Regular Article

MYELOID NEOPLASIA

High *NPM1*-mutant allele burden at diagnosis predicts unfavorable outcomes in de novo AML

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KEY POINTS

- **High *NPM1*-mutant allele burden at diagnosis is associated with poor clinical outcome in de novo AML.**
- **The adverse effect of high *NPM1*-mutant allele burden is independent of comutations and clinical variables.**

Acute myeloid leukemia (AML) with mutated *NPM1* is a newly recognized separate entity in the revised 2016 World Health Organization classification and is associated with a favorable prognosis. Although previous studies have evaluated *NPM1* in a binary fashion, little is known about the significance of its mutant allele burden at diagnosis, nor has the effect of comutations (other than *FLT3*) been extensively evaluated. We retrospectively used targeted sequencing data from 109 patients with de novo AML with mutated *NPM1* to evaluate the potential significance of *NPM1* variant allele frequency (VAF), comutations, and clinical parameters with regard to patient outcomes. We observed that high *NPM1* VAF (uppermost quartile) correlated with shortened overall survival (median, 12.1 months vs not reached; *P* < .0001) as well as event-free survival (median, 7.5 vs 65.44 months; *P* < .0001) compared with the other *NPM1*-mutated cases. In both univariate and multivariable analyses, high *NPM1* VAF had a particularly adverse prognostic effect in the subset of patients treated with stem-cell transplantation in first remission (*P* = .0004) and in patients with mutated *DNMT3A* (*P* < .0001). Our findings indicate that the prognostic effect of *NPM1* mutation in de novo AML may be influenced by the relative abundance of the mutated allele. (*Blood*. 2018;131(25):2816-2825)

Introduction

Acute myeloid leukemia (AML) with mutated nucleophosmin 1 (*NPM1*) gene is recognized as a separate entity in the revised 2016 World Health Organization (WHO) classification of myeloid neoplasms, after previous inclusion as a provisional entity in the 2008 WHO classification.[1] *NPM1* is one of the most commonly observed mutations in AML; it is detected in ~30% of all cases and in 50% to 60% of those with normal cytogenetics.[1-6] Although AML with mutated *NPM1* is considered a distinct entity,[1] *NPM1* mutation alone seems to be insufficient for leukemogenesis, and it typically occurs in association with founder mutations such as *DNMT3A*.[7-9] Similarly, internal tandem duplication (ITD) mutations in *FLT3* are twice as frequent in *NPM1*-mutant AML as compared with AML with wild-type *NPM1*.[10-14] *NPM1* mutation is generally associated with a favorable clinical outcome; however, *FLT3*-ITD comutation has been shown to diminish the favorable *NPM1* effect,[15] particularly in the presence of mutant *DNMT3A*.[7] Conversely, RAS pathway comutations may positively influence outcome of AML with mutated *NPM1*.[16]

Among patients with AML with *FLT3*-ITD, a high mutational burden using a polymerase chain reaction–based assay has been associated with an inferior survival in *NPM1*-mutated AML.[17] However, assessment of the *FLT3*-ITD–mutated allele burden is not currently performed in many centers. Conversely, the influence of *NPM1* mutational load on outcome has yet to be investigated, particularly within the setting of *FLT3*-ITD and/or *DNMT3A* comutation. Previous mutational landscape–focused studies have evaluated *NPM1* in a binary fashion, without emphasis on quantitative mutational burden at the time of diagnosis.

Patients with *NPM1*-mutated AML without *FLT3*-ITD, or with a low *FLT3*-ITD allelic burden, comprise a prognostically favorable subgroup and are often treated with induction chemotherapy and an intensive chemotherapy-based consolidation regimen rather than allogeneic stem-cell transplantation (SCT). The decision to perform SCT in first complete remission (CR1) is complex, and even *NPM1*-mutated/*FLT3*-ITD–low or *FLT3* wild-type patients may be referred for SCT based on older age or presence of unfavorable comutations, adverse cytogenetics, or measurable residual disease (MRD).[18-20] Nonetheless, outcomes in patients who have detectable *NPM1* mutations after chemotherapy are poor, even when treated with subsequent SCT.[19-21] It remains unclear whether MRD negativity after induction chemotherapy can obviate the need for SCT in a patient whose disease exhibits unfavorable biology at diagnosis or whether MRD positivity is

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Table 1. Characteristics of patients with de novo AML with mutated *NPM1* (N = 109)

