Journal of Hematopathology (2020) 13:165–168
https://doi.org/10.1007/s12308-020-00398-5

CASE REPORT

The molecular analysis of four coexistent mutations in additional sex combs like 1 (ASXL1) gene in a patient with acute myeloid leukemia

Aleksandra Leszczyńska[1] · Witold Prejzner[1] · Jolanta Grzenkowicz-Wydra[2] · Maria Bieniaszewska[1] · Jan Maciej Zaucha[1]

Received: 19 February 2020 / Accepted: 12 May 2020 / Published online: 27 May 2020

Abstract

Mutations in the additional sex combs like 1 (*ASXL1*) gene are frequently involved in clonal hematopoiesis and are connected with an increased risk of hematologic cancer. These aberrations are frequently detected in a number of different hematological neoplasms including acute myeloid leukemia (AML). Patients harboring *ASXL1* mutations tend to have a poor prognosis and poor response to therapy. Here, we report the coexistence of four different mutations in *ASXL1* gene in a patient with AML. Such accumulation of mutations, in this gene, has not been described yet. Our findings suggest that accumulations of mutations in *ASXL1* gene may play an important role in the development and/or progression of AML.

Keywords ASXL1 mutation · Acute myeloid leukemia · Cloning · Sequencing · Clonal hematopoiesis

Introduction

ASXL1 (additional sex combs like 1) gene encodes a highly conserved, nuclear protein that is involved in the epigenetic regulation of gene expression [1]. Mutations in this gene occur frequently in several hematological neoplasms including acute myeloid leukemia (AML). According to the latest (2017) European Leukemia Network recommendation, *ASXL1* mutations are used for risk stratification of AML patients. Patients with *ASXL1* mutations have a poor prognosis, not responding to primary treatment, and are classified as the high-risk category [2, 3].

ASXL1 mutations are also frequently detected in healthy individuals with clonal hematopoiesis with indeterminate potential (CHIP) which is considered a precursor for malignant states.

Here, we report four different aberrations in exon 12 *ASXL1* gene, of which one deletion and one substitution have not been described yet, detected during a routine work-up in a male patient with AML most likely secondary to the preexisting myeloproliferative neoplasm.

Clinical history

A 67-year-old patient was hospitalized in December 2015 at the Department of Hematology with symptoms of fatigue secondary to severe anemia (hemoglobin level 4.8 g/dL). At admission, his WBC was 3.0 G/l, with 24% of blasts. The platelet count was 105 G/L, and there was no leukoerythroblastosis observed at initial diagnosis. Except for an increased lactate dehydrogenase activity (289 U/l upper normal range, 240 U/l), no other significant abnormalities in other laboratory tests were found. The physical examination was unremarkable; however, in the ultrasound of the abdomen, the enlarged spleen was noted (158 × 75 cm). Bone marrow aspiration was consistent with AML (25% myeloblast). The trephine biopsy revealed single megakaryocytes in the areas of fibrosis, outside them megakariocytes localized in small, compact clusters. Fibrosis grade 3 (reticulin + collagen type) was present. The changes suggested preexisting myeloproliferative neoplasm. Cytogenetic test revealed normal karyotype—46XY [20]. No other molecular tests were available at the time of diagnosis. The first cycle of induction chemotherapy adjusted for the patient age (> 60 years old: ara-c 100 mg/m^2 c.i. for 7 days and daunorubicin 45 mg/m^2 for 3 days) was

✉ Aleksandra Leszczyńska
aleksandra.leszczynska@gumed.edu.pl

[1] Department of Hematology and Transplantology, Medical University of Gdansk, Gdansk, Poland

[2] Pomeranian Science and Technology Park, Gdynia, Poland

complicated on day 6 by bacterial sepsis (*E. coli*) successfully treated with antibiotics. After chemotherapy, transient hepatic toxicity was observed. The patient achieved only partial response with 8% of myeloblasts in the bone marrow aspiration and was switched to hypomethylating therapy (azacytidine 75 mg/m^2 for 7 days every 28 days) that was continued until progression for 2 years (25 cycles). There were no major complications of hypomethylating therapy.

