VOLUME 33 · NUMBER 18 · JUNE 20 2015

JOURNAL OF CLINICAL ONCOLOGY

ORIGINAL REPORT

Simpson's Paradox and the Impact of Different *DNMT3A* Mutations on Outcome in Younger Adults With Acute Myeloid Leukemia

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Published online ahead of print at www.jco.org on May 11, 2015.

Supported by Leukaemia and Lymphoma Research, UK and the United Kingdom Medical Research Council. The work was done at University College London Hospitals/University College London, which received a proportion of funding from the United Kingdom Department of Health's National Institute for Health Research Biomedical Research Centres funding scheme.

Presented at the 56th Annual Meeting of the American Society of Hematology, San Francisco, CA, December 6-9, 2014.

Authors' disclosures of potential conflicts of interest are found in the article online at www.jco.org. Author contributions are found at the end of this article.

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0732-183X/15/3318w-2072w/$20.00

DOI: 10.1200/JCO.2014.59.2022

ABSTRACT

Purpose
To evaluate the impact of *DNMT3A* mutations on outcome in younger patients with cytogenetic intermediate-risk acute myeloid leukemia.

Patients and Methods
Diagnostic samples from 914 patients (97% < 60 years old) were screened for mutations in *DNMT3A* exons 13 to 23. Clinical outcome was evaluated according to presence or absence of a mutation and stratified according to type of mutation (R882, non-R882 missense, or truncation).

Results
DNMT3A mutations (*DNMT3A*^{MUT}) were identified in 272 patients (30%) and associated with a poorer prognosis than wild-type *DNMT3A*, but the difference was only seen when the results were stratified according to *NPM1* genotype. This example of Simpson's paradox results from the high coincidence of *DNMT3A* and *NPM1* mutations (80% of patients with *DNMT3A*^{MUT} had *NPM1* mutations), where the two mutations have opposing prognostic impact. In the stratified analyses, relapse in patients with *DNMT3A*^{MUT} was higher (hazard ratio, 1.35; 95% CI, 1.07 to 1.72; $P = .01$), and overall survival was lower (hazard ratio, 1.37; 95% CI, 1.12 to 1.87; $P = .002$). The impact of *DNMT3A*^{MUT} did not differ according to *NPM1* genotype (test for heterogeneity: relapse, $P = .4$; overall survival, $P = .9$). Further analysis according to the type of *DNMT3A* mutation indicated that outcome was comparable in patients with R882 and non-R882 missense mutants, whereas in those with truncation mutants, it was comparable to wild-type *DNMT3A*.

Conclusion
These data confirm that presence of a *DNMT3A* mutation should be considered as a poor-risk prognostic factor, irrespective of the *NPM1* genotype, and suggest that further consideration should be given to the type of *DNMT3A* mutation.

J Clin Oncol 33:2072-2083. © 2015 by American Society of Clinical Oncology

INTRODUCTION

The development of high-throughput technologies has had a major influence on uncovering the molecular landscape of tumors and, in acute myeloid leukemia (AML), has revealed a hitherto unrecognized degree of complexity and heterogeneity that presents considerable challenges for the stratification of risk-directed therapy. Whole-genome/exome sequence data from 200 patients with AML showed that the incidence of the majority of recurrently mutated genes detected was less than 10% of all patients, but *FLT3* internal tandem duplications (*FLT3*^{ITD}) and mutations in the *NPM1* (*NPM1*^{MUT}) and DNA methyltransferase 3A (*DNMT3A*^{MUT}) genes were much more frequent, each occurring in more than 25% of patients.[1] All three mutations predominate in patients with cytogenetically intermediate-risk (IR) AML, and they are often coincident.[2-9] *FLT3* and *NPM1* are now routinely screened at diagnosis, with general consensus that *FLT3*^{ITD} is associated with an adverse impact and *NPM1*^{MUT} with a favorable impact on prognosis,[10,11] and patients with wild-type (WT) *FLT3* and *NPM1*^{MUT} are not recommended for stem-cell transplantation in first remission.[12,13] The role of *DNMT3A*^{MUT} in prognostication is less clear. Although most studies have reported that mutations are associated with worse overall survival (OS), reviewed in recent meta-analyses,[14,15] the largest study to date that included 1,060 younger adult patients with IR cytogenetics found that *DNMT3A*^{MUT} had no significant impact on survival end points, either overall or in the normal karyotype (NK) subgroup.[9] Furthermore, in

studies where an adverse impact has been recorded, there are variable results for the different *FLT3*/*NPM1* genotypic subgroups.[3-7,9] Of particular importance to patient management, there is controversy on the impact of the mutations in low-risk patients with IR/NK cytogenetics and $FLT3^{WT}NPM1^{MUT}$ genotype, with studies reporting either no impact[4,7,9] or adverse impact.[5,6]

DNMT3A is a DNA methyltransferase that is responsible for de novo methylation of CpG dinucleotides. It has three conserved functional domains, the C-terminal methyltransferase catalytic domain encoded by exons 16 to 23, an N-terminal PWWP domain (exons 8 and 9) that binds to specific lysine residues in methylated histones, and an ADD zinc finger domain (exons 13 to 15) that interacts with nonmethylated H3K4.[16] Mutations were first identified in AML from array-based or whole-genome/exome sequencing of patient samples.[2,17,18] Missense mutations at residue R882 are particularly frequent, occurring in approximately two thirds of mutated cases.[2-9,18] Other mutations are found throughout the gene, predominantly in exons 13 to 23. They include missense, nonsense, frameshift, and splice site mutations and are usually heterozygous.

There is limited information on the impact of the different mutations, with reports of either no difference between R882 and non-R882 mutations[2,4,6,7] or variable impact in selected groups[8,9] and no data comparing the impact of missense and truncating mutations, although they may have differing functional consequences. The major R882H mutation disrupts the DNMT3A homodimer interface and acts in a dominant-negative manner, leading to reduced homotetramer formation and much lower catalytic activity.[19-21] Other missense mutations also occur at the dimer interface, but some are located at the tetramer interface and may have more modest effects on catalytic activity but reduce overall methylation.[22] Truncating mutations, however, are more likely to lead to nonsense-mediated decay[23] and haploinsufficiency than dominant-negative activity. In mouse models, heterozygous *Dnmt3a* deletion had no apparent effect on phenotype,[24] and primary transplant recipients of *Dnmt3a*-null hematopoietic stem cells did not have evident changes in hematopoiesis.[25,26] Furthermore, high-resolution methylation analysis has now shown focal hypomethylation at specific CpG residues in R882-mutated patients that is not seen in the non–R882-mutated patients, with a distinct hierarchical clustering of the R882-mutated patients away from the non–R882-mutated patients.[21]

Because knowledge of *DNMT3A* mutations and their impact on clinical outcome may influence both gene screening strategies and therapy risk stratification, we have screened samples from a large cohort of 914 younger adult IR patients in whom there is extensive follow-up data and evaluated outcome according to the type of *DNMT3A* mutation detected.

PATIENTS AND METHODS

Patient Cohort

Genomic DNA was available from diagnostic samples of 914 patients with IR cytogenetics, as defined by the Medical Research Council classification,[27] uniformly treated on the United Kingdom Medical Research Council AML10 and AML12 trials between 1988 and 2002; 54% of samples were from bone marrow, 44% were from peripheral blood, and for 2%, the source was not known. Ethical approval for the trials and tissue collection for research was obtained from the Multi-Centre Research Committee of Wales. Informed consent was obtained in accordance with the Declaration of Helsinki. Patient characteristics are listed in Table 1. Median age at presentation was 43 years (range, 15 to 68 years); only 24 patients (3%) were ≥ 60 years old. All samples had known $FLT3^{ITD}$ and *FLT3* tyrosine kinase domain, *NPM1*, CCAAT/enhancer binding protein-α (*CEBPA*), and isocitrate dehydrogenase (*IDH) 1* and *IDH2* genotype.[28-32] Compared with the 1,996 IR patients treated on these trials who were not included in the study, the investigated patients had significantly higher WBC counts ($P < .001$) but no differences in median age, sex, incidence of secondary disease, or proportion who received a stem-cell transplantation in first remission (Appendix Table A1, online only). The investigated cohort, compared with patients not included in the study, had a borderline higher remission rate (87% *v* 84%, respectively; $P = .03$) and better 5-year OS (39% *v* 34%, respectively; $P = .02$), but there was no significant difference in cumulative incidence of relapse (CIR; 49% *v* 53%, respectively; $P = .1$).

DNMT3A *Screening and Mutant Quantification*

Amplicons of *DNMT3A* exons 13 to 23 were screened by denaturing high-performance liquid chromatography (Appendix and Appendix Table A2, online only). The common R882H and R882C mutations were confirmed by restriction enzyme digestion; other samples with abnormal chromatograms were sequenced. The relative mutant level for samples with R882H and R882C mutations was quantified using pyrosequencing and expressed as a relative proportion of total *DNMT3A* alleles.

Statistical Methods and Clinical End Points

Details of the trial protocols have been previously published.[33,34] The AML12 trial is registered at http://www.controlled-trials.com under ISRCTN17833622. Of the 914 patients, 100 underwent allogeneic transplantation from a sibling donor and 17 underwent transplantation from a matched unrelated donor in first remission.

