original research report

Development of a flow cytometric method to detect the presence of mutated nucleophosmin 1 in acute myeloid leukemia

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OBJECTIVE/BACKGROUND: Nucleophosmin 1 (NPM1) plays multiple roles in cell growth and proliferation. Deletion/insertion mutations in exon 12 of *NPM1* (*NPM1-DIM*), commonly found in patients with acute myeloid leukemia (AML), alter the C-terminal amino acids and disrupt the normal nucleocytoplasmic shuttling function of the protein, which in turn leads to disease pathogenesis. However, this altered function as a result of *NPM1-DIM* positivity is actually associated with a significantly better response to therapy and overall survival, and thus it is of clinical relevance to investigate the mutation status at diagnosis. Our objective was to design a reliable flow cytometry assay to detect mutated NPM1 in peripheral blood (PB) samples from AML patients, using a polyclonal mutation-specific antibody.

METHODS: A commercially available NPM1 mutation-specific polyclonal antibody in combination with a secondary goat antirabbit antibody was used to detect the C-terminal-mutated NPM1 by flow cytometry. OCI/AML3 (+) cell line and clinical PB controls were used to optimize the assay and determine sensitivity, reliability, and reproducibility parameters. The assay was then tested on a small cohort of 12 AML patients at diagnosis and compared with *NPM1-DIM* testing on a standard polymerase chain reaction (PCR) platform.

RESULTS: Flow cytometry using the polyclonal antibody was able to reliably detect mutated NPM1 populations of at least 10%. Using an objective analysis of the mean fluorescent intensity, clear positive and negative mutated cell populations could be distinguished using the clinical AML samples. From the analysis of 12 patients, 2 were found to be positive using this assay, which corresponded with conventional PCR methodology.

CONCLUSIONS: Flow cytometry may be used to detect NPM1 C-terminal mutations in AML patients using a polyclonal anti-NPM1 antibody, allowing rapid mutation status determination at diagnosis.

KEYWORDS: Acute myeloid leukemia; Flow cytometry; Nucleophosmin 1

Acute myeloid leukemia (AML) is characterized by the rapid proliferation and accumulation of immature myeloid precursor cells in the bone marrow, resulting in disruption of normal blood cell production.[1] AML actually encompasses a heterogeneous group of diseases that traditionally relied on morphology, cytochemistry, and cytogenetic analysis for classification into various subtypes and in turn into different prognostic categories.[1,2] In those AML subtypes with recurrent cytogenetic abnormalities, patients classified under the "poor prognostic" category would be considered for an allogeneic stem cell transplant, whereas the others may not.[3] Up to 50% of AML patients, however, demonstrate a

normal cytogenetic profile with conventional cytogenetics,[4] making it difficult to assign these patients to any prognostic category. In 2008, the World Health Organization (WHO) introduced a new category called "AML with gene mutations," which allows classification of AML into prognostic subgroups depending on the specific gene mutations or combinations of mutations.[1] Mutations in this category include mutations in Fms-like tyrosine kinase 3 (*FLT3*), nucleophosmin 1 (*NPM1*), and chloramphenicol acetyltransferase-box enhancer-binding protein alpha (*CEBPA*), which carry a high prognostic value.[5] In general, *NPM1* deletion/insertion mutations (*NPM1-DIM*)[6] and *CEBPA* point mutations[7] are considered to convey a good prognosis; by contrast, *FLT3* internal tandem duplications (*FLT3-ITD*) are considered to convey a poor prognosis.[8] The combination of mutations also alters the prognostic value of the individual mutations, with coexistence of *FLT3-ITD* negating the positive prognostic effect of *NPM1-DIM*.[9] It is thus of great importance to be able to rapidly identify these mutations before initiating treatment protocols.

In cytogenetically normal AML, the most common *NPM1* mutations involve deletions or insertions in exon 12 (*NPM1-DIM*), with six major variants being described (A–F), although the most common mutations involve the insertion of 4 bp.[10] Despite variations in the exact positioning of the mutations, all resulting mutant *NPM1* translation products contain the same five C-terminal amino acids. This results in changes in their nuclear localization signal[11] and a shift in the balance of nuclear export, leading to the accumulation of NPM1 in the cytoplasm.[10,12,13] This nucleocytoplasmic shuttling protein plays an important role in regulation of ribosomal biogenesis, as well as cell-cycle regulation during oncogenic stress,[12,14,15] centrosome duplication, and regulation of apoptosis.[16] Its ability to shuttle between the nucleolus, nucleus, and cytoplasm is paramount to these functions, which are severely inhibited by the *NPM1* mutations, thus affecting the growth of the mutated cells. This actually offers a good prognosis in AML clones, as tumor growth is negatively affected.[10]

