

**The development of a flow cytometric method to
detect the presence of mutated nucleophosmin in
Acute Myeloid Leukaemia**

By

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The development of a flow cytometric method to detect the presence of mutated nucleophosmin in Acute Myeloid Leukaemia

LA Du Pisani; K Shires – National Health Laboratory Service – Groote Schuur and the University of Cape Town

Background:

Nucleophosmin (NPM1) is an acidic, nucleo-cytoplasmic, shuttling protein with predominant nucleolar localisation that plays multiple roles in cell growth and proliferation. Deletion insertion mutations of *NPM1* (*NPM1 DIM*) seem to disrupt its normal physiologic role as a molecular chaperone, which likely leads to its oncogenic potential. *NPM1* if present alone (not associated with *FLT3 internal tandem duplications (FLT3-ITD)*) is associated with significantly better overall survival and disease free survival in AML and has been entered as a provisional category in the World Health Organisation (2008) classification of Acute Myeloid Leukaemia with recurrent genetic abnormalities. Current methodology uses reverse transcriptase polymerase chain reaction (RT-PCR) and genomic deoxyribonucleic acid (DNA) PCR techniques to detect *NPM1 DIM*. Although these methods are robust and relatively easy to perform they can be expensive, labour intensive and not universally available. Six major variants of *NPM1 DIM* (Types A-F) have been described all leading to frame shift. All six types share the same last five amino acids in the C-terminal. The aim of this study was to develop a robust flow cytometry methodology that could be used in the routine assessment of AML samples to determine the mutational state of NPM, using a commercially available polyclonal antibody against the mutated NPM1.

Methods:

A commercially available NPM1 mutation specific antibody was used to develop a diagnostic method by flow cytometry. The antibody is polyclonal and of rabbit origin but is unconjugated. A secondary antibody, Alexafluor 488 goat anti-rabbit, was used to detect the primary antibody binding. Cells were first labeled on the surface with CD45 PerCP for the purpose of gating. Red cells were lysed using FACS Lyse and the specimen was permeabilised using FACS PERM (Becton-Dickinson). Optimal antibody concentration, blocking technique for non-specific binding as well as intracellular staining methodology was established. Flow cytometry was performed using a BD FACS Canto II flow cytometer and gating was performed on CD45 expression and side scatter. The technique was tested on a commercial cell line, OCI/AML3, with a known Type A *NPM1DIM*. The NPM1 mutation was confirmed on PCR. The minimum detectable concentration of NPM1 mutated cells was determined by doing dilution studies with normal peripheral blood. Samples from twelve newly diagnosed AML patients were used to correlate the NPM1 flow cytometry assay with DNA based PCR. Analysis was performed using Kaluza™ software. Median Fluorescence Intensity ratio (patient : negative control) of greater than 1.8 was established to optimally differentiate positive and negative patients on flow cytometry.

Results:

The flow cytometric technique effectively detected the presence of mutant NPM1 protein in the positive cell line with minimal non-specific binding when using an optimised intracytoplasmic staining and blocking technique as well as antibody concentration. Flow cytometry was able to detect blast populations of just less than 10% on dilution studies. Two of the twelve patients showed *NPM1 DIM* on both flow and PCR and ten were negative.

Conclusions:

Flow cytometry may be utilised as a relatively cost effective, rapid alternative to PCR to test for *NPM1 DIM* and may be applicable to laboratories that do not have access to molecular services however continued validation of this technique is required.

PART A: RESEARCH PROTOCOL

Principle investigator

Name: Karen Shires^a

HPCSA Registration number: MW0010197

University of Cape Town – Department of Haematology

Project leader

Name: Louis Almero Du Pisani

Degree registered: Masters in medicine

Year initially registered: 2006 (January)

Project title

Developing a novel flow cytometric method for determining the mutational status of *Nucleophosmin (NPM1)* in Acute Myeloid Leukaemia (AML)

Short description of project

This project aims to develop a simple method using flow cytometry to determine the mutational status of *NPM1* in AML patients, by specifically detecting the mutated carboxy-terminal of this protein, using a mutation specific antibody. This may then in future form part of the standard antibody panel for Acute Leukaemia, as it has established prognostic significance.

^aPrinciple investigator was changed from Maureen Stein (due to resignation from NHLS) to Karen Shires (see collaborators)

Collaborators

Prof. N. Novitzky

University of Cape Town - Head of Division of Haematology

Project

Aims & Objectives

Develop and validate a simple, cost effective, novel method using flow cytometry to determine the mutational status of *NPM1*. This aim will be achieved through the following objectives:

- a) Establish flow cytometry analysis of *NPM1* molecules, using a *NPM1* mutated specific antibody conjugated to FITC and a *NPM1* mutant control.^b
- b) Establish the methodology for the DNA based PCR analysis of *NPM1* mutations, which is currently the most widely used technique, using a positive control cell line. This will serve as a comparison to the new flow cytometry method.^c
- c) Flow cytometry and PCR analysis of *NPM1* will be conducted on various routine patients' samples with newly diagnosed AML to establish the validity of this novel method. Results will also be correlated with cytogenetic results where appropriate.

^bConjugating the mutated *NPM1* specific antibody was cost prohibitive and would have created stability problems for storage of the antibody after conjugation. It was therefore decided to rather use a secondary antibody to detect the primary one.

^cPCR was performed at Groote Schuur Hospital NHLS laboratory

d) **Background**

NPM1: Wild type normal physiological function

NPM1 is an acidic, nucleo-cytoplasmic, shuttling protein with predominant nucleolar localisation that plays multiple roles in cell growth and proliferation. In general the expression level is proportional to the cell growth rate (review) [1]. One of its roles is as a molecular chaperone that prevents protein aggregation in the nucleolus [2]. It also regulates the assembly and transport of pre-ribosomal particles through the nuclear membrane [3] thus playing a role in ribosomal biogenesis. Regulation of p14-Alternate reading frame (ARF) p53 pathway also takes place [4]. ARF is regulated by NPM1 in the nucleus. During oncogenic stress NPM1 is up regulated thus causing accumulation of ARF. The function of ARF is to stabilise Murine double minute 2 (MDM2) and thus prevent its attachment and destruction of p53 [5,6] over all leading to increased p53 and cell cycle arrest (see fig. 1). NPM1 initiates centrosome duplication and thus assists in mitosis [7].

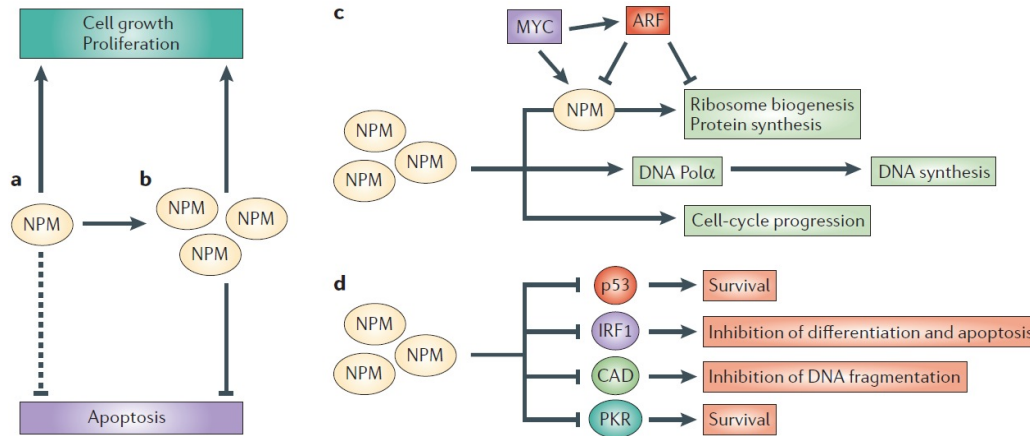


Figure 1. NPM over expression in tumour cells leads to increased proliferation and inhibition of apoptosis. (Reproduced from Nature Reviews Cancer, 2006) [8]

a) In normally proliferating tissues, nucleophosmin (NPM) is expressed at physiological levels, and the balance between cell proliferation and apoptosis is preserved. **b)** In tumour cells, NPM is often over expressed, and can exert its oncogenic potential both by stimulating cell proliferation and preventing apoptosis. **c)** NPM over expression can lead to increased cell growth and proliferation through hyperactivation of the ribosome machinery, in view of its role as a ribosome biogenesis factor. NPM, together with a number of ribosomal proteins, is a transcriptional target of MYC. The altered activity of MYC in tumour cells correlates with increased protein synthesis. In addition, NPM is a putative stimulatory factor for DNA polymerase- α (DNA Pol α), and contributes to cell-cycle progression. **d)** Over expressed NPM shows oncogenic potential by opposing apoptosis through different mechanisms: it prevents the DNA-binding activity of the transcription factor interferon regulatory factor 1 (IRF1); it binds to and inhibits the catalytic function of eukaryotic initiation factor 2 kinase PKR; it opposes the DNA-fragmentation activity of caspase-activated DNase (CAD) in neural cells, thereby suppressing its pro-apoptotic activity; and finally, it interacts with and inhibits p53 in response to apoptotic stimuli.

Studies of NPM1 functional domains reveals a nuclear localisation signal (NLS) that drives NPM1 from the cytoplasm to the nucleoplasm where it is translocated to the nucleolus through its nucleolar-binding domain, particularly tryptophans 288 and 290 [9]. Nuclear export of NPM1 is mediated by evolutionary conserved Exportin 1, the export receptor of proteins containing leucine-rich nuclear export signal (NES) motifs with the generally accepted loose consensus L-x(2,3)-(LIVFM)-x(2,3)-L-x-(LI), a core of closely spaced leucines or other large hydrophobic amino acids. Two such highly conserved NES motifs have been

identified in the wild type NPM1 protein: one with the sequence I-xx-P-xx-L-x-L within residues 94 to 10228 and another at the N-terminus within amino acids 42 to 61, with leucines 42 and 44 as critical nuclear export residues. However, despite the NES motifs, NPM1 remains localised in nucleoli. Thus, in physiologic conditions, nuclear import of wild type NPM1 greatly predominates over export, since wild type NPM1 NES motifs exhibit the general property of NES motifs (ie, weak interaction with Exportin 1) [10].

Effects and types of mutations in NPM1

NPM1 mutations have recently been associated with AML [11]. Mutations of *NPM1* generate an additional leucine-rich NES motif reinforcing nuclear export [12]. They also cause loss of tryptophan residues 288 and 290 (or 290 alone), which determine the NLS [4]. Evidence exists that wild type NPM1 is recruited by mutated form causing delocalisation into the nucleus and cytoplasm from the nucleolus [13].

Knockout mice models of NPM1 led to the development of a pre-leukemic Myelodysplastic syndrome (MDS) like disease. This may be due to improper control of centrosome duplication leading to genomic instability. Although acute leukaemia does not develop in these mice, it is thought that secondary mutations such as Fms like tyrosine kinase 3 internal tandem duplications (FLT3 ITD), which are also commonly associated with AML are the second-hit

required for leukemic transformation [7] implying that *NPM1* mutation may be a primary event in leukaemogenesis.

However mutated *NPM1* remains effective in regulating centrosome duplication and *NPM1* mutations are extremely rare in humans with MDS [14] therefore other mechanisms must be involved. Delocalisation of *NPM1* and ARF from nucleolus to cytoplasm leading to an unopposed MDM2 degradation of p53 may cause cell cycle progression [15].

AML is a heterogeneous group of diseases, which relies on cytogenetic analysis to subtype and in turn prognosticate. Up to 50% are however normal with conventional cytogenetics [16]. With the development of molecular diagnostics and the strong association of AML with certain mutations, the World Health Organisation (WHO) has recently introduced a new molecular provisional entity into the classification of AML called “AML with gene mutations” and includes *NPM1*, as well as *CCAT box enhancer binding protein alpha (CEBPA)* mutations [17].

