

ASXL1 mutations in younger adult patients with acute myeloid leukemia: a study by the German-Austrian Acute Myeloid Leukemia Study Group

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ABSTRACT

We studied 1696 patients (18 to 61 years) with acute myeloid leukemia for *ASXL1* mutations and identified these mutations in 103 (6.1%) patients. *ASXL1* mutations were associated with older age ($P < 0.0001$), male sex ($P = 0.041$), secondary acute myeloid leukemia ($P < 0.0001$), and lower values for bone marrow ($P < 0.0001$) and circulating ($P < 0.0001$) blasts. *ASXL1* mutations occurred in all cytogenetic risk-groups; normal karyotype (40%), other intermediate-risk cytogenetics (26%), high-risk (24%) and low-risk (10%) cytogenetics. *ASXL1* mutations were associated with *RUNX1* ($P < 0.0001$) and *IDH2*^{R140} mutations ($P = 0.007$), whereas there was an inverse correlation with *NPM1* ($P < 0.0001$), *FLT3*-ITD ($P = 0.0002$), and *DNMT3A* ($P = 0.02$) mutations. Patients with *ASXL1* mutations had a lower complete remission rate (56% versus 74%; $P = 0.0002$), and both inferior event-free survival (at 5 years: 15.9% versus 29.0%; $P = 0.02$) and overall survival (at 5 years: 30.3% versus 45.7%; $P = 0.0004$) compared to patients with wildtype *ASXL1*. In multivariable analyses, *ASXL1* and *RUNX1* mutation as a single variable did not have a significant impact on prognosis. However, we observed a significant interaction ($P = 0.04$) for these mutations, in that patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} had a higher risk of death (hazard ratio 1.8) compared to patients without this genotype. *ASXL1* mutation, particularly in the context of a coexisting *RUNX1* mutation, constitutes a strong adverse prognostic factor in acute myeloid leukemia.

Introduction

The *additional sex combs-like 1* (*ASXL1*) gene on chromosomal band 20q11¹ is one of three human homologs of the *additional sex combs* (*Asx*) gene of drosophila.² Somatic *ASXL1* mutations were found in a broad variety of myeloid malignancies including chronic myelomonocytic leukemia (CMML, 45%), myelodysplastic syndromes (MDS, 16%), primary myelofibrosis (35%), and acute myeloid leukemia (AML, secondary AML 30%; *de novo* AML 6.5%).³ Although the exact role of *ASXL1* in normal hematopoiesis and the contribution of mutated *ASXL1* to the development of hematopoietic malignancies have not been fully delineated yet, there are emerging data suggesting that *ASXL1* is a tumor suppressor. An animal study has shown that *Asx1* deletion or haploinsufficiency constitutes a sufficient condition for the development of myeloid neoplasia reminiscent of MDS and MDS/myeloproliferative neoplasms.⁴ In another study the transplantation of bone marrow cells expressing oncogenic

NRAS^{G12D} together with knocked-down *Asx1* into lethally irradiated mice promoted myeloproliferation.⁵ The animals with additional loss of *Asx1* showed accelerated myeloproliferation and shorter survival than animals expressing *Asx1*.⁵ In fact, *in vitro*, *ASXL1* mutations cause a loss of Polycomb-repressive complex 2 (PRC2)-mediated repression of leukemogenic target genes including those from the posterior *HOXA* cluster.⁵

The first studies on *ASXL1* mutations in adult AML are remarkably consistent with respect to the increasing incidence of these mutations with age and their association with distinct clinical and genetic features.⁶⁻⁹ In addition, *ASXL1* mutations in AML appear to have an adverse impact on induction success and long-term outcome.⁶⁻¹⁰ However, there are only limited data on the prognostic relevance of *ASXL1* mutations in younger patients with AML.^{10,11}

In our study, representing the largest AML cohort (n=1696) studied for *ASXL1* mutations, we focused on younger patients with AML (≤ 61 years) and assessed the incidence

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and clinical impact of these mutations in the context of other clinical and genetic factors in a well-defined population of patients intensively treated in trials of the German-Austrian AML Study Group (AMLSG).

