Clinical characteristics and prognostic implications of *NPM1* mutations in acute myeloid leukemia

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Recently, somatic mutations of the nucleophosmin gene *(NPM1)*, which alter the subcellular localization of the product, have been reported in acute myeloid leukemia (AML). We analyzed the clinical significance of *NPM1* mutations in comparison with cytogenetics, *FLT3, NRAS,* and *TP53* mutations, and a partial tandem duplication of the *MLL* gene (MLL-TD) in 257 patients with AML. We found *NPM1* mutations, including 4 novel sequence variants, in 64 of 257 (24.9%) patients. *NPM1* mutations were associated with normal karyotype and with internal tandem duplication (ITD) and D835 mutations in *FLT3*, but not with other mutations. In 190 patients without the M3 French-American-British (FAB) subtype who were treated with the protocol of the Japan Adult Leukemia Study Group, multivariate analyses showed that the *NPM1* mutation was a favorable factor for achieving complete remission but was associated with a high relapse rate. Sequential analysis using 39 paired samples obtained at diagnosis and relapse showed that *NPM1* mutations were lost at relapse in 2 of the 17 patients who had *NPM1* mutations at diagnosis. These results suggest that the *NPM1* mutation is not necessarily an early event during leukemogenesis or that leukemia clones with *NPM1* mutations are sensitive to chemotherapy. (Blood. 2005;106:2854-2861)

Introduction

Acute myeloid leukemia (AML) is characterized by autonomous proliferation and impaired differentiation of hematopoietic progenitors but is a genetically and phenotypically heterogeneous disease. A number of genetic mutations, such as point mutations, gene rearrangements, and chromosomal translocations, which are involved in the pathogenesis of leukemia, have been documented. Recently, it was suggested that AML is the consequence of 2 broad complementation classes of mutations: those that confer a proliferative or a survival advantage to hematopoietic progenitors (class 1)—including activating mutations in tyrosine kinases such as *BCR-ABL1*, *ETV6-PDGFRB*, *KIT,* and *FLT3* or their downstream effectors such as *NRAS*—and those that impair hematopoietic differentiation and confer properties of self-renewal (class 2)—including rearrangements or point mutations of core binding factor *(CBF)* genes and *PML-RARA*.[1] Mutations in *FLT3*, *NRAS,* and *KIT* have been found in approximately 30% to 35%, 15% to 20%, and 5% to 10% of adult patients with AML, respectively, indicating that mutations in these 3 genes are the most frequent genetic alterations in AML.[2-12] *FLT3* and *KIT* mutations are often found in AML patients with *PML-RARA* and *CBF* gene translocations, respectively,[13,14] whereas *FLT3* mutations have been preferentially found in AML patients with normal karyotype. Because mutated FLT3 reportedly induces myeloproliferative disease (MPD), but not AML, in primary hematopoietic progenitors in the murine bone marrow transplantation model and MPD does not have serial transplantability, *FLT3* mutations alone are not sufficient for the development of AML.[15] Therefore, it has been suggested that additional mutations are involved in the pathogenesis of AML with *FLT3* mutations.

Recently, Falini et al[16] reported that the nucleophosmin gene *(NPM1)* is mutated in a high proportion of adults with AML, resulting in an aberrant cytoplasmic localization of the product

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Submitted April 28, 2005; accepted June 12, 2005. Prepublished online as *Blood* First Edition Paper, June 30, 2005; DOI 10.1182/blood-2005-04-1733.

Supported by Grants-in-Aid from the Ministry of Health, Labor, and Welfare and from the Scientific Research and the 21st Century COE Program "Integrated Molecular Medicine for Neuronal and Neoplastic Disorders" of the Ministry of Education, Culture, Sports, Science, and Technology, Japan.

T.S., H.K., R.U., and T.N. designed the research protocol; T.S., H.K., K.O., A. Tomita, and S.Y. performed the genetic analysis; T.S., H.K., R.S., and T.N. analyzed the data; H.K., K.O., R.S., Y.K., S.M., N.A., K.K., F.Y., C.S., H.A., M.N., T.M., K.S., A. Takeshita, R.U., T.K., N.E., and T.N. collected samples and managed clinical data; and T.S., H.K., and T.N. wrote the paper.

The online version of this article contains a data supplement.

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(NPMc+). Of note is that NPMc+ is associated with a wide spectrum of morphologic subtypes of AML, a normal karyotype, and *FLT3* mutations. Furthermore, NPMc+ AML is clinically associated with better responsiveness to induction chemotherapy, although its prognostic implications for long-term outcome remain unclear. Although the prevalence and significance of several genetic abnormalities in patients with AML have been reported to date, the most powerful prognostic factor in AML has been the karyotype of the leukemia cells.[17] Three cytogenetic risk groups (favorable, intermediate, and poor) are widely accepted, but there is a practical limitation to the definition of cytogenetic risk, especially in patients in the intermediate group. Additional prognostic factors are, therefore, required. We and several groups[9,10,12,18] have demonstrated that *FLT3* mutations are a strong prognostic factor in AML, especially in patients with normal karyotype, but they do not affect responsiveness to induction chemotherapy. Therefore, *NPM1* mutations seem to characterize a distinct disease entity not only of AML with normal karyotype but also of AML with *FLT3* mutations.

