Cytoplasmic mutated nucleophosmin is stable in primary leukemic cells and in a xenotransplant model of NPMc+ acute myeloid leukemia in SCID mice

Brunangelo Falini,[1] Maria Paola Martelli,[1] Cristina Mecucci,[1] Arcangelo Liso,[2] Niccolò Bolli,[1] Barbara Bigerna,[1] Alessandra Pucciarini,[1] Stefano Pileri,[3] Giovanna Meloni,[4] Massimo F. Martelli,[1] Torsten Haferlach,[5] and Susanne Schnittger[5]

[1]Institute of Hematology, University of Perugia, Perugia, Italy; [2]Institute of Hematology, University of Foggia, Foggia, Italy; [3]Hematopathology Section, Policlinico S. Orsola, University of Bologna, Bologna, Italy; [4]Institute of Hematology, University "La Sapienza", Rome; [5]Munich Leukemia Laboratory GmbH, Munich, Germany

ABSTRACT

We investigated the *NPM1* mutation status or subcellular expression of NPM protein (nuclear *vs.* aberrant cytoplasmic) at diagnosis and relapse in 125 patients with acute myeloid leukemia from Italy and Germany. All 52 patients with acute myeloidleukemia carrying at diagnosis mutated or cytoplasmic NPM (NPMc+ acute myeloid leukemia) retained this feature at relapse. Notably, cytoplasmic mutated NPM has now been retained for eight years in a xenotransplant model of NPMc+ acute myeloid leukemia in immunodeficient mice. None of 73 acute myeloid leukemia patients carrying at diagnosis wild-type *NPM1* gene or showing at immunohistochemistry nucleus-restricted expression of nucleophosmin (NPMc– acute myeloid leukemia), which is predictive of *NPM1* gene in germline configuration, acquired cytoplasmic mutated NPM at relapse. This finding further confirms that NPMc+ acute myeloid leukemia represents a primary event rather than a transformation stage of NPMc– acute myeloid leukemia. The stability of cytoplasmic mutated NPM in patients with acute myeloid leukemia, even at relapse in extramedullary sites, and in a xenotransplant model, suggest this event is crucial for leukemogenesis and represents the rationale for monitoring minimal residual disease and molecular targeted therapy in NPMc+ acute myeloid leukemia.

Key words: acute myeloid leukemia, nucleophosmin, *NPM*, mutations, antibodies, immunohistochemistry.

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Introduction

Nucleophosmin (*NPM1*) gene mutations represent the most common genetic alteration in adult acute myeloid leukemia (AML), accounting for about one-third of cases and 50-60% of AML with normal karyotype.[1] AML carrying *NPM1* mutations is characterized by aberrant cytoplasmic expression of nucleophosmin in the cytoplasm of leukemic cells[1,2] (hence the term NPMc+ AML), and shows distinctive biological, clinical and prognostic features,[3,4] as well as a unique gene expression[5] and microRNA[6] profile. Over the past two years, much information has become available on the molecular heterogeneity of *NPM1* mutations (with identification of about 40 variants to date),[3] and the structural and functional properties of encoded NPM leukemic mutants in AML at diagnosis.[3,4] However, little is known about *NPM1* gene status during the course of AML.[7,8] Data on *NPM1* gene mutation status during disease mainly come from Asian AML patients[7,8] and, because of ethnic differences, may not be suitable for extrapolation to AML in Western countries. The recent report of *NPM1* stability in AML samples from Italy[9] does not conclusively answer this question, because of the small number of patients investigated. Furthermore, none of these studies provided information on the status of nucleophosmin expression and nucleo-cytoplasmic transport during the course of AML.

To address these issues, after selecting a series of 125 AML from Germany and Italy as representative of patients from Western countries, we investigated *NPM1* mutation status and subcellular NPM expression at diagnosis and relapse, using either molecular analysis or immunohistochemistry. We also used a xenotransplant model of NPMc+ AML in immunodeficient mice to investigate the status of the NPM1 gene and protein over an extensive period of time.