Methods

Patients

A total of 1696 younger AML patients (18 to 61 years) were studied. Patients were enrolled in prospective treatment protocols of the AMLSG, namely AML HD98A¹² (n=733; NCT00146120), AMLSG 07-04¹³ (n=893; NCT00151242), and APL HD95¹⁴ (n=70) for the patients with acute promyelocytic leukemia (APL). The clinical studies were approved by the local ethics review committees and all patients gave informed consent for both treatment and cryopreservation of leukemia samples according to the Declaration of Helsinki. The only criterion to include patients in our study was the availability of a pretreatment bone marrow or peripheral blood specimen for analysis of *ASXL1* mutations. Cytogenetic and additional molecular analyses were performed as previously described.¹⁵⁻¹⁹

ASXL1 mutation analysis

A detailed description of the *ASXL1* mutation analysis is provided in the *Online Supplementary Material*. Briefly, genomic DNA was used as a template for polymerase chain reactions to amplify several fluorescently-labeled DNA fragments covering the entire exon 12 (AMLSG 07-04) or regions within exon 12 (AML HD98A and APL HD95) identified as main mutation clusters in AML.^{6,20} Amplicons were screened for mutations by a GeneScan-based fragment analysis (*Online Supplementary Figures S1 and S2*). Samples classified as mutated after the GeneScan analysis (*Online Supplementary Figure S2*) were further analyzed by direct sequencing to validate the mutation and to determine the mutation type.

Statistical analysis

Statistical analyses of clinical outcome were performed according to previous reports.¹⁶ The median follow-up for survival was calculated according to the method of Korn.²¹ The definition of complete remission (CR), event-free survival (EFS), relapse-free survival (RFS), and overall survival (OS) as well as cytogenetic categorization into favorable-, intermediate-, and adverse-risk groups followed recommended criteria.²² Pairwise comparisons between patients' characteristics (covariates) were performed using the Mann-Whitney test for continuous variables and the Fisher exact test for categorical variables. The Kaplan-Meier method was used to estimate the distribution of EFS, RFS and OS.²³ Estimation of confidence intervals for the survival curves was based on the Greenwood formula for standard error estimation. A logistic regression model was used to analyze associations between baseline characteristics and the achievement of CR. A Cox model was used to identify prognostic variables.²⁴ In addition to *ASXL1* mutation status, age, sex, hemoglobin level, logarithm of white blood cell count, type of AML (*de novo*, secondary AML, therapy-related AML), percentage of peripheral blood and bone marrow blasts, cytogenetic risk group,²² and mutational status of *NPM1*, *FLT3* (ITD and TKD), *CEBPA* (*CEBPA* double-mutated, *CEBPA*^{dm}), *IDH1*, *IDH2* (*IDH2*^{R140}, *IDH2*^{R172}), *RUNX1*, *MLL* (PTD), and *DNMT3A* were included as explanatory variables in the regression analyses, as indicated, without further selection (full models). The full model presentation was chosen to allow estimation of the relative impact of the new marker (*ASXL1* mutational status) in the context of the already known prognostic and predictive markers. For

multivariable analyses a missing value imputation technique was used as recommended for the situation termed *missing at random*.²⁵ We estimated missing data for covariates by using 50 multiple imputations in chained equations that incorporated predictive mean matching.²⁶ All statistical analyses were performed with the statistical software environment R version 2.14.0, using the R packages *rms* version 3.3-1, *survival* version 2.36-8, and *cmprsk* version 2.2-2.33.

Results

Demographics, clinical baseline characteristics and outcomes of the entire study population

The median age at diagnosis in the entire study cohort (n=1696) was 48.3 years (range, 18-61 years). The patients' baseline characteristics are summarized in *Online Supplementary Table S2*. The CR rate was 73.1% (1230 of 1683 patients). With a median follow-up for survival of 5.6 years (95%-confidence interval, 5.4 to 5.9 years), the estimated 5-year rates for EFS, RFS and OS in the entire cohort were 28.2%, 42.6% and 40.2%, respectively (*Online Supplementary Table S2*). In total, 422 patients underwent allogeneic hematopoietic stem cell transplantation in first CR.

