ASXL1 mutations identify a high-risk subgroup of older patients with primary cytogenetically normal AML within the ELN Favorable genetic category

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The associations of mutations in the enhancer of trithorax and polycomb family gene *ASXL1* with pretreatment patient characteristics, outcomes, and gene-/microRNA-expression profiles in primary cytogenetically normal acute myeloid leukemia (CN-AML) are unknown. We analyzed 423 adult patients for *ASXL1* mutations, other prognostic gene mutations, and gene-/microRNA-expression profiles. *ASXL1* mutations were 5 times more common in older (≥ 60 years) patients (16.2%) than those younger than 60 years (3.2%; *P* < .001). Among older patients, *ASXL1* mutations associated with wild-type *NPM1* (*P* < .001), absence of *FLT3*-internal tandem duplications (*P* = .002), mutated *CEBPA* (*P* = .01), and with inferior complete remission (CR) rate (*P* = .04), disease-free survival (DFS; *P* = .03), overall survival (OS; *P* = .006), and event-free survival (EFS; *P* = .002). Within the European LeukemiaNet (ELN) genetic categories of older CN-AML, *ASXL1* mutations associated with inferior CR rate (*P* = .02), OS (*P* < .001), and EFS (*P* < .001) among ELN Favorable, but not among ELN Intermediate-I patients. Multivariable analyses confirmed associations of *ASXL1* mutations with unfavorable CR rate (*P* = .03), DFS (*P* < .001), OS (*P* < .001), and EFS (*P* < .001) among ELN Favorable patients. We identified an *ASXL1* mutation-associated gene-expression signature, but no microRNA-expression signature. This first study of *ASXL1* mutations in primary CN-AML demonstrates that *ASXL1*-mutated older patients, particularly within the ELN Favorable group, have unfavorable outcomes and may be candidates for experimental treatment approaches. (*Blood*. 2011;118(26):6920-6929)

Introduction

The *additional sex combs like-1* (*ASXL1*) gene, located in chromosome band 20q11, encodes a highly conserved protein that belongs to the enhancer of trithorax and polycomb (ETP) genes, a gene family with dual functions in both epigenetic activation and repression of gene transcription.[1] In mice, *Asxl1* is necessary for proper regulation of *Hox* gene expression during embryogenesis.[2] Human *ASXL1* is involved in an unbalanced chromosomal rearrangement [dic(9;20)(p13;q11)] recurrent in acute lymphoblastic leukemia.[3] More recently, mutations in *ASXL1* exon 12 were identified in patients with myelodysplastic syndromes,[4] myeloproliferative neoplasms,[5] and acute myeloid leukemia (AML).[6,7] The reported prevalence of *ASXL1* mutations in primary (de novo) AML ranges between 5% and 30%, whereas some studies suggest a higher prevalence in secondary AML.[6-10] Cytogenetically normal (CN) AML represents the largest cytogenetic subgroup of adult AML,[11] and the one that has been most extensively studied regarding the clinical implications of various gene mutations.[12,13] To date, no study has specifically addressed the association of *ASXL1* mutations with clinical and molecular patient characteristics and outcomes in CN-AML.

In a cytogenetically heterogeneous cohort, Chou et al[8] reported that *ASXL1*-mutated (mut) AML patients had a lower complete remission (CR) rate and shorter overall survival (OS) than *ASXL1*–wild-type (wt) patients. *ASXL1* mutation status was no longer significantly associated with outcomes in multivariable models adjusting for other prognostic factors. Although more than one-half of all *ASXL1* mutations in that study were found in patients 60 years of age or older, 80% of the patients included in the outcome analyses were 60 years of age or younger. It thus remains unclear whether *ASXL1* mutation status has independent prognostic relevance in older patients or in specific cytogenetic groups, such as CN-AML.

Therefore, we studied *ASXL1* mutations and their associations with baseline patient characteristics, other genetic alterations, and outcomes in relatively large and well-characterized cohorts of younger (< 60 years) and older (≥ 60 years) primary CN-AML patients. We also examined genome-wide gene and microRNA (miR) expression profiles and report the first *ASXL1* mutation-associated gene-expression signature in CN-AML, which will help to gain insights into the biology of *ASXL1*-mut myeloid neoplasms.

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Methods

Patients

Pretreatment BM or peripheral blood (PB) samples containing more than 20% leukemic blasts were obtained from 423 primary CN-AML patients (including 189 patients younger than 60 years and 234 patients 60 years of age or older). The diagnosis of normal cytogenetics was based on 20 or more analyzed metaphase cells in BM specimens subjected to 24- and/or 48-hour culture. Cytogenetic analyses were performed by Cancer and Leukemia Group B (CALGB)-approved institutional laboratories and confirmed by central karyotype review.[14] All patients received cytarabine/daunorubicin-based first-line therapy on CALGB trials (supplemental Appendix, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). As stipulated in the treatment protocols for both younger and older patients, no patient received allogeneic stem cell transplantation in first CR. Study protocols were in accordance with the Declaration of Helsinki and approved by the institutional review boards at each center, and all patients provided written informed consent.

Mutational analyses

Exon 12 of the *ASXL1* gene was amplified from genomic DNA by PCR and analyzed by direct sequencing (supplemental Methods). Patients were also characterized for *FLT3*-internal tandem duplications (*FLT3*-ITD),[15] *FLT3*-tyrosine kinase domain mutations (*FLT3*-TKD),[16] *MLL*-partial tandem duplications (*MLL*-PTD),[17,18] and mutations in *NPM1*,[19] *CEBPA*,[20] *TET2*,[21] *IDH1*/*IDH2*,[22] and *WT1*[23] as previously reported. All mutational analyses were performed at The Ohio State University Comprehensive Cancer Center.

Microarray experiments

Gene-expression profiling was performed using Affymetrix HG-U133 plus Version 2.0 oligonucleotide microarrays; miR-expression profiling was performed using a custom microarray platform (OSU_CCC miR array Version 4.0), as previously reported.[19,21] See supplemental Methods for details on microarray analyses. All microarray data are available on ArrayExpress under accession number E-TABM-1208.

Statistical analyses

Baseline characteristics were compared between *ASXL1*-mut and *ASXL1*-wt patients using Fisher exact test for categorical variables and the Wilcoxon rank-sum test for continuous variables. Definitions of clinical end points (ie, CR, disease-free survival [DFS], OS, and event-free survival [EFS]) and details on outcome analyses are provided in supplemental Methods. Briefly, for time-to-event analyses, we calculated survival estimates using the Kaplan-Meier method, and compared groups by the log-rank test. 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 endpoints. All analyses were performed by the Alliance for Clinical Trials in Oncology Statistics and Data Center.

