by flow cytometry are performed in the context of erythroid lysis and reported as a percentage of total leukocytes, whereas sequencing is reported as a percentage of total reads, which includes DNA sampled from nucleated erythroid cells that are excluded in flow cytometry.

Table 2
Variability of next-generation sequencing of *NPM1*-mutation

Percent Abundance *NPM1* mutated cells	Average inter-run Coefficient of Variation	Average intra-run Coefficient of Variation
10%	0.0373	0.0345
1%	0.148	0.0607
0.1%	0.125	0.0660
0.01%	0.407	0.216
0.001%	0.324*	0.314*

*Values calculated from replicates where mutant NPM1 reads were detected