Design and Methods

Leukemic samples

Bone marrow biopsies for immunohistochemical studies at diagnosis and relapse were obtained from 47 AML patients followed at the Institutes of Hematology, Universities of Perugia and Rome ("La Sapienza"). *NPM1* mutation status was analyzed

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Correspondence: Brunangelo Falini, MD, Institute of Hematology, University of Perugia, Perugia, Italy. E-mail: faliniem@unipg.it

at diagnosis and relapse in bone marrow leukemic cells from 78 AML patients collected at the Munich Leukemia Laboratory (MLL). All AML cases from Munich carried a normal karyotype and this allowed us to investigate the status of *NPM1* in the context of this cytogenetic category. Cytogenetics and/or molecular analysis for the major recurrent genetic abnormalities were available in 33 out of 47 AML patients from Perugia and Rome (17 out of 23 *NPM1*-mutated and 16 out of 24 *NPM1*-unmutated). Sixteen out of the 17 *NPM1*-mutated AML cases showed a normal karyotype and one case carried a secondary chromosomal aberration (+8). Three out of the 16 NPM1-unmutated AML cases carried a normal karyotype while the remaining 13 cases showed the following chromosomal abnormalities: t(8;21)/*AML1-ETO* (n=2); t(15;17)/*PML-RARα* (n=2); t(6;11) (n=1); t(6;9)/*DEK-CAN* (n=1); t(1;3) (n=1); t(3;5) (q21;q31)/*NPM-MLF1* (n=1); del(9q) (n=1); monosomy 7 (n=2); iso17q (n=1) and a complex karyotype (n=1).

Immunohistochemical studies

NPM subcellular expression was detected in paraffin-sections from B5-fixed/EDTA decalcified bone marrow trephines or formalin-fixed extramedullary bioptic samples (n=5 myeloid sarcomas) using anti-NPM specific antibodies and the highly sensitive alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, as previously described.[1] The rationale for this approach derives from our previous observations that immunohistochemistry is fully predictive of NPM1 mutation status.[10] In fact, cases of AML with aberrant NPM cytoplasmic expression (NPMc$^+$) always carry *NPM1* mutations, while cases showing nucleus-restricted NPM positivity (NPMc$^-$ AML) consistently harbor a wild-type *NPM1* gene.

Mutational analysis of the NPM1 gene

Mononucleated cells were isolated by standard Ficoll-Hypaque density gradient centrifugation. Nucleic acid isolation, cDNA synthesis and screening for *NPM1* gene mutations were performed using a melting curve-based LightCycler assay, as previously described.[11] AML samples with an aberrant melting curve underwent nucleotide sequence analysis.

Western blot analysis

NPM mutant protein expression was detected by Western Blot analysis on whole cell lysates of NPMc$^+$ AML cells from xenotransplanted mice (see below) using an affinity-purified rabbit polyclonal antibody (anti-NPMm, Sil-A), which specifically recognizes the NPM leukemic mutant protein, as previously described.[12]

Establishment of human NPMc$^+$ AML xenografts in immunodeficient mice

Xenotransplant of NPMc$^+$ AML in immunodeficient mice was established from the leukemic cells of a 36-year-old female (*M.A.*) with AML-M4 carrying cytoplasmic mutated NPM (mutation A). Besides *NPM1* mutation, the leukemic cells harbored *FLT3* internal tandem duplication (*FLT3*-ITD). The disease was resistant to treatment, even after a haploidentical peripheral blood stem cell transplant performed at the Hematology Institute, Perugia University, in April 1999. When the patient relapsed (July 1999), bone marrow leukemic cells were isolated by standard Ficoll-Hypaque density gradient centrifugation and used to inject mice. Five 4-6 week old C.B-17 SCID/SCID non-irradiated mice were injected subcutaneously in the flank with between 50 and 100×10^6 NPMc$^+$ leukemic cells. Injected cells produced palpable tumors in all mice by 3-4 weeks. The presence of cytoplasmic mutated NPM at the first and second passage proved that xenograft tumors were derived from the patient's leukemic sample. To propagate xenografts, tumors were removed, minced in Dulbecco's phosphate buffered saline (D-PBS) and re-injected subcutaneously into immunodeficient mice. The *NPM1* gene and protein status of xenografted NPMc$^+$ AML tumors was investigated at established time-points using molecular techniques, immunohistochemistry and Western blot analysis.