After the 25th cycle of azacytidine, the patient's general condition worsened (progression from performance status 1 to 3 according to the Eastern Cooperative Oncology Group) with increasing splenomegaly (15 cm below the costal margin) and hepatomegaly. At that time the molecular testing was available and revealed *CALR*, *MPL,* and *ASXL1* mutations. The following mutations were described: CALR c.1099-1150del (L367fs*46) and MPL c.1543T>A (W515R). This finding strongly suggests that AML in our case most likely developed from the blastic transformation of a preexisting myeloproliferative disorder. Interestingly, no mutation in *JAK2* and *BCR-ABL* gene was found. Azacytidine was discontinued, and the patient was lost to follow-up in June 2018.

Materials and methods

DNA isolation Genomic DNA, at the time of progression, was extracted from bone marrow using commercially available mini spin columns, according to the manufacturer's instructions.

ASXL1 mutation analysis To amplify exon 12 of the *ASXL1* gene, the PCR reaction was performed using primers previously described by Pratcorona et al. [3]. The purified PCR fragments were subjected to direct sequencing using fluorescence-labeled dideoxynucleotide chain terminator cycle-sequencing kit according the manufacturer's protocol. The products of sequencing reaction were separated in an 8-capillary ABI Prism 3500 Genetic Analyzer. Sequences were analyzed by using Chromas Lite free software (Technelysium Pty Ltd., Australia).

TA cloning and plasmid sequence analysis PCR products were purified with gel-out extraction kit and inserted into pGEM-T Easy Vector System I (Promega, USA) according to the manufacturer's instructions. High-efficiency JM109 Competent Cell was transformed with obtained plasmid constructions. Selection of transformants was done on LB plates with ampicillin. Two independent transformations, with two PCR products, obtained in separate reactions, were done to verify possible polymerase mistakes.

Two independent cloning procedures from two separate PCR reactions were performed. Twenty randomly selected transformants from each reaction were grown in LB broth with ampicillin with overnight culture. The plasmids were extracted with the plasmid spin column kit and separated in 1% agarose electrophoresis. Plasmid sequencing was performed with the same primers that were used for PCR amplification.

Results

The direct sequencing of the *ASXL1* PCR product showed at least 3 signals. Since the chromatograms were completely unreadable, identification of mutants was not possible. To identify the existing aberrations, cloning and transformant analysis were performed.

After cloning, 40 randomly selected transformants were grown with overnight culture. Plasmids were extracted and separated in 1% gel electrophoresis. Six plasmid constructs appeared not to have inserted PCR fragment, so the remaining 34 clones were subjected to sequencing reaction. As a result of clone sequencing, four mutations were identified: c.1817G>T, c.1857_1867delCCGTGCTCTGC, c.1870_1874delGTCCG, and c.1935delT. On the basis of number of clones detected, we can assume that the most frequent variant was 5 nucleotide deletion (26.5%). This mutation was sequenced in 9 out of 34 clones. The 11 nucleotide deletion and 1 nucleotide deletion and substitution were detected in 17.6%, 8.8%, and 5.9%, respectively. These mutations were obtained in more than one clone and were confirmed in two different transformations which were performed with two separate PCR fragments. Therefore, we are confident that identified aberrations are real changes. Moreover, c.1937delG mutation was also identified in the repetitive mononucleotide motif. But because of controversy, regarding mutant identification in the region of mononucleotide repeats, we were cautious to acknowledge this change with absolute certainty, since this mutant was present in only one clone and was visible as a mixed sequence and the signal height from mutant allele was at the very low level. All other mutations were obtained as homogeneous sequences (Fig. 1).

Screening the literature and according to COSMIC database, we found that 5 nucleotide deletion (c.1870_1874delGTCCG) and substitution (c.1817G>T) have not been described yet. Only one mutation was the missense point substitution with aminoacid change (p.R606L). All remaining changes were deletions (11 nucleotide, 5 nucleotide, and 1 nucleotide) generating the type of frameshift mutations.