Frequency and types of ASXL1 mutations

ASXL1 mutations were detected in 103 (6.1%) of the 1696 patients. The types of mutation at the DNA and protein levels are described in *Online Supplementary Table S3*. The most common mutation, found in 60% (62/103) of the mutated cases, was a duplication of guanine at cDNA position 1934 (c.1934dupG). Other *ASXL1* mutations found in more than one patient were c.1900_1922del (n=17) and c.1934delG (n=6). All frame shift mutations resulted in premature stop codons with consecutive loss of the c-terminal plant-homeo-domain. The wild-type allele was retained in all mutated samples. Of note, the majority (>90%) of the mutations clustered within or around a glycine-rich domain located between amino acids 642 and 685.²⁷

Clinical and genetic characteristics of acute myeloid leukemia with ASXL1 mutations

Patients with *ASXL1* mutations were older ($P<0.0001$), more frequently males ($P=0.04$), and more often had a history of MDS ($P<0.0001$) (Table 1). *ASXL1* mutations were associated with lower values for white blood cell count ($P=0.002$), lactate dehydrogenase in serum ($P=0.0008$), proportion of bone marrow ($P<0.0001$) and circulating ($P<0.0001$) blasts (Table 1).

Among the *ASXL1* mutated cases with informative cytogenetics (n=99), 40 (40.4%) patients had a normal karyotype; *ASXL1* mutations tended ($P=0.08$) to be more frequent in AML with adverse-risk cytogenetics and less frequent in AML with favorable-risk cytogenetics compared to wild-type *ASXL1* (Table 1); in core-binding factor-AML, *ASXL1* mutations were only found in AML with t(8;21)(q22;q22) (9/91; 9.9%); none of the 106 cases with inv(16)(p13.1q22) had an *ASXL1* mutation. *ASXL1* mutations were further associated with trisomy 8 ($P=0.0001$), and with del(7q)-7 ($P=0.01$) within a non-complex karyotype and in the absence of balanced translocations.

ASXL1 mutations showed several associations with

other gene mutations (Table 2). *RUNX1* ($P<0.0001$) and *IDH2*^{R140} mutations ($P=0.007$) co-occurred more frequently with *ASXL1* mutations than with wild-type *ASXL1*. Conversely, *ASXL1* mutations were only rarely detected in patients with *NPM1* ($P<0.0001$), *FLT3*-ITD ($P=0.0002$), and *DNMT3A* ($P=0.02$) mutations. Of note, 31 of 103 (30%) patients with *ASXL1* mutations had a genetic abnormality involving the *RUNX1* gene on chromosome 21q22, i.e., these patients had either a somatic *RUNX1* mutation ($n=22$) or $t(8;21)(q22;q22)$; *RUNX1*-*RUNX1T1* fusion ($n=9$).

Twenty-two patients had both *ASXL1* and *RUNX1* mutations; among these double-mutated cases 15 presented with *de novo* AML, five with secondary AML, and one with therapy-related AML. The distribution of cytogenetically defined risk-groups among the *ASXL1*^{mutated}/*RUNX1*^{mutated} patients was as follows: 18 had intermediate-risk and three had high-risk cytogenetics; no information on cytogenetic risk group was available at diagnosis for one patient.

Table 1. Clinical and cytogenetic characteristics according to the *ASXL1* mutation status.

Characteristic	<i>ASXL1</i> status <i>ASXL1</i> mutated n=103	<i>ASXL1</i> wildtype n=1593	P
Median age, years (range)	53 (36-61)	48 (16-61)	<0.0001
Male sex, n. (%)	63 (61.2)	805 (50.5)	0.04
AML history, n. (%)			<0.0001
<i>De novo</i>	83 (81)	1446 (91.0)	
Secondary	15 (15)	59 (3.7)	
Therapy-related	4 (4)	84 (5.3)	
Unknown	1	4	
Median WBC count, x10 ⁹ /L (range)	6.5 (0.7-126.5)	14.2 (0.2-532.7)	0.002
Missing data, n.	2	25	
Median platelet count, x10 ⁹ /L (range)	66 (5-563)	52 (2-933)	0.15
Missing data, n.	2	26	
Median hemoglobin, g/dL (range)	9.0 (4.9-15.1)	9.0 (2.5-17.6)	0.39
Missing data, n.	2	26	
Median LDH, U/L (range)	297 (84-5215)	432 (79-15098)	0.0008
Missing data, n.	2	43	
Median % blood blasts (range)	18 (0-95)	38 (0-100)	<0.0001
Missing data, n.	10	127	
Median % bone marrow blasts (range)	52 (13-100)	79 (2-100)	<0.0001
Missing data, n.	6	133	
Cytogenetic risk*, n. (%)			0.08
Favorable	10 (10.1)	257 (17.5)	
Intermediate	65 (65.7)	954 (64.7)	
Adverse	24 (24.2)	263 (17.9)	
Missing data, n.	4	119	
Chromosomal aberration, n. (%)			
None	40 (40.4)	711 (48.2)	0.15
<i>t(8;21)</i>	9 (9.1)	82 (5.6)	0.18
<i>inv(16)/t(16;16)</i>	0	106 (7.2)	0.001
<i>t(15;17)</i>	1 (1)	69 (4.7)	0.12
Trisomy 8 in non-complex karyotype	14 (14.1)	61 (4.1)	0.0001
<i>del(7q)-7</i> in non-complex karyotype	11 (11.1)	68 (4.6)	0.01
Complex karyotype	7 (7.1)	149 (10.1)	0.39