Results and Discussion

The results of immunohistochemical studies on bone marrow and extramedullary site biopsies in Italian AML patients are shown in Table 1 and Figure 1. All 24 NPMc$^+$ AML cases with aberrant cytoplasmic NPM expression at diagnosis retained this abnormal staining pattern at relapse (Figure 1). The percentage of NPMc$^+$ leukemic cells and the intensity of NPM cytoplasmic positivity were usually the same at first diagnosis and relapse (*data not shown*).

In cases of early relapse, small clusters of re-emerging NPMc$^+$ leukemic blasts co-existed with a population of normal hemopoietic cells showing nucleus-restricted NPM expression (Figure 1, top left and right). Conversely, none of the 24 AML cases with nucleus-restricted NPM positivity (which is predictive of *NPM1* gene in a germline configuration) at diagnosis showed cytoplasmic NPM expression at relapse (*data not shown*). All AML relapsing as myeloid sarcoma retained, even in extranodal sites, the same NPM staining pattern as at diagnosis (nucleus-restricted or cytoplasmic) (Figure 1, bottom left and right). Molecular investigations into *NPM1* mutation status at diagnosis and relapse in German patients with AML provided identical results (Table 1). At relapse, all cases showed the same *NPM1* mutation as found at diagnosis.

The time lapse between diagnosis and relapse in AML is usually very short (median one year), which raises the question of whether patients were observed for enough time to detect changes in *NPM1* mutation status and NPM protein expression. Our xenotransplant model of NPMc$^+$ AML in SCID mice provides evidence of *NPM1* mutation stability and aberrant cytoplasmic expression of NPM over time, and indicates they are intrinsic features of the leukemic process. At present (2007), after 53 passages, NPMc$^+$ AML cells from patient M.A. (Figure 2, top left) that were xenografted into SCID mice in 1999 still maintain their original features. Engraftment occurs in 100% of animals and subcutaneous tumors develop in 3-4 weeks, usually remaining outside the abdominal fascia (*data not shown*). The morphology and phenotype (expression of myeloperoxidase and macrophage-

restricted CD68) of the subcutaneously growing leukemic cells appear the same as the patient's myelomonocytic blasts (Figure 2, top left). Most importantly, they show the same biological features as the patient's leukemic cells, i.e i) aberrant cytoplasmic expression of NPM (Figure 2, middle left), but nucleus-restricted positivity for C23/nucleolin (Figure 2, middle right); and ii) presence of a mutated NPM protein (Figure 2, bottom) and *NPM1* mutation A (*data not shown*).

Evidence that the stability of mutated *NPM1* gene and protein persists over a long period of time also comes from other sources. We previously reported a case of NPMc$^+$ myeloid sarcoma 20 years after diagnosis of AML.[13] Furthermore, the OCI-AML3 cell line, which was generated in 1989 from a 57 year-old male with AML FAB-M4 (karyotype not established),[14] harbors two distinguishing characteristics of NPMc$^+$ AML., i.e. *NPM1* mutation A and aberrant cytoplasmic expression of NPM.[12]

The results presented in this paper have important bio-

Table 1. Cytoplasmic mutated nucleophosmin in 125 cases of acute myeloid leukemia at diagnosis and relapse.

	NPM mutations/expression#		
AML (N.)	*Diagnosis*	*Relapse 1*	*Relapse 2*
Perugia			
20*	Nuclear	Nuclear	
3	Nuclear	Nuclear	Nuclear
23°	Cytoplasmic	Cytoplasmic	
1	Cytoplasmic	Cytoplasmic	Cytoplasmic
Munich			
41	Unmutated	Unmutated	
9	Unmutated	Unmutated	Unmutated
26	Mutated	Mutated	
2	Mutated	Mutated	Mutated

**In 2 cases relapse was at bone marrow and extramedullary sites; °in 3 cases relapse was at bone marrow and extramedullary sites; #nuclear expression of NPM indicates unmutated NPM1, cytoplasmic expression of NPM indicates mutated NPM1.*

