NPM1 mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities: a comparative analysis of 2562 patients with acute myeloid leukemia

Brunangelo Falini,[1] Cristina Mecucci,[1] Giuseppe Saglio,[2] Francesco Lo Coco,[3] Daniela Diverio,[4] Patrick Brown,[5] Fabrizio Pane,[6] Marco Mancini,[4] Maria Paola Martelli,[1] Stefano Pileri,[7] Torsten Haferlach,[8] Claudia Haferlach,[8] and Susanne Schnittger[8]

[1]Institute of Hematology, University of Perugia, Perugia, Italy; [2]Department of Clinical and Biological Sciences, University of Turin, San Luigi Hospital, Orbassano, Turin, Italy; [3]Department of Biopathology and Diagnostic Imaging, Policlinico Tor Vergata, Rome, Italy; [4]Department of Cellular Biotechnologies and Hematology, Policlinico "La Sapienza", Rome, Italy; [5]Sidney Kimmel Comprehensive Cancer Center at Johns Hopkins, Baltimore, USA; [6]Department of Biochemistry and Medical Biotechnologies, University "Federico II", Neples, Italy; [7]Laboratory of Hematopathology, University of Bologna, Policlinico S. Orsola, Bologna, Italy and [8]Munich Leukemia Laboratory GmbH, Munich, Germany

ABSTRACT

Acute myeloid leukemia carrying *NPM1* mutations and cytoplasmic nucleophosmin (NPMc+ acute myeloid leukemia) represents one-third of adult *AML* (50-60% of all acute myeloid leukemia with normal karyotype) and shows distinct biological, pathological and clinical features. We confirm in 2562 patients with acute myeloid leukemia our previous observation that *NPM1* mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities. Taken together, these findings make NPMc+ acute myeloid leukemia a good candidate for inclusion in the upcoming World Health Organization classification.

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

Citation: Falini B, Mecucci C, Saglio G, Lo Coco F, Brown P, Diverio D, Pane F, Mancini M, Martelli MP, Pileri S, Haferlach T, Haferlach C, and Schnittger S. NPM1 mutations and cytoplasmic nucleophosmin are mutually exclusive of recurrent genetic abnormalities: a comparative analysis on 2562 patients with acute myeloid leukemia. Haematologica 2008 Mar; 93(3):439-442. doi: 10.3324/haematol.12153

Introduction

One of the most controversial issues in the WHO classification of myeloid malignancies is *acute myeloid leukemias not otherwise characterized*.[1] This category accounts for 60-70% of AML, including cases with normal cytogenetics (AML-NC) which are characterized by a great molecular, pathological and clinical heterogeneity. *AML not otherwise characterized* is currently defined according to FAB criteria which are, however, insufficient to characterize AML-NC, since they do not identify distinct disease entities within this heterogeneous group. Recognition of underlying molecular alterations is crucial for the improvement of AML-NC classification.

We previously identified nucleophosmin (*NPM1*) mutations, causing aberrant cytoplasmic expression of NPM,[2-4] as the most frequent genetic alteration associated with AML-NC (50-60% of cases). *NPM1* mutations and cytoplasmic NPM appear to be mutually exclusive of recurrent genetic abnormalities[2] and identify a leukemia subgroup called NPMc+ (cytoplasmic-positive) AML that show distinct biological, pathological and clinical features,[5,6] as well as a unique gene expression profile.[7] For these reasons, NPMc+ AML (as well as AML with CEBPA mutations that also appear to be mutually exclusive with recurrent genetic abnormalities) is a good candidate for inclusion as a new entity in the forthcoming WHO classification. However, since a few studies[8-11] have reported very rare cases of *NPM1* mutations coinciding with either inv(16), t(8;21) or t(9;22), we extended our analysis to 2562 patients with acute myeloid leukemia.

