ORIGINAL ARTICLE

Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype

Brunangelo Falini, M.D., Cristina Mecucci, M.D., Ph.D., Enrico Tiacci, M.D., Myriam Alcalay, M.D., Ph.D., Roberto Rosati, Ph.D., Laura Pasqualucci, M.D., Roberta La Starza, M.D., Ph.D., Daniela Diverio, M.D., Emanuela Colombo, Ph.D., Antonella Santucci, M.D., Barbara Bigerna, Roberta Pacini, Alessandra Pucciarini, Ph.D., Arcangelo Liso, M.D., Marco Vignetti, M.D., Paola Fazi, M.D., Natalia Meani, Ph.D., Valentina Pettirossi, Ph.D., Giuseppe Saglio, M.D., Franco Mandelli, M.D., Francesco Lo-Coco, M.D., Pier-Giuseppe Pelicci, M.D., Ph.D., and Massimo F. Martelli, M.D., for the GIMEMA Acute Leukemia Working Party*

ABSTRACT

BACKGROUND

Nucleophosmin (NPM), a nucleocytoplasmic shuttling protein with prominent nucleolar localization, regulates the ARF-p53 tumor-suppressor pathway. Translocations involving the *NPM* gene cause cytoplasmic dislocation of the NPM protein.

METHODS

We used immunohistochemical methods to study the subcellular localization of NPM in bone marrow–biopsy specimens from 591 patients with primary acute myelogenous leukemia (AML). We then correlated the presence of cytoplasmic NPM with clinical and biologic features of the disease.

RESULTS

Cytoplasmic NPM was detected in 208 (35.2 percent) of the 591 specimens from patients with primary AML but not in 135 secondary AML specimens or in 980 hematopoietic or extrahematopoietic neoplasms other than AML. It was associated with a wide spectrum of morphologic subtypes of the disease, a normal karyotype, and responsiveness to induction chemotherapy, but not with recurrent genetic abnormalities. There was a high frequency of *FLT3* internal tandem duplications and absence of CD34 and CD133 in AML specimens with a normal karyotype and cytoplasmic dislocation of NPM, but not in those in which the protein was restricted to the nucleus. AML specimens with cytoplasmic NPM carried mutations of the *NPM* gene that were predicted to alter the protein at its C-terminal; this mutant gene caused cytoplasmic localization of NPM in transfected cells.

CONCLUSIONS

Cytoplasmic NPM is a characteristic feature of a large subgroup of patients with AML who have a normal karyotype, *NPM* gene mutations, and responsiveness to induction chemotherapy.

From the Institute of Hematology (B.F., C.M., E.T., R.R., L.P., R.L.S., A.S., B.B., R.P., A.P., M.F.M.) and the Department of Biochemistry and Molecular Biotechnology (V.P.), University of Perugia, Perugia, Italy; the European Institute of Oncology, Milan, Italy (M.A., E.C., N.M., P.-G.P.); the Institute for Cancer Genetics, Columbia University, New York (L.P.); the Institute of Hematology, University La Sapienza, Rome (D.D., F.M.); the Institute of Hematology, University of Foggia, Foggia, Italy (A.L.); the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) Data Center, Rome (M.V., P.F.); the Division of Internal Medicine and Hematology, Ospedale S. Luigi, Orbassano-Turin, Italy (G.S.); and the Department of Biopathology, University of Tor Vergata, Rome (F.L.-C.). Address reprint requests to Dr. Falini at the Institute of Hematology, Policlinico Monteluce, 06122 Perugia, Italy, or at faliniem@unipg.it.

Drs. Tiacci, Alcalay, and Rosati contributed equally to this article.

*The centers and investigators participating in the GIMEMA Study are listed in the Appendix.

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Acute myelogenous leukemia (AML), the most common form of acute leukemia in adults, is a heterogeneous group of diseases that are curable in about 30 percent of cases. Cytogenetic analysis assigns AML to different prognostic groups[1]; some subtypes, such as acute promyelocytic leukemia with a t(15;17) translocation (subtype M3 according to the French–American–British [FAB] classification), are amenable to specific therapies. However, no chromosomal abnormality is visible by conventional karyotyping in 40 to 50 percent of cases. Attempts to stratify such cases on the basis of microarrays of complementary DNA[2,3] have succeeded in associating gene-expression patterns with differences in responses to treatment, but no specific genetic subgroups have emerged from these studies.

Nucleophosmin (NPM), a protein that shuttles between the nucleus and cytoplasm,[4] is most prominent in nucleoli.[5] NPM is a molecular chaperone[6] that may prevent protein aggregation in the nucleolus and regulate the assembly and transport of preribosomal particles through the nuclear membrane.[4] It is also a target of CDK2–cyclin E complexes in centrosome duplication[7] and has been implicated in the regulation of the alternate-reading-frame protein (ARF)–p53 tumor-suppressor pathway.[8-10]

The *NPM* gene is a partner in the chromosomal translocations of leukemias and lymphomas that result in fusion proteins containing only the NPM N-terminal region[11,12] — namely, NPM–anaplastic lymphoma kinase (NPM-ALK),[13] NPM–retinoic acid receptor α (NPM-RARα),[14] and NPM–myeloid leukemia factor 1 (NPM-MLF1).[15] NPM appears to contribute to oncogenesis by activating the oncogenic potential of the fused protein partner (ALK, MLF1, or RARα).[16] Since NPM is thought to have a tumor-suppressor function, perturbations in its movement from the nucleus to the cytoplasm may be critical for malignant transformation. Such changes in the subcellular distribution of NPM and NPM-containing fusion protein can be detected by immunohistochemical methods.[12,17]

In this study, we identified a large subgroup of patients with AML who had cytoplasmic NPM in leukemic blasts, a mutated *NPM* gene, a normal karyotype, and a relatively good response to induction chemotherapy.

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METHODS

TUMOR SAMPLES

Immunohistochemical studies were performed on 1835 paraffin-embedded tumor specimens from the following patients: 591 patients (15 to 60 years of age) with primary AML other than FAB subtype M3 who had been enrolled in Italy either in the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) Leucemia Acuta Mieloide Protocollo begun in 1999 (LAM99P) (316 patients), or in the GIMEMA/European Organization for Research and Treatment of Cancer (GIMEMA/EORTC) AML12 trials (275 patients) (details available from the National Auxiliary Publications Service [NAPS]*); 129 patients with primary AML (including 70 with FAB subtype M3), and 135 with secondary AML who had not been participants in the GIMEMA study (details available from NAPS*); and 980 with hematopoietic and extrahematopoietic neoplasms other than AML. Specimens from 25 patients with AML were investigated by immunohistochemistry, both at diagnosis and at remission.

After written informed consent had been obtained at each participating center, a bone marrow–biopsy specimen from each patient was fixed for 2.5 hours in B5, transferred to a 70 percent alcohol solution, and delivered to the Institute of Hematology at the University of Perugia, Perugia, Italy. The specimens were decalcified and processed for paraffin embedment.

ANTIBODIES

Immunohistochemical studies were performed with the use of monoclonal antibodies against ALK and NPM,[5,11,12] including two new anti-NPM monoclonal antibodies (clones 322 and 376) that produce strong staining in paraffin sections. Other monoclonal antibodies used were antinucleolin (anti-C23) (Santa Cruz Biotechnology), antiglycophorin, anti-CD34 (DakoCytomation), and anti-CD133 (Miltenyi Biotec).