Clinical end points are defined in the Appendix. Mantel-Haenszel and χ^2 tests were used to test for differences in demographic and clinical data by *DNMT3A* status. Kaplan-Meier curves were constructed for survival data and compared by means of the log-rank test, with standard tests for heterogeneity between subgroups.[35] Surviving patients were censored on August 9, 2010, with follow-up complete for 98% of patients. Median follow-up was 13.4 years (range, 5.2 to 21.9 years). Multivariable logistic regression analysis was used to find the factors most closely associated with complete remission, and multivariable Cox analysis was used for CIR and OS. Results were adjusted for age, WHO performance status, log(WBC), secondary disease, $FLT3^{ITD}$, and *NPM1* genotype. Odds ratios or hazard ratios (HRs) and 95% CIs are quoted for end points. In all cases, a ratio of less than 1 indicates benefit for a mutation. All *P* values are two-tailed.

RESULTS

DNMT3A *Mutation Analysis*

Overall, 278 mutations were detected in 272 (30%) of 914 patients (Fig 1A and Appendix Table A3, online only); 175 (63%) were missense R882 mutations. The remaining mutations were distributed throughout the gene and included 63 missense mutations, 37 mutations classified as truncations (12 nonsense, 13 frameshift, 12 at splice sites), and three in-frame deletions. Six patients had homozygous mutations, all except one non-R882 missense substitutions, and six patients had two mutations, all non-R882. Therefore, of the 272 $DNMT3A^{MUT}$ patients, 175 (64%) had R882 mutations, 59 (22%) had non-R882 missense mutations, 35 (13%) had truncations or in-frame deletions, and three (1%) had two mutations of differing types.

The median *DNMT3A* mutant level for 172 patients with R882H (n = 123) or R882C (n = 49) mutations was 47% (range, 15% to 85%; Appendix Fig A1, online only), indicative of a heterozygous mutation in nearly all cells. $NPM1^{MUT}$ levels were available from 147 of these

Table 1. Patient Demographic and Clinical Characteristics

Characteristic	All Patients (No.)	No. of Patients (%)						*P*†				
		WT	MUT	R882	Non-R882	Missense*	Truncating*	MUT *v* WT	WT *v* R882 *v* Non-R882	WT *v* R882 *v* Non-R882 Missense *v* Truncation	R882 *v* Non-R882	R882 *v* Non-R882 Missense *v* Truncation
All patients	914	642	272	175	97	59	35					
Trial								.1	.1	.01	.2	.02
AML10	311	231 (36)	80 (29)	47 (27)	33 (34)	26 (44)	7 (20)					
AML12	603	411 (64)	192 (71)	128 (73)	64 (66)	33 (56)	28 (80)					
Age, years								< .001‡	< .001	< .001	.2‡	.6
15-29	174	157 (24)	17 (6)	12 (7)	5 (5)	3 (5)	2 (6)	< .001§	< .001§	< .001§	.1§	.2§
30-39	185	130 (20)	55 (20)	40 (23)	15 (15)	9 (15)	6 (17)					
40-49	265	178 (28)	87 (32)	55 (31)	32 (33)	19 (32)	10 (29)					
50-59	266	161 (25)	105 (39)	61 (35)	44 (45)	27 (46)	17 (49)					
60+	24	16 (2)	8 (3)	7 (4)	1 (1)	1 (2)	0					
Median	43	41	48	47	49	49	49					
Range	15-68	15-68	18-67	20-67	18-64	18-64	25-59					
Sex								.004	.01	.02	.2	.4
Female	475	314 (49)	161 (59)	99 (57)	62 (64)	38 (64)	23 (66)					
Male	439	328 (51)	111 (41)	76 (43)	35 (36)	21 (36)	12 (34)					
Diagnosis								.8	.7	.9	.5	.8
De novo	850	596 (93)	254 (93)	162 (93)	92 (95)	56 (95)	33 (94)					
Secondary	64	46 (7)	18 (7)	13 (7)	5 (5)	3 (5)	2 (6)					
WHO PS								.7‡	.8	.8	.9‡	.7
0	369	262 (41)	107 (39)	71 (41)	36 (37)	18 (31)	15 (43)					
1	240	162 (25)	78 (29)	49 (28)	29 (30)	18 (31)	11 (31)					
2	200	140 (22)	60 (22)	35 (20)	25 (26)	18 (31)	7 (20)					
3	92	68 (11)	254 (9)	17 (10)	7 (7)	5 (8)	2 (6)					
4	13	10 (2)	3 (1)	3 (2)	0	0	0					
WBC, $\times 10^9$/L								< .001‡	< .001	< .001	.004‡	.01
0-9.9	250	199 (31)	51 (19)	25 (15)	26 (28)	11 (20)	13 (37)	< .001§	< .001§	< .001§	.005§	.01§
10-19.9	144	107 (17)	37 (14)	24 (14)	13 (14)	5 (9)	8 (23)					
20-49.9	207	139 (22)	68 (26)	43 (25)	25 (27)	20 (36)	5 (14)					
50-99.9	156	105 (17)	51 (19)	35 (20)	16 (17)	11 (20)	5 (14)					
≥ 100	145	86 (14)	59 (22)	45 (26)	14 (15)	9 (16)	4 (11)					
Unknown	12	6	6	3	3	3	0					
Median	26.85	22.4	37.3	46.7	25.2	29.4	18.7					
Range	0.4-480	0.4-480	0.7-439	0.7-388	0.9-439	1.2-439	0.9-213					
FAB type								< .001	< .001	< .001	.01	.001
M0	32	29 (5)	3 (1)	0	3 (3)	1 (2)	1 (3)					
M1	186	151 (25)	35 (14)	20 (12)	15 (17)	10 (19)	4 (12)					
M2	236	177 (29)	59 (23)	34 (20)	25 (28)	13 (25)	12 (35)					
M4	237	142 (23)	96 (37)	67 (39)	28 (31)	18 (35)	9 (26)					
M5	134	77 (13)	57 (22)	44 (26)	13 (15)	9 (17)	4 (12)					
M6	22	19 (3)	3 (1)	0	3 (3)	0	3 (9)					
M7	8	4 (1)	4 (2)	3 (2)	1 (1)	0	1 (3)					

(continued on following page)

Table 1. Patient Demographic and Clinical Characteristics (continued)

Characteristic	All Patients (No.)	No. of Patients (%)						*P*†				
		WT	MUT	R882	Non-R882	Missense*	Truncating*	MUT *v* WT	WT *v* R882 *v* Non-R882	WT *v* R882 *v* Non-R882 Missense *v* Truncation	R882 *v* Non-R882	R882 *v* Non-R882 Missense *v* Truncation
Bilineage	1	1 (< 0.5)	0	0	0	0	0					
RAEB/RAEB-t	13	10 (2)	3 (1)	2 (1)	1 (1)	1 (2)	0					
Unknown	45	32	13	5	8	7	1					
Cytogenetics								< .001	< .001	< .001	.9	.9
Normal	645	413 (64)	232 (85)	149 (85)	83 (86)	50 (85)	31 (89)					
Abnormal	269	229 (36)	40 (15)	26 (15)	14 (14)	9 (15)	4 (11)					
FLT3/ITD								< .001	.001	< .001	.4	.2
WT	620	459 (72)	161 (59)	100 (57)	61 (63)	33 (56)	26 (74)					
MUT	294	183 (29)	111 (41)	75 (43)	36 (37)	26 (44)	9 (26)					
FLT3/TKD								.3	.2	.3	.1	.3
WT	823	582 (91)	241 (89)	151 (86)	90 (93)	55 (93)	32 (91)					
MUT	91	60 (9)	31 (11)	24 (14)	7 (7)	4 (7)	3 (9)					
NPM1								< .001	< .001	< .001	.01	.1
WT	455	401 (62)	54 (20)	27 (15)	27 (28)	14 (24)	11 (31)					
MUT	459	241 (38)	218 (80)	148 (85)	70 (72)	45 (76)	24 (69)					
ITD/*NPM1*								< .001	< .001	< .001	.1	.2
$ITD^{WT}NPM1^{WT}$	357	312 (49)	45 (17)	22 (13)	23 (24)	12 (20)	9 (26)					
$ITD^{WT}NPM1^{MUT}$	263	147 (23)	116 (43)	78 (45)	38 (39)	21 (36)	17 (49)					
$ITD^{MUT}NPM1^{WT}$	98	89 (14)	9 (3)	5 (3)	4 (4)	2 (3)	2 (6)					
$ITD^{MUT}NPM1^{MUT}$	196	94 (15)	102 (38)	70 (40)	32 (33)	24 (41)	7 (20)					
CEBPA								< .001‡	.002	.004	.9‡	.1
WT	829	566 (88)	263 (97)	169 (97)	94 (97)	59 (100)	32 (91)					
1 MUT	31	26 (4)	5 (2)	4 (2)	1 (1)	0	1 (3)					
2 MUT	54	50 (8)	4 (1)	2 (1)	2 (2)	0	2 (6)					
IDH1								.004	.01	.02	.5	.7
WT	831	595 (93)	236 (87)	150 (86)	86 (89)	51 (86)	32 (91)					
MUT	83	47 (7)	36 (13)	25 (14)	11 (11)	8 (14)	3 (9)					
IDH2								.01	.003	.1	.1	.7
WT	798	575 (90)	223 (82)	143 (82)	80 (82)	50 (85)	29 (83)					
R140 MUT	91	54 (8)	37 (14)	27 (15)	10 (10)	6 (10)	4 (11)					
R172 MUT	25	13 (2)	12 (4)	5 (3)	7 (7)	3 (5)	2 (6)					

Abbreviations: AML, acute myeloid leukemia; FAB, French-American-British; ITD, internal tandem duplication; MUT, mutant; PS, performance status; RAEB, refractory anemia with excess blasts; RAEB-t, refractory anemia with excess blasts in transformation; TKD, tyrosine kinase domain; WT, wild type.