	All patients (N = 109)	*NPM1* VAF ≤ 0.43 (n = 85)	*NPM1* VAF ≥ 0.44 (n = 24)
Patient characteristics			
Median age (range), y	60 (15-83)	60 (19-75)	63 (15-83)
Male/female ratio	0.84	0.79	1
Clinical parameters			
Median WBC (range), $\times 10^9$/L	24.0 (0.8-340)	22.8 (0.8-340)	44.3 (1.5-309)
Median PB blasts (range), %	26 (0-98)	26 (0-97)	48 (1-98)
Median BM blasts (range), %	71 (21-96)	73 (21-95)	70 (29-96)
Abnormal cytogenetics, N (%)	14 (13)	10 (12)	4 (17)
Median *NPM1* VAF (range)	0.39 (0.04-0.54)	0.38 (0.04-0.43)	0.46 (0.44-0.54)
FLT3-ITD positive, N (%)	42 (39)	31 (36)	11 (46)
Comutations by pathway, N (%)			
DNA methylation			
DNMT3A	55 (50)	45 (53)	10 (42)
IDH1	24 (22)	17 (20)	7 (29)
IDH2	12 (11)	11 (13)	1 (4)
TET2	29 (27)	26 (31)	3 (13)
Epigenetic regulation			
ASXL1	2 (2)	2 (2)	0 (0)
EZH2	0 (0)	0 (0)	0 (0)
BCOR	2 (2)	2 (2)	0 (0)
SETBP1	0 (0)	0 (0)	0 (0)
BCORL	1 (1)	1 (1)	0 (0)
SH2B3	2 (2)	1 (1)	1 (4)
SETD2	0 (0)	0 (0)	0 (0)
CREBBP	1 (1)	1 (1)	0 (0)
Transcriptional regulation			
WT1	7 (7)	5 (6)	2 (9)
PHF6	1 (1)	1 (1)	0 (0)
CEBPA	5 (5)	2 (2)	3 (13)
RUNX1	1 (1)	1 (1)	0 (0)
ETV6	0 (0)	0 (0)	0 (0)
Cohesin complex			
STAG2	2 (2)	1 (1)	1 (4)
PDS5B	2 (2)	1 (1)	1 (4)
RAD21	4 (4)	3 (4)	1 (4)
RAS pathway			
KRAS	4 (4)	4 (5)	0 (0)
NRAS	26 (24)	23 (27)	3 (13)
FLT3 (non-ITD)	26 (24)	19 (23)	7 (29)
KIT	1 (1)	0 (0)	1 (4)
CBL	3 (3)	2 (2)	1 (4)
RIT1	4 (4)	3 (4)	1 (4)
PTPN11	26 (24)	23 (27)	3 (13)
NF1	3 (3)	3 (4)	0 (0)
JAK2	0 (0)	0 (0)	0 (0)
Spliceosome			
U2AF1	0 (0)	0 (0)	0 (0)
ZRSR2	0 (0)	0 (0)	0 (0)
PRPF40b	0 (0)	0 (0)	0 (0)
SRSF2	5 (5)	4 (5)	1 (4)
SF3B1	0 (0)	0 (0)	0 (0)

BM, bone marrow; PB, peripheral blood.

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Table 1. (continued)

	All patients (N = 109)	*NPM1* VAF ≤ 0.43 (n = 85)	*NPM1* VAF ≥ 0.44 (n = 24)
Other			
CSF3R	2 (2)	1 (1)	1 (4)
BRAF	0 (0)	0 (0)	0 (0)
GATA2	1 (1)	0 (0)	1 (4)
TP53	0 (0)	0 (0)	0 (0)
Outcome, N (%)			
Relapsed	39 (36)	24 (29)	15 (63)
Alive at last follow-up	73 (67)	62 (74)	10 (42)
Underwent SCT, N (%)	59 (54)	49 (58)	10 (42)
SCT in CR1	45 (41)	38 (45)	7 (29)
Conditioning (all SCTs)			
Reduced intensity	36 (61)	32 (65)	4 (40)
Myeloablative	23 (39)	17 (35)	6 (60)
SCT type (all SCTs), N (%)			
Matched related donor	18 (31)	14 (29)	4 (40)
Matched unrelated donor	31 (53)	27 (55)	4 (40)
Mismatched unrelated donor	3 (5)	3 (6)	0 (0)
Haploidentical	5 (8)	3 (6)	2 (20)
Cord blood	2 (3)	2 (4)	0 (0)

BM, bone marrow; PB, peripheral blood.

truly an indication for SCT in a patient with good-risk disease. Moreover, sensitive *NPM1* MRD assessment is a specialized test that is not available in most centers at the current time. Thus, additional independent predictive markers at diagnosis may still be useful in AML with mutated *NPM1*.

We examined patient outcomes in a series of patients with *NPM1*-mutated de novo AML treated with induction chemotherapy, with or without subsequent SCT, focusing on the influence of commonly occurring comutations and *NPM1* variant allele frequency (VAF) at the time of diagnosis.

Methods

Case selection

After institutional review board approval, we identified 109 cases of newly diagnosed de novo AML with mutated *NPM1* from the pathology archives of Brigham and Women's Hospital/Dana-Farber Cancer Institute and Massachusetts General Hospital (2008-2017) that fulfilled 2016 WHO classification criteria for AML with mutated *NPM1*; thus, we excluded patients who had received prior cytotoxic therapy, carried a prior diagnosis of any myeloid neoplasm, or had WHO-defined recurrent cytogenetic abnormalities. All patients were treated with standard anthracycline-cytarabine induction chemotherapy, with or without subsequent allogeneic SCT.

NGS studies

For all patients meeting inclusion criteria, we reviewed data from targeted sequencing studies performed on bone marrow aspirates (n = 93) or peripheral blood specimens (n = 16) at the time of diagnosis, as previously described.[22-24] Target regions of 87 genes (hybrid capture system; Agilent Technologies, Santa Clara, CA) were evaluated in 46 patients, 95 genes (rapid heme panel [RHP]; Illumina TruSeq custom amplicon kit; San Diego, CA) in 56 patients, and 54 genes (Massachusetts General Hospital; SNaPshot; Illumina TruSeq) in 7 patients; genes were selected based on pathogenic involvement in myeloid malignancies. Genes assessed in all platforms were *NPM1*, *FLT3*, *DNMT3A*, *IDH1*, *IDH2*, *TET2*, *ASXL1*, *EZH2*, *BCOR*, *SETBP1*, *BCORL*, *SH2B3*, *SETD2*, *CREBBP*, *WT1*, *PHF6*, *CEBPA*, *RUNX1*, *ETV6*, *STAG2*, *PDS5B*, *RAD21*, *KRAS*, *NRAS*, *KIT*, *CBL*, *RIT1*, *PTPN11*, *NF1*, *JAK2*, *U2AF1*, *ZRSR2*, *PRPF40b*, *SRSF2*, *SF3B1*, *CSF3R*, *BRAF*, *GATA2*, and *TP53*. Variant calls were made using minimum criteria of either 10 variant reads with VAF >2% or 5 to 9 variant reads with VAF >33% and a minimum total read depth of 50. All cases were evaluated for average read count across all amplicons and also for the read count and VAF of *NPM1*. We classified variants as pathogenic mutations based on mutation type, position, and frequency in publicly available single-nucleotide polymorphism databases. Mutations were additionally segregated by pathway as previously described[25]: DNA methylation, epigenetic regulation, transcription factor, cohesin complex, RAS, and spliceosome. *FLT3*-ITD was detected by either a sizing assay based on polymerase chain reaction amplification followed by fragment analysis capillary electrophoresis or a next-generation sequencing (NGS)–based methodology (RHP).[24] *FLT3*-ITD mutation load was segregated into high (>50% VAF) or low (<50%) level for the subset cases in which these data were available.