NPM is a ubiquitously expressed phosphoprotein, continuously shuttles between the nucleus and the cytoplasm,[19,20] and is involved in the oncogenesis of some types of leukemia and lymphoma because the *NPM* gene is a partner in several tumor-associated chromosomal translocations.[21-23] However, it is also thought to have a tumor-suppressor function and to regulate the p53 pathway through its chaperoning activity.[24-26] It is suggested that a loss of nuclear NPM function caused by mutation might impair the p53 pathway and that a lack of p53 might induce genetic instability[27]; hence, *NPM1* mutations seem to cause AML cells to acquire additional genetic alterations.

In this study, we analyzed the prevalence and clinical characteristics of *NPM1* mutations in comparison with cytogenetics, *FLT3, NRAS,* and *TP53* mutations and a partial tandem duplication of the *MLL* gene (MLL-TD) in 257 patients with newly diagnosed de novo AML. The prognostic implications of *NPM1* mutations were evaluated in 190 patients with AML, excluding those with the M3 FAB subtype, who were treated according to the protocol of the Japan Adult Leukemia Study Group (JALSG). Furthermore, to clarify the stability of *NPM1* mutations and the potential effect on genetic instability in AML cells during disease progression, we compared the mutational status of these genes in 39 paired samples obtained at initial diagnosis and first relapse.

Patients, materials, and methods

Patients and samples

The diagnosis of AML was based on the French-American-British (FAB) classification. The study population included 257 patients with newly diagnosed de novo AML, as follows: 9 with the M0, 54 with the M1, 89 with the M2, 15 with the M3, 54 with the M4, 19 with the M5, 8 with the M6, and 9 with the M7 FAB subtype. In 39 of the 257 AML patients, paired samples obtained at diagnosis and first relapse were available. Furthermore, in 6 patients, samples obtained at diagnosis and complete remission (CR) were available. Bone marrow (BM) samples from patients with AML were subjected to Ficoll-Hypaque (Pharmacia LKB, Uppsala, Sweden) density gradient centrifugation. All samples taken at diagnosis or relapse were confirmed to contain more than 90% leukemia cells after enrichment by centrifugation. Informed consent was obtained from all patients to use their samples for banking and molecular analysis, and approval for these studies was obtained from the Nagoya University institutional review board.

Cytogenetic G-banding analysis was performed according to standard methods. In this study, cytogenetic risk groups were stratified according to the criteria adopted by the Medical Research Council.[28]

Screening for mutations of the *FLT3, NRAS,* and *TP53* genes and of MLL-TD

High molecular weight DNA and total RNA were extracted from the samples using standard methods. *FLT3* gene mutations of the ITD (FLT3/ITD) and activation loop (FLT3/D835Mt), *NRAS* gene mutations of codons 12, 13, and 61, and *TP53* gene mutations of exons 5 to 8 were examined as reported and were confirmed by the sequencing procedure.[4,29,30] MLL-TD was examined by reverse transcription–polymerase chain reaction (RT-PCR), as described previously.[31]

Screening for mutations of the *NPM1* gene

For the screening of *NPM1* mutations, we amplified genomic DNA corresponding to exon 12 of *NPM1* by PCR using the primers NPM1-F, 5′-TTAACTCTCTGGTGGTAGAATGAA-3′ and NPM1-R, 5′-CAAGACTATTTGCCATTCCTAAC-3′, as previously reported.[16] Amplified products were separated through agarose gel, purified using a QIAquick gel extraction kit (Qiagen Inc, Chatsworth, CA), and directly sequenced on a DNA sequencer (310; Applied Biosystems, Foster City, CA) using a BigDye terminator cycle sequencing kit (Applied Biosystems). If mutations were found by direct sequencing, the fragments were cloned into a pGEM-T Easy vector (Promega, Madison, WI), then transfected into the *Escherichia coli* strain DH5α. At least 4 recombinant colonies were selected, and plasmid DNA was prepared using a QIAprep Spin Miniprep Kit (Qiagen Inc) and sequenced.

Analysis of clinical characteristics

It was necessary to analyze the clinical characteristics in a well-documented group. Among the 257 patients analyzed, 15 had acute promyelocytic leukemia (APL). APL has been considered a separate disease entity among AML, and the introduction of all-*trans* retinoic acid (ATRA) has dramatically improved its clinical outcome.[32] In addition, 52 patients with AML, excluding those with APL, were treated with independent regimens. We, therefore, analyzed the clinical characteristics of 190 patients with AML, excluding those with APL, who were treated with the AML87, AML89, and AML92 protocols of JALSG.[33-35] (Each protocol is presented in Document S1; see the Supplemental Document link at the top of the online article, at the *Blood* website.)

Statistical analysis

Differences in continuous variables were analyzed using the Mann-Whitney *U* test for distribution between 2 groups. Analysis of frequencies was performed using the Fisher exact test for 2×2 tables or the Pearson χ^2 test for larger tables. Multivariate analysis to identify risk factors for achieving CR was performed using the logistic regression model. Survival probabilities were estimated by the Kaplan-Meier method, and differences in survival distributions were evaluated using the log-rank test. Overall survival was defined as the time from the first day of therapy to death or last visit. Relapse-free survival was defined as the time from the first day of CR to relapse, death, or last visit. Patients undergoing hematopoietic stem cell transplantation were censored at the time of transplantation. The prognostic significance of the clinical variables was assessed using the Cox proportional hazards model. These statistical analyses were performed with StatView-J 5.0 (Abacus Concepts Inc, Berkeley, CA). For all analyses, the *P* values were 2-tailed, and $P < .05$ was considered statistically significant.