Although *NPM1-DIM* are routinely detected by polymerase chain reaction (PCR) techniques, an antibody that can detect the resulting mutated NPM1 protein could potentially be used in combination with flow cytometry to provide a relatively quick, simple, and cost-effective method for detecting these mutations in newly diagnosed AML patients. It could also be potentially included in the immunophenotyping diagnostic panel with minimal additional effort. Oelschlaegel et al.[17] developed a flow cytometric method to detect the cytoplasmic localization of NPM1. However, the monoclonal antibody used in their study was capable of detecting both mutated and nonmutated forms, potentially leading to false positivity or reduced sensitivity. Monoclonal antibodies, raised and selected specifically to detect the mutant protein, have been developed by 2 independent groups[18,19]; however, inconsistent results were obtained and these antibodies are not available commercially. One polyclonal antibody to detect mutated NPM1 protein is commercially available and has previously been validated for use in Western hybridization assays, but has not been extensively studied in the application of flow cytometry. The aim of this study was to develop a flow cytometric method to detect mutated NPM1 protein in AML patients using this polyclonal antibody.

METHODS AND MATERIALS

Cell lines and clinical samples

The OCI/AML3 cell line, which harbors a confirmed Type A exon 12 *NPM1* mutation,[20] was used as a positive control in the development of the flow cytometry assay (kindly donated by Dr. P. Szankasi, University of Utah, Salt Lake City, UT, USA). Cells were cultured in suspension using α-minimum essential medium/20% fetal bovine serum (FBS; Gibco, USA). A Jurkat T-lymphoblastic cell line (ATCC TIB-152) with wild-type (WT) *NPM1* genetics was used as a negative control for the antibody titration experiments and was cultured using RPMI-1640/10% FBS (Gibco, USA). Both cell lines were grown under standard conditions (37 °C, 5% CO_2, and 80–90% humidity).

For the analysis of clinical samples, 5 mL peripheral blood (PB) samples were collected into EDTA-coated tubes over a 6-month period from all newly diagnosed AML patients (excluding those with acute promyelocytic leukemia), who were referred to the National Health Laboratory Services at Groote Schuur Hospital, Cape Town, South Africa, for flow cytometric analysis. This produced 12 individual patients, with no specific AML subtype selected, and included those who had both normal and abnormal cytogenetic profiles (Table 1). As clinical *NPM1* WT controls, 5 mL PB was randomly obtained from routine patients with normal full blood count results and PCR-negative *NPM1-DIM* results. Informed consent was obtained from each of the participants

Table 1. AML patient characteristics.

Identifier	Age (y) and sex	WBC $\times 10^9$/l	% Blasts	Leukemia immunophenotyping[a]	WHO classification[1]	Cytogenetics[b]	FLT3-ITD[c]
AML1	46 and M	192	56	117/13/33	AML with maturation	Normal	Negative
AML2	54 and F	7	32	34/117/13/33	AML without maturation	Dup(1)q23q42	Negative
AML3	21 and F	93	93	34/117/11c/33	AML without maturation	Normal	Negative
AML4	64 and F	186	95	117/13/33	AML without maturation	No growth	Positive
AML5	23 and F	NA	80	117/3/11c/11b/13/14/33/36	AML	Normal	Positive
AML6	18 and F	165	95	117/13/33	AML without maturation	Normal	Positive
AML7	58 and F	2	20	34/117/13/33	AML with maturation	Normal	Negative
AML8	18 and M	144	75	34/117/11c/13/33/64	AML without maturation	Normal	Negative
AML9	18 and F	9	42	34/117/13/33	AML t(3;3)	t(3;3)+	Negative
AML10	69 and M	62	77	34/117/13/33	AML	No growth	Negative
AML11	35 and F	NA	>70	117/33	AML without maturation	Normal	Negative
AML12	45 and F	34	41	117/7/13/33	AML with MDS-related changes	Normal	Negative

Note: AML = acute myeloid leukemia; MDS = myelodysplastic syndrome; NA = Not available due to referral specimen being analyzed; PB = peripheral blood; WBC = white blood cell; WHO = World Health Organization.
[a]All new AML patients PB blast populations were analyzed with the following leukemia flow cytometry panel: CD2, 4, 7, 10, 19, 13, 14, 33, 34, 35, 45, 56. >20% positivity was regarded as positive.[b]Standard karyotype only reported.
[c]*FLT3-ITD* mutation status was determined by National Health Laboratory Service Molecular Haematology-accredited diagnostic laboratory.