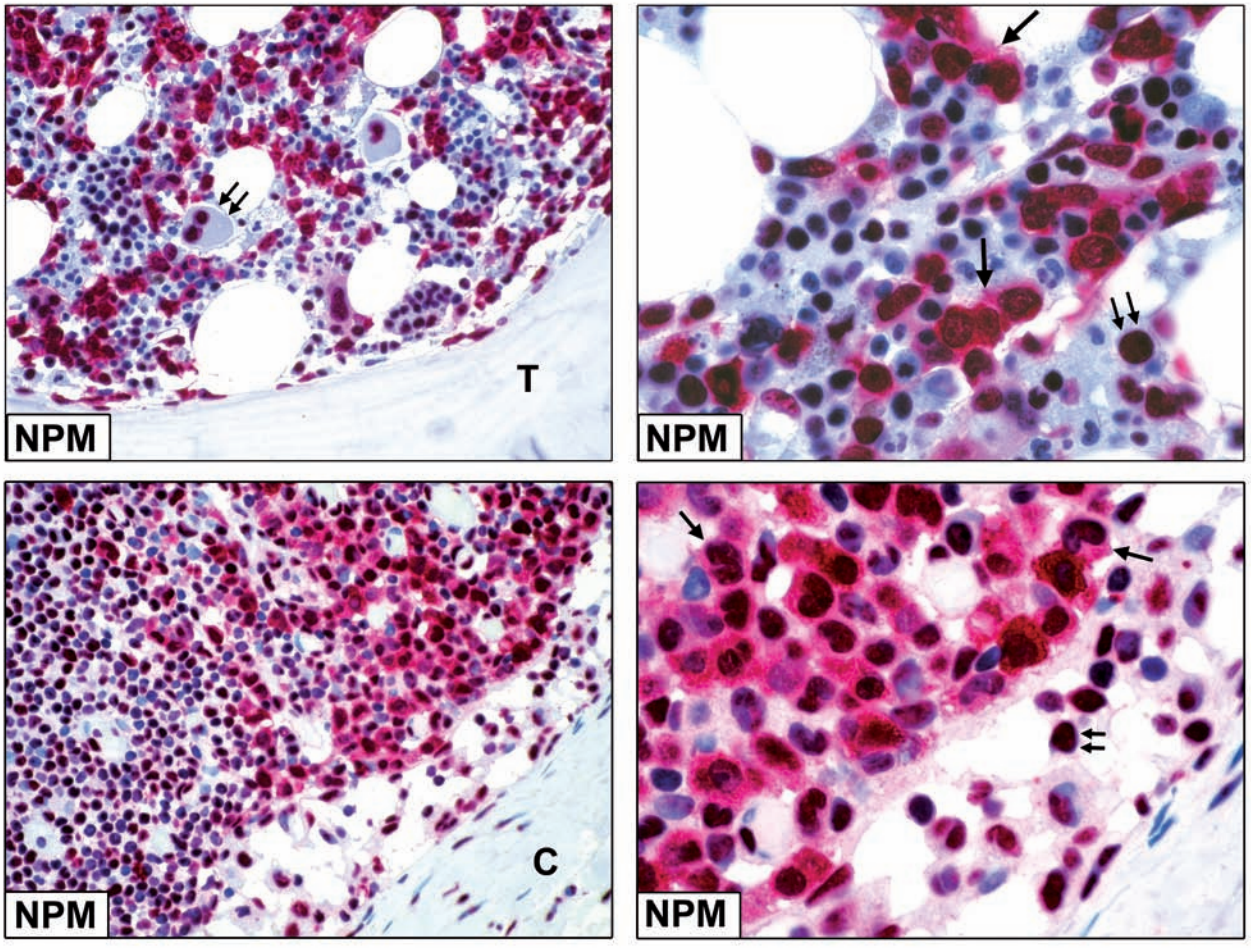

Figure 1. Medullary and extramedullary relapse of NPMc$^+$ AML. Top, left. Early bone marrow relapse by NPMc$^+$ AML. Clusters of NPM cytoplasmic positive blasts co-exist with normal hemopoietic cells with nucleus-restricted positivity (double arrows point to a normal megakaryocyte); T indicates a bone trabecula (Bone marrow trephine; APAAP technique; ×400). Top right. Higher magnification from a different field of the bone marrow biopsy. The single arrows indicate AML cells with aberrant cytoplasmic expression of NPM, while the double arrows point to a normal hemopoietic cell (Bone marrow trephine; paraffin section; APAAP technique; ×800). Bottom, left. Partial infiltration of the lymph node by myeloid leukemic cells showing aberrant cytoplasmic expression of nucleophosmin (upper right area). Residual lymphoid elements exhibit the expected nucleus-restricted NPM positivity (lower left area). C. Indicates the lymph node capsule (lymph node paraffin section; APAAP technique; ×400). Bottom, right. Higher magnification from a different field of the lymph node showing aberrant expression of NPM in the cytoplasm of leukemic cells (arrows). The double arrows point to a residual lymphoid cell with NPM positivity restricted to the nucleus (lymph node paraffin section; APAAP technique; ×800).