Results

Prevalence of *ASXL1* mutations in primary CN-AML

We studied the coding sequence of *ASXL1* exon 12 in 423 primary CN-AML patients 18 to 83 years of age. In previous studies, all *ASXL1* mutations were found in this exon, which contains more than 60% of the entire *ASXL1* coding sequence.[4,5] Among 189 younger patients, only 6 carried protein-truncating (nonsense or frame shift) *ASXL1* mutations (3.2%). In contrast, among 234 older patients, we identified 39 heterozygous nonsense or frame shift mutations affecting 38 patients (16.2%; Figure 1A; $P < .001$ for the comparison between younger and older patients). In agreement with previous reports,[4-9] duplication of a guanine nucleotide at coding sequence position 1934 (c.1934dupG) accounted for approximately half of the *ASXL1* mutations we identified. The remaining patients carried a variety of other nonsense or frame shift mutations. Supplemental Table 1 provides detailed information on all mutations identified in the entire cohort of 423 patients. Besides these mutations, we also detected missense variations leading to exchanges of single amino acids that have not been annotated as single nucleotide polymorphisms (SNPs) in the dbSNP database Version 132 (supplemental Table 2). Such variations were present in 13 younger patients (6.9%) and in 16 older patients (6.8%, 2 also had a nonsense or frame shift mutation). Some of these variants have recently been shown to be germline SNPs.[24,25] Patients who only had missense variations in *ASXL1* exon 12 (13 younger and 14 older patients) were excluded because of the uncertain pathogenic relevance of these changes. This is consistent with previous studies.[5-8,26] Baseline clinical characteristics and treatment outcomes of these patients were not significantly different from patients with wild-type *ASXL1* (supplemental Figure 1).

Since *ASXL1* mutations were rare in younger primary CN-AML patients and since younger and older patients were enrolled on separate CALGB trials that differed in their treatment intensity, we restricted all subsequent analyses of baseline associations and treatment outcomes to older patients (n = 220). Although the small number of *ASXL1*-mut patients younger than 60 years (n = 6) did not allow a detailed evaluation in this age group, the clinical and molecular characteristics of these patients generally resembled our findings in older *ASXL1*-mut patients (supplemental Results).

Clinical and molecular characteristics of *ASXL1*-mut older primary CN-AML patients

Patients with *ASXL1* mutations (n = 38) tended to be more frequently male ($P = .08$), and had lower pretreatment white blood cell counts (WBC; $P = .02$), lower PB ($P < .001$) and BM ($P = .04$) blast percentages, and a trend toward higher platelet counts ($P = .06$), compared with *ASXL1*-wt patients (n = 182; Table 1). *ASXL1* mutations rarely occurred concurrently with *NPM1* mutations ($P < .001$) or *FLT3*-ITD ($P = .002$; Table 1; Figure 1B). On the other hand, 26% of *ASXL1*-mut patients also carried *CEBPA* mutations, compared with only 9% of *ASXL1*-wt patients ($P = .01$). Figure 1B visualizes coexisting mutations in *NPM1*, *FLT3*, *CEBPA* and 5 other genes among the 38 *ASXL1*-mut older CN-AML patients. In 8 of these 38 patients (21%), no further mutations were detected in any of the other 8 genes analyzed.

According to the current European LeukemiaNet (ELN) guidelines for the diagnosis and management of AML, CN-AML patients are classified into 2 genetic categories based on their *NPM1*, *FLT3*-ITD, and *CEBPA* mutation status.[27] The ELN Favorable category of CN-AML is defined as patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (ie, those with wild-type *CEBPA*, and wild-type *NPM1* with or without *FLT3*-ITD or mutated *NPM1* with *FLT3*-ITD) form the ELN Intermediate-I category. *ASXL1*-mut patients tended to fall into the ELN Favorable category less frequently than into the ELN Intermediate-I category ($P = .08$; Table 1). Of the 12 ELN Favorable/*ASXL1*-mut patients, 10 carried *CEBPA* mutations, and the other 2 had mutated *NPM1* without *FLT3*-ITD (Figure 1B).

Association between *ASXL1* mutations and treatment outcomes in older primary CN-AML patients

Due to the baseline associations between *ASXL1* mutations and established prognostic gene mutations (ie, *NPM1*, *FLT3*-ITD, and *CEBPA*

