# Acquired mutations in *ASXL1* in acute myeloid leukemia: prevalence and prognostic value

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## ABSTRACT

Somatic mutations in the additional sex comb-like 1 (*ASXL1*) gene have been described in various types of myeloid malignancies, including acute myeloid leukemia. Analysis of novel markers, such as *ASXL1* mutations, in independent clinical trials is indispensable before considering them for clinical decision-making. We analyzed 882 well-characterized acute myeloid leukemia cases to determine the prevalence and prognostic impact of *ASXL1* exon12 mutations. Truncating *ASXL1* mutations were present in 46 cases (5.3%). *ASXL1* mutations were inversely associated with *FLT3* internal tandem duplications and mutually exclusive with *NPM1* mutations. *ASXL1* mutations were an unfavorable prognostic factor as regards survival (median overall survival 15.9 months *vs.* 22.3 months; *P*=0.019), with a significantly lower complete response rate (61% *vs.* 79.6%; *P*=0.004). In multivariate analyses, *ASXL1* mutations were independently associated with inferior poor overall survival (HR 1.52, *P*=0.032). In conclusion, *ASXL1* mutations are common mutations in acute myeloid leukemia and indicate a poor therapy outcome.

Key words: acute myeloid leukemia, *ASXL1* mutations, prognosis.

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## Introduction

The Polycomb Group (PcG) and trithorax Group (trxG) proteins are epigenetic regulators.[1] PcG proteins are required to epigenetically silence their target genes, whereas trxG proteins maintain transcriptional activation.[2] *Additional sex comb-like 1* (*ASXL1*) belongs to the Enhancer of trithorax and Polycomb genes, which are responsible for maintaining activation and silencing of PcG and trxG proteins.[2] *ASXL1* is a highly conserved gene consisting of an N-terminal ASX homology domain and a C-terminal plant homeodomain finger region (PHD).[3] Somatic mutations in the *ASXL1* gene have been described in various types of myeloid malignances: 11% myelodysplastic syndrome (MDS),[4] 8% myeloproliferative neoplasia,[5] 49% of chronic myelomonocytic leukemia,[6] 10.8% of *de novo* acute myeloid leukemia (AML)[7] and 23% of post-MDS AML.[8] Mutations were all found in exon 12 of the gene and cause truncation of the protein with loss of the PHD domain.[4-8]

The prevalence and prognostic value of *ASXL1* mutations (*ASXL1*$^{mut}$) in AML have not been extensively described.[7] However, thorough analyses of novel markers in independent clinical trials are warranted before implementation of these markers in treatment decision-making can be considered. In this study, we determined the *ASXL1* mutations in a cohort of 882 cases of AML. We investigated their distribution in relationship with cytogenetic and molecular risk categories, and we evaluated the impact of these mutations on survival and relapse.

## Design and Methods

Bone marrow aspirates or peripheral blood samples of cohorts of patients with various hematologic malignancies were collected after obtaining written informed consent in accordance with the Declaration of Helsinki. This research has been reviewed and approved by the Erasmus University Medical Center Medical Ethical Review Committee (MEC-2004-030). AML patients were treated according to the HOVON (Dutch-Belgian Hematology-Oncology Cooperative group) protocols HO04, HO04A, HO29, HO42, HO42A and HO43 (*http://www.hovon.nl*).[9-12]

Mutation detection in *FLT3* (internal tandem duplications (ITD) or tyrosine kinase domain mutations (TKD)), *NPM1*, *N-RAS*, *IDH1* and *IDH2*, as well as *EVI1* overexpression were performed as described previously.[13-17]

*ASXL1* mutations in AML were determined by cDNA amplifica-

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tions using Fw-ASXL1-Ex12 5'-CCACCCTGGGTGGTTAAAG-3' and Rev-ASXL1-Ex12 5'-TCGCTGTAGATCTGACGTAC-3'. By this approach 83% of all known exon 12 *ASXL1* mutations are identified (range P575 to R687 of ASXL1).[7]

