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ORIGINAL REPORT

Age-Related Prognostic Impact of Different Types of *DNMT3A* Mutations in Adults With Primary Cytogenetically Normal Acute Myeloid Leukemia

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ABSTRACT

Purpose
To determine the frequency of *DNMT3A* mutations, their associations with clinical and molecular characteristics and outcome, and the associated gene- and microRNA-expression signatures in primary cytogenetically normal acute myeloid leukemia (CN-AML).

Patients and Methods
Four hundred fifteen previously untreated adults were analyzed for *DNMT3A* mutations and established prognostic gene mutations and expression markers. Gene- and microRNA-expression profiles were derived using microarrays.

Results
Younger (< 60 years; n = 181) and older (≥ 60 years; n = 234) patients had similar frequencies of *DNMT3A* mutations (35.3% *v* 33.3%). Missense mutations affecting arginine codon 882 (R882-*DNMT3A*) were more common (n = 92; 62%) than those affecting other codons (non–R882-*DNMT3A*). *DNMT3A*-mutated patients did not differ regarding complete remission rate, but had shorter disease-free survival (DFS; $P = .03$) and, by trend, overall survival (OS; $P = .07$) than *DNMT3A*–wild-type patients. In multivariable analyses, *DNMT3A* mutations remained associated with shorter DFS ($P = .01$), but not with shorter OS. When analyzed separately, the two *DNMT3A* mutation types had different significance by age group. Younger patients with non–R882-*DNMT3A* mutations had shorter DFS ($P = .002$) and OS ($P = .02$), whereas older patients with R882-*DNMT3A* mutations had shorter DFS ($P = .005$) and OS ($P = .002$) after adjustment for other clinical and molecular prognosticators. Gene- and microRNA-expression signatures did not accurately predict *DNMT3A* mutational status.

Conclusion
DNMT3A mutations are frequent in CN-AML, and their clinical significance seems to be age dependent. *DNMT3A*-R882 mutations are associated with adverse prognosis in older patients, and non–R882-*DNMT3A* mutations are associated with adverse prognosis in younger patients. Low accuracy of gene- and microRNA-expression signatures in predicting *DNMT3A* mutation status suggested that the role of these mutations in AML remains to be elucidated.

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INTRODUCTION

Acute myeloid leukemia (AML) is a genetically heterogeneous disease characterized by nonrandom cytogenetic aberrations[1,2] and, at the submicroscopic level, recurrent gene mutations and changes in gene expression.[3] Cytogenetic and molecular alterations not only define distinct biologic entities, but are also relevant for disease classification and treatment guidance.[4]

Cytogenetically normal (CN) AML, comprising 45% to 50% of adults with primary disease,[5] is one of the best molecularly characterized cytogenetic groups. Some gene mutations recurrent in CN-AML are strong, independent prognosticators (eg, *FLT3* internal tandem duplications [*FLT3*-ITD],[6,7] *CEBPA*,[8,9] and *WT1*[10,11] mutations), whereas others affect outcome in distinct molecular or clinical subsets of CN-AML (eg, *NPM1*,[12,13] *TET2*,[14] and *IDH1*/*IDH2*[15,16] mutations) or are of uncertain significance (eg, *FLT3*-tyrosine kinase domain mutations [*FLT3*-TKD][17,18]). More intense treatment may modify the prognostic weight of some molecular markers in CN-AML, such as *MLL*

partial tandem duplication (*MLL*-PTD)[19,20] or *FLT3*-ITD.[21] Additionally, altered expression of genes (eg, high *BAALC*,[22,23] *ERG*,[23,24] and *MN1*[25-27] levels) and microRNAs (eg, low *miR-181a* level[28]) identify high-risk CN-AML patients.

The *DNMT3A* gene encodes one of the three DNA methyltransferase (DNMT) isoforms. Among these, DNMT1 is the most abundant and preferentially replicates existing DNA methylation patterns, whereas DNMT3A and DNMT3B are responsible for establishing de novo DNA methylation. The process of DNA methylation consists of an enzymatic addition of a methyl group at the carbon 5 position of cytosine in the context of cytosine-guanine dinucleotides. When occurring in the promoter region of a coding gene, it generally results in gene silencing. In AML, all three DNMT enzymes are reportedly overexpressed in malignant blasts compared with normal bone marrow (BM) cells and contribute to leukemogenesis by mediating tumor suppressor gene silencing.[29] Somatic *DNMT3A* mutations in AML were first described by Yamashita et al[30] and subsequently by other groups.[31,32] Ley et al[31] first reported that *DNMT3A* mutations conferred worse outcome in AML. However, the patients analyzed were heterogeneous for biologic and clinical characteristics and treatment received, and the prognostic value of *DNMT3A* mutations was not fully evaluated within the context of other known molecular prognosticators.[31] Recently, Thol et al[33] reported that *DNMT3A* mutations are associated with shorter overall survival (OS) in cytogenetically diverse patients with AML who are younger than 60 years and with lower complete remission (CR) rates and shorter OS in a CN-AML subset. However, this study included patients with secondary disease and those who received allogeneic stem-cell transplantation (SCT) and did not analyze the prognostic impact of different types of *DNMT3A* mutations.[33]

