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ORIGINAL ARTICLE

Prognostic and biologic significance of *DNMT3B* expression in older patients with cytogenetically normal primary acute myeloid leukemia

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DNMT3B encodes a DNA methyltransferase implicated in aberrant epigenetic changes contributing to leukemogenesis. We tested whether *DNMT3B* expression, measured by NanoString nCounter assay, associates with outcome, gene and microRNA expression and DNA methylation profiles in 210 older (⩾60 years) adults with primary, cytogenetically normal acute myeloid leukemia (CN-AML). Patients were dichotomized into high versus low expressers using median cut. Outcomes were assessed in the context of known CN-AML prognosticators. Gene and microRNA expression, and DNA methylation profiles were analyzed using microarrays and MethylCap-sequencing, respectively. High *DNMT3B* expressers had fewer complete remissions (CR; $P = 0.002$) and shorter disease-free (DFS; $P = 0.02$) and overall (OS; $P < 0.001$) survival. In multivariable analyses, high *DNMT3B* expression remained an independent predictor of lower CR rates ($P = 0.04$) and shorter DFS ($P = 0.04$) and OS ($P = 0.001$). High *DNMT3B* expression associated with a gene expression profile comprising 363 genes involved in differentiation, proliferation and survival pathways, but with only four differentially expressed microRNAs (*miR-133b*, *miR-148a*, *miR-122, miR-409-3p*) and no differential DNA methylation regions. We conclude that high *DNMT3B* expression independently associates with adverse outcome in older CN-AML patients. Gene expression analyses suggest that *DNMT3B* is involved in the modulation of several genes, although the regulatory mechanisms remain to be investigated to devise therapeutic approaches specific for these patients.

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INTRODUCTION

Acute myeloid leukemia (AML) is a heterogeneous disease presenting with a wide spectrum of prognostically relevant cytogenetic aberrations, gene mutations and abnormal expression of genes and microRNAs. Cytogenetically normal AML (CN-AML) patients, constituting 40 to 50% of all AML patients,[1] are the largest and molecularly best characterized cytogenetic subset in primary (*de novo*) AML.[1–3] Although leukemic blasts of these patients do not contain microscopically detectable chromosome abnormalities, they harbor prognostically relevant mutations and aberrantly expressed genes and microRNAs.[2–16] In addition to these genetic alterations, epigenetic changes have recently been shown to participate in myeloid leukemogenesis and be pharmacologically targetable.[17,18] Notably, some genes whose mutations are prognostic in CN-AML encode proteins that are implicated in epigenetic regulation of gene transcription, namely *IDH2, ASXL1* and *DNMT3A*. The latter is among the most frequently mutated genes in primary CN-AML patients, being found mutated in 29 to 34% of the patients.[9,19]

DNMT3A encodes DNA methyltransferase 3A (DNMT3A), which is involved in epigenetic gene silencing through DNA hypermethylation.[20] In addition to DNMT3A, DNMT1 and DNMT3B also mediate DNA methylation in normal and malignant cells, and may represent potential therapeutic targets in cancer and leukemia.[21–24] However, in contrast to *DNMT3A*, no recurrent mutations of *DNMT1* and *DNMT3B* genes have been reported in AML.[25] Instead, one study has indicated that higher expression of *DNMT3B* is associated with worse outcome in AML.[26] However, the patient cohort analyzed was cytogenetically diverse and heterogeneous for clinical features and treatment received. Thus, it is

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unknown whether *DNMT3B* expression is an independent prognostic factor and can be used for stratification guidance in CN-AML.

Thus, we analyzed the clinical significance of *DNMT3B* expression in the context of a comprehensive panel of molecular prognosticators in a relatively large cohort of older (aged $\geqslant 60$ years) patients with CN-AML who were similarly treated on cytarabine/daunorubicin-based protocols. To gain biologic insights, we also derived genome-wide *DNMT3B*-associated gene and microRNA expression and DNA methylation profiles. We studied older patients because both the incidence of AML and the role of epigenetics increase with age. Moreover, we have recently reported a favorable clinical response to hypomethylating agents in this age group of AML patients.[27]

PATIENTS AND METHODS

Patients, treatment and cytogenetic studies

Pretreatment bone marrow or blood samples were obtained from 210 patients with primary CN-AML aged 60 to 83 years (median, 68 years) who received intensive first-line therapy on Cancer and Leukemia Group B (CALGB) trials.[28–32] All patients received cytarabine–daunorubicin-based induction chemotherapy, and no patient received allogeneic hematopoietic stem cell transplantation during first complete remission (CR). For details regarding treatment protocols and sample collection, see Supplementary Information. All patients were enrolled on companion CALGB/Alliance protocols: 8461 (cytogenetic analyses), 9665 (tissue banking) and 20 202 (molecular analyses).

Cytogenetic analyses were performed in institutional CALGB/Alliance cytogenetics laboratories. For the patient's karyotype to be considered normal, $\geqslant 20$ metaphase cells from short-term cultures of pretreatment bone marrow specimens had to have been analyzed and the normal result confirmed by central karyotype review.[33] All patients provided written informed consent for participation in these studies; study protocols were in accordance with the Declaration of Helsinki and approved by local Institutional Review Boards.