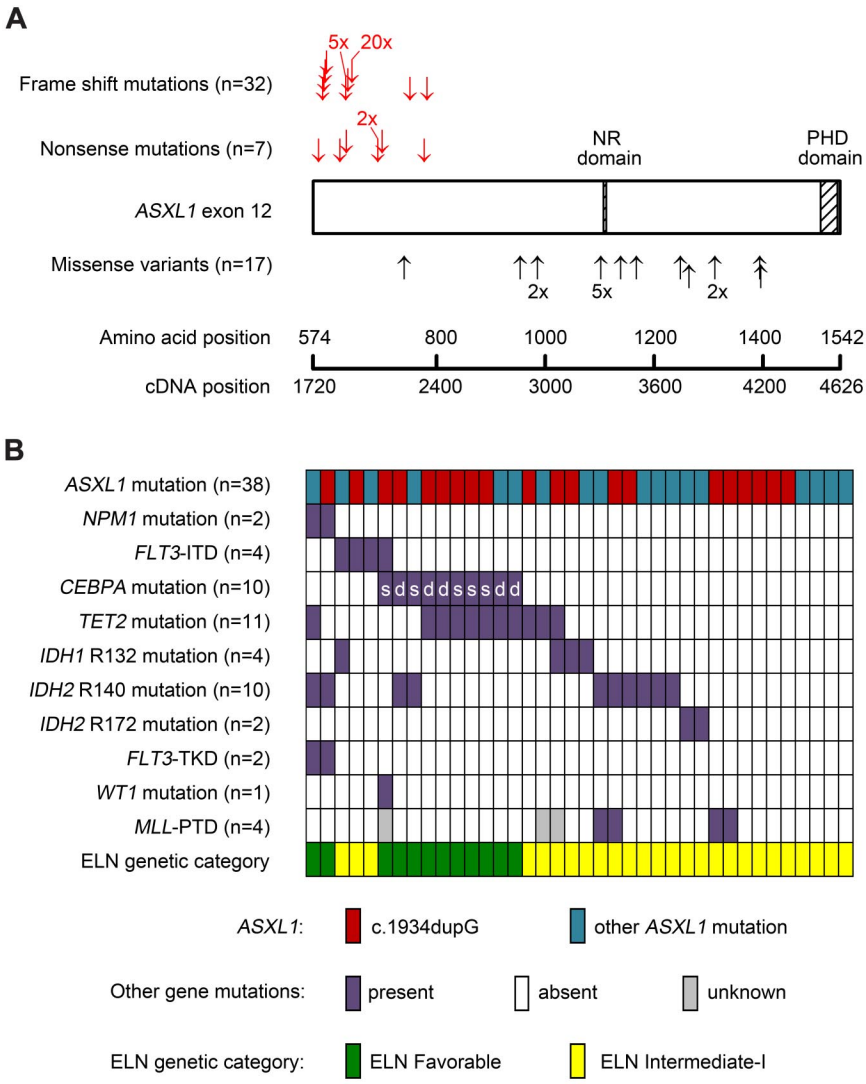

Figure 1. *ASXL1* exon 12 mutations in primary CN-AML patients 60 years of age or older. (A) Localization of sequence variations within *ASXL1* exon 12 found among 234 older CN-AML patients. Each arrow represents one of the nonsynonymous changes, except for known SNPs, which are not displayed. Top part of the panel: nonsense and frame shift mutations leading to truncation of the protein-coding sequence (indicated by red arrows). All these alterations were considered pathogenic mutations for the analysis of clinical outcomes. One patient had both a nonsense and a frame shift mutation. Bottom part: missense variations that alter single amino acids (indicated by black arrows). Patients who only had such missense variations were excluded from analyses of clinical correlations and outcomes. Known functional domains in *ASXL1* exon 12 are designated by the shaded areas. NR indicates the RAR-binding nuclear receptor domain; and PHD, the plant homeodomain. (B) Relationship between *ASXL1* mutations and other common gene mutations in 38 older CN-AML patients. Each column represents 1 patient. The topmost row indicates whether a patient had c.1934dupG (red) or another *ASXL1* mutation (blue). The following rows represent 10 different types of mutations in 8 different genes that were found together with mutated *ASXL1*: purple represents the presence of the mutation; white, absence of the mutation; and gray fields, missing data. For *CEBPA*-mut patients, "s" indicates single and "d" double (biallelic) *CEBPA* mutations. The bottom row displays the ELN genetic category for each patient[27]: green represents ELN Favorable; and yellow, ELN Intermediate-I.

mutations), it was important to study the prognostic relevance of mutated *ASXL1* not only in our entire cohort of older CN-AML patients, but also in the genetic subsets defined by the ELN classification (Table 2). To adjust for other, potentially confounding risk factors, we also constructed multivariable models (Table 3). Overall, *ASXL1*-mut patients had a lower CR rate (53%) than *ASXL1*-wt patients (71%, $P = .04$). Even within the ELN Favorable group, only 50% of the *ASXL1*-mut patients achieved CR, compared with 82% of *ASXL1*-wt patients ($P = .02$). In the ELN Intermediate-I genetic category, no significant difference in CR rates was observed between *ASXL1*-mut and *ASXL1*-wt patients (Table 2). In a multivariable logistic regression model for achievement of CR, ELN Favorable/*ASXL1*-mut patients had lower odds of achieving a CR, compared with ELN Favorable/ASXL1-wt patients ($P = .03$; odds ratio [OR] = 0.24; Table 3), whereas *ASXL1* mutations were not associated with CR rate in ELN Intermediate-I patients ($P = .47$). Other factors associated with a lower CR rate were a higher WBC ($P = .01$; OR = 0.73), higher platelet count ($P = .03$; OR = 0.81), and the presence of extramedullary involvement ($P = .05$; OR = 0.49).

Among patients who achieved CR, those with *ASXL1* mutations had shorter DFS than *ASXL1*-wt patients ($P = .03$; 3-year DFS, 10% vs 19%; Table 2; Figure 2A). All 6 ELN Favorable/*ASXL1*-mut patients achieving CR relapsed within 13 months, whereas 27% of the ELN Favorable/*ASXL1*-wt patients were alive and disease-free at 3 years. *ASXL1* mutations were not associated with DFS in ELN Intermediate-I patients ($P = .95$; Table 2). A multivariable Cox model for DFS showed that ELN Favorable patients had a more than 4-fold increased risk of relapse or death if they carried an *ASXL1* mutation ($P < .001$; hazard ratio [HR] = 4.38; Table 3). In contrast, *ASXL1* mutations were not associated with DFS in ELN Intermediate-I patients in this model ($P = .65$). The P value for interaction between *ASXL1* mutation status and ELN category was .01, confirming that the impact of *ASXL1* mutations on DFS differed between ELN Favorable and Intermediate-I patients. The only other factor associated with shorter DFS was extramedullary involvement ($P = .03$; HR = 1.65).

ASXL1-mut patients had shorter OS than *ASXL1*-wt patients ($P = .006$; 3-year OS, 5% vs 23%; Table 2; Figure 2B). A negative impact of *ASXL1* mutations on OS was also observed within the ELN Favorable subset ($P < .001$): all *ASXL1*-mut patients died within 18 months from diagnosis, whereas 34% of *ASXL1*-wt patients were alive at 3 years (Table 2; Figure 3A). In the ELN Intermediate-I category, *ASXL1* mutation status was not significantly associated with OS ($P = .73$; Table 2, Figure 3B). A multivariable model for OS confirmed that patients in the ELN Favorable category with mutated *ASXL1* had a more than 4 times higher risk of death than *ASXL1*-wt patients ($P < .001$; HR = 4.43; Table 3), whereas *ASXL1* mutations were not associated with OS in the ELN Intermediate-I group ($P = .79$; $P < .001$ for interaction

Table 1. Comparison of clinical and molecular characteristics by *ASXL1* mutation status in primary CN-AML patients 60 years of age or older

Variable	*ASXL1*-mut (n = 38)	*ASXL1*-wt (n = 182)	*P*
Age, y			.43
Median	68	69	
Range	61-83	60-82	
Male sex, no. (%)	24 (63)	85 (47)	.08
Race, no. (%)			.75
White	34 (89)	164 (92)	
Nonwhite	4 (11)	15 (8)	
WBC, × 10^9/L			.02
Median	10.6	28.1	
Range	0.9-173.1	0.9-450.0	
Percent of blood blasts			< .001
Median	12	52	
Range	0-88	0-99	
Percent of bone marrow blasts			.04
Median	52	67	
Range	8-93	4-97	
Hemoglobin, g/dL			.74
Median	9.4	9.4	
Range	6.0-11.7	5.4-15.0	
Platelet count, × 10^9/L			.06
Median	85	65	
Range	14-510	4-850	
FAB category, no. (%)*			—
M0	1 (4)	2 (2)	
M1	2 (8)	31 (25)	
M2	12 (50)	36 (29)	
M4	6 (25)	27 (22)	
M5	3 (13)	24 (20)	
M6	0 (0)	3 (2)	
Extramedullary involvement, no. (%)	7 (18)	42 (23)	.67
***NPM1*, no. (%)**			< .001
Mutated	2 (5)	125 (69)	
Wild-type	36 (95)	57 (31)	
***FLT3*-ITD, no. (%)**			.002
Positive	4 (11)	66 (36)	
Negative	34 (89)	116 (64)	
***CEBPA*, no. (%)**			.01
Mutated	10 (26)	17 (9)	
Single mutated	5	11	
Double mutated	5	6	
Wild-type	28 (74)	165 (91)	
ELN genetic category, no. (%)†			.08
Favorable	12 (32)	87 (48)	
Intermediate-I	26 (68)	95 (52)	
***TET2*, no. (%)**			1.0
Mutated	11 (29)	55 (30)	
Wild-type	27 (71)	126 (70)	
***IDH1*, no. (%)**			1.0
Mutated	4 (11)	22 (12)	
Wild-type	34 (89)	159 (88)	
***IDH2*, no. (%)**			.21
IDH2 mutated	12 (32)	39 (22)	
Codon R140 mutation	10	31	
Codon R172 mutation	2	8	
Wild-type	26 (68)	142 (78)	
***FLT3*-TKD, no. (%)**			.54
Present	2 (5)	18 (10)	
Absent	36 (95)	164 (90)	
***WT1*, no. (%)**			.48
Mutated	1 (3)	14 (8)	
Wild-type	37 (97)	168 (92)	
***MLL*-PTD, no. (%)**			.28
Present	4 (11)	9 (6)	
Absent	31 (89)	139 (94)	

FAB indicates French-American-British classification; and —, not applicable.