WBC: white blood cell; LDH: lactate dehydrogenase. *Cytogenetic categorization into favorable, intermediate, and adverse-risk groups as recommended by the European LeukemiaNet.²²

Response to induction therapy

The CR rate was significantly lower in patients with *ASXL1* mutations than in patients with wild-type *ASXL1* (56.3% versus 74.2%; $P=0.0002$; Table 3); exclusion of APL patients did not change the unfavorable ($P=0.0002$) impact of *ASXL1* mutations. The inferior CR rate in *ASXL1* mutated cases was due to both a higher rate of resistant disease (29.1% versus 18.2%; $P=0.03$) and a higher rate of early deaths (14.6% versus 7.7%; $P=0.02$). Results were similar in the subset of cytogenetically normal AML (data not shown). Among the 91 AML patients with $t(8;21)$, the CR rate did not differ between patients with ($n=9$) and without ($n=82$) *ASXL1* mutation (89% versus 94%; $P=0.47$). Because of the association of *ASXL1* with *RUNX1* mutations, we assessed response according to the various *ASXL1/RUNX1* genotypes: patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} had an inferior ($P=0.0001$) CR rate (45.5%) compared to patients with any other *ASXL1/RUNX1* genotype (Table 4). Patients with the *ASXL1*^{wildtype}/*RUNX1*^{wildtype} genotype had the highest CR rate (75.2%); CR rates were comparable in patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{wildtype} (62.3%) and *ASXL1*^{wildtype}/*RUNX1*^{mutated} (63.9%). In multivariable analysis *ASXL1* mutations were not a significant factor for achievement of CR (Online Supplementary Table S4).

Survival analyses

In univariable analyses, *ASXL1* mutations were associated with inferior EFS ($P=0.02$) and OS ($P=0.0004$), while there was no significant effect on RFS ($P=0.58$) (Table 3; Figure 1); the impact of *ASXL1* mutations on outcome (EFS, $P=0.028$; RFS, $P=0.69$; OS, $P=0.0006$) was retained after exclusion of patients with APL. The estimated 5-year

Table 2. Co-occurrence of gene mutations in *ASXL1* mutated cases.

Mutation	<i>ASXL1</i> status <i>ASXL1</i> mutated n=103	<i>ASXL1</i> wildtype n=1593	P
<i>RUNX1</i> , n. (%)	22 (22.2)	108 (6.9)	<0.0001
Missing data, n.	4	30	
<i>IDH2</i> ^{R140} , n. (%)	14 (13.7)	88 (6.2)	0.007
Missing data, n.	1	20	
<i>IDH2</i> ^{R172} , n. (%)	6 (5.9)	43 (2.7)	0.12
Missing data, n.	1	20	
<i>NPM1</i> , n. (%)	6 (5.9)	467 (29.7)	<0.0001
Missing data	2	22	
<i>FLT3</i> -ITD, n. (%)	8 (8.1)	361 (23.2)	0.0002
Missing data, n.	4	38	
<i>DNMT3A</i> , n. (%)	11 (11.8)	325 (21.9)	0.02
Missing data, n.	10	111	
<i>IDH1</i> , n. (%)	11 (10.8)	104 (6.6)	0.11
Missing data, n.	1	13	
<i>CEBPA</i> , n. (%)	3 (3.2)*	121 (8.1)†	0.11
Missing data, n.	10	101	
<i>FLT3</i> -TKD, n. (%)	4 (4.3)	120 (7.9)	0.23
Missing data, n.	9	96	
<i>MLL</i> -PTD, n. (%)	7 (8.0)	77 (5.6)	0.34
Missing data, n.	15	220	

To estimate the frequency in percentage of an additional gene mutation in the study cohort, data on missing cases are considered in the denominator. *One case with *CEBPA* double mutant. †Seventy-six cases with *CEBPA* double mutant.