To our knowledge, our study is the first to investigate the prognostic impact of *DNMT3A* mutations in a large population of patients diagnosed exclusively with primary CN-AML, comprehensively characterized for other molecular prognosticators, and receiving intensive chemotherapy. Additionally, we analyzed the differential impact of *DNMT3A* mutations by age group (younger [< 60 years] *v* older [≥ 60 years]) and mutation type (missense mutations at codon R882 [hereafter called R882-*DNMT3A*] *v* mutations at other locations [denoted non–R882-*DNMT3A*]). Furthermore, to gain insights into the biologic role of *DNMT3A* mutations in CN-AML, we derived genome-wide *DNMT3A* mutation-associated gene- and microRNA-expression signatures.

PATIENTS AND METHODS

Patients, Treatment, and Cytogenetic Studies

Pretreatment BM or blood samples were obtained from 415 patients with primary CN-AML, 18 to 83 years of age (181 younger and 234 older), who received intensive first-line therapy on Cancer and Leukemia Group B trials.[34-42] Patients received cytarabine-daunorubicin-based induction chemotherapy; most younger patients received consolidation with high-dose chemotherapy and autologous SCT. Per protocol, no patient received allogeneic SCT during first CR. For details regarding treatment protocols and sample collection, see the Data Supplement. The diagnosis of normal cytogenetics was based on centrally reviewed analysis of ≥ 20 metaphases in BM specimens.[43] All patients provided written informed consent; study protocols were in accordance with the Declaration of Helsinki and approved by local institutional review boards.

Mutational Analyses

For *DNMT3A* mutational analysis, the sequences of exons 18, 19, 21, 22, and 24 to 26 (GenBank reference NM_175629) were analyzed from genomic DNA by polymerase chain reaction and direct sequencing. Patients were also characterized for *FLT3*-ITD,[7,44] *FLT3*-TKD,[17] *MLL*-PTD,[20,45] mutations in *NPM1*,[13] *CEBPA*,[8] *WT1*,[10,11] *TET2*[14] and *IDH1*/*IDH2*,[15] and expression levels of *ERG*[23,24] and *BAALC*,[22,23] as previously reported. Molecular analyses were performed at The Ohio State University.

Microarray Experiments

Gene-expression profiling was performed using oligonucleotide microarrays (Affymetrix, Santa Clara, CA), and microRNA-expression profiling was performed using a custom microarray, as previously reported.[13,14,46] Expression signatures were identified by comparing *DNMT3A*-mutated and *DNMT3A*–wild-type (*DNMT3A*-wt) patients, and analyses to predict *DNMT3A* mutation status were performed (Data Supplement).

Statistical Analyses

Baseline characteristics were compared between *DNMT3A*-mutated and *DNMT3A*-wt patients using Fisher's exact test for categorical and the Wilcoxon rank sum test for continuous variables. Clinical end points were defined according to published recommendations (Data Supplement).[47] For time-to-event analyses, survival estimates were calculated using the Kaplan-Meier method, and groups were compared using the log-rank test. In addition to analyzing all *DNMT3A*-mutated cases as a combined group, we also evaluated the prognostic significance of R882-*DNMT3A* and non–R882-*DNMT3A* mutations separately, in the entire cohort and in the younger and older groups.

In models considering both age groups, we adjusted for an age-group effect (≥ 60 years *v* < 60 years). We constructed multivariable logistic regression models to analyze factors influencing achievement of CR and multivariable Cox proportional hazards models for factors associated with survival end points (Data Supplement). All analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