Results

NPM1 mutations were frequently found in AML

We first screened for mutations within exon 12 of the *NPM1* gene through direct sequencing in 257 patients with AML, then confirmed

each type of mutation by cloning. We found the *NPM1* mutation in 64 of 257 (24.9%) patients (Table 1). Importantly, direct sequencing revealed that all AML cells with *NPM1* mutations retained the wild-type allele. Given that all samples contained more than 90% AML cells, all mutations seemed to occur in only one allele. Previously, 6 kinds of mutants, designated mutations A to F, were identified. In this study, we found 49 mutations of type A, 7 mutations of type B, 4 mutations of type D, and 4 novel mutants that were designated mutations G to J (Figure 1). All novel mutations included distinct 4-bp insertions (mutation G, TTTG; mutation H, CTTG; mutation I, TAAG; mutation J, TATG) at position 960, resulting in the same frameshift as mutations A to D. Predicted mutant proteins of mutations G and H and of mutation J were the same as those of mutations A and B, respectively. The protein of mutation I contained a lysine residue at position 289, although the other residues were conserved.

Table 1. Relationship among *NPM1* mutation, FAB type, and genetic alterations in AML

	Total no. patients	*NPM1* Mutation, no. (%)*	*NPM1* Wild type, no. (%)†	*P*
FAB subtype				.005
M0	9	2 (22.2)	7 (77.8)	
M1	54	17 (31.5)	37 (68.5)	
M2	89	12 (13.5)	77 (86.5)	
M3	15	0 (0)	15 (100)	
M4	54	21 (38.9)	33 (61.1)	
M5	19	9 (47.3)	10 (52.7)	
M6	8	2 (25.0)	6 (75.0)	
M7	9	1 (11.1)	8 (88.9)	
Cytogenetics				< .001
t(8;21)	31	0 (0)	31 (100)	
inv(16)	8	1 (12.5)	7 (87.5)	
t(15;17)	15	0 (0)	15 (100)	
t(9;22)	3	1 (33.3)	2 (66.7)	
del(5)	7	0 (0)	7 (100)	
del(7)	5	0 (0)	5 (100)	
Others	43	5 (11.6)	38 (88.4)	
Normal	97	46 (47.4)	51 (52.6)	
Unknown	48	11 (22.9)	37 (77.1)	
FLT3				
Mutations, total	67	39 (58.2)	28 (41.8)	< .001
ITD	58	35 (60.3)	23 (39.7)	< .001‡
D835	9	4 (44.4)	5 (55.6)	.028‡
Wild type	190	25 (13.2)	165 (86.8)	
TP53				NS
Mutation	16	2 (12.5)	14 (87.5)	
Wild type	227	58 (25.6)	169 (74.4)	
Not done	14	5 (35.7)	10 (64.3)	
NRAS				NS
Mutation	34	9 (26.5)	25 (73.5)	
Wild type	202	48 (23.8)	154 (76.2)	
Not done	21	7 (33.3)	14 (66.7)	
MLL-TD				NS
Mutation	17	2 (11.8)	15 (88.2)	
Wild type	130	33 (25.4)	97 (74.6)	
Not done	110	29 (26.4)	81 (73.6)	

Numbers and percentages of 257 patients with AML are shown by FAB type; cytogenetics; and *FLT3, TP53, NRAS,* and MLL-TD mutations, according to *NPM1* mutation.

NS indicates not significant.

*n = 64; 24.9%.

†n = 193; 75.1%.

‡These variables were compared with those of the wild type.

In 2 patients whose leukemia cells had *NPM1* mutations at diagnosis, the mutations were lost at CR, indicating that these were somatic mutations.

Morphologic and genotypic characteristics of AML with *NPM1* mutations

NPM1 mutations were found in patients with AML of all FAB subtypes except M3 (Table 1). Cytogenetic data were available for 209 patients. Consistent with findings of a previous report,[16] the *NPM1* mutation was preferentially found in patients with normal karyotype (46 of 97; 47.4%), but it was not found in patients with t(8;21), t(15;17), del(5), or del(7). In total, there was a significant difference in the frequency of the *NPM1* mutation among patients with (7 of 112; 6.3%) and without (46 of 97; 47.4%) cytogenetic abnormalities ($P < .001$). Moreover, an *NPM1* mutation was found in each of 8 and 3 patients with inv(16) and t(9;22), respectively.

FLT3/ITD and FLT3/D835Mt were found in 58 (22.6%) and 9 (3.5%) of 257 patients, respectively (Table 1). Both *FLT3* mutations were significantly associated with *NPM1* mutations: 35 of 58 (60.3%) FLT3/ITD ($P < .001$) and 4 of 9 (44.4%) FLT3/D835Mt ($P = .028$) were found in the patients with *NPM1* mutations. *TP53*, *NRAS,* and MLL-TD mutations were found in 16 of 243 (6.6%), 34 of 236 (14.4%), and 17 of 147 (11.6%) patients, respectively, although there was no significant correlation between these mutations and the *NPM1* mutation (Table 1).

Clinical characteristics and prognoses of AML patients with or without *NPM1* mutations

Among the 257 patients with AML, 190 patients (excluding those with M3 who were treated with the AML87, AML89, and AML92 protocols of the JALSG) were evaluated for clinical characteristics and initial response to therapy (Table 2). Of these patients, 49 (25.8%) had *NPM1* mutations. The presence of *NPM1* mutations was related neither to sex nor to the occurrence of hepatosplenomegaly or extramedullary involvement. Patients with *NPM1* mutations (median, 58 years; range, 15-77 years) were significantly older than those without mutations (median, 47 years; range, 15-85 years) ($P = .003$). White blood cell (WBC) counts and peripheral blood blast cells were significantly higher in the *NPM1* mutation group than in the wild-type group ($P = .002$ and $P = .029$, respectively). According to the FAB classification, the *NPM1* mutation was infrequent in the M2 subtype ($P = .004$). According to the cytogenetic risk groups, the *NPM1* mutation was preferentially found in the intermediate risk group ($P < .001$). *NPM1* mutations were associated with FLT3/ITD ($P < .001$) and FLT3/D835Mt ($P = .018$), but not with *TP53* and *NRAS* mutations. Because we could analyze MLL-TD in only 118 of the 190 patients, we excluded MLL-TD from the variables for statistical analysis.