EFS and OS rates in patients with *ASXL1* mutations were 15.9% and 30.3%, respectively, whereas in patients with wild-type *ASXL1* they were 29.0% and 45.7%, respectively (Table 3; Figure 1). The adverse effect of *ASXL1* mutations on EFS and OS was also present in the subset of cytogenetically normal-AML (*data not shown*). In the subset of AML with t(8;21), none of the survival endpoints was impacted by the presence of *ASXL1* mutations (EFS, $P=0.77$; RFS, $P=0.74$; OS, $P=0.81$). The one patient with t(15;17) and an *ASXL1* mutation is still alive and in complete remission with a follow-up of 4 years. Among the 58 patients with *ASXL1* mutations achieving a CR, 24 underwent allogeneic hematopoietic stem cell transplantation in first CR; Mantel-Byar analyses of allogeneic hematopoietic stem cell transplantation did not reveal a significant impact on either RFS ($P=0.87$) or OS ($P=0.66$).

We also conducted an exploratory analysis of the composite *ASXL1/RUNX1* genotypes. Patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} had a significantly worse EFS ($P<0.0001$) compared to patients with any other *ASXL1/RUNX1* genotype (Table 4, Figure 2). Of note, 73%

of patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} experienced an event within the first year. The dismal EFS in patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} translated into a significantly inferior OS ($P<0.0001$) compared to that of patients with any other *ASXL1/RUNX1* genotype (Table 4, Figure 2). Among the ten patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} who achieved a CR, four underwent hematopoietic stem cell transplantation in first CR. The small number of patients in this molecular subgroup prevented a meaningful analysis of the impact of allogeneic stem cell transplantation in first CR.

Both *ASXL1* and *RUNX1* mutations as a single variable did not remain a significant factor for any clinical endpoint (EFS, RFS, and OS) after multivariable analyses (*Online Supplementary Tables S5 and S6*; Table 5). However, a significant interaction ($P=0.04$) for *ASXL1* and *RUNX1* mutations was observed in terms of OS (Table 5); the risk of death was almost twice as high in patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} than in patients without this genotype.

Discussion

We here present the largest study assessing the incidence and clinical impact of *ASXL1* mutations in younger (≤ 61 years) adults with newly diagnosed AML.

ASXL1 mutations were detected in 6.1% of the patients. In line with previous studies in AML,^{6-9,11,27,28} we found that c.1934dupG (p.G646WfsX12) was the most frequent *ASXL1* mutation, accounting for 60% of the *ASXL1* mutated cases. In a prior, large study of 501 patients with unselected AML, Chou *et al.* found *ASXL1* mutations in 10.8% of the patients.⁶ The incidence of *ASXL1* mutations in that study increased significantly with age; *ASXL1* mutations occurred in 6.4% of patients <60 years, but in 18% of patients ≥ 60 years.⁶ In accordance, a Cancer and Leukemia Group B (CALGB) study on 423 adults with cytogenetically normal-AML reported a 5-fold higher frequency of *ASXL1* mutations in patients ≥ 60 years than in those <60 years (16.2% versus 3.2%).⁷ A German study on 740 unselected AML cases with a median age of 67 years identified *ASXL1* mutations in 17.2%

Table 3. Univariable outcome analyses according to *ASXL1* mutation status.

Clinical endpoint	<i>ASXL1</i> ^{mutated} n=103	<i>ASXL1</i> ^{wildtype} n=1580	P
CR rate, %	56.3	74.2	0.0002
EFS			0.02
Median, years	0.48	0.83	
5-year EFS (%)	15.9	29.0	
95%-CI	9.7-25.9	26.8-31.4	
RFS	n=58	n=1170	0.58
Median, years	2.2	2.2	
5-year RFS (%)	33.9	43.0	
95%-CI	22.9-50.1	40.1-46.0	
OS	n=103	n=1580	0.0004
Median, years	1.8	3.3	
5-year OS (%)	30.3	45.7	
95%-CI	22.3-41.1	43.2-48.3	

CR: complete remission; EFS: event-free survival; CI: confidence interval; RFS: relapse-free survival; OS: overall survival.

Table 4. Univariable outcome analyses in the entire AML cohort according to combined *ASXL1/RUNX1* genotype.

