

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 malignancies: 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 MgCl₂, 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).

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.

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

	<i>ASXL1</i> ^{mutant}	<i>ASXL1</i> ^{wt}	<i>P</i>
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, ×10 ⁹ /L (range)	13 (1.1-220)	23.4 (0.3-510)	0.007
Median platelets at diagnosis, ×10 ⁹ /L (range)	66 (13-400)	61 (3-998)	
Mean bone marrow blasts at diagnosis, percentage (range)	51 (13-93)	62 (0-98)	
AML <i>de novo</i> (%)	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. (%)			
<i>FLT3</i> ITD	4 (2)	200 (98)	0.014
<i>FLT3</i> TKD	2 (4)	73 (96)	
<i>NPM1</i>	—	259	<0.0001
N-RAS	7 (7)	86 (93)	
<i>IDH1</i>	4 (7)	51 (93)	
<i>IDH2</i>	5 (5)	91 (95)	
<i>EVI1</i>	6 (6)	101 (94)	

WBC: White blood cell count at diagnosis; FAB: French American British classification; ^aKaryotypes 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.

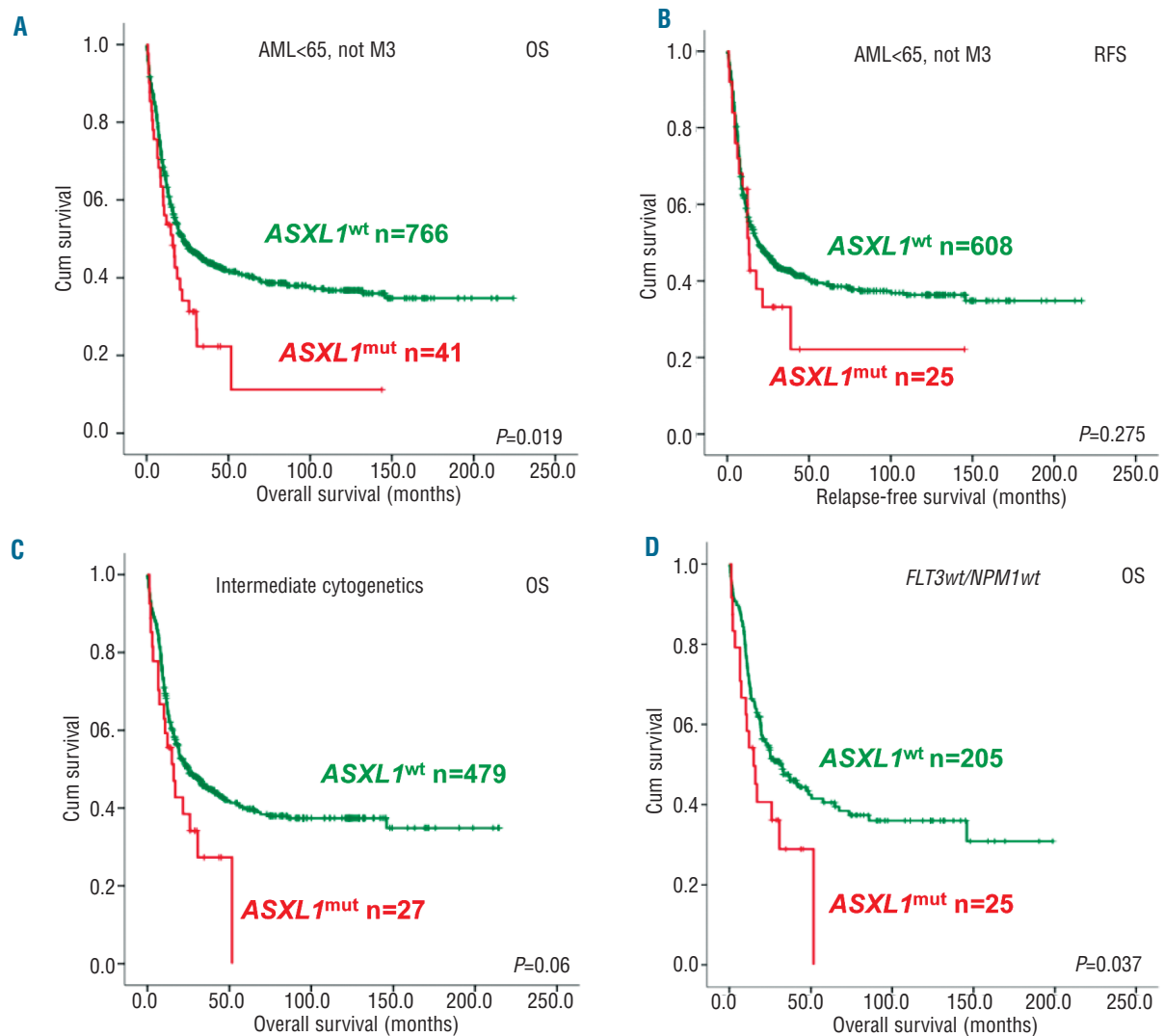


Figure 1. Kaplan-Meier survival analyses of patients with AML with or without *ASXL1* mutations. (A) OS for all AML patients (<65 years). (B) RFS for all AML patients (<65 years). (C) OS for patients with intermediate-risk AML. (D) OS for patients with intermediate-risk and *FLT3*^{wt} and *NPM1*^{wt}. 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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References