Of the 190 patients, 139 (73.2%) achieved CR after induction chemotherapy. The CR rate was significantly higher in the patients with *NPM1* mutations (42 of 49; 85.7%) than without them (97 of 141; 68.8%) ($P = .025$). In addition, Fisher exact test showed that FAB subtypes other than M2, cytogenetic findings other than good risk, and the presence of *NRAS* and *TP53* mutations were unfavorable factors for achieving CR ($P < .001$, $P = .002$, $P = .030$, and $P = .034$, respectively). Age (older than 60), WBC count (more than 100×10^9/L), and presence of the *FLT3* mutation were not associated with the CR rate. Multivariate logistic regression analysis showed that wild-type *NPM1* ($P < .001$), FAB subtypes other than M2 ($P = .008$), and cytogenetic findings other than good

A

Type of mutation	Sequence	Predicted protein	number
Wild type	GATCTCTG····GCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLWQWRKSL	193
Mutation A	GATCTCTGTCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCLAVEEVSLRK	49
Mutation B	GATCTCTGCATGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCMAVEEVSLRK	7
Mutation D	GATCTCTGCCTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCLAVEEVSLRK	4
Mutation G	GATCTCTGTTTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCLAVEEVSLRK	1
Mutation H	GATCTCTGCTTGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCLAVEEVSLRK	1
Mutation I	GATCTCTGTAAGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCKAVEEVSLRK	1
Mutation J	GATCTCTGTATGGCAGTGGAGGAAGTCTCTTTAAGAAAATAG	DLCMAVEEVSLRK	1

B

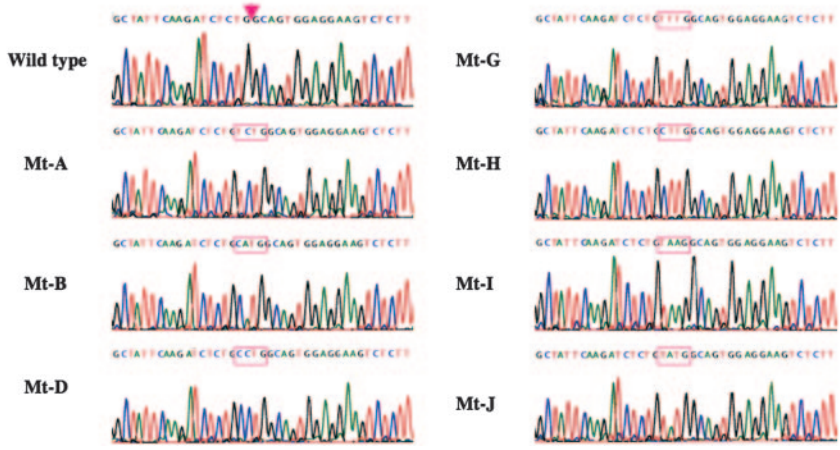

Figure 1. Mutations in NPM1 exon 12. (A) The mutated nucleotide and the predicted amino acid sequence in *NPM1* exon 12 found in the present study are shown in comparison with the wild-type sequence. Type of mutation (mutations A, B, D) is designated according to a previous report. Four novel mutant variants are designated mutations G, H, I, and J. Red and blue indicate nucleotide insertions and termination codons, respectively. Green indicates the conserved residues in mutant NPM. Purple indicates putative residues of a consensus nuclear export signal (Lx[1-3]Vx[2-3]VxL; x indicates any residue). (B) Sequence results after cloning. Boxes indicate inserted 4-bp nucleotides. Arrowhead indicates the position of the insert at nucleotide 960 of the *NPM1* gene.

risk ($P = .039$) were independent unfavorable factors for achieving CR (Table 3).

Kaplan-Meier analyses according to *NPM1* mutation are shown in Figure 2A. Univariate analysis showed that the poor prognostic factors for overall survival were age 60 or older ($P = .001$), mutation of *TP53* ($P < .001$), FAB subtypes other than M2 ($P = .005$), FLT3/ITD ($P = .010$), high WBC count (more than 100×10^9/L) ($P = .019$), and cytogenetic findings (poor vs others) ($P = .024$). Multivariate Cox regression analysis with stepwise selection showed that the mutation of *TP53* (odds ratio, 4.002 [95% confidence interval (CI), 1.876-8.538]; $P < .001$), age 60 or older (odds ratio, 1.651 [95% CI, 1.131-2.410]; $P = .009$), and FAB subtypes other than M2 (odds ratio, 1.643 [95% CI, 1.127-2.396]; $P = .010$) were independent poor prognostic factors for overall survival (Table 4). When adjusted with age 60 years or older, FLT3/ITD, FAB subtype other than M2, high WBC count (more than 100×10^9/L), and mutation of *NRAS*, mutation of *NPM1* was an adverse prognostic factor (odds ratio, 1.949 [95% CI, 1.164-3.268]; $P = .011$). Although careful assessment and further investigation are needed, the mutation of *NPM1* may act as a prognostic factor for overall survival.