Clinical endpoint	Genotype <i>ASXL1</i> ^{mutated} / <i>RUNX1</i> ^{mutated} n=22	<i>ASXL1</i> ^{mutated} / <i>RUNX1</i> ^{wildtype} n=77	<i>ASXL1</i> ^{wildtype} / <i>RUNX1</i> ^{mutated} n=108	<i>ASXL1</i> ^{wildtype} / <i>RUNX1</i> ^{wildtype} n=1442	P
CR rate, %	45.5	62.3	63.9	75.2	0.0001
EFS					<0.0001
Median, years	0.17	0.71	0.31	0.87	
5-year EFS (%)	0.0	21.1	13.9	30.5	
95%-CI		13.4-33.5	8.5-22.6	28.1-33.0	
RFS					0.13
Median, years	1.34	2.53	1.40	2.39	
5-year RFS (%)	N.A.	38.3	28.6	44.1	
95%-CI		26.0-56.2	19.2-42.8	41.2-47.3	
OS					<0.0001
Median, years	0.49	1.43	1.20	2.75	
5-year OS (%)	17.1	35.9	35.7	46.6	
95%-CI	6.6-44.3	26.3-48.9	27.6-46.3	44.0-49.3	

CR: complete remission; EFS: event-free survival; CI: confidence interval; RFS: relapse-free survival; OS: overall survival.

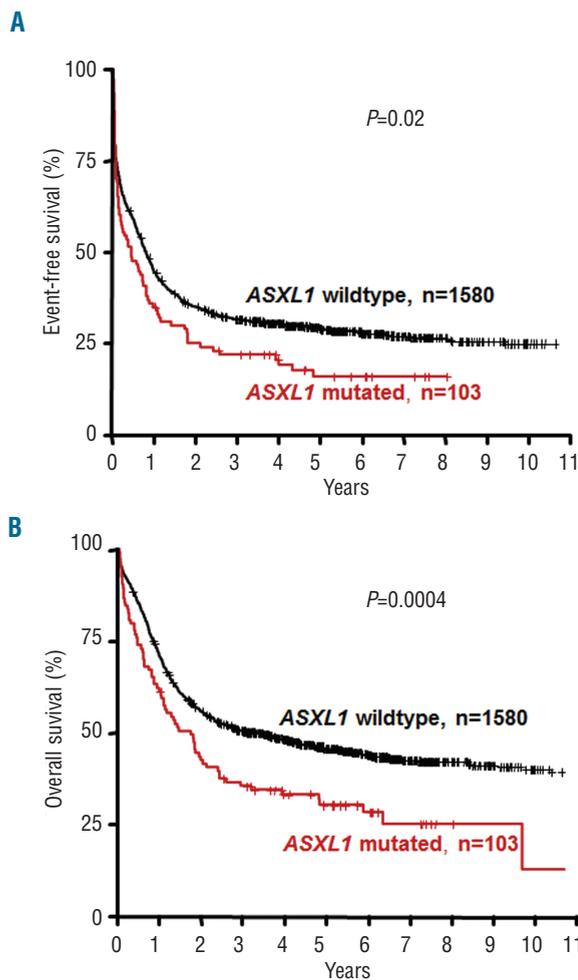


Figure 1. Impact of *ASXL1* mutations on (A) event-free survival and (B) overall survival.

