



Late-onset *NPM1* mutation in a *MYC*-amplified relapsed/refractory acute myeloid leukemia patient treated with gemtuzumab ozogamicin and glasdegib

by Sonia Jaramillo, Michael Scherer, Chelsea Szu-Tu, Sergi Beneyto-Calabuig, Carsten Mueller-Tidow, Richard F. Schlenk, Michael Hundemer, Lars Velten, and Caroline Pabst

Received: January 10, 2024.

Accepted: May 20, 2024.

Citation: Sonia Jaramillo, Michael Scherer, Chelsea Szu-Tu, Sergi Beneyto-Calabuig, Carsten Mueller-Tidow, Richard F. Schlenk, Michael Hundemer, Lars Velten, and Caroline Pabst. Late-onset *NPM1* mutation in a *MYC*-amplified relapsed/refractory acute myeloid leukemia patient treated with gemtuzumab ozogamicin and glasdegib. *Haematologica*. 2024 May 30. doi: 10.3324/haematol.2023.284922 [Epub ahead of print]

Publisher's Disclaimer.

E-publishing ahead of print is increasingly important for the rapid dissemination of science. Haematologica is, therefore, E-publishing PDF files of an early version of manuscripts that have completed a regular peer review and have been accepted for publication.

E-publishing of this PDF file has been approved by the authors.

After having E-published Ahead of Print, manuscripts will then undergo technical and English editing, typesetting, proof correction and be presented for the authors' final approval; the final version of the manuscript will then appear in a regular issue of the journal.

All legal disclaimers that apply to the journal also pertain to this production process.

Late-onset *NPM1* mutation in a MYC-amplified relapsed / refractory acute myeloid leukemia patient treated with gemtuzumab ozogamicin and glasdegib

Sonia Jaramillo¹, Michael Scherer², Chelsea Szu-Tu², Sergi Beneyto-Calabuig², Carsten Müller-Tidow^{1,3}, Richard F. Schlenk^{1,4}, Michael Hundemer¹, Lars Velten^{2,5}, Caroline Pabst^{1,3}

Affiliations

¹Department of Internal Medicine V, Heidelberg University Hospital, Germany.

²Centre for Genomic Regulation (CRG), The Barcelona Institute of Science and Technology, Barcelona, Spain.

³Molecular Medicine Partnership Unit (MMPU), University of Heidelberg and European Molecular Biology Laboratory, Heidelberg, Germany.

⁴NCT-Trial Center, National Center of Tumor Diseases, Heidelberg University Hospital and German Cancer Research Center, Germany.

⁵Universitat Pompeu Fabra (UPF), Barcelona, Spain.

CORRESPONDING AUTHOR

Dr. med. Caroline Pabst

Caroline.Pabst@med.uni-heidelberg.de

Tel. +49 6221 56-34121 | Fax. +49 6221 56-5813

DATA SHARING STATEMENT:

The authors welcome requests for the original data related to the laboratory analysis results.

FUNDING

This research was funded by the José Carreras Leukaemia Foundation (DJCLS 01SP/ (12)2022). C.P. is supported by a Max-Eder grant of German Cancer Aid (70114435). The authors gratefully

acknowledge the data storage service SDS@hd supported by the Ministry of Science, Research and the Arts Baden-Württemberg (MWK) and the German Research Foundation (DFG) through grant INST 35/1314-1 FUGG and INST 35/1503-1 FUGG. M.S. was supported through the Walter Benjamin Fellowship funded by Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) – 493935791.

AUTHORS' CONTRIBUTIONS

CP and SJ designed the study, performed clinical data analysis and wrote the manuscript. MS, LV, CST, and SBC performed single cell sequencing and data analysis. MH performed the clinical diagnostics. SJ, RS, and CMT designed the clinical study. All authors contributed to writing the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors report no conflicts of interest.