*Three patients are excluded because they have two mutations of differing types.

†Tests are χ^2, except where noted otherwise.

‡Mantel-Haenszel test for trend over ordered categories.

§Wilcoxon rank sum/Kruskal-Wallis test.

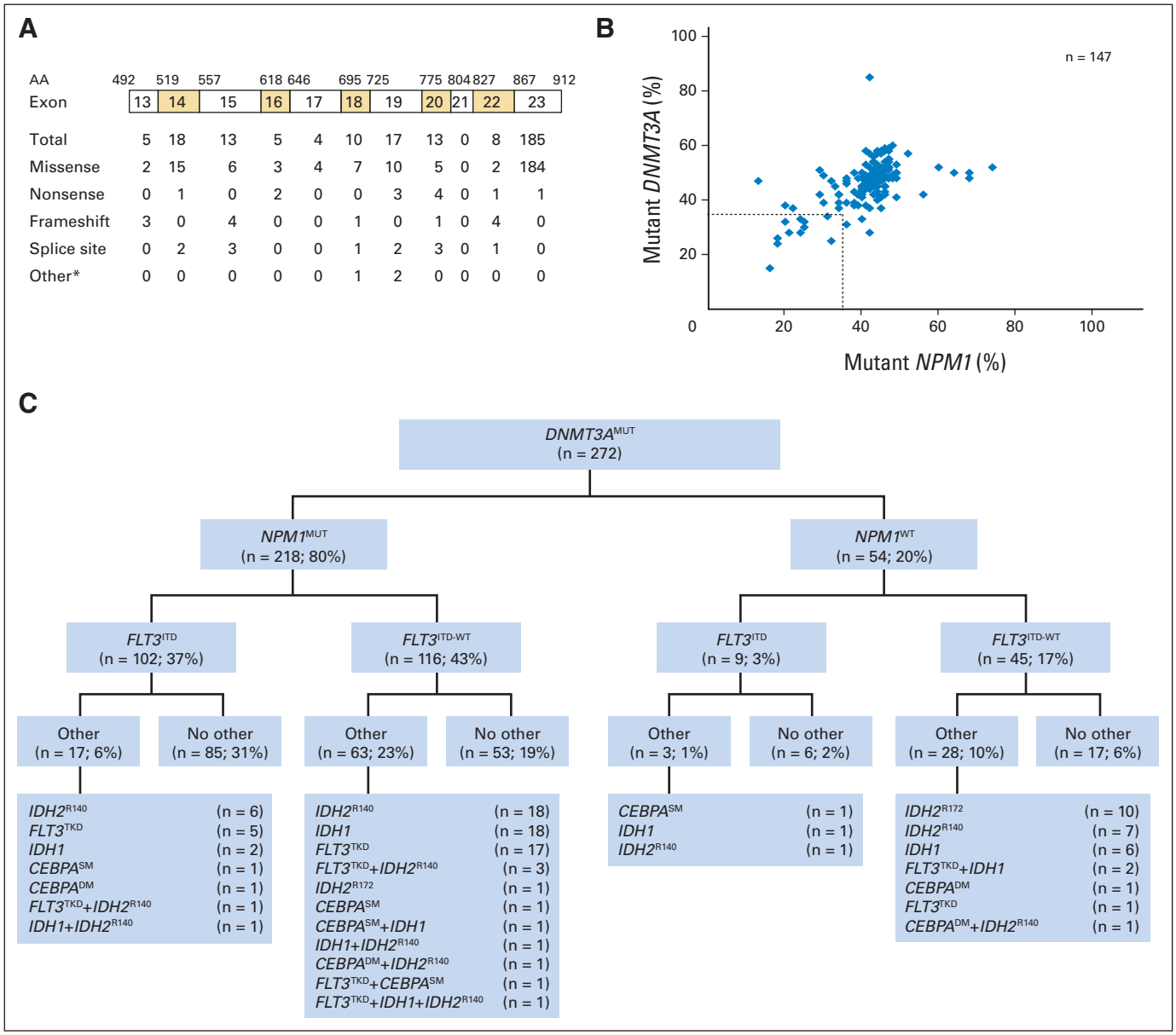

Fig 1. *DNMT3A* mutations detected: mutant type, level, and coincidence with other recurrent mutations. (A) Exonic location and the mutant type of all detected mutations. The amino acid (AA) given is the first AA of the exon, except for the last AA (912). (*) In-frame deletions. (B) Comparison of *DNMT3A* and *NPM1* mutant levels in 147 patients with R882H or R882C mutations. The dotted line indicates a mutant level of 35%. (C) Coincidence of other mutations in the cohort of 272 *DNMT3A*-mutated patients. DM, double mutant; ITD, internal tandem duplication; SM, single mutant; TKD, tyrosine kinase domain; WT, wild type.

patients, and of the 12 patients with a $DNMT3A^{MUT}$ level less than 35%, nine also had an $NPM1^{MUT}$ level less than 35% (Fig 1B). Assuming that an *NPM1* mutation is found in virtually all leukemic cells, this suggests that the instances of low-level $DNMT3A^{MUT}$ were a result of nonleukemic cell contamination rather than the mutation being present only in a subclone of leukemic cells.

Patient Characteristics According to DNMT3A *Genotype*

$DNMT3A^{MUT}$ patients were significantly older than $DNMT3A^{WT}$ patients ($P < .001$) and more likely to be female ($P = .004$) and have a higher presenting WBC ($P < .001$; Table 1). There was a significant difference across morphologic subgroups ($P < .001$), with higher rates in M4 (40%) and M5 (43%), which represented 37% and 22%, respectively, of all $DNMT3A^{MUT}$ patients. $DNMT3A^{MUT}$ patients were significantly more likely than $DNMT3A^{WT}$ patients to have an NK rather than an IR abnormal karyotype ($P < .001$). Coincidence with other recurrent mutations showed a positive correlation between $DNMT3A^{MUT}$ and $NPM1^{MUT}$ ($P < .001$), $FLT3^{ITD}$ ($P < .001$), $IDH1^{MUT}$ ($P = .004$), and $IDH2^{MUT}$ ($P = .01$), but a negative correlation with $CEBPA^{MUT}$ ($P < .001$; Table 1; Fig 1C). Of note, 80% of the $DNMT3A^{MUT}$ samples were $NPM1^{MUT}$, 37% were $FLT3^{ITD}NPM1^{MUT}$, and 43% were $FLT3^{ITD\text{-}WT}NPM1^{MUT}$. Conversely, 47% of $NPM1^{MUT}$ samples were $DNMT3A^{MUT}$.

There was no difference in age, sex, and type of leukemia between $DNMT3A^{MUT}$ patients with R882 ($DNMT3A^{R882}$) and

