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

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APPENDIX: *DNMT3A* mutations in adult primary CN-AML

Supplemental Methods

Definition of Clinical Endpoints

Complete remission (CR) was defined as recovery of morphologically normal bone marrow and blood counts (ie, neutrophils ≥1,500/μl and platelets >100,000/μl), and no circulating leukemic blasts or evidence of extramedullary leukemia. Disease-free survival (DFS) was measured from the date of CR until the date of relapse or death; patients alive and in CR were censored at last follow-up. Overall survival (OS) was measured from the date of study entry until the date of death, and patients alive at last follow-up were censored.

Treatment

Patients with cytogenetically normal acute myeloid leukemia (CN-AML) younger than 60 years were treated on Cancer and Leukemia Group B (CALGB) trials 9621 or 19808. Patients enrolled on CALGB 19808 (n=92) were randomly assigned to receive induction chemotherapy with cytarabine, daunorubicin, and etoposide with or without PSC-833 (valspodar), a multidrug resistance protein inhibitor.[1] On achievement of CR, patients were assigned to intensification with high-dose cytarabine and etoposide for stem-cell mobilization followed by myeloablative treatment with busulfan and etoposide supported by autologous peripheral blood stem-cell transplantation. Patients enrolled on CALGB 9621 (n=89) were treated similarly to those on CALGB 19808, as previously reported.[2,3] Older patients (≥60 years) were all treated with cytarabine/daunorubicin-based induction therapy followed by cytarabine-based consolidation therapy. Patients on CALGB 8525 (n=24) were treated with induction chemotherapy consisting of cytarabine in combination with daunorubicin and were randomly assigned to consolidation with different doses of cytarabine followed by maintenance treatment.[4] Patients on CALGB 8923 (n=21) were treated with induction chemotherapy consisting of cytarabine in combination with

daunorubicin and were randomly assigned to receive postremission therapy with cytarabine alone or in combination with mitoxantrone.[5] Patients on CALGB 9420 (n=5) and 9720 (n=110) received induction chemotherapy consisting of cytarabine in combination with daunorubicin and etoposide, with (CALGB 9420) or with/without (CALGB 9720) the multidrug resistance protein modulator PSC-833.[6,7] Patients on CALGB 9420 received postremission therapy with cytarabine (2 g/m^2/d) alone, and patients on CALGB 9720 received a single cytarabine/daunorubicin consolidation course and were then randomly assigned to low-dose recombinant interleukin-2 maintenance therapy or none.[8] Patients on CALGB 10201 (n=74) received induction chemotherapy consisting of cytarabine and daunorubicin, with or without the *BCL2* antisense oblimersen sodium. The consolidation regimen included two cycles of cytarabine (2 g/m^2/d) with or without oblimersen.[9]

When the outcomes of patients studied for *DNMT3A* mutations were compared with patients enrolled on the relevant CALGB protocols who were not included in our study (n=473), differences in outcome did not reach statistical significance.

Multivariable Models

Multivariable logistic regression models were generated for attainment of CR, and multivariable proportional hazards models were constructed for DFS and OS, using a limited backwards elimination procedure. Variables that were considered for univariable analyses in addition to *DNMT3A* mutations were age, sex, race, hemoglobin, platelet count, white blood cell count (WBC), mutation status of *NPM1*, *CEBPA*, *WT1*, *TET2*, *IDH1* and *IDH2*, presence/absence of *FLT3*-internal tandem duplications (*FLT3*-ITD) and tyrosine kinase domain mutations (*FLT3*-TKD), and *MLL* partial tandem duplications (*MLL*-PTD), and *BAALC* and *ERG* expression status (high *v* low). Variables significant at α=.20 from the univariable analyses were considered for multivariable analyses. For the time-to-event endpoints, the proportional hazards assumption was checked for each variable individually. All models considering both age groups were

adjusted for an age-group effect (≥60 years *v* <60 years).

Gene- and microRNA-Expression Profiling

To establish a signature of genes differentially expressed between *DNMT3A*-mutated and *DNMT3A* wild-type (*DNMT3A*-wt) patients, we evaluated gene-expression profiles obtained using Affymetrix HG-U133plus2.0 oligonucleotide microarrays (Affymetrix, Santa Clara, CA). Details regarding sample preparation, array hybridization, and signal computation have been published previously.[10] A total of 24,995 probe-sets that passed a filtering criterion were analyzed. Normalized expression values were compared between *DNMT3A*-mutated (n=92) and *DNMT3A*-wt (n=186) patients, and a univariable significance level of .001 was used to identify differentially expressed probe-sets. A global test of significance based on a permutation procedure was performed to determine whether or not the number of differentially expressed probe sets was more than expected by chance; if not, no signature is reported for the comparison.