IMMUNOHISTOCHEMICAL STAINING

Immunostaining was performed with use of the alkaline phosphatase monoclonal anti–alkaline phosphatase technique[18] (details available from NAPS*). The subcellular distribution of NPM (i.e., restriction to the nucleus or presence in the cytoplasm) was assessed without knowledge of the FAB subtype, cytogenetic features, or molecular find-

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ings. Cases were classified as either NPMc+ (positive for cytoplasmic NPM) or NPMc− (negative for cytoplasmic NPM). All sections were stained in parallel for nucleolin (C23), another nucleolar antigen, which in NPMc+ cases was required to be restricted to the nucleus.

CYTOGENETIC AND MOLECULAR ANALYSES

Cytogenetic investigations were performed after short-term culture. Karyotypes, analyzed after G-banding, were described according to the International System for Human Cytogenetic Nomenclature.[19] Fluorescence in situ hybridization (FISH) investigations were carried out as previously described.[20] The following analyses were also carried out as previously described: reverse-transcriptase–polymerase-chain-reaction (RT-PCR) analysis for promyelocytic leukemia (PML)–RARα, acute myelogenous leukemia 1–eight twenty-one (AML1-ETO), core binding factor B–myosin heavy chain 11 (CBFB-MYH11), breakpoint cluster region–v-Abl Abelson murine leukemia viral oncogene homologue 1 (BCR-ABL), and DEK–nucleoporin 214 kD (DEK-CAN); Southern blotting and FISH for rearrangements in the mixed-lineage leukemia gene (*MLL*); and mutational analysis of the Fms-like tyrosine kinase gene (*FLT3*) and *MLL*.[21-24]

MUTATIONAL ANALYSIS OF *NPM*

We investigated 161 specimens for *NPM* mutations: 52 specimens of NPMc+ AML, 56 of NPMc− AML, 9 of chronic myelogenous leukemia, and 44 of lymphoid neoplasms (Table 1). Specimens from five patients with NPMc+ AML were analyzed both at diagnosis and at remission.

For the *NPM* coding-region analysis, 1 μg of RNA was retrotranscribed with use of the Thermoscript RT-PCR System (Invitrogen). Then cDNA sequences were amplified with primers *NPM1*_25F (5′GGTTGTTCTCTGGAGCAGCGTTC3′) and *NPM1*_1112R (5′CCTGGACAACATTTATCAAACACGGTA3′) with use of the Expand High-Fidelity[PLUS] PCR System (Roche Applied Science). To amplify the sequence of *NPM1* exon 12 from genomic DNA, two oligonucleotides were designed to anneal to the flanking intron sequences (*NPM1-F* [5′TTAACTCTCTGGTGGTAGAATGAA3′] and *NPM1-R* [5′CAAGACTATTTGCCATTCCTAAC3′]). PCR products, purified

Table 1. *NPM* Mutations in 161 Specimens of Myelogenous Leukemias and Lymphoid Neoplasms.*

Tumor Type	No. of Specimens	FAB Subtype	CD34+	*FLT3* Mutation	*NPM* Mutation
				no./total no.	
NPMc+ AML	52				
Normal karyotype	49	All but M3, M4eo, and M7	2/49	25/46	48/49
Abnormal karyotype	3	M1, M5b	1/3	1/3	3/3
NPMc− AML	56				
Normal karyotype	12	All but M3, M4eo, and M7	8/10	4/12	0/12
Abnormal karyotype†	44	M1–M6	5/8	3/8	0/44‡
CML	9	NA	0/9	ND	0/9
Lymphoid neoplasms§	44	NA	ND	ND	0/44

* *NPM* denotes the gene encoding nucleophosmin, FAB the French–American–British classification, *FLT3* the gene encoding Fms-like tyrosine kinase, NPMc+ positive for cytoplasmic nucleophosmin, AML acute myelogenous leukemia, NPMc−negative for cytoplasmic nucleophosmin, CML chronic myelogenous leukemia, NA not applicable, and ND not done.

† The abnormal karyotypes included 9 with t(15;17), 12 with t(8;21), 13 with inv(16), 1 with rearrangement of the mixed-lineage leukemia gene (*MLL*), 1 with inv(3), 1 with t(6;9), 6 with other chromosomal abnormalities, and 1 with a complex karyotype.

‡ One specimen with an inv(16) karyotype showed a trinucleotide deletion in *NPM1* exon 6 at positions 583, 584, and 585, without involvement of the 3′ terminus.

§ The lymphoid neoplasms included acute lymphoblastic leukemia (7 specimens), B-cell chronic lymphocytic leukemia (7), mantle-cell lymphoma (5), follicular lymphoma (5), diffuse large B-cell lymphoma (10), Burkitt's lymphoma (5), and multiple myeloma (5).

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by standard methods, were sequenced directly from both strands. Mutations were confirmed by assessing independent PCR products and, in representative cases, by cloning with pGEM-T Easy Vector Systems (Promega) and sequencing.

EXPRESSION VECTORS AND TRANSFECTION ASSAYS

Antihuman NPM monoclonal antibodies also react with NPM from other species (including mouse). To track the localization of exogenous NPM, we generated plasmids expressing wild-type (pEGFPc1-*NPM*wt) or mutant (pEGFPc1-*NPM*mA) *NPM* alleles fused to the enhanced green fluorescent protein (EGFP). *NPM* cDNA sequences in an NPMc+ AML specimen from a patient carrying a heterozygous mutation in exon 12 were amplified with the use of primers *NPM1*_89F_*Bam*HI (GCCACGGATCCGAAGATTCGATGGAC) and *NPM1*_ 1044R_*Eco*RI (ATCAAGAATTCCAGAAATGAAATAAGACG) and subcloned in frame into the pEGFPc1 vector (BD Biosciences Clontech). Sequencing analysis confirmed that there were no Taq-introduced errors in either plasmid.

NIH-3T3 cells were transiently transfected with pEGFPc1-*NPM*wt, pEGFPc1-*NPM*mA, and empty pEGFPc1 vector with the use of Lipofectamine 2000 reagents (Invitrogen). Transfection efficiency was monitored by Western blotting. Images were obtained with a confocal microscope (Bio-Rad MRC-1024), with the use of Imaris software for three-dimensional reconstruction.

STATISTICAL ANALYSIS

Chi-square analysis with two-way contingency tables was used to test the association between categorical variables. Statistical differences between means were analyzed by the t-test. A multivariate logistic model was used to analyze associations among the white-cell count at presentation, subcellular NPM localization, *FLT3* mutations, and the response to induction therapy in 126 assessable patients with AML and a normal karyotype (79 NPMc+ and 47 NPMc–) who were treated according to the GIMEMA LAM99P protocol (induction, consolidation, and postconsolidation therapy [as described in Supplementary Appendix 1A, available with the full text of this article at www.nejm.org]). Analyses were performed with SAS software (version 8.2). Two-sided P values of less than 0.05 were considered to indicate statistical significance.