NPM1 mutation, if present alone (not associated with *Flt3-ITD*) is associated with significantly better overall survival and disease free survival [18].

Mutated *NPM1* is also strongly associated with a normal karyotype. In one recent study of 1395 patients with AML, 686 had an abnormal karyotype, with

only 8% (58/686) of these patients having *NPM1* mutations. The remaining 709 patients with a normal karyotype showed *NPM1* mutation rate of 46% (324/709). The 8% of patients with *NPM1* mutations and abnormal karyotype showed mostly single genetic abnormalities like trisomies, monosomies and deletions. Only very rarely (<1%) was there an association with t(8;21) and inv(16) [18]. This was confirmed in a large study using 1393 patient with AML. 835 showed a normal karyotype and 558 abnormal. Of the 558 patients with the abnormal karyotype 246 showed reciprocal translocations confirmed by RT-PCR or FISH. None of the 246 patients with the reciprocal translocations had *NPM1* mutation on PCR. Reciprocal translocations included t(8;21), inv(16), t(15;17), Chromosome 11q23 rearrangements and t(6;9) [19]. Hence the *NPM1* mutated state may be used as a predictor that the patient is unlikely to have a reciprocal translocation, dispelling the need for expensive tests such as FISH and PCR for identifying specific translocation if standard cytogenetics testing fails [19].

Six major variants of *NPM1* mutations (Types A-F) have been described all leading to frame shift mutations [4]. Type A is a duplication of TCTG (956 – 959) resulting in a frame shift causing the last 7 amino acids to be replaced with 11 different ones. [4]. Type A mutations are by far the most common and account for 75 – 85% of cases with mutations B and D observed in 10% and 5% respectively [10]. Other mutations are very rare [10] (Types B, C and D results in a 4bp insertion at position 960 again causing a frame shift mutation, which has a similar effect as Type A. Type E and F occur as a result of a 5bp GGAGG (965-

969) deletion, as well as a 9bp insertion in this position leading to a total gain of 4bp. All six types amazingly share the same last 5 amino acids in the C-terminal VSLRK [4]. Various other mutations have been described, the vast majority leading to the common amino acid C-terminal [13].

Current methodology uses various RT-PCR and genomic DNA PCR techniques to detect *NPM1* mutations. Although these methods are robust and relatively easy to perform they are expensive and labour intensive. Detection methods include capillary zone electrophoresis, polyacrylamide gel electrophoresis, denaturing high performance liquid chromatography as well as new assays for specific mutations (Type A) using fluorescence resonance energy transfer (FRET) probes [20-22]. The turnaround time for these assays is also usually more than 24 hours. A flow cytometric technique should be simple, cheap and done on the same day as the bone marrow giving immediate results and thus the ability to decide whether to cancel cytogenetics testing if *NPM1* is mutated. Flow cytometry will be a novel technique and has never been evaluated for this particular purpose. The mutation specific antibody has however been used to detect the *NPM1* mutated protein in Western Blotting techniques [13].

Research Plan

The research strategy is to compare the new flow cytometry methodology with a well-established *NPM1* mutation detection technique: PCR - capillary electrophoresis. This will involve establishing both methodologies and comparing the results obtained from both when used to analyse patients simultaneously.

This project will run over 12 to 18 months. The first 6 months will be dedicated to setting up the particular tests using flow cytometry and PCR and validating these assays with a commercial cell line positive (*NPM1* mutant) control. All equipment required is available either in the C17 Core Laboratory or C21 molecular laboratory.

The antibody will then be used in routine flow cytometry performed on all new acute leukaemias (peripheral blood or bone marrow) and information accumulated and correlated with PCR results and routine cytogenetics results. This will take place over the following 12 months.

Detailed Methodology

Study Population

The study population will consist of all patients of any age group presenting with acute leukaemia that would receive routine bone marrow biopsies, immunophenotyping by flow cytometry, cytogenetics and routine molecular

investigations. The project will be reviewed by the local ethics committee and approval obtained. Written informed consent (see attached consent form) will be obtained from test subjects as part of the routine consent given for bone marrow biopsy.

Laboratory Assays

The cell line OCI-AML3 obtained from the German Collection of microorganisms and cell cultures (GCMZ) will be cultured in alpha-minimal essential medium (Invitrogen) combined with 20% fetal bovine serum (Gibco). These cells contain the following mutation: Type A *NPM1* and will be used as a positive control for the establishment of the PCR and flow detection methods.^d

For PCR^e analysis of *NPM1*, DNA will be extracted from the tissue culture using standard spin column extraction procedures (Invivoscribe). PCR will be established using the protocol described by Szankasi and co-workers [23]. Briefly, the following *NPM1* primers (0.2 μmol/L): *NPM-F*, 6-FAM-5'GATGTCTATGAAGTGTGTTGTGGTTCC-3' and *NPM-R*, 5'GGACAGCCAGATATCAACTG-3' will be used in a 20μl PCR reaction containing 100 ng of genomic DNA, deoxynucleoside-5'-triphosphates (0.2 mmol/L each), 1X cloned *Pfu* buffer (Stratagene), 1.25units of *Pfu* Turbo (Stratagene), MgCl₂ (3.5 mmol/L final), where 1.5 mmol/L MgCl₂ is contributed

^dDue to difficulty in importing the cell line from Germany and this company directly, a group in the United States of America donated the cells.

^eUltimately a different DNA based PCR methodology was used, as this method did not give clear results – see Part 3 of the dissertation

by the cloned Pfu buffer. PCR program as follows: initial denaturation at 94°C for 2 minutes, 35 cycles of 94°C for 20 seconds, 60°C for 20 seconds, 72°C for 20 seconds, followed by a hold at 72°C for 2 minutes. The 6-FAM labeled PCR products will be diluted fivefold in water, and 1µl mixed with 9 µl of HiDi formamide (Applied Biosystems). GeneScan ROX 350 internal size standards (Applied Biosystems) will be added (0.5µl) and the samples heated to 95°C for 2 minutes. The samples will be run on an ABI 3130xl Genetic Analyzer using 36-cm capillaries and POP-7 polymer. The samples will be injected at 2 kV for 5 seconds and run at 15 kV for 950 seconds at 60°C. Raw data will be analysed with GeneMapper v4.0 software (Applied Biosystems, Inc.)

A new method using flow cytometry and NPM1 mutation specific antibody (ABCAM) will be developed. The antibody is polyclonal and of rabbit origin but is not conjugated. A FITC conjugation kit will be used for conjugation purposes. Cells will be permeabilised using FACS PERM (Becton-Dickinson) and labeled with the antibody using standard intracellular staining protocols. Optimum antibody concentrations as well as staining methodology will have to be established. Flow cytometry will be performed using a 4 colour FACS Calibur (Becton-Dickinson).^f

^fFACS Calibur was replaced with a FACS Canto II during the study period

Envisaged outputs/outcomes

It is envisaged that this study will provide a novel, rapid and cost-effective method for evaluating the *NPM1* mutation status of a patient with AML. The application may be used in smaller laboratories without molecular facilities. This has prognostic implications in cytogenetically normal AML patients.

Impact

Capacity development:

Training of research workers:

Dr Du Pisani will be the project leader. He is currently training as a Registrar in Haematology and is registered for the MMed Degree at UCT. The project will provide training in flow cytometry and molecular techniques and will be the basis for the dissertation towards the MMed degree.

Institutional approval

This proposal will be submitted for ethics approval to the University of Cape Town Research Ethics Committee.

Budget

Consumable		Total Price
OCI/AML3 Cell line		10000
Tissue culture consumables		15000
Mutation specific NPM1 polyclonal rabbit antibody (US\$300 x 3)		10000
Conjugation Kit (FITC)		5000
Permeabilising reagent		5000
Flow consumables		10000
PCR consumables		20000
DNA extraction kits (Invivoscribe)		5000
Capillary electrophoresis		10000
	TOTAL	90000

Funding

The NHLS Research Trust Fund, subject to ethics approval, will provide funding.

No other funding has been approved for this project.

Previous NHLS research trust funding

No previous NHLS research trust funding has been received.

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PART B: LITERATURE REVIEW

The development of a flow cytometric method to detect the presence of mutated nucleophosmin in Acute Myeloid Leukaemia

Objectives

Review literature pertaining to Nucleophosmin deletion/insertion mutations and their role in Acute Myeloid Leukaemia pathogenesis including:

1. Normal physiological function
2. Types of mutations and their effects
3. Methods of detection
4. Utilising *NPM1* mutation detection in Minimal Residual Disease monitoring
5. Conclusions and further areas of study

Literature search strategy

Pubmed was used as the primary search engine. Articles in languages other than English and those not available via the University of Cape Town were excluded. Referencing was performed using RefWorks

Search parameters included:

1. Nucleophosmin
2. NPM1
3. Nucleophosmin 1 deletion/insertion mutations
4. Acute myeloid leukemia/leukaemia
5. AML

Summary of literature

Nucleophosmin 1 (NPM1) – structure and normal physiologic functions

Nucleophosmin (occasionally referred to as B23 [1], NO38 [2] or numatrin [3]) is an acidic nucleolar phosphoprotein with a predominant nucleo-cytoplasmic shuttling function [4]. The protein is encoded by the *NPM1* gene located on chromosome 5q35. It consists of 12 exons spanning a 30kb region (OMIM / NCBI data base) and 3 mRNA splice variants can be produced, although only one protein isoform dominates in the cell, the 37kDa protein referred to as NPM1 [5,6]. This protein is present predominantly in the nucleolus, but can shuttle between the nucleus and the cytoplasm [7,8]. This shuttling is extremely important in maintaining cellular processes and changes in this function have clinical implications and will be discussed later in this review [9].

A study of NPM1 functional domains revealed a nuclear localisation signal (NLS), nucleolar localisation motif (NoLS) (including two essential tryptophan amino acids at position 288 and 290), and 3 nuclear export signals (NES) (see fig. 1). These motifs allow movement of NPM1 between the cytoplasm, nucleoplasm and nucleolus of the cell [10]. Exportin 1 is a nuclear export factor, which binds to proteins containing leucine-rich NES motifs, such as NPM1 and mediates nuclear export of these proteins back into the cytoplasm. Despite the NES motifs however, NPM1 remains largely localised in the nucleolus under normal physiological conditions. This is due to the weak interaction of the wild

type NPM1 NES motifs with Exportin1, which leads to nuclear import of wild type NPM1 predominating over export [11].

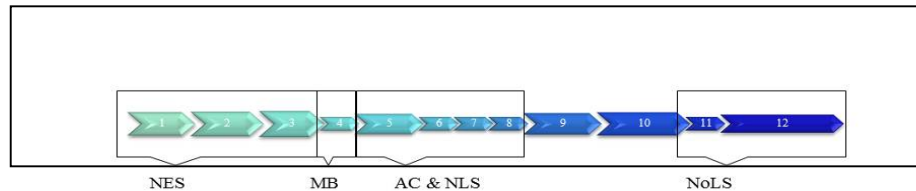


Figure 1: Basic functional domains of NPM1 protein (adapted from Falini et al., 2007) [11]
Nuclear export signal (NES) motifs and a metal-binding (MB) domain shown at the N-terminal. Nuclear localisation signal (NLS) and acid stretches (AC) for histone binding present in the middle. C-terminus with ribonuclease activity and involved in nucleic-acid binding. NPM1 specifically contains two very important tryptophan residues (288 and 290), required for nucleolar localisation of the protein (NoLS). Numbers refer to different exons.

NPM1 has multiple functions in the cell, some of which can be regarded as promoting proliferation and are anti-apoptotic, while others are tumour suppressive, participating in pathways that control the cell cycle and promote apoptosis. These specific functions are dependent upon the localisation of the protein, which is achieved due to its shuttling capabilities (as described earlier). NPM1 can also act as a molecular chaperone, helping to transport other proteins or molecules across the nuclear membrane. Fig. 2 highlights some of the major functions of NPM1 in the various cellular components.