Statistical analyses

We performed multivariable linear regression for the following variables with respect to *NPM1* VAF: total number of mutations, presence or absence of a subclone, *DNMT3A*, *NRAS*/*KRAS*,

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Table 2. Clinical rationale for SCT in CR1 (n = 45)

Clinical rationale	N of patients (%)
Age >60 y	17 (38)
FLT3-ITD	25 (56)
Abnormal cytogenetics	3 (7)
Unfavorable molecular profile	6 (13)
Extramedullary disease	3 (7)

Some cases had multiple clinical rationales.

PTPN11, *TET2*, *IDH1*/*IDH2*, *FLT3* mutations other than ITD, *FLT3*-ITD and *DNMT3A* and *FLT3*-ITD comutations (genes mutated and gene combinations comutated in >10% of the cohort), highest VAF value of all mutations for each case, age, sex, white blood cell (WBC) count, hemoglobin, platelet count, bone marrow cellularity, bone marrow blast percentage, peripheral blood blast percentage, and karyotype (normal vs abnormal).

We evaluated patients' overall survival (OS) and event-free survival (EFS), defined as previously reported.[23,26] Briefly, OS was defined as the time in months from the date of diagnosis to last follow-up or death, and EFS was defined as the time in months from the date of diagnosis to relapse, death, or last follow-up (the latter was used as the end point in those who did not relapse). We examined the following variables: age, sex, WBC count, hemoglobin, platelet count, bone marrow cellularity, bone marrow blast percentage, peripheral blood blast percentage (all as continuous variables), karyotype (normal vs abnormal), SCT in CR1 status, *NPM1* VAF assessed in quartiles and as a continuous variable, *FLT3*-ITD status, and mutation status of any genes mutated or gene combinations comutated in >10% of the cohort. Univariate analysis (log-rank test) followed by multivariable analysis (Cox proportional hazards model) was performed for OS and EFS for the entire cohort as well as for EFS of the entire cohort censoring patients at the time of SCT. The same variables were also analyzed for effect on EFS from the time of diagnosis in the subset of patients undergoing SCT in CR1. Statistical analyses were performed using XLSTAT (version 2017.5) and Prism 7.0c (GraphPad) software packages.

Results

Patient characteristics

We identified 109 patients with AML with *NPM1* mutations (male/female ratio, 0.84) who met inclusion criteria, with a median age of 60 years (range, 15-83 years) and median follow-up time of 18.1 months for all patients. All patients were treated with standard induction chemotherapy, and 59 (54%) underwent SCT, including 45 (41%) in CR1, before any AML relapse (Table 1). In the patients undergoing SCT in CR1, the SCT occurred a median of 3.7 months after AML diagnosis (range, 1.4-12.4 months). Clinical reasons for SCT varied and are shown in Table 2. Thirty-nine patients (36%) relapsed, and 73 (67%) were alive at last follow-up. Of note, 57% of the *FLT3*-ITD$^+$ patients received *FLT3*-inhibitor therapy before any relapse (sorafenib, n = 14; midostaurin, n = 7; quizartinib, n = 1; gilteritinib, n = 1; crenolanib, n = 1).[7]

Cytogenetic and molecular characteristics

Most patients (95 [87%] of 109) had a normal karyotype; the karyotype abnormalities are listed in Table 3. The *NPM1* read count (total depth) was >100 in 98% of cases, with 2 cases having <100 reads (64 and 81 total reads, respectively). The median for average coverage across all tested loci was 1222 (range, 161-4606). The median VAF for *NPM1* was 0.39 (range, 0.04-0.54; supplemental Table 1, available on the *Blood* Web site). We did not identify any effect of *NPM1* read count on *NPM1* VAF (P = .86). There was no significant difference in *NPM1* VAF measured between the 3 testing platforms (median, 0.39 [n = 46], 0.395 [n = 56], and 0.42 [n = 7] for hybrid capture, RHP, and SNaPshot, respectively; Kruskall-Wallis P = .71) or between blood (median, 0.395 [n = 16]) and bone marrow (median, 0.39 [n = 93]) samples (Mann-Whitney P = .45). *NPM1* VAF exhibited no significant correlation with patient age (P = .37) but correlated positively with WBC (r = 0.26; P = .005), peripheral blast percentage (r = 0.29; P = .002), and percentage of marrow blasts (r = 0.25; P = .008).