with ethics approval from the Faculty of Health Science Ethics Committee (HREC REF: 086/2009)

To determine the assay sensitivity, OCI/AML3 cells were serially diluted into PB from the WT control patients (both sample types standardized to a final white cell count of 10×10^9/L). Dilutions ranged from 100% to 1% of OCI/AML3-positive cells. These samples were then analyzed by flow cytometry. The 10% dilution was individually prepared 14 times (dilution and antibody labeling) and analyzed on 2 separate occasions to assess reproducibility.

Overview of final flow cytometry assay

All samples were diluted in phosphate-buffered saline (PBS) to a standard nucleated cell count of 10×10^9/L. Cell surface staining was performed on 100 µL of the prepared sample using 10 µL of anti-CD45 peridinin–chlorophyll–protein complex (Becton Dickinson, USA). After incubating the cells with anti-CD45 for 15 min, erythrocytes were lysed using 1 mL FACS Lyse (Becton Dickinson, USA) for 10 min at room temperature (RT). Samples were then washed once using 2 mL of 0.5% bovine serum albumin (BSA) in PBS and the cell pellets were collected by centrifugation at 400*g* for 5 min. Cell permeabilization with FACS perm II (0.5 mL; Becton Dickinson, USA) was performed for 10 min at 4 °C and the cells were washed one more time with 0.5% BSA/PBS. The cell pellets were collected as described earlier. Optimal blocking of nonspecific binding sites was performed by resuspending the cell pellets in 1 mL of 3:2 ratio of human AB serum to 0.5% BSA/PBS and incubating at 37 °C for 2 h. The sample was then incubated with 2 µL of NPM1 mutation-specific polyclonal rabbit antihuman primary antibody (0.3 mg/mL stock; Abcam, UK) for 30 min at RT. It was then washed two times with 0.5% BSA/PBS and resuspended in 100 µL of the 3:2 AB serum/BSA mixture. Staining with 2 µL stock solution of secondary goat antirabbit antibody (Alexa Fluor 488 conjugate, Invitrogen, USA; 0.2 mg/mL) was performed for another 30 min at RT. Following this secondary antibody labeling step, the samples were finally washed two times with 2 mL of 0.5% BSA/PBS and the cell pellets were resuspended in 1 mL PBS.

Labeled samples were analyzed on an FACS Canto II instrument (Becton Dickinson, USA), and analyzed with Kaluza software (Beckman-Coulter). Blast populations were difficult to isolate in most AML cases based on CD45 and side-scatter characteristics alone,[21] and CD34 gating was not performed due to the limited CD34 positivity in some AML cases (Table 1).[17] Instead, the CD45-positive population, excluding the lymphocyte population, was analyzed, with a minimum of 10,000 events being acquired in

this gate. For the analysis of all clinical samples, a positive control (OCI/AML3) and negative control sample (both obtained on the same day) were simultaneously analyzed with the AML patient samples. NPM1 positivity was determined by analyzing the Alexa Fluor 488 fluorescence of the gated population in a histogram format. The median fluorescence intensity (MFI) of the NPM1 peak for the AML patients was determined and divided by the MFI of the NPM1 peak for the simultaneously labeled WT control sample to generate the normalized NPM1 MFI value. A normalized NPM1 MFI value of 2.0 and above was determined to correspond with *NPM1-DIM* PCR positivity.

PCR analysis of NPM1 exon 12 mutation status

The PCR detection of *NPM1-DIM* was performed in the Molecular Haematology Diagnostic Unit of the National Health Laboratory Services at Groote Schuur Hospital using a validated and South African National Accreditation System-accredited methodology. In brief, primers developed by Laughlin et al.[22] were used to amplify the relevant area of exon 12 of NPM1/NPM1F: TTTTCCAGGCTATTCAAGATC and NPM1R: GGACAGCCAGATATCA ACTG [21]. PCR products were analyzed by capillary electrophoresis (ABI 3100 genetic analyzer, Applied Biosystems, USA). GeneMapper 4.1 (Life Technologies, USA) was used to size the peaks, with a minimum peak height defined as 50 relative fluorescence units. This assay was found to be reliable to detect up to 5% of mutated alleles in our laboratory setting.