Single-gene expression analyses

The expression of *DNMT3B* transcript was assessed by NanoString nCounter assays (NanoString Technologies, Seattle, WA, USA; Supplementary Information).[34] These assays measured global expression of the *DNMT3B* gene, and did not allow for quantification of isoform-specific expression of *DNMT3B*. *DNMT3B* expression levels were normalized using *ABL* as an internal control. We also used NanoString nCounter assays to measure expression of *BAALC*, *ERG* and *miR-155*, and real-time reverse transcription-PCR to measure *miR-3151* expression, all of which have been previously shown to affect prognosis of older CN-AML patients.[10,15,16]

Mutational analyses

The presence or absence of *FLT3* internal tandem duplication (*FLT3*-ITD),[35,36] *FLT3* tyrosine kinase domain mutations (*FLT3*-TKD),[37] *MLL* partial tandem duplication (*MLL*-PTD),[38] and mutations in the *NPM1*,[5] *CEBPA*,[39] *WT1*,[40] *IDH1* and *IDH2*,[7] *TET2*,[41] *ASXL1*,[8] *DNMT3A*[9] and *RUNX1*[42] genes were determined centrally as previously described.

Gene and microRNA expression profiling

The gene and microRNA expression profiling were assessed using the Affymetrix U133 plus 2.0 array (Affymetrix, Santa Clara, CA, USA) and The Ohio State University custom microRNA array (OSU_CCC Version 4.0, The Ohio State University, Columbus, OH, USA), respectively, as previously reported,[5,43] and detailed in the Supplementary Information. For *DNMT3B*, the Affymetrix U133 plus 2.0 arrays measured global *DNMT3B* expression levels, and did not quantify expression of the individual *DNMT3B* isoforms. For the gene and microRNA expression profiling, summary measures of gene and microRNA expression were computed, normalized and filtered (Supplementary Information). A *DNMT3B* expression-associated signature (see Supplementary Information for details) was derived by comparing gene expression between high and low *DNMT3B* expressers in the CALGB/Alliance cohort and in two additional sets of CN-AML patients with microarray and RNAseq gene expression data publicly available (German AML Cooperative Group (AMLCG)[44] and The Cancer Genome Atlas (TCGA)[25]). For comparison of the high *DNMT3B* expression signature with the *FLT3*-ITD signature we used gene set enrichment analysis (for details see Supplementary Information). For the microRNA expression signature, only the CALGB/Alliance patients were used. Univariable significance levels of $P < 0.001$ (false discovery rates < 0.01) were used to select genes and microRNAs that constituted the signatures. To assess enrichment of genes in the *DNMT3B* gene expression-associated signature in distinct biologic processes, a Gene Ontology analysis was performed using the Database for Annotation, Visualization and Integrated Discovery (DAVID).[45] We identified as statistically significant 'annotation clusters' those clusters of Gene Ontology terms with enrichment scores of >2.0, *P*-values $\leqslant 0.001$ and Benjamini corrected *P*-values $\leqslant 0.05$. All molecular analyses were performed centrally at The Ohio State University.

DNA methylation

Genome-wide DNA methylation and levels of DNA methylation across the genome's functional regions (that is, genomic features) were measured using the MethylCap-seq assay as previously reported.[18]

Statistical analyses

The patients were dichotomized into high and low expressers using the median cut. This cut was supported by significant results of the trend test applied to outcome of patients divided into quartiles by *DNMT3B* expression ($P \leqslant 0.001$). We compared pretreatment features and outcome between patients with high and low *DNMT3B* expression. Definitions of clinical end points (that is, CR rates, disease-free (DFS) and overall (OS) survival) are provided in the Supplementary Information. Baseline characteristics between high and low *DNMT3B* expressers were compared using the Fisher's exact test for categorical and the Wilcoxon rank-sum test for continuous variables.[46] The categorical variables included the European LeukemiaNet (ELN) Genetic Groups.[47] The ELN guidelines classify CN-AML patients within the Favorable or Intermediate-I Genetic Groups based on *CEBPA, NPM1* and *FLT3* mutational status. The ELN Favorable Genetic Group consists of CN-AML patients with *CEBPA* mutation and/or *NPM1* mutation without *FLT3*-ITD, whereas the Intermediate-I Genetic Group is comprised of patients with wild-type *CEBPA* and *FLT3*-ITD with or without *NPM1* mutation, or wild-type *NPM1* without *FLT3*-ITD.[47]

For time-to-event analyses, we calculated survival estimates using the Kaplan–Meier method, and compared groups by the log rank test.[46] In order to provide the odds ratios and hazard ratios and associated confidence intervals, logistic regression and Cox proportional hazards models were generated to compare outcomes between high and low *DNMT3B* expressers for CR and survival end points (DFS, OS), respectively, and *P*-values from the Wald test are reported. We constructed multivariable logistic regression models to analyze factors associated with the achievement of CR, and multivariable Cox proportional hazards models for factors associated with survival end points,[46] the details of which are provided in the Supplementary Information. All analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

RESULTS

Associations of *DNMT3B* expression with pretreatment clinical and molecular characteristics

At diagnosis, high *DNMT3B* expressers had higher white blood counts (WBC; $P = 0.004$), and percentages of blood ($P = 0.004$) and marrow ($P = 0.02$) blasts than low *DNMT3B* expressers. Concerning molecular features, high *DNMT3B* expressers were more often *FLT3*-ITD-positive ($P < 0.001$) and classified in the ELN Intermediate-I Genetic Group ($P = 0.02$). *IDH2*-R140 mutations were less frequent in high *DNMT3B* expressers, whereas six of seven *IDH2*-R172 mutations were detected in this patient group. High *DNMT3B* expressers also had higher *ERG* ($P < 0.001$), *BAALC* ($P = 0.002$) and *miR-155* ($P = 0.006$) expression than low expressers (Table 1, Supplementary Figure S1).