The total mutation count per case ranged from 1 to 8 (including the *NPM1* mutations, which were present in all cases), with a median of 4. *NPM1* was the sole mutation in a small subset of cases (2 [1.9%] of 109). The most common cooccurring mutations included *DNMT3A* (50%), *FLT3*-ITD (39%), *TET2* (27%), *FLT3* non-ITD (24%), *NRAS* (24%), and *PTPN11* (24%), similar to previous reports.[7] DNA methylation pathway mutations were overall the most frequent (78%), followed by RAS pathway mutations including *FLT3*-ITD (74%), other RAS pathway mutations (44%), and epigenetic regulation (8%), cohesin complex (7%), transcriptional regulation (7%), and spliceosome (5%) pathway mutations (Figure 1; Table 1).

Table 3. Cases with abnormal cytogenetics (n = 14)

Case	Karyotype
1	46,XX, t(1;13)(q42;q14)[19]/46,XX[1]
2	47,XX,+mar[13]/48,idem,+21[7]
3	47,XY,+8[13]/46,XY[7]
4	46,XY,der(1)t(1;2)(p34;q1?3)[5]/45,idem,10[10]/46,XY[5]
5	47,XY,+4[3]/46,XY[17]
6	46,XY,del(3)(q21),add(9)(p22)[2]/46,XY[18]
7	48,XX,+4,+8[17]/46,XX[3]
8	47,XX,+4[19]/46,XX[1]
9	46,XY,-6,+r[cp17]/46,XY[3]
10	47,XY,+8[7]/48,idem,+5[2]/46,XY[cp11]
11	46,XX,i(21)(q10)[6]/46,XX,idem,?inv(20)(p11.2q12)[6]/46,XX[8]
12	46,XY,t(3;18)(q26;q21),del(6)(q13q26)[18]/46,XY[2]
13	46,XX,del(9)(q12q22)[20]
14	47,XX,+8 [20]

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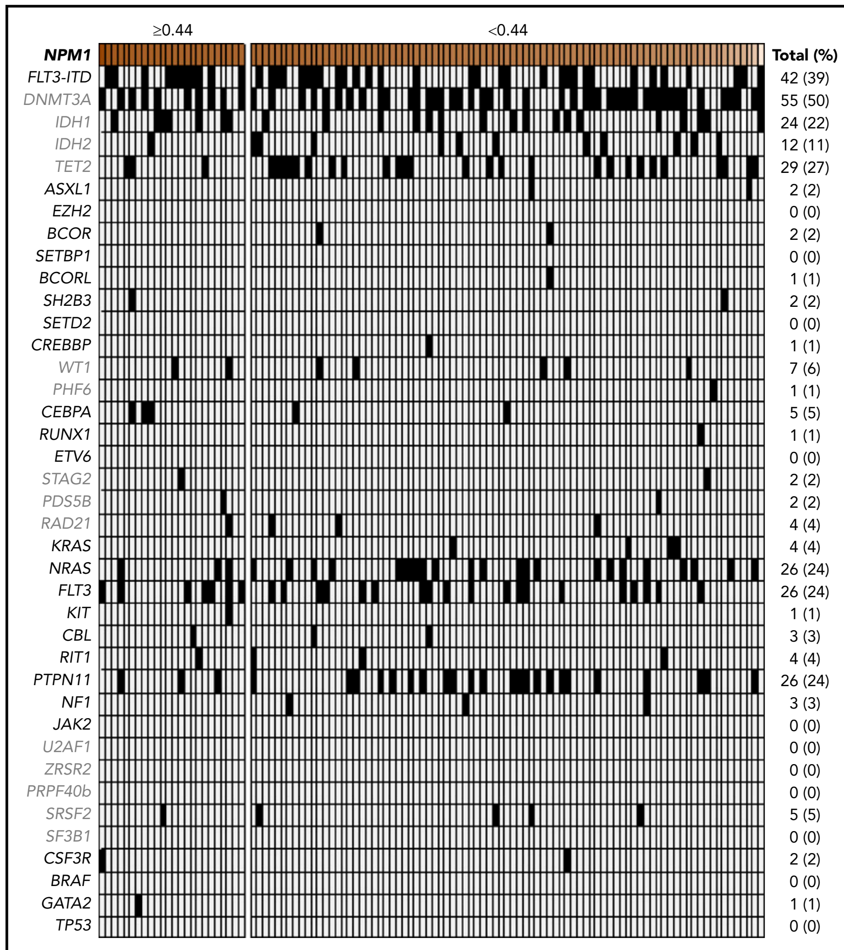

Figure 1. Comutational profiles for all patients with de novo AML with mutated *NPM1*. All cases of de novo AML with mutated *NPM1* evaluated (n = 109). Each column represents an individual patient. Intensity gradient corresponds to *NPM1* VAF, and patients in highest VAF quartile (≥0.44) are shown. All comutations, including *FLT3*-ITD status, provided in binary format.

Clinical and comutational variables

In univariate analyses for OS, older patient age was significantly associated with shorter OS (P = .03), and SCT in CR1 was associated with longer OS (P = .009; Figure 2A). A *DNMT3A* mutation was significantly associated with shorter OS (P = .04). In univariate analyses for EFS, SCT in CR1 was associated with longer EFS (P = .0002; Figure 2B). In univariate analysis for EFS censoring patients at the time of any SCT in CR1, older age was significantly associated with shorter EFS (P = .015). Stratification of the cohort at the median for total mutational number (>4 vs ≤4) revealed no significant difference in OS between these groups (P = .93). There was also no significant effect of mutation number as a continuous variable on OS (P = .27), although there was borderline association of higher mutation number as a continuous variable with shorter EFS (P = .07). *FLT3*-ITD (P = .043) and *FLT3*-ITD plus *DNMT3A* mutations (P = .029; Figure 2C) were associated with shorter EFS, whereas *NRAS/KRAS* mutations were associated with longer EFS (P = .044; Figure 2D) in the analysis that censored patients at the time of SCT. There were no significant associations of any variables with EFS in the 45 patients receiving SCT in CR1. No statistically significant associations with OS, EFS, or EFS censored at the time of SCT were identified with peripheral blood blast percentage or comutations in epigenetic regulation, transcription factor, spliceosome, or cohesin complex pathways or specifically with *TET2*, non-ITD *FLT3*, or *PTPN11* mutations (genes mutated in >10% of the total cohort). Ten percent of patients harbored mutations associated with secondary AML,[27] and there was no association of this subset with OS, EFS, EFS censored at the time of SCT, or EFS of patients undergoing SCT in CR1.