NPM1 is present predominantly in the nucleus and thus the majority of its functions take place here. This largely entails cell cycle regulation and other processes involved in cellular proliferation. Nucleoplasmic NPM1 regulates the p14-Alternate reading frame (ARF) / p53 pathway by promoting nucleolar localisation and stability of ARF, which is in turn involved in cell-cycle control

(see fig. 2) [9]. During oncogenic stress, NPM1 expression is upregulated, thus causing accumulation and stabilisation of ARF in the nucleus. The function of ARF is to stabilise Murine Double Minute 2 (MDM2) protein and prevent its attachment to and destruction of p53 [12,13]. Overall, this leads to increased p53 and cell cycle arrest. Loss of NPM1 expression or functional changes of the protein due to mutation will abrogate this effect and may contribute to tumourigenesis [14].

Other nuclear functions include involvement with DNA repair mechanisms, as NPM1 is markedly upregulated after UV radiation [15] and shows chromatin/histone binding functions when DNA double-stranded breaks are present utilising the acid stretch regions (see fig. 1) [16]. NPM1 also initiates centrosome duplication, the formation of two daughter centrosomes just before cellular mitosis, by acting as a substrate for cyclin dependant kinase 2/cyclin E and thus assists in this process [17]. NPM1 seems to protect the cell from centrosome hyperamplification and allows the cell to progress from G2 to M phase of cell cycle [18-20].

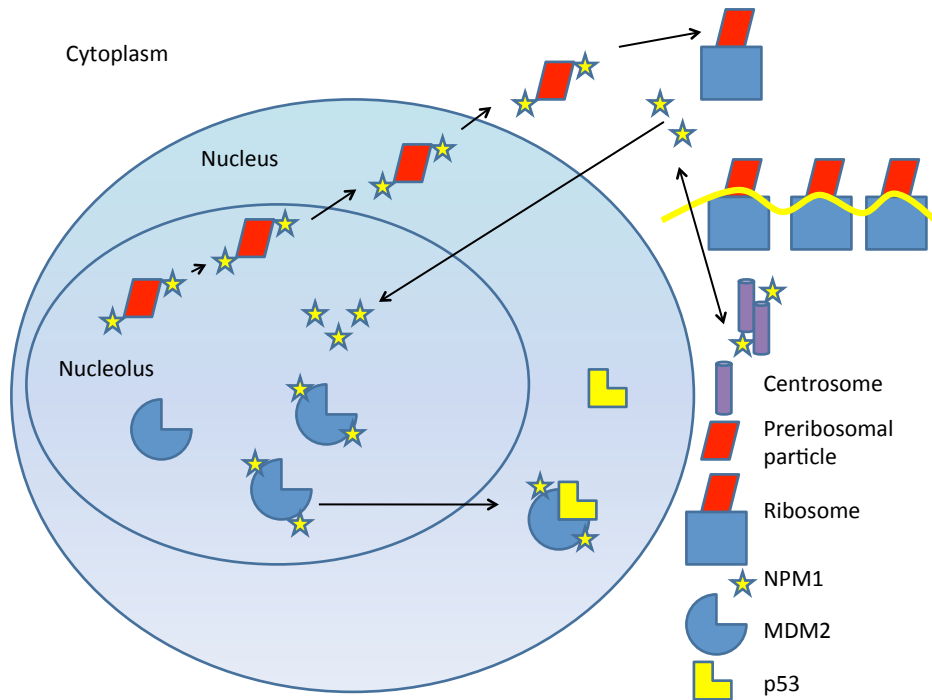


Figure 2: Basic functions of NPM1(adapted from Falini et al., 2007)[21]

NPM1 moves between the nucleus and cytoplasm. Shuttling itself is important for ribosome biogenesis via transport of pre-ribosomal particles. In the cytoplasm, NPM1 binds to the centrosome - this regulates its duplication during cell mitosis. NPM1 also interacts with and regulates ARF-p53-HDM2 pathway in the cell cycle

Although predominantly in the nucleus, NPM1 has one cytoplasmic function. It interacts with Breast Cancer Type 1 (BRCA1) protein and BRCA-associated RING domain 1 (BARD1). The NPM1 and BRCA1-BARD1 complex localises at the centrosomes, which seems important for maintaining the mitotic spindle poles, genomic stability and integrity during mitosis [17].

NPM1 also has molecular chaperone functions by virtue of its ability to shuttle across the nuclear membrane. Molecular chaperones are defined by their ability

to assist in de novo protein folding, stabilisation of proteins under stress conditions and maintaining polypeptide chain components in a loosely folded state for translocation across organellar membranes [22]. As a molecular chaperone, NPM1 is involved in ribosome biosynthesis. Ribosomes play a central role in protein synthesis, growth and development. NPM1 regulates the assembly and transport of pre-ribosomal particles across the nuclear membrane, via its nucleo-cytoplasmic shuttling and ability to bind nucleic acids [7,23,24] thus playing a role in ribosomal biogenesis. NPM1 also expresses ribonuclease activity and can process pre-ribosomal ribonucleic acid molecules [25,26]. NPM1 also acts as a molecular chaperone for GADD45, as it lacks its own intrinsic NLS [27]. GADD45 α is a protein induced by genotoxic stress, with its primary roles including DNA repair and cell cycle arrest [28] via the p53 pathway.

Types of *NPM1* mutations and their effects

Deletion/Insertion mutations in exon 12 of *NPM1* gene (*NPM1 DIM*) are commonly seen in Acute Myeloid Leukemia (AML). These mutations result in the loss of the two fundamentally important tryptophan residues within the NoLS and the creation of an extra NES motif, which subsequently disrupts the shuttling function and protein localisation. Rare cases of mutations within other exons have also been reported [29-31].

Six major variants of *NPM1 DIM* (Types A-F) have been described, all of which lead to frame shift mutations [9]. Type A is a duplication of TCTG at nucleotide 956 – 959. This effectively leads to a frame shift, which causes the last seven amino acids to be exchanged with eleven different ones [9]. Types B, C and D results in a four base pair (bp) insertion at nucleotide position 960, again causing a frame shift mutation, which has a similar effect to the type A insertion. Type E and F occur as a result of a five bp GGAGG (nucleotide 965-969) deletion, as well as a nine bp insertion in this position leading to a total gain of four bp. All six major mutation types result in the same last five amino acids in the C-terminal: VSLRK [9]. Type A mutations are by far the most common and account for 75 – 85% of cases with mutations B and D observed in 10% and 5% respectively [11]. Other mutations are very rare [11].

Despite the various combinations of deletions and insertions that may occur in exon 12, the alteration of the last five amino acids in these mutants results in an additional leucine-rich NES motif, which favours export from the nucleus to the cytoplasm [32,33]. It is important to note that it also causes the loss of tryptophan residues 288 and/or 290, which forms part of the NoLS [9]. Essentially these mutations change NPM1 from a protein that is preferentially imported into the nucleus and the nucleolus, to one that is predominantly exported to the cytoplasm. As described above, this will seriously change the predominant role that NPM1 plays in the cell, abrogating nuclear functions.

To further compound the effects of the mutations on cellular function, there is evidence that wild type NPM1 protein is recruited by the mutated isoform to create heterodimers, which results in the delocalisation of the remaining wild type protein into the cytoplasm [34]. This essentially causes a dominant negative effect when a heterozygous mutation is present and negates the effect of the normal remaining NPM1 chaperone functions

In conclusion, NPM1 plays an important role in regulation of the cell cycle via its interaction with ARF-p53 pathway. Displacing it into the cytoplasm due to the mutation may result in the loss of this function, resulting in down regulation of p53, cell cycle progression and prevention of apoptosis. Disruption of the shuttling of NPM1 and thus its ability to act as a chaperone will also result in ribosomal biogenesis failure, which will have an impact on growth, development and differentiation of cells.

Characteristics of AML with *NPM1* *DIM*: effecton prognosis and treatment

AML is a neoplasm of a specific lineage of blood cells, characterised by the rapid proliferation and accumulation of immature myeloid precursor cells in the bone marrow, with disruption of the normal blood cell production [35].

AML actually encompasses a heterogeneous group of diseases, which traditionally relied on morphology, cytochemistry and cytogenetic analysis to subtype and in turn prognosticate [35] (review). In those AML subtypes with

recurrent cytogenetic abnormalities, three broad prognostic categories were established [36] (review):

- a) Favourable, consisting of the core binding factor leukaemias - t(8;21), inv(16)
- b) Intermediate, consisting of t(9;11)
- c) Poor, associated with inv(3), t(3;3), t(6;9), other 11q23 rearrangements, complex karyotypes, -5, del(5q), -7 or abn(17p)

Up to 50% of AML's however demonstrate a normal cytogenetic profile with conventional cytogenetics (CN-AML), [37] and as a consequence, a new category named "AML with gene mutations" was introduced by the World Health Organisation (WHO) in 2008, to try to sub-categorise these patients and aid with therapy decisions. AML cases in this category have normal cytogenetic profiles and at least one of the mutations listed by the WHO, which includes mutations in Fms-like tyrosine kinase 3 (FLT3), NPM1 and CAT box enhancer binding protein alpha (CEBPA). The prognosis of cases within these "molecular" groups differs and is dependent upon the mutation type found. *CEBPA* mutations, *NPM1 DIM* and *FLT3 internal tandem duplications (ITD)* seem to have the highest prognostic value [38]. Generally *NPM1 DIM* [30] and *CEBPA* mutations [39] are considered to convey a good prognosis and *FLT3-ITD* [40] a poor prognosis. The combination of mutations also alters the prognostic value of the individual mutations, with co-existence of *FLT3-ITD* negating the positive prognostic effect of *NPM1 DIM* [41].

A large number of CN-AML cases have *NPM1 DIM* and the opposite is true as well – *NPM1 DIM* is strongly associated with a normal karyotype in AML. Only a small minority of patients with *NPM1 DIM* mutations have single genetic abnormalities like trisomies, monosomies and deletions [30] and this has been confirmed in several large studies [42,43]. No AML, with a WHO recognised recurrent cytogenetic abnormality, was detected in the group of AML cases with *NPM1 DIM* [42,43]. Hence the *NPM1* mutated state may be used as a predictor that the patient is unlikely to have a reciprocal translocation, dispelling the need for expensive tests such as FISH and PCR for identifying specific translocation if standard cytogenetics testing fails [42].

NPM1 DIM are overall more common in females with a median age of 46 years and are associated with some degree of monocytic differentiation in AML, as well as lower expression of CD34 [44-46].

Patients with AML *NPM1 DIM* alone generally show an improved prognosis in relation to other AMLs (as discussed previously). CN-AML with *NPM1 DIM* show high blast counts [47] and dysplasia [48]. These characteristics have classically been interpreted as poor prognostic indicators [49], however they do not seem to apply in this setting showing the importance of establishing the status of this mutation. The mechanism by which *NPM1 DIM*, when present without *FLT3-ITD*, convey a better prognosis is not well understood [50].

CN-AML is a very heterogeneous group of AML's in which only high-risk groups benefit from an allogeneic stem cell transplant as this type of treatment carries an inherent risk of mortality. Here the mutation status of the patient plays an important role in determining therapy. Patients younger than 60 years with CN-AML, wild type *NPM1* and wild type *CEBPA*, but mutated *FLT3 (FLT3-ITD)* should be considered for allogeneic stem cell transplant [38] as the risk of relapse outweighs the risk of mortality and morbidity associated with the transplant. *NPM1 DIM* or *CEBPA* mutations, without *FLT3-ITD* however is associated with significantly better overall survival and disease free survival [30] and allogeneic stem cell transplant does not result in improved survival compared to post remission consolidation with chemotherapy [46]. Patients older than 60 years with CN-AML show similar improvement in prognosis when associated with *NPM1 DIM* alone. These AML cases also expressed a similar microRNA and gene expression profiles compared to younger patients with *NPM1 DIM* [51] and this is likely the same disease. Elderly patients who are medically fit and have CN-AML with no *FLT3-ITD* or *NPM1 DIM* only, should be considered for post remission therapy (e.g. Allogeneic stem cell transplant) [52].