Associations of *DNMT3B* expression with clinical outcome in the entire patient cohort

With a median follow-up of 5.1 years (range, 2.3–11.6 years) for patients who are alive, high *DNMT3B* expressers had lower CR

Table 1. Comparison of clinical and molecular characteristics of patients with cytogenetically normal acute myeloid leukemia with high versus low *DNMT3B* expression

Characteristic	*High DNMT3B* (n = *105*)	*Low DNMT3B* (n = *105*)	P-*value*
Age, years			0.82
Median	68	68	
Range	60–83	60–81	
Sex, n *(%)*			0.58
Male	58 (55)	53 (50)	
Female	47 (45)	52 (50)	
Race, n *(%)*			0.48
White	96 (92)	92 (89)	
Nonwhite	8 (8)	11 (11)	
Hemoglobin, g/dl			0.71
Median	9.4	9.3	
Range	6.5–12.4	5.4–15.0	
Platelet count, x 10^9/l			0.87
Median	68	71	
Range	4–850	11–510	
WBC, x 10^9/l			0.004
Median	43.7	21.8	
Range	1.0–450.0	0.8–249.3	
Blood blasts, %			0.004
Median	64	40	
Range	0–99	0–97	
Bone marrow blasts, %			0.02
Median	72	64	
Range	21–97	4–97	
Extramedullary involvement, n *(%)*	27 (27)	24 (23)	0.63
NPM1, n *(%)*			0.31
Mutated	67 (66)	60 (59)	
Wild type	34 (34)	42 (41)	
FLT3-ITD, n *(%)*			< 0.001
Present	54 (53)	21 (21)	
Absent	48 (47)	81 (79)	
CEBPA, n *(%)*			0.83[a]
Mutated	13 (13)	12 (12)	
Single mutated	10	5	
Double mutated	3	7	
Wild type	88 (87)	90 (88)	
ELN Genetic Group, n *(%)*[b]			0.02
Modified Favorable	38 (38)	56 (55)	
Intermediate-I	63 (62)	45 (45)	
FLT3-TKD, n *(%)*			0.83
Present	12 (12)	11 (11)	
Absent	89 (88)	91 (89)	
WT1, n *(%)*			0.41
Mutated	8 (8)	5 (5)	
Wild type	93 (92)	97 (95)	
TET2, n *(%)*			1.00
Mutated	32 (32)	31 (32)	
Wild type	68 (68)	67 (68)	
MLL-PTD, n *(%)*			1.00
Present	5 (6)	5 (6)	
Absent	74 (94)	81 (94)	
IDH1, n *(%)*			0.18
Mutated	14 (14)	8 (8)	
Wild type	86 (86)	94 (92)	
IDH2, n *(%)*			0.05
Mutated	18 (18)	31 (30)	
R140	12	30	0.005[c]
R172	6	1	0.13[d]
Wild type	82 (82)	71 (70)	
RUNX1, n *(%)*			0.31
Mutated	17 (18)	11 (12)	
Wild type	79 (82)	81 (88)	
ASXL1, n *(%)*			0.69
Mutated	13 (13)	15 (15)	
Wild type	87 (87)	84 (85)	
DNMT3A, n *(%)*			0.65
Mutated	35 (35)	31 (32)	
R882	19	20	1.00[e]
Non-R882	16	11	0.40[f]
Wild type	64 (65)	66 (68)	
ERG expression group, n *(%)*[g,h]			< 0.001
High	65 (62)	40 (38)	
Low	40 (38)	65 (62)	
BAALC expression group, n *(%)*[g,h]			0.002
High	64 (61)	41 (39)	
Low	41 (39)	64 (61)	
miR-155 expression group, n *(%)*[g,h]			0.006
High	63 (60)	42 (40)	
Low	42 (40)	63 (60)	
miR-3151 expression group, n *(%)*[g,i]			0.76
High	42 (49)	39 (46)	
Low	43 (51)	46 (54)	

Abbreviations: 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; n, number; WBC, white blood count. [a]The *P*-value pertains to a comparison of frequencies of *CEBPA* mutations (single and double combined) versus *CEBPA* wild-type between high and low *DNMT3B* expressers. [b]The ELN modified Favorable Genetic Group is defined as CN-AML patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (that is, those with wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD, or mutated *NPM1* with *FLT3*-ITD) belong to the ELN Intermediate-I Genetic Group.[47] [c]The *P*-value pertains to a comparison of frequencies of *IDH2*-R140 mutations versus *IDH2* wild-type between high and low *DNMT3B* expressers. [d]The *P*-value pertains to a comparison of frequencies of *IDH2*-R172 mutations versus *IDH2* wild-type between high and low *DNMT3B* expressers. [e]The *P*-value pertains to a comparison of frequencies of *DNMT3A*-R882 mutations versus *DNMT3A* wild-type between high and low *DNMT3B* expressers. [f]The *P*-value pertains to a comparison of frequencies of *DNMT3A* non-R882 mutations versus *DNMT3A* wild-type between high and low *DNMT3B* expressers. [g]The median expression value was used as a cut point. [h]Data was assessed by the NanoString nCounter assay. [i]Data was assessed by real-time reverse transcription-PCR.

rates ($P = 0.002$, Wald test; 58% vs 78%), and shorter DFS ($P = 0.02$, Wald test) and OS ($P < 0.001$, Wald test) than *DNMT3B* low expressers (Table 2, Figure 1).