RESULTS

CYTOPLASMIC DISLOCATION OF NPM

Of the 591 specimens from patients with AML in the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials, 208 (35.2 percent) were NPMc+ (Fig. 1A and 1B). All the other tumors were NPMc– (Fig. 1B). In NPMc+ leukemic cells, nucleolin (C23) remained restricted to the nucleus (Fig. 1A). Cytoplasmic NPM was usually found in all leukemic cells, except in FAB subtype M5b (monocytic leukemia) specimens, where it was detected in 30 to 60 percent of the most immature cells of monocytic lineage (data not shown). NPMc– AML specimens contained only a few NPMc+ leukemic cells, usually tumor cells undergoing mitosis (Fig. 1A).

The NPMc+ pattern was found at diagnosis and in relapse in the 25 patients studied at those times. The NPMc+ pattern also was seen only in those with primary AML; specimens from the 135 patients with secondary AML contained NPM restricted exclusively to the nucleus (Fig. 1B).

FEATURES OF NPMc+ AML

Morphology

The NPMc+ pattern was found in AML specimens of all FAB subtypes except M3 (acute promyelocytic leukemia), M4eo (acute myelomonocytic leukemia with eosinophilia), and M7 (acute megakaryocytic leukemia) (Fig. 1C). The frequency of the finding ranged from 13.6 percent in M0 tumors (minimally differentiated AML) to 87.5 percent in M5b specimens (acute monocytic leukemia). Most NPMc+ AML tumors of the M5b and M6 subtypes (acute erythroid leukemia) and about 30 percent of NPMc+ tumors of the M1 (AML without maturation), M2 (AML with maturation), and M4 (acute myelomonocytic leukemia) subtypes showed cytoplasmic NPM in erythroid precursors, particularly proerythroblasts (Fig. 1D), and less frequently in megakaryocytes (data not shown).

This multilineage distribution of the NPMc+ pattern prompted an investigation of CD34 and CD133 antigens, which occur on hematopoietic stem cells. Twelve of 159 NPMc+ AML specimens (7.5 percent) contained at least 20 percent CD34-positive cells, as compared with 227 of 317 NPMc– AML specimens (71.6 percent) ($P<0.001$) (Fig. 2A through 2E). CD34-negative NPMc+ AML specimens also did not contain CD133 (Fig. 2F).

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Karyotypes

Cytogenetic data were available for 493 of the 591 patients with AML (166 NPMc+ and 327 NPMc−). The karyotype was normal in 142 of the 166 patients with NPMc+ AML (85.5 percent), as compared with 88 of the 327 patients with NPMc− AML (26.9 percent) (P<0.001) (Fig. 3A; details available from NAPS*). Thus, 142 of the 230 AML specimens with a normal karyotype (61.7 percent) were NPMc+ (Figure 3B). Of the 24 specimens of NPMc+ AML with an abnormal karyotype, 12 had cells in normal and abnormal metaphase (details available from NAPS*). No case of AML associated with specific genetic abnormalities was NPMc+ (Fig. 3B, 3C, and 3D) (details available from NAPS*).

FLT3 Mutations

Internal tandem duplication of the *FLT3* gene was detected in 59 of 219 patients with AML who had a normal karyotype (26.9 percent), and a mutation at aspartic acid residue 835 (D835) in FLT3 was detected in 13 of 202 such patients (6.4 percent). One patient carried both an internal tandem duplication and the D835 mutation. Internal tandem duplication of this gene was twice as frequent in cases of NPMc+ disease as it was in cases of NPMc− disease (P<0.003) (see Supplementary Appendix 1B). A multivariate logistic-regression model adjusted for age and cytogenetic features established an independent association between cytoplasmic NPM (the dependent variable) and internal tandem duplication in *FLT3*. No statistical association was found between D835 mutations in *FLT3* and the subcellular localization of NPM, possibly because of the small number of cases involving a D835 mutation.

Response to Induction Therapy

Between 1999 and 2002, 539 patients with AML were enrolled in the GIMEMA LAM99P trial. All the patients received the same induction therapy (see Supplementary Appendix 1A). The association between the subcellular localization of NPM and the response to induction therapy was evaluated in 126 patients with a normal karyotype for whom NPM immunostaining and clinical information were available. There were 63 men and 63 women, the median age at diagnosis was 49 years (range, 19 to 60), and the median white-cell count at diagnosis was 28.4×10^3 per cubic millimeter (range, 0.6×10^3 to 400.0×10^3). The distribution of FAB subtypes was as follows: M0 (5 patients), M1 (23), M2 (42), M4 (25), M5 (28), and M6 (3). An *FLT3* mutation was

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Figure 1 (facing page). Specific Association of Cytoplasmic Expression of Nucleophosmin with a Large Subgroup of AML.

Panel A shows subcellular patterns of expression of nucleophosmin (NPM) in specimens from patients with acute myelogenous leukemia (AML) (alkaline phosphatase monoclonal anti–alkaline phosphatase [APAAP] technique). In NPMc+ AML (two left-hand images), most leukemic cells (arrow) show cytoplasmic NPM expression in addition to nuclear NPM expression; the arrowhead indicates a residual hematopoietic cell with the expected pattern of nucleus-restricted NPM. In NPMc+ AML, C23 is always restricted to the nucleus. In the two right-hand images, leukemic cells that are negative for cytoplasmic NPM (NPMc−) have the expected nuclear expression of NPM and nucleolin (C23); the arrows indicate mitotic figures with the expected cytoplasmic expression of NPM and C23. Panel B presents data, based on the subcellular expression of NPM in paraffin sections from 1706 human tumors, indicating that cytoplasmic expression of NPM is specific to primary AML. Cytoplasmic expression of NPM was restricted to 35.2 percent of the specimens from patients with primary AML who were enrolled in the GIMEMA LAM99P and GIMEMA/EORTC AML12 trials. (One hundred twenty-nine patients with primary AML not enrolled in the GIMEMA/EORTC studies were excluded from this analysis.) All the other specimens, including those from 135 patients with secondary AML, showed nucleus-restricted NPM expression. ALL denotes acute lymphoid leukemia, CML chronic myelogenous leukemia, MDS myelodysplastic syndrome, and NHL non-Hodgkin's lymphoma. As indicated by the data in Panel C, NPMc+ AML represents a wide morphologic spectrum of French–American–British (FAB) subtypes. The graph shows the correlation between subcellular NPM expression and morphologic subtype in specimens from 591 patients with primary AML in the GIMEMA/EORTC study plus 70 patients with AML of the M3 subtype with a t(15;17) translocation who were not enrolled in the trial. Panel D shows NPMc+ AML specimens of the M6 subtype with cytoplasmic NPM expression in erythroid and myeloid cell lineages (APAAP technique). In the left-hand image, the marrow is infiltrated by myeloid blasts (arrowhead) and erythroid blasts (arrow). In the middle image, abnormal erythroid precursors (arrow) and clusters of myeloid blasts (double arrow) show cytoplasmic as well as nuclear NPM expression; the arrowhead indicates a residual hematopoietic cell with nucleus-restricted NPM expression, and the arrow in the inset indicates double-staining of leukemic cells for surface glycophorin (brown) and cytoplasmic and nuclear NPM (blue). In the right-hand image, leukemic cells show nucleus-restricted nucleolin (C23) expression; the arrow in the inset indicates double-staining of leukemic cells for surface glycophorin (brown) and nucleus-restricted C23 (blue).