Case Report

In March 2020, a 72-year-old male patient with no relevant medical history presented with four weeks of asthenia and adynamia, 10 kg weight loss and petechias on both hands. Examination of the bone marrow (BM) revealed 80% infiltration by immature blastoid cells with the following immune phenotype: low-intermediate CD45 expression, CD34+/-, CD117+, HLA-DR+, CD38+, CD13+/-, CD33+/-, myeloperoxidase (MPO)-/+, CD15+/-, nuTdt+/-, CD123+/-, CD7+. In the Next-Generation-Sequencing (NGS) AML panel only one mutation in TET2 p.Y1245Lfs*22 was reported. Fluorescence in situ hybridization (FISH) identified a *MYC* amplification, characterized by double minutes (dmin) involving chromosome band 8q24 in 71% of uncultured BM cells. The disease was classified as acute myelogenous leukemia not otherwise specified (AML-NOS) according to ELN 2017 criteria (1). Intensive treatment within the “GnG” trial was initiated after obtaining the patient’s written informed consent comprising gemtuzumab-ozogamicin and “7+3” during induction and the hedgehog inhibitor glasdegib during consolidation and maintenance therapy (2). The course of events is described in detail in **Figure 1**. Glasdegib maintenance therapy was prematurely discontinued after 3 cycles due to persistent dysgeusia. For this specific type of AML, no targeted maintenance therapy had been approved in the EU at that time. However, cytologic relapse occurred three months after the suspension of glasdegib with 60% BM blasts (**Figure 1**). NGS revealed several new molecular mutations including a typical *NPM1* 4bp insertion (**Table 1**). FISH analysis showed the initial *MYC* amplification in 1% of uncultured BM cells, however a new trisomy 8 involving the *MYC* locus (8q24) occurred. We initiated a salvage therapy with azacitidine and venetoclax (aza-ven) (3) and prepared the patient for allogeneic stem cell transplantation. However, BM aspiration after the second cycle revealed a refractory disease with 60% blasts and persistent *NPM1* mutational load. Another salvage therapy with high-dose cytarabine and mitoxantrone (HAM) was initiated, but the disease was refractory with novel extramedullary skin lesions. Considering the poor prognosis of this chemo-refractory disease, as well as the patient’s age and wish a best supportive care therapy was initiated. The patient died two months after the appearance of the skin lesions.

To gain a deeper understanding of the clonal composition and evolution of this unusual course of the disease, we performed single-cell DNA sequencing (scSeq) on four time points indicated in **Figure 1** and **Table 1** using the Mission Bio Tapestry Single-Cell DNA and protein sequencing platform investigating genes covered in Mission Bio’s myeloid panel (4) with v2 of the protocol. Additionally, we used antibodies against four surface proteins to simultaneously generate a single-cell protein library. Libraries were sequenced to a DNA/protein read depth of 324/31 M (ID), 267/31 M (CRi-1), 292/37 M (CRi-2) and 225/37 M (Rel-1) on an Illumina NextSeq2000 sequencer (P3, 2x150bp). Sequencing details are available upon request.

Using scSeq, we were able to confirm the *MYC* amplification at initial diagnosis in a large fraction of CD34+ blasts. To discern *MYC*-amplified from wildtype cells, we used *CloneTracer* to assign clonal probabilities to each cell (5). Those cells being assigned to the *MYC* clone were termed *MYC*-amplified (*MYCamp*) (**Figure 2a**). Notably, healthy T-cells were reliably identified as non-*MYCamp* as expected (**Figure 2a**). We identified an *EZH2* missense mutation p.T683A in CD34+ cells with aberrant CD7 expression, which seemed to be present in the *MYCamp* and also non-*MYCamp* CD34+CD7+ cells, but not in healthy CD3+CD34- T cells. This suggested that the *EZH2* mutation occurred in an ancestral committed progenitor cell giving rise to the *MYCamp* leukemic clone (**Figure 2b, c**). Moreover, an *ASXL1* p.E635Rfs frameshift mutation was detected in around 1% of the cells not harboring the *MYC* amplification (**Figure 2b, c**).