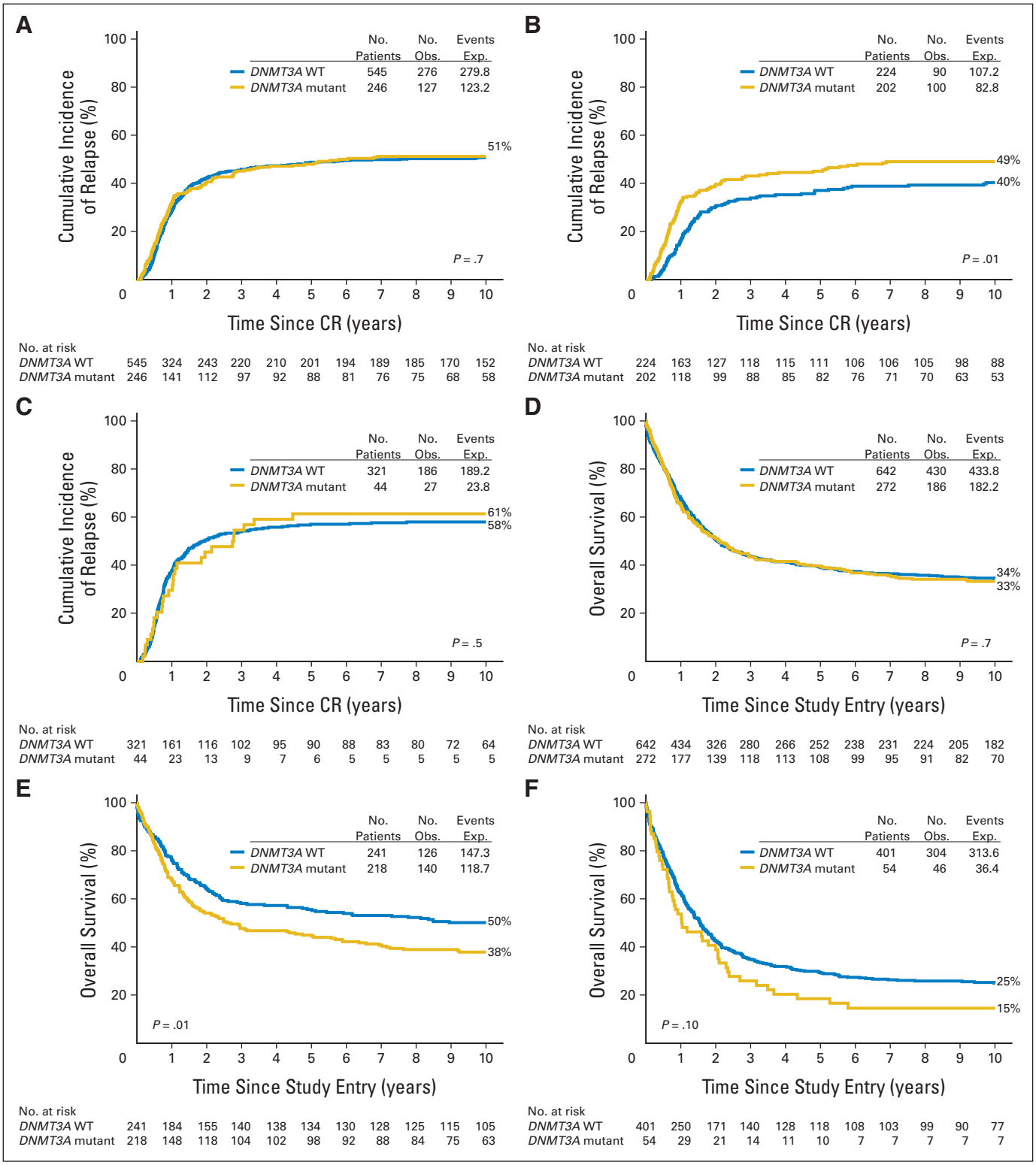

Fig 2. Kaplan-Meier curves for outcome stratified according to *DNMT3A* genotype. Cumulative incidence of relapse in (A) the total cohort, (B) $NPM1^{MUT}$ patients, and (C) $NPM1^{WT}$ patients. Overall survival in (D) the total cohort, (E) $NPM1^{MUT}$ patients, and (F) $NPM1^{WT}$ patients. CR, complete remission; Exp., expected; MUT, mutation; Obs., observed; WT, wild type.

$DNMT3A^{MUT}$ patients with other mutations ($DNMT3A^{non\text{-}R882}$), irrespective of the type of non-R882 mutation (Table 1). However, $DNMT3A^{R882}$ patients had a significantly higher WBC than $DNMT3A^{non\text{-}R882}$ patients ($P = .005$) and a significantly higher correlation with $NPM1^{MUT}$ ($P = .01$). In the $DNMT3A^{non\text{-}R882}$ patients, those with missense mutations had a higher median WBC and more coincidence with $NPM1^{MUT}$, $FLT3^{ITD}$, and $IDH1^{MUT}$ than those with truncations (Table 1).

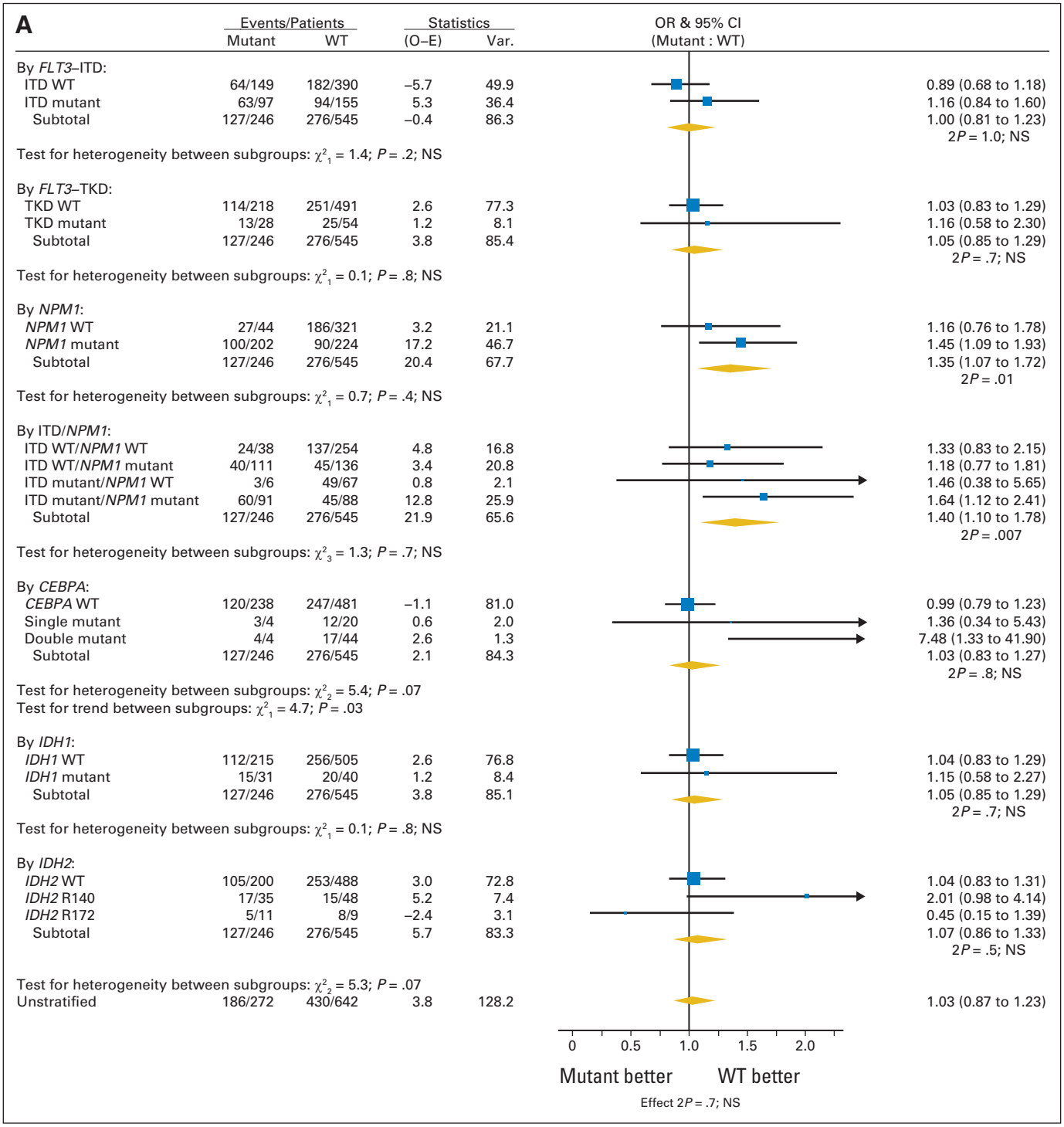

Fig 3. Forest plots for the impact of a *DNMT3A* mutation on outcome in different molecular groups. (A) Relapse. (B) Overall survival. E, expected; ITD, internal tandem duplication; NS, not significant; O, observed; OR, odds ratio; TKD, tyrosine kinase domain; WT, wild type.

Response to Therapy and Long-Term Outcome Stratified According to DNMT3A *Genotype*

There was a slightly but significantly higher remission rate in $DNMT3A^{MUT}$ patients than $DNMT3A^{WT}$ patients (90% *v* 85%, respectively; $P = .03$). However, this can probably be attributed to the association with $NPM1^{MUT}$, and the significance was not maintained in multivariable analysis taking into account patient characteristics and other molecular markers (odds ratio, 0.74; 95% CI, 0.43 to 1.28; $P = .3$; Appendix Table A4, online only). In the total group, neither CIR nor OS differed according to *DNMT3A* genotype ($P = .7$ for both; Figs 2A and 2D). However, these results suggesting that *DNMT3A* mutations are not associated with a poor prognosis must be interpreted with