Design and Methods

Leukemic samples

The aim of the study for AML patients enrolled in the GIMEMA LAM99P and the GIMEMA/EORTC AML12 trials

Acknowledgments: we would like to thank Roberta Pacini and Manola Carini for performing the immunohistochemical stainings and Mrs. Claudia Tibidò for her secretarial assistance. Funding: This work was supported by the Associazione Italiana per la Ricerca sul Cancro (A.I.R.C.).
Manuscript received August 24, 2007. Manuscript accepted December 17, 2007.
Correspondence: Brunangelo Falini, MD, Institute of Hematology, University of Perugia, Perugia, Italy. E-mail: faliniem@unipg.it

was to compare the subcellular expression of NPM with the results of molecular studies for recurrent genetic alterations. The rationale for this approach derives from our previous observations that immunohistochemistry is fully predictive of *NPM1* mutation status.[12] Bone marrow biopsies for immunohistochemical studies were available from 1073 unselected, consecutively observed AML cases, 591 of whom had been previously reported.[2] Ninety-six acute promyelocytic leukemia cases (not included in the GIMEMA LAM 99P and GIMEMA/EORCT-AML12) were also available for comparative immunohistochemical and molecular analysis. In addition, 1403 unselected AML cases were investigated at the Munich Leukemia Laboratory (MLL). Cytogenetics failed in 10 cases and these were excluded from the present study. Therefore, the Munich cohort consisted of 1393 consecutively observed, unselected newly diagnosed AML for which complete cytogenetic/molecular studies were available. In these cases, we compared the results of *NPM1* mutational analysis[13] and cytogenetic/molecular studies. Written informed consents for the study was obtained from each participating center.

Immunohistochemical studies

Immunohistochemistry for NPM was available in 1073 AML cases, 591 of whom had been previously reported.[2] NPM subcellular expression was detected in paraffin-sections from B5-fixed/EDTA decalcified bone marrow trephines using a specific anti-NPM monoclonal antibody (clone 376) and the highly sensitive alkaline phosphatase anti-alkaline phosphatase (APAAP) technique, as previously described.[2] Monoclonal antibody against nucleolin/C23 was purchased from Santa Cruz Biotechnology.

Cytogenetics and molecular studies for recurrent genetic abnormalities

Cytogenetic investigation was performed after short-term culture. Karyotypes were analyzed after G-banding and described according to the International System for Human Cytogenetic Nomenclature.[14] Fluorescence *in situ* hybridization was carried out according to standard techniques. Reverse-transcriptase-polymerase-chain-reaction (RT-PCR) for *AML1-ETO*, *CBFB-MYH11*, *DEK-CAN* and *BCR-ABL,* performed as previously reported,[2] was available in 1019/1073, 1020/1073, 936/1073 and 1015/1073 AML cases respectively. Southern blotting and FISH for rearrangements of the mixed-lineage leukemia gene (*MLL*)[2] was carried out in 728/1073 patients.

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.[13] AML samples with an aberrant melting curve underwent nucleotide sequence analysis.

Results and Discussion

Of the 1073 bone marrow biopsies from patients of the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials, 368 (34.2%) were characterized by aberrant cytoplasmic expression of nucleophosmin (NPMc+) associated with nucleus-restricted positivity for nucleolin (C23) (Figure 1). As previously reported,[12] this staining pattern is fully predictive of a mutated *NPM1* gene. All other biopsies showed a nucleus-restricted positivity for nucleophosmin (NPMc-) which is fully predictive of *NPM1* gene in a germline configuration.[12] Comparison of immunohistochemistry with the results of molecular studies for the recurrent genetic abnormalities is shown in Table 1. Remarkably, all 300 AMLs carrying various types of recurrent genetic abnormalities showed a nucleus-restricted expression of NPM. Representative examples of these staining patterns in AML carrying the most

Table 1. Correlation between recurrent genetic abnormalities and NPM subcellular expression in the GIMEMA/EORTC AML12 trial.

*Genetic alteration**	*(N=)*	*NPMc+*	*NPMc–*
AML1-ETO	67	0/67	67/67
CBFB-MYH11	82	0/82	82/82
*PML-RARA**	95	0/95	95/95
*PLZF-RARA**	1	0/1	1/1
MLL-self fusion	12	0/12	12/12
MLL-Rearr.	14	0/14	14/14
DEK-CAN	14	0/14	14/14
BCR-ABL	15	0/15	15/15
Total	300	0/300	300/300

**Results from the molecular analysis of AML (1073 AML and 96 acute promyelocytic leukemias) for which NPM immunostaining was available (see details in the text). NPMc+: cytoplasmic NPM (fully predictive of mutated NPM1 gene); NPMc-: expected nucleus-restricted positivity for NPM (fully predictive of unmutated NPM1 gene).*

Table 2. Correlation between recurrent genetic abnormalities and *NPM1* mutations in acute myeloid leukemia cases from the Munich Leukemia Laboratory.