In the remission sample post induction (CRi-1), which was considered MRD negative by flow cytometry, the *EZH2* mutation was present in the majority of CD3 negative BM cells, but the *MYC* amplification was detectable in a minority of cells (**Figure 2d**). However, we identified a *JAK2* V617F mutation typically found in myeloproliferative neoplasms (MPN) and AML (6), in 9% of the cells, and the *ASXL1* mutation had increased in frequency. Notably, we observed a high allelic dropout rate of *EZH2*, suggesting that many cells called as *EZH2* wild type were actually mutant, and that the overall fraction of *EZH2* mutant myeloid cells was very high. Considering also that the false positive rate of the applied technology is very low (7) (8), the abundant co-occurrence of the *ASXL1* and *EZH2* mutations in the first remission time point (**Figure 2d**) strongly suggested that *ASXL1* occurred downstream of the *EZH2* mutation.

In the second remission sample post consolidation 2 (CRi-2) the *MYC* amplification was detectable at a very low rate by scSeq, while FISH was still negative. The *JAK2* clone increased in abundance (25%), continued to co-occur with the *EZH2* p.T683A variant, but did not overlap with *MYCamp* cells (**Table 1**). In addition, a new *PHF6* mutation (p.Y103stop) was detected in 10% of cells. These cells shared the *EZH2* p.T683A, but they were devoid of the *JAK2* mutation and *MYC* amplification. Of note, blasts were below 5% at this stage and flow cytometry did not detect the leukemia-associated immunophenotype from initial diagnosis, so that the patient was considered flow MRD negative.

In the cytologic relapse sample, which was acquired 6 months later, *MYCamp* was detectable again by scSeq (12%, **Figure 2e, 2f**). The *JAK2* clone had decreased from 25% to 7%, while the *PHF6*-mutated cells represented the second largest clone at relapse downstream of the *EZH2* mutation (22%). An *NPM1* TCTG-insertion was found in 15% of cells. Interestingly, the *NPM1*-mutated cells harbored the *ASXL1* mutation, which was already present at diagnosis independently of the other three clones (**Figure 2e, 2f**).

Several observations are remarkable in this scSeq study: an *EZH2* missense mutation p.T683A was detectable already at initial diagnosis and was shared by the initial *MYC*-amplified clone, but also by two distinct *JAK2*- and *PHF6*-mutated clones emerging during cytologic remission. Based on the in-depth scSeq information this AML would have been classified as high-risk AML with myelodysplasia-related gene mutations (AML-MR) according to the new World Health Organization (WHO) and European Leukemia Net (ELN) 2022 classification, so that a therapy with CPX-351 would have been recommended (1).

While the *JAK2*-mutated clone decreased from remission to relapse, the *PHF6*-mutated clone became the second largest subclone downstream of the *EZH2* mutation at relapse potentially pointing towards clonal selection during the maintenance therapy with glasdegib. *PHF6* mutations in AML are often frameshift or nonsense mutations (9). As *PHF6* is an X-linked gene, these alterations are predominantly found in males like in our patient. *PHF6* mutations are associated with immature AML (FAB subtypes M0–M2) (9). Moreover, *PHF6* along with other epigenetic regulators such as *DNMT3A*, *TET2*, and *ASXL1*, is frequently altered in patients with clonal hematopoiesis (9).

Genomic amplifications, such as double minutes (dmin), homogeneously staining regions (hsr), are rare in leukemia, accounting for less than 1% of cytogenetically abnormal hematological malignancies and are associated with elderly patients and poor prognosis (10). While we found *MYC*-amplification in form of dmin at initial diagnosis by FISH, the relapse sample revealed a novel trisomy 8 involving the *MYC* locus confirming the hypothesis that different *MYC*-amplification mechanisms can coexist within the same leukemic cell population as previously described (11). scSeq was able to detect *MYC* amplification in both conditions, and the fraction of cells highly corresponded to the fractions detected by FISH or cytogenetics. *MYC* mutations have been reported to coincide with other mutations in AML, such as *FLT3*, *NPM1*, and *DNMT3A* mutations (12). The interesting observation in this case is, that the additional clones developing during therapy did not occur in the *MYC*amp clone as expected. This might suggest, that the *MYC*amp clone facilitated development of the other AML clones by paracrine mechanisms, e.g. via BM niche remodeling.