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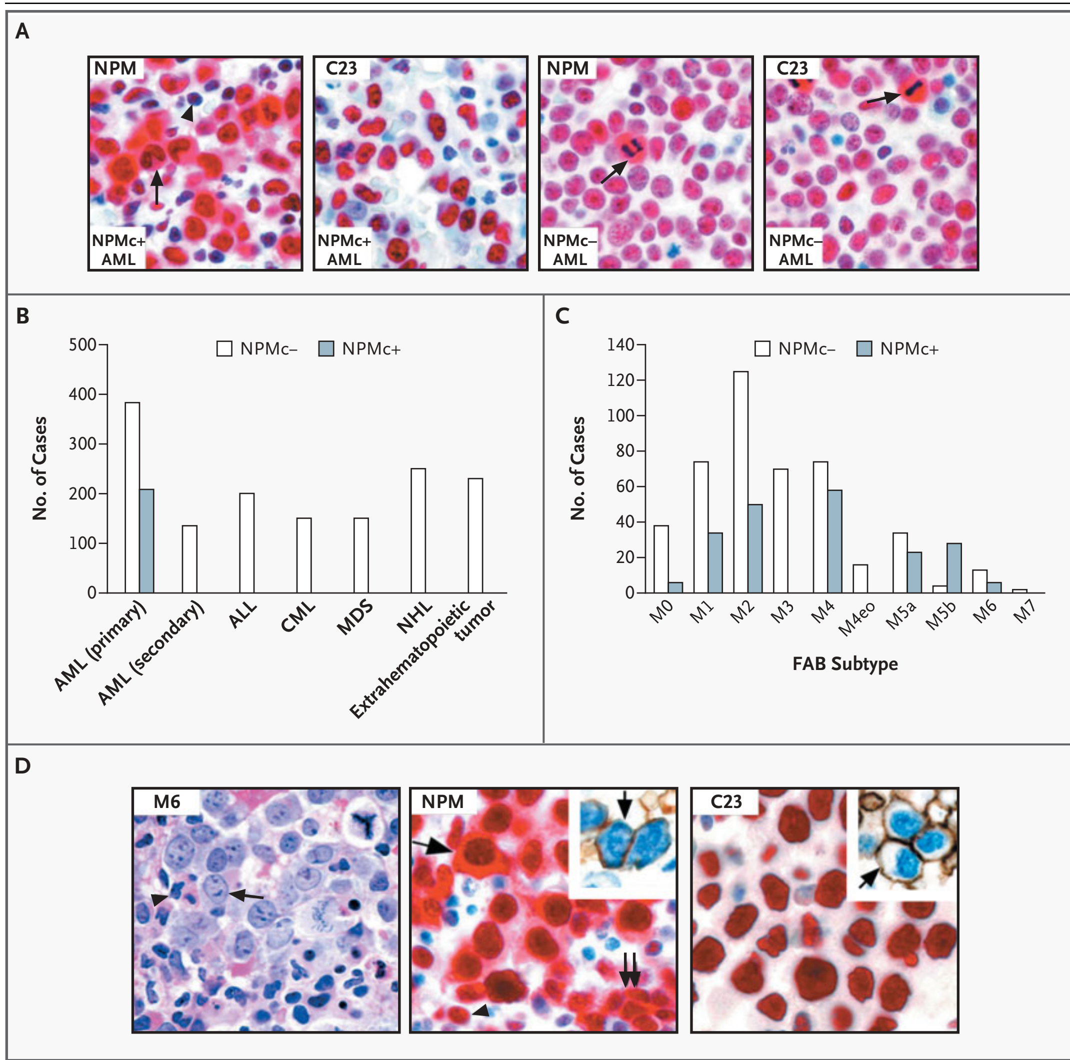

present in 45 patients (36 percent), and cytoplasmic NPM was present in 79 (63 percent).

The median ages of patients with NPMc+ and NPMc– tumors were 51.8 and 41.9 years, respectively ($P<0.001$). There were no significant differences between the two groups at presentation in terms of sex, white-cell count, FAB subtypes, *FLT3* status, and clinical features.

Of the 126 patients, 90 (71 percent) had complete remission after induction therapy. In a univariate analysis, a lower white-cell count at presentation was associated with a higher rate of complete response (78 percent among patients with a white-cell count of 80×10^3 per cubic millimeter or below vs. 50 percent among those with a white-cell count above 80×10^3 per cubic millimeter) ($P<0.003$). There was no statistically significant difference in the rates of complete response between patients with NPMc+ tumors (77 percent) and those with NPMc– tumors (62 percent) ($P=0.070$). There was resistance to treatment in 9 percent of patients with NPMc+ tumors and in 23 percent of those with NPMc– tumors. Sex, age, FAB subtype, and *FLT3* status were not associated with the rate of complete response.

A multivariate logistic-regression model that included the white-cell count, age, NPM localization (nuclear or cytoplasmic), and the presence or absence of an *FLT3* mutation showed that a lower white-cell count and cytoplasmic expression of NPM are independent prognostic factors for a complete remission. A white-cell count above 80×10^3 per cubic millimeter was found to have a negative effect ($P=0.006$; odds ratio, 0.27 [95 percent confi-