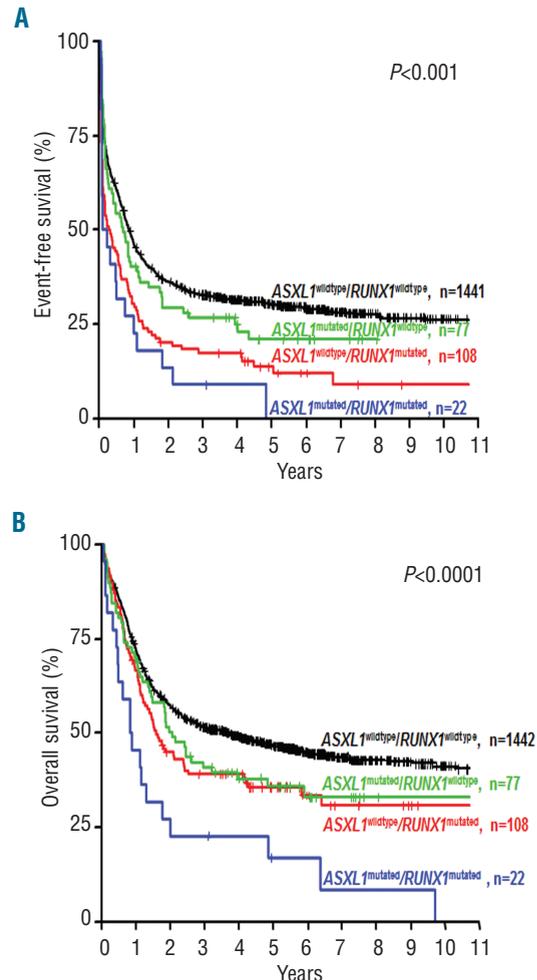


Figure 2. Impact of *ASXL1/RUNX1* genotypes on (A) event-free survival and (B) overall survival.

of the cases,⁹ whereas a study from the Dutch-Belgian Hematology-Oncology Cooperative Group (HOVON) on 882 unselected AML patients with a median age <55 years found *ASXL1* mutations in 5.3% of the cases.⁸ Finally, the most recent study from the UK Medical Research Council (MRC) on 367 adults with AML identified *ASXL1* mutations in 9% of the cases.¹¹ In line with other studies the frequency of *ASXL1* mutations in this MRC study increased with age, and was twice as high in older AML patients (≥ 60 years; 12%, 18/148) than in younger ones (15-59 years; 6%, 14/219).¹¹ Based on our data and the results from previous studies,^{6,8,9,11} *ASXL1* mutations account for less than 10% of cases in younger adults with AML, whereas in older patients the incidence increases over 10%. Base exchanges resulting in stop codons (non-sense mutations) are not detected with our screening method. However, as already reported in previous studies, which used direct sequencing for mutational analysis of *ASXL1*, non-sense mutations appear to be infrequent molecular alterations, at least in the younger AML population; Metzeler *et al.*⁷ did not find any non-sense mutations in 189 younger (<60 years) AML patients with a normal karyotype, and Chou *et al.*⁶ detected only one (0.3%) non-sense mutation in 312 younger (<60

years) patients with unselected AML. In addition, the overall incidence of *ASXL1* mutations in our study is very much in line with the data obtained for younger AML patients in other studies. Thus, we obviously did not miss a substantial proportion of *ASXL1* mutations in younger AML patients by using our screening approach.

As in other studies, we found that *ASXL1* mutations were more frequent in males than in females,^{6,7,9} and we also observed an association of *ASXL1* mutations with both lower white blood cell count⁷⁻⁹ and blast count,⁷ as well as with a prior phase of MDS.^{9,28} This is in line with the finding that *ASXL1* mutations are frequently detected already at the stage of a MDS or CMML.^{20,29,30} In a recent study of 48 AML patients with myelodysplasia-related changes *ASXL1* mutations were detected in one third of the cases.³¹

In our study *ASXL1* mutations were associated with distinct genetic characteristics. More than 60% of the *ASXL1* mutated cases belonged to the cytogenetically intermediate-risk group. Among patients with core-binding factor-AML *ASXL1* mutations were almost exclusively detected in AML with t(8;21), a finding which is concordant with that of other studies.^{6,8} In line with previous reports, we found that *ASXL1* mutations were frequently associated

with *RUNX1*^{16,9,32} and *IDH2*^{9,11} mutations, whereas they were only rarely detected in patients with *FLT3*-ITD^{6,9} or *NPM1* mutations.^{6,9,11,28} In contrast to two other studies,^{7,9} we and others^{6,11} did not observe any significant association of *ASXL1* mutations with *CEBPA* or *FLT3*-TKD mutations. However, the association of *ASXL1* mutations with *CEBPA* mutations was only reported in older patients with cytogenetically normal AML⁷ and for *FLT3*-TKD mutations in an AML cohort with a relatively high median age of 67 years.⁹ Thus, the selection of the AML cases and age difference might in part contribute to the differences among the results of the studies. In our study, 31 (30%) of the 103 *ASXL1* mutated cases had a concurrent genetic abnormality involving the *RUNX1* gene, i.e., a somatic *RUNX1* mutation or t(8;21)(q22;q22); *RUNX1*-*RUNX1T1*. Similar data can be obtained from the publication by Chou *et al.* comprising 54 *ASXL1* mutated cases.⁶