In a multivariable model for CR, *DNMT3B* expression remained prognostic ($P = 0.04$), after adjustment for *BAALC* expression status ($P < 0.001$), WBC ($P = 0.007$) and age ($P = 0.02$) (Table 3). High *DNMT3B* expressers were half as likely to achieve a CR as low expressers. In multivariable analysis for DFS, high *DNMT3B* expression associated with shorter DFS ($P = 0.04$), once adjusted for *BAALC* expression ($P = 0.004$), *DNMT3A*-R882 mutation status ($P = 0.009$) and ELN Genetic Groups ($P = 0.03$). The risk of experiencing relapse or death was 46% higher for high *DNMT3B*

Table 2. Outcomes of patients with cytogenetically normal acute myeloid leukemia according to *DNMT3B* expression status

End point	*High DNMT3B (I)*	*Low DNMT3B (II)*	P-*value*[a]	*OR/HR (95% CI) I vs II*
All patients, n	105	105		
Complete remission, *n* (%)	61 (58)	82 (78)	0.002	0.39 (0.21–0.71)
Disease-free survival			0.02	1.55 (1.09–2.20)
Median, years	0.6	1.1		
% Disease-free at 3 years (95% CI)	13 (6–23)	22 (14–31)		
% Disease-free at 5 years (95% CI)	11 (5–21)	15 (8–23)		
Overall survival			< 0.001	1.85 (1.38–2.47)
Median, years	0.8	1.5		
% Alive at 3 years (95% CI)	11 (6–18)	29 (20–37)		
% Alive at 5 years (95% CI)	8 (4–14)	21 (14–29)		
Patients in the ELN modified Favorable Genetic Group[b], n	38	56		
Complete remission, *n* (%)	27 (71)	47 (84)	0.14	0.47 (0.17–1.28)
Disease-free survival			0.10	1.54 (0.92–2.57)
Median, years	0.9	1.3		
% Disease-free at 3 years (95% CI)	15 (5–30)	30 (18–43)		
% Disease-free at 5 years (95% CI)	11 (3–26)	21 (11–34)		
Overall survival			0.002	2.04 (1.29–3.22)
Median, years	1.3	2.1		
% Alive at 3 years (95% CI)	18 (7–31)	39 (27–52)		
% Alive at 5 years (95% CI)	12 (4–24)	30 (19–43)		
Patients in the ELN Intermediate-I Genetic Group[b], n	63	45		
Complete remission, *n* (%)	31 (49)	33 (73)	0.01	0.35 (0.15–0.80)
Disease-free survival			0.25	1.36 (0.81–2.27)
Median, years	0.5	0.7		
% Disease-free at 3 years (95% CI)	13 (4–27)	9 (2–22)		
% Disease-free at 5 years (95% CI)	13 (4–27)	3 (0–13)		
Overall survival			0.03	1.57 (1.06–2.33)
Median, years	0.6	1.1		
% Alive at 3 years (95% CI)	8 (3–16)	16 (7–28)		
% Alive at 5 years (95% CI)	6 (2–14)	9 (3–19)		

Abbreviations: CI, confidence interval; ELN, European Leukemia Net; HR, hazard ratio; n, number; OR, odds ratio. [a]*P*-values provided are generated by logistic regression and Cox proportional hazards models to compare outcome of patients for CR and survival end points (DFS, OS), respectively, using the Wald test. [b]The ELN modified Favorable Genetic Group is defined as CN-AML patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (that is, those with wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD, or mutated *NPM1* with *FLT3*-ITD) belong to the ELN Intermediate-I Genetic Group.[47]

expressers than for low expressers. *DNMT3B* expression also remained prognostic for OS ($P = 0.001$), after adjustment for *BAALC* ($P < 0.001$), *miR-3151* ($P = 0.02$) and *miR-155* ($P = 0.02$) expression. The risk of death was 72% higher for high *DNMT3B* expressers compared with low expressers (Table 3).

Associations of *DNMT3B* expression with clinical outcome in ELN Genetic Groups

We analyzed the associations of *DNMT3B* expression with outcome separately within the ELN Favorable and Intermediate-I Genetic Groups. Within the Favorable Group ($n = 94$), there was no significant difference in CR rates (71% vs 84%, $P = 0.14$, Wald test) or DFS ($P = 0.10$, Wald test) between high and low *DNMT3B* expressers. However, high expressers had shorter OS ($P = 0.002$, Wald test) than low expressers (Table 2, Figures 2a and b). In multivariable analyses for the ELN Favorable Genetic Group (Table 3), *DNMT3B* expression remained significant for OS ($P = 0.003$) after adjustment for *BAALC* expression ($P = 0.01$). High *DNMT3B* expressers were twice as likely to die as low expressers.