*FAB categories are centrally reviewed

†Within CN-AML patients, the ELN Favorable group is defined as patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (ie, those with wild-type *CEBPA*, and *FLT3*-ITD and/or wild-type *NPM1*) belong to the ELN Intermediate-I category.[27]

between *ASXL1* mutations and ELN category). The only other factor associated with OS was higher WBC ($P = .01$; HR = 1.13).

Similarly, EFS of *ASXL1*-mut patients was worse compared with *ASXL1*-wt patients ($P = .002$; 3-year EFS, 5% vs 14%; Table 2; Figure 2C). Within the ELN Favorable genetic category, *ASXL1*-mut patients had worse EFS than *ASXL1*-wt patients ($P < .001$; 3-year EFS, 0% vs 22%; Table 2; Figure 3C), whereas no significant difference was observed in ELN Intermediate-I patients ($P = .68$; Figure 3D). In a multivariable model for EFS, ELN Favorable patients with mutated *ASXL1* had a 3.6-fold higher risk of an event than *ASXL1*-wt patients ($P < .001$; HR = 3.61; Table 3). In this multivariable model, *ASXL1* mutations were not associated with EFS in the ELN Intermediate-I group ($P = .44$; $P = .005$ for interaction between *ASXL1* mutations and ELN category). Higher WBC ($P = .005$; HR = 1.18) and extramedullary involvement ($P = .02$; HR = 1.52) were also associated with shorter EFS. Of note, none of the *ASXL1*-mut and only 5 *ASXL1*-wt patients died while in first CR (Table 2), indicating that the observed survival differences reflect differences in the course of the disease rather than treatment toxicity or other unrelated causes.

In summary, *ASXL1* mutations were associated with lower CR rate and shorter survival, particularly within the ELN Favorable genetic category and thus identify a previously unrecognized high-risk subgroup among older primary CN-AML patients.

Exploratory analyses of *ASXL1* mutations in the context of other gene mutations

As mentioned previously, *ASXL1* mutations were significantly associated with mutated *CEBPA* in older primary CN-AML. Of 27 *CEBPA*-mut patients, 10 also were *ASXL1*-mut. Five *CEBPA*-mut/*ASXL1*-mut patients (50%) achieved a CR, compared with 13 of 17 *CEBPA*-mut/*ASXL1*-wt patients (76%, $P = .21$; supplemental Table 3). We could not formally analyze DFS because of inadequate sample size. However, all 5 *CEBPA*-mut/*ASXL1*-mut patients who achieved CR relapsed within 13 months, whereas 31% of *CEBPA*-mut/*ASXL1*-wt patients were still alive and disease-free at 3 years. The OS ($P < .001$; supplemental Figure 2A) and EFS ($P = .02$; supplemental Figure 2C) of *CEBPA*-mut/*ASXL1*-mut patients were significantly worse than those of *CEBPA*-mut/*ASXL1*-wt patients (supplemental Table 3). Recently, it has been reported that only double, but not single, *CEBPA* mutations are associated with favorable outcomes.[28,29] We were unable to formally study the prognostic relevance of *ASXL1* mutations in patients with single or double *CEBPA* mutations separately because there were only 5 *ASXL1*-mut patients with single *CEBPA* mutations and 5 *ASXL1*-mut patients with biallelic *CEBPA* mutations. However, whereas 4 of 5 *CEBPA*-double-mutated/*ASXL1*-mutated patients achieved CR, all 4 relapsed within 13 months. All 5 *CEBPA*-double-mutated/*ASXL1*-mutated patients died within 18 months from study inclusion. Thus, in our cohort, patients with double *CEBPA* mutations and mutated *ASXL1* appeared to have unfavorable outcomes. Among *CEBPA*-wt patients, those with *ASXL1* mutations tended to have a lower CR rate (54% vs 70%; $P = .09$), showed no significant difference in DFS ($P = .17$) or OS ($P = .11$; supplemental Figure 2B), but had shorter EFS ($P = .03$; supplemental Figure 2D) compared with *ASXL1*-wt patients (supplemental Table 3). These exploratory analyses suggest that *ASXL1* mutations may have a particularly unfavorable impact in older CN-AML patients harboring *CEBPA* mutations.

In an analysis including both younger and older CN-AML patients, we have recently demonstrated that *TET2* mutations also adversely affect patient outcomes in the ELN Favorable genetic subset.[21] Therefore, we analyzed the outcomes of older ELN

Table 2. Univariable analyses of outcomes according to *ASXL1* mutation status in primary CN-AML patients aged 60 years or older

	ASXL1-mut	*ASXL1*-wt	*P*	OR/HR (95% CI)
All patients (n = 220)				
No. of patients	38	182		
CR, no. (%)	20 (53)	129 (71)	.04	0.46 (0.22-0.93)
Death in CR, no. (%)	0/20 (0)	5/129 (4)	1.0	—
DFS			.03	1.71 (1.06-2.77)
Median, y	0.6	1.0		
% disease-free at 3 y (95% CI)	10 (2-27)	19 (12-26)		
OS			.006	1.64 (1.14-2.43)
Median (y)	0.9	1.2		
% alive at 3 y (95% CI)	5 (1-15)	23 (17-39)		
EFS			.002	1.74 (1.22-2.49)
Median, y	0.4	0.7		
% event-free at 3 y (95% CI)	5 (1-16)	14 (9-18)		
ELN Favorable group* (n = 99)				
No. of patients	12	87		
CR, no. (%)	6 (50)	71 (82)	.02	0.23 (0.06-0.79)
DFS			ND†	—
Median, y	0.4	1.2		
% disease-free at 3 y (95% CI)	0	27 (17-37)		
OS			< .001	5.15 (2.63-10.10)
Median, y	0.8	1.6		
% alive at 3 y (95% CI)	0	34 (25-44)		
EFS			< .001	4.01 (2.09-7.68)
Median, y	0.2	1.1		
% event-free at 3 y (95% CI)	0	22 (14-31)		
ELN Intermediate-I group* (n = 121)				
No. of patients	26	95		
CR, no. (%)	14 (54)	58 (61)	.51	0.74 (0.31-1.78)
DFS			.95	1.02 (0.56-1.84)
Median, y	0.7	0.6		
% disease-free at 3 y (95% CI)	14 (2-37)	10 (4-20)		
OS			.73	0.93 (0.60-1.44)
Median, y	1.1	0.8		
% alive at 3 y (95% CI)	8 (1-22)	13 (7-20)		
EFS			.68	1.09 (0.71-1.69)
Median, y	0.5	0.4		
% event-free at 3 y (95% CI)	8 (1-22)	6 (3-12)		

The median follow-up for those alive is 5.0 years (range, 2.3-11.6 years). The median follow-up for those who have not had an event is 5.1 years (range, 3.7-11.6 years). An OR > 1.0 (< 1.0) means a higher (lower) CR rate for *ASXL1*-mut patients compared with *ASXL1*-wt patients. A HR > 1.0 (< 1.0) corresponds to a higher (lower) risk of an event for *ASXL1*-mut patients compared with *ASXL1*-wt patients.