All PCR reactions were carried out at an annealing temperature of 60°C in the presence of 25mM dNTP, 15 pmol primers, 2mM $MgCl_2$, Taq polymerase and 1 x buffer (Invitrogen Life Technologies, Breda, The Netherlands). Cycling conditions were as follows: 1 cycle 5' at 94°C, 35 cycles 1' at 94°C, 1' at 60°C, 1' at 72°C, and 1 cycle 7' at 72°C. All *ASXL1* RT-PCR products were subjected to denaturing high performance liquid chromatography (dHPLC) analyses using a Transgenomics (Omaha, NE, USA) WAVE system. Samples were run at 64.1°C. PCR products showing aberrant dHPLC profiles were purified using the Multiscreen-PCR 96-well system (Millipore, Bedford, MA, USA) followed by direct sequencing with the appropriate forward and reversed primers using an ABI-PRISM3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). PCR products were sequenced with Fw-ASXL1-Ex12 and Rev-ASXL1-Ex12. We validated this strategy using 272 cases of AML that had been previously analyzed using PCR on genomic DNA followed by direct sequencing.

Information on the *ASXL1* mutation status of all AML cases is available in Table 1 and of all AML cases for whom gene expression profiling has been performed (*www.ncbi.nlm.nih.gov/geo*, accession number GSE6891).

The relation between *ASXL1* mutations and various patients' characteristics were determined by Student's t-test, equal variances not assumed (continuous variables), Wilcoxon's rank order test (continuous variables not normally distributed) and Fisher's exact test (categorical variables).

We distinguished the following cytogenetic risk categories: (I) favorable, t(8;21), inv(16) or t(15;17); (II) unfavorable, inv(3)/t(3;3), t(6;9), 11q23 abnormalities other than t(9;11), -5, 5q-, -7, 7q-t(9;22) or monosomal karyotypes (MK); (III) intermediate risk, cytogenetically normal (CN) and the remaining AML cases (CA rest).

Overall survival (OS) end points were death (failure) and alive at last follow up (censored), as measured from entry onto trial. Relapse-free survival (RFS) end points were relapse, or death from any cause, measured from the date of achievement of CR. Distribution estimations and survival distributions of OS and RFS were calculated by the Kaplan-Meier method and the log rank test.

## Results and Discussion

*ASXL1* mutations were determined in 882 cDNA samples of AMLs by RT-PCR/dHPLC followed by direct sequencing (Table 1). *ASXL1*$^{mut}$ were identified in 46 AML cases (5.3%): in 35 out of 775 *de novo AML* cases (4.5%), in 3 out of 24 post-MDS AML cases (11%), and in 4 out of 37 therapy-related AML cases (10%) (Table 2).

All the mutations found were frame shift mutations that caused a disruption of the PDH domain, and the most prevalent was p.Gly646TrpfsX12 (25 of 46, 54%). Recently, the ASXL1 p.Gly646TrpfsX12 mutation had been suspected to represent a PCR artifact rather than a somatic mutation.[18] In the latter report, buccal samples from AML patients with this mutation all showed the ASXL1 mutation p.Gly646TrpfsX12. Furthermore, this particular mutation has also been reported in approximately 25% of healthy individual samples.[18] For this reason, we set out to confirm the ASXL1 p.Gly646TrpfsX12 mutation in all our 25 samples, *i.e.* in a second independent sample of the same patient. Furthermore, in 2 available remission samples of these AML patients, the p.Gly646TrpfsX12 mutation in *ASXL1* was absent. We also found the mutation in the Kasumi-1 cell line with the

