Can cytoplasmic nucleophosmin be detected by immunocytochemical staining of cell smears in acute myeloid leukemia?

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ABSTRACT

Mutations in the C-terminal region of nucleophosmin in acute myeloid leukemia (AML) result in aberrant cytoplasmic nucleophosmin (cNPM) in leukemic blast cells which is detectable by immunocytochemistry in bone marrow trephine (BMT) biopsy sections. We tested whether cNPM is detectable by immunocytochemistry in air-dried smears of AML with *nucleophosmin1* (*NPM1*) mutations. An immuno-alkaline phosphatase method was developed using the OCI-AML3 cell line, known to have mutated *NPM1*, and assessed on blood and marrow smears of 60 AML cases. NPM was detectable in all blast cell nucleoli and cNPM in 21 of 31 of *NPM1* mutated and 15 of 29 wild-type cases. Paired air-dried smears and BMT biopsies from the same case (mutated and wild-type) gave discrepancies in cNPM expression and there was no correlation in 10 of 22 cases. Due to the high false positive and negative rates for cNPM in cell smears, this method should not be used as a surrogate for *NPM1* mutations in AML.

Key words: nucleophosmin, AML, blood smears, OCI-AML3, mutation.

Citation: Mattsson G, Turner SH, Cordell J, Ferguson DJP, Schuh A, Grimwade LF, Bench AJ, Weinberg OK, Marafioti T, George TI, Arber DA, Erber WN, and Mason DY. Can cytoplasmic nucleophosmin be detected by immunocytochemical staining of cell smears in acute myeloid leukemia? Haematologica. 2010;95:670-673. doi:10.3324/haematol.2009.011817

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Introduction

Approximately one-third of cases of *de novo* acute myeloid leukemia (AML) carry mutations in the C-terminal region of the nucleophosmin (*NPM1*) gene.[1-2] AML with mutated *NPM1* shows distinctive biological and clinical features, including female gender, monocytoid morphology, CD34-negativity and a unique gene expression profile.[3] These features are irrespective of whether NPM1-mutated AML carries a normal karyotype (about 85% of cases) or secondary chromosomal aberrations (about 15%), thus reinforcing the view that *NPM1* mutation is a founder genetic lesion.[4] In the absence of *FLT3*-ITD mutations, AML with a normal karyotype and *NPM1* mutation confers a significantly better prognosis than other AML cases with a normal karyotype.[5-8] A recent study indicates that these prognostic considerations also apply to *NPM1*-mutated AML patients carrying secondary chromosomal aberrations.[4] Frame-shift *NPM1* mutations create a new leucine-rich sequence at the protein's C-terminus which serves as a nuclear export signal. This, accompanied by loss of tryptophan residues, is responsible for the increased nuclear export and aberrant cytoplasmic accumulation of the *NPM1* leukemic mutants.[9]

NPM1 mutations were initially detected following the observation that in B5-fixed/EDTA decalcified bone marrow trephine (BMT) biopsies, NPM protein was aberrantly expressed in the cytoplasm of leukemic cells of about one-third of AML patients.[1] This anomalous staining pattern is closely correlated with the presence of mutated *NPM1*.[9] As BMT biopsies are not always performed at diagnosis of AML, the ability to detect cNPM on cell smears would be advantageous. We have established a technique to assess NPM expression in routine hematologic smear samples and assessed its correlation with *NPM1* mutation status.

Design and Methods

Samples

Cell lines FL18 and L428 were cultured with recommended media and OCI-AML3 (DSMZ, Braunschweig, Germany) as described else-

Acknowledgments: we are indebted to Dr Ioannis Kakkas, Dr Andrew Lister, and Dr Jude Fitzgibbon for providing AML blood samples. We are grateful to Bridget Watson for her expert help in the preparation of this manuscript and Dr Brunangelo Falini for valuable comments and suggestions when preparing this manuscript. The authors regret the untimely and sad death of Professor David Y Mason during the preparation of this manuscript. He is missed by us all. Funding: this work was supported by a Project Grant (N. 06077) and a Programme Grant (N. 04061) from Leukaemia Research; and by the European Commission under the Marie Curie Programme Transfer of Knowledge (MTKI-CT-2004-014555). The work was carried out in the Leukaemia Research Immunodiagnostics Unit, Nuffield Department of Clinical Laboratory Sciences, University of Oxford, John Radcliffe Hospital, Oxford, UK.
Manuscript received on May 27, 2009. Revised version arrived on September 18, 2009. Manuscript accepted on September 21, 2009.
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where.[10] OCI-AML3 were diluted into normal whole blood at approximately 4-5×10^{6} cells/mL.