Discussion

The analysis revealed the occurrence of four different mutations in the *ASXL1* gene. Such accumulation of mutations has

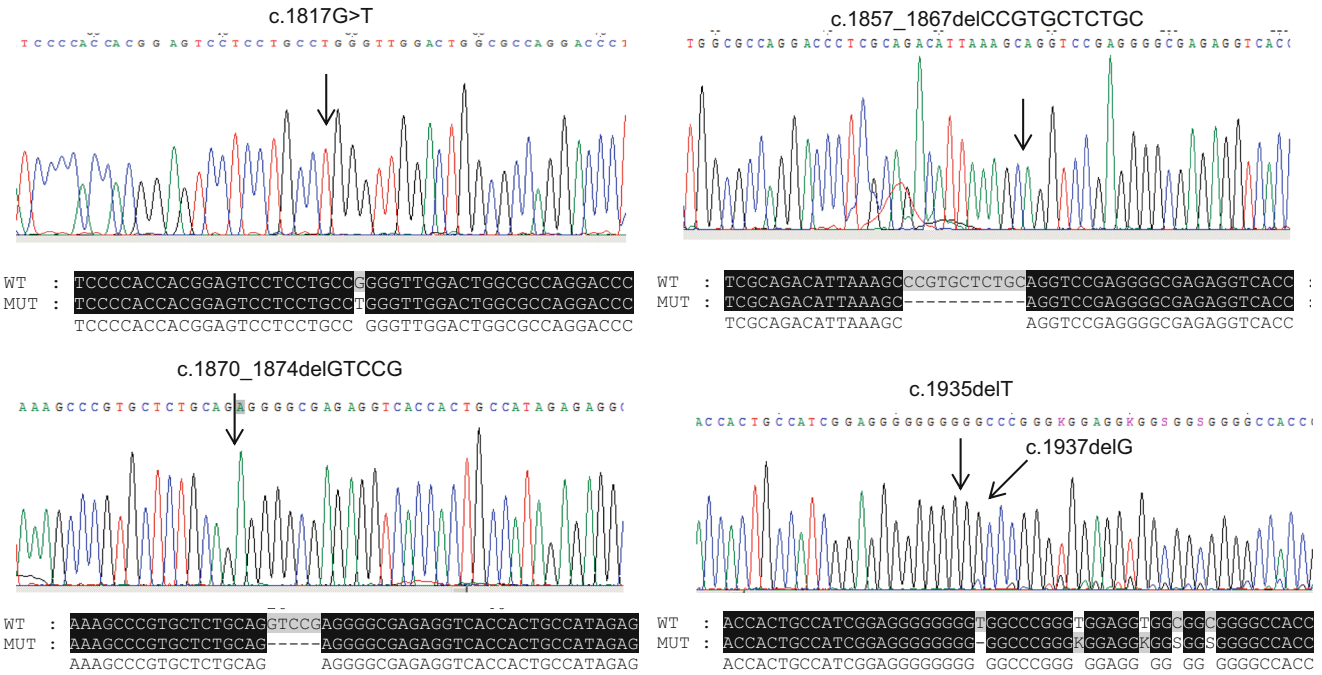

Fig. 1 DNA sequence chromatograms demonstrating *ASXL1* mutations with comparison of wild-type (WT) sequences to the mutant sequences (MUT). The arrows indicate the sites of mutations

not been described yet. Co-occurrence of two *ASXL1* mutations was previously reported by few studies, and the existence of three *ASXL1* variants was described only in one case [4]. In our case, the existence of four *ASXL1*variants suggests the co-occurrence of at least several pathogenic clones.

This patient had also a rare coexistence of mutations in *CALR* and *MPL* genes. Both *CALR* and *MPL* alternations are regarded as driver mutations in Philadelphia negative myeloid neoplasms and seem to be mutually exclusive. However, reports describing co-occurrence of these genes have been published in rare cases [5, 6].

Data regarding the frequency of *ASXL1* mutation show that the most common mutation in AML patients is c.1934dupG. This variant occurs in more than 60% AML patients, reaching 84.6% in some studies [7]. In contrast to these data, our patient did not have this most frequent variant and any other most common alternations. Moreover, two mutations have not been described yet (c.1870_1874delGTCCG and c.1817G>T). The remaining mutations (c. 1857_1867delCCGTGCTCTGC, c.1935delT, and c.1937delG) were reported in COSMIC database in rare cases.