**Table 1. Types of *ASXL1* mutations in 46 cases of acute myeloid leukemia.**

| # | Mutation DNA | Mutation protein |
|---|---|---|
| 2190 | c.1934dupG | p.Gly646TrpfsX12 |
| 2220 | c.1934dupG | p.Gly646TrpfsX12 |
| 2222 | c.1900_1922del | p.Glu635ArgfsX15 |
| 2223 | c.1934dupG | p.Gly646TrpfsX12 |
| 2245 | c.1934dupG | p.Gly646TrpfsX12 |
| 2267 | c.1934dupG | p.Gly646TrpfsX12 |
| 3278 | c.2005_2011dup | p.Ala671GlyfsX2 |
| 3315 | c.1900_1922del | p.Glu635ArgfsX15 |
| 3481 | c.1900_1922del | p.Glu635ArgfsX15 |
| 3485 | c.1998_1999del | p.Asp667TrpfsX3 |
| 3488 | c.1934dupG | p.Gly646TrpfsX12 |
| 5364 | c.1900_1922del | p.Glu635ArgfsX15 |
| 6452 | c.1900_1922del | p.Glu635ArgfsX15 |
| 6453 | c.1934dupG | p.Gly646TrpfsX12 |
| 6457 | c.1900_1922del | p.Glu635ArgfsX15 |
| 6887 | c.1934dupG | p.Gly646TrpfsX12 |
| 7070 | c.1934dupG | p.Gly646TrpfsX12 |
| 7076 | c.1934dupG | p.Gly646TrpfsX12 |
| 7147 | c.1934dupG | p.Gly646TrpfsX12 |
| 7167 | c.1934dupG | p.Gly646TrpfsX12 |
| 7177 | c.1934dupG | p.Gly646TrpfsX12 |
| 7183 | c.1773C>G | p.Tyr591X |
| 7326 | c.1900_1922del | p.Glu635ArgfsX15 |
| 7409 | c.2030_2046del | p.Pro677HisfsX35 |
| 7420 | c.1934dupG | p.Gly646TrpfsX12 |
| 9629 | c.1934dupG | p.Gly646TrpfsX12 |
| 9643 | c.1934dupG | p.Gly646TrpfsX12 |
| 11674 | c.1772dupA | p.Tyr591X |
| 13174 | c.1774C>T | p.Gln592X |
| 13759 | c.1934dupG | p.Gly646TrpfsX12 |
| 14330 | c.1933insC | p.Gly645ArgfsX12 |
| 14369 | c.1934dupG | p.Gly646TrpfsX12 |
| 16047 | c.1934dupG | p.Gly646TrpfsX12 |
| 16052 | c.1934dupG | p.Gly646TrpfsX12 |
| 16077 | c.1900_1922del | p.Glu635ArgfsX15 |
| 16105 | c.1934dupG | p.Gly646TrpfsX12 |
| 16115 | c.1900_1922del | p.Glu635ArgfsX15 |
| 16143 | c.1934dupG | p.Gly646TrpfsX12 |
| 16157 | c.1934dupG | p.Gly646TrpfsX12 |
| 16161 | c.1934dupG | p.Gly646TrpfsX12 |
| 16168 | c.1957_1964del | p.Gly652ArgX2 |
| 16180 | c.1934dupG | p.Gly646TrpfsX12 |
| 16197 | c.1934dupG | p.Gly646TrpfsX12 |
| 16206 | c.1934dupG | p.Gly646TrpfsX12 |
| 16238 | c.1945G>T | p.Gly649X |
| 16254 | c.1927insA | p.Gly643ArgfsX15 |

translocation t(8;21). In addition, we screened 91 samples from healthy individuals, and none of them revealed a mutation in exon 12 of *ASXL1*. Based on these results, we regard the p.Gly646TrpfsX12 mutation as a real somatic mutation.

*ASXL1*$^{mut}$ AML were more frequent at older age ($P$=0.004) and present with lower WBC count than AML with *ASXL1* wild type (ASXL1$^{wt}$) ($P$=0.014) (Table 2). *ASXL1*$^{mut}$ AML was associated with FAB M0 subtype ($P$=0.027), but inversely related to FAB M4 subtype ($P$=0.023), *NPM1* mutations ($P$<0.0001; *NPM1*$^{mut}$) and *FLT3*$^{ITD}$ ($P$=0.014) (Table 2).