ND indicates not determined; and —, not applicable.

*Within CN-AML, the ELN Favorable group is defined as patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (ie, those with wild-type *CEBPA*, and *FLT3*-ITD and/or wild-type *NPM1*) belong to the ELN Intermediate-I category.[27]

†A *P* value for DFS in the ELN Favorable group was not calculated because only 6 ELN Favorable/*ASXL1* mutated patients achieved CR.

Favorable CN-AML patients when taking both *ASXL1* and *TET2* mutation status into consideration. *ASXL1*-mut patients (irrespective of their *TET2* status) had a significantly worse OS compared with either *ASXL1*-wt/*TET2*-wt patients ($P < .001$) or *ASXL1*-wt/*TET2*-mut patients ($P < .001$; supplemental Figure 3A). Likewise, EFS of ELN Favorable/*ASXL1*-mut patients was shorter than EFS of *ASXL1*-wt/*TET2*-wt ($P < .001$) or *ASXL1*-wt/*TET2*-mut patients ($P = .004$; supplemental Figure 3B). ELN Favorable patients with *ASXL1*-wt and *TET2*-wt had the best outcomes in these analyses, with a 3-year OS of 39% and a 3-year EFS of 26%. Because of small patient numbers, we could not analyze *ASXL1*-mut/*TET2*-wt and *ASXL1*-mut/*TET2*-mut patients separately.

Analysis of gene- and miR-expression profiles associated with *ASXL1* mutations in older primary CN-AML patients

To gain insight into the biologic consequences of *ASXL1* mutations in CN-AML, we studied genome-wide gene-expression signatures associated with these mutations. In our cohort, *ASXL1* mutations were almost mutually exclusive with *NPM1* mutations and *FLT3*-ITD, which themselves have strong, characteristic gene-expression signatures.[15,19] To avoid confounding due to the effects of *NPM1* mutations and *FLT3*-ITD on gene expression, we confined our analyses to *NPM1*-wt/*FLT3*-ITD-negative patients. Within this subset, we compared 26 *ASXL1*-mut to 39 *ASXL1*-wt patients and identified a signature of 92 differentially expressed probe sets, corresponding to 67 named genes (supplemental Table 4). Among the genes most strongly up-regulated in *ASXL1*-mut patients were *LRP6* (a WNT signaling pathway coreceptor[30]), cytochrome P450 *CYP1B1* (associated with chemotherapy resistance[31]), and gap junction protein α 1 (*GJA1,* connexin 43). *GJA1* couples early hematopoietic and stromal cells in the BM, is important for regeneration of hematopoiesis after chemotherapy,[32] and mediates secretion of stroma-derived factor 1 (*CXCL12*), a chemokine essential for hematopoietic stem cell homing and function.[33] On the other hand, *KISS1R* (a G-protein-coupled receptor suppressing CXCL12-CXCR4 signaling[34]) was down-regulated in *ASXL1*-mut CN-AML. Also down-regulated was *MLLT10* (*AF10*, a fusion partner in recurrent chromosomal translocations in AML[35]). Figure 4 visualizes the expression of the 92 differentially expressed probe sets in all 185 older CN-AML patients with available microarray data.

Since about half of all *ASXL1*-mut patients had one specific type of mutation (c.1934dupG), whereas the other half had a variety of other mutations, we looked for potential biologic differences between these 2 groups by comparing the gene-expression profiles of patients with c.1934dupG versus other *ASXL1* mutations. No significant expression signature was identified, suggesting that c.1934dupG and other *ASXL1* mutations share a similar gene-expression profile.

Gene-set analysis was performed to identify sets of genes, representing canonical biologic pathways, which were deregulated in patients with mutated *ASXL1*. Ten gene sets were found to be significantly deregulated (supplemental Table 5), 7 of which were up-regulated in *ASXL1*-mut patients compared with *ASXL1*-wt. Up-regulated biologic pathways include the cytochrome P450 system and gene sets related to glycoprotein and glycolipid biosynthesis and transmembrane transport of metabolites. Three gene sets were down-regulated, including one representing pyrimidine nucleotide metabolism.

In an approach similar to the analyses of gene expression, we also studied genome-wide miR-expression profiles. We compared miR expression in the subgroup of *NPM1*-wt/*FLT3*–ITD-negative patients (24 *ASXL1*-mut and 38 *ASXL1*-wt patients) but did not identify a signature of significantly deregulated miRs associated with *ASXL1* mutations.

Discussion

Our study of 423 patients represents, to our knowledge, the largest cohort of primary CN-AML patients analyzed for *ASXL1* mutations so far. We identified 45 *ASXL1* exon 12 nonsense or frame shift mutations in 44 patients, for an overall prevalence of 10.4%. *ASXL1* mutations were 5 times more common in patients 60 years

Table 3. Multivariable models for the associations between patient characteristics and treatment outcomes

Variable	OR (95% CI) or HR (95% CI)	*P*
CR	**OR**	
ASXL1 (mutated vs wild-type)		
ELN Favorable patients*	0.24 (0.06-0.90)	.03
ELN Intermediate-I patients	0.71 (0.28-1.79)	.47
Interaction between *ASXL1* mutations and ELN category		.18
WBC (continuous, per 50-unit increase)	0.73 (0.57-0.94)	.01
Platelet count (continuous, per 50-unit increase)	0.81 (0.67-0.98)	.03
Extramedullary involvement (present vs absent)	0.49 (0.24-0.99)	.05
DFS	**HR**	
ASXL1 (mutated vs wild-type)		
ELN Favorable patients	4.38 (1.84-10.42)	< .001
ELN Intermediate-I patients	1.15 (0.63-2.09)	.65
Interaction between *ASXL1* mutations and ELN category		.01
Extramedullary involvement (present vs absent)	1.65 (1.06-2.56)	.03
OS	**HR**	
ASXL1 (mutated vs wild-type)		
ELN Favorable patients	4.43 (2.35-8.33)	< .001
ELN Intermediate-I patients	0.94 (0.60-1.47)	.79
Interaction between *ASXL1* mutations and ELN category		< .001
WBC (continuous, per 50-unit increase)	1.13 (1.03-1.23)	.01
EFS	**HR**	
ASXL1 (mutated vs wild-type)		
ELN Favorable risk patients	3.61 (1.93-6.75)	< .001
ELN Intermediate-I risk patients	1.19 (0.76-1.87)	.44
Interaction between *ASXL1* mutations and ELN category		.005
WBC (continuous, per 50-unit increase)	1.18 (1.05-1.32)	.005
Extramedullary involvement (present vs absent)	1.52 (1.08-2.13)	.02

An OR > 1.0 (< 1.0) means a higher (lower) CR rate for the higher values of the continuous variables and the first category listed for the categorical variables. An HR > 1.0 (< 1.0) 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 $P < .2$. See the supplemental Appendix for a full list of variables evaluated in univariable analyses. Since *NPM1*, *FLT3*-ITD, and *CEBPA* mutations are integrated in the ELN genetic classification, they were not additionally considered as individual variables. Variables that were significant at $P < .2$ in the univariable models and considered for model inclusion were as follows: In the model for achievement of CR: *ASXL1* mutations, ELN genetic group, and their interaction term; WBC, platelet count, age, and extramedullary involvement. In the model for DFS: *ASXL1* mutations, ELN group, and their interaction term; WBC, age, and extramedullary involvement. In the model for OS: *ASXL1* mutations, ELN group, and their interaction term; WBC, platelet count, and extramedullary involvement. In the model for EFS: *ASXL1* mutations, ELN group, and their interaction term; WBC, platelet count, and extramedullary involvement.