The mutated *NPM1* protein itself may in future be useful as a treatment target. *NPM1 DIM* is stable throughout the disease, probably a founder mutation/primary genetic event and is restricted to the leukaemic cells [53]. All TransRetinoicAcid (ATRA) administration after completion of three days of induction chemotherapy has been used in elderly patients with CN-AML with

NPM1 DIM and these patients showed an improved outcome compared to those than received standard chemotherapy alone [54]. This result may be due to the enhanced cytoplasmic NPM1 function as a possible co-repressor during retinoic acid-induced cell differentiation, when this protein is mutated. Studies supporting this hypothesis showed that ATRA, in pharmacological doses, induced cell cycle arrest and apoptosis in OCI/AML3 a NPM1 mutated cell line [54]. Demethylating (5-azacytidine) agents have also recently been successfully used to suppress *NPM1* levels at relapse [55], the mechanism of which is largely unknown.

Detecting *NPM1 DIM* in AML

Polymerase chain reaction (PCR)

Various RNA-based reverse transcriptase PCR (RT-PCR) and genomic DNA based PCR techniques to detect *NPM1 DIM* are currently being reported in the literature. Detection methods include direct sequencing [9], capillary electrophoresis [56], polyacrylamide gel electrophoresis [57], denaturing high performance liquid chromatography [58] as well as new assays for specific mutations (Type A) using fluorescence resonance energy transfer (FRET) probes and real-time PCR approaches [59,60]. Although these methods are robust and relatively easy to perform, they can be expensive and labour intensive depending upon the methodology employed. Sensitivity of assays ranges from 20% with direct Sanger sequencing [9] to 0.0001% with semi-nested allele specific oligonucleotide RT-PCR techniques [58]. The more

sensitive assays allow for the monitoring of minimal residual disease (discussed below) [58,61,62].

Antibody mediated protein detection

As an alternative approach, the detection of the abnormal protein product can either be achieved by detecting it in an abnormal location (the cytoplasm) or by detecting the specific change in amino acid structure due to the mutation.

Immunohistochemistry (IHC)

As previously mentioned, mutated NPM1 protein localisation is skewed towards that of the cytoplasm. The mutated protein, due to dimerisation, also causes the wild type NPM1 to be predominantly present in the cytoplasm. This provides the opportunity to use this physical property for diagnostic purposes. IHC, using an antibody that detects both WT and mutated *NPM1* has previously been used on bone marrow trephines to detect the abnormal cytoplasmic localisation of NPM1 and thus predict if *NPM1* is mutated or not [74]. Reliable and consistent results could not however be obtained when using smears and cytopsin preparations [63]. One study [64] disputed the accuracy of the IHC results even on the bone marrow trephines, showing some discordance between cytoplasmic localisation of NPM1 detected with IHC compared to mutation screening by PCR, concluding that PCR cannot be replaced by IHC. This may be due to NPM1 diffusion during tissue fixation and the use of different fixatives influencing the IHC technique [64].

Flow cytometry

Recently an Italian group [63] developed an unconjugated NPM1 monoclonal antibody (mAb) called T26. As previously mentioned, NPM1 mutated proteins have a novel C-terminal sequence of eleven amino acids, with the last five (VLSRK) being common to all currently described mutants [9]. In this study, a 19-amino acid C-terminal peptide was used to generate the mAb containing eight amino acids from *WT NPM1* and the last five commonly shared amino acids from the mutated NPM1 (CQEAIQDLCLAVEE-VLSRK) [9,63]. To confirm that the mAb specificity was restricted to the *NPM1* mutated sequence, a study correlating *NPM1 DIM* detection via PCR with flow cytometry, using cells from 39 de Novo AML patients was performed - 24 tested negative and 15 positive on flow cytometry with perfect concordance with PCR. The range of *NPM1 DIM* mutation types was however limited in this study, with 93% of the positive patients showing the type A mutation. Sensitivity of detection using this antibody was evaluated by performing serial dilutions using a positive *NPM1 DIM* cell line (OCI/AML3) and a promyelocytic leukaemia cell line with wild type *NPM1* (HL-60). Detection of the positive cells was possible even as low as 0.001%. However, this could be criticised as performing dilutions with a single negative cell line may not truly be representative of flow cytometry on a bone marrow or peripheral blood sample. In these scenarios many more populations of different cell types may be present; some overlapping with commonly used gating strategies. Tan *et al* (2011) attempted a similar study and developed their own antibody (2G3), but showed significant cross reaction with the wild type NPM1

[65], bringing into question the usefulness and robustness of antibody-based techniques. Imaging flow cytometry to assess the localisation of NPM1 in the cell after using an antibody for NPM1 protein, have also been successfully performed, this technology is unfortunately not commonly available at the moment [75]. Flow cytometry is however relatively easy to perform, provides same day results and may be applicable to settings where molecular testing laboratories and equipment is not available. This technique thus requires further study.

Minimal residual disease (MRD) monitoring in CN-AML by monitoring *NPM1*

DIM

AML patients with recognised cytogenetic abnormalities such as translocations are relatively easy to monitor either by FISH or by RNA-based transcript monitoring. However, due to the lack of these hallmark genetic rearrangements, most CN-AML cases are not amenable to the same techniques. Phenotypic shifts and lack of aberrant markers may also hamper MRD monitoring by flow cytometry.

In the subgroup of “AML with gene mutations”, several of these smaller mutations have been investigated as MRD markers. *NPM1 DIM* was found to be more consistently present at relapse than *FLT3-ITD* and thus represented a more stable marker for monitoring residual disease in AML than *FLT3* mutations [66]. However not all studies have demonstrated this stability and loss of *NPM1*

DIM in up to 9% of patients has been reported during the evolution of the disease [67,68]. Caution is thus required when using this mutation as an MRD marker.

A number of studies which looked at large patient groups with *NPM1 DIM* [68-70] showed that clinical and morphological relapses could be predicted several weeks earlier by monitoring *NPM1* levels using sensitive mutation-specific real-time quantitative polymerase chain reaction (QRT-PCR). Schnittger *et al* (2009) also reported that the *NPM1* mRNA level at various time points was predictive of event free survival for patients receiving first line therapy, second line therapy and allogeneic stem cell transplant for AML [68].

One study focusing specifically on overall survival (OS) and remission duration, evaluated 245 patients with *NPM1 DIM*. *NPM1DIM* mRNA transcript levels were measured at multiple time points during therapy and found that at all time points, patients with higher *NPM1 DIM* transcript levels were associated with higher relapse rates and shorter survival. The most significant predictive time points were after the second induction and at completion with a level of >200 *NPM1 DIM* transcripts/ 10^4 ABL predictive of relapse [69]. Another study showed that failure to obtain at least a two log reduction in *NPM1 DIM* transcript levels after consolidation was associated with worse OS and shorter relapse free survival [71].

Normal immunophenotyping can be used for monitoring residual disease in AML [72] however this can at times be taxing. Incorporating *NPM1* mutation flow cytometry may assist in performing these flow cytometry MRD assays when the mutation is present. The assay and antibody developed for detecting mutated *NPM1* by Gruszka *et al* (2010) researched sensitivity levels of 0.001%, appropriate for these applications [63]. Flow cytometry results may be available quicker than QRT-PCR, assisting in making immediate management decisions.

Conclusion and identification of needs for further research

NPM1 is a nucleo-cytoplasmic shuttling protein with numerous functions relating to cellular proliferation. The protein is predominately located in the nuclear compartment and has a sophisticated structure controlling the movement through the nucleosome and cytoplasm to fulfill its various functions. When the balance between nuclear import and cytoplasmic export is disturbed (as in the case of mutations), this can lead to the disruption of normal *NPM1* functions, causing dysregulation of a number of cellular functions involved with cell cycle regulation and ribosomal protein assembly.

NPM1 *DIM* have been found in CN-AML and have been shown to be associated with a better prognosis when occurring in isolation. The presence of these mutations not only serves as a prognostic tool and guide for treatment, it also serves to eliminate unnecessary expensive additional testing (i.e.: FISH and

cytogenetics), as it is mutually exclusive from the AML-associated translocations. Only *FLT3-ITD* testing is additionally required.

A number of methods are available to detect these mutations including PCR based assays and detecting the mutated protein directly or its abnormal position within the cell, using immunohistochemistry or flow cytometry. Antibodies developed against specific mutated proteins provide the unique opportunity to detect genetic mutations at the translational level. Using antibodies on a flow cytometry platform provides the added benefit that it is fast and relatively easy to use. As all of the relevant exon 12 mutations for NPM1 result in the alteration of the last five amino acids, theoretically a single antibody can be used to detect all the variants. Although a few groups have developed their own monoclonal antibodies against the mutated protein, their results on the efficiency and specificity of the assays are contradictory and more work is needed to establish if this technology is applicable. Unfortunately monoclonal antibodies developed by these groups are not yet commercially available, making it difficult to evaluate these studies directly. A mutation specific unconjugated polyclonal antibody for NPM1 is however commercially available and this provides the opportunity for future study and development of a technique that can be implemented by any laboratory with expertise and availability of a flow cytometer.

Although PCR-based assays are currently used more often, the use of a flow cytometric methodology at diagnosis, when flow cytometry panels are routinely

used to characterise the leukemia, would not only be cost-effective and more rapid (than PCR), but also a more efficient use of labour. Additionally it may negate the need for expensive cytogenetics and FISH analysis in the situation that the *NPM1* mutation is present as virtually all AML patients, that prove to have this mutation, have normal cytogenetics. Many smaller laboratories may have access to flow cytometry but do not have extensive molecular facilities or expertise in this field. The development of bead-based assays to detect chromosomal translocations in Haematology, using flow cytometry, echoes this sentiment [73]. Therefore the development of a reliable flow cytometry assay will make the detection of these mutations more accessible.

The aim of this study was therefore to develop a robust flow cytometry methodology that could be used in the routine assessment of AML samples to determine the mutational state of NPM, using a commercially available polyclonal antibody against the mutated NPM1. To achieve this aim, the study was composed of several objectives, including the determination of the optimal concentration of both primary and secondary antibodies and finalising a protocol; characterising the assay performance in terms of sensitivity, repeatability and robustness and finally evaluating the assay using patient samples and comparing results to the classical PCR method of *NPM1 DIM* detection.

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The development of a flow cytometric method to detect the presence of mutated nucleophosmin in Acute Myeloid Leukaemia

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Key words: NPM1, nucleophosmin, AML, acute myeloid leukaemia, flow cytometry

Running title: The development of a flow cytometric method to detect the presence of mutated nucleophosmin in Acute Myeloid Leukaemia

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Abstract

The development of a flow cytometric method to detect the presence of mutated nucleophosmin in Acute Myeloid Leukaemia

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Introduction:

NPM1 is a protein that plays multiple roles in cell growth and proliferation. Deletion insertion mutations of NPM1 seem to disrupt its normal physiologic role as a molecular chaperone. NPM1, if present alone (not associated with Flt3-ITD) however, is associated with significantly better overall. The aim of this study was to develop a flow cytometry methodology to detect *NPM1 DIM*.

Methods:

NPM1 mutation specific antibody was used to develop a diagnostic method by flow cytometry. Optimal antibody concentration, blocking technique for non-specific binding as well as intracellular staining methodology was established. Samples from twelve newly diagnosed AML patients were used to correlate the NPM1 flow cytometry assay with DNA based PCR. Median Fluorescence Intensity ratio (patient :negative control) of greater than 1.8 was established to optimally differentiate positive and negative patients on flow cytometry.