It is rather believed that *ASXL1* mutation is regarded as an early event in the process of leukemogenesis. To predict clinical significance of described alternations, Mutation Tester software was used (www.mutationtaster.org). Analysis revealed that all tested mutations were potentially pathogenic.

Since several mutations occurred in our patient, we speculate that at least one mutation could occur at the very early stage of clonal hematopoiesis preceding AML. The remaining alternations could arise in subclones, as a consequence of genomic instability of cancer cells. The number of mutations, in our patient, raises the question about the mechanism of mutations acquiring and could suggest that DNA repair system may play an important role. As it was shown by Novotna et al., *ASXL1* mutations can influence mitochondrial activity and reactive oxygen species (ROS) overproduction. Elevated ROS level results in increased DNA damage [8]. In physiological conditions, oxidative DNA damage should be efficiently repaired via the base excision repair (BER) pathway. Dysfunction in this repair system potentially contributes to the genomic instability and disease progression.

All mutations, described in our patient, do not commonly occur and cover the same region of the gene. It is reported that mutations in this region lead to truncation of exon 12 as a consequence to deletion of plant homeodomain (PHD) localized on C-terminal end of ASXL1 protein. The PHD finger is involved in an interaction with transcription activators and repressors affecting the function of regulating chromatin [9]. The fact that the mutations occur in the last exon causes that mutated mRNA is not eliminated by nonsense-mediated decay (NMD) pathway and a stable, although truncated protein is expressed. As it was shown in mice model, the expression of truncated Asxl1 protein results in a gain-of-function role in leukemogenesis. The shortened form of Asxl1 protein acquires the ability of interaction with BRD4, multifunctional protein involved in transcription, DNA replication, and epigenetic regulation [10].

It is known that polymerase can generate mistakes in mono- and dinucleotide repeats and there is a controversy about the real nature of nucleotide changes within such simple sequence repeats. In exon 12 of the *ASXL1* gene, 8 base-pair

guanine repeats occur, and there is a possibility that mutation, in these repetitive sequences, is rather a PCR artifact than a real somatic mutation. Some researchers suggest that even the most frequently occurring *ASXL1* alternation (c.1934dupG) is caused by slippage of polymerase and is not a real somatic alternation [11]. Since one mutation described in our patient (c.1937delG) was present in only one clone and was visible as a mixed sequence in repetitive motif with the low signal height from the mutant allele, we are cautious to define this variant as a real mutation. But, on the other hand, the effect of polymerase slippage was not observed in any of the 34 sequenced clones. We could not exclude that this variant was present in a very small number of hematopoietic cells and that is why it was detected only in one clone transformant.

The presence of *ASXL1* mutations is reported to be a negative prognostic factor in AML [12]. Unfortunately, we do not know if *ASXL1* mutations were present at the time of the initial diagnosis. Such an information could help to clarify if the good response to AZA occurred in the presence of *ASXL1* mutant clones and the order how the subsequent mutations occurred during leukomogenesis.

We conclude that accumulation of four different mutations in *ASXL1* gene, as in our case, may suggest that mutations in this gene are important events for the development and/or progression of AML.

Acknowledgments We would like to thank Klaudia Pinkasiewicz from BioLaboratory, Pomeranian Science, and Technology Park Gdynia for technical assistance in laboratory work.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts of interest.

Statement of ethics The patient gave his written informed consent to use his clinical material and data in research study.