Because only 1 of 139 patients who achieved CR died during CR, we compared the probability of relapse between patients with and without *NPM1* mutations (Figure 2A). Univariate analysis showed that the unfavorable factors for relapse were mutation of *NPM1* ($P = .006$), FLT3/ITD ($P = .007$), cytogenetic findings (poor vs others) ($P = .007$), high WBC count (more than 100×10^9/L) ($P = .016$), FAB subtypes other than M2 ($P = .024$), and age 60 or older ($P = .024$). Multivariate Cox regression analysis with stepwise selection identified that cytogenetic findings (poor vs others) (odds ratio, 3.876 [95% CI, 1.718-8.772]; $P = .001$) and mutation of *NPM1* (odds ratio, 2.106 [95% CI, 1.324-3.350]; $P = .002$) were independent unfavorable factors for relapse (Table 5).

In addition, we analyzed the prognostic value of *NPM1* mutations in 79 patients with normal karyotype. *NPM1* mutations were found in 37 of 79 (46.8%) patients. Of these 79 patients, 59 (74.7%) achieved CR after induction chemotherapy. The CR rate was significantly higher in the patients with *NPM1* mutations (32 of 37; 86.4%) than in those without (27 of 42; 64.3%) ($P = .037$ by Fisher exact test). Multivariate logistic regression analysis, including wild-type *NPM1*, FAB subtypes (other than M2), presence of *FLT3, NRAS,* and *TP53* mutations, age (older than 60 years), and WBC count (more than 100×10^9/L) showed that wild-type *NPM1* was the only independent unfavorable factor for achieving CR (odds ratio, 4.908 [95% CI, 1.011-23.824]; $P = .048$). Kaplan-Meier curves according to *NPM1* mutation in this group are shown in Figure 2B. Multivariate Cox regression analysis with stepwise selection showed that age 60 or older was the only poor prognostic factor for overall survival (odds ratio, 2.068 [95% CI, 1.175-3.641]; $P = .012$). Mutation of *NPM1* was not a significant prognostic factor for overall survival regardless of whatever factors were used for adjustment by means of Cox analysis. The relapse risk was analyzed in 59 patients who achieved CR. Multivariate Cox regression analysis with stepwise selection identified that the *NPM1* mutation was the only independent unfavorable factor for relapse (odds ratio, 2.096 [95% CI, 1.050-4.186]; $P = .036$). However, because the patient number of this group was small, larger-scale analysis was required.

FLT3/ITD has been identified as an unfavorable prognostic factor in patients with AML, although the prognostic implications of FLT3/D835Mt remain unclear. Given that the *NPM1* mutation was associated with FLT3/ITD, it was important to define the role of *NPM1* mutations alone or in combination with FLT3/ITD for long-term prognosis. Therefore, we compared the clinical impact of *NPM1* mutations in patients with and without FLT3/ITD. Interestingly, mutation of *NPM1* was an independent favorable prognostic factor for CR in patients with FLT3/ITD (odds ratio, 20.8 [95% CI, 2.0-200]; $P = .011$ by multivariate logistic regression analysis), but it did not affect the CR rate in patients without FLT3/ITD. In addition, mutation of *NPM1* was a favorable prognostic factor for overall survival in patients with FLT3/ITD ($P = .045$), but not in those without FLT3/ITD. However, mutation

Table 2. Characteristics of 190 patients with AML, excluding patients with the M3 subtype, treated according to JALSG protocols

		NPM1		
	Total N = 190	Mutation, n = 49	Wild type, n = 141	*P*
Age, y	50 (15-85)	58 (15-77)	47 (15-85)	.003
WBC count, × 10^9/L	24.5 (0.9-372)	52.2 (1.0-372)	23.3 (0.9-337.6)	.002
PB blast count, × 10^9/L	14.9 (0.017-357)	29.6 (0.093-357)	12.6 (0.017-324)	.029
FAB subtype				.004
M0	3	1	2	
M1	44	12	32	
M2	78	10	68	
M4	46	16	30	
M5	14	8	6	
M6	4	1	3	
M7	1	1	0	
Cytogenetics				< .001
Favorable	33	1	32	
t(8;21)	27	0	27	
inv(16)	6	1	5	
Intermediate	119	42	77	
Normal	79	37	42	
Others	40	5	35	
Poor	14	1	13	
t(9;22)	3	1	2	
del(5) or del(7)	11	0	11	
Unknown	24	5	19	
Outcome				.025
CR	139	42	97	
Failure	51	7	44	
FLT3				
Mutation, total	51	31	20	< .001
ITD mutation	43	27	16	< .001*
D835 mutation	8	4	4	.018*
Wild type	139	18	121	
P53				NS
Mutation	8	1	7	
Wild type	182	48	134	
NRAS				NS
Mutation	26	8	18	
Wild type	164	41	123	

Clinical characteristics of the 190 patients with AML, excluding those with the M3 subtype, who were treated according to the JALSG protocols, are shown. Median values (with range in parentheses) are indicated for age, WBC count, and PB blast count. Numbers of patients are shown by FAB type, cytogenetics, outcome, and gene mutation. The favorable risk group was defined by t(8;21) or inv(16); the poor risk group by t(9;22), del(5), or del(7); and the intermediate risk group by normal or other karyotypes.

PB indicates peripheral blood; NS, not significant.

*Compared with wild-type variables.

of *NPM1* was not associated with the high relapse rate in patients with FLT3/ITD, whereas it tended to be a worse factor for relapse in those without FLT3/ITD ($P = .084$) (Figure 3).