RESULTS

Assay development

The following 2 key factors were investigated to optimize the differentiation of positive and negative mutated NPM1 populations (*NPMI-DIM* associated): (a) primary/secondary antibody ratio, with 2 µL primary antimutated NPM1 antibody (final concentration 6 µg/mL) and 2 µL secondary antibody (final concentration 7 µg/mL) in a final labeling volume of 104 µL, resulting in the best population discrimination; (b) Reduction of nonspecific antibody binding, which is common in dual-labeling scenarios. Although plastic adherence was attempted to reduce the monocyte population, and various concentrations of BSA were used to block the Fc receptors on both monocyte and neutrophil populations,[23] optimal blocking of Fc receptor sites and thus nonspecific positivity was only achieved using a combination of human AB Rh-negative serum and BSA 0.5%/PBS (3:2 ratio) for 2 h at 37 °C. Figure 1 shows the reduction in nonspecific antibody binding using this approach, whereas the optimal separation of the anti-NPM1-labeled populations can be seen in Figure 2A.

Assay sensitivity and reproducibility

Using the optimized parameters, the assay sensitivity was assessed by diluting the positive control cell line (OCI/AML3) into PB of WT clinical controls, to provide a more realistic cellular profile, as opposed to a negative control cell line only. The following dilutions were assayed in duplicate: 50%, 20%, 10%, and 5%. Gating was performed on the entire CD45-positive population, as OCI/AML3 cells were not easily distinguishable from granulocytes, monocytes, and lymphocytes in these mixed samples. Figure 2 shows the results from the analysis of the 20%, 10%, and 5% mixes. The population of *NPM1-DIM* positive cells could be clearly identified at the 50%, 20%, and 10% level on the relevant histogram; however, it became progressively more difficult to differentiate the *NPM1-DIM*-positive cells from the negative population at lower levels (as is seen in Figure 1C, 5% positive cells). Reproducibility was evaluated at this 10% sensitivity limit, with 14 individually prepared 10% mixtures labeled and analyzed on two separate occasions. All 10% OCI/AML3 cell mixtures showed clear discrimination between the two peaks, with the positive *NPM1-DIM* population showing a mean MFI of 37.9 compared with the WT peak of 6.7. The MFI of the mutated NPM1 peak showed minimal variation with interexperimental and intraexperimental variability calculated at 6.1% and 5%, respectively, for the 28 samples analyzed.

Clinical assessment

To determine the specificity and performance of the assay in a clinical setting, PB samples from 12 newly diagnosed AML patients were assayed for *NPM1-DIM* using both the proposed flow cytometry approach (C-terminal-mutated NPM1 detection) and a routine PCR assay.

The PCR assay indicated that only 2/12 patients were positive for the exon 12 mutation (type not defined; Table 2, normal cytogenetic profiles). For the flow cytometric analysis, patient samples were analyzed on separate days and were coanalyzed with a 100% positive control OCI/AML3 sample and a clinically negative PB control sample, to control for variation in antibody labeling efficiencies and instrument fluctuations. Because of the high percentage of