To derive microRNA expression signatures associated with *DNMT3A* mutations, younger and older patients with cytogenetically normal acute myeloid leukemia (CN-AML) were analyzed separately In order to avoid confounding batch effects. For younger CN-AML patient samples, the Ohio State University custom microRNA array version 3.0 platform was used. Details regarding sample preparation, array hybridization, and signal computation have been published previously.[10] 305 unique human probes that passed filtering criteria were analyzed. Normalized expression values were compared between *DNMT3A*-mutated (n=29) and *DNMT3A*-wt (n=53) patients, and a univariable significance level of .005 was used to identify differentially expressed probes. For older CN-AML patient samples, the Ohio State University custom microRNA array version 4.0 platform was used. Details regarding sample preparation, array hybridization, and signal computation have been published previously.[11] Four-hundred-sixty unique human probes

that passed filtering criteria were analyzed. Normalized expression values were compared between *DNMT3A*-mutated (n=53) and *DNMT3A*-wt (n=125) patients, and a univariable significance level of .005 was used to identify differentially expressed probes. For each comparison, a global test of significance based on a permutation procedure was performed to determine whether or not the number of differentially expressed probes was more than expected by chance; if not, no signature is reported for the comparison.

We implemented compound covariate prediction using leave-one-out cross-validation to predict *DNMT3A* mutation status of patients from gene- and microRNA-expression profiles.[12] Each patient, one at a time, was removed from analysis and the expression profiles of the remaining patients were compared to derive a gene- or microRNA-expression signature. A compound covariate was then computed for each patient based on this signature: the value of the compound covariate for patient i was $c_i = \Sigma\ w_j\ x_{ij}$, where x_{ij} is the log-transformed expression value for probe j in patient i and w_j is the weight assigned to probe j (in this case, w_j was set equal to the two-sample t-statistic for the comparison of the *DNMT3A*-mutated and *DNMT3A*-wt groups for probe j). The sum is over all j probes included in the signature. A classification threshold was computed to be the midpoint of the means of the compound covariate values for the *DNMT3A*-mutated and *DNMT3A*-wt groups. The compound covariate was then calculated for the left-out patient and its *DNMT3A* status was predicted by comparing its value to the classification threshold. This entire process was repeated until every patient had been left out one time and its mutation status predicted. The overall accuracy of the prediction is indicated, as are the sensitivity and specificity for prediction of *DNMT3A* mutations. All microarray analyses were performed using BRB-ArrayTools Version 3.8.1, developed by Richard Simon, DSc, and Amy Peng Lam.

Supplemental References

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Supplemental Tables

Table A1. *DNMT3A* Mutations Detected in 415 Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia

Mutations	No.
Patients with any *DNMT3A* mutations	142
Total number of *DNMT3A* mutations	148
Mutations at codon R882	92
- R882H	49*
- R882C	36†
- R882P	3
- R882S	3
- R882G	1‡
Nonsense mutations	8
Frame shift mutations	9§
Mutations affecting splice sites	5‖
Missense mutations not affecting codon R882¶	32#
Short in-frame deletions	2

* One patient had both an R882H mutation and a splice site mutation.
† One patient had both an R882C mutation and a non-R882 missense mutation.
‡ This patient had both an R882G mutation and a frame shift mutation.
§ One patient had both a frame shift mutation and a non-R882 missense mutation.
‖ One patient had both a splice site mutation and a non-R882 missense mutation.
¶ Two of these mutations were demonstrated by Ley et al[13] to be somatically acquired mutations.
# One patient had two different non-R882 missense mutations.

Table A2. Comparison of Molecular Characteristics of Younger (<60 Years) Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia with R882-*DNMT3A* Mutations Versus *DNMT3A* Wild Type.

