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