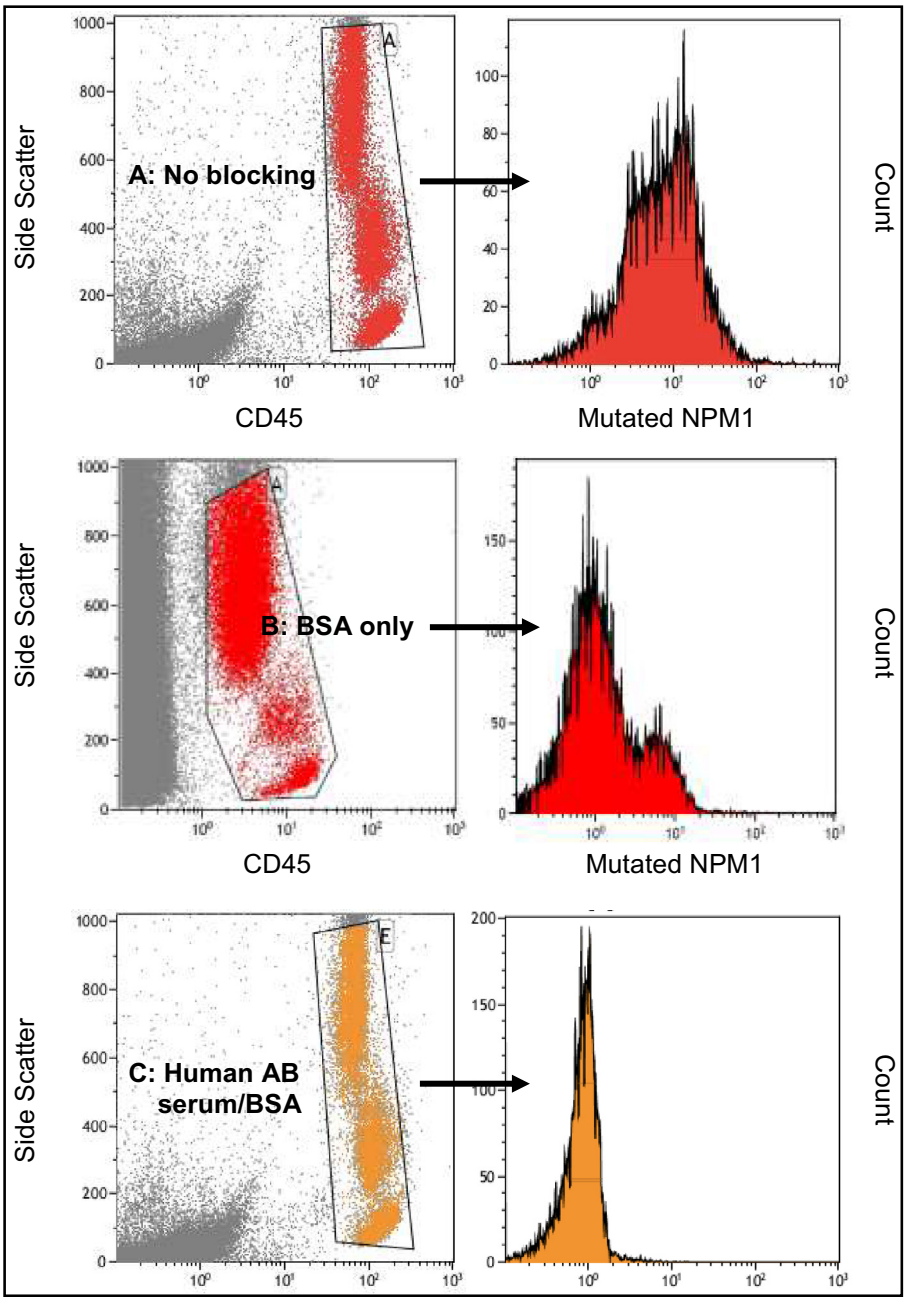

Figure 1. Reduction in nonspecific binding. WT PB was labeled with primary anti-NPM1-DIM/secondary Alexa-488 goat antirabbit antibodies and analyzed by flow cytometry. The CD45-positive population was gated (left panel) and then the false NPM1-DIM positivity was assessed with a histogram of Alexa-488 positivity versus count (right panel). (A) Generation of a single fluorescent ``false-positive'' peak in the absence of any Fc blocking. (B) Effect of 0.5% BSA blocking prior to antibody labeling, with the false positivity still evident. (C) Combining human AB Rh-negative serum with 0.5% BSA (3:2 ratio) removes the false positivity by effectively blocking the Fc receptors. It must be noted that the negative peak was adjusted to a median fluorescence intensity of 5–10 for all subsequent analysis.

blasts in the majority of the patients (Table 1), the CD45 gating strategy produced a single peak for all AML patients (examples shown in Figure 3). While the MFI values of the PCR-positive patients were significantly different from the PCR-negative AML cases ($p < .005$), unexpectedly these values were also significantly different from the positive control OCI/AML3 (i.e., 23.1 AML12 vs. 45.1 OCI/AML3) in the same experiment, which made it difficult to use the positive control as a gating marker for determining NPM1 mutation positivity. It is postulated that this discrepancy may be because the