*Genetic abnormality**	*(N=)*	*NPM1mut*	*NPM1germ*
t(8;21)/*AML1-ETO*	105	0/105	105/105
inv16/*CBFB-MYH11*	97	0/97	97/97
t(15;17)/*PML-RARA*	20	0/20	20/20
MLL/11q23	19	0/19	19/19
t(6;9)/*DEK-CAN*	5	0/5	5/5
Total	246	0/246	246/246

**Results from the cytogenetic analysis of 1393 AML cases. NPM1mut: NPM1 mutated; NPM1germ: NPM1 in germline configuration.*

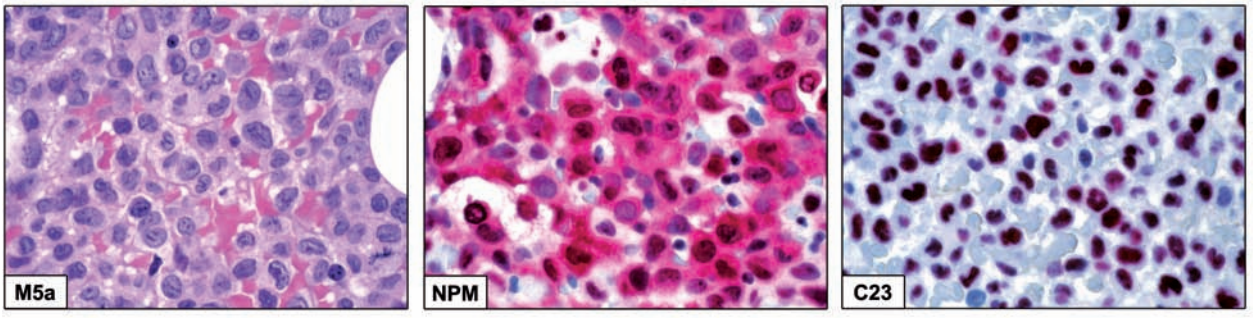

Figure 1. Expression pattern of nucleophosmin in a case of typical NPMc+ AML. Left panel: LAM FAB-M5a showing diffuse infiltration by monoblasts (bone marrow trephine; hematoxylin-eosin; ×800). Middle panel: leukemic cells show aberrant cytoplasmic expression of nucleophosmin (NPM). Bone marrow trephine immunostained with anti-NPM monoclonal antibody (APAAP technique; hematoxylin counterstain; ×800). Right panel: leukemic cells show nucleus-restricted positivity for nucleolin/C23 (bone marrow trephine; APAAP technique; hematoxylin counterstain; ×800).

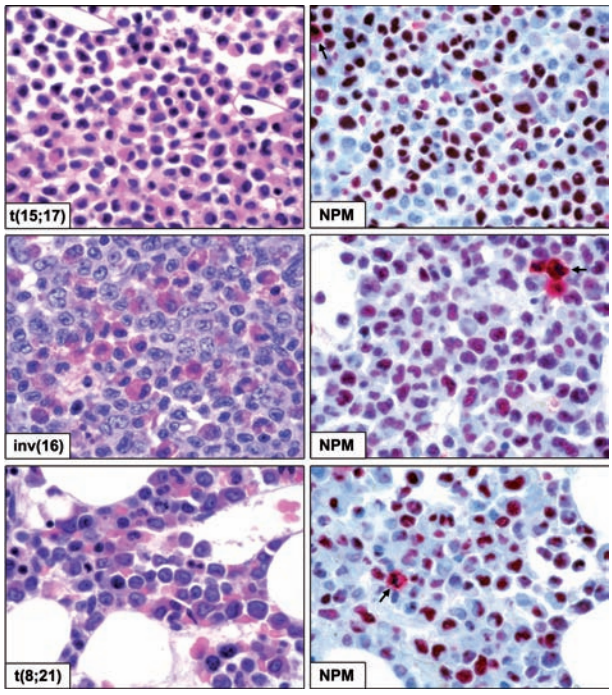

Figure 2. Expression pattern of nucleophosmin (NPM) in AML with recurrent genetic abnormalities. Left panels: typical morphological appearance of acute promyelocytic leukemia with t(15;17) (top), LAM FAB-M4eo with inv(16) (middle) and LAM FAB-M2 with t(8;21) (bottom). All cases are bone marrow trephines (hematoxylin-eosin; ×800). Right panels: leukemic cells in all AML cases with recurrent genetic abnormalities show a nucleus-restricted positivity for nucleophosmin which is indicative of wild-type ***NPM1*** gene. The arrows points to mitotic figures showing the expected cytoplasmic positivity. Bone marrow trephines immunostained with anti-NPM monoclonal antibody (APAAP technique; hematoxylin counterstain; ×800).