Characteristic	**R882-*DNMT3A* Mutated (n=47)**	***DNMT3A* Wild Type (n=117)**	***P****
NPM1, no. (%)			.02
Mutated	36 (77)	66 (56)	
Wild type	11 (23)	51 (44)	
FLT3-ITD, no. (%)			.03
Present	24 (51)	38 (32)	
Absent	23 (49)	79 (68)	
CEBPA, no. (%)			< .001
Mutated	2 (4)	33 (28)	
Single mutated	2	10	
Double mutated	0	23	
Wild type	45 (96)	84 (72)	
ELN Genetic Group[†], no. (%)			.06
Favorable	19 (40)	68 (58)	
Intermediate-I	28 (60)	49 (42)	
FLT3-TKD, no. (%)			1.00
Present	4 (9)	10 (9)	
Absent	43 (91)	105 (91)	
WT1, no. (%)			.02
Mutated	1 (2)	19 (16)	
Wild type	46 (98)	98 (84)	
TET2, no. (%)			.49
Mutated	6 (13)	21 (18)	
Wild type	41 (87)	94 (82)	
MLL-PTD, no. (%)			1.00
Present	3 (6)	8 (7)	
Absent	44 (94)	109 (93)	
IDH1, no. (%)			.16
R132 Mutated	7 (15)	9 (8)	
Wild type	39 (85)	107 (92)	
IDH2, no. (%)			
Mutated	7 (15)	9 (8)	.16
Codon R140	5	8	
Codon R172	2	1	
Wild type	39 (85)	107 (92)	

Characteristic	R882-*DNMT3A* Mutated (n=47)	*DNMT3A* Wild Type (n=117)	*P**
ERG expression group‡ no. (%)			.04
High	23 (68)	37 (45)	
Low	11 (32)	45 (55)	
BAALC expression group‡, no. (%)			.17
High	14 (39)	51 (54)	
Low	22 (61)	43 (46)	

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; ELN, European LeukemiaNet; *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.[14] The ELN Intermediate-I genetic group comprises the remaining CN-AML patients, 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.

Table A3. Comparison of Molecular Characteristics in Younger (<60 Years) Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia with non-R882-*DNMT3A* Mutations Versus *DNMT3A* Wild Type.

Characteristic	**non-R882-*DNMT3A* Mutated (n=17)**	***DNMT3A* Wild Type (n=117)**	***P****
NPM1, no. (%)			.02
Mutated	15 (88)	66 (56)	
Wild type	2 (12)	51 (44)	
FLT3-ITD, no. (%)			.11
Present	9 (53)	38 (32)	
Absent	8 (47)	79 (68)	
CEBPA, no. (%)			.07
Mutated	1 (6)	33 (28)	
Single mutated	0	10	
Double mutated	1	23	
Wild type	16 (94)	84 (72)	
ELN Genetic Group[†], no. (%)			.20
Favorable	7 (41)	68 (58)	
Intermediate-I	10 (59)	49 (42)	
FLT3-TKD, no. (%)			.60
Present	0 (0)	10 (9)	
Absent	14 (100)	105 (91)	
WT1, no. (%)			1.00
Mutated	2 (12)	19 (16)	
Wild type	15 (88)	98 (84)	
TET2, no. (%)			.74
Mutated	4 (24)	21 (18)	
Wild type	13 (76)	94 (82)	
MLL-PTD, no. (%)			.60
Present	0 (0)	8 (7)	
Absent	17 (100)	109 (93)	
IDH1, no. (%)			.06
R132 Mutated	4 (24)	9 (8)	
Wild type	13 (76)	107 (92)	
IDH2, no. (%)			
Mutated	4 (24)	9 (8)	.06
Codon R140	4	8	
Codon R172	0	1	
Wild type	13 (76)	107 (92)	

Characteristic	**non-R882-*DNMT3A* Mutated (n=17)**	***DNMT3A* Wild Type (n=117)**	***P****
ERG expression group‡, no. (%)			.57
High	7 (54)	37 (45)	
Low	6 (46)	45 (55)	
BAALC expression group‡, no. (%)			1.00
High	5 (56)	51 (54)	
Low	4 (44)	43 (46)	

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; ELN, European LeukemiaNet; *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.[14] The ELN Intermediate-I genetic group comprises the remaining CN-AML patients, 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.

Table A4. Comparison of Molecular Characteristics in Older (≥60 Years) Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia with R882-*DNMT3A* Mutations Versus *DNMT3A* Wild Type.