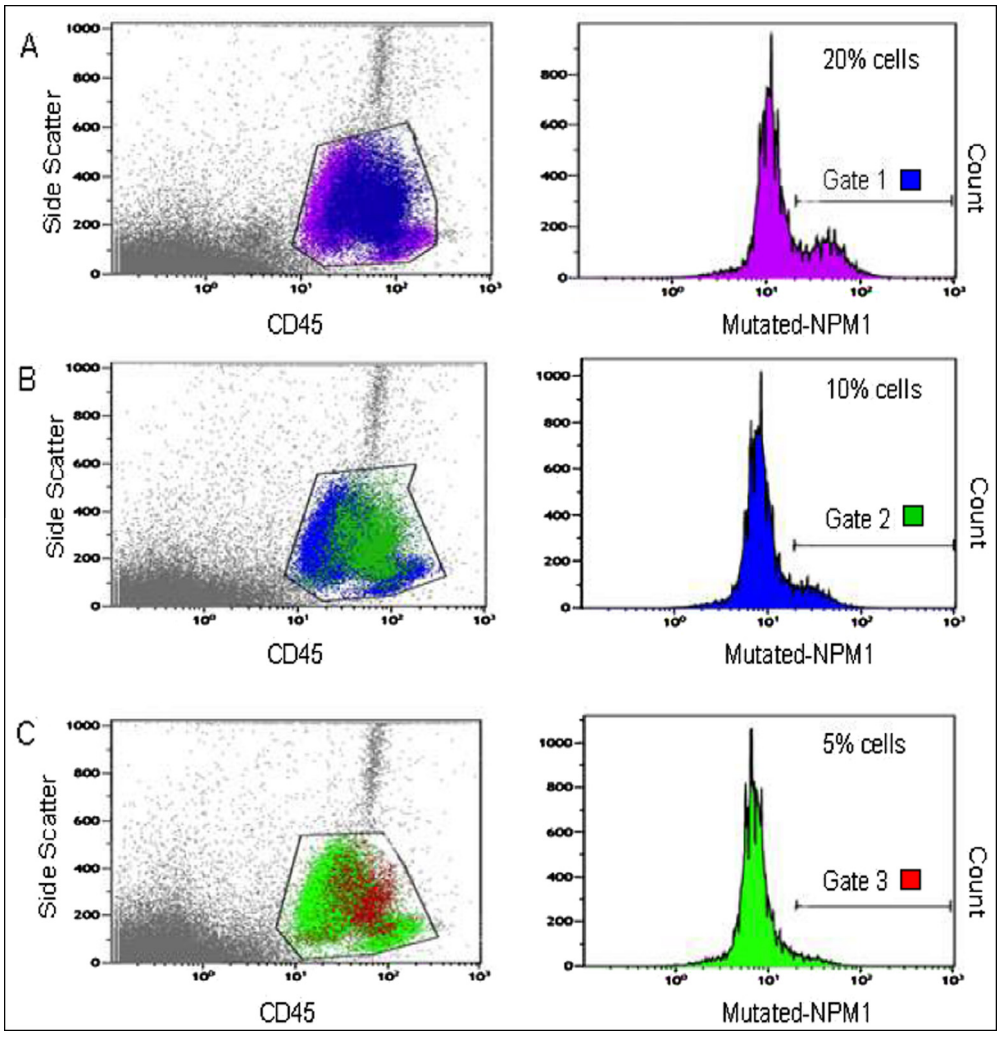

Figure 2. Determination of assay sensitivity. OCI/AML3 cells and wild-type (WT) peripheral blood were mixed in various percentages, blocked with human AB serum/bovine serum albumin, labeled with primary anti-NPM1-DIM antibody and secondary Alexa-488 goat antirabbit antibody and then analyzed by flow cytometry for NPM1-DIM detection. The CD45-positive population was gated (left panel) and then the NPM1-DIM population was assessed with a histogram of Alexa-488 positivity versus count (right panel). Back-gating of the mutated NPM1 population (Gates 1–3, with corresponding color changes) shows the location of a specific CD45-positive population (left panel). (A) 20% OCI/AML3, (B) 10% OCI/AML3 and (C) 5% OCI/AML3 cells.

OCI/AML3 cells are much larger than clinically mutated blasts and have a higher fluorescence due to the increased content of the mutated protein; however, this remains to be proven.

In the absence of a fixed gating strategy, to objectively determine *NPM1-DIM* positivity, we adopted a similar approach used by Oelschlaegel et al.,[17] where the MFI of each patient's single peak was normalized to the WT-negative control peak MFI, to control for labeling efficiency issues between runs. Table 2 presents the results of this analysis, indicating that the 10 *NPM1-DIM* PCR-negative (WT) AML patients had a normalized peak MFI range of 1–1.8 (minimum–maximum). By contrast, the two *NPM1-DIM*-positive patients had normalized MFI values clearly above 1.8 (AML11, 2.5; and AML12, 2.4). Although the data set was limited, a receiver operating characteristic curve analysis indicated that a normalized MFI of 2.0 and above would give the best specificity and sensitivity to discriminate between *NPM1-DIM*-positive and *NPM1-DIM*-negative AML patients using this polyclonal antibody specific for the C-terminal-mutated NPM1 associated with *NPM1-DIM*.