Features of high *NPM1* VAF

NPM1 VAF showed significant association with shorter OS and EFS, when analyzed as a continuous variable (P = .042 and P = .026, respectively), stratified at the median (≥0.40 or <0.40; P = .0015) and as quartile groupings (VAF, <0.36, 0.36-0.39, 0.40-0.43, and ≥0.44; P = .0004 for both OS [supplemental Figure 1] and EFS). Patients in the uppermost quartile (high *NPM1* VAF, ≥0.44; n = 24 patients) had shortened OS (median, 12.1 months vs not reached; P < .0001) and EFS (median, 7.5 vs 65.44 months; P < .0001; Figure 3A-B). High *NPM1* VAF correlated with shortened OS both in patients age >60 years (n = 51; P = .01) and in those age ≤60 years (n = 58; P = .006). High *NPM1* VAF was also significantly associated with shorter EFS in patients censored at the time of any SCT (median, 6.7 vs 31.0 months; P = .006; Figure 3C). In the 45 patients treated with SCT in CR1, *NPM1* VAF as a continuous variable was associated with shortened EFS (P = .02), and high *NPM1* VAF (n = 7) was also associated with shortened EFS (median, 11.0 months vs not reached; P = .0004; Figure 3D). These results remained significant on exclusion of the 2 cases with *NPM1* read counts of <100 (data not shown).

High *NPM1* VAF status was not significantly associated with the presence of comutations in *FLT3*-ITD (P = .48), *FLT3* non-ITD

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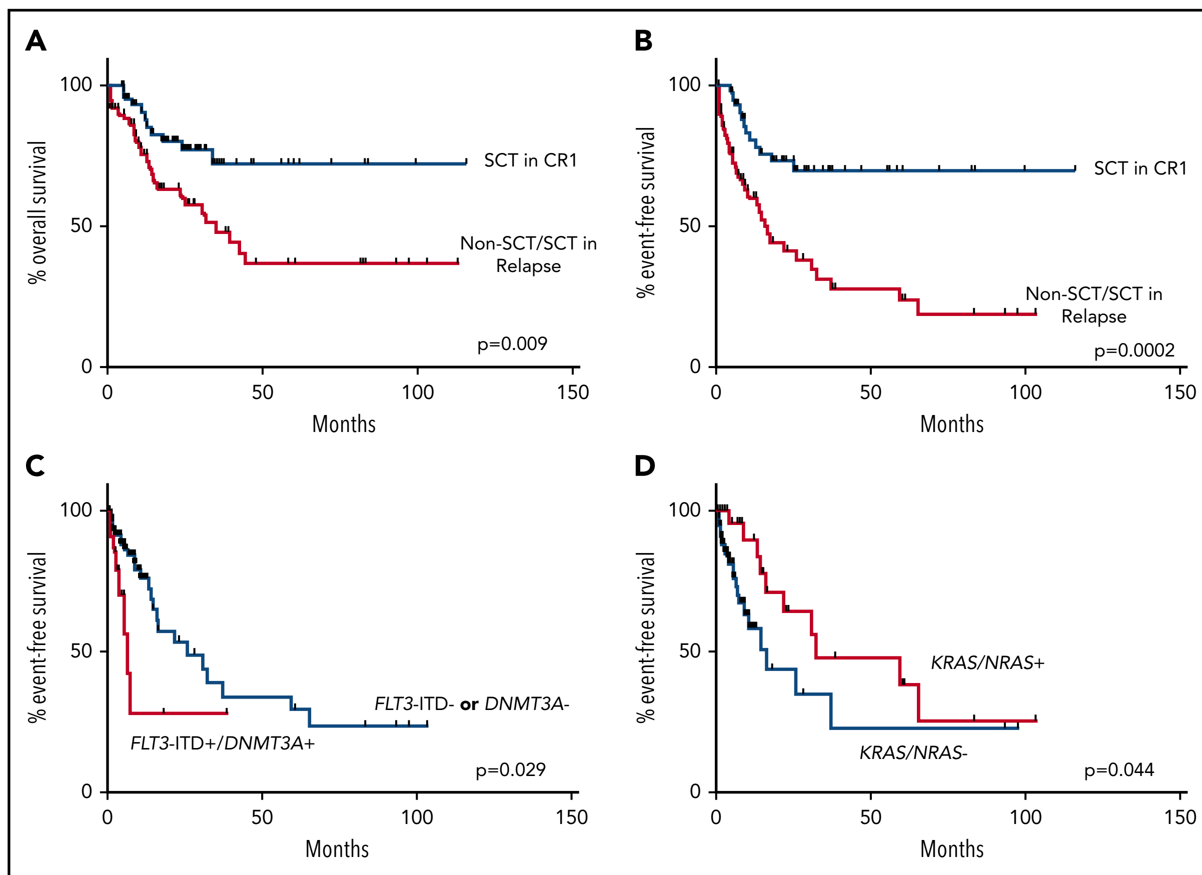