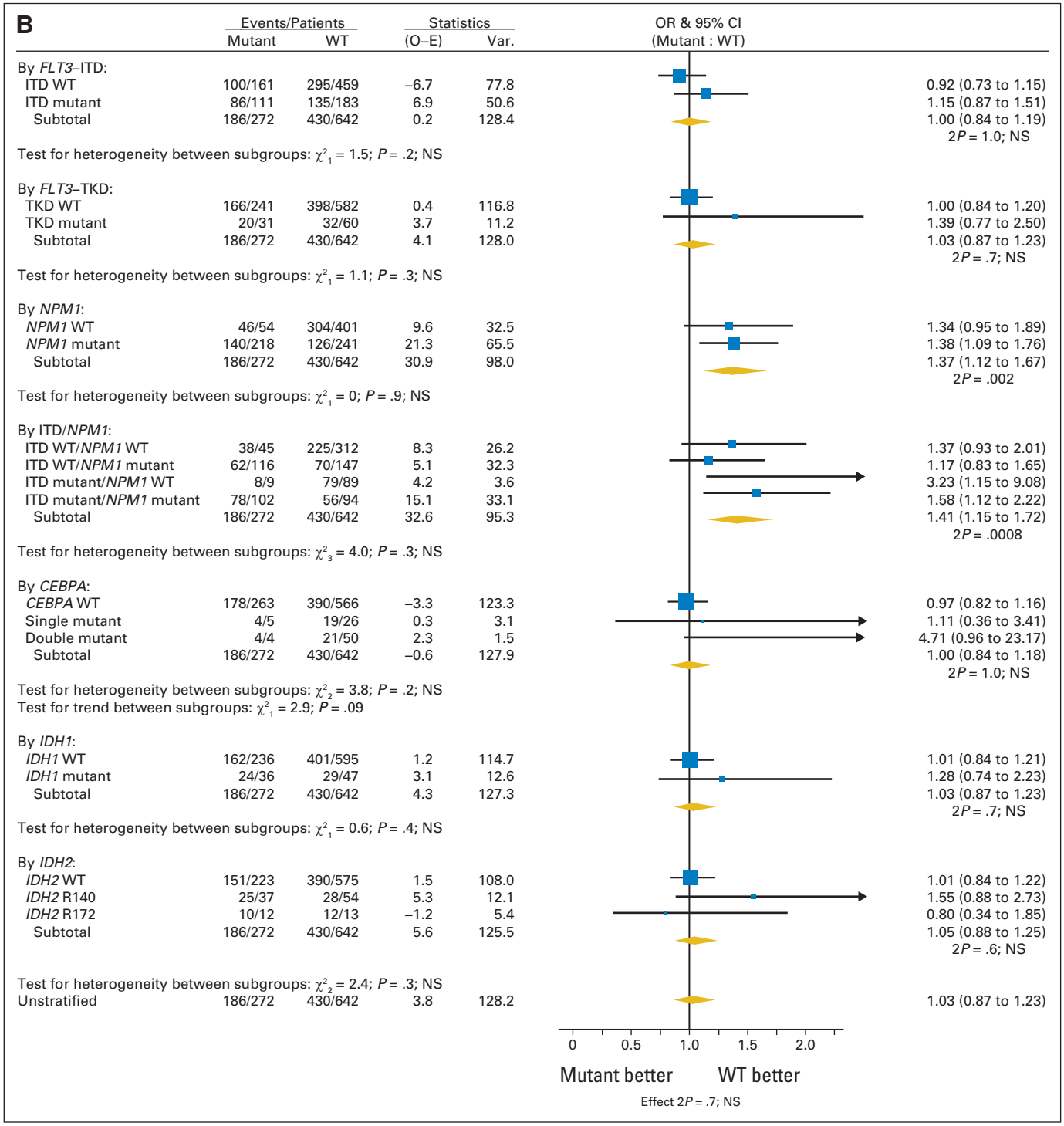

Fig 3. (continued).

caution because of the high level of concordance with *NPM1*^{MUT}, which is associated with a good prognosis. Therefore, we performed a subgroup analysis of *NPM1*^{MUT} and *NPM1*^{WT} patients.

The remission rate in *NPM1*^{MUT} patients was 93% for both *DNMT3A*^{MUT} and *DNMT3A*^{WT}; in *NPM1*^{WT} patients, the remission rates were 81% and 80% for *DNMT3A*^{MUT} and *DNMT3A*^{WT}, respectively. *DNMT3A*^{MUT} was associated with higher CIR in both *NPM1*^{MUT} and *NPM1*^{WT} patients when analyzed separately (Figs 2B and 2C). Although this was not significant for the *NPM1*^{WT} *DNMT3A*^{MUT} patients, in the analysis stratified for *NPM1* genotype, the overall impact of *DNMT3A*^{MUT} was significantly adverse (HR, 1.35; 95% CI, 1.07 to 1.72; $P = .01$), and testing for heterogeneity showed that the impact did not significantly differ between *NPM1*^{MUT} and *NPM1*^{WT} patients ($P = .4$; Fig 3A). There was no difference in the

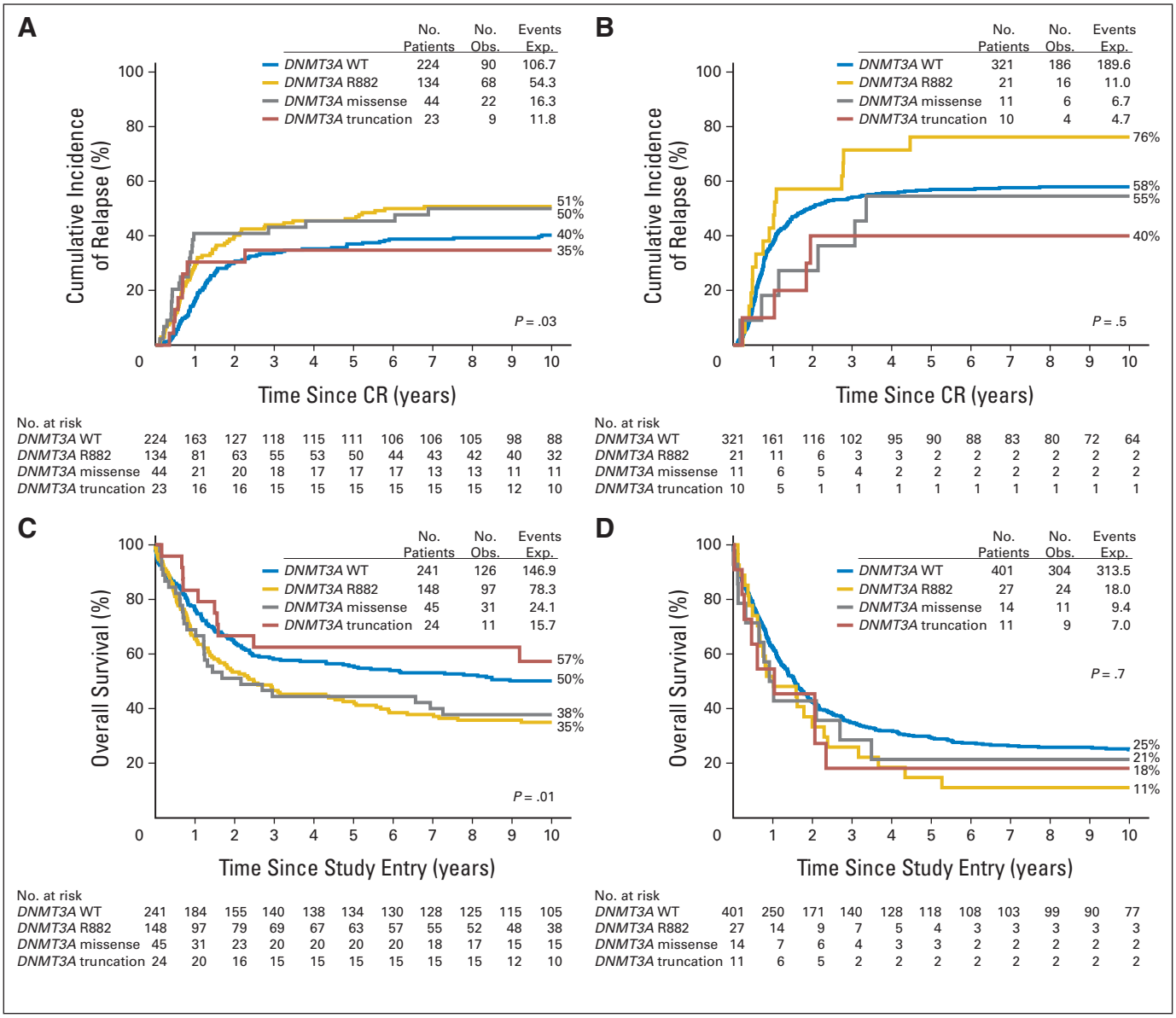

Fig 4. Kaplan-Meier curves for outcome stratified according to type of *DNMT3A* mutation. Cumulative incidence of relapse in (A) $NPM1^{MUT}$ patients and (B) $NPM1^{WT}$ patients. Overall survival in (C) $NPM1^{MUT}$ patients and (D) $NPM1^{WT}$ patients. CR, complete remission; Exp., expected; Obs., observed; WT, wild type.

impact stratified according to $FLT3^{ITD}$ status. Further analysis of the four subgroups defined by *NPM1* and $FLT3^{ITD}$ genotype similarly showed no heterogeneity between the groups of the poor prognostic impact on CIR associated with $DNMT3A^{MUT}$ ($P = .7$; Fig 3A). In accord with these results, $DNMT3A^{MUT}$ patients had worse OS than $DNMT3A^{WT}$ patients with either $NPM1^{MUT}$ or $NPM1^{WT}$ genotype (Figs 2E and 2F), and although not significant for the $NPM1^{WT}$ $DNMT3A^{MUT}$ patients, the overall impact in the stratified analysis was highly significant (HR, 1.37; 95% CI, 1.12 to 1.87; $P = .002$; Fig 3B). There was no evidence of heterogeneity of the impact of a *DNMT3A* mutation between $NPM1^{MUT}$ and $NPM1^{WT}$ patients ($P = .9$) or between the four *NPM1*/$FLT3^{ITD}$ genotypes ($P = .3$; Fig 3B). Of note, four of the 54 patients with double *CEBPA* mutations ($CEBPA^{DM}$) were also $DNMT3A^{MUT}$. All four patients attained remission but experienced relapse, which represents a significantly higher CIR compared with $CEBPA^{DM}DNMT3A^{WT}$ patients ($P = .03$; Fig 3A). There was also a trend to lower OS in these $CEBPA^{DM}DNMT3A^{MUT}$ patients ($P = .1$; Fig 3B).