Comparison of mutational status of the *NPM1* gene at diagnosis and relapse

Mutational status of the *NPM1* and *FLT3* genes was compared between diagnosis and relapse in 39 AML patients. *NRAS* and *TP53*

Table 3. Logistic regression analysis results of the effect of unfavorable factors for CR in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Variable	*P*	Odds ratio	95% CI
NPM1, wild type	< .001	8.434	2.562-27.77
FAB, other than M2	.008	3.247	1.353-7.813
Cytogenetic, other than good risk	.039	5.240	1.091-25.16
NRAS, mutation	.056	2.667	0.978-7.299
TP53, mutation	.058	6.024	0.941-38.46
WBC count, more than 100 × 10^9/L	.090	2.427	0.872-6.757
FLT3, mutation	.279	1.719	0.645-4.583
Age, older than 60 y	.960	2.427	0.446-2.340

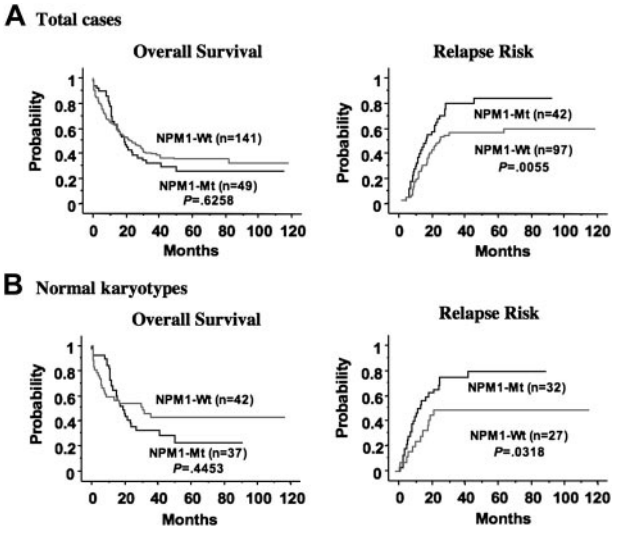

Figure 2. Kaplan-Meier curves according to NPM1 mutation. (A) Overall survival and relapse risk in all patients. (B) Overall survival and relapse risk in patients with normal karyotype. Statistical difference was evaluated with the log-rank test.

Table 4. Unfavorable prognostic factors for overall survival in 190 patients, excluding those with the M3 subtype, treated according to JALSG protocols

Prognostic factors	*P*	Odds ratio	95% CI
TP53 mutation	.003	4.002	1.876-8.538
Age, older than 60 y	.009	1.651	1.131-2.410
FAB, other than M2	.010	1.643	1.127-2.396

mutations were also analyzed in 20 and 29 patients, respectively (Table 6). An *NPM1* mutation was found in 17 of 39 patients at diagnosis. Of the 17 patients with *NPM1* mutations at diagnosis, 15 carried the same mutation at relapse, although 2 patients (unique patient number [UPN] 16 and UPN 17) lost the mutation at relapse. In UPN 16, the karyotype of leukemia cells was normal (46XY) at diagnosis, although it changed to 46XYdel(20)(q1?) at relapse. In contrast, *FLT3, NRAS,* and *TP53* were of the wild type at both stages. In UPN 17, leukemia cells showed normal karyotype (46XX), FLT3/ITD, and wild-type *NRAS* and *TP53* mutations at diagnosis, and these were retained at relapse.

An *FLT3* mutation was found in 11 of 39 patients at diagnosis, and all mutations were FLT3/ITD. The *FLT3* mutation was lost at relapse in 1 patient, but it emerged at relapse in 4 patients who did not have the *FLT3* mutation at diagnosis. An *NRAS* mutation was found in 5 of 20 patients at diagnosis. The *NRAS* mutation was lost at relapse in 2 patients, but it emerged at relapse in 1 patient. A *TP53* mutation was found in 3 of 28 patients at diagnosis. The *TP53* mutation was lost at relapse in 1 patient, but it emerged at relapse in 1 patient. Taken together, in 11 of 39 (28.2%) patients, the gene status of *NPM1, FLT3, NRAS,* and *TP53* was different at diagnosis than it was at relapse (Table 6). However, *NPM1* gene status was relatively stable, in contrast to that of *FLT3*, and was not related to inducing a genotypic change in leukemia cells at relapse. In addition, age and CR duration were not associated with the genotypic change of leukemia cells at relapse. Importantly, the *NPM1* mutation was not associated with achieving second CR after first CR.

Discussion

In this study, we found *NPM1* mutations in 64 of 257 (24.9%) adult patients with de novo AML. Of the 64 *NPM1* mutations, 60 were variants reported previously, and 4 were novel. All the novel variants had a 4-bp insertion at position 960, resulting in the same frameshift as previously reported. The C-terminal of NPM is important to the nuclear localization of NPM,[36] and tryptophan residues at positions 288 and 290 are reportedly associated with the nucleolar localization of NPM.[37] Interestingly, predicted mutant proteins contain a nuclear export signal (NES) motif at the C-terminal, which may be a reason for the cytoplasmic dislocation of mutant NPM.[38,39] All novel variants found in the present study also contained the NES motif. Therefore, we believe that the generation of the NES motif by the insertion mutation is strongly associated with the cytoplasmic dislocation of mutant NPM.