Results:

Flow cytometry was able to detect NPM1 DIM positive blast populations of just less than 10% on dilution studies. Two of the twelve patients were positive for NPM1 on both flow and PCR and ten were negative.

Conclusions:

Flow cytometry may be utilised as a relatively cost effective, rapid alternative to PCR but the study needs further validation.

Introduction

Acute Myeloid Leukaemia (AML) is a neoplasm of a specific lineage of blood cells, characterised by the rapid proliferation and accumulation of immature myeloid precursor cells in the bone marrow, with disruption of the normal blood cell production [1].

AML actually encompasses a heterogeneous group of diseases, which traditionally relied on morphology, cytochemistry and cytogenetic analysis to subtype and in turn prognosticate [1]. In those AML subtypes with recurrent cytogenetic abnormalities, three broad prognostic categories were established [2] (review): A) Favourable, consisting of the core binding factor leukaemias - t(8;21), inv(16), B) Intermediate, consisting of t(9;11) and C) Poor, associated with inv(3), t(3;3), t(6;9), other 11q23 rearrangements, complex karyotypes, -5, del(5q), -7 or abn(17p). Generally patients in poor prognostic categories would be considered for allogeneic stem cell transplant while 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.

As a consequence, the World Health Organisation (WHO) introduced a new category named "AML with gene mutations" in 2008, which is further divided into prognostic groups depending upon the specific gene mutations or combinations of mutations [1]. Mutations listed by the WHO in this category include mutations

in Fms-like tyrosine kinase 3 (*FLT3*), Nucleophosmin 1 (*NPM1*) and CAT box enhancer binding protein alpha (*CEBPA*), which carry a high prognostic value[5]. Generally *NPM1 deletion/insertion mutations (NPM1 DIM)* [6] and *CEBPA* point mutations [7] are considered to convey a good prognosis, while *FLT3 internal tandem duplications (FLT3-ITD)* a poor prognosis [8]. The combination of mutations also alters the prognostic value of the individual mutations, with co-existence 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 treatment protocols are initiated.

In cytogenetically normal AML (CN-AML), the most common *NPM1* mutations involve deletions or insertions in exon 12 (*NPM1 DIM*), with six major variants being described (A-F) [10]. All of these *NPM1* mutations cause similar changes to the last five amino acids at the C-terminus of the protein. This results in changes in its nuclear localisation signal [11] and a shift in the balance of nuclear export leading to the accumulation of *NPM1* in the cytoplasm [10,12,13]. This nucleo-cytoplasmic 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 then severely inhibited by the *NPM1* mutations [10].

While *NPM1 DIM* are currently detected via PCR techniques, a single C-terminus, mutation specific antibody could potentially be used for detecting the majority of *NPM1 DIM* subtypes (A-F) and be combined 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.

Monoclonal antibodies, raised and selected specifically to detect this mutant protein, have been developed by two groups [17,18], however discrepant results were obtained and these antibodies are not available commercially. One polyclonal antibody to detect mutated NPM1 protein is available from a commercial company (ABCAM) and has previously been validated in Western blotting techniques but has not been extensively studied in the application of flow cytometry (personal communication with ABCAM).

The aim of this study was to develop a flowcytometric method to detect mutated NPM1 protein in AML patients. The assay development, testing sensitivity, repeatability and specificity were assessed using a combination of cell lines and clinical samples; in conjunction with *NPM1 DIM* PCR assessment. The assay was also assessed from a cost perspective to evaluate its use in a diagnostic laboratory setting.

Methods and materials

Cell lines and clinical samples

The OCI/AML3 cell line, which harbors a confirmed Type A exon 12 *DIM* mutation of *NPM1*[19] 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, Utah, USA)). Cells were cultured in suspension using α MEM (Gibco) and 20% FBS (Gibco). A Jurkat T lymphoblastic cell line (ATCC: TIB-152), with wild type *NPM1* genetics, was used as a negative control for the antibody titration experiments and was cultured using RPMI 1640 (Gibco), 10% FBS (Gibco). Both cell lines were grown under standard conditions: 37°C, 5% CO₂ and 80-90% humidity.

For the analysis of clinical samples, 5ml peripheral blood (PB) (EDTA) samples were collected over a three-month period from all newly diagnosed AML patients (no specific subtype was selected or excluded from the analysis) who had samples sent for flow cytometry analysis at the National Health Laboratory Service – Groote Schuur Hospital. A total of 12 patients were analysed. As clinical normal controls, 5 ml PB (EDTA) was randomly obtained from routine patients with normal full blood count results. Informed consent was obtained from each of the participants, and ethics approval was obtained from the University of Cape Town Ethics review board (086/2009).

To determine the assay sensitivity, OCI/AML3 cells were serially diluted into PB from the control patients (both sample types standardised to a final white cell count of $10 \times 10^9/l$). Dilutions ranged from 100% to 1% OCI/AML3 positive cells. These samples were then analysed via flow cytometry. To assess the reproducibility of the assay sensitivity, the 10% dilution was individually prepared fourteen times (dilution and sample preparation) and the samples analysed on two separate occasions.

Overview of final developed flow cytometry assay

Samples (cell line/ clinical samples) were diluted in phosphate buffered saline (PBS) to a standard nucleated cell count of $10 \times 10^9/l$ (as determined using the Siemens Advia 2120). The samples were protected from light exposure throughout the antibody staining process, which involved both cell surface and intracellular staining. Cell surface staining was performed on 100ul of the prepared sample using 10ul of anti-CD45-Peridinin Chlorophyll Protein Complex (PerCP) (Beckton-Dickenson). After anti-CD45 incubation for 15 minutes, erythrocytes were lysed using 1ml FACS Lyse (Beckton-Dickenson) for 10 minutes at RT. Samples were then washed once using 2ml 0.5% Bovine Serum Albumin (BSA) in PBS and the cell pellet collected via centrifugation at 400g for 5 minutes. Cell permeabilisation with FACS perm II (0.5ml) (Beckton-Dickenson) was performed for 10 minutes at 4°C and the cells were again washed once with 0.5% BSA and the cell pellet collected as described. Optimal blocking of non-specific binding sites was performed by re-suspending the cell pellet in 1ml of

3:2 ratio of human AB serum and 0.5% BSA/PBS and incubation at 37°C for 2 hours. The sample was then incubated with 2µl of NPM1 mutation specific polyclonal rabbit antihuman primary antibody (0.3mg/ml stock) (ABCAM) for 30 minutes at room temperature. It was then washed twice using 0.5% BSA / PBS and re-suspended in 100µl of the 3:2 AB serum / BSA mixture. Staining with 2µl stock solution of secondary goat anti-rabbit antibody (Alexafluor-488 conjugate, Invitrogen) (0.2mg/ml) was performed for a further 30 minutes at RT. Following this secondary antibody labeling step, the samples were finally washed twice with 2ml 0.5% BSA/PBS and the cell pellet re-suspended in 1ml PBS.

Labeled samples were analysed on a FACS Canto II instrument (Beckton Dickenson), which was calibrated daily using Cytometer Setup and Tracking beads (Beckton Dickenson). Compensation was deemed unnecessary, as PerCP and AlexaFluor-488 do not show spectral overlap. Analysis of raw data was performed with Kaluza™ software (Beckman-Coulter). To improve the assay sensitivity when using the clinical samples, the blasts were gated on using the CD45 dim and low side scatter characteristics of this cell population[20]. If no differentiation between granulocytes and blasts could be made, then the entire CD45 positive population was gated except for the lymphocytes. A minimum of 10 000 events was acquired in this gate. For the analysis of all clinical samples, a positive control (OCI/AML3) and negative control (control PB acquired the same day as the AML patients) sample was simultaneously analysed with the AML patient samples. In the absence of clear NPM1

positive/negative peak differentiation, 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 normal PB control sample (NPM1 MFI ratio). A NPM1 MFI ratio of >1.8 was determined to correspond with *NPM1* 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, Cape Town, using validated and SANAS accredited methodology. Briefly, DNA was extracted and purified from PB using the Maxwell 16 nucleic acid extractor (Promega, USA) and diluted to 25ng/μl with nuclease-free water (Promega, USA). Primers developed by Laughlin et al (2008) were used to amplify an 117bp fragment of exon 12 of NPM1 and a 121bp fragment containing the *NPM1 DIM*, if present - NPM1F: TTTTCCAGGCTATTCAAGATC, NPM1R: GGACAGCCAGATATCAACTG[21]. The following PCR conditions were used for the amplification on a GeneAmp 9700: 35 cycles - 95°C/60s, 56°C/60s, 72°C/60s with a final 72°C/7mins. PCR products were diluted 1/10 with Hi-dye formamide, mixed with ILS600 standard/Hi-Dye (Life technologies, USA) and separated on a POP4/37cm column using the ABI3100 genetic analyzer (capillary electrophoresis) (Applied Biosystems, UK). GeneMapper 4.1 (Life technologies, USA) was used to size the peaks, with a minimum peak height defined as 50 relative light units. Patient samples were

analysed in duplicate, along with DNA from OCI/AML3 (50% *NPM1 DIM* alleles), a control clinical sample (sequence confirmed) and the appropriate DNA extraction control. This assay was found to be able to reliably detect *NPM1 DIM* when present in 10% of the mutated cells (5% mutated alleles).

Results

Assay development

Antibody titration

An antibody titration experiment was designed to determine the optimal primary and secondary antibody concentrations for the assay, which would produce the highest discriminatory power between the positive and negative populations. OCI/AML3 (positive for mutated *NPM1*) and Jurkat cells (negative for *NPM1*) were used in a 1:1 ratio with a final white cell count of $10 \times 10^9/l$. The volume of the primary and secondary antibodies was varied sequentially from 1 – 5 μ l, in 100 μ l of cell mixture, until acceptable differentiation could be made between the positive and negative population groups. The cell mixtures were analysed by creating a histogram for each combination of primary and secondary antibody by plotting mutated *NPM1* against the number of cell events. Optimum volumes of primary and secondary antibody were found to be: 2 μ l primary (final concentration 6 μ g/ml) antibody and 2 μ l secondary antibody (final concentration 7 μ g/ml) in a final labeling volume of 104 μ l. Fig. 1 shows the optimal negative/positive population discrimination, using this combination.

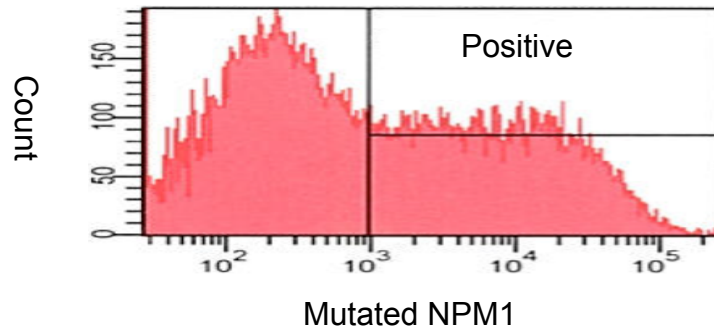


Figure 1: Optimal mutated-NPM1 detection.^g

1:1 mix of OCI/AML3 (NPM1DIM positive) and Jurkat (NPM1DIM negative) cells stained with 2 μ l of the primary antibody (anti-mutated NPM1) and 2 μ l of the secondary antibody (Alexafluor 488 goat anti-rabbit) in a final reaction mixture of 104 μ l. Histogram shows optimal separation of these cell lines.

Minimising non-specific antibody binding

Non-specific binding and thus false positive results are common in assays using primary and secondary antibodies. This is mostly due to binding by Fc receptors on the monocytes and neutrophils [22]. While this problem was not experienced with the labeling of the cultured cells (due to the limited cell differentiation), analysis of clinical normal PB samples demonstrated a significant non-specific binding issue (Fig. 2A), resulting in false mutated-NPM1 positivity.