Blood and bone marrow smears (n=45) from patients with AML were obtained from John Radcliffe Hospital, Oxford, UK, Addenbrooke's Hospital, Cambridge, UK, and Stanford University, Stanford, USA. BMT sections were obtained from 22 of these patients. Frozen mononuclear cell samples from AML cases (n=15) were obtained from St. Bartholomew's Hospital, London, UK. Blood samples from a patient with chronic lymphocytic leukemia (CLL) and healthy normal volunteer donors were obtained from John Radcliffe Hospital, Oxford, UK. Conventional smears were made.

All experiments had institutional ethics committee approval from the University of Oxford and all samples were collected from individuals who had given consent.

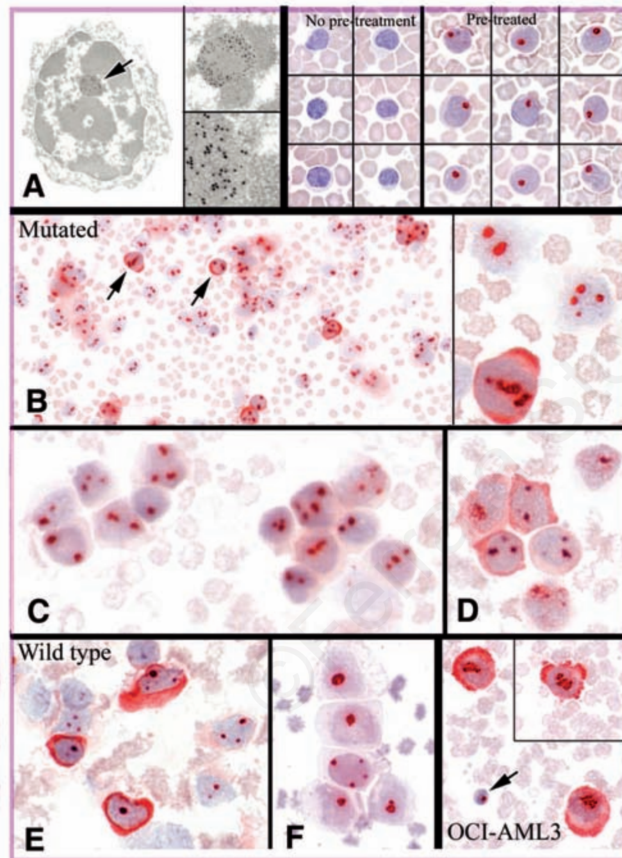

Figure 1. (A) Immunoelectron microscopy images showing nucleolar NPM (Clone 376) in a lymphocyte (arrow), also shown at higher magnification, then a range of leukocytes from normal volunteer donors stained with NPM (Clone 376) with no pre-treatment and after pre-treatment (positive nucleoli staining shown in red). (B) A case of AML with *NPM1* mutation showing NPM immunostaining (red, Clone 376) (arrows indicate blast cells with cytoplasmic staining) and at higher magnification. (C-D) Two cases of AML with *NPM1* mutated showing nucleolar NPM immunostaining (red, Clone 376) with both positive and negative cytoplasmic labeling. (E-F) Two cases of AML with *NPM1* wild-type stained for NPM (red, Clone 376), both with positive nucleoli. (E) Also shows positive cytoplasmic labeling. Then OCI-AML3 cell line (mixed with normal blood) shows positive NPM immunostaining (red, Clone 376) in the nucleoli and cytoplasm of the OCI-AML3 cell line (the arrow indicates a stained normal white blood cell).

Fixation and decalcification

Cell smear fixation: acetone (ten minutes); acetone/methanol (50:50 v/v; three minutes); ethanol/acetic acid (95:5 v/v; ten minutes); 4% buffered formalin (three minutes); acetone/methanol/formalin (19:19:2; ten minutes); and buffered formaldehyde-acetone (three minutes).

BMT biopsies were processed into paraffin by fixation in neutral buffered formalin for six hours and decalcification in EDTA for 24 hours, or fixation in Bouin's solution for six hours and acid decalcification in Rapid Decal (American Master Tech Scientific, USA) for 30-60 minutes.

Antibodies and immunocytochemical staining

Mouse anti-NPM antibodies NA24 (author's laboratory; DYM)[11] and Clone 376 (Dako A/S, Glostrup, Denmark) were used.[1,9,11-13] Fixed air-dried cell smears underwent pre-treatment in a pressure cooker with Target retrieval solution, pH9 (Dako A/S), rinsed, incubated with an anti-NPM antibody for 30 minutes, stained by the “enhanced” APAAP-technique and Fast red substrate.[14]

BMT sections (3-4μm) were dewaxed and stained with the same protocol as for the cell smears or using a BondmaX™ immunostainer and Bond Polymer Refine Detection kit (Leica Biosystems, UK). The protocol included 30 minutes pre-treatment with the Bond Epitope Retrieval Solution 1 (Leica Biosystems), incubation with antibodies NA24 (1:20) and Clone 376 (0.05ng/mL). All cases were reviewed by at least two observers.