RESULTS

Prevalence and Spectrum of DNMT3A Mutations in Primary CN-AML

Excluding known single-nucleotide polymorphisms, 148 nonsynonymous sequence variations (mutations) in *DNMT3A* were found in 142 (34.2%) of 415 patients (Data Supplement). The frequencies of these mutations were similar in younger (35.3%) and older (33.3%) patients. Six patients had two mutations each, and four mutations appeared homozygous. Ninety-two mutations (62%) were missense changes in codon R882, leading to an amino acid exchange from arginine to histidine (R882H, n = 49), cysteine (R882C, n = 36), proline (R882P, n = 3), serine (R882S, n = 3), or glycine (R882G, n = 1). R882-*DNMT3A* missense mutations were the most common mutation type among both younger (26%) and older (19%) patients. The remaining non–R882-*DNMT3A* mutations (n = 56; 38%) included 22 nonsense, frameshift, and splice-site mutations found in 22 different patients. These mutations are predicted to either trigger nonsense-mediated RNA decay or result in a truncated protein and thus are likely to impair protein function.[48] Two of these 22 patients concomitantly had an R882-*DNMT3A* mutation (for outcome analyses, these patients were included in the R882-*DNMT3A* mutation group), and two others concomitantly had another non–R882-*DNMT3A* missense mutation. Furthermore, there were 32 missense mutations not affecting codon R882 and two short in-frame deletions. All 32 non-R882 missense mutations were predicted to be "disease causing" by the MutationTaster software,[49] a

Table 1. Clinical and Molecular Characteristics of 415 Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status

Characteristic	*DNMT3A* Mutated (n = 142) No.	%	*DNMT3A* Wild Type (n = 273) No.	%	*P**
Age, years					.46
Median	61		62		
Range	22-82		18-83		
Age, years					.68
< 60	64	45	117	43	
≥ 60	78	55	156	57	
Female sex	72	51	135	49	.84
Race					.49
White	124	89	247	91	
Nonwhite	16	11	25	9	
Hemoglobin, g/dL					.80
Median	9.4		9.4		
Range	4.8-14.5		4.6-15		
Platelet count, $\times 10^9$/L					.55
Median	66		61		
Range	4-481		7-850		
WBC, $\times 10^9$/L					< .001
Median	43.4		22.4		
Range	0.9-434.1		0.9-450		
Percentage of blood blasts					.83
Median	58		57		
Range	0-97		0-99		
Percentage of bone marrow blasts					.03
Median	70		66		
Range	4-97		7-96		
FAB category					< .001
M0	1	1	7	4	
M1	29	27	54	27	
M2	18	17	71	36	
M4	33	31	41	21	
M5	26	24	21	11	
M6	0	0	3	2	
NPM1					< .001
Mutated	107	75	146	53	
Wild type	35	25	127	47	
FLT3-ITD					.01
Present	62	44	85	31	
Absent	80	56	188	69	
CEBPA					< .001
Mutated	7	5	58	21	
Single mutated	4		26		
Double mutated	3		32		
Wild type	135	95	215	79	
ELN genetic group†					.10
Favorable	60	42	140	51	
Intermediate-I	82	58	133	49	
FLT3-TKD					1.00
Present	10	7	21	8	
Absent	128	93	245	92	
WT1					.37
Mutated	10	7	28	10	
Wild type	132	93	245	90	

(continued in next column)

Table 1. Clinical and Molecular Characteristics of 415 Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status (continued)

Characteristic	*DNMT3A* Mutated (n = 142) No.	%	*DNMT3A* Wild Type (n = 273) No.	%	*P**
TET2					.54
Mutated	31	22	67	25	
Wild type	110	78	202	75	
MLL-PTD					1.00
Present	7	6	15	6	
Absent	116	94	228	94	
IDH1					.07
R132	22	16	26	10	
Wild type	118	84	246	90	
IDH2					
Mutated	24	17	51	19	.79
Codon R140	18		44		
Codon R172	6		7		
Wild type	116	83	221	81	
ERG expression group‡					.39
High	56	55	92	49	
Low	45	45	94	51	
BAALC expression group‡					.38
High	45	47	104	53	
Low	51	53	93	47	

Abbreviations: FAB, French-American-British classification; ELN, European LeukemiaNet; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutation in the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

*P values for categorical variables are from Fisher's exact test; *P* values for continuous variables are from the Wilcoxon rank sum test.

†The ELN favorable genetic group includes patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD.[4] The ELN intermediate-I risk group comprises the remaining patients with CN-AML who had wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD.

‡The median expression value was used as a cut point.

computational algorithm that evaluates the disease-causing potential of gene mutations on the basis of evolutionary conservation and structural protein features.

Associations of DNMT3A *Mutations With Pretreatment Clinical and Molecular Characteristics*

No differences in age, sex, or race were observed between patients with and without *DNMT3A* mutations. However, *DNMT3A*-mutated patients had higher WBC counts ($P < .001$) and BM blasts percentages ($P = .03$) and harbored *NPM1* mutations ($P < .001$) and *FLT3*-ITD ($P = .01$) more often and *CEBPA* mutations ($P < .001$) less often than those with *DNMT3A*-wt (Table 1).