Monocytes and neutrophils can either be removed from the specimen, systematically gated out during analysis or the Fc receptors effectively blocked to resolve this issue. A number of techniques are available and were attempted to achieve this goal. This included plastic adsorption of monocytes (and thus removing them from the specimen), however this will not resolve the presence of

^gNote that all diagrams have been inserted into the text according to University of Cape Town guidelines. This is in contrast with the instruction of the author guidelines of the chosen journal

the neutrophils, and blocking the Fc receptor with varying concentrations of BSA (0.5% - 5% in PBS) on its own or in combination with human AB Rh negative serum (AB serum: BSA/PBS ranged from 1:1 to 3:2) [22]. An initial attempt to remove the monocytes by plastic absorbance did not significantly reduce the number of cells showing non-specific binding and using BSA alone also had minimal effect (results not shown). Optimal blocking was finally achieved using a combination of human AB Rh negative serum and BSA 0.5%/PBS (3:2 ratio) for 2 hours at 37°C. Fig. 2B shows the expected mutated-NPM1 negativity in the control sample when using this blocking approach.

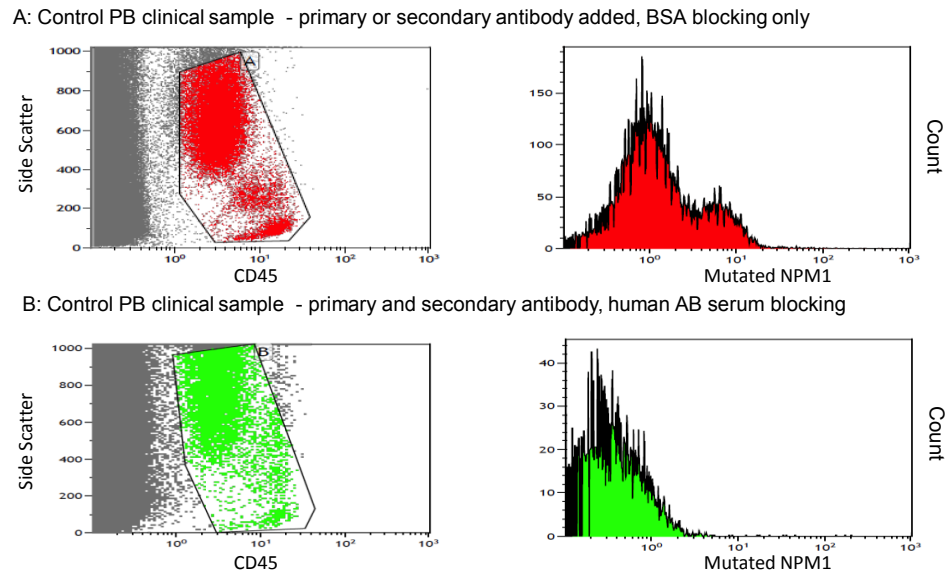


Figure 2: Optimal blocking technique.

A) Control PB clinical sample with primary anti-mutated NPM1 and secondary antibody (goat anti-rabbit Alexa-488) added. Only 0.5% BSA blocking was performed on this specimen. CD45 positive population was gated on the dot plot. This population was then analysed with a histogram of mutated-NPM1. B) Control PB clinical sample with primary anti-mutated NPM1 and secondary antibody (Alexa-488 goat anti-rabbit) added. Blocking was performed on this specimen using human AB Rh negative serum and BSA 0.5%/PBS (3:2 ratio) for 2 hours at 37°C. CD45 positive population was gated on the dot plot. This population was then analysed with a histogram of mutated-NPM1.

Testing the sensitivity and reproducibility

A sensitivity experiment was performed by diluting the positive control cell line (OCI/AML3) into PB of the normal clinical controls, to provide a more realistic cellular profile compared to only using a negative control cell line. The following dilutions were assayed in duplicate: 100%, 50%, 20%, 10% and 5%. Gating was performed on the entire CD45 positive population, as OCI/AML3 cells were not easily distinguishable from granulocytes and monocytes in these mixed samples.

Sensitivity of the assay was established at 10%, using the mutated-NPM1 histogram. Fig. 3 shows the results from the analysis of the 20%, 10% and 5% mixes. The population of NPM1 positive cells could be clearly identified at the 10% level on the histogram (fig. 3B); however it became progressively more difficult to differentiate the NPM1 positive cells from the negative population at lower levels (fig. 3C).

Reproducibility was evaluated at this sensitivity limit, by analysing fourteen 10% sample preparations on two separate occasions. This was performed to establish if the assay could reproducibly detect the NPM1 mutated cells in the control clinical samples. The MFI of the mutated NPM1 peak was calculated and showed minimal variation (see Table 1), with intra and inter-experimental variability calculated at 6.1% and 5% respectively.

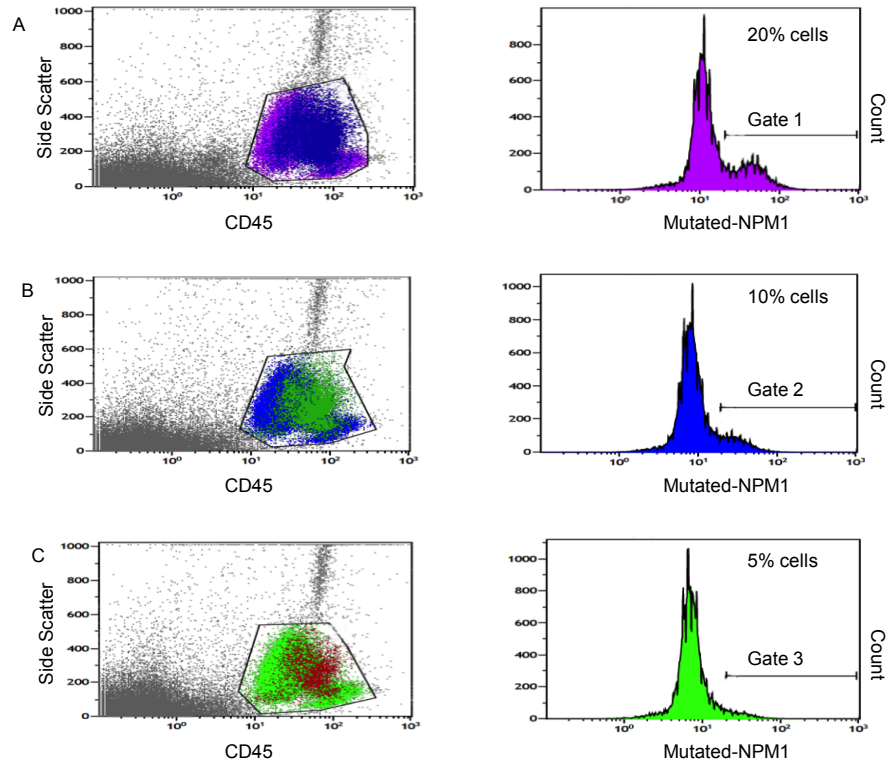


Figure 3: Sensitivity of assay.

(A) 20% NPM1 mutated positive control cell line mixed with clinically normal PB. CD45 positive population was gated on a dot plot. The histogram displays this CD45 gated population with mutated NPM1 (Alexa-488 positivity) vs count. The NPM1 mutated cells population was then gated on the histogram (gate 1) and back gating shown in a different colour on the CD45 dot plot, (B) 10% (gate 2) and (C) 5% positive cells (gate 3).

Table 1: Reproducibility and precision of assay using 10% NPM1 DIM positive cells^h

	MFI	MFI
Individually prepared samples containing 10% positive control cells	Day1	Day2
1	39	36.87
2	40.52	40.51
3	37.4	36.52
4	39.99	40.7
5	38.96	37.16
6	36.56	36.35
7	37.24	36.64
8	36.14	36.69
9	33.3	33.29
10	36.23	37.48
11	41.4	36.78
12	39.59	42.18
13	40.26	37.8
14	35.34	40.27
Mean	38.00	37.80
Standard deviation	2.32	2.32
Coefficient of variation	6.12	6.14
Positive control only	51.32	51.38
Negative control only	6.74	6.73

^hThis table will not be included in the article and will be submitted as part of the appendix. This was done to comply with the IJLH restriction on figures and tables

Clinical assessment:

To determine the specificity and performance of the assay in a clinical setting, PB samples from twelve newly diagnosed AML patients were assayed for the *NPM1 DIM* using both this flow cytometry and a routine PCR assay. The PCR assay indicated that only 2/12 patients were positive for the exon 12 mutation (type not defined) (see Table 2). Both patients showed normal cytogenetics.

For the flow cytometric analysis, patient samples were analysed on separate days and were co-analysed with a 100% positive control OCI/AML3 sample and a clinical negative control sample, to control for variation in antibody labeling efficiencies and instrument fluctuations. What was immediately evident was that although the single peaks obtained for the PCR positive *NPM1 DIM* patients and the positive control cell line gave distinctly different MFIs compared to those of clinically negative control samples, the MFI of the PCR-positive patients was significantly different from the positive control (MFI: 23.13 & 10.36 vs. 44.95) (shown in fig. 4). This made it difficult to use the positive control as a marker for determining *NPM1* mutation positivity. It is postulated that this discrepancy may be due to the fact that the cells of the positive cell line (OCI/AML3) 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.

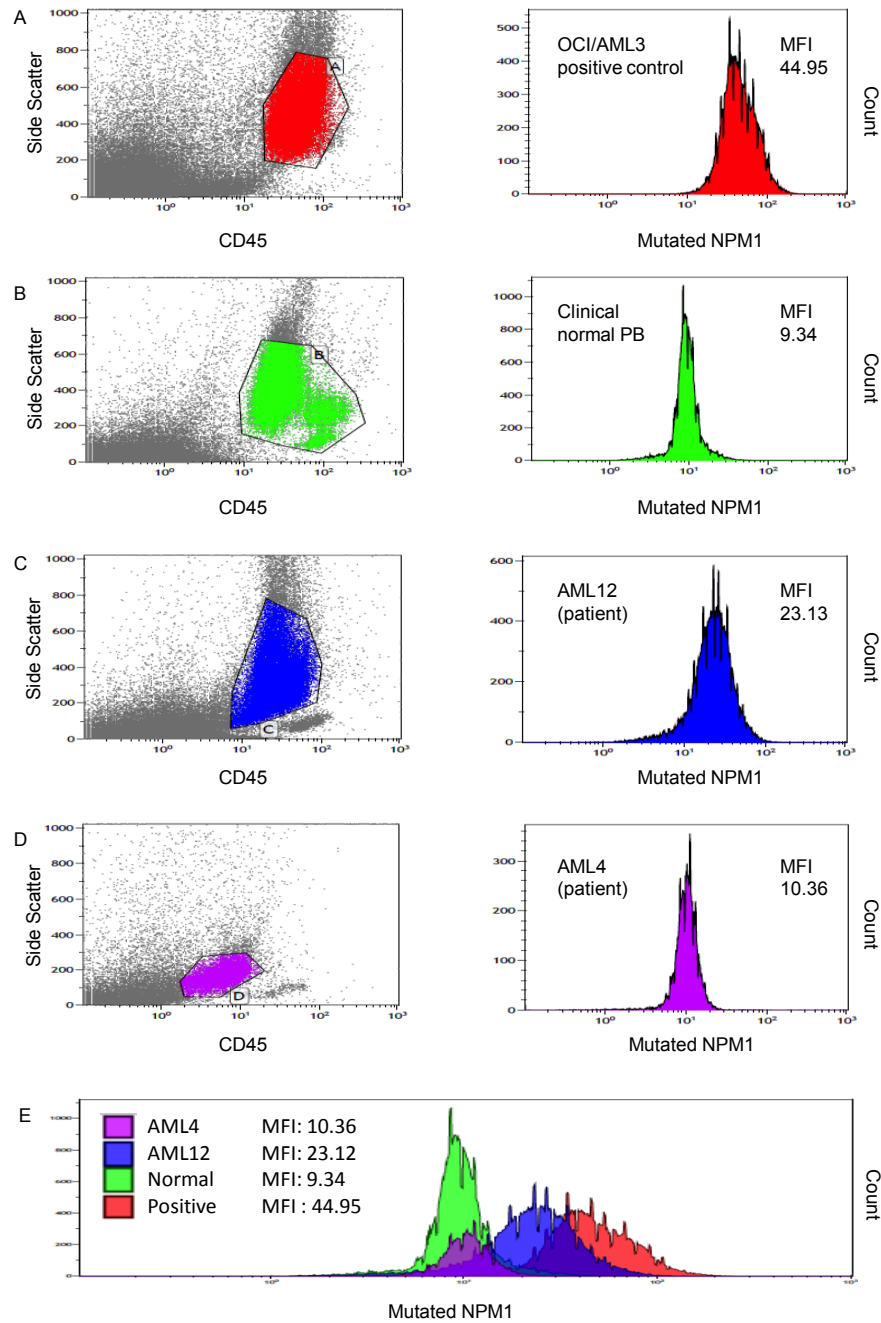


Figure 4: Clinical evaluation of AML patients and controls.