Figure 2. Kaplan-Meier curves for effects of SCT and selected comutations. (A) Effect of SCT in CR1 (n = 45) on OS (35.4 months vs not reached; P = .009). (B) Effect of SCT in CR1 on EFS (16.1 months vs not reached; P = .0002). (C) Effect of combined *FLT3*-ITD and *DNMT3A* mutations (n = 22) on EFS in comparison with cases with *FLT3*-ITD$^-$/*DNTM3A*$^+$, *FLT3*-ITD$^+$/*DNMT3A*$^-$, or *FLT3*-ITD$^-$/*DNMT3A*$^-$ (6.6 vs 25.8 months; P = .029), censoring patients at the time of SCT. (D) Effect of either *KRAS* or *NRAS* mutation (n = 30) on EFS (16.3 vs 32.4 months; P = .044), censoring patients at the time of SCT.

(P = .59), *DNMT3A* (P = .36), *TET2* (P = .12), or *PTPN11* (P = .18) and was borderline associated with a lack of *KRAS/NRAS* mutations (Fisher's exact test P = .07). High *NPM1* VAF correlated with shortened EFS in both *FLT3*-ITD$^+$ (5.9 months vs not reached; P = .018) and *FLT3*-ITD$^-$ (10.6 vs 37.4 months; P = .002) patients and in both *DNMT3A*-mutated (5.7 vs 37.4 months; $P < .0001$) and *DNMT3A* wild-type (11.0 months vs not reached; P = .04) patients (Figure 4A-B). High *NPM1* VAF borderline correlated with shortened OS (12.1 months vs not reached; P = .052) in the *FLT3*-ITD$^+$ patients and significantly correlated with shortened OS in patients who harbored *DNMT3A* mutation (10.0 vs 44.5 months; $P < .0001$). In the 22 patients who harbored both *FLT3*-ITD and *DNMT3A* mutations, high *NPM1* VAF (n = 5) trended toward a correlation with shortened OS (P = .071).

The *NPM1* variant had the highest VAF of all mutated genes in 26 cases (24%). Of note, there was no association between the highest VAF among all mutated genes in each case and EFS in the entire cohort (P = .41). Among the 55 patients with *DNMT3A* mutation, higher *DNMT3A* VAF was borderline associated with shorter EFS (P = .093), and *DNMT3A* VAF was positively correlated with *NPM1* VAF (r = 0.356; P = .008). Among the 22 *FLT3*-ITD cases in which the mutant level could be determined, 10 had high *FLT3*-ITD and 12 had low *FLT3*-ITD; there was no significant difference in *NPM1* VAF between *FLT3*-ITD–high and *FLT3*-ITD–low cases (Mann-Whitney P = .94). Using previously published criteria for defining subclones,[28] we identified NPM1 as subclonal in 29 cases (27%). Using the log-rank test, there were no significant differences in OS (P = .44) or EFS (P = .53) based on whether or not the *NPM1* mutation was subclonal.

In multivariable linear regression analyses to evaluate for variables associated with *NPM1* VAF, the final model showed that higher peripheral blood blasts ($P < .005$) was the only variable significantly associated with higher *NPM1* VAF, whereas the presence of *DNMT3A* mutation (P = .12) and presence of *FLT3* mutations other than ITD (P = .09) were borderline associated with higher *NPM1* VAF.

In multivariable analyses with respect to outcome in the entire patient cohort, high *NPM1* VAF and *DNMT3A* mutation were each independently associated with OS, whereas high *NPM1* VAF, *DNMT3A* mutation, and SCT in CR1 were each independently associated with EFS (Table 4). When censoring at the time of any SCT, age and combined *FLT3*-ITD and *DNMT3A* comutation were independently associated with EFS (Table 4). *DNMT3A* VAF was not independently associated with OS or EFS in any of the models, either when considered as a continuous variable or when using cutoffs at the median or highest quartile. In the cohort of patients treated with SCT in CR1, high *NPM1* VAF (P = .002) and *NPM1* VAF as a continuous variable (P = .021) were each significantly associated with EFS in multivariable