Another striking observation in this case is the occurrence of a therapy-associated *NPM1* mutation. Approximately 15% of therapy-related AML cases carry *NPM1* mutations, frequently accompanied by a normal karyotype and *DNMT3A* mutations, and rarely associated with chromosome aberrations (13). Cases of *NPM1*-mutated AML following chemotherapy for previous lymphoid malignancies appear to arise from a background of *DNMT3A*- or *TET2*-driven clonal hematopoiesis (CH) rather than being a direct result of cytotoxic therapy (14). It is therefore remarkable, that the *NPM1* mutation in this case occurred in cells harboring mutations in two other epigenetic

regulators, ASXL1 and EZH2, suggesting a common mechanism. The *ASXL1*-mutated background and the co-existence of *NPM1* wild type clones might explain the non-response to aza-ven in this case.

In summary we present a very unusual AML case with emergence of multiple parallel genetic clones during remission after treatment with intensive chemotherapy and targeted drugs including the hedgehog inhibitor glasdegib and the CD33 directed antibody "GO". The complex clonal evolution observed in this case resulted in a very aggressive and multi-refractory disease, which raises the question, whether and how the agents applied might have contributed to clonal selection or exit from dormancy. These findings underscore the importance of employing scSeq to dissect individual cases, which can offer valuable mechanistic insights and help optimize therapeutic strategies.

REFERENCES

1. Dohner H, Wei AH, Appelbaum FR, et al. Diagnosis and Management of AML in Adults: 2022 ELN Recommendations from an International Expert Panel. *Blood*. 2022;140(12):1345-1377.
2. Jaramillo S, Krisam J, Le Cornet L, et al. Rationale and design of the 2 by 2 factorial design GnG-trial: a randomized phase-III study to compare two schedules of gemtuzumab ozogamicin as adjunct to intensive induction therapy and to compare double-blinded intensive postremission therapy with or without glasdegib in older patients with newly diagnosed AML. *Trials*. 2021;22(1):765.
3. DiNardo CD, Pratz K, Pullarkat V, et al. Venetoclax combined with decitabine or azacitidine in treatment-naive, elderly patients with acute myeloid leukemia. *Blood*. 2019;133(1):7-17.
4. Pellegrino M, Sciambi A, Treusch S, et al. High-throughput single-cell DNA sequencing of acute myeloid leukemia tumors with droplet microfluidics. *Genome Res*. 2018;28(9):1345-1352.
5. Beneyto-Calabuig S, Merbach AK, Kniffka JA, et al. Clonally resolved single-cell multi-omics identifies routes of cellular differentiation in acute myeloid leukemia. *Cell Stem Cell*. 2023;30(5):706-721.
6. Percy MJ, McMullin MF. The V617F JAK2 mutation and the myeloproliferative disorders. *Hematol Oncol*. 2005;23(3-4):91-93.
7. Bianchi A, Scherer M, Zaurin R, Quililan K, Velten L, Beekman R. scTAM-seq enables targeted high-confidence analysis of DNA methylation in single cells. *Genome Biol*. 2022;23(1):229.
8. Miles LA, Bowman RL, Merlinsky TR, et al. Single-cell mutation analysis of clonal evolution in myeloid malignancies. *Nature*. 2020;587(7834):477-482.
9. Van Vlierberghe P, Patel J, Abdel-Wahab O, et al. PHF6 mutations in adult acute myeloid leukemia. *Leukemia*. 2011;25(1):130-134.
10. Huh YO, Tang G, Talwalkar SS, et al. Double minute chromosomes in acute myeloid leukemia, myelodysplastic syndromes, and chronic myelomonocytic leukemia are associated with micronuclei, MYC or MLL amplification, and complex karyotype. *Cancer Genet*. 2016;209(7-8):313-320.
11. L Abbate A, Tolomeo D, Cifola I, et al. MYC-containing amplicons in acute myeloid leukemia: genomic structures, evolution, and transcriptional consequences. *Leukemia*. 2018 Oct;32(10):2152-2166.
12. Papaemmanuil E, Gerstung M, Bullinger L, et al. Genomic Classification and Prognosis in Acute Myeloid Leukemia. *N Engl J Med*. 2016;374(23):2209-2221.
13. SanMiguel JM, Eudy E, Loberg MA, et al. Cell origin-dependent cooperativity of mutant Dnmt3a and Npm1 in clonal hematopoiesis and myeloid malignancy. *Blood Adv*. 2022;6(12):3666-3677.
14. Othman J, Meggendorfer M, Tiacci E, et al. Overlapping features of therapy-related and de novo NPM1-mutated AML. *Blood*. 2023;141(15):1846-1857.