Because AML biology and treatment regimens differ between younger and older patients, and it is unclear whether R882-*DNMT3A* and non–R882-*DNMT3A* mutations are functionally and clinically equivalent, we performed subgroup analyses taking age and *DNMT3A* mutation types into account. Younger R882-*DNMT3A*–mutated patients more often had *NPM1* mutations ($P = .02$) and *FLT3*-ITD ($P = .03$) and less often had *CEBPA* mutations ($P < .001$), *WT1* mutations ($P = .02$), and low *ERG*

Table 2. Age Group–Adjusted Analysis of Outcomes of Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia According to *DNMT3A* Mutation Status

End Point	*DNMT3*-mut (n = 142)	R882-*DNMT3A* (n = 92)	non–R882-*DNMT3A* (n = 50)	*DNMT3A*-wt (n = 273)	*P* (*DNMT3A*-mut *v* *DNMT3A*-wt)	*P* (R882-*DNMT3A* *v* *DNMT3A*-wt)	*P* (non–R882-*DNMT3A* *v* *DNMT3A*-wt)
Complete remission					.42	.85	.21
Odds ratio	1.22	1.05	1.62	Reference group			
95% CI	0.75 to 1.96	0.61 to 1.83	0.76 to 3.43				
Disease-free survival					.03	.03	.19
Hazard ratio	1.34	1.42	1.30	Reference group			
95% CI	1.02 to 1.75	1.03 to 1.96	0.88 to 1.90				
Overall survival					.07	.05	.39
Hazard ratio	1.25	1.32	1.16	Reference group			
95% CI	0.98 to 1.57	1.01 to 1.74	0.83 to 1.63				

Abbreviations: mut, mutated; wt, wild type.

expression ($P = .04$) than *DNMT3A*-wt patients (Data Supplement). Younger patients with non–R882-*DNMT3A* mutations were more frequently *NPM1*-mutated ($P = .02$) and showed trends toward a higher frequency of *FLT3*-ITD ($P = .11$) and lower frequency of *CEBPA* mutations ($P = .07$; Data Supplement). Among older patients, those with R882-*DNMT3A* mutations showed trends toward a higher frequency of *NPM1* mutations ($P = .09$) and *FLT3*-ITD ($P = .15$) and lower frequency of *CEBPA* mutations ($P = .14$; Data Supplement). Older patients with non–R882-*DNMT3A* mutations were more likely *NPM1*-mutated ($P = .003$) and, by trend, *WT1*-mutated ($P = .07$) and less likely *CEBPA*-mutated ($P = .05$; Data Supplement).

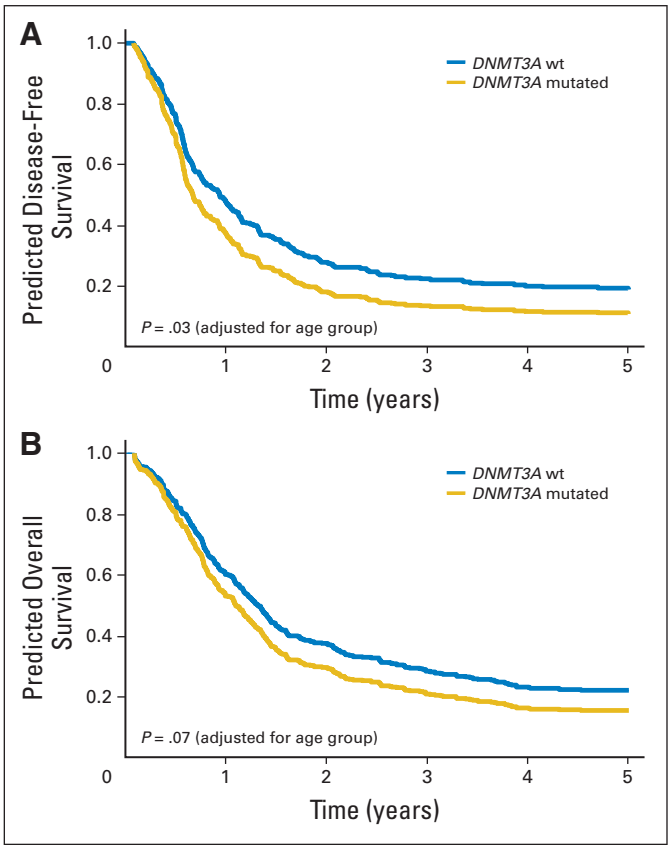

Fig 1. Age group–adjusted clinical outcome for patients with and without *DNMT3A* mutations. (A) Disease-free survival. (B) Overall survival. The curves are adjusted for age group. wt, wild type.