Table 5. Unfavorable prognostic factors for relapse in 139 patients with AML, excluding those with the M3 subtype, who achieved CR

Prognostic factors	*P*	Odds ratio	95% CI
Poor cytogenetics	.001	3.876	1.718-8.772
NPM1 mutation	.002	2.106	1.324-3.350

FLT3, TP53, and *NRAS* mutations are the most frequent genetic alterations involved in the pathogenesis of AML. It was reported that the *NPM1* mutation is closely associated with FLT3/ITD. We found that it is also associated with FLT3/D835Mt, but a large-scale study is necessary to confirm this association because of the small number of patients with FLT3/D835Mt in this study. It is noteworthy that the *NPM1* mutation was not associated with *TP53, NRAS,* and *MLL-TD* mutations. In fact, the *NPM1* mutation was found in only 2 of 16 patients with the *TP53* mutation, although this was not statistically significant. Because the *TP53* mutation is known to be present in patients with karyotypic abnormalities,[40,41] the *NPM1* mutation seems to be infrequent in this group. The *NRAS* mutation is negatively related to FLT3/ITD but is found in all patients with AML, regardless of karyotypic abnormalities.[6,42] In contrast, a possible association between MLL-TD and FLT3/ITD was reported.[43] Therefore, to identify factors associated with *NPM1* mutations, we performed multivariate logistic regression analysis that included the presence or absence of *FLT3, TP53, NRAS,* and *MLL-TD* mutations and karyotypic abnormality in 119 patients in whom all these genetic alterations were determined. The analysis showed that the normal karyotype ($P < .001$; odds ratio, 26.0 [95% CI, 6.231-108.73]) and the *FLT3* mutation ($P < .001$; odds ratio, 15.2 [95% CI, 3.690-62.50]) were independently associated with *NPM1* mutation. These results suggested that the *NPM1* mutation might be involved in the pathogenesis of AML with and without *FLT3* mutations.

The most important finding in the present study is that in patients with AML, excluding those with the M3 subtype, the *NPM1* mutation is a favorable factor for achieving CR after induction chemotherapy, but it implicates a high relapse rate. FLT3/ITD is clinically demonstrated to be a poor prognostic factor in patients with AML, especially those in the intermediate-risk group. FLT3/ITD confers autonomous proliferation to hematopoietic progenitors through its constitutive kinase activity, though alone it is not sufficient for the development of AML. In the murine APL model, the transduction of FLT3/ITD into *PML-RARA* transgenic bone marrow cells resulted in a shortened latency with

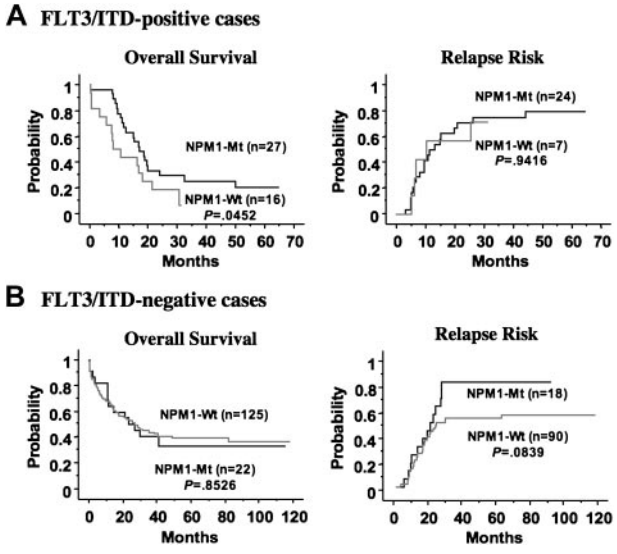

Figure 3. Kaplan-Meier curves according to NPM1 mutation in the FLT3/ITD-positive and -negative patients. (A) In the FLT3/ITD-positive group, the *NPM1* mutation was a favorable prognostic factor for overall survival, but did not affect the relapse risk. (B) In the FLT3/ITD-negative group, the *NPM1* mutation did not affect overall survival. The *NPM1* mutation tended to be a worse factor for relapse in this group, although it was not statistically significant. Statistical difference was evaluated with the log-rank test.