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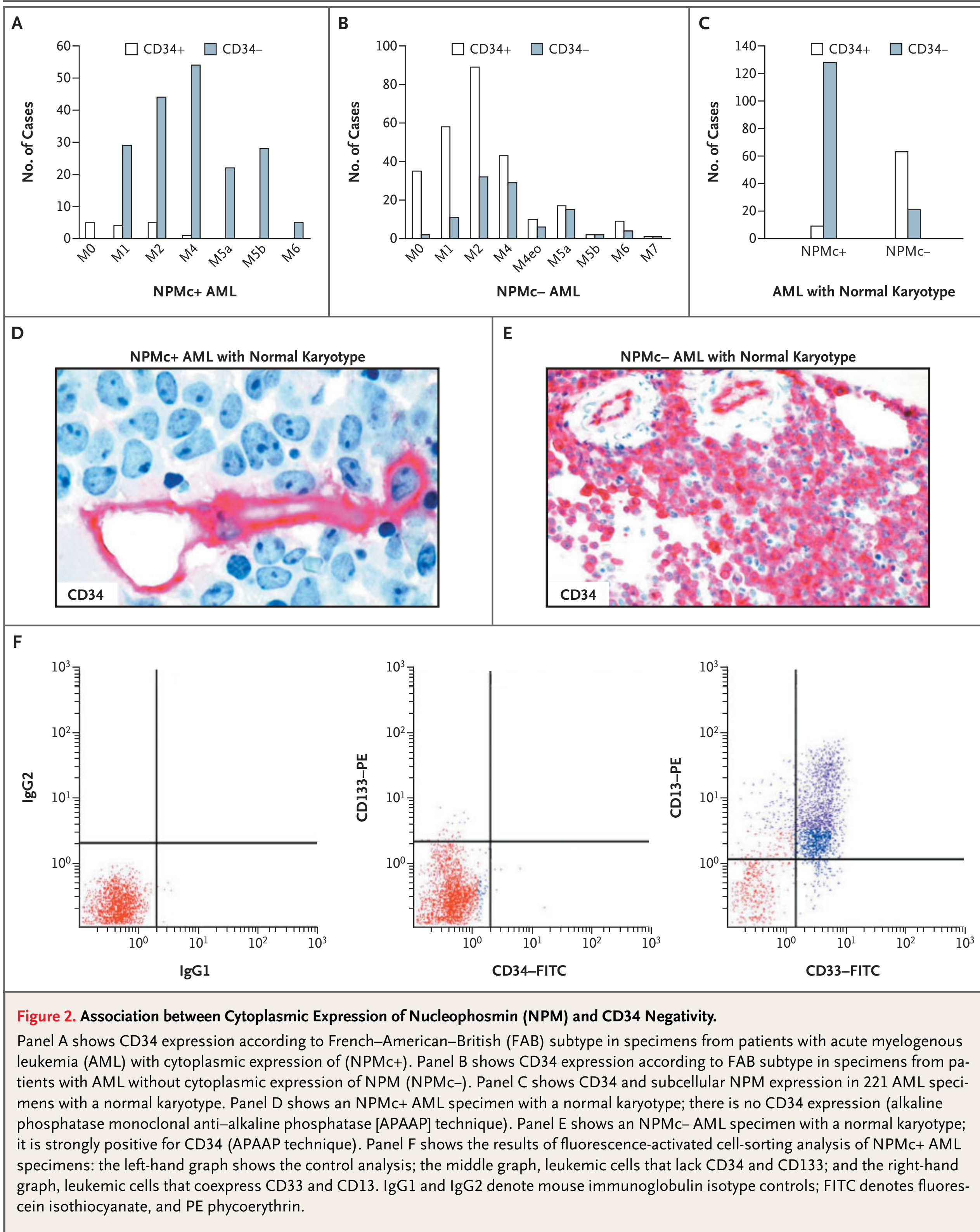

Figure 2. Association between Cytoplasmic Expression of Nucleophosmin (NPM) and CD34 Negativity.

Panel A shows CD34 expression according to French–American–British (FAB) subtype in specimens from patients with acute myelogenous leukemia (AML) with cytoplasmic expression of (NPMc+). Panel B shows CD34 expression according to FAB subtype in specimens from patients with AML without cytoplasmic expression of NPM (NPMc–). Panel C shows CD34 and subcellular NPM expression in 221 AML specimens with a normal karyotype. Panel D shows an NPMc+ AML specimen with a normal karyotype; there is no CD34 expression (alkaline phosphatase monoclonal anti–alkaline phosphatase [APAAP] technique). Panel E shows an NPMc– AML specimen with a normal karyotype; it is strongly positive for CD34 (APAAP technique). Panel F shows the results of fluorescence-activated cell-sorting analysis of NPMc+ AML specimens: the left-hand graph shows the control analysis; the middle graph, leukemic cells that lack CD34 and CD133; and the right-hand graph, leukemic cells that coexpress CD33 and CD13. IgG1 and IgG2 denote mouse immunoglobulin isotype controls; FITC denotes fluorescein isothiocyanate, and PE phycoerythrin.

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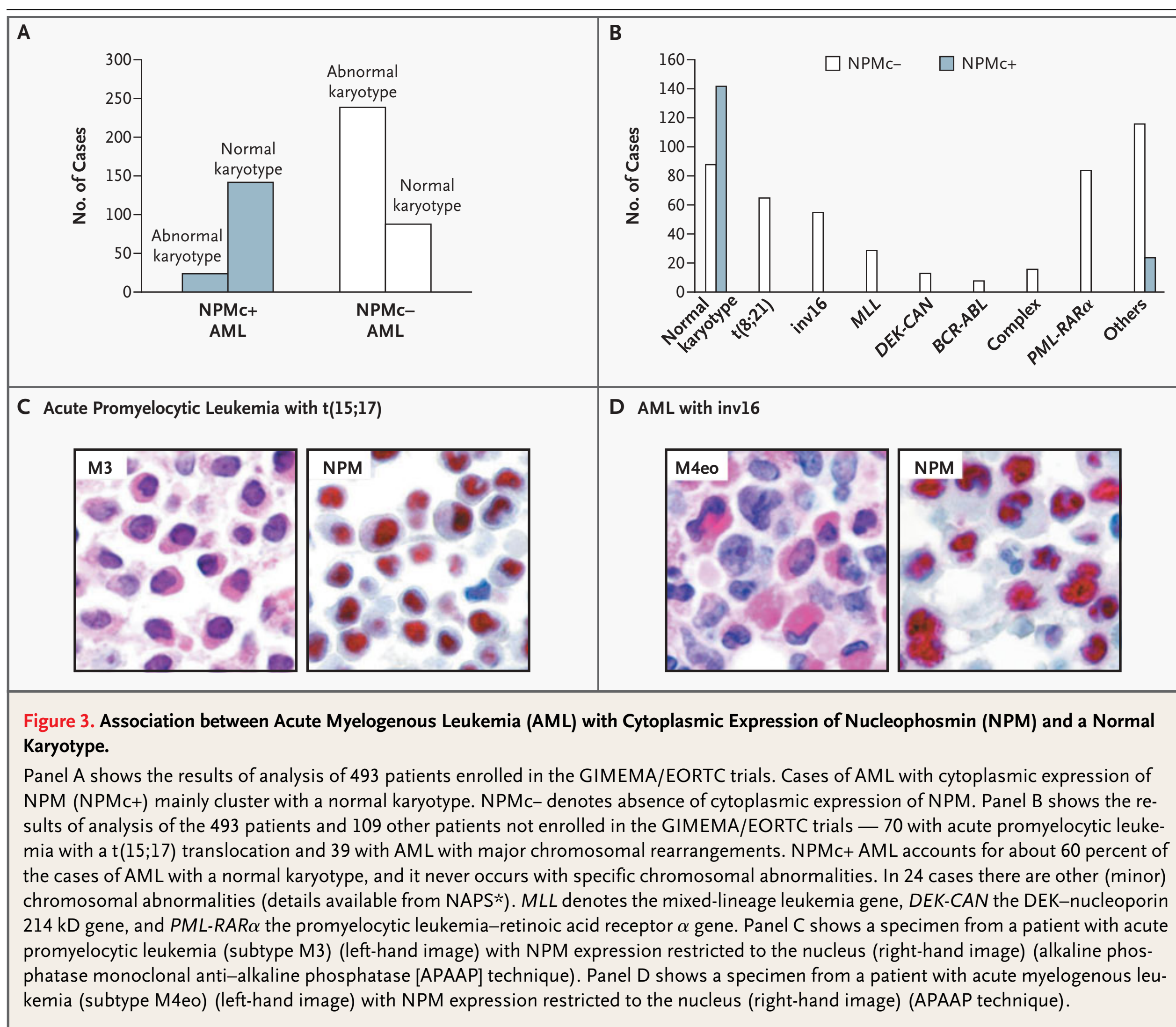

Figure 3. Association between Acute Myelogenous Leukemia (AML) with Cytoplasmic Expression of Nucleophosmin (NPM) and a Normal Karyotype.