In multivariable analysis, $DNMT3A^{MUT}$ was a significant adverse risk factor for CIR (HR, 1.27; 95% CI, 1.01 to 1.61; $P = .04$) and a borderline adverse factor for OS (HR, 1.19; 95% CI, 0.98 to 1.45; $P = .1$; Appendix Table A4). In a forward selection model considering all clinically relevant factors, *DNMT3A* genotype entered as a significant factor for relapse but not complete remission or OS (Appendix Table A5, online only).

Outcome Stratified According to Type of DNMT3A *Mutation*

To assess the impact of different *DNMT3A* mutations, outcome was considered according to the type of mutation—R882, non-R882

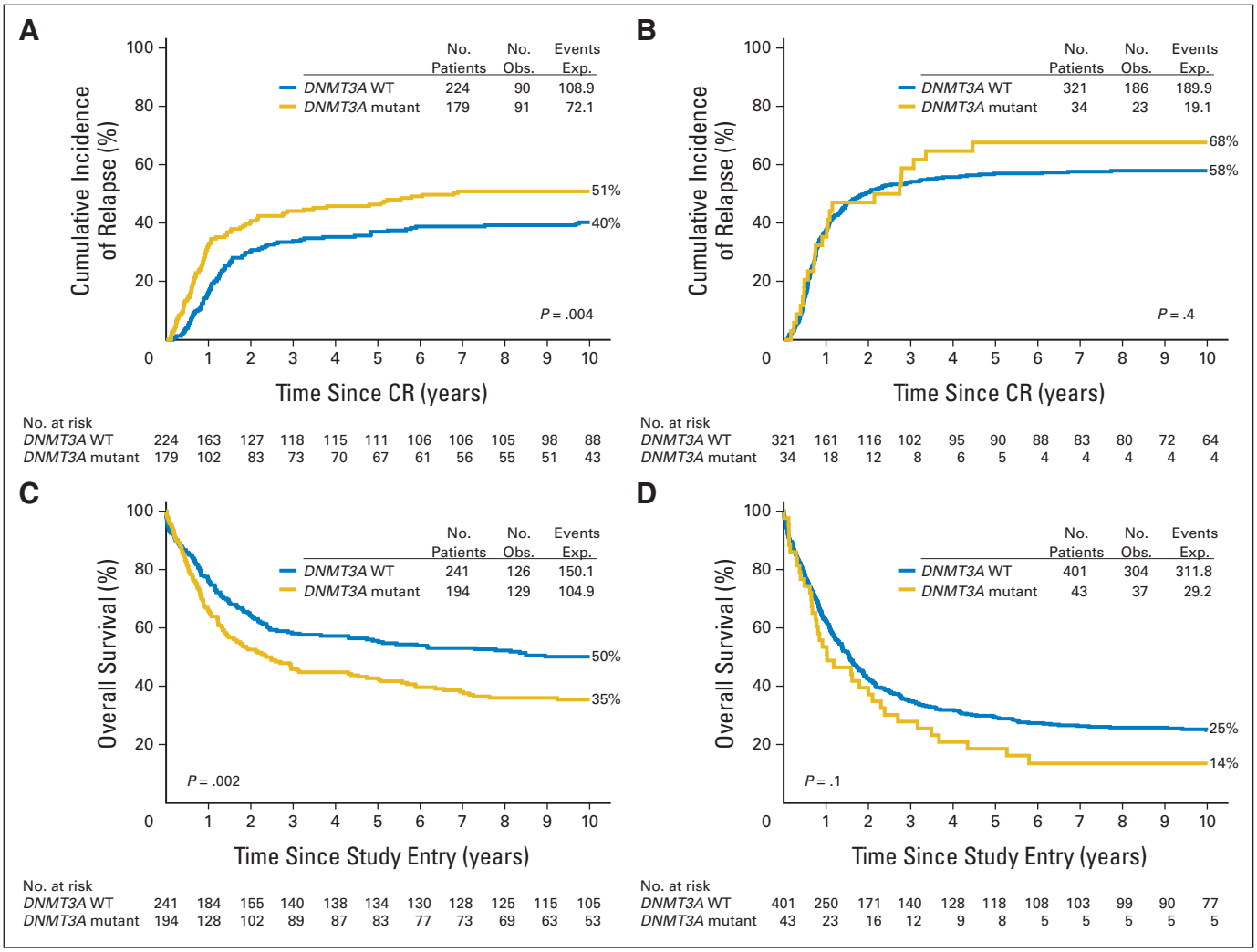

Fig 5. Kaplan-Meier curves for outcome stratified according to *DNMT3A* genotype excluding truncation mutations. Cumulative incidence of relapse in (A) $NPM1^{MUT}$ patients and (B) $NPM1^{WT}$ patients. Overall survival in (C) $NPM1^{MUT}$ patients and (D) $NPM1^{WT}$ patients. CR, complete remission; Exp., expected; Obs., observed; WT, wild type.

missense, or truncation. Three patients were excluded from the analysis because they had two mutations of differing types.

The remission rate did not differ significantly according to mutation type ($P = .4$ across the three groups) in either $NPM1^{MUT}$ ($P = .2$) or $NPM1^{WT}$ ($P = .6$) patients. CIR was higher in $DNMT3A^{R882}$ patients than $DNMT3A^{WT}$ patients with either $NPM1^{MUT}$ or $NPM1^{WT}$ genotype, whereas patients with truncations had similar CIR to those with $DNMT3A^{WT}$ among $NPM1^{MUT}$ patients and lower CIR than those with $DNMT3A^{WT}$ among $NPM1^{WT}$ patients (Figs 4A and 4B). Non-R882 missense mutations were associated with higher CIR than $DNMT3A^{WT}$ in $NPM1^{MUT}$ patients, equivalent to $DNMT3A^{R882}$ patients, and similar CIR to $DNMT3A^{WT}$ in $NPM1^{WT}$ patients. In accord with this, patients with $DNMT3A^{R882}$ had worse OS than patients with $DNMT3A^{WT}$ and either $NPM1^{MUT}$ or $NPM1^{WT}$ genotype (Figs 4C and 4D). Truncations were associated with nonsignificantly better OS in $NPM1^{MUT}$ patients and were similar to $DNMT3A^{WT}$ in $NPM1^{WT}$ patients. Non-R882 missense mutations were associated with poorer OS in $NPM1^{MUT}$ patients, similar to R882 mutations, but seemed to have a lesser impact in $NPM1^{WT}$ patients, although there were only 12 patients in this category.

Because these results suggested that *DNMT3A* truncation and missense mutations may differ in their impact on outcome, the data were reanalyzed excluding patients with truncations. The results were comparable to the initial results, but the differences were of slightly greater significance (Figs 5A to 5D).

DISCUSSION

In this large cohort of cytogenetically IR younger patients with AML, a *DNMT3A* mutation was associated with poorer prognosis, but this difference was only observed if the results were analyzed separately according to *NPM1* genotype, where worse OS was seen in $DNMT3A^{MUT}$ patients with both $NPM1^{MUT}$ and $NPM1^{WT}$ genotype. This is an example of Simpson's paradox.[36] It results from the strong association between *DNMT3A* and *NPM1* mutations and the opposing prognostic impact of the two mutants. In our cohort, 80% of $DNMT3A^{MUT}$ patients were also $NPM1^{MUT}$, which leads to a marked inequality in the proportion of $DNMT3A^{MUT}$ in the $NPM1^{MUT}$ and $NPM1^{WT}$ patients. Because outcome of the total group reflects the

relative proportion of the different genotypic subgroups, the effect that is seen separately in the groups is masked when data from the two groups are combined. This is illustrated in Appendix Table A6 (online only). This paradox may explain, at least in part, some of the differences reported to date in studies examining the prognostic impact of *DNMT3A* mutations, in particular where the genotypic subgroups are small and likely to be less statistically robust.[3-7,9] Although there was a significant association between $DNMT3A^{MUT}$ and $FLT3^{ITD}$ (41% of $DNMT3A^{MUT}$ patients were $FLT3^{ITD}$), no such differences were observed when $DNMT3A^{MUT}$ patients were stratified according to $FLT3^{ITD}$ status because both are associated with poorer outcome.

Studies have shown that different mutations within the same gene may be associated with variable patient characteristics and outcome (eg, *IDH2* R140 and R172 mutations).[32,37] Therefore, consideration needs to be given to the variety of mutations identified in the *DNMT3A* gene and their potentially differing functional consequences. In our cohort, 64% of $DNMT3A^{MUT}$ patients had a mutation at residue R882, comparable to the incidence reported in other studies (mean, 68%; range, 60% to 83%; test for heterogeneity, $P = .7$).[2,4-9,38] However, the remaining mutations were scattered across the zinc finger and methyltransferase domains and included not only other missense mutations (22% of mutated patients), but also truncations (13%). Analysis in the three mutant categories suggested that, whereas R882 and non-R882 missense mutations were associated with a similar adverse outcome, the truncations seemed to have a different functional impact on the R882 mutations and should be considered as equivalent to $DNMT3A^{WT}$ from a prognostic standpoint. These data require corroboration from other large cohorts, because there were only 35 patients with a truncation, which therefore results in limited statistical power when split into the $NPM1^{MUT}$ and $NPM1^{WT}$ groups. If total loss of protein from the mutated allele leads to haploinsufficiency, then the data are compatible with the lack of impact on hematopoiesis observed for *Dnmt3a* loss in mouse models,[24-26] rather than the dominant-negative effect that has been demonstrated for the R882 mutant.[20] However, it does raise the issue of the role of such mutations in leukemogenesis, and further studies will be required to investigate this.