- Fisher CL, Randazzo F, Humphries RK, Brock HW. Characterization of *Asxl1*, a murine homolog of Additional sex combs, and analysis of the *Asx*-like gene family. *Gene*. 2006;369:109-18.
- Fisher CL, Pineault N, Brookes C, Helgason CD, Ohta H, Bodner C, et al. Loss-of-function Additional sex combs like 1 mutations disrupt hematopoiesis but do not cause severe myelodysplasia or leukemia. *Blood*. 2010;115(1):38-46.
- Fisher CL, Berger J, Randazzo F, Brock HW. A human homolog of Additional sex combs, ADDITIONAL SEX COMBS-LIKE 1, maps to chromosome 20q11. *Gene*. 2003;306:115-26.
- Gelsi-Boyer V, Trouplin V, Adelaide J, Bonansea J, Cervera N, Carubbia N, et al. Mutations of polycomb-associated gene *ASXL1* in myelodysplastic syndromes and chronic myelomonocytic leukaemia. *Br J Haematol*. 2009;145(6):788-800.
- Carubbia N, Murati A, Trouplin V, Brecqueville M, Adelaide J, Rey J, et al. Mutations of *ASXL1* gene in myeloproliferative neoplasms. *Leukemia*. 2009;23(11):2183-6.
- Gelsi-Boyer V, Trouplin V, Roquain J, Adelaide J, Carubbia N, Esterni B, et al. *ASXL1* mutation is associated with poor prognosis and acute transformation in chronic myelomonocytic leukaemia. *Br J Haematol*. 2010;151(4):365-75.
- Chou WC, Huang HH, Hou HA, Chen CY, Tang JL, Yao M, et al. Distinct clinical and biological features of de novo acute myeloid leukemia with additional sex comb-like 1 (*ASXL1*) mutations. *Blood*. 2010;116(20):4086-94.
- Boultonwood J, Perry J, Pellagatti A, Fernandez-Mercado M, Fernandez-Santamaria C, Calasanz MJ, et al. Frequent

- mutation of the polycomb-associated gene ASXL1 in the myelodysplastic syndromes and in acute myeloid leukemia. *Leukemia*. 2010;24(5):1062-5.
9. Lowenberg B, Boogaerts MA, Daenen SM, Verhoef GE, Hagenbeek A, Vellenga E, et al. Value of different modalities of granulocyte-macrophage colony-stimulating factor applied during or after induction therapy of acute myeloid leukemia. *J Clin Oncol*. 1997;15(12):3496-506.
 10. Lowenberg B, van Putten W, Theobald M, Gmur J, Verdonck L, Sonneveld P, et al. Effect of priming with granulocyte colony-stimulating factor on the outcome of chemotherapy for acute myeloid leukemia. *N Engl J Med*. 2003;349(8):743-52.
 11. Ossenkoppele GJ, Graveland WJ, Sonneveld P, Daenen SM, Biesma DH, Verdonck LF, et al. The value of fludarabine in addition to ARA-C and G-CSF in the treatment of patients with high-risk myelodysplastic syndromes and AML in elderly patients. *Blood*. 2004;103(8):2908-13.
 12. Lowenberg B, Pabst T, Vellenga E, van Putten W, Schouten HC, Graux C, et al. Cytarabine dose for acute myeloid leukemia. *N Engl J Med*. 2011;364(11):1027-36.
 13. Abbas S, Lugthart S, Kavelaars FG, Schelen A, Koenders JE, Zeilemaker A, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood*. 2010;116(12):2122-6.
 14. Care RS, Valk PJ, Goodeve AC, Abu-Duhier FM, Geertsma-Kleinekoort WM, Wilson GA, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol*. 2003;121(5):775-7.
 15. Valk PJ, Bowen DT, Frew ME, Goodeve AC, Lowenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). *Haematologica*. 2004;89(1):106.
 16. Verhaak RG, Goudswaard CS, van Putten W, Bijl MA, Sanders MA, Hagens W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood*. 2005;106(12):3747-54.
 17. Gröschel S, Lugthart S, Schlenk RF, Valk PJ, Eiwien K, Goudswaard C, et al. High EVI1 expression predicts outcome in younger adult patients with acute myeloid leukemia and is associated with distinct cytogenetic abnormalities. *J Clin Oncol*. 2010;28(12):2101-7.
 18. Abdel-Wahab O, Kilpivaara O, Patel J, Busque L, Levine RL. The most commonly reported variant in ASXL1 (c.1934dupG;p.Gly646TrpfsX12) is not a somatic alteration. *Leukemia*. 2010;24(9):1656-7.