*The ELN Favorable group is defined as patients with mutated *CEBPA* and/or mutated *NPM1* without *FLT3*-ITD. All remaining CN-AML patients (ie, those with wild-type *CEBPA*, and *FLT3*-ITD and/or wild-type *NPM1*) belong to the ELN Intermediate-I category.[27]

of age or older (16.2%) than in younger patients (3.2%), the largest difference in the proportion of mutated cases between age groups for any gene mutation that we have studied so far. Our findings are in agreement with the report by Chou et al, who found *ASXL1* mutations in 5.4% of cytogenetically heterogeneous younger AML patients, 18.0% of cytogenetically heterogeneous older patients, and in 8.9% (17 of 192) of CN-AML patients across both age groups.[8] To our knowledge, our study is the first to show that *ASXL1* mutations are associated with lower WBC and blast percentages and with mutated *CEBPA* in CN-AML patients. Our results also confirm previous reports showing that *ASXL1* mutations very rarely occur together with *NPM1* mutations or *FLT3*-ITD.[7,8]

We are not aware of any published data regarding the prognostic relevance of *ASXL1* mutations in primary CN-AML, and particularly in older patients who account for the majority of AML cases and have the highest prevalence of *ASXL1* mutations. Chou et al[8] reported data on treatment outcomes in a cytogenetically heterogeneous cohort of 360 patients, including 202 with intermediate-risk cytogenetics. In multivariable analyses, *ASXL1* mutations had no impact on OS in the entire cohort or in cytogenetic intermediate-risk patients. Of note, more than 80% of patients in the outcome analyses in that report were younger than 60 years, and only two-thirds of the patients with intermediate-risk cytogenetics had CN-AML. CN-AML not only is the most extensively studied AML subgroup on the molecular level, it also constitutes the largest cytogenetic subgroup of adult AML.[11-13,15-23,29] Therefore, it is

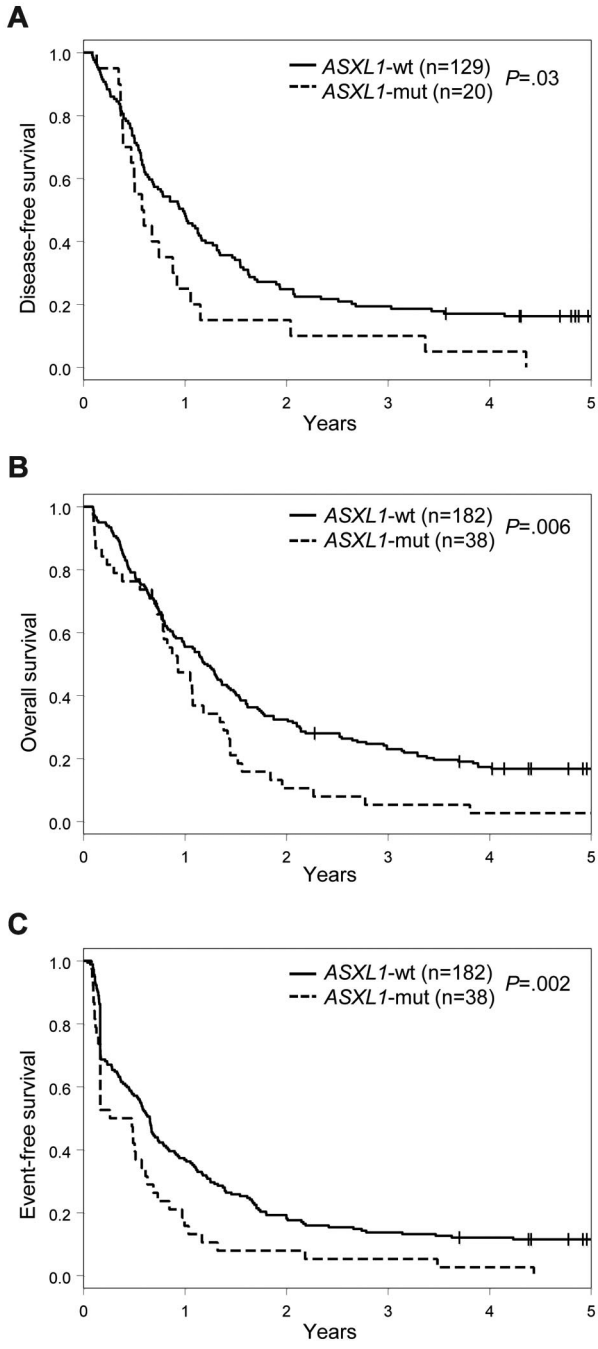

Figure 2. Survival of CN-AML patients 60 years of age or older, according to *ASXL1* mutation status. (A) DFS. (B) OS. (C) EFS.

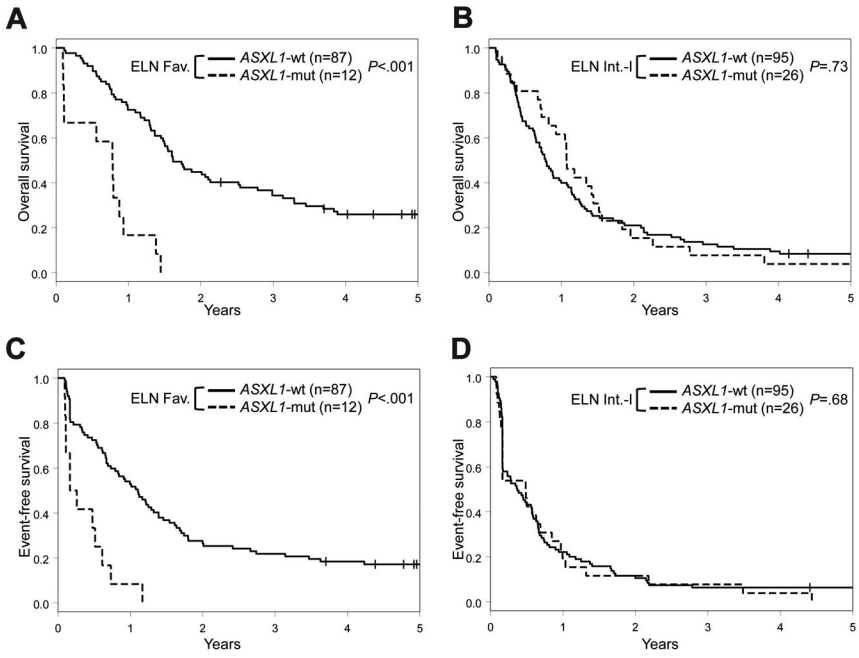

Figure 3. Survival of older CN-AML patients in the ELN Favorable and ELN Intermediate-I genetic groups, according to *ASXL1* mutation status. (A) OS of ELN Favorable group patients. (B) OS of ELN Intermediate-I group patients. (C) EFS of ELN Favorable group patients. (D) EFS of ELN Intermediate-I group patients. ELN Fav. indicates ELN Favorable category; and ELN Int.-I, ELN Intermediate-I category.