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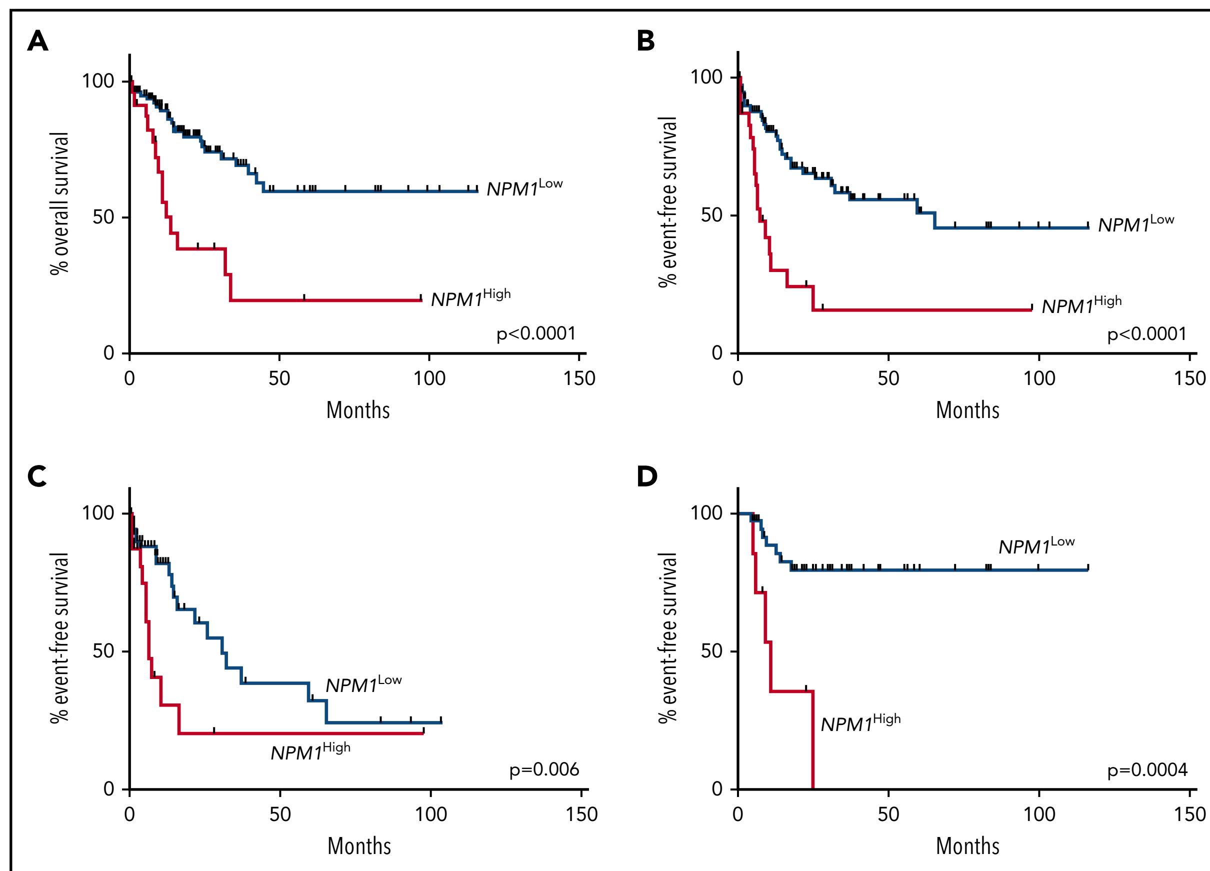

Figure 3. Kaplan-Meier curves showing effect of *NPM1* VAF. (A) Effect of high *NPM1* VAF on OS in the entire cohort (12.1 months vs not reached; $P < .0001$). (B) Effect of high *NPM1* VAF on EFS in the entire cohort (7.5 vs 65.44 months; $P < .0001$). (C) Effect of high *NPM1* VAF on EFS in patients censored at the time of any SCT in CR1 (6.7 vs 31.0 months; $P = .006$). (D) Effect of high *NPM1* VAF (n = 7) on EFS in patients treated with SCT in CR1 (n = 45; 11.0 months vs not reached; $P = .0004$).

analyses; other tested variables were not significant in this patient cohort.

Discussion

In this study, we investigated the potential effects of clinical variables, comutations, and *NPM1* mutational burden at diagnosis in de novo AML with mutated *NPM1*. The frequencies of various comutations in our cohort were similar to those reported by other groups.[7] Our results confirm previous findings that older patient age as well as concomitant *FLT3*-ITD or *DNMT3A* mutations may offset the otherwise favorable effect of *NPM1* mutation; of note, the frequent use of FLT3 inhibitor therapy in the *FLT3*-ITD–mutated cases may have dampened the negative effect of the latter variable in our cohort.[29,30] We also found that patients in our cohort with either *KRAS* or *NRAS* mutations experienced longer relapse-free survival in a univariate analysis that censored for SCT.[16] As previously reported, we found that the combination of *FLT3*-ITD and *DNMT3A* mutations conferred a negative effect on outcome when censoring for SCT,[7] but these mutations did not influence the outcome of patients treated with SCT in CR1.

Interestingly, we identified a powerful negative effect on survival of high *NPM1* mutational burden at diagnosis. Although high *NPM1* VAF was correlated with higher WBC and blast percentages, other variables classically associated with higher-risk disease, its effect on prognosis was independent of these factors. We did not observe differential outcomes based on our VAF-based mathematical approximation of the position of *NPM1* within the clonal hierarchy (ie, clone vs subclone); however, we are limited in our ability to validate this finding in the absence of single-cell sequencing data. Because *NPM1* mutation has been shown to be present in nonblast maturing hematopoietic cells in *NPM1*-mutated AML,[31] the VAF may in fact capture the true disease burden more effectively than blast percentage in blood or bone marrow. Importantly, the prognostic effect of *NPM1* VAF was observed across our entire cohort, including in subset analyses of patients whose samples were run on either the hybrid capture (n = 46) or amplicon-based (RHP; n = 56) NGS platforms (data not shown). The effect was also seen in subset analyses of patients with and without *FLT3*-ITD comutation but was especially prominent in patients with *DNMT3A* comutation and in patients who underwent SCT in CR1. Although the high *NPM1* VAF variable showed only borderline association with OS in subset analyses of *FLT3*-ITD–mutated and *FLT3*-ITD/*DNMT3A* comutated cases (possibly because of diminishing sample sizes), these findings nevertheless raise the possibility that *DNMT3A* comutation may potentiate ability of high-burden *NPM1*-mutated hematopoiesis to persist after induction chemotherapy. Higher *NPM1* mutant allele burden may be less amenable to eradication by induction chemotherapy, resulting in a higher likelihood of MRD, which has been associated with relapse in patients treated with SCT.[32]