**Table 2. Distribution of *ASXL1* mutations in 882 cases of acute myeloid leukemia..**

| | ASXL1$^{mutant}$ | *ASXL1*$^{wt}$ | *P* |
|---|---|---|---|
| Total, n. (%) | 46 (5) | 836 (95) | |
| Female, n. (%) | 19 (4) | 417 (96) | |
| Male, n. (%) | 27 (6) | 419 (94) | |
| Median age at diagnosis, years (range) | 54 (15-74) | 47 (15-77) | 0.002 |
| Median WBC at diagnosis, $\times10^9$/L (range) | 13 (1.1-220) | 23.4 (0.3-510) | 0.007 |
| Median platelets at diagnosis, $\times10^9$/L (range) | 66 (13-400) | 61 (3-998) | |
| Mean bone marrow blasts at diagnosis, percentage (range) | 51 (13-93) | 62 (0-98) | |
| AML *de novo* (%) | 36 (5) | 759 (95) | |
| AML post MDS (%) | 3 (11) | 24 (89) | |
| AML therapy-related (%) | 4 (10) | 37 (90) | |
| MDS (%) | 3 (16) | 16 (84) | |
| **FAB, n. (%)** | | | |
| M0 | 6 (13) | 39 (87) | 0.027 |
| M1 | 10 (6) | 158 (94) | |
| M2 | 12 (5) | 222 (95) | |
| M3 | 1 (5) | 20 (95) | |
| M4 | 2 (1) | 141 (99) | 0.023 |
| M5 | 5 (3) | 166 (97) | |
| M6 | 1 (5) | 17 (95) | |
| M7 | — | 1 | |
| RAEB | 3 (16) | 16 (84) | |
| Unknown | 6 (10) | 56 (90) | |
| **Cytogenetics, n. (%)[a]** | | | |
| t(8;21) | 4 (8) | 48 (92) | |
| inv(16) | — | 52 | |
| t(15;17) | 1 (5) | 20 (95) | |
| CA unfavorable | 5 (4) | 113 (96) | |
| MK | 4 (5) | 78 (95) | |
| CN | 21 (5) | 369 (95) | |
| CA rest | 10 (7) | 136 (93) | |
| Unknown | 1 (4) | 25 (96) | |
| **Mutations, n. (%)** | | | |
| *FLT3* ITD | 4 (2) | 200 (98) | 0.014 |
| *FLT3* TKD | 2 (4) | 73 (96) | |
| *NPM1* | — | 259 | <0.0001 |
| N-RAS | 7 (7) | 86 (93) | |
| *IDH1* | 4 (7) | 51 (93) | |
| *IDH2* | 5 (5) | 91 (95) | |
| EVI1 | 6 (6) | 101 (94) | |

*WBC: White blood cell count at diagnosis; FAB: French American British classification; [a]Karyotypes were centrally reviewed. CA unfavorable: inv(3)/t(3;3), t(6;9), 11q23 abnormalities except t(9;11), -5, 5q-, -7, 7q- or t(9;22); MK: monosomal karyotypes; CN: normal cytogenetics or -X or –Y as single abnormalities only; CA rest: any other abnormal cytogenetics not included in any of the other categories.*

To investigate the prognostic value of *ASXL1*$^{mut}$, 807 patients with non-APL AML and those under 65 years of age were considered for survival analysis. The median follow up of these patients was 46.0 months (range 1.9-224 months). In this cohort, univariate survival analyses with several known recurrent aberrations in AML demonstrated that *FLT3*$^{ITD}$ ($P$<0.0001, unfavorable), *NPM1*$^{mut}$ ($P$=0.025, favorable), *IDH2*$^{mut}$ ($P$=0.02, favorable) and *EVI1* overexpression ($P$<0.001, unfavorable) were significantly associated with OS, whereas *FLT3*$^{TKD}$ ($P$=0.214), *NRAS*$^{mut}$ ($P$=0.53), and *IDH1*$^{mut}$ ($P$=0.81) were not.