Association of DNMT3A *Mutation Status With Clinical Outcome*

When younger and older patients were considered together in analyses adjusted for age group, *DNMT3A* mutations were not associated with the probability of CR attainment ($P = .42$; Table 2). With a median follow-up of 7.5 years (range, 2.3 to 12.4 years) for patients alive, those harboring *DNMT3A* mutations had shorter disease-free survival (DFS; $P = .03$) and a trend toward shorter OS ($P = .07$) than *DNMT3A*-wt patients (Table 2; Fig 1). In a multivariable analysis for DFS (Table 3), *DNMT3A* mutations were associated with a 47% increased risk of relapse or death ($P = .01$), once adjusted for *FLT3*-ITD, *WT1* mutations, *MLL*-PTD status, and age group. In contrast, once adjusted for other clinical and molecular prognosticators, there was no association of *DNMT3A* mutation status with OS.

Association of Different DNMT3A *Mutation Types With Clinical Outcome*

We tested the association of the two types of *DNMT3A* mutations with outcome of younger and older patients separately because these age groups were treated on Cancer and Leukemia Group B protocols that differ in chemotherapy intensity (Data Supplement). Neither type of *DNMT3A* mutation had an impact on the probability of achieving CR in younger or older patients.

In younger patients, R882-*DNMT3A* mutations were not significantly associated with DFS or OS (Table 4, Figs 2A and 2B). In contrast, patients harboring non–R882-*DNMT3A* mutations had a significantly shorter DFS ($P = .007$; 3-year rates, 20% *v* 49%; Fig 2A) and a trend toward shorter OS ($P = .09$; 3-year rates, 29% *v* 52%; Fig 2B) than *DNMT3A*-wt patients (Table 4). In a multivariable analysis for DFS (Table 3), patients with non–R882-*DNMT3A* mutations had an almost three-fold increased risk of relapse or death ($P = .002$), once adjusted for *FLT3*-ITD and mutations in *NPM1*, *CEBPA*, and *WT1*. Likewise, in a multivariable model for OS (Table 3), the risk of death of non–R882-*DNMT3A*–mutated patients was more than twice that of *DNMT3A*-wt patients ($P = .02$) after adjustment for *FLT3*-ITD, *NPM1*, *CEBPA*, and *WT1* mutation status.

Table 3. Multivariable Analyses for Outcome in Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia

Group	Disease-Free Survival			Overall Survival		
	HR	95% CI	*P*	HR	95% CI	*P*
All patients						
DNMT3A, mutated *v* wild type	1.47	1.08 to 2.00	.01	*DNMT3A* mutation status was not significantly associated with OS upon adjusting for other variables		
FLT3-ITD, ITD *v* no ITD	1.82	1.34 to 2.48	< .001			
WT1, mutated *v* wild type	2.17	1.26 to 3.72	.005			
MLL-PTD, present *v* absent	1.95	1.12 to 3.39	.02			
Age group, older *v* younger	2.53	1.89 to 3.39	< .001			
Patients, age < 60 years						
DNMT3A, non-R882-mutated *v* wild type	2.78	1.45 to 5.36	.002	2.24	1.17 to 4.30	.02
NPM1, mutated *v* wild type	0.52	0.29 to 0.95	.03	0.38	0.23 to 0.64	< .001
FLT3-ITD, ITD *v* no ITD	1.79	1.07 to 3.02	.03	1.70	1.06 to 2.76	.03
CEBPA, double-mutated *v* single-mutated or wild type	0.21	0.09 to 0.50	< .001	0.15	0.06 to 0.35	< .001
WT1, mutated *v* wild type	4.88	2.37 to 10.04	< .001	5.91	3.20 to 10.90	< .001
Patients, age ≥ 60 years						
DNMT3A, R882-mutated *v* wild type	1.85	1.20 to 2.84	.005	1.76	1.24 to 2.49	.002
NPM1, mutated *v* wild type	0.54	0.36 to 0.80	.002	0.48	0.35 to 0.66	< .001
FLT3-ITD, ITD *v* no ITD	2.00	1.34 to 2.98	< .001	1.80	1.31 to 2.47	< .001
Age, each 10 year increase	0.96	0.93 to 0.99	.02			

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; HR, hazard ratio; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

In older patients, R882-*DNMT3A* mutations were associated with significantly shorter DFS ($P = .006$; 3-year rates, 3% *v* 21%; Fig 2C) and OS ($P = .01$; 3-year rates, 4% *v* 24%; Fig 2D), whereas non–R882-*DNMT3A* mutations were not (Table 4, Figs 2C and 2D). In a multivariable model for DFS (Table 3), R882-*DNMT3A* mutations remained associated with an 85% increased risk of relapse or death ($P = .005$) after adjustment for *NPM1* mutation and *FLT3*-ITD status and age. Similarly, in a multivariable model for OS (Table 3), R882-*DNMT3A* mutations were associated with a 76% increased risk of death ($P = .002$) once adjusted for *NPM1* mutation and *FLT3*-ITD status.