important to understand the prognostic implications of mutated *ASXL1* within this group. Novel molecular markers need to be studied in cytogenetically defined and molecularly well-characterized, homogeneously treated patient cohorts because the prognostic relevance of a gene mutation may vary between different cytogenetic groups,[36] depend on the context of other gene mutations present in the leukemic clone,[21,37,38] or be modified by treatment regimens.[18,39]

In our cohort of older primary CN-AML patients, univariable analyses revealed that *ASXL1* mutations were associated with a lower CR rate and shorter DFS, OS, and EFS. At the same time, *ASXL1* mutations showed baseline associations with other gene mutations, which themselves have well-recognized prognostic impact. *ASXL1* mutations very rarely occurred together with *NPM1* mutations,[7-9] which confer a favorable prognosis among older CN-AML patients.[19] In addition, *ASXL1*-mut patients rarely carried *FLT3*-ITD (a marker associated with shorter DFS and OS in older patients[15]), but more often harbored *CEBPA* mutations (a favorable prognostic marker studied mainly in younger patients[20]) than *ASXL1*-wt patients. These complex associations make it difficult to interpret the results of univariable outcome analyses concerning the entire cohort of older CN-AML patients. To better delineate the relevance of *ASXL1* mutations in the context of other gene mutations, we explored their prognostic impact within the molecular subgroups defined in the recent ELN guidelines for the diagnosis and management of AML.[27] According to these internationally accepted guidelines, CN-AML patients are stratified into the ELN Favorable and ELN Intermediate-I genetic categories based on their *NPM1, FLT3*-ITD, and *CEBPA* mutation status, which are the only 3 mutations that showed baseline associations with *ASXL1* mutations. *ASXL1* mutations were relatively rare among older CN-AML patients belonging to the ELN Favorable group. However, those ELN Favorable patients who carried mutated *ASXL1* had unfavorable outcomes, including a significantly lower CR rate and shorter OS and EFS compared with ELN Favorable/*ASXL1*-wt patients. Multivariable models supported these findings, confirming that *ASXL1* mutations remained associated with a lower CR rate and shorter DFS, OS, and EFS among ELN Favorable patients after adjusting for prognostic pretreatment characteristics and gene mutations. Further exploratory analyses suggested that *ASXL1* mutations have a strong negative prognostic impact in older CN-AML patients with mutated *CEBPA*, including those with double *CEBPA* mutations, but these results require confirmation because of the small patient numbers. Among ELN Intermediate-I older CN-AML patients, *ASXL1* mutations did not impact outcomes. The ELN Intermediate-I group consists of 3 genetic subsets (mutated *NPM1* and *FLT3*-ITD, wild-type *NPM1* and *FLT3*-ITD, and wild-type *NPM1* without *FLT3*-ITD).[27] All but 3 ELN Intermediate-I, *ASXL1*-mut patients belonged to the last of these subsets (Figure 1B), reflecting the negative association of *ASXL1* mutations with *NPM1* mutations and *FLT3*-ITD and precluding further subgroup analyses.

Our results exemplify how novel genetic markers can be useful to refine existing, clinically applicable classification schemes. In this respect, our findings add to our previous report on *TET2* mutations, which included both younger and older CN-AML patients, and also identified a subset of the ELN Favorable group with inferior outcomes.[21] When we combined our data on *ASXL1* and *TET2* mutations with the established ELN classification system, we identified a genetically defined subgroup of older primary CN-AML patients (ELN Favorable, *ASXL1*-wt, *TET2*-wt) that achieved a 3-year OS of 39% after cytarabine/daunorubicin-based chemotherapy regimens as used in our trials (supplemental Figure 3A). In contrast, all ELN Favorable/*ASXL1*-mut patients died of their disease within 18 months from study inclusion. Thus, *ASXL1* mutations identify a previously unrecognized high-risk subgroup among older CN-AML patients who would be categorized as low-risk based on currently used classification systems. ELN Favorable/*ASXL1*-mut patients might therefore be considered candidates for experimental treatment approaches.

Recently, our group reported that, in older CN-AML patients, high expression of *BAALC* (measured in PB) is associated with a lower CR rate and shorter DFS and OS and that high *ERG* expression is associated with shorter OS.[40] Information on *BAALC* and *ERG* expression in PB was unavailable for one-third of patients in the present study because of lack of adequate material. This, along with the limited number of *ASXL1*-mutated patients, precluded inclusion of *BAALC* and *ERG* expression levels in our