Panel A shows the results of analysis of 493 patients enrolled in the GIMEMA/EORTC trials. Cases of AML with cytoplasmic expression of NPM (NPMc+) mainly cluster with a normal karyotype. NPMc– denotes absence of cytoplasmic expression of NPM. Panel B shows the results of analysis of the 493 patients and 109 other patients not enrolled in the GIMEMA/EORTC trials — 70 with acute promyelocytic leukemia with a t(15;17) translocation and 39 with AML with major chromosomal rearrangements. NPMc+ AML accounts for about 60 percent of the cases of AML with a normal karyotype, and it never occurs with specific chromosomal abnormalities. In 24 cases there are other (minor) chromosomal abnormalities (details available from NAPS*). *MLL* denotes the mixed-lineage leukemia gene, *DEK-CAN* the DEK–nucleoporin 214 kD gene, and *PML-RARα* the promyelocytic leukemia–retinoic acid receptor α gene. Panel C shows a specimen from a patient with acute promyelocytic leukemia (subtype M3) (left-hand image) with NPM expression restricted to the nucleus (right-hand image) (alkaline phosphatase monoclonal anti–alkaline phosphatase [APAAP] technique). Panel D shows a specimen from a patient with acute myelogenous leukemia (subtype M4eo) (left-hand image) with NPM expression restricted to the nucleus (right-hand image) (APAAP technique).

dence interval, 0.11 to 0.68]) and the NPMc+ pattern to have a positive effect (P=0.019; odds ratio, 2.98 [95 percent confidence interval, 1.2 to 7.43]) (Table 2).

MUTATIONS IN *NPM* EXON 12 IN NPMc+ AML

In none of the NPMc+ AML specimens was NPM-ALK,[13] NPM-RARα,[14] NPM-MLF1,[15] or any other NPM-containing fusion protein found. Transcripts of the corresponding fusion genes were not found by FISH or RT-PCR, and FISH did not detect other *NPM* translocations. Western blotting showed only the expected 38-kD NPM polypeptide. The finding of cytoplasmic positivity with a monoclonal antibody directed against the NPM C-terminal (which is not retained in NPM fusion proteins)[5] further supported the presence of full-length NPM in the cytoplasm of leukemic cells.

RT-PCR and direct sequencing of the *NPM* coding region revealed mutations affecting exon 12 in all but one case of NPMc+ disease (Table 1 and Supplementary Appendix 1C). Figure 4 shows a schematic diagram of the *NPM* gene and summarizes the mutations. Six sequence variants were observed, all leading to a frame shift in the region encoding the C-terminal of the NPM protein. The most frequent mutation (which we have called mutation A) was a duplication of a TCTG tetranucleotide at positions 956 through 959 of the reference sequence

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Table 2. Logistic-Regression Analysis of the Effect of Prognostic Factors for a Response to Induction Therapy in 126 Patients with Acute Myelogenous Leukemia.*

Variable	Analysis of Maximum Likelihood Estimates				Odds Ratio (95% CI)		
	Degrees of Freedom	Estimate	Chi-Square	P Value			
FLT3 (mutated vs. unmutated)	1	−0.4020±0.4541	0.7836	0.3760	0.67	0.20	1.60
NPM (cytoplasmic vs. nuclear)	1	1.0946±0.4647	5.5486	0.0185	2.98	1.20	7.43
White-cell count (≤80 vs. >80×10³/mm³)†	1	−1.3123±0.4748	7.6399	0.0057	0.27	0.11	0.68
Age (≤48 vs. >48 yr)	1	−0.3405±0.4498	0.5732	0.4490	0.71	0.20	1.70

* Plus–minus values are means ±SE. The 126 patients were treated according to the GIMEMA LAM99P protocol. The chi-square values and the confidence intervals for the odds ratios were calculated by the Wald test. CI denotes confidence interval.

† White-cell counts were categorized as above or below the 75th percentile.

(GenBank accession number NM_002520) (Fig. 4B and 4C); the resulting shift in the reading frame is predicted to alter the C-terminal portion of the NPM protein by replacing the last seven amino acids (WQWRKSL, where the amino acids are designated by their single-letter codes) with 11 different residues (CLAVEEVSLRK). Three additional mutations (called B, C, and D) included distinct 4-bp insertions at position 960, resulting in the same frame shift as mutation A. In the last two mutations (called E and F), nucleotides 965 through 969 (GGAGG) were deleted and two different 9-bp sequences were inserted, leading to the same frame shift and to a distinct C-terminal consisting of nine amino acids. All six NPM mutant proteins showed mutations in at least one of the tryptophan residues at positions 288 and 290 and shared the same last five amino acid residues (VSLRK) (Fig. 4B). Thus, despite genetic heterogeneity, all *NPM* gene mutations result in a distinct sequence in the NPM protein C-terminal. The sequences of the six mutated *NPM* alleles have been deposited in the National Center for Biotechnology Information database (GenBank accession numbers AY740634 through AY740639).

Mutations in exon 12 of *NPM* and their specific association with cases of NPMc+ AML were con-

Figure 4 (facing page). Mutations in Exon 12 of the Nucleophosmin (*NPM*) Gene and in the Encoded Protein.

Panel A shows a schematic representation of the *NPM* gene as deduced from GenBank sequences NM_002520, NM_199185, and AB042278. Green indicates coding sequences, and yellow 3' and 5' untranslated regions. MB denotes metal-binding domain, Ac acidic domain, NLS nuclear localization signal, and NAB nucleic acid–binding domain. Primers for amplification of genomic DNA (NPM1-F and NPM1-R [blue arrowheads]) and complementary DNA (NPM1_25F and NPM1_1112R [red arrowheads]) are shown in their approximate positions above the map. In Panel B, the wild-type NPM sequence (nucleotides 952 through 989) is aligned with six mutant variants, called A to F. Red type indicates nucleotide insertions. The predicted protein is also shown, with boxed areas indicating the positions of the two C-terminal tryptophan (W) residues; the wild-type tryptophan residue, is shown in yellow, and the mutated residues are shown in gray. The new amino-acid sequence common to all the mutated proteins is shown in green. For each variant, the number and percentage of affected cases, of the 52 total NPMc+ cases, are given. Panel C shows sequencing results from one patient bearing mutation A, as obtained by direct sequencing (top diagram) and after cloning and sequencing of the two individual alleles (middle [wild-type] and bottom [mutated allele] diagrams). The arrow and the dashed line indicate the position where the two alleles diverge, and the box indicates the most frequently mutated nucleotides. As shown in the images in Panel D, the mutated NPM protein is dislocated in the cytoplasm. The images are tridimensional reconstructions of confocal micrographs of NIH-3T3 cells transfected with plasmids encoding wild-type and mutant NPM alleles tagged with enhanced green fluorescent protein; the nuclei were counterstained with propidium iodide (which appears as red). The wild-type protein is located in the nucleoli and nuclear membrane, whereas the mutated NPM shows aberrant cytoplasmic localization.