Gene- and microRNA-Expression Signatures Associated With DNMT3A *Mutations*

To gain insights into the biology of *DNMT3A*-mutated CN-AML, we studied mutation-associated gene-expression signatures in a subset of patients (n = 278) with available material. Clinical and

Table 4. Outcome of Patients With Primary Cytogenetically Normal Acute Myeloid Leukemia, According to Age Group and Type of *DNMT3A* Mutation

End Point	*DNMT3A*-mut	R882-*DNMT3A*	non–R882-*DNMT3A*	*DNMT3A*-wt	*P* (*DNMT3A*-mut *v* *DNMT3A*-wt)	*P* (R882-*DNMT3A* *v* *DNMT3A*-wt)	*P* (non–R882-*DNMT3A* *v* *DNMT3A*-wt)
Patients < 60 years of age, no.	64	47	17	117			
Complete remission rate, %	81	79	88	96 (82)	1.00	.66	.74
Disease-free survival					.16	.68	.007
Median, years	1.1	1.3	0.7	2.9			
% Disease-free at 3 years	37	43	20	49			
95% CI	24 to 49	27 to 58	5 to 42	39 to 58			
Overall survival					.36	.78	.09
Median, years	1.4	3.5	1.3	3.6			
% Alive at 3 years	45	51	29	52			
95% CI	33 to 57	36 to 64	11 to 51	43 to 61			
Patients ≥ 60 years of age, no.	78	45	33	156			
Complete remission rate,%	73	71	76	66	.30	.59	.31
Disease-free survival					.11	.006	.91
Median, years	0.7	0.7	1.0	1.0			
% Disease-free at 3 years	11	3	20	21			
95% CI	4 to 20	1 to 14	7 to 37	14 to 30			
Overall survival					.10	.01	.96
Median, years	1.0	0.9	1.1	1.3			
% Alive at 3 years	12	4	24	24			
95% CI	6 to 21	1 to 13	11 to 39	17 to 31			

Abbreviations: mut, mutated; wt, wild type.