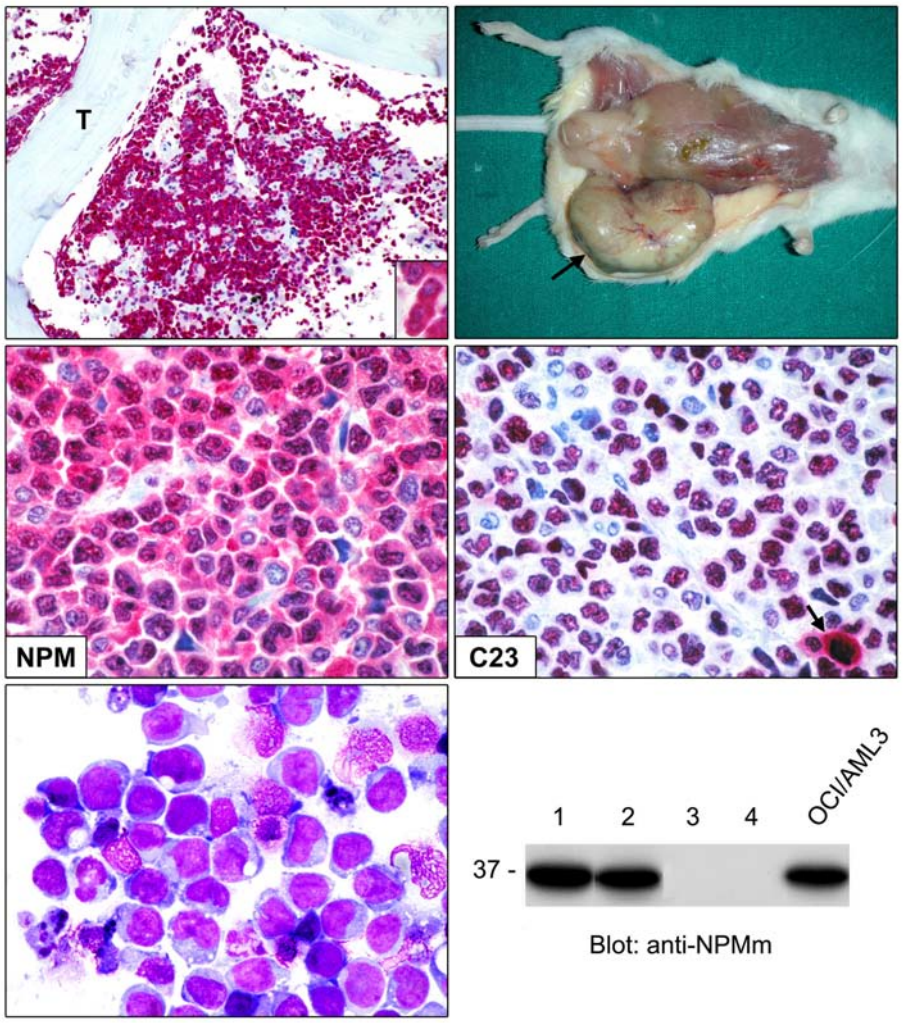

Figure 2. Xenotransplantation model of NPMc$^+$ AML in SCID mice. Top, left. Bone marrow biopsy from patient M.A. at the time of relapse (June 1999). Leukemic cells show aberrant cytoplasmic expression of nucleophosmin which is indicative of *NPM1* mutation (APAAP technique; ×200); T indicates a bone trabecula. Inset: leukemic cells at higher magnification (×800). Top, right. Leukemic cells from the NPMc$^+$ AML patient M.A. growing as subcutaneous mass in the mouse right flank (arrow). The tumor cells show aberrant cytoplasmic expression of NPM (middle, left) and nucleus-restricted positivity for C23/nucleolin (middle, right) (paraffin sections from a tumor mass at the 53rd passage in mice; ×800); the arrow in panel middle-right indicates a mitotic figure. Bottom, left. Leukemic cells from the mass at the 53rd passage in mice showing a myelo monocytic appearance (imprint of the mass; May-Grünwald-Glemsa; ×800). Bottom, right. Western blotting showing the presence of a mutated NPM protein in lysates from leukemic cells at the 53rd passage in mice. A clear band at 37 kDa molecular weight corresponding to NPM mutant protein is evident in whole cell lysate from mice tumor mass cells (lane 1). The OCI/AML3 cell line and an NPMc$^+$ AML patient (lane 2) are included here as positive controls for expression of NPM mutant protein. Negative controls are represented by cells from two NPMc$^-$ AML patients (lanes 3 and 4).

logical and clinical implications. The inability of AML with a normal karyotype to transform from a NPMc$^-$ to a NPMc$^+$ status reinforces the concept that NPMc$^+$ AML represents a clearly distinct entity and it is not a transformation stage of NPMc$^-$ AML. This was also shown by gene expression profile analysis.[5,15] This finding, together with the observation that cytoplasmic mutated NPM is stable in AML patients and in a xenotransplant model of NPMc$^+$ AML, suggests *NPM1* mutations are a founder genetic lesion in AML. This view is also in agreement with previous findings that *NPM1* mutations are mutually exclusive with other recurrent genetic abnormalities.