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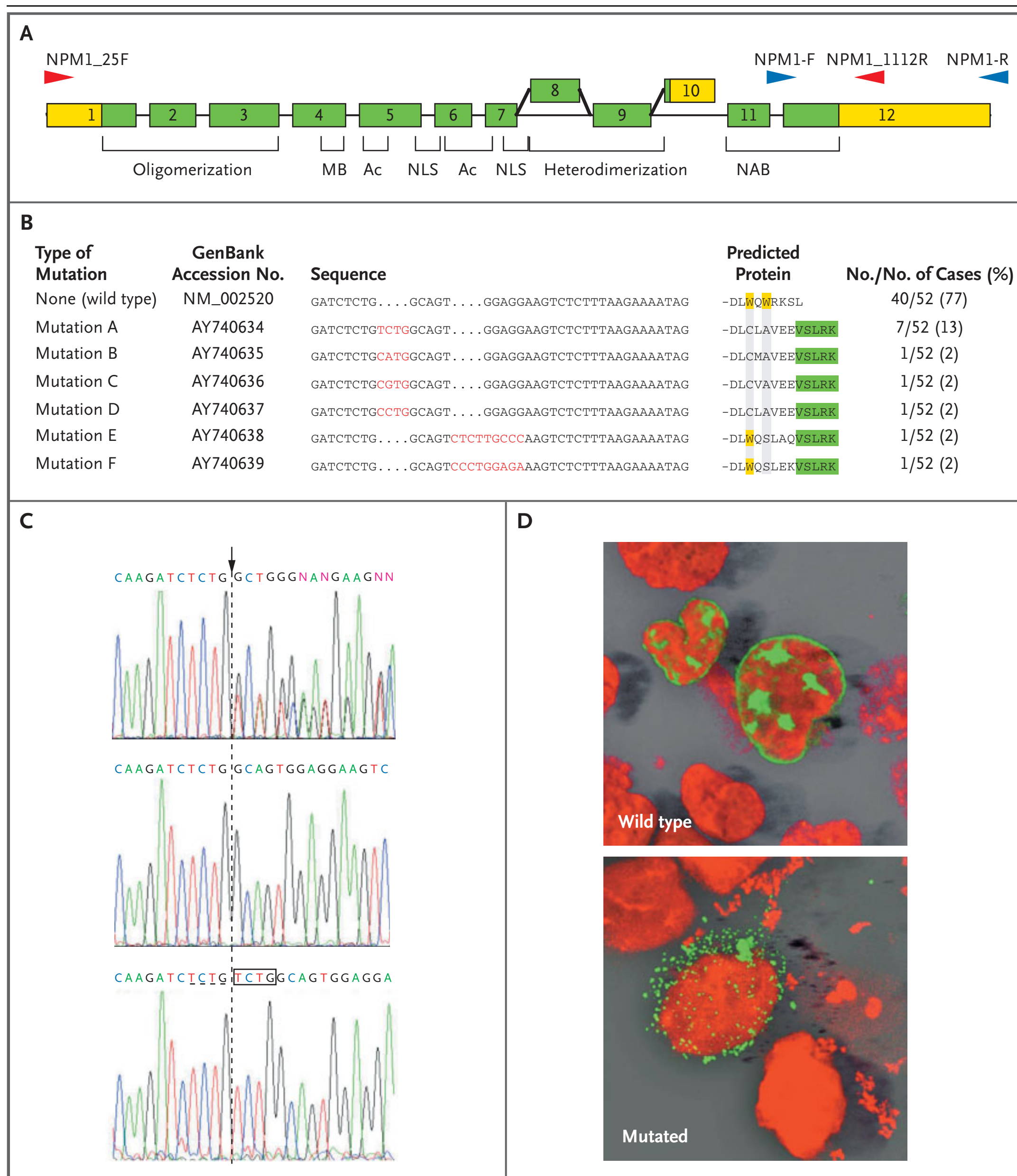
firmed by sequence analysis of genomic DNA in 11 available specimens. The mutations were heterozygous and were related only to the malignant clone, since they were not present in bone marrow specimens from the five patients in complete remission. Mutations were observed in a wide variety of FAB categories of NPMc+ AML and even in cases with abnormal karyotypes or CD34 expression (Table 1 and Supplementary Appendix 1C). All NPMc– AML tumors and neoplasms other than AML had wild-type NPM sequences.

TRANSFECTION OF MUTATED *NPM*

To test whether mutations in *NPM* exon 12 caused cytoplasmic dislocation of NPM, NIH-3T3 cells were transiently transfected with expression vectors encoding wild-type and mutant alleles fused with EGFP. Confocal microscopy showed nucleolar localization of the EGFP–NPM wild-type protein. The mutant form of NPM was dislocated into the cytoplasm (Fig. 4D and Supplementary Appendix 1D) — a finding that suggested that the genetic event correlated with the subcellular localization of NPM.



Type of Mutation	GenBank Accession No.	Sequence	Predicted Protein	No./No. of Cases (%)
None (wild type)	NM_002520	GATCTCTG....GCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLWQWRKSL	40/52 (77)
Mutation A	AY740634	GATCTCTGTCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK	7/52 (13)
Mutation B	AY740635	GATCTCTGCATGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCMAVEEVSLRK	1/52 (2)
Mutation C	AY740636	GATCTCTGCGTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCVAVEEVSLRK	1/52 (2)
Mutation D	AY740637	GATCTCTGCCTGGCAGT....GGAGGAAGTCTCTTTAAGAAAATAG	-DLCLAVEEVSLRK	1/52 (2)
Mutation E	AY740638	GATCTCTG....GCAGTCTCTTGCCCAAGTCTCTTTAAGAAAATAG	-DLWQSLAQVSLRK	1/52 (2)
Mutation F	AY740639	GATCTCTG....GCAGTCCCTGGAGAAAGTCTCTTTAAGAAAATAG	-DLWQSLEKVSLRK	1/52 (2)

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DISCUSSION

We found that cytoplasmic NPM is the hallmark of a distinct type of AML that constitutes about one third of the cases of primary AML in adults (excluding FAB subtype M3). Patients with this type of AML have a normal karyotype, a mutant *NPM* gene, and a relatively good response to induction chemotherapy. Moreover, of the patients with AML who had a normal karyotype, cytoplasmic NPM was found in about 60 percent. Our findings are important because no chromosomal rearrangement is visible by standard karyotyping in 40 to 50 percent of cases of AML,[1] and uncertainty clouds some of the biologic and clinical features in these patients.

Mutations in *NPM* exon 12 and the resulting shift of NPM into the cytoplasm are the most specific and frequent events we have found in AML with a normal karyotype. Mutations of the *FLT3*[25,26] and *CEBPA*[27] genes or *MLL* self-fusion[28] also occurs in primary AML with a normal karyotype or recurrent genetic abnormalities[23,29] and in secondary AML.[30] The chromosomal abnormalities we found in about 14 percent of cases of NPMc+ AML are probably secondary, as indicated by the frequent occurrence of cells with an abnormal karyotype as subclones within the population with a normal karyotype and similarity with the type and incidence of secondary chromosomal changes in AML with recurrent genetic abnormalities.[31]

Internal tandem duplication of *FLT3* was seen twice as often in cases of NPMc+ AML as it was in cases of NPMc– AML, suggesting that the *FLT3* and *NPM* mutations are mechanistically linked. NPMc+ AML is CD34-negative, has a wide morphologic spectrum, and demonstrates multilineage involvement. These features might reflect derivation of this type of AML from the few lineage-marker–negative, CD34-negative, CD38-negative hematopoietic stem cells in bone marrow.[32] Alternatively, CD34 might be down-regulated as result of the leukemic transformation.