The presence of *ASXL1* mutations in patients with a clinically preleukemic condition such as MDS or CMML and the association of *ASXL1* mutations with secondary AML suggest that these mutations represent a relatively early event in leukemogenesis. One recent study backtracked *ASXL1* mutations in four patients with secondary AML and demonstrated that these mutations were already present in all patients at the stage of the preceding MDS.⁹ Other studies provide additional evidence that *ASXL1* mutations favor the development of AML from MDS or CMML. Thol *et al.* reported that the presence of *ASXL1* frameshift mutations in patients with lower-risk MDS, i.e., those with an International Prognostic Scoring System classification of low or intermediate-1, was associated with a shorter time to AML progression,³³ and a more recent study by Papaemmanuil *et al.* of 595 MDS cases found an inferior leukemia-free survival in patients with *ASXL1* mutations (n=81).³⁰ A study by Gelsi-Boyer *et al.* of 51 CMML patients identified *ASXL1* mutations as an unfavorable factor for the progression to AML.³⁴ Of note, in that study only CMML patients with an *ASXL1* mutation (11/25; 44%) did progress to AML, whereas none of the patients with wild-type *ASXL1* (n=26) developed AML.³⁴

Chou *et al.* found in a group of 360 unselected AML cases that *ASXL1* mutations had an unfavorable impact on CR and OS in univariable, but not in multivariable analyses.⁶ In a subsequent CALGB study in older patients with cytogenetically normal AML, *ASXL1* mutations were associated with inferior CR, EFS, DFS, and OS in univariable analyses,⁷ but on multivariable analyses *ASXL1* mutations were revealed as a relevant factor for CR, EFS, DFS, and OS only in cytogenetically normal AML classified as favorable-risk (n=220) according to the European LeukemiaNet (ELN) recommendations.⁷ A HOVON study of 807 AML patients <65 years showed a lower CR rate and shorter OS in patients with *ASXL1* mutations than in those with wild-type *ASXL1*; multivariable analysis in this study identified *ASXL1* mutation as a relevant factor for

shorter survival.⁸ Schnittger *et al.* evaluated 481 unselected AML patients, 50% of whom were ≥60 years, and found that *ASXL1* mutations were a relevant factor for a shorter survival.⁹ In a study by the Eastern Cooperative Oncology Group (ECOG) including younger (<60 years) AML patients (n=398), *ASXL1* mutations also conferred inferior survival.¹⁰ In a recent MRC study including 367 AML cases, *ASXL1* mutations were found to be an unfavorable factor with regards to the cumulative incidence of relapse and overall survival in univariable, but not in multivariable analyses.¹¹ However, the high proportion of older patients in this study [148/367 (40%) ≥60 years] might have been a confounding factor overriding the impact of *ASXL1* mutations in multivariable analyses.¹¹ In our study, which focuses on younger patients with AML, the presence of *ASXL1* mutations was associated with inferior CR, EFS, and OS. We describe for the first time an additive, prognostically unfavorable effect for the combined genotype *ASXL1*^{mutated}/*RUNX1*^{mutated}. While multivariable analysis did not identify *ASXL1* as well as *RUNX1* mutation as a single marker to be a relevant factor for long-term outcome, a significant interaction was found for these two mutations, in that patients with the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated} had almost twice the risk of death compared to patients lacking this double mutated *ASXL1*/*RUNX1* genotype.

We report here the largest study in AML assessing the incidence and prognostic relevance of *ASXL1* mutations in younger patients with AML. We confirm that *ASXL1* mutations constitute a recurrent molecular alteration in AML and are associated with distinct clinical and genetic features and, most importantly, with an adverse prognosis. We extend the results of previous studies by the finding that the combined genotype *ASXL1*^{mutated}/*RUNX1*^{mutated}, which can be detected in up to 30% of *ASXL1* mutated AML,⁶ is associated with a particularly dismal rate of CR achievement and long-term outcome. Thus, allogeneic hematopoietic stem cell transplantation might be evaluated prospectively as one therapeutic option in younger AML patients carrying the genotype *ASXL1*^{mutated}/*RUNX1*^{mutated}. In addition, since *ASXL1* mutations lead to a loss of H3K27me₃, in particular at the *HOXA* locus, pharmacological inhibition of H3K27 demethylation³⁵ might be explored as a potential therapeutic approach in *ASXL1* mutated AML.

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