For prognostication purposes, combined analysis of the patients with non-R882 missense mutations suggested that they should be considered together with R882-mutated patients as having worse outcome than $DNMT3A^{WT}$ patients. However, most are unique, and they comprise a highly heterogeneous group of mutations that disrupt different domains of the enzyme. Although both the SIFT and PolyPhen algorithms predict that the majority of mutations in the zinc finger and methyltransferase domains are likely to be deleterious, there is evidence that they differ in their functional and cellular significance. For example, G543 was the second most commonly mutated amino acid, detected in 12 patients (4%). It is close to the histone H3K4 binding surface and does not affect DNA methylation activity but has increased in vitro ability to interact with histone H3.[18] Similarly, differences in mean methylation levels and methylated CpGs have been reported between patients with R882 and non-R882 mutations.[21] More detailed structural and functional analysis of these mutants may thus provide information about the normal roles of DNMT3A and its interactions with other proteins and how disruption of these processes may lead to AML.

From a therapeutic viewpoint, our data confirm that $DNMT3A^{MUT}$ should be treated as a poor-risk factor. This is of particular relevance to management of patients currently considered as favorable risk, in particular those with $CEBPA^{DM}$, where $DNMT3A^{MUT}$ patients were significantly more likely to experience relapse, and those with $NPM1^{MUT}FLT3^{ITD\text{-}WT}$, where the lack of heterogeneity between subgroups indicated that these patients should not be considered differently from the other *NPM1*/*FLT3* genotype groups. The adverse outcome associated with the non-R882 missense mutations also indicates that screening strategies cannot be limited to analysis of exon 23 alone. It is important to note that the need for subgroup analysis in prognostication, as in this case, requires cautious interpretation of the data because of the pitfalls of multiple comparisons and potential bias. The controversy over determining how mutant status should be used for risk stratification, even for the three most commonly mutated genes (*DNMT3A*, *NPM1*, and *FLT3*), reflects the increasing challenge for determining outcome analysis in the face of the extensive genotypic heterogeneity and variable interaction between coincident mutations that is becoming apparent in AML.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Disclosures provided by the authors are available with this article at www.jco.org.

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AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

Simpson's Paradox and the Impact of Different *DNMT3A* Mutations on Outcome in Younger Adults With Acute Myeloid Leukemia

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Travel, Accommodations, Expenses: Gilead Sciences

Acknowledgment

We are grateful to the clinical investigators who entered and managed patients in these trials.

Appendix

Mutation Screening

Polymerase chain reaction (PCR) products for *DNMT3A* exons 13 to 23 were amplified from genomic DNA using Optimase Polymerase (Transgenomic, Glasgow, United Kingdom) or Phusion High-Fidelity DNA Polymerase (New England BioLabs, Hitchin, United Kingdom) according to manufacturer's instructions, with 35 cycles of amplification and primers and annealing temperatures as specified (Table A2). Products were denatured, reannealed slowly to allow heteroduplex formation, and then analyzed on a denaturing high-performance liquid chromatography WAVE platform (Transgenomic, Glasgow, United Kingdom) at optimal melting temperatures calculated using Transgenomic's Navigator software (Table A2). Samples with abnormal WAVE chromatograms were sequenced.

For screening of R882H and R882C mutations, PCR products were prepared using BIOTAQ DNA Polymerase (BIOLINE, London, United Kingdom) with 32 cycles of amplification and then digested with restriction enzymes (New England BioLabs) according to manufacturer's instructions. For R882H, the primers were 23F2 and mismatch primer R882H MM (R; Table A2), and the products were digested at 65°C for 3 hours with BsaAI. Wild-type products remained uncut (172 base pairs [bp]), and mutant products were digested to 143 + 29 bp. For R882C, the primers were 23F and 23R, and the products were digested overnight at 37°C with AluI. Wild-type products remained uncut (318 bp), and mutant products were digested to 193 + 125 bp.

Quantification of Mutant Level

For R882H and R882C mutations, the relative mutant level was quantified using pyrosequencing of biotinylated PCR products prepared using GoTaq Hot Start Polymerase (Promega, Southampton, United Kingdom), 50 cycles of amplification, primers Pyro F and biotinylated Pyro R (Table A2), and an annealing temperature of 63°C. The 184-bp products were sequenced on a PyroMark MD system (Qiagen, Crawley, United Kingdom) using PyroMark Q96 reagents and protocols (Qiagen) and primer Pyro sequence (Table A2). The pyrosequencing software calculated mutant level as a percentage of the total alleles for a particular mutation.

Definition of Clinical End Points

Complete remission (CR) was defined as a normocellular bone marrow containing less than 5% blasts and showing evidence of normal maturation of other marrow elements. CR with incomplete blood count recovery was classified as CR with residual neutropenia or thrombocytopenia. Persistence of myelodysplastic features did not preclude the diagnosis of CR. Remission failures were classified by the clinicians as either a result of induction death (death related to treatment and/or hypoplasia within 30 days of trial entry) or resistant disease (failure to eliminate disease, including partial remission with 5% to 15% blasts in the bone marrow). Where the clinician's evaluation was not available, deaths within 30 days of entry were classified as induction deaths, and deaths later than 30 days after entry were classified as resistant disease. Overall survival was defined as the time from random assignment to death. For patients achieving CR, cumulative incidence of relapse was the incidence of relapse after CR with death in CR as a competing risk.

Table A1. Comparison of Patients in the Two Trials Who Were Included and Excluded From the Analysis

Characteristic	*DNMT3A* Cohort (n = 914)	Patients Excluded From Analysis (n = 1,996)	*P*
Median age, years	43	45	.1
Male, %	48	49	.7
Median WBC, $\times 10^9$/L	26.85	11.1	< .001
WHO PS ≥ 2, %	33	36	.1
Secondary disease, %	7	9	.1
SCT in CR1, %	26	23	.1
CR/CRi, %	87	84	.03
OS at 5 years, %	39	34	.02
CIR at 5 years, %	49	53	.1

Abbreviations: CIR, cumulative incidence of relapse; CR, complete remission; CR1, first complete remission; CRi, complete remission with incomplete hematologic recovery; OS, overall survival; PS, performance status; SCT, stem-cell transplantation.

Table A2. Details of Polymerase Chain Reaction Primers and Experimental Conditions for Mutation Screening and Mutant Quantification

Exon and Primer	Primer Sequence	Size (bp)	Annealing Temperature (°C)	WAVE Temperature (°C)
13				
F	5′-GCTGGTCTGGTGCTGGGCTC-3′	228	65	63.3
R	5′-CACAGTCAGCCAGAAGGCCGA-3′			
14				
F	5′-TGAGGCCAGGTGTGGAGCCTC-3′	241	67	64.1
R	5′-TGGGGCCCAGCTAAGGAGACCA-3′			
15				
F	5′-TCCATTCCAGGTAGCACACCTTG-3′	338	63	63.4, 64.0
R	5′-ACCCTGCGCACAGCTCAGGC-3′			
16				
F	5′-GACACCGCTGGGCCTGCATC-3′	221	66	62.6
R	5′-ACCATCATTTCGTTTTGCCAGAGTTGC-3′			
17				
F	5′-TGCCGAGACCAGGGTGCCAG-3′	269	66	64.1
R	5′-CTCCAGGTGCTGAGTGTGCAG-3′			
18				
F	5′-CTGGGTCTCCTCTCTTTCGTG-3′	252	63	62.6
R	5′-GCACCAGCTGAGAAGGTGGAG-3′			
19				
F	5′-AGCCACACCACTGTCCTATGC-3′	304	63	61.5, 62.7
R	5′-TCCCCAGCTCCACAATGCAGAT-3′			
20				
F	5′-CTTTAAGGCTCGACCCCAGCA-3′	241	64	62.2
R	5′-GCTTCCCCACTATGGGTCATC-3′			
21				
F	5′-GAGGGAGGGGAGTCGTGCA-3′	267	63	62.4
R	5′-GCATTCTCCACACTAGCTGGAGA-3′			
22				
F	5′-GAGTACCTGGCATATTTGGTAGAC-3′	297	64	59.0
R	5′-CAAGTCAGGTGGGAAAGGCAG-3′			
23				
F	5′-CCTGCTGTGTGGTTAGACGGCT-3′	318	64	60.8, 63.6
R	5′-CTCTCCATCCTCATGTTCTTGGTG-3′			
F2	5′-CTGGTCCTCCGGGTCCTGC-3′	172	64	NA
R882H MM (R)	5′-GACCGGCCCAGCAGTCTCTGCCTCGCCACG-3′*			
Pyro F	5′-TGTGTGGTTAGACGGCTTCC-3′	184	63	NA
Pyro R (biotin)	5′-GAAGAGGTGGCGGATGACT-3′			
Pyro sequence	5′-TGACGTCTCCAACATGA-3′			

Abbreviations: bp, base pair; F, forward; NA, not applicable; R, reverse.
*Mismatch underlined.