Table 1. Clonal composition and evolution of the disease

	clinical time point	karyotype	blast count bone marrow (%)	MYCamp (FISH)	MYCamp scSeq	ASXL1 p.Glu635Rfs	ASXL1 p.Glu635fs COSM36165	EZH2 p.Thr683Ala	EZH2 p.Tyr646Cys	JAK2 p.Val617Phe COSM12600	NPM1 p.Trp288fs COSM17559	PHF6 p.Tyr103stop	TET2 p.Try1245fs COSM87129
clinical diagnostics	diagnosis (ID)	46,XY,del(16)(p13.1),1~19dmin[13]/46,XY,del(16)(p13.1)[3]/46,XY,2~15dmin[3]/46,XY[1]	80%	71%				48% *					49%
	relapse (Rel-1)	48,XY,+8,+19,2~4dmin[11]/49,sl,+8[2]/46,XY,inv(16)(p11.2q22)[4]/46,XY[3]	60%	2%		3%		34%		9%	11%		48%
	progressive disease		65%	18% +8				20%	27%	10%	31%		49%
scSeq	diagnosis (ID)		67% PB		72%		1%	80%					
	CRi-1		4%		0%		42%	47%		8%			
	CRi-2		<5%		1%		6%	64%		23%		10%	
	relapse (Rel-1)		60%		12%		18%	51%		6%	15%	19%	

*not reported initially by clinic, probably because no COSMIC ID and near 50% so considered SNP, PB: peripheral blood

Figure Legends

Figure 1. Timeline of therapy, clinical response, and sampling time points.

The patient was randomized to receive gemtuzumab ozogamicin (GO) in addition to “7+3” during induction therapy as follows: cytarabine 200 mg/m² administered via continuous intravenous (IV) infusion for a total of 7 days and daunorubicin 60 mg/m² days 1, 2, and 3; in addition GO 3 mg/m² IV over 1 h, on days 1, 4, and 7. During the consolidation phase, the patient received two cycles of cytarabine (1.0 g/m²) administered by IV infusion every 12 h on days 1, 2, and 3. Glasdegib 100 mg was administered from day 1 to 28 of each consolidation cycle. Maintenance therapy with glasdegib 100mg was started after the end of the 2nd consolidation therapy cycle for a total of 3 additional cycles of 28 days each (2). The patient achieved complete hematological remission with incomplete hematological recovery (CRi) after induction. Minimal residual disease (MRD) assessed by flow cytometry remained positive. After the first consolidation therapy, CR with MRD negativity was achieved. At this point, an allogeneic transplantation was disclaimed by the patient. Thus, a second cycle of consolidation therapy was administered. Maintenance therapy with glasdegib was initiated five months after induction therapy. Glasdegib was prematurely discontinued after 3 cycles of maintenance therapy due to the patient’s wish because of adverse effects (dysgeusia and muscle cramps grade II). Cytologic relapse occurred three months after the suspension of glasdegib. BM examination revealed 60% blasts. Flow cytometry analysis showed a leukemia population with the following characteristics: CD34-, CD117-, HLA-DR+, CD38+, CD33+, CD13-/+, CD64+, NG2+, CD15+ and a second population displaying the initial immunophenotype. NGS revealed molecular aberrations not detected at initial diagnosis. The *NPM1*-mut/*ABL* ratio quantified by real-time PCR was 520%. Salvage therapy with azacitidine and venetoclax (aza-ven) was initiated. After one cycle, blast percentage dropped to 9%. A second cycle of aza-ven was administered and the patient was prepared for allogeneic transplantation. BM aspiration after the second cycle of aza-ven revealed a refractory disease with 60% blasts and an *NPM1*-mut/*ABL* ratio of 416%. Another salvage therapy with high-dose cytarabine and mitoxantrone (HAM) was initiated. Day +15 BM aspiration revealed only 1% blasts but MRD positivity for *NPM1*. The patient did not achieve hematological recovery, and on day 35, the patient presented with 23% BM blasts. In addition, he developed progressive skin papules suspicious of chloroma covering the entire body, and skin biopsy confirmed AML cell infiltration. Best supportive care therapy was initiated. The patient died two months later. CRi: Complete remission with incomplete hematological recovery, ID: Initial diagnosis; CRi-1: Complete remission with incomplete hematological recovery one, CRi-2: Complete remission with incomplete hematological recovery two, Rel-1: Relapse one, PD: progressive disease, RD: refractory disease.