The complete response (CR) rate was significantly reduced in patients with *ASXL1*$^{mut}$ (61% *vs.* 79.6%; $P$=0.004). The median OS of patients with AML with or without *ASXL1*$^{mut}$ was 15.9 months *vs.* 22.3 months ($P$=0.019; Figure 1A). The relapse free survival was similar between the two groups once they achieved a CR ($P$=0.27; Figure 1B).

In multivariate analyses, trial-stratified and considering other prognostic variables that were significantly associated with OS in univariate analysis, such as age, WBC, cytogenetics, *NPM1*$^{mut}$, *FLT3*$^{ITD}$, *IDH2*$^{mut}$ and *EVI1* overexpression, the presence of *ASXL1*$^{mut}$ was an independent prognostic factor for poor OS (HR 1.64, 95% CI= 1.12 - 2.4, $P$=0.010). Recently, Chou *et al.*[7] were unable to demonstrate that *ASXL1* mutations were an independent factor for prognosis in AML. Apart from population differences, this is likely due to the lower number of patient samples, both *ASXL1* wild-type and mutant, in the survival analyses and the inclusion of other covariables in the multivariate analyses, such as *RUNX1* mutations, which in fact appeared to be associated with *ASXL1* mutations.[7] When comparing both studies, it should be noted that the hazard ratios are similar, but that the confidence interval in the study by Chou *et al.*[7] is greater as a result of decreased power. It is worthy of note that Chou and co-workers indicate that although *ASXL1* mutations in their analysis did not represent a significant independent factor for prognosis, it is highly associated with a worse survival and lower CR rate.

The subgroup of AML patients with intermediate-risk cytogenetics showed a trend for worse OS for those with *ASXL1*$^{mut}$ AML (median OS: 15.9±7.4 *vs.* 24.7±7.9 months; $P$=0.06; Figure 1C), whereas there is no association between OS and the *ASXL1*$^{mut}$ genotype in normal karyotype AML ($P$=0.36). The trend for inferior OS in intermediate-risk cytogenetics, is significantly worse when AML patients with *FLT3* wild-type (*FLT3*$^{wt}$) and *NPM1* wild-type (*NPM1*$^{wt}$) genotypes are considered (median OS: 14.9±7.2 *vs.* 32.3±17.7 months; $P$=0.037; Figure 1D). In the other *FLT3/NPM1* composite genotypes, the frequencies of *ASXL1*$^{mut}$ AML are too low to allow a meaningful analysis of the prognostic value of *ASXL1*$^{mut}$.

The current study demonstrates that *ASXL1* gene mutations are common in AML. These mutations are inversely associated with *FLT3*$^{ITD}$, and mutually exclusive with *NPM1*$^{mut}$. The presence of *ASXL1*$^{mut}$ in AML is an independent poor risk factor for OS mostly due to a higher initial resistance to chemotherapy. The adverse prognostic impact of *ASXL1*$^{mut}$ was confirmed in the subset of patients harboring intermediate-risk cytogenetics and lacking *NPM1* and *FLT3* mutations, and might contribute to refine prognosis in this subgroup of patients with uncertain outcome.