frequent recurrent genetic abnormalities, i.e. t(15;17), inv(16) and t(8;21), are shown in Figure 2. Completely overlapping results were obtained in cases from the Munich Leukemia Laboratory, by comparing the mutation status of the *NPM1* gene with the results on cytogenetic/molecular studies for recurrent genetic abnormalities. In total, we investigated 1393 AML cases, 835 of which had a normal and 558 an abnormal karyotype. *NPM1* mutations were detected in 420 out of 1393 patients (30.1%) while the other cases harboured a *NPM1* gene in a germline configuration. In 246 of 1393 patients, reciprocal translocations and the respective fusion genes were detected; RT-PCR and/or FISH were performed for all respective fusion genes. The results are shown in Table 2. Notably, none of the 246 AMLs carrying different types of genetic abnormalities harbored *NPM1* mutations. These results were obtained independently from a total of 2562 AMLs from two large series using different approaches, support our original finding that, cytoplasmic mutated NPM is mutually exclusive of recurrent genetic abnormalities.[2] Our findings raise several issues surrounding the significance of the sporadic cases of *NPM1* mutations coinciding with recurrent genetic abnormalities, as reported in some studies.[8-10] First, none of these studies documented whether *NPM1* mutations and concurrent genetic abnormalities occur in the same or different leukemic cell populations. Another major concern is specificity control in large multi-center trials. This is addressed in Falini B. *et al.*[2] (Supplementary Materials). In particular, 4 patients in the GIMEMA EORTC AML12 trial were diagnosed as having cytoplasmic mutated NPM together with inv(16), t(8;21) or MLL rearrangement. In-depth re-investigation of these cases revealed errors in sample registration or PCR contamination. Because of the presence of *NPM1* mutation in association with a t(16;16)*/MYH11-CBFB* transcript, 1 patient from the CCG-2961 AML pediatric trial was also re-investigated and found not to have a *NPM1* mutation (Patrick Brown, unpublished results). Therefore, in our experience, re-analysis according to strict criteria showed that *NPM1* mutations are mutually exclusive of recurrent genetic abnormalities. Whether the occurrence of two or more specific genetic markers in exceptional cases is just coincidental or represents a true association is still not understood. Rare examples include t(15;17) plus t(8;21),[15] t(15;17) plus t(9;22),[16] and t(9;22) or BCR-ABL plus inv(16).[17,18] Interestingly, 1 case reported by Thiede *et al.*[9] carried both a t(8;21) and an inv(16) in addition to *NPM1* mutations with a *NPM1* mut/wt ratio of only 0.37 (*Christian Thiede, personal communication on February 6,*

2006). More recently, coexistence of *JAK2V617F* and *BCR-ABL* has also been reported in chronic myeloproliferative disorders[19,20] and association of *JAK2V617F* with t(8;21) in AML therapy-related[21] or secondary to a myeloproliferative syndrome.[22] In conclusion, our data unequivocally confirm in a large series of patients with acute myeloid leukemia that *NPM1* mutations are mutually exclusive of other recurrent genetic abnormalities and that *NPM1* mutations identify a distinct acute myeloid leukemia genetic entity which should be considered for inclusion in the upcoming WHO Classification.

Authorship and Disclosures

BF is responsible for the study concept and wrote the manuscript; GS, FLC, DD, FP, CM, MM were involved in the molecular and cytogenetic studies of patients from GIMEMA/ EORTC AML12; MPM and SP were involved in the immunohistochemical study of NPM expression in AML specimens. TH, CH and SS carried out the molecular analysis of cases from the Munich Leukemia Laboratory. BF and CM applied for a patent on the clinical use of *NPM1* mutants.