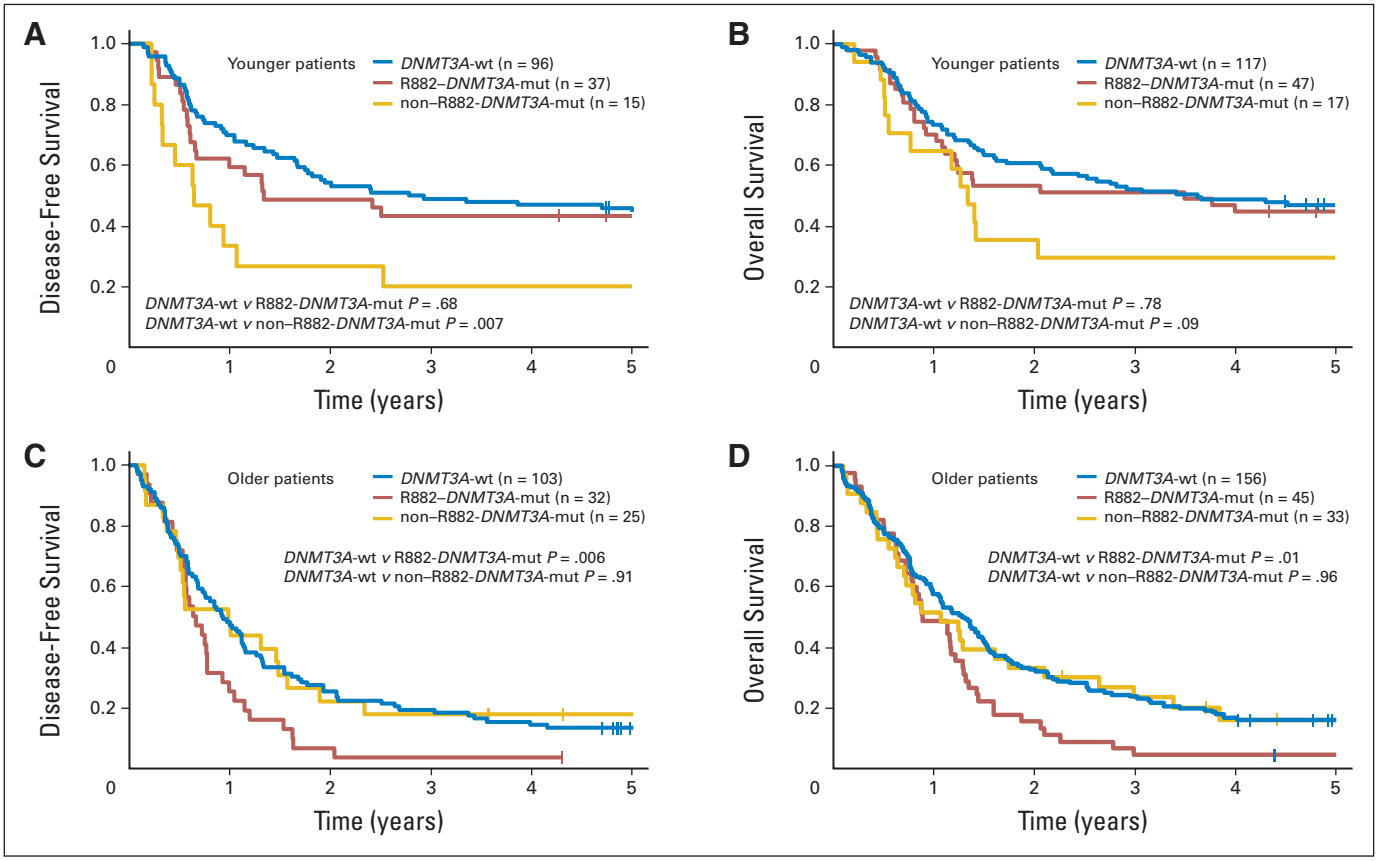

Fig 2. Kaplan-Meier survival curves according to *DNMT3A* mutation type (R882-*DNMT3A* v non–R882-*DNMT3A* mutations v *DNMT3A* wild type). (A) Disease-free survival and (B) overall survival of younger (< 60 years) patients. (C) Disease-free survival and (D) overall survival of older (≥ 60 years) patients. mut, mutated; wt, wild type.

molecular characteristics and outcome of this subset were similar to those of patients not analyzed.

A gene-expression signature associated with *DNMT3A* mutations comprised 1,886 differentially expressed probe sets: 1,323 were upregulated and 563 downregulated in *DNMT3A*-mutated patients (Data Supplement). The most upregulated known gene was *VCAN*, encoding a protein involved in cell adhesion, proliferation, migration, and angiogenesis; the most downregulated gene was *ALAS2*, involved in the heme biosynthetic pathway. However, the signature had an overall cross-validated accuracy of only 67% for predicting *DNMT3A* mutation status (62% sensitivity; 70% specificity), thereby suggesting the contributing effect of other associated molecular aberrations.

When we attempted to derive gene-expression signatures associated with specific types of *DNMT3A* mutations, no significant signature separated patients harboring non–R882-*DNMT3A* mutations (n = 32) from those with *DNMT3A*-R882 mutation (n = 60).

For microRNA profiling, younger and older patients were analyzed separately to avoid confounding batch effects. Testing for differentially expressed microRNAs revealed no signature associated with *DNMT3A* mutations in the younger group. In contrast, we derived a signature consisting of 12 microRNAs associated with *DNMT3A* mutations in older patients (Data Supplement), with four microRNA probes upregulated, including a member of the *miR-10* family reportedly associated with *NPM1* mutations,[13] and eight microRNA probes downregulated, including *miR-181c*, a member of the *miR-181* family associated with *CEBPA* mutations.[8] However, these features might reflect confounding as a result of the significant positive association of *DNMT3A* mutations with *NPM1* mutations and the negative association with *CEBPA* mutations. This microRNA-expression signature had an overall accuracy of only 58% for predicting *DNMT3A* mutation status (49% sensitivity; 62% specificity).

DISCUSSION

Advanced sequencing technologies have allowed analysis of the whole genome of AML blasts. Application of these technologies has recently identified two novel recurrent gene mutations in CN-AML, first *IDH1* mutations[50] and, more recently, *DNMT3A* mutations.[31] As this approach becomes broadly used, it is likely that previously unrecognized mutations in AML will continue to emerge. Because these mutations have the potential to contribute to myeloid leukemogenesis and become prognostic factors and/or therapeutic targets, it is imperative to rapidly test their biologic and clinical impact on patients with AML. However, from previously discovered mutated or aberrantly expressed genes in CN-AML, we have learned that only rarely is testing for a single genetic alteration sufficient for accurate outcome prediction and treatment guidance.[13] Instead, the clinical impact of most molecular markers is influenced by other, concurrent molecular

aberrations.[12,14-16,21] Therefore, to fully understand the clinical significance of emerging molecular markers, such as *DNMT3A* mutations, they need to be evaluated in large series of patients homogeneous for age and type of disease (primary *v* secondary or treatment-related AML), similarly treated and fully characterized for established prognostic markers. To our knowledge, our study analyzed *DNMT3A* mutations in the largest CN-AML patient cohort to date and is first to report subgroup analyses and multivariable models considering different types of *DNMT3A* mutations in distinct age groups.