DISCUSSION AND CONCLUSION

Flow cytometry is gaining importance as an alternative method to PCR for detection of fusion proteins

Table 2. *NPM1-DIM* clinical assessment: AML patient analysis.

Identifier	Patient MFI[a]	Positive control MFI	Negative control MFI	Normalized MFI[b]	*NPM1-DIM* PCR
AML1	8.4	27.8	7.7	1.1	Negative
AML2	6.0	29.9	5.1	1.2	Negative
AML3	6.4	29.9	5.1	1.3	Negative
AML4	10.2	48.0	6.1	1.7	Negative
AML5	10.4	48.0	6.1	1.7	Negative
AML6	10.7	48.0	6.1	1.8	Negative
AML7	7.8	33.7	6.9	1.1	Negative
AML8	6.9	33.7	6.9	1.0	Negative
AML9	6.9	33.7	6.9	1.0	Negative
AML10	10.5	34.2	6.3	1.7	Negative
AML11	11.3	19.8	4.7	2.4	Positive
AML12	23.1	45.1	9.4	2.5	Positive

[a]MFI of single peak generated.
[b]Calculated as MFI positive peak in sample/MFI peak of clinical negative control.

in hematological malignancies.[24–27] The aim of this study was to develop an alternative method to PCR for specifically detecting the mutated NPM1 protein utilizing flow cytometry and commercially available reagents.

We developed a flow cytometric assay using the commercially available Abcam polyclonal antibody to the mutated NPM1 (associated with *NPM1-DIM*) and tested it with relevant cell lines and a small cohort of clinical AML PB samples. We found that we were able to correctly identify patients expressing this mutated protein using a normalized MFI as an objective indicator of positivity and that the assay had a 10% cellular limit with good reproducibility.

While 2 groups have previously tested monoclonal antibodies for detecting the mutated C-terminal of NPM1,[18,19] and the studies demonstrated variable success, with the antibody from Gruszka's research group[18] showing high specificity for the mutated NPM1 protein, whereas Tan et al.[19] experienced significant cross-reactivity with the WT protein. This appears to be due to the Fc receptor blocking, which we optimized for the polyclonal antibody use, but which was not taken into consideration by Tan et al.[19] who only tested the antibody's performance on an immunohistochemistry platform, using a peroxidase-labeled secondary antibody. The Fc receptor blocking in this complex intracellular secondary-antibody labeling methodology is therefore essential to ensure the prevention of false positivity.

Gruszka et al.[18] found the sensitivity of their assay to be as low as 0.001%, whereas that of our assay was 10%. Unlike our study, Gruszka et al.[18] only tested the assay performance using single-population HL60 and OCI/AML3 cell line mixtures, which do not simulate the normal scenario faced when dealing with clinical specimens containing different cell types. They also used a monoclonal antibody, which allows for a higher specificity and thus higher sensitivity than polyclonal antibodies. We feel that our study using normal PB as the diluent for the OCI/AML3 cells better represents the sensitivity in the clinical setting. The use of this polyclonal antibody raised against the mutated NPM1 also offers better specificity compared with the method of Oelschlaegel et al.[17] which relies on the localization of the protein and thus the preservation of cell integrity to prevent false positivity. While our assay would not be suitable for minimal residual disease detection after therapy or transplantation, a sensitivity level of 10% is clinically acceptable because most diagnostic AML samples will contain in excess of 20% blasts, all of which should be positive for the *NPM1-DIM*.[1] No additional personnel or equipment is required to perform the flow cytometry in a laboratory where these functions have already been established and it is possible to incorporate this