Table 1. Cytoplasmic nucleophosmin (cNPM) staining results for the 22 acute myeloid leukemia cases stained in blood (PB) or bone marrow (BM) smears and paired bone marrow trephines (BMT).

NPM1 Status	PB/BM Smears % cNPM positive cells	BMT + = cNPM detected
NPM1 mutated		
1	<1%	+
2	<1%	–
3	<1%	+
4	<1%	+
5	<1%	+
6	0	+
7	0	+
8	0	+
9	0	+
10*	10%	+
11*	0	+
NPM1 wild-type		
12	0	–
13	0	–
14	0	–
15	0	–
16	0	–
17	0	–
18	0	+
19	<1%	–
20	<1%	–
21*	<1%	–
22*	0	–

*Footnote * Cases processed in neutral buffered formalin and EDTA decalcification; nucleophosmin gene (*NPM1*).*

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Images were captured with an Axiocam system and Photoshop processed.[15] A Nikon E800 Eclipse microscope was used with 40x magnification.

Detection of nucleophosmin mutations

Genomic DNA was extracted from blood or bone marrow aspirate samples, and mutations within exon 12 of *NPM1* detected by PCR and fragment analysis.[16] Two protocols were used; the fluorescently labeled forward 5'-(dyeD4)TTCCATACATACTTAAAACCAAGCA-3' and reverse 5'-TGGTTCCTTAACCACATTTCTTT-3' primers were used to amplify exon 12 of *NPM1*.[17] Fluorescent PCR products were subjected to capillary electrophoresis on denaturing polyacrylamide gel before being analyzed. The second protocol was performed with PCR, restriction enzyme detection and capillary electrophoresis.[18] The primers used were NPM1 12F 5'NED/TGTCTATGAAGTGTTGTGGTTCC-3', and NPM1 12R 5'-CTGGACAACATTTATCAAACACG-3'.

Immunoelectron microscopy

Suspensions of FL18, L428, OCI-AML3 and leukocytes from a CLL case were fixed overnight in 2% (vol/vol) formaldehyde in 0.1 mol/L phosphate buffer. After washing samples were rapidly dehydrated in ethanol, embedded in LR White resin and processed.[19] Sections were placed on grids, incubated with NA24 (undiluted) or Clone 376 (1:80), and a secondary antibody (10nm gold particles), counterstained and examined in a Joel 1200EX transmission electron microscope (Joel UK Ltd, Welwyn Garden City, UK) at 80 kV.[19]

Results and Discussion

The immunostaining protocol to detect NPM was optimized on smears prepared of normal blood mixed with OCI-AML3 cells, which carries the *NPM1* C-terminal mutation.[20] Optimal morphological preservation and NPM antigenicity was obtained with acetone/methanol/ formalin fixation and pre-treatment. This resulted in cytoplasmic NPM (cNPM) detectable in more than 98% of the OCI-AML3 cells as well as the nucleolus (Figure 1).

Immunoelectron microscopy showed positive labeling in the nucleoli of CLL leukocytes (Figure 1A), OCI-AML3, FL18 and L428 cells (*data not shown*), in line with earlier published electron microscopy results.[21] The nucleolar localization of NPM in cytological samples contrasts with previous reports that demonstrate pan-nuclear expression pattern.[1,11] The OCI-AML3 cell line did not show cNPM by immunoelectron microscopy. We postulate that this difference in localization is due to differences in sample processing, including the acetone/methanol/formalin fixative, or that the cytoplasmic NPM is non-specific. The phenomenon of alterations in staining localization in differently processed tissue samples has been observed for other proteins, e.g. ALK1 which, in fresh tissue, shows predominantly nucleolar labeling, but has a cytoplasmic pattern in formalin-fixed paraffin-embedded material.[22] Alternatively, cytoplasmic localization of NPM may be non-specific (as has been previously reported by Falini *et al.*)[23] in apoptosis or with extensive necrosis.