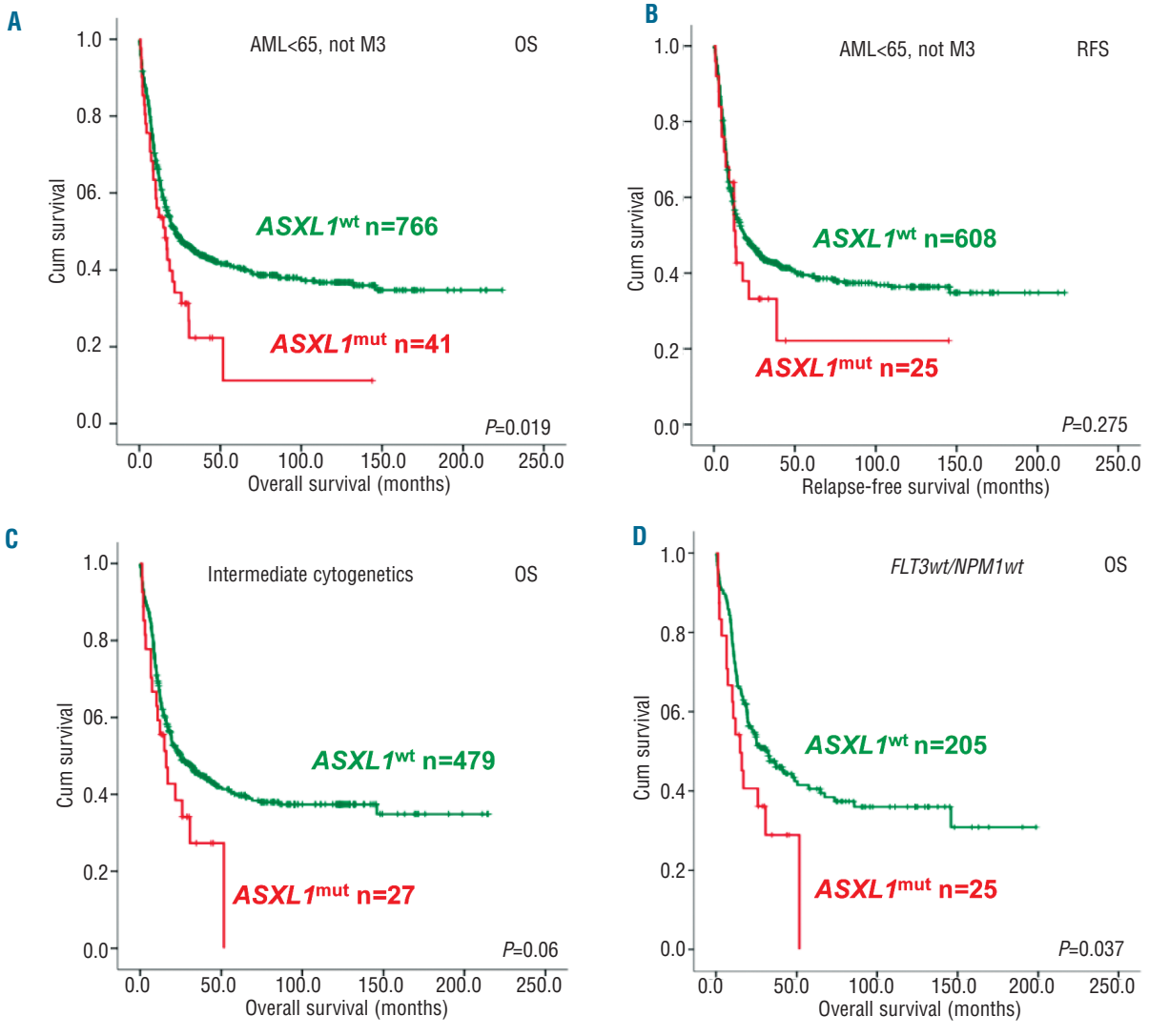


**Figure 1.** Kaplan-Meier survival analyses of patients with AML with or without *ASXL1* mutations. (A) OS for all AML patients (<65 years). (B) RFS for all AML patients (<65 years). (C) OS for patients with intermediate-risk AML. (D) OS for patients with intermediate-risk and *FLT3*$^{wt}$ and *NPM1*$^{wt}$. Survival curves in red refer to cases with *ASXL1*$^{mut}$; those in green to *ASXL1*$^{wt}$. The log rank *P* value is indicated per Kaplan-Meier analysis.

## Authorship and Disclosures

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