In the Intermediate-I Group ($n = 108$), high *DNMT3B* expressers had a lower CR rate (49% vs 73%, $P = 0.01$, Wald test) and shorter OS ($P = 0.03$, Wald test) than low *DNMT3B* expressers, but there was no significant difference in DFS between the groups (Table 2, Figures 2c and d). In multivariable analyses within the ELN Intermediate-I Genetic Group (Table 3), *DNMT3B* expression was significant for OS ($P = 0.02$), after adjustment for *BAALC* expression ($P = 0.03$), *miR-3151* expression ($P = 0.03$) and WBC ($P = 0.03$). High *DNMT3B* expressers were 1.7 times more likely to die than low expressers.

Genome-wide gene expression profiles associated with *DNMT3B* expression

To gain biologic insights into the role of *DNMT3B*, we derived a *DNMT3B*-associated gene expression profile using three independent sets of CN-AML patients, that is, CALGB/Alliance ($n = 177$), AMLCG ($n = 75$) and The Cancer Genome Atlas ($n = 88$). We identified 195 upregulated genes and 168 downregulated genes that were significantly associated with higher *DNMT3B* expression in each of the three cohorts (Supplementary Table S1). As high *DNMT3B* expression was associated with the presence of *FLT3*-ITD (Table 1), we performed gene set enrichment analysis to test whether a set of 195 genes that are upregulated in high *DNMT3B* expressers was associated with a set of genes differentially expressed between patients who harbored *FLT3*-ITD versus those who did not (Supplementary Information). We found a significant correlation between the high *DNMT3B* expression and *FLT3*-ITD signatures ($P = 0.006$; false discovery rate = 0.006; Supplementary Figure S2). Among the genes upregulated in high *DNMT3B* expressers, we noted a variety of genes previously involved in AML including *CDK6* and *WT1* that encode cyclin kinase and transcription factor proteins, respectively. Among the down-regulated genes, we noted genes involved with both normal

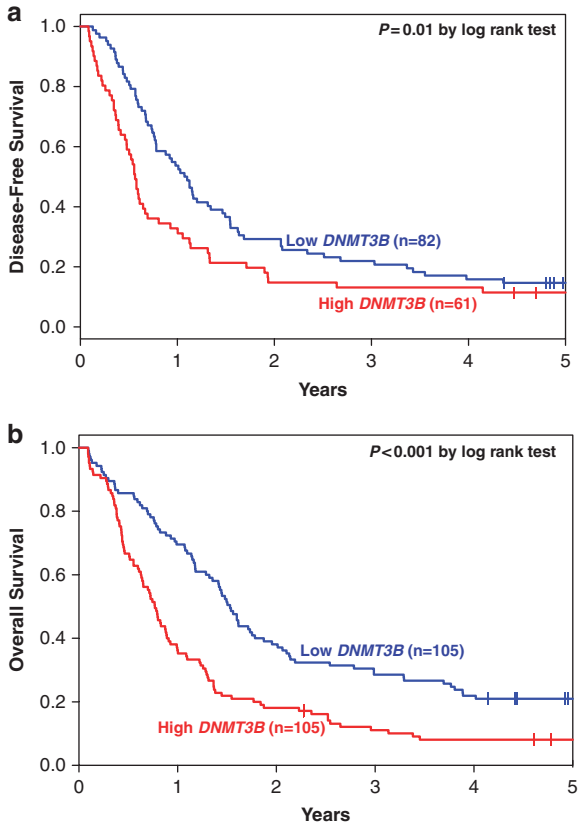

Figure 1. Clinical outcome of CN-AML patients with high and low *DNMT3B* expression. Kaplan–Meier survival curves for (**a**) disease-free survival and (**b**) overall survival. *P*-values presented are from the log rank test.

monocyte/macrophage differentiation and immune function including *CD14*, *TLR4*, *CEBPB* and *TLR8*.

Gene Ontology was used to assess the biologic features of the *DNMT3B* expression profile (Table 4). For *DNMT3B*-associated upregulated genes, there were three Gene Ontology terms comprising genes involved in nucleotide biosynthetic processes and metabolism and included in annotation cluster 1 that had a trend for statistical significance (Benjamini *P*-value < 0.1). For *DNMT3B*-associated downregulated genes, cellular processes included lysosome biology, endocytosis and membrane signaling. These results may be interpreted as consistent with the previously noted dysregulated genes involved in monocyte/macrophage differentiation and activity.

Genome-wide microRNA profiles associated with *DNMT3B* expression

The influence of *DNMT3B* expression on microRNA genome-wide profiles could be evaluated in 162 patients. In contrast to coding genes, only four microRNAs were differentially expressed between high and low *DNMT3B* expressers ($P \leqslant 0.001$). High *DNMT3B* expression was associated with *miR-133b* upregulation, and *miR-148a*, *miR-122* and *miR-409-3p* downregulation. *miR-133b* upregulation in high *DNMT3B* expressers was somewhat surprising as this microRNA was reported to have tumor suppressor activity in other cancers.[48,49] However, consistent with the downregulated gene expression profile as discussed above, *miR-133b* has recently been shown to target granulocyte-macrophage colony-stimulating factor (GM-CSF), a cytokine involved in granulocyte–monocyte/macrophage differentiation.[50] Among the downregulated microRNAs, *miR-148a* was reported to target *DNMT3B* and to be itself a target of aberrant hypermethylation in cancer.[51,52] Lower expression of *miR-122* has been associated with aggressive hepatocellular carcinoma and *miR-409-3p* with cell invasion and metastasis in gastric cancer[53–56]; however, a role for these microRNAs in AML is currently unknown.