All samples prepared using AB serum / BSA blocking methodology. Population of CD45 positive cells gated on dot plot of CD45 vs Side Scatter (lymphocytes excluded from analysis in patients). This population was then evaluated using a histogram of mutated NPM1 (Alexafluor-488) vs Count. The MFI of the peak was determined. A) Positive control (MFI: 44.73) B) Negative control (MFI: 9.38) C) PCR positive for NPM1 DIM patient (MFI: 23.1) and D) PCR negative patient (MFI: 10.2). E) Overlay of A-D NPM1 DIM

To more objectively indicate the NPM1 mutational state, the MFI of the ten *NPM1 DIM*-PCR negative wild type NPM1 AML patients was determined. The MFI of each patient single peak was normalised to the negative control, which had been co-analysed with each patient sample. Table 2 shows the results of this analysis, indicating the wild type *NPM1* MFI ratio range to be 1 – 1.8 (minimum – maximum) using the ten AML *NPM1 DIM* negative patients. The presence of the NPM1 mutated protein would therefore be indicated by a NPM1 MFI ratio of greater than 1.8. The two PCR positive cases were subsequently evaluated using this methodology. Table 2 shows this result, indicating a NPM1 MFI ratio of 2.5 for AML11 and 2.4 for AML 12, both then indicating the presence of the mutated NPM1 protein. Using this analysis methodology, we were able to get full concordance between both the PCR and flow cytometric assays.

Table 2: NPM1 DIM clinical assessment – AML patient analysis

Identifier	Patient MFI [#]	Positive Control	Negative Control	MFI ratio*	PCR
		MFI	MFI		
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	10.4	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

[#]: MFI of single peak generated

* Calculated as MFI positive peak in sample/MFI peak of clinical negative control

Cost and analysis time:

The comparative cost and time needed to generate a reportable patient result using either the PCR or flow cytometry methodology was calculated. For flow cytometry analysis, the costs included the preparation of a single patient tube, as well as a positive and negative control. A result could be generated for under R500 (R470.73/\$45.75), within the same day as obtaining the sample (1 hour hands on time / 4.5 hours total). The assay does not require more labour or additional instrumentation compared to the standard immunophenotyping that is performed on all cases of acute leukaemia and can be easily incorporated into the routine flow cytometry on these patients without requiring additional time.

The PCR analysis would include a single patient DNA extraction, PCR reactions of the patient DNA in duplicate, positive/negative and extraction blank PCR controls and fragment analysis via capillary electrophoresis. The total cost of this three-step process was significantly cheaper than flow cytometry, being able to generate a result for under R200 (R193.06/\$18.39). The total time required for this analysis would be at least 4 hours, with a hands-on time of about 30 minutes. While this is slightly faster than the flow cytometry, in practice this assay would be spread over at least two days due to the standard practice of batching samples for automated DNA extraction (C21 NHLS laboratory).

Table 3: Cost analysis of the NPM1 DIM PCR and flow cytometry assays to generate a single patient result.ⁱ

Flow cytometry Consumables	Price in Rand/Dollar*	PCR consumables	Price in Rand/Dollar*
CD45 gating antibody	52.77/5.02	DNA extraction (automated)	45.25/4.31
NPM1 primary antibody	244.14/4.20	PCR primers	4.20/0.40
Alexa-Fluor 488 secondary antibody	122.85/11.70	dNTPs/nuclease-free water	15.00/1.43
FACS Perm II	16.53/1.57	PCR tubes/tips	13.36/1.27
FACS Lyse	25.20/2.40	Formamide/ILS600	15.25/1.45
Pipette tips/tubes	4.92/0.47	ABI analysis	100.00/9.52
PBS	4.3/20.41		
Total	470.73/45.75	Total	193.06/18.39

* Rand / Dollar exchange @R10.50 / \$

Discussion:

Flow cytometry is gaining importance as an alternative method to PCR for detection of fusion proteins in haematological malignancies [23-26]. The aim of the project was to develop an alternative method to PCR for specifically

ⁱThis table will not be included in the article and will be submitted as part of the appendix. This was done to comply with the IJLH restriction on figures and tables

detecting the mutated NPM1 protein utilising flow cytometry and commercially available reagents.

We successfully developed and optimised an assay using positive and negative control cell lines to detect *NPM1 DIM* on flow cytometry and partially validated this, using clinical samples. The sensitivity of the assay was established at 10%, showing good reproducibility. The assay showed 100% concordance with a PCR assay to detect these mutations, but because of low numbers of positive patients requires further study and validation before it can be implemented diagnostically.

Two groups have developed and tested mutated NPM1 protein specific antibodies for flow cytometry [17,18], however the antibodies that were used are not yet commercially available. The studies demonstrated variable success, with the antibody from Gruszka's research group [17] showing high specificity for mutated NPM1 protein, but the other from Tan et al [18] showing significant cross reactivity with WT NPM1 protein. While both research groups used secondary antibodies to detect the antibody to mutated NPM1 protein [17,18], Tan et al [18] only tested the antibody's performance on an immunohistochemistry platform using a peroxidase labeled secondary antibody without blocking steps to prevent non-specific binding. Gruszka et al [17] tested their antibody on multiple platforms including flow cytometry and utilising purified IgG1 for the purpose of blocking. This study was therefore in agreement with

ours that it is essential to block the cells before secondary-antibody labelling to ensure NPM1-labelling specificity.

Gruszka et al [17] found the sensitivity of their assay to be as sensitive as 0.001%, however this was performed using positive and negative cell line (HL60 and OCI/AML3) mixtures, which does not simulate the normal scenario faced when dealing with clinical specimens containing a range of different cell types (including red cells). This is in contrast to the sensitivity limit established in our study; however our experiments were conducted using normal PB as the NPM1 WT specimen (diluting factor) and therefore more likely represents the sensitivity in the clinical setting. Sensitivity of the assay was deemed to be acceptable at 10%, as most diagnostic AML samples will contain in excess of 20% blasts [1]. However, this technique cannot be used for residual disease monitoring post-transplant or therapy where a much higher sensitivity is required (reviewed by Paietta [27]).

The assay presented here however requires further validation as only a small number of AML patients with *NPM1 DIM* were tested. It is envisaged that at least 20 additional patients with AML with eight showing positivity, will be required to ensure the validity of this assay. This will also mean that a larger selection of *NPM1 DIM* subtypes is evaluated. Unfortunately the PCR methodology used in this study cannot differentiate the different subtypes of *NPM1 DIM*. This may pose a hurdle in future studies when there is discordance

between the PCR and the flow cytometry assay, to establish which subtypes were not detected (if applicable). Unfortunately due to lack of funding more patient samples could not be performed at this time.

The presence of *NPM1* *DIM* in patients with AML is strongly associated with a normal cytogenetic karyotype [28]. Cytogenetic analysis is a labour intensive process with long turn-around times and is costly[29]. No cases of AML with a *NPM1* mutation have ever been shown to have recurrent cytogenetic abnormalities as described in the WHO[28], potentially obviating the need to perform cytogenetics when this mutation is present. Immediate information relating to the mutational status of *NPM1* can thus assist in decision making to process cytogenetics or not, leading to cost saving. This may also be an advantage for laboratories without a laboratory to process cytogenetics and need to transport samples over great distances to gain access to these facilities often resulting in failure of the assay due to age of the specimens [29]. Although the cost of the assay is somewhat more than that of PCR, one must take into account the additional labour specifically required to perform the PCR. No additional personal or equipment is required to perform the flow cytometry in a laboratory where these functions have already been established. The flow cytometry assay delivers same day results and patient management may potentially be commenced faster.

In summary, we have developed and partially validated a flow cytometry assay to detect *NPM1 DIM* at the translational level by using commercially available reagents. This assay should be reproducible in any laboratory with expertise in flow cytometry but requires further validation.

Acknowledgements

This work was possible due to a grant from the National Health Laboratory Service research trust. Special thanks to Dr P Szankasi (University of Utah, Salt Lake City, Utah, USA) for donating the OCI/AML3 cell line. Dr. K Shires acted as supervisor for the project and performed the molecular work. Dr. LA du Pisani performed the flow cytometry, cell culture and writing of the paper. The authors have no competing interests.

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PART D: SUPPORTING DOCUMENTATION

Annexure A: University of Cape Town ethics approval.



UNIVERSITY OF CAPE TOWN

Health Sciences Faculty
Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: shuretta.thomas@uct.ac.za

25 February 2009

REC REF: 086/2009

Dr M Stein
Division Haematology
C17

Dear Dr Stein

PROJECT TITLE: DEVELOPING A NOVEL FLOW CYTOMETRIC METHOD FOR DETERMINING THE MUTATIONAL STATUS OF NUCLEOPHOSMIN (NPM1) IN ACUTE MYELOID LEUKAEMIA (AML).

Thank you for submitting your study to the Research Ethics Committee for review.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted for one year till the 26th February 2010.

Please submit an annual progress report if the research continues beyond the expiry date. Please submit a brief summary of findings if you complete the study within the approval period so that we can close our file.

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

signature removed

PROFESSOR M BLOCKMAN
CHAIRPERSON, HSE HUMAN ETHICS

Federal Wide Assurance Number: FWA00001637.
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lemjedi

Annexure B:



CONSENT

I, _____ hereby give consent that a sample of my bone marrow may be used in the development of a new laboratory technique for the determination of Nucleophosmin mutational status, a protein involved in the cause and prognosis of leukaemia. Studies will be conducted at the National Health Laboratory Service – Groote Schuur Hospital.

The samples will be anonymous and will not affect your diagnosis or treatment at all.

I understand that I may withdraw my consent for any aspect of the above study at any time without affecting my future medical care.

All of the above have been explained to me in a language that I understand and my questions have been answered.

Participant/Legal guardian signature:

Date

Informed consent obtained by: _____

Signature: _____

Annexure C:

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Early View

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Revised Author Guidelines – July 2013

Annexure D

MMed / MPhil Minor dissertation guidelines
Approved via Dean's Circular Med 12/2009 AND amended as per PMC meeting held on 14 Sept 2011, spell check, etc. – 3 Nov 2011



The MMed minor dissertation is one of three examination components of the MMed degree. In the case of sub-specialities, the MPhil minor dissertation is one of two components of the MPhil degree. This minor dissertation carries one third of the weight of a full master's dissertation in terms of its credit weighting, i.e. 60 credits which approximate 600 hours of work. In order to register as a specialist (or sub-specialist) in South Africa, the Health Professions Council of South Africa (HPCSA) and the Colleges of Medicine of South Africa (CMSA) now require all specialist trainees who register for training after 1 January 2011 to have completed a relevant research study.