We found that *DNMT3A* mutations were among the most common mutations in CN-AML, occurring in 34% of patients, with a similar frequency among younger and older patients, and were significantly associated with *NPM1* mutations, *FLT3*-ITD, and wild-type *CEBPA*. Regarding prognostic significance, we showed that *DNMT3A* mutations had worse DFS and OS, after adjustment for age. Moreover, we observed that the prognostic significance of *DNMT3A* mutations depended both on age and the type of mutation (R882-*DNMT3A v* non–R882-*DNMT3A*) considered concurrently (see Fig 2 and also Data Supplement). In younger patients, only non–R882-*DNMT3A* mutations were associated with worse clinical outcome, whereas R882-*DNMT3A* mutations had no prognostic significance. Conversely, in older patients, only R882-*DNMT3A* mutations, not non–R882-*DNMT3A* mutations, were independently associated with worse outcome. The reasons why the prognostic significance of different *DNMT3A* mutation types varies in younger and older patients are currently unknown. One could postulate that this is related to their association with other prognosticators. Thus, in older patients, the potentially negative prognostic significance of non–R882-*DNMT3A* mutations might have been somewhat offset by a high incidence (79%) of accompanying *NPM1* mutations, known to favorably affect prognosis of older patients.[13] However, two thirds of older patients with the prognostically adverse R882-*DNMT3A* mutations also harbored *NPM1* mutations, and slight differences in frequencies of other molecular markers between patients harboring R882-*DNMT3A* and non–R882-*DNMT3A* mutations, both in the older and younger age groups, do not seem sufficient to account for the differential association of the two *DNMT3A* mutation types on treatment outcome.

Our results differ somewhat from those reported by Ley et al,[31] who found a strong, independent association of *DNMT3A* mutations with OS, and those by Thol et al,[33] who studied only patients younger than 60 years and who found that in the CN-AML subgroup, *DNMT3A* mutations were associated with a lower CR rate and shorter OS in multivariable analyses. These discrepancies may be related to differences in the patient populations analyzed with respect to their size, cytogenetics, molecular markers, age, disease type, and treatment. Furthermore, previous studies did not include older patients[33] or included only a small proportion of older patients and did not present data on CR rates, DFS, or multivariable analyses for patients with CN-AML.[31] Therefore, a direct comparison of the findings across studies is not possible.

We also report the first gene- and microRNA-expression signatures associated with *DNMT3A* mutations. However, the accuracy of the gene-expression signature in predicting *DNMT3A* mutational status was only 67%. These results are consistent with an unsupervised analysis of gene-expression array data reported by Ley et al,[31] where no patient cluster was clearly linked to *DNMT3A* mutation status. Similarly, a microRNA-expression signature derived in older patients with CN-AML comprised microRNAs strongly associated with other markers (ie, *NPM1* mutations and wild-type *CEBPA*) and was not accurate in predicting *DNMT3A* mutational status. These results suggest that *DNMT3A* mutations have no strong impact on genome-wide gene- and microRNA-expression profiles in CN-AML. The signatures we identified might at least partially reflect the association of *DNMT3A* mutation status with other molecular markers that are themselves associated with characteristic gene- and microRNA-expression signatures.

The mechanisms through which *DNMT3A* mutations contribute to leukemogenesis are not yet characterized. Although two studies[31,33] found no differences in global DNA methylation or changes in gene methylation patterns in *DNMT3A*-mutated patients, other reports[30,32] suggested that most of the *DNMT3A* mutations decrease the enzymatic activity of the encoded protein. Uncovering how *DNMT3A* mutations affect DNA methylation and epigenetic regulation of gene expression may have ramifications for treatment selection because DNA hypomethylating agents, such as decitabine, are increasingly used for up-front or salvage therapies in older patients with AML,[51] and response to these drugs may be affected by alterations in *DNMT3A* function.[52]

In summary, testing for the mutations in *DNMT3A* may provide a new tool for refining age-related risk classification of CN-AML. The strongest prognostic significance was found in older patients harboring R882-*DNMT3A* mutations, whereas non–R882-*DNMT3A* mutations were associated with relapse risk in younger patients. The gene- and microRNA-expression signatures were not accurate in predicting *DNMT3A* mutational status likely because they are affected by other, concurrent molecular markers. Thus the contribution of the *DNMT3A* mutations to myeloid leukemogenesis requires further investigation, as does the usefulness of *DNMT3A* mutations for risk stratification both in patients with CN-AML and in other cytogenetic and molecular subsets of AML.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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