Genome-wide methylation profiling associated with *DNMT3B* expression

As *DNMT3B* encodes a methyltransferase that mediates *de novo* DNA methylation, we assessed whether high and low *DNMT3B* expressers differed in DNA methylation patterns. Surprisingly, we found no significant differences in genome-wide DNA methylation levels or in the numbers of differentially methylated regions[18] in distinct functional genomic regions (for example, gene promoters) when high versus low *DNMT3B* expressers were compared.

DISCUSSION

In this study, we report that high *DNMT3B* expression associates with lower CR rates and shorter DFS and OS in chemotherapy-treated CN-AML patients aged $\geqslant 60$ years. High *DNMT3B* expression was associated with such adverse prognostic factors as *FLT3*-ITD, high *ERG*, *BAALC* and *miR-155* expression and the ELN Intermediate-I Genetic Group; nevertheless the association of *DNMT3B* expression with clinical outcome is independent from the aforementioned and other established molecular and clinical prognosticators for all outcome end points studied.

Our findings are consistent to some extent with the only, to our knowledge, previous study that assessed the prognostic value of *DNMT3B* expression.[26] Although, in the subset of 93 CN-AML patients, Hayette *et al.*[26] did not find significant differences in event-free survival or OS between high and low *DNMT3B* expressers, high *DNMT3B* expressers had a shorter event-free survival than low *DNMT3B* expressers in the whole cytogenetically diverse cohort of 191 AML patients analyzed. It is difficult to directly compare their results with ours as approximately one-half of the patients analyzed by Hayette *et al.*[26] had various abnormal karyotypes, more than two-thirds of the patients were younger than 60 years and a quarter underwent allogeneic hematopoietic stem cell transplantation in first CR. Thus, although the two studies are not comparable, they both conclude that higher *DNMT3B* expression is associated with worse outcome in AML.

Recently, the ELN reporting system,[47] which for CN-AML is based on only three molecular markers (that is, *FLT3*-ITD, *CEBPA* and *NPM1* mutations), was shown to provide important prognostic information in AML.[57] However, we and others have shown that additional molecular markers, such as *TET2*,[41] *ASXL1*,[8] *RUNX1*[42] and *DNMT3A*[58] mutations and expression of *MN1*,[12] *miR-155*[15] and *miR-3151*,[16] may refine outcome prediction of CN-AML patients within the ELN Genetic Groups. Hence, in the current study, we investigated whether considering *DNMT3B* expression as a novel prognosticator could alter patient classification within the ELN Genetic Groups. In the Favorable Group, we found that low *DNMT3B* expression identified a subset of CN-AML patients with a significantly longer OS, thus making *DNMT3B* expression the third molecular marker, in addition to *ASXL1* mutations[8] and *miR-155* expression,[15] capable of refining prognostication of older patients in this ELN Genetic Groups. We also observed a significant difference in OS between high and low *DNMT3B* expressers classified in the ELN Intermediate-I Genetic Group. Previously, *RUNX1* mutations[42] and expression levels of *MN1*,[12] *miR-155*[15] and *miR-3151*[16] were demonstrated to add prognostic information in this ELN Genetic Group.

We report the first, to our knowledge, *DNMT3B*-associated gene and microRNA expression and DNA methylation profiles in CN-AML. We were able to derive a strong gene expression profile

Table 3. Multivariable analyses of CN-AML patients according to *DNMT3B* expression status

Variable	*Complete remission*		*Disease-free survival*		*Overall survival*	
	OR (95% CI)	P-*value*	*HR (95% CI)*	P-*value*	*HR (95% CI)*	P-*value*
All patients						
DNMT3B expression, high vs low	0.49 (0.25–0.97)	0.04	1.47 (1.01–2.13)	0.04	1.72 (1.24–2.38)	0.001
BAALC expression, high vs low	0.21 (0.10–0.43)	< 0.001	1.79 (1.21–2.65)	0.004	1.98 (1.39–2.80)	< 0.001
WBC, per 50-unit increase	0.69 (0.52–0.90)	0.007				
Age, per 10-year increase	0.49 (0.27–0.89)	0.02				
DNMT3A[a]						
R882 mutated vs wild type			1.85 (1.17–2.95)	0.009		
non-R882 mutated vs wild type			0.92 (0.52–1.61)	0.76		
ELN Genetic Group, Favorable vs Intermediate-I[b]			0.65 (0.45–0.96)	0.03		
miR-3151 expression, high vs low					1.51 (1.07–2.13)	0.02
miR-155 expression, high vs low					1.47 (1.06–2.05)	0.02
Patients in the ELN modified Favorable Genetic Group[b]						
DNMT3B expression, high vs low	No models including a significant term for *DNMT3B* expression				1.99 (1.26–3.15)	0.003
BAALC expression, high vs low	were found				1.81 (1.13–2.92)	0.01
Patients in the ELN Intermediate-I Genetic Group[b]						
DNMT3B expression, high vs low	No models including a significant term for *DNMT3B* expression				1.73 (1.10–2.72)	0.02
BAALC expression, high vs low	were found				1.88 (1.05–3.35)	0.03
miR-3151 expression, high vs low					1.79 (1.05–3.07)	0.03
WBC, per 50-unit increase					1.16 (1.01–1.33)	0.03