Table 6. Comparisons of mutations between diagnosis and relapse

					NPM1		*FLT3*		*NRAS*		*TP53*		
UPN	Age, y	Sex	FAB	CR, d	D	R	D	R	D	R	D	R	2nd CR
1	56	M	M4	255	Mt	Mt	Mt	Mt	WT	WT	WT	WT	No
2	38	F	M2	574	Mt	Mt	Mt	Mt	WT	WT	WT	WT	Yes
3	62	M	M1	328	Mt	Mt	Mt	Mt	ND	ND	WT	WT	Yes
4	55	M	M5	408	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
5	15	F	M2	181	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
6	62	F	M1	521	Mt	Mt	Mt	Mt	ND	ND	WT	WT	No
7	50	M	M2	264	Mt	Mt	Mt	Mt	ND	ND	ND	ND	Yes
8	66	M	M1	608	Mt	Mt	*Mt*	*WT*	WT	WT	WT	WT	Yes
9	67	F	M4	483	Mt	Mt	*WT*	*Mt*	WT	WT	WT	WT	Yes
10	57	F	M1	603	Mt	Mt	WT	WT	*Mt*	*WT*	WT	WT	Yes
11	54	M	M1	175	Mt	Mt	WT	WT	WT	WT	WT	WT	No
12	53	F	M1	214	Mt	Mt	WT	WT	WT	WT	WT	WT	No
13	54	M	M4	1026	Mt	Mt	WT	WT	ND	ND	ND	ND	Yes
14	53	M	M4	542	Mt	Mt	WT	WT	ND	ND	ND	ND	No
15	70	M	M1	773	Mt	Mt	WT	WT	ND	ND	WT	WT	No
16	64	M	M4	489	*Mt*	*WT*	WT	WT	WT	WT	WT	WT	No
17	44	F	M0	147	*Mt*	*WT*	Mt	Mt	WT	WT	Mt	Mt	No
18	59	M	M1	222	WT	WT	Mt	Mt	ND	ND	ND	ND	No
19	55	M	M5	128	WT	WT	Mt	Mt	ND	ND	ND	ND	Yes
20	16	M	M4	308	WT	WT	*WT*	*Mt*	Mt	Mt	WT	WT	No
21	34	F	M4	370	WT	WT	*WT*	*Mt*	Mt	Mt	WT	WT	No
22	56	F	M4	161	WT	WT	*WT*	*Mt*	WT	WT	*Mt*	*WT*	No
23	29	M	M0	361	WT	WT	WT	WT	Mt	Mt	WT	WT	No
24	26	F	M4	430	WT	WT	WT	WT	*Mt*	*WT*	WT	WT	Yes
25	48	M	M2	525	WT	WT	WT	WT	*WT*	*Mt*	WT	WT	Yes
26	74	F	M1	147	WT	WT	WT	WT	WT	WT	*WT*	*Mt*	Yes
27	44	F	M1	137	WT	WT	WT	WT	WT	WT	WT	WT	No
28	39	F	M2	200	WT	WT	WT	WT	WT	WT	WT	WT	Yes
29	22	M	M1	168	WT	WT	WT	WT	WT	WT	WT	WT	Yes
30	68	M	M2	208	WT	WT	WT	WT	WT	WT	WT	WT	No
31	23	M	M5	133	WT	WT	WT	WT	ND	ND	Mt	Mt	No
32	62	M	M2	271	WT	WT	WT	WT	ND	ND	WT	WT	No
33	55	F	M2	281	WT	WT	WT	WT	ND	ND	WT	WT	Yes
34	32	M	M1	257	WT	WT	WT	WT	ND	ND	ND	ND	No
35	60	F	M5	429	WT	WT	WT	WT	ND	ND	ND	ND	Yes
36	47	M	M2	435	WT	WT	WT	WT	ND	ND	ND	ND	Yes
37	19	M	M2	389	WT	WT	WT	WT	ND	ND	ND	ND	Yes
38	58	M	M3	412	WT	WT	WT	WT	ND	ND	ND	ND	Yes
39	44	F	M4	266	WT	WT	WT	WT	ND	ND	ND	ND	Yes

Mutational status of *NPM1, FLT3, NRAS,* and *TP53* mutations was analyzed in 39 paired samples obtained at diagnosis and relapse. Italics in data field indicate a change in mutational status.

D indicates diagnosis; R, relapse; Mt, mutation; WT, wild type; ND not done.

increasing penetration of APL-like disease.[44] The *NPM1* mutation is essentially absent in patients with APL or CBF leukemia, in contrast to the high frequency of FLT3/ITD and *KIT* mutations in patients with APL and CBF leukemia, respectively. Only the mutation of *NPM1* was an independent favorable prognostic factor for achieving CR and a marginally favorable prognostic factor for overall survival in patients with FLT3/ITD. However, it did not affect the CR rate, and its prognostic value for relapse was not significant in patients without FLT3/ITD. Mutant NPM, therefore, might be involved in the pathogenesis of AML by its conferring a differentiation block or properties of self-renewal, or both, to the hematopoietic progenitors in concert with mutant FLT3, and it might be associated with sensitivity to chemotherapy. More recently, it was reported that several genes putatively involved in the maintenance of a stem cell phenotype, such as *HOX* and *JAG1* genes, were up-regulated in NPMc+ AML cells,[45] supporting this hypothesis. However, the exact role of mutant NPM in the pathogenesis of AML, especially without FLT3/ITD, has not been fully resolved. Our sequential analysis using the paired samples obtained at diagnosis and relapse demonstrated that the *NPM1* mutation is not related to acquiring additional genetic changes in *FLT3, TP53,* and *NRAS* genes. In addition, the *NPM1* mutation was not a favorable factor for achieving second CR after relapse, suggesting that unknown genetic or epigenetic changes might additionally occur in AML cells with the *NPM1* mutation during disease progression. It is notable that *NPM1* mutations were lost at relapse in 2 of the 17 patients who had *NPM1* mutations at initial diagnosis. Loss of the mutation at relapse has been repeatedly observed in the mutations of *FLT3* and *NRAS*, and these mutations are thought of as late or secondary events in leukemogenesis. Loss of the *NPM1* mutation at relapse suggests that it is not necessarily an earlier event and that it is not needed for continuance of the disease. However, because NPM is involved in ribosome assembly/transport, cytoplasmic/nuclear trafficking, regulation of DNA polymerase alpha activity, centrosome duplication, acute response of mammalian cells to environmental stress, and stabilization of the p53 pathway,[25,26,46-48] it is possible that mutant NPM also has a multifunctional role in the pathogenesis of AML. Alternatively,

mutant NPM might use its multiple functions according to the genetic state of leukemia progenitors. Further biologic studies are necessary to clarify how mutant NPM is involved in the pathogenesis of AML and what kinds of genetic or epigenetic alterations cooperate with mutant NPM for the development of AML.

Acknowledgments

This study was performed in cooperation with JALSG. We thank Manami Kira for secretarial assistance.

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2005 106: 2854-2861
doi:10.1182/blood-2005-04-1733 originally published online June 30, 2005

Clinical characteristics and prognostic implications of *NPM1* mutations in acute myeloid leukemia

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Blood (print ISSN 0006-4971, online ISSN 1528-0020), is published weekly by the American Society of Hematology, 2021 L St, NW, Suite 900, Washington DC 20036.