The dissertation must be the result of independent work of the candidate conducted under the guidance and direction of a supervisor(s) and should demonstrate evidence of an ability to undertake research, to interpret results adequately and to review the relevant literature comprehensively and critically. Although the research need not necessarily be original, the findings must be seen to advance scientific understanding. A case report is not acceptable for the dissertation, as it cannot meet these requirements. **The topic, study design and scope of research will depend on the particular disciplines and must be agreed on in consultation with the supervisor(s).**

The dissertation may be presented in one of two formats:

- I: Publication-ready format;
- II: Monograph format.

As disciplines differ in their requirements, it is important that the format chosen is acceptable to the discipline and appropriate College within the CMSA.

Research protocol

Candidates intending to register for the MMed Part III / MPhil Part II are required to submit a full research protocol for approval to their respective **Departmental Research Committee (DRC)**. The candidate must then obtain **UCT Research Ethics Committee (REC)** approval prior to conducting their research; studies that involve the audit of clinical records or services also require formal REC approval. Any primary research taking place in a provincial or local authority health facility, such as public sector hospitals or clinics, must also be submitted to the provincial government for approval, after the UCT Research Ethics Committee approval has been obtained. **Approval to access public sector facilities for research is needed for all provincial and local authority facilities.** There are five points where approval for research can be applied for - the three teaching hospitals, the local authorities and "all other province". Teaching hospitals and the local authorities approve research projects in-house. "All other province" approvals are done via the Directorate: Health Impact Assessment (Sub-directorate: Research) at provincial head office. If research crosses these boundaries, up to five approvals may be needed. Further details can be found at http://www.capegateway.gov.za/other/2011/3/phrc_approval_guidelines_november_2010.pdf. The Provincial Health Research Committee does not approve research proposals itself, but oversees this approval process by reviewing difficult applications on referral (Chair: Prof Rodney Ehrlich).

The research protocol should outline the scope and content of the dissertation and must include the title of the proposed dissertation, name of the supervisor(s) and their brief curriculum vitae(s). This full research protocol together with a copy of the REC approval letter and completed **Form D1** must be submitted to the postgraduate administration office, for approval by the **Professional Masters Committee Chair** and the **Board of the Faculty of Health Sciences**, prior to commencement of the research.

Timelines

Submission of the research protocol for approval should generally be made within the first 18 months of the registrar programme. Heads of Departments or Divisions should meet with their registrars at least annually to review progress towards their research project. Unless otherwise stipulated by your Division / Department or constituent College of the CMSA, the research project should generally be completed by the end of Year 2. For a number of constituent Colleges, the dissertation must be submitted 6-months before writing the Part II examination.

Supervisors

The importance of identifying a dissertation supervisor as early as possible cannot be overemphasized. The supervisor should be an individual who can relate to the candidate's research project, be available for frequent and regular discussion and advice, and someone with whom the candidate can develop a good working relationship. Where specialised equipment and/or laboratory work is required for the study, the supervisor should assist in facilitating access to appropriate facilities.

The primary supervisor may be based outside the candidate's home department, faculty or university. In such a case, an internal (co-)supervisor will also be required in addition to the primary supervisor, to serve as a guide and link to UCT faculty and discipline-specific procedures. Primary supervisors retain responsibilities to the candidate and the university until the dissertation process is complete. The supervisor and student must complete form D3, which describes the contractual agreement of supervision.

Please note: in order to assist a candidate with a master's research topic the supervisor should hold a master's degree or equivalent (such as a Fellowship of one of the constituent Colleges of the CMSA), and have relevant research experience. If the primary supervisor does not hold such a higher qualification, then a secondary supervisor who has a higher degree will need to be appointed in addition to the primary supervisor.

The dissertation

Submission of the dissertation should include the following:

The title page should contain the candidate's name, dissertation title and the name of the university. It must also state the degree, e.g. Master of Medicine (MMed) in Public Health Medicine, Occupational Medicine, Family Medicine, Surgery, etc. The title page should also include a statement to the effect that the research reported is based on independent work performed by the candidate and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree to any other university. It must also state that this work has not been reported or published *prior to registration* for the abovementioned degree.

The abstract should summarise the study rationale, methods, results, discussion and conclusion in fewer than 500 words.

The remainder of the dissertation may be presented in one of two formats:

- I: Publication-ready format;
- II: Monograph format.

I: Publication-ready Format:

The body of the dissertation, which must be structured to include the following:

Part A: The protocol as approved by the Departmental Research Committee and UCT Faculty Research Ethics Committee (REC). The protocol should not exceed 4000 words. Should the protocol approved by the REC exceed 4000 words, the candidate should provide a shortened version of this protocol in not more than 4000 words.

Part B: A structured literature review appropriate to the subject matter and methods of the dissertation. The literature review must, amongst other things, show that the student is sufficiently acquainted with, and is able to conduct a critical appraisal of the relevant literature. If appropriate for the topic, candidates should demonstrate a good understanding of evidence-based medicine.

The structured literature review should be between 3 000 and 4 000 words.

A suggested structure for the literature review is as follows:

- a) Objectives of literature review;
- b) Literature search strategy, including inclusion and exclusion criteria;
- c) Quality criteria. These will vary with the nature of the dissertation;
- d) Summary and interpretation of literature, and its implications for the research;
- e) Identification of gaps or needs for further research;
- f) References (which will overlap with but will not be the same lists as in the journal article and protocol).

Part C: Publication-ready Manuscript: The results of the study must be presented in the form of a manuscript of an article for a **named peer reviewed journal**, meeting all the requirements set out in the "Instructions for Authors" of that journal, including the word count and referencing style. Unless specially motivated, the journal chosen will need to allow for at least 3000 words excluding abstract, tables, figures and references. The "Instructions to Authors" of the journal must be appended. The co-authors should be listed in the appropriate order, and each of their contributions to the manuscript stated. The journal chosen for publication must be appropriate to the subject matter of the dissertation and listed in the citation index of the Institute for Scientific Information (ISI) or accredited by the Department of Education:

(<http://www.lib.uct.ac.za/medical/index.php?html=/libs/accredjnls.htm&libid=24>)

Important note: The candidate need not have submitted the article for publication, nor is the acceptance of the article for publication a requirement for passing the degree. However, the norm is to publish the study with the supervisor(s) as co-author(s), and candidates are strongly encouraged to submit their manuscript for publication either before or shortly after examination of the minor dissertation. Submitting the manuscript for publication before submitting the minor dissertation has the advantage that addressing the peer reviewers' comments improves the standard of the manuscript included in the dissertation. A candidate who fails to submit a manuscript for publication within one year of examination of the minor dissertation must accept that their supervisor(s) may publish their data with him/her as co-author.

For a systematic review, Parts B and C are combined in the publication-ready manuscript.

Part D: Appendices All supporting documents including:

- Acknowledgements, including a description of the role played by each person who would be expected to be an author on a published article arising from the dissertation. In a dissertation derived from work started by others, e.g. analysis of data collected for another project, the candidate's contribution must have been made after his/her registration for the degree and therefore under supervision. In a manuscript from a multi-

authored project suitable for submission towards a minor dissertation, the candidate would be expected to be first author;

- Questionnaire/data capture instrument(s) (if not appended to protocol in Part A)
- Consent forms and any related participant information sheets (if not appended to protocol in Part A)
- Technical appendices, including, if considered necessary, any additional tables not included in the main manuscript for the examiner to have available. These should be accompanied by a brief narrative.
- Official Ethics approval letter from the Faculty Research Ethics Committee and any other approvals required (e.g. Provincial Government).

II: Standard monograph format:

Some disciplines and constituent Colleges of the CMSA require a standard monograph format, which should be 16 000 to 20 000 words in length, and presented in a comprehensive and scholarly style.

The dissertation must contain the following:

- a) A structured and comprehensive review of the literature relevant to the study (see guidelines under Section I-B above);
- b) Material and methods of the study must be fully described and factually presented and must evidence familiarity with the laboratory and/or clinical methods used;
- c) Results, discussion and conclusions.

Language and writing

Candidates should refer to the document D4, Guidelines on the Layout and Style of the Dissertation or Thesis. Clear, grammatically correct English is essential. As long as the dissertation is readable and internally consistent, any of a number of styles is acceptable. Supervisors may assist candidates in developing scientific communication skills but they are not required to do detailed editing or correction of spelling, grammar, or style. They may refer candidates elsewhere for this, at the candidate's own expense. Candidates who may have difficulties are encouraged to seek help from the writing support facilities on main campus (see: <http://www.ched.uct.ac.za/adp/writing/>).

The Harvard style for referencing is recommended. In this style, referencing is by first author in parentheses in the text and the bibliography is listed alphabetically (rather than using numerical superscripts in the text). NOTE: For Section C (Publication-ready manuscript) references should be formatted according to the instructions to authors for the journal selected, and candidates may prefer to use the same style throughout their dissertation. For reference management, Refworks can be downloaded from the ICTS or UCT library websites.

It is suggested that candidates look at previous examples of Master's dissertations in the library for appealing layouts. Master's dissertations are available in the Health Sciences Library. A search will need to be done to obtain a list of titles and authors. This search can be done using search words (e.g. dissertation, health, health sciences, etc.). The librarian should be asked for assistance. Some of these dissertations are available online at http://srvrhldig001.uct.ac.za/R/R3CAKV8FM3PHV23A363D7J4F947AN4AXGRBTHIPM2L62RSUXD M-02943?func=collections&collection_id=1526 but this site does not yet differentiate MMed, MPhil and MSc dissertations within the faculty of Health Sciences, so candidates will have to open each dissertation to identify whether it is relevant to their minor dissertation.

Submission of dissertations

On completion, the dissertation should be submitted to the Faculty Postgraduate Office. The candidate should inform the Faculty Officer one month in advance of the intention to submit, using **Form D8 (Intention to submit)**. Supervisors will be requested by the Faculty Postgraduate Officer to submit a letter supporting submission, and clearly specifying whether the dissertation will be submitted in a "Publication-ready" or "Monograph" format, so that the appropriate instructions are sent to the examiners. This letter should be supplied by the primary supervisor. If this supervisor is external, the internal supervisor must be kept informed at every stage of the process.

The candidate must submit 2 copies of the dissertation, in temporary binding (e.g. plastic ring) and an electronic copy in a universally readable format (e.g. pdf) on a compact disc. The candidate must **clearly** state which of the formats has been chosen ("Publication-ready" or "Monograph"), so that the appropriate instructions are sent to the examiners. Specific submission requirements may be set by individual disciplines or constituent Colleges of the CMSA, and registrars are obliged to ensure that their research projects and dissertations meet these specific requirements.

UCT Dissertation Submission deadlines:

1. March 15th for June graduation
2. August 15th for December graduation

Note on fees: To avoid attracting fees, dissertations need to be submitted before the beginning of the first quarter (first day of academic year), and before the start of the second semester (mid July) to qualify for a 50% fee rebate.

Examiners

The full dissertation will be submitted for examination through the Postgraduate Office of our Faculty to two external examiners (nominated by the supervisors and HOD).

It is the supervisors' responsibility to submit names of three potential examiners to the Faculty Officer when the candidate is ready to submit. Of the three examiners nominated, two are invited to examine, and one is held as an alternate. All examiners must all be external to UCT, and appointment of examiners from outside South Africa is encouraged. These nominations need to be approved by the Deputy Dean: Postgraduate Affairs on behalf of the Faculty Board and submitted to the Faculty Board for ratification via a Dean's Circular.

The examiners will be well briefed regarding the specific requirements and criteria for submission and examination of the minor dissertation. Such criteria will clearly explain the difference between the minor dissertation and a Master's degree by dissertation alone, and between the monograph and the "publication-ready" format of dissertation.

Details required for each examiner are: academic qualifications, postal and/or physical address, telephone and fax numbers and e-mail address, and one paragraph description of their standing in the relevant field (drawn from their CV if need be.)

The candidate may not be informed of the identity of the examiners. After the outcome of the minor dissertation has been finalised, the examiners' identities are made known if the examiners have indicated that they do not object to this.