References

1. Vardiman JW, Harris NL, Brunning RD. The World Health Organization (WHO) classification of the myeloid neoplasms. Blood 2002;100:2292-302.
2. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med 2005; 352:254-66.
3. Falini B, Bolli N, Shan J, Martelli MP, Liso A, Pucciarini A, et al. Both carboxy-terminus NES motif and mutated tryptophan(s) are crucial for aberrant nuclear export of nucleophosmin leukemic mutants in NPMc+ AML. Blood 2006; 107:4514-23.
4. Bolli N, Nicoletti I, De Marco MF, Bigerna B, Pucciarini A, Mannucci R, et al. Born to be exported: COOH-terminal nuclear export signals of different strength ensure cytoplasmic accumulation of nucleophosmin leukemic mutants. Cancer Res 2007; 67:6230-7.
5. Falini B, Nicoletti I, Martelli MF, Mecucci C. Acute myeloid leukemia carrying cytoplasmic/mutated nucleophosmin (NPMc+ AML): biologic and clinical features. Blood 2007; 109: 874-85.
6. Falini B, Nicoletti I, Bolli N, Martelli MP, Liso A, Gorello P, et al. Translocations and mutations involving the nucleophosmin (NPM1) gene in lymphomas and leukemias. Haematologica 2007;92: 519-32.
7. Alcalay M, Tiacci E, Bergomas R, Bigerna B, Venturini E, Minardi SP, et al. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. Blood 2005;106:899-902.
8. Suzuki T, Kiyoi H, Ozeki K, Tomita A, Yamaji S, Suzuki R, et al. Clinical characteristics and prognostic implications of NPM1 mutations in acute myeloid leukemia. Blood 2005;106: 2854-61.
9. Thiede C, Koch S, Creutzig E, Steudel C, Illmer T, Schaich M, et al. Prevalence and prognostic impact of NPM1 mutations in 1485 adult patients with acute myeloid leukemia (AML). Blood 2006;107:4011-20.
10. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hugens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. Blood 2005;106: 3747-54.
11. Palmisano M, Grafone T, Ottaviani E, Testoni N, Baccarani M, Martinelli G. NPM1 mutations are more stable than FLT3 mutations during the course of disease in patients with acute myeloid leukemia. Haematologica 2007;92: 1268-9.
12. Falini B, Martelli MP, Bolli N, Bonasso R, Ghia E, Pallotta MT, et al. Immunohistochemistry predicts nucleophosmin (NPM) mutations in acute myeloid leukemia. Blood 2006; 108:1999-2005.
13. Schnittger S, Schoch C, Kern W, Mecucci C, Tschulik C, Martelli MF, et al. Nucleophosmin gene mutations are predictors of favorable prognosis in acute myelogenous leukemia with a normal karyotype. Blood 2005;106:3733-9.
14. Mitelman F. ISCN 1995: an international system for human cytogenetic nomenclature. Basel, Switzerland; 1995.
15. Bonomi R, Giordano H, del Pilar Moreno M, Bodega E, Landoni AI, Gallagher R, et al. Simultaneous PML/RARalpha and AML1/ETO expression with t(15;17) at onset and relapse with only t(8;21) in an acute promyelocytic leukemia patient. Cancer Genet Cytogenet 2000;123: 41-3.
16. Scolnik MP, Palacios MF, Acevedo SH, Castuma MV, Larripa IB, Palumbo A, et al. Promyelocytic blast crisis of chronic myelogenous leukaemia with translocations (9;22) and (15;17). Leuk Lymphoma 1998; 31:231-6.
17. Mecucci C, Noens L, Aventin A, Testoni N, Van den Berghe H. Philadelphia-positive acute myelomonocytic leukemia with inversion of chromosome 16 and eosinobasophils. Am J Hematol 1988;27:69-71.
18. Svaldi M, Lanthaler A, Venturi R, Coser P, Mitterer M. Simultaneous occurrence of bcr-abl and inv16 in a case of M1 acute myeloid leukemia. Leukemia 2001;15:695.
19. Hussein K, Bock O, Seegers A, Flasshove M, Henneke F, Buesche G, et al. Myelofibrosis evolving during imatinib treatment of a chronic myeloproliferative disease with coexisting BCR-ABL translocation and JAK2V617F mutation. Blood 2007;109:4106-7.
20. Kramer A, Reiter A, Kruth J, Erben P, Hochhaus A, Muller M, et al. JAK2-V617F mutation in a patient with Philadelphia-chromosome-positive chronic myeloid leukaemia. Lancet Oncol 2007;8:658-60.
21. Schnittger S, Bacher U, Kern W, Haferlach C, Haferlach T. JAK2 seems to be a typical cooperating mutation in therapy-related t(8;21)/AML1-ETO-positive AML. Leukemia 2007;21:183-4.
22. Schneider F, Bohlander SK, Schneider S, Papadaki C, Kakadyia P, Dufour A, et al. AML1-ETO meets JAK2: clinical evidence for the two hit model of leukemogenesis from a myeloproliferative syndrome progressing to acute myeloid leukemia. Leukemia 2007;21: 2199-201.