An initial immunocytochemical analysis of NPM localization was performed of 38 AML cell smear samples, 20 *NPM1* mutated and 18 wild-type. Strong nucleolar staining was seen in all cases with both antibodies and there was no difference between fresh and freeze-thawed samples (Figure 1B-F). cNPM of variable intensity was seen in 15 of 20 (75%) AML cases with mutated *NPM1* (range <1-50%; mean 11%) and 12/18 (67%) wild-type cases (range <1-50%; mean 7%) (Figure 1B-D). No case showed clear cytoplasmic immunostaining of constant intensity in all AML blast cells, as reported for BMT biopsies. These results, therefore, show that in the cell smears cNPM positivity appeared to be independent of *NPM1* mutation status.[1,9]

To determine whether the discrepancies between *NPM1* mutation status and cNPM in the cell smears was by chance, paired smears and BMT biopsies of a further 22 AML cases were analyzed. The results for the smears were as described above with positive staining in the nucleoli and minimal cytoplasmic positivity (Table 1). In the BMT biopsies all cells showed pan-nuclear NPM staining with the strongest signal being within the nucleolus and some cNPM (Figure 2). There was no correlation between the percentage of cNPM positive cells in cell smears and the BMT result. There were 2 BMT cases (Table 1; n. 2 and n. 18) where cNPM and the *NPM1* mutation status did not agree. It is of note that all publications to date that demonstrate a positive correlation between *NPM1* mutations and cNPM by immunocytochemistry on BMT biopsies have used B5 fixation.[1,23] This fixative is rarely used routinely in hematopathology with a recent report showing B5 was only used by 18% of laboratories for BMT biopsies.[24] As different fixatives are known to have different effects on tissue antigens this could explain the 2 "false negative" Bouin-fixed BMT results in the present series.[24]

Overall, for all 60 cell smears assessed, the false positive rate for cNPM compared with *NPM1* genotype was 41.2% (15 of 36 cNPM-positive cases) and false negative rate 41.7% (10 of 24 cNPM-negative cases). We hypothesize that the discrepancies are due to the sample type and methodological differences in processing and are not case-specific. Specifically, BMT have undergone fixation, decalcification and processing into paraffin, whereas cell smears have been air-dried and fixed immediately prior to

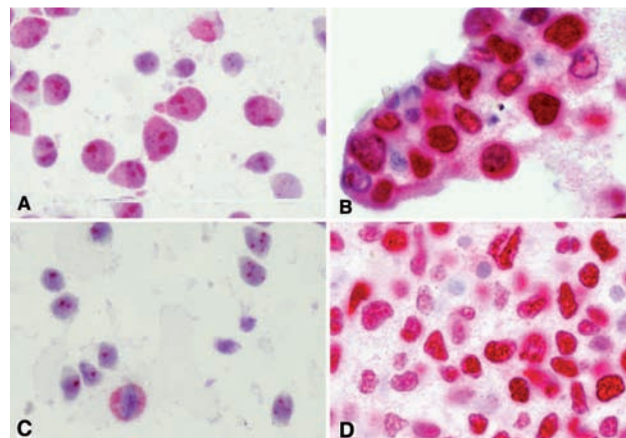

Figure 2. The case numbers correspond to Table 1. (A-B) AML with *NPM1* mutation (case 1): (A) is the cell smear (NA24) and (B) the corresponding bone marrow trephine with cytoplasmic and nuclear labeling (clone 376). (C-D) AML NPM1 wild-type (case 20). (C) The cell smear with positive nucleoli and one cell with cytoplasmic labeling. (D) The corresponding bone marrow trephine with nuclear labeling (Clone 376).

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immunocytochemical analysis. Furthermore, in smears the cell is intact and the entire cytoplasm is visible and can easily be distinguished from the nucleus (Figure 1). This is relevant, as a morphological characteristic of blast cells in AML is that they have minimal cytoplasm and a high nuclear to cytoplasmic ratio. The small size of some blast cells and minimal cytoplasm is acknowledged as one of the problems encountered in the interpretation of the subcellular localization of NPM in BMT biopsies.[23]

We have developed a method capable of detecting cNPM in cell smears by immunocytochemistry, but the staining results bear no correlation with *NPM1* mutation status. We therefore recommend not to use this method as a surrogate marker for *NPM1* mutations. B5-fixed/EDTA decalcified BMT biopsies, therefore, remain the only suitable specimen for the immunocytochemical detection of cNPM. Flow cytometric methods may prove to be a useful alternative for assessing blood and bone marrow aspirate samples.[25]

Authorship and Disclosures

GM performed research, analyzed data and wrote the paper. JC created one of the antibodies. DJPF carried out all electron microscopy research and edited the manuscript. SHT, AS and AJB collected valuable samples and performed research. LFG, OKW, TM, TIG and DAA analyzed data and edited the manuscript. WNE and DYM directed the research, analyzed data and wrote the paper. No potential conflicts of interests relevant to this article were reported.

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