Characteristic	**R882-*DNMT3A*-Mutated (n=45)**	***DNMT3A* Wild Type (n=156)**	***P****
NPM1, no. (%)			.09
Mutated	30 (67)	80 (51)	
Wild type	15 (33)	76 (49)	
FLT3-ITD, no. (%)			.15
Present	19 (42)	47 (30)	
Absent	26 (58)	109 (70)	
CEBPA, no. (%)			.14
Mutated	3 (7)	25 (16)	
Single mutated	1	16	
Double mutated	2	9	
Wild type	42 (93)	131 (84)	
ELN Genetic Group†, no. (%)			.39
Favorable	17 (38)	72 (46)	
Intermediate-I	28 (62)	84 (54)	
FLT3-TKD, no. (%)			.74
Present	2 (5)	11 (7)	
Absent	42 (95)	140 (93)	
WT1, no. (%)			1.00
Mutated	2 (4)	9 (6)	
Wild type	43 (96)	147 (94)	
TET2, no. (%)			1.00
Mutated	13 (30)	46 (30)	
Wild type	31 (70)	108 (70)	
MLL-PTD, no. (%)			.69
Present	3 (8)	7 (6)	
Absent	33 (92)	119 (94)	
IDH1, no. (%)			.44
R132 Mutated	7 (16)	17 (11)	
Wild type	38 (84)	139 (89)	
IDH2, no. (%)			
Mutated	9 (20)	42 (27)	.44
Codon R140	7	36	
Codon R172	2	6	
Wild type	36 (80)	114 (73)	

Characteristic	**R882-*DNMT3A*-Mutated (n=45)**	***DNMT3A* Wild Type (n=156)**	***P****
ERG expression group‡, no. (%)			.69
High	19 (58)	55 (53)	
Low	14 (42)	49 (47)	
BAALC expression group‡, no. (%)			.84
High	15 (48)	53 (51)	
Low	16 (52)	50 (49)	

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; ELN, European LeukemiaNet; *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.[14] The ELN Intermediate-I genetic group comprises the remaining CN-AML patients, 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.

Table A5. Comparison of Molecular Characteristics in Older (≥60 Years) Patients with Primary Cytogenetically Normal Acute Myeloid Leukemia with non-R882-*DNMT3A* Mutations Versus *DNMT3A* Wild Type.

Characteristic	**non-R882-*DNMT3A* Mutated (n=33)**	***DNMT3A* Wild Type (n=156)**	***P****
NPM1, no. (%)			.003
Mutated	26 (79)	80 (51)	
Wild type	7 (21)	76 (49)	
FLT3-ITD, no. (%)			1.00
Present	10 (30)	47 (30)	
Absent	23 (70)	109 (70)	
CEBPA, no. (%)			.05
Mutated	1 (3)	25 (16)	
Single mutated	1	16	
Double mutated	0	9	
Wild type	32 (97)	131 (84)	
ELN Genetic Group†, no. (%)			.70
Favorable	17 (52)	72 (46)	
Intermediate-I	16 (48)	84 (54)	
FLT3-TKD, no. (%)			.48
Present	4 (12)	11 (7)	
Absent	29 (88)	140 (93)	
WT1, no. (%)			.07
Mutated	5 (15)	9 (6)	
Wild type	28 (85)	147 (94)	
TET2, no. (%)			.67
Mutated	8 (24)	46 (30)	
Wild type	25 (76)	108 (70)	
MLL-PTD, no. (%)			1.00
Present	1 (4)	7 (6)	
Absent	22 (96)	119 (94)	
IDH1, no. (%)			.76
R132 Mutated	4 (13)	17 (11)	
Wild type	28 (87)	139 (89)	
IDH2, no. (%)			
Mutated	4 (13)	42 (27)	.11
Codon R140	2	36	
Codon R172	2	6	
Wild type	28 (87)	114 (73)	

Characteristic	**non-R882-*DNMT3A* Mutated (n=33)**	***DNMT3A* Wild Type (n=156)**	***P****
ERG expression group‡, no. (%)			.15
High	7 (33)	55 (53)	
Low	14 (67)	49 (47)	
BAALC expression group‡, no. (%)			.81
High	11 (55)	53 (51)	
Low	9 (45)	50 (49)	

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; ELN, European LeukemiaNet; *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.[14] The ELN Intermediate-I genetic group comprises the remaining CN-AML patients, who had wild-type *CEBPA,* wild type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD.

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

Table A6. *DNMT3A* Mutation-Associated Gene-Expression Signatures

see separate Excel file

Table A7. *DNMT3A* Mutation-Associated microRNA-Expression Signatures

see separate Excel file

Supplemental Figure Legend

Fig. A1*:* Clinical outcome for patients with and without *DNMT3A* mutations, according to age group. (A) Disease-free survival and (B) overall survival of patients aged <60 years. (C) Disease-free survival and (D) overall survival of patients aged ≥60 years.

Supplemental Figure

Figure A1*:*