Abbreviations: CI, confidence interval; ELN, European LeukemiaNet; HR, hazard ratio; OR, odds ratio; WBC, white blood count. An odds ratio < 1 means a lower CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. A hazard ratio > 1 (< 1) corresponds to a higher (lower) risk of an event for higher values of continuous variables and the first category listed of a dichotomous variable. Variables were considered for inclusion in the multivariable models if they had a univariable *P*-value of $\leqslant 0.20$. See the Supplementary Information for a full list of variables evaluated in univariable analyses. As *NPM1*, *FLT3*-ITD and *CEBPA* mutations are integrated in the ELN genetic classification, they were not additionally considered as individual variables. In the entire patient cohort, variables considered for inclusion in the model for achievement of CR were *DNMT3B*, *ERG*, *BAALC*, *miR-155* and *miR-3151* expression, ELN Genetic Groups, *WT1* and *ASXL1* mutation status, WBC, age and extramedullary involvement. In the model for DFS, we considered *DNMT3B*, *ERG*, *BAALC* and *miR-3151* expression, ELN Genetic Groups, *FLT3*-TKD, *ASXL1*, *DNMT3A*-R882 and *DNMT3A* non-R882 mutation status and extramedullary involvement; and in the model for OS, *DNMT3B*, *ERG*, *BAALC, miR-155* and *miR-3151* expression, ELN Genetic Groups, *MLL*-PTD, *WT1*, *ASXL1*, *DNMT3A*-R882 and *DNMT3A* non-R882 mutation status, WBC and extramedullary involvement. For patients in the ELN modified Favorable Genetic Group, variables considered for inclusion in the model for OS were *DNMT3B*, *ERG*, *BAALC* and *miR-155* expression, *ASXL1* and *TET2* mutation status and extramedullary involvement. For patients in the ELN Intermediate-I Genetic Group, variables considered for inclusion in the model for OS were *DNMT3B*, *ERG*, *BAALC*, *miR-155* and *miR-3151* expression, *RUNX1*, *IDH1*, *DNMT3A*-R882 and *DNMT3A* non-R882 mutation status, and WBC and hemoglobin. [a]The types of *DNMT3A* mutations detected in our cohort are provided in Supplementary Table S2. [b]The ELN modified Favorable Genetic Group is defined as CN-AML patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (that is, those with wild-type *CEBPA* and wild-type *NPM1* with or without *FLT3*-ITD, or mutated *NPM1* with *FLT3*-ITD) belong to the ELN Intermediate-I Genetic Group.[47]

comprising 363 genes by overlapping the microarray results from three independent sets of patients. The profile was quite heterogeneous, comprising genes encoding for proteins involved in multiple biologic processes that play a role in leukemia cell differentiation, proliferation and survival. Among the down-regulated genes, we noted enrichment of genes involved in monocyte/macrophage differentiation and activity, suggesting a role of *DNMT3B* in impairing differentiation of the leukemic blasts into cells with normal innate immunity activity. Using gene set enrichment analysis, we found a significant correlation between the high *DNMT3B* expression and *FLT3*-ITD signatures (Supplementary Figure S2). This, along with the increased frequency of *FLT3*-ITD in high *DNMT3B* expressers (Table 1), suggests the existence of a functional association between high expression of the *DNMT3B* gene and *FLT3*-ITD.

In contrast, the *DNMT3B*-associated microRNA profile was relatively weak, comprising only four microRNAs that were differentially expressed in high versus low *DNMT3B* expressers. Nevertheless, the unique upregulation of expression of *miR-133b*, recently reported to target GM-CSF,[50] in *DNMT3B* high expressers was somewhat consistent with the enrichment of the gene expression profile in multiple downregulated genes involved in the differentiation and activity of hematopoietic cells participating in innate immunity.

Surprisingly, despite the fact that *DNMT3B* encodes a DNA methyltransferase, we observed no significant association of high *DNMT3B* levels and DNA methylation changes. No difference in global DNA methylation levels and number of differentially methylated regions could be identified between *DNMT3B* high and low expressers. Our results are reminiscent of a recent report showing that changes in *DNMT3B* expression did not affect methylation levels of putative DNMT3B target genes.[59] Moreover, Russler–Germain *et al.*[60] have recently demonstrated that DNA methylation levels in leukemic blasts from CN-AML patients are not influenced by *DNMT3B* expression as mainly inactive splice variants of *DNMT3B* are expressed in these cells. Overall, therefore, these data may suggest that although overexpressed *DNMT3B* is a potentially valuable predictive marker for response to conventional chemotherapy, it does not necessarily identify subsets of older AML patients characterized by aberrant DNA methylation who might be responsive to hypomethylating azanucleosides.