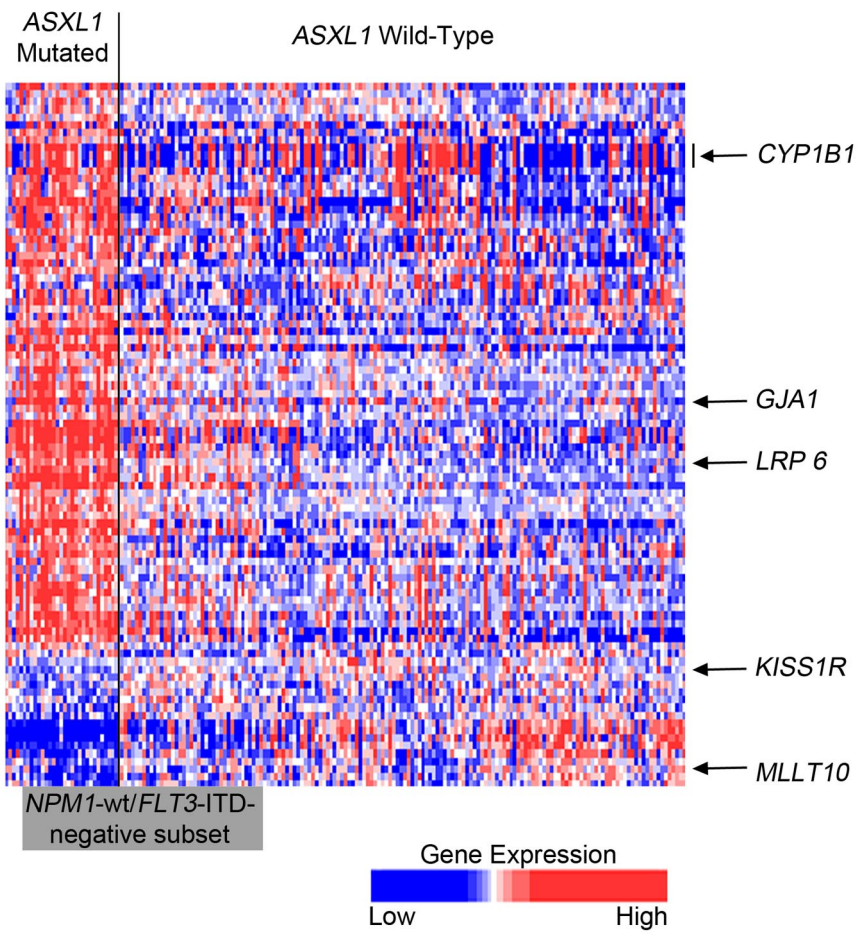

Figure 4. Heat map of the gene-expression signature associated with *ASXL1* mutations in older patients with primary CN-AML. Differential gene expression was studied within the subset of *NPM1*-wt/*FLT3*-ITD-negative patients, to avoid confounding by those gene mutations. In this subset, a comparison of *ASXL1*-mut (n = 26) and *ASXL1*-wt (n = 39) patients identified 92 differentially expressed probe sets. The heat map shows expression levels of these 92 probe sets in all 185 older CN-AML patients with available microarray data. The *NPM1*-wt/*FLT3*-ITD-negative subset where the signature was derived is indicated by the gray bar at the bottom of the heat map. Rows represent probe sets; and columns, individual patients. Patients are grouped by *ASXL1* mutation status, and genes are ordered by hierarchical cluster analysis. Expression values of the probe sets are represented by color: blue represents expression less than the median value for the given probe set; and red, expression greater than the median value for the given probe set. Arrows identify genes that are discussed in the text, with the vertical bar indicating multiple probe sets representing the same gene (*CYP1B1*).

multivariable analyses. It is therefore unclear whether *BAALC* or *ERG* expression offers additional prognostic information in patients characterized for *ASXL1* mutation status, or vice versa. However, prospective clinical use of quantitative measurements of gene expression requires extensive standardization and calibration efforts,[41] whereas analyses of gene mutations can be more easily transferred into routine use. Therefore, *ASXL1* mutations may be a clinically valuable prognostic marker in older patients.

Similar to previous studies in patients with various myeloid neoplasias,[4-9] one specific variant (c.1934dupG) accounted for more than half of the *ASXL1* mutations we found. A variety of other frame shift and nonsense mutations were detected in the remaining patients. One previous report raised doubts as to the pathogenic relevance of the c.1934dupG mutation by suggesting it was a false-positive finding, possibly representing an artifact introduced during PCR amplification.[42] We believe that c.1934dupG is indeed a true somatic mutation based on 3 lines of evidence. First, using a DNA polymerase enzyme that is resistant to this particular type of artifacts for PCR,[43] we reproducibly observed c.1934dupG in patients' BM or PB samples, but not in germline DNA from matched buccal swabs (supplemental Figure 4). Second, c.1934dupG, like other *ASXL1* mutations, was strongly associated with older age, wild-type *NPM1*, and absence of *FLT3*-ITD. These associations, which were statistically highly significant and consistent between our study and other reports,[7-9] would be unlikely to occur if c.1934dupG was a technical artifact occurring ex vivo. Moreover, outcomes of older CN-AML patients with c.1934dupG were not significantly different from those with other *ASXL1* mutations (data not shown). Third, *ASXL1* mutations were characterized by a common gene-expression signature, which was shared between patients with c.1934dupG and other *ASXL1* mutations, but was distinct from *ASXL1*-wt.

The function of *ASXL1* in normal hematopoiesis and the role of *ASXL1* mutations during leukemogenesis are not well understood. *Asxl1* knockout in mice leads to defects in B- and T-lineage lymphopoiesis but does not cause severe myelodysplastic changes or leukemias.[44] However, this model may not adequately reflect the situation in human myeloid cancers, where heterozygous *ASXL1* mutations are predicted to result in a truncated protein with possible gain-of-function or dominant-negative effects.[44] The ASXL1 protein modulates gene expression induced by retinoic acid receptors (RARs) and peroxisome proliferator-activated receptors, at least in part through affecting histone methylation.[45,46] Thereby, ASXL1 is involved in pathways controlling cell proliferation and differentiation in the hematopoietic system and other tissues.[44] Most reported *ASXL1* mutations are predicted to truncate the protein before the RAR-binding domain (Figure 1A), but it is largely unknown how this affects gene expression in AML. We thus studied *ASXL1* mutation-associated gene- and miR-expression profiles, using precautions to avoid confounding by other gene mutations, and identified an *ASXL1* mutation-associated gene-expression signature composing 67 named genes. These include several genes involved in pathways related to hematopoietic stem cell maintenance, such as the WNT and CXCL12-CXCR4 pathways.

Two other groups previously studied gene-expression signatures associated with *ASXL1* mutations in myeloid disorders.[6,47] Boultwood et al, in a cohort of 36 patients with myelodysplastic syndromes or chronic myelomonocytic leukemia, identified 30 differentially expressed genes, and Ingenuity Pathway Analysis suggested deregulation of the RAR pathway.[6] Thol et al applied

Gene Set Enrichment Analysis (a method similar to the gene-set analysis algorithm we used) in 30 AML patients, and found activation of the immune response pathway and down-regulation of cell adhesion and phosphatase pathways.[47] In our larger set of patients, we did not find deregulation of these or closely related pathways. Because both previous reports[6,47] provide no lists of individual differentially expressed genes, we are unable to assess their concordance with our own results in more detail. Finally, we did not detect a significant miR-expression signature associated with *ASXL1* mutations, suggesting that deregulation of miRs is not a prominent feature of CN-AML with mutated *ASXL1*.

In conclusion, we have shown that *ASXL1* mutations are rare in younger primary CN-AML patients, but that they affect 16.2% of patients 60 years of age or older. *ASXL1* mutations are mainly found in older CN-AML patients without *NPM1* mutations or *FLT3*-ITD. We found that *ASXL1* mutations are associated with mutated *CEBPA*, and we report signatures of differentially expressed genes in *ASXL1*-mut CN-AML. Importantly, we demonstrated that *ASXL1* mutations associate with inferior response rates and survival, particularly in the ELN Favorable genetic category. Thereby, *ASXL1* mutations may be useful to refine existing genetic classification systems and identify a group of older patients who may be considered candidates for experimental treatment approaches.

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Authorship

Contribution: K.H.M., K. Maharry, M.D.R., J.K., K. Mrózek, G.M., and C.D.B. designed the study, analyzed the data, and wrote the manuscript; K.H.M., H.B., S.P.W., Y.-Z.W., and S.S. carried out laboratory-based research; K. Maharry, M.D.R., J.K., and D.N. performed statistical analyses; B.L.P., T.H.C., M.W., J.O.M., J.E.K., M.R.B., A.J.C., R.A.L., M.A.C., G.M., and C.D.B. were involved directly or indirectly in the care of patients and/or sample procurement; and all authors approved the final version of the manuscript.

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A complete list of the Cancer and Leukemia Group B institutions, principal investigators, and cytogeneticists participating in this study can be found in the online supplemental Appendix.

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