References

1. Scheuermann JC, de Ayala Alonso AG, Oktaba K, Ly-Hartig N, McGinty RK, Fraterman S, Wilm M, Muir TW, Müller J (2010) Histone H2A deubiquitinase activity of the Polycomb repressive complex PR-DUB. Nature. 465(7295):243–247. https://doi.org/10.1038/nature08966
2. Döhner H, Estey E, Grimwade D, Amadori S, Appelbaum FR, Büchner T, Dombret H, Ebert BL, Fenaux P, Larson RA, Levine RL, Lo-Coco F, Naoe T, Niederwieser D, Ossenkoppele GJ, Sanz M, Sierra J, Tallman MS, Tien HF, Wei AH, Löwenberg B, Bloomfield CD (2017) Diagnosis and management of AML in adults: 2017 ELN recommendations from an international expert panel. Blood. 129(4):424–447. https://doi.org/10.1182/blood-2016-08-733196
3. Pratcorona M, Abbas S, Sanders MA, Koenders JE, Kavelaars FG, Erpelinck-Verschueren CAJ, Zeilemakers A, Lowenberg B, Valk PJM (2012) Acquired mutations in ASXL1 in acute myeloid leukemia: prevalence and prognostic value. Haematologica. 97(3):388–392. https://doi.org/10.3324/haematol.2011.051532
4. Montes-Moreno S, Routbort MJ, Lohman EJ, et al. Clinical molecular testing for ASXL1 c.1934dupG p.Gly646fs mutation in hematologic neoplasms in the NGS era. Balusu R, ed. *PLoS One*. 2018;13(9):e0204218. https://doi.org/10.1371/journal.pone.0204218
5. Bernal M, Jiménez P, Puerta J, Ruíz-Cabello F, Jurado M (2017) Co-mutated CALR and MPL driver genes in a patient with myeloproliferative neoplasm. Ann Hematol 96(8):1399–1401. https://doi.org/10.1007/s00277-017-3023-9
6. Tashkandi H, Moore EM, Tomlinson B, Goebel T, Sadri N (2017) Co-occurrence of type I CALR and two MPL mutations in patient with primary myelofibrosis. Ann Hematol 96(8):1417–1418. https://doi.org/10.1007/s00277-017-3022
7. Kakosaiou K, Panitsas F, Daraki A, Pagoni M, Apostolou P, Ioannidou A, Vlachadami I, Marinakis T, Giatra C, Vasilatou D, Sambani C, Pappa V, Manola KN (2018) ASXL1 mutations in AML are associated with specific clinical and cytogenetic characteristics. Leuk Lymphoma 59(10):2439–2446. https://doi.org/10.1080/10428194.2018.1433298
8. Novotna B, Bagryantseva Y, Siskova M, Neuwirtova R (2009) Oxidative DNA damage in bone marrow cells of patients with low-risk myelodysplastic syndrome. Leuk Res 33(2):340–343. https://doi.org/10.1016/j.leukres.2008.07.005
9. Brecqueville M, Rey J, Bertucci F, Coppin E, Finetti P, Carbuccia N, Cervera N, Gelsi-Boyer V, Arnoulet C, Gisserot O, Verrot D, Slama B, Vey N, Mozziconacci MJ, Birnbaum D, Murati A (2012) Mutation analysis of *ASXL1, CBL, DNMT3A, IDH1, IDH2, JAK2, MPL, NF1, SF3B1, SUZ12,* and *TET2* in myeloproliferative neoplasms. Genes Chromosom Cancer 51(8):743–755. https://doi.org/10.1002/gcc.21960
10. Yang H, Kurtenbach S, Guo Y, Lohse I, Durante MA, Li J, Li Z, al-Ali H, Li L, Chen Z, Field MG, Zhang P, Chen S, Yamamoto S, Li Z, Zhou Y, Nimer SD, Harbour JW, Wahlestedt C, Xu M, Yang FC (2018) Gain of function of ASXL1 truncating protein in the pathogenesis of myeloid malignancies. Blood. 131(3):328–341. https://doi.org/10.1182/blood-2017-06-789669
11. Abdel-Wahab O, Kilpivaara O, Patel J, Busque L, Levine RL (2010) The most commonly reported variant in ASXL1 (c.1934dupG;p.Gly646TrpfsX12) is not a somatic alteration. Leukemia. 24(9):1656–1664. https://doi.org/10.1038/leu.2010.144
12. Schnittger S, Eder C, Jeromin S, Alpermann T, Fasan A, Grossmann V, Kohlmann A, Illig T, Klopp N, Wichmann HE, Kreuzer KA, Schmid C, Staib P, Peceny R, Schmitz N, Kern W, Haferlach C, Haferlach T (2013) ASXL1 exon 12 mutations are frequent in AML with intermediate risk karyotype and are independently associated with an adverse outcome. Leukemia. 27(1):82–91. https://doi.org/10.1038/leu.2012.262