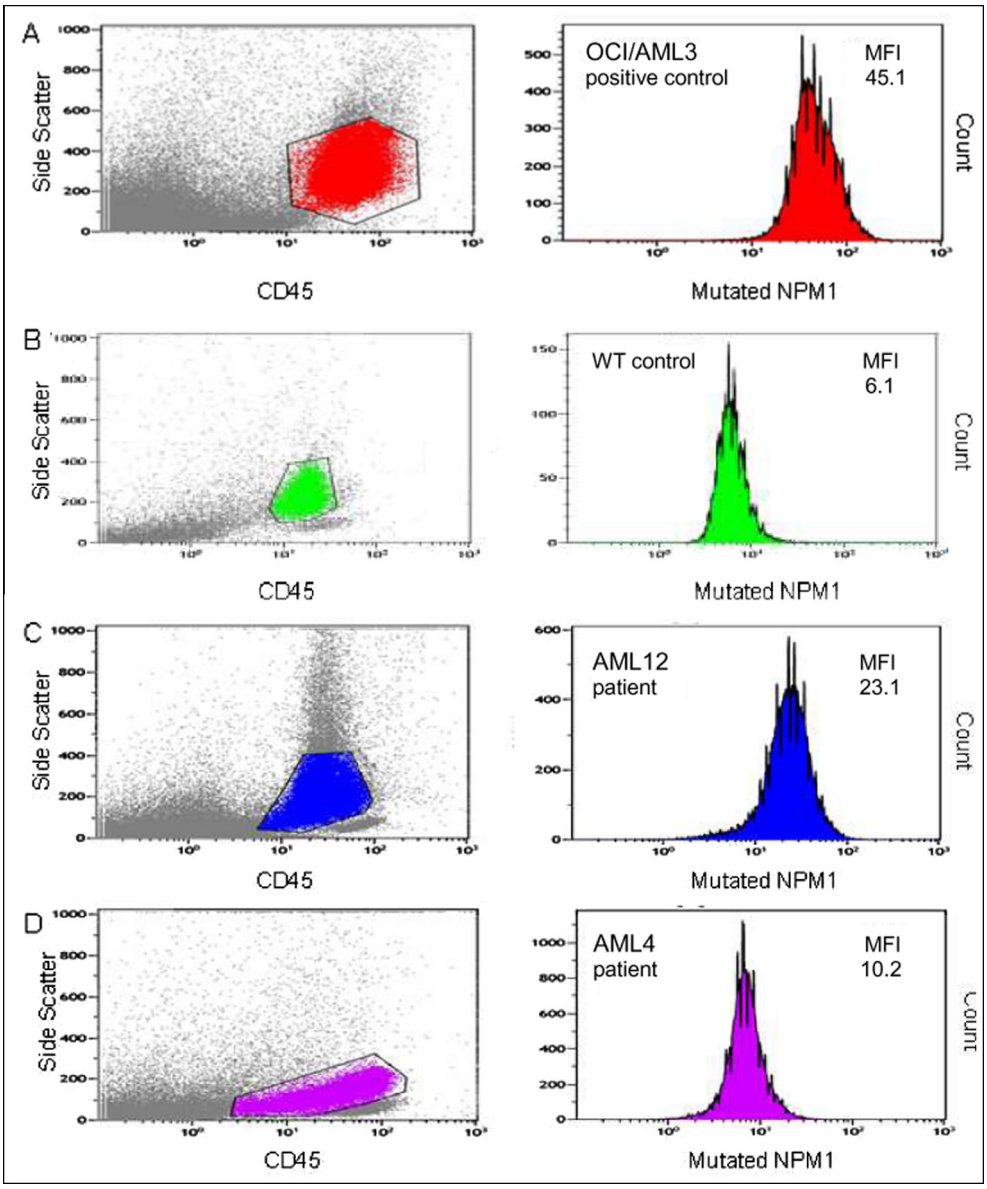

Figure 3. Clinical evaluation of AML patients and controls. CD45-positive cells (excluding lymphocytes; left panel) were evaluated for mutated NPM1 using a histogram of Alexa Fluor 488 fluorescence. The MFI of the observed peak is indicated. (A) 100% OCI/AML3-positive control, (B) WT PB-negative control, (C) *NPM1-DIM* PCR-positive patient AML12 and (D) *NPM1-DIM* PCR-negative patient AML4.

assay into an existing leukemia flow panel and obtain results within 24 h.

The presence of *NPM1-DIM* in patients with AML is strongly associated with a normal cytogenetic karyotype.[28] Cytogenetic analysis is a labor-intensive process with long turnaround times and is costly.[29] No cases of AML with an *NPM1* mutation have yet been shown to have the recurrent cytogenetic abnormalities described in the WHO AML classification,[28] potentially obviating the need to perform cytogenetic testing when this mutation is present. This may also be an advantage for laboratories that do not have the necessary facilities to perform cytogenetic testing, which then requires transporting samples over great distances to facilitate these tests. Consequently, this often results in assay failure due to aging of samples.[29] The cost of this assay was also found to be 10 times cheaper than the standard cytogenetic panel, offering a potential cost-saving option when performed first. The flow cytometric assay described in this study is therefore of potential use in a resource-restricted diagnostic environment, especially in

nonfirst world countries. Further validation with a larger AML cohort is required, and it may provide a key mutation detection alternative method.

CONFLICTS OF INTEREST

All contributing authors declare no conflicts of interest.

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