Figure 2. Single-cell DNA-Seq analysis.

a) Dot plots showing total counts on the x-axis and *MYC* counts on the y-axis, as well as highlighted in color the cell type (*left panels*: red: T cells, gray: other cells) or mutational status (*right panels*: blue: wild type, yellow: *MYC*-amplified) at the indicated time points initial diagnosis (top) and relapse (bottom). b) Dot plots

showing total counts on the x-axis and *MYC* counts on the y-axis, as well as highlighted in color the mutational status (*blue*: wild type, *yellow*: mutant) at the indicated time points initial diagnosis (top) and relapse (bottom). c-e) Heatmaps showing the mutational status for the indicated genes at first diagnosis (c), remission 1 (d), and at relapse (e). Cells with dropout in any of the genes were removed, and the cells were additionally annotated with the expression of surface proteins (CD34, CD7, CD3, CD14) measured with CITE-seq. g) Schematic of the clonal evolution as detected through scDNA-seq.

Figure 1

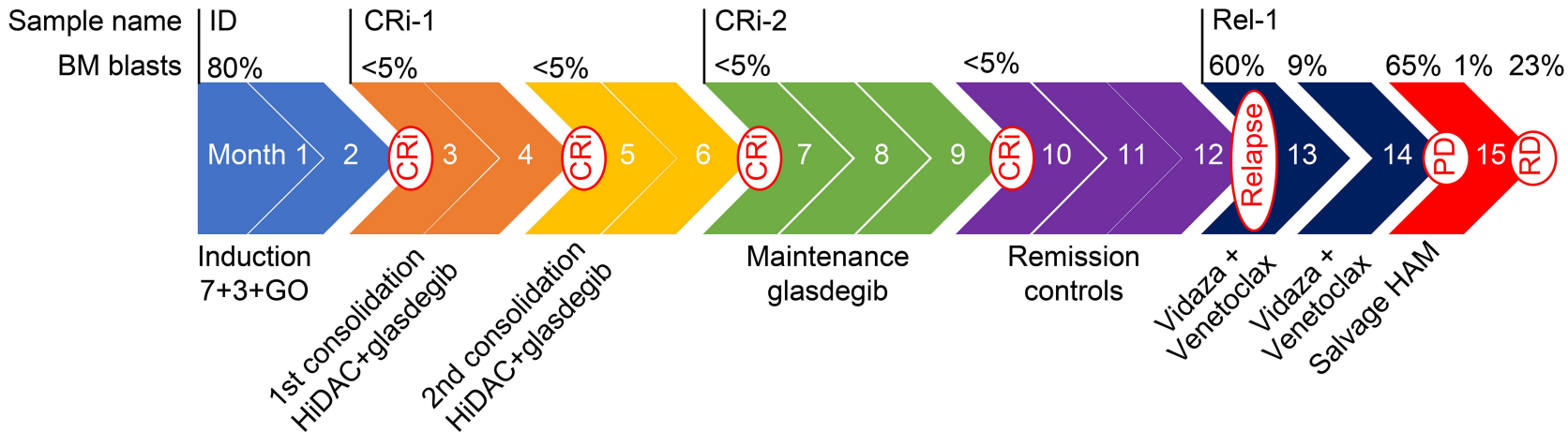


Figure 2