Table A3. Details of *DNMT3A* Mutations Detected

Exon	Total No. of Mutations	Mutation Type	Missense		Nonsense		Frameshift		Splice Site	Other	
			Nucleotide Change	AA Change	Nucleotide Change	AA Change	Nucleotide Change	AA Change		Nucleotide Change	AA Change
13	5	2 missense 3 frameshift	1482C>G 1490G>A	C494W C497Y			1507dupA 1510delC 1516dupC	T503fs L504fs H506fs			
14	18	15 missense 1 nonsense 2 splice site	1627G>T 1628G>C 1628G>A 1640T>A 1640T>G	G543C (n = 8) G543A (n = 3) G543D L547H (n = 2) L547R	1560C>A	C520X			(+1) G>T (+1) G>A		
15	13	6 missense 4 frameshift 3 splice site	1670G>A 1676G>A 1723G>C 1743G>C 1748_1749GG>CT	C557Y C559Y A575P W581C W581S (n = 2)			1701_1704dup 1705delC 1710_1720del 1742_1743ins58	G568dup P569fs A571fs W581fs	(+1) G>A (+2) delT (+5) A>G		
16	5	3 missense 2 nonsense	1904G>A 1906G>A 1919T>C	R635Q V636M F640S	1885G>T 1918_1919delTT	E629X F640X					
17	4	4 missense	2006C>T 2023G>T 2053G>A	S669F V675L G685R (n = 2)							
18	10	7 missense 1 frameshift 1 splice site 1 other	2096G>A 2119G>A 2141C>G 2158C>G 2167C>G	G699D G707S S714C (n = 3) R720G L723V			2085_2088delCCAG	I695fs	c.2083-11_2096del	2129_2140del	C710_L713del
19	17	10 missense 3 nonsense 2 splice site 2 other	2185C>T 2204A>G 2206C>T 2207G>A 2213T>A 2245C>T 2254T>G 2312G>T 2314T>A	R729W Y735C (n = 2) R736C R736H L738Q R749C F752V R771L F772I	2259G>A 2311C>T	W753X R771X (n = 2)			(+1) G>A (+1) G>T	2189_2191delTCT 2251_2253delTTC	F731del F751del
20	13	5 missense 4 nonsense 1 frameshift 3 splice site	2330C>G 2339T>C 2369G>C 2398G>A 2401A>G	P777R I780T R790T G800S M801V	2357delC 2379C>A	S786X (n = 2) Y793X (n = 2)			(−2) A>G (−1) Gdel (+2) T>G		
21	0										

(continued on following page)

Table A3. Details of *DNMT3A* Mutations Detected (continued)

Exon	Total No. of Mutations	Mutation Type	Missense		Nonsense		Frameshift			Other	
			Nucleotide Change	AA Change	Nucleotide Change	AA Change	Nucleotide Change	AA Change	Splice Site	Nucleotide Change	AA Change
22	8	2 missense	2489T>C	V830A	2560G>T	E854X	2481delC	F827fs	(−1) G>A		
		1 nonsense	2503A>G	T835A			2571delC	D857fs (n = 2)			
		4 frameshift					2574_2576delinsTT	I858fs			
		1 splice site									
23	185	184 missense	2603T>G	F868C (n = 2)	2622T>A	Y874X					
		1 nonsense	2623A>G	T875A							
			2632T>C	S878P							
			2635A>G	N879D (n = 2)							
			2644C>G	R882G							
			2644C>T	R882C (n = 49)							
			2645G>A	R882H (n = 122)							
			2645G>C	R882P (n = 3)							
			2689G>T	V897F							
			2711C>T	P904L (n = 2)							

Table A4. Multivariable Analysis of Outcome in the Total Cohort

Outcome	MUT *v* WT HR (95% CI)	MUT *v* WT *P*	*P*: WT *v* R882 *v* Others	*P*: WT *v* R882 *v* Non-R882 Missense *v* Truncation	R882 *v* Others HR (95% CI)	R882 *v* Others *P*	R882 *v* Non-R882 Missense *v* Truncation, *P*
CR/CRi	0.74 (0.43 to 1.28)	.3	.2	.4	1.97 (0.67 to 5.77)	.2	.5
OS	1.19 (0.98 to 1.45)	.1	.1	.1	1.19 (0.87 to 1.64)	.3	.2
CIR	1.27 (1.01 to 1.61)	.04	.1	.05	1.25 (0.85 to 1.85)	.3	.2

NOTE: Results were adjusted for age, WHO performance status, log(WBC), secondary disease, *FLT3*^{ITD}, and *NPM1* genotype.
Abbreviations: CIR, cumulative incidence of relapse; CR, complete remission; CRi, complete remission with incomplete hematologic recovery; HR, hazard ratio; MUT, mutant; OS, overall survival; WT, wild type.

Table A5. Significant Variables in Order of Entry in a Forward Selection Model

Variable	HR (95% CI)	*P*
CR		
NPM1^{MUT}	0.21 (0.13 to 0.34)	< .001
Age	1.05 (1.03 to 1.07)	< .001
WHO PS	1.52 (1.26 to 1.83)	< .001
Log(WBC)	2.19 (1.52 to 3.14)	< .001
Secondary disease	2.51 (1.31 to 4.81)	.005
CIR		
FLT3^{ITD}	2.14 (1.73 to 2.65)	< .001
NPM1^{MUT}	0.39 (0.31 to 0.49)	< .001
Age	1.01 (1.00 to 1.02)	.003
CEBPA^{DM}	0.57 (0.36 to 0.89)	.01
DNMT3A^{MUT}	1.27 (1.00 to 1.61)	.05
OS		
NPM1^{MUT}	0.42 (0.35 to 0.50)	< .001
Age	1.02 (1.02 to 1.03)	< .001
FLT3^{ITD}	1.55 (1.30 to 1.85)	< .001
WHO PS	1.13 (1.05 to 1.22)	< .001
CEBPA^{DM}	0.43 (0.28 to 0.65)	< .001
Log(WBC)	1.26 (1.10 to 1.45)	.001

NOTE. Models were fitted using forward selection, with variables added to the model if they had a $P < .05$, derived using the deviance statistic. Variables entered were age, WHO PS, log(WBC), secondary disease, *FLT3*^{ITD}, *FLT3*^{TKD}, *NPM1*, *CEBPA*^{DM}, *IDH1*, *IDH2*, and *DNMT3A* genotype. *DNMT3A* was entered as mutant/not, WT *v* R882 *v* other, WT *v* R882, and missense *v* truncation.
Abbreviations: CIR, cumulative incidence of relapse; CR, complete remission; DM, double mutant; HR, hazard ratio; ITD, internal tandem duplication; MUT, mutant; OS, overall survival; PS, performance status; TKD, tyrosine kinase domain; WT, wild type.

Table A6. Contingency Table for OS in the Different Genotype Groups

Genotype	*DNMT3A*^{WT} No. of Patients (% of *DNMT3A* subgroup)	*DNMT3A*^{WT} Observed 10-Year OS Rate (%)	*DNMT3A*^{MUT} No. of Patients (% of *DNMT3A* subgroup)	*DNMT3A*^{MUT} Observed 10-Year OS Rate (%)	Total No. of Patients
NPM1^{WT}	401 (62)	25	54 (20)	15	455
NPM1^{MUT}	241 (38)	50	218 (80)	38	459
Total	642		272		914

NOTE. The survival for each *DNMT3A* group (*DNMT3A*^{WT} and *DNMT3A*^{MUT}) is obtained by adding together the proportion of *NPM1*^{WT} patients multiplied by their survival and the proportion of *NPM1*^{MUT} patients multiplied by their survival. For *DNMT3A*^{WT}, OS = (0.62 × 25%) + (0.38 × 50%) = 34%. For *DNMT3A*^{MUT}, OS = (0.20 × 15%) + (0.80 × 38%) = 33%.
Abbreviations: MUT, mutant; OS, overall survival; WT, wild type.

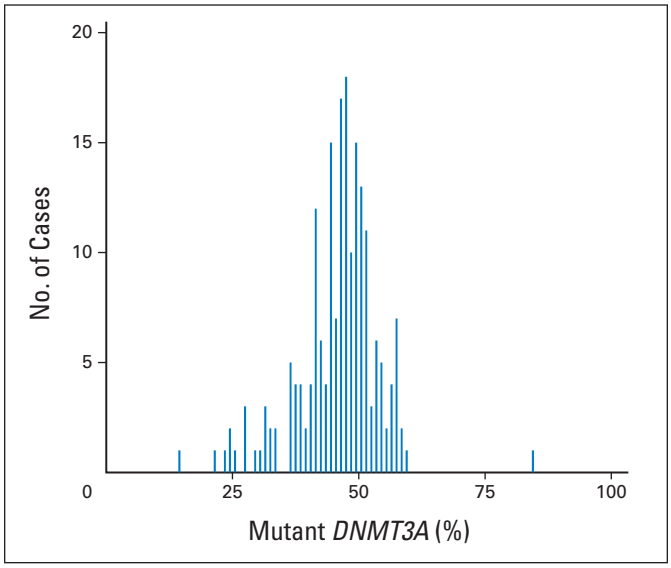

Fig A1. Distribution of relative mutant level quantified in 172 patients with *DNMT3A* R882H or R882C mutations.