Our transfection experiments established a causal relationship between *NPM* mutations and NPM cytoplasmic dislocation, in keeping with data from mutant rat NPM showing that the C-terminal region (in particular, the tryptophan residues at positions 286 and 288) is necessary for nucleolar localization of NPM.[33] All predicted proteins encoded by the six variant mutations in our patients were altered in at least one of the critical tryptophan residues at positions 288 and 290.

NPMc+ AML might originate from a small, undetectable subset of pluripotent hematopoietic stem cells that normally harbor a mutated form of NPM. It is more likely, however, that mutation of NPM and cytoplasmic dislocation of NPM are primary leukemogenic events. The mutation might interfere with normal NPM functions — including stabilization of p53 in the nucleus when DNA is damaged[9,10] and direct interaction with the tumor-suppressor gene *ARF* to regulate the cell cycle[34] — by sequestering the protein in the cytoplasm. The mutation might also perturb other NPM functions that have been mapped within its C-terminal region, such as nucleic acid binding,[35] ATP binding,[36] and stimulation of DNA polymerase α activity.[37]

Our findings have several diagnostic and prognostic implications. Detection of cytoplasmic NPM by a sensitive, specific, simple, inexpensive, and rapid assay done on paraffin sections from bone marrow trephine specimens or marrow clots could be used to rule out recurrent chromosomal abnormalities. Cytoplasmic NPM also appears to be a reliable predictor of mutations in *NPM* exon 12 in AML with a normal karyotype.

The identification of the NPMc+ AML genetic subtype within the heterogeneous World Health Organization category of primary AML "not otherwise characterized"[38] may have repercussions for AML classification. Cases of AML are currently assigned to prognostic groups according to cytogenetic and molecular findings.[1,25,39] AML with a normal karyotype that cannot be classified by cytogenetic means (as in about 20 percent of the cases in this study) because of the lack of a specimen, deterioration of the specimen, or absence of mitoses could be assigned to the intermediate-risk category by detection of cytoplasmic NPM. Moreover, the association of cytoplasmic NPM with primary AML has prognostic significance, since secondary AMLs usually carry a poor prognosis.

Finally, cytoplasmic NPM is associated with responsiveness to induction therapy, although its role (alone or in combination with FLT3) in predicting the outcome of AML with a normal karyotype after remission remains to be defined. Immunohistochemistry plus mutational analysis of NPM may assist in the monitoring of minimal residual disease in a setting (normal karyotype and CD34

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negativity) in which no molecular or immunophenotypic markers are available. Understanding the mechanisms leading to leukemogenesis in NPMc+ AML may lead to more specific antileukemia therapies.[40]

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APPENDIX

The centers and investigators contributing to the GIMEMA Study were as follows (listed in order of the number of cases provided): Istituto di Ematologia, Università La Sapienza, Rome (G. Meloni); Divisione di Ematologia, Ospedale V. Cervello, Palermo (F. Fabbiano); Cattedra di Ematologia, Bari (V. Liso); Divisione di Ematologia, Azienda USL di Pescara, Pescara (M. Sborgia); Ospedale Ferrarotto S. Bambino, Catania (F. Di Raimondo); Divisione di Ematologia, Ospedale S. Eugenio, Rome (A. Venditti); Divisione di Ematologia e Oncologia Clinica, Catanzaro (D. Magro); Dipartimento di Emato-Oncologia, Azienda Ospedaliera Bianchi-Melacrino-Morelli, Reggio Calabria (F. Nobile); Istituto di Ematologia, Università Federico II, Naples (B. Rotoli); Azienda Ospedaliera S.G. Moscati, Avellino (N. Cantore); Divisione di Ematologia, Ospedale Casa Sollievo della Sofferenza, S. Giovanni Rotondo (L. Melillo); Istituto di Ematologia, Ospedale A. Businco, Cagliari (E. Angelucci); Istituto di Ematologia, Policlinico Monteluce, Perugia (A. Tabilio); Cattedra di Ematologia, Ospedale S. Chiara, Pisa (M. Petrini); Policlinico Gemelli, Rome (S. Sica); Università di Ancona, Ancona (P. Leoni); Sezione di Medicina Interna, Oncologia ed Ematologia, Dipartimento Scienze Mediche, Oncologiche e Radiologiche, Modena (G. Torelli); Divisione di Ematologia, Ospedale SS. Antonio e Biagio, Alessandria (A. Levis); Divisione di Ematologia, Fondazione Centro S. Raffaele del Monte Tabor, Milan (L. Camba); Divisione di Ematologia, Ospedale S. Carlo, Potenza (F. Ricciuti); Divisione di Ematologia, Ospedale S. Giovanni Bosco, Naples (E. Miraglia); Azienda Ospedaliera A. Di Summa, Brindisi (G. Quarta); Divisione di Ematologia, Ospedale S. Francesco, Nuoro (A. Gabbas); Divisione di Ematologia con Trapianto Midollo Osseo, Università di Palermo, Palermo (M.E. Mitra); Cattedra di Ematologia–Centro Trapianto Midollo Osseo, Università di Parma, Parma (V. Rizzoli); Divisione Ematologica di Muraglia, Ospedale S. Salvatore, Pesaro (G. Sparaventi); Divisione di Ematologia, Azienda Ospedaliera Cremona, Cremona (S. Moranti); Divisione di Ematologia, Ospedale S. Croce, Cuneo (A. Gallamini); Divisione di Medicina Interna, Ospedale S. Luigi, Orbassano (A. Serra); Sezione Ematologia, Dipartimento Scienze Biomediche, Arcispedale S. Anna, Ferrara (P.-L. Castaldi); Divisione di Ematologia, Università di Sassari, Sassari (F. Dore); Divisione di Medicina, Azienda Ospedaliera E. Morelli, Sondalo (E. Epis); Divisione Medicina 1, Ospedale S. Antonio Abate, Gallarate (R. Mozzana); Divisione Medica, Ospedale Maggiore, Lodi (G. Nalli); Azienda Sanitaria Locale Salerno 1, Medicina Interna, Ematologia–Oncologia, Nocera Inferiore (A.M. D'Arco); Divisione di Ematologia, Policlinico Careggi, Florence (P.-L. Rossi Ferrini); Unità Operativa Ematologia, Ospedale di Foggia, Foggia (M. Monaco); Divisione di Ematologia, Ospedale di Messina, Messina (M. Brugiatelli); and S. Vincenzo, Ospedale di Taormina, Divisione di Ematologia, Taormina (M. Russo) — all in Italy.

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CORRECTION

Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype

Cytoplasmic Nucleophosmin in Acute Myelogenous Leukemia with a Normal Karyotype . On page 263, in Panel B of Figure 4, the data under the column heading ``No./No. of Cases (%)´´ are incorrect. They should read, according to the type of mutation, as follows: for none (wild-type), no data; for mutation A, 40/51 (78); for mutation B, 7/51 (14); for mutation C, 1/51 (2); for mutation D, 1/51 (2); for mutation E, 1/51 (2); and for mutation F, 1/51 (2).

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