In summary, we have demonstrated that *DNMT3B* expression constitutes an independent prognostic factor in older CN-AML patients treated intensively, and could also refine the ELN classification. Furthermore, we have provided some insights into the biologic activity of *DNMT3B* in CN-AML, which is seemingly independent from mechanisms of DNA hypermethylation and/or microRNA-dependent gene repression. Further studies focused on gaining more clinical and mechanistic insights into the leukemogenic role of *DNMT3B* expression are

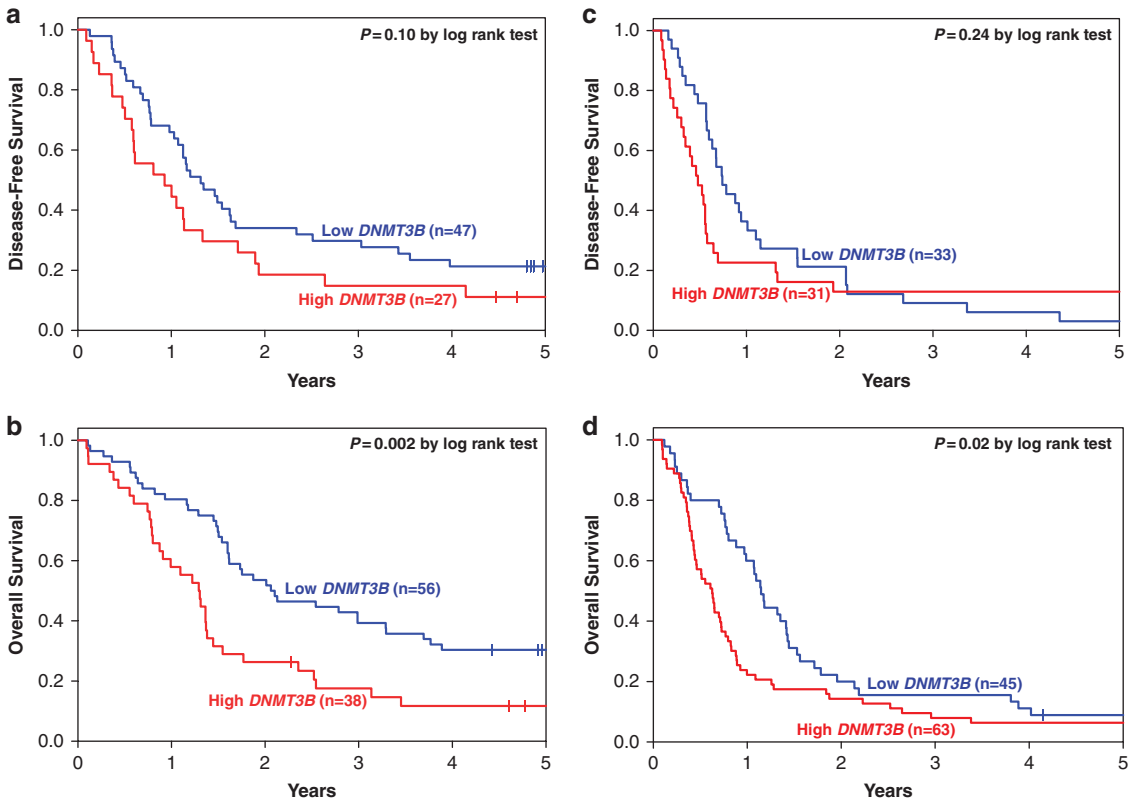

Figure 2. Clinical outcome of CN-AML patients with high and low *DNMT3B* expression classified into ELN Genetic Groups. Kaplan–Meier survival curves for (**a**) disease-free survival and (**b**) overall survival of patients in the ELN modified Favorable Genetic Group; (**c**) disease-free survival and (**d**) overall survival of patients in the ELN Intermediate-I Genetic Group. *P*-values presented are from the log rank test.

Table 4. Gene Ontology terms associated with differentially expressed genes in the high *DNMT3B* expression group

Biologic process or cellular component	*Number of genes*	*Benjamini P-value*
Associations with genes upregulated in the high DNMT3B expression group		
Annotation Cluster 1		
GOTERM_BP: nucleotide biosynthetic process	10	0.098
GOTERM_BP: nucleobase, nucleoside and nucleotide biosynthetic process	10	0.066
GOTERM_BP: nucleobase, nucleoside, nucleotide and nucleic acid biosynthetic process	10	0.066
Associations with genes downregulated in the high DNMT3B expression group		
Annotation Cluster 1		
GOTERM_CC: vacuole	12	0.0052
GOTERM_CC: lytic vacuole	10	0.012
GOTERM_CC: lysosome	10	0.012
Annotation Cluster 2		
GOTERM_BP: endocytosis	11	0.0091
GOTERM_BP: membrane invagination	11	0.0091
GOTERM_BP: membrane organization	12	0.089
Annotation Cluster 3		
GOTERM_CC: intrinsic to membrane	74	0.028

Abbreviations: BP, biologic process; CC, cellular component; GO, Gene Ontology, see also Huang da *et al.*[45]

warranted to design active therapeutic strategies for high *DNMT3B* expressers in CN-AML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

CN, JK, SV, KMr, GM and CDB designed the study, analyzed the data and wrote the manuscript, and all authors agreed on the final version; SPW, KHM, A-KE, PY, DF, HB, SS, JHM, JPC, Y-ZW, and RB carried out laboratory-based research; JK, KMa, SV and DN performed statistical analyses; and AJC, MRB, BLP, JEK, JOM, THC, RAL, RMS, KMr, GM and CDB were involved directly or indirectly in the care of patients and/or sample procurement.

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Supplementary Information accompanies this paper on the Leukemia website (http://www.nature.com/leu)