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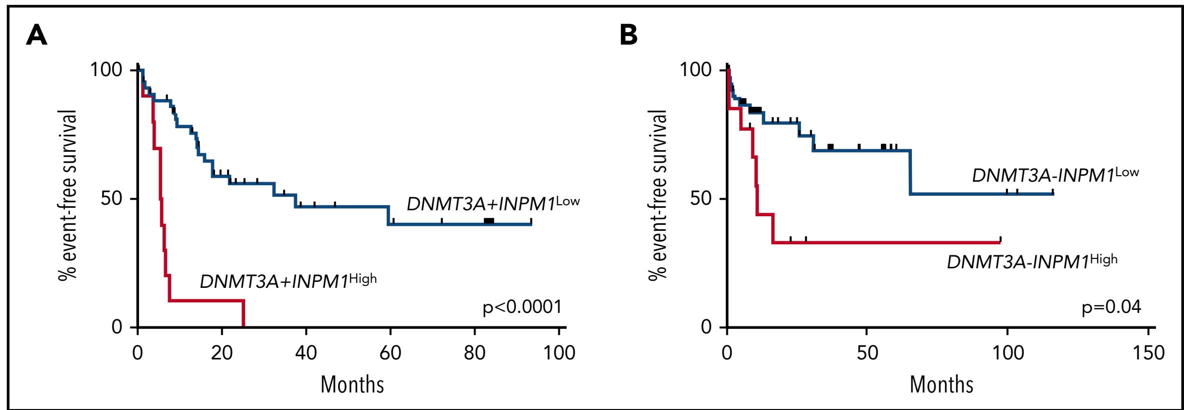

Figure 4. Kaplan-Meier curves showing effect of *NPM1* VAF within subpopulations defined by *DNMT3A* comutation. (A) Effect of high *NPM1* VAF (n = 10) on EFS in *DNTM3A*-mutated patients (n = 55; 5.7 vs 37.4 months; $P < .0001$). (B) Effect of high *NPM1* VAF (n = 14) on EFS in *DNMT3A* wild-type patients (n = 54; 11.0 months vs not reached; $P = .04$).

Alternatively, high *NPM1*-mutant allele burden may indicate the presence of disease in hematopoietic cell populations that are resistant to chemotherapy and foster relapse after SCT. Unlike detection of low-level MRD after therapy, which requires specialized high-sensitivity testing that is not currently available in most laboratories, *NPM1* VAF information is readily available from most NGS testing platforms. In our cohort, NGS was performed in only a subset of patients posttreatment; 2 of 4 cases with high *NPM1* VAF and 9 of 17 cases without high *NPM1* VAF had persistent *NPM1* mutations identified after induction therapy.

Although we attempted to control for all potential effect-bearing variables in multivariable analyses, our study is limited by its retrospective nature, the relatively small number of examined cases, and the variable application of SCT in our patient cohort, reflecting the controversial nature of the need for SCT in the setting of *NPM1*-mutated AML. Although the *NPM1* VAF values were derived from more than one molecular testing platform and included testing of both blood and bone marrow samples, we did not observe significant differences in the VAF based on the platform used or sample type. VAF may be subject to variability because of skewed amplification, particularly in cases with low read numbers; however, it should be noted that such variability is inherent in any quantitative prognostic marker, such as blast count strata, which are used routinely to risk stratify myelodysplastic syndromes. We recognize that although our data suggested a VAF cutoff of ≥0.44, this value likely represents an approximation of a true biologic cutoff, given variation among assays and testing methodologies. We also acknowledge that the VAF cutoff of 0.44 is based on a relatively small series of patients and should be evaluated in other patient cohorts. Until now, *NPM1* VAF has not generally been investigated as a risk factor in AML; we feel that the results of our study could stimulate additional investigation of this variable in terms of its effect on outcome in other independent patient cohorts with *NPM1*-mutated AML. Further study in larger patient cohorts and using different testing platforms will be necessary to optimize a VAF cutoff indicating higher-risk disease.

In summary, we have shown in this cohort of patients with AML with mutated *NPM1*, diagnosed according to current WHO criteria, that high *NPM1*-mutant allele burden at diagnosis is an independent predictor of unfavorable clinical outcomes, particularly in patients treated with SCT and in the subset of patients with *DNMT3A* comutation. These findings raise the possibility that the biology of this leukemia subtype might differ based on *NPM1* clone size. Although these results require validation in a

Table 4. Multivariable analysis of factors influencing OS, EFS, and EFS censoring patients at the time of SCT

Variable	*P*	Hazard ratio	95% CI
OS of all patients			
High (≥0.44) *NPM1* VAF	<.0001	4.043	2.037-8.024
DNMT3A mutation	.017	2.381	1.165-4.869
EFS of all patients			
High (≥0.44) *NPM1* VAF	<.0001	3.777	1.946-7.333
DNMT3A mutation	.023	2.088	1.107-3.939
SCT in CR1	.003	0.365	0.188-0.711
EFS of all patients censored at time of SCT			
Age (continuous, per year)	.01	1.044	1.010-1.078
Combined *FLT3*-ITD and *DNMT3A* mutation	.02	2.71	1.171-6.273

CI, confidence interval.

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larger patient cohort, they suggest that routine quantification of *NPM1* mutational burden at diagnosis may provide important prognostic information for patients with de novo AML and help guide subsequent management.

Authorship

Contribution: S.S.P., R.P.H., and O.K.W. analyzed clinical databases, performed statistical analyses, and wrote the manuscript; F.C.K., C.J.G., and V.N. performed select data analyses; M.W., R.J.S., D.P.S., R.M.S., E.P.A., D.J.D., Y.-B.A.C., A.T.F., A.M.B., and T.A.G. provided patients and contributed critical clinical insight; and all authors reviewed and approved the final manuscript.

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Footnotes

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