[16] In addition, our immunohistochemical studies show that, during disease evolution, *NPM1* mutated genes efficiently continue to encode NPM mutated proteins which are aberrantly exported from nucleus to cytoplasm. Accumulation of NPM mutants in leukemic cell cytoplasm at relapse, even at extramedullary sites, strongly suggests cytoplasmic dislocation plays a crucial role in leukemogenesis. This view is also supported by our findings that *NPM1* mutations aim to achieve massive exportation of leukemic mutants from the nucleus (with consequent accumulation in the cytoplasm) by using different strength C-terminus NES motifs.[17] Notably, this phenomenon also occurs with very rare mutations that involve exon-9[18] and exon-11[19-21] of the *NPM1* gene. Furthermore, we recently showed that, in human genome, the generation of a nuclear export signal through duplication appears unique to *NPM1* mutations and is restricted to AML.[22]

Demonstrating that *NPM1* mutations are stable in AML patients not only from Asia but also from Western countries provides a rationale for widespread minimal residual disease monitoring in NPMc$^+$ AML by means of quantitative PCR assessment of *NPM1* mutant transcripts.[23] Results appeared predictive of early relapse and long-term prognosis in patients from Taiwan,[24] and preliminary observations from the Munich Leukemia Laboratory indicate the approach is feasible in patients from Western countries (*Schnittger S. et al., manuscript in preparation*).

Finally, finding that leukemic cells exhibit aberrant NPM cytoplasmic expression at all disease stages provides a rationale for developing drugs designed to interfere with this abnormal NPM nucleo-cytoplasmic traffic and redirect the protein to the nucleolus, its physiological site.[25] In this regard, our xenograft model for human NPMc$^+$ AML may, in combination with the OCI-AML3 cell line,[12] serve as a valuable pre-clinical *in vivo* assay for the development of new anti-leukemic drugs.

Authorship and Disclosures

BF designed the study, wrote the paper and analyzed data; MPM and AL were involved in biochemical studies and immunohistochemical analysis; NB, BB and AP were involved in generation and study of the mouse model; SP, MFM, TH and SS performed diagnosis and provided patients' samples. TH and SS performed molecular studies. BF and CM applied for a patent on the clinical use of *NPM1* mutants. The other authors reported no potential conflicts of interest.

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