



Scientific Letter

Experiences with Next-Generation Sequencing in Relapsed Acute Myeloid Leukemia: A Patient Case Series

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To the editor.

Next-generation sequencing (NGS) has opened the opportunity for assessing the unique clonal composition of patients with acute myeloid leukemia (AML), not only at the beginning but also within the course of the disease, and particularly at relapse. The molecular genetic characterization of the clonal composition has therapeutic relevance, as a number of molecularly directed treatment options have recently become available. Since relapse of AML remains a major clinical challenge, comprehensive diagnostics during follow-up and at relapse has become an increasingly important pillar of clinical decision making. In the current manuscript, we have applied myeloid NGS panel sequencing to compare the genetic profiles of six illustrative AML patients at initial diagnosis and at relapse. We found that NGS has the potential to identify clonal molecular stability, evolution, and devolution in addition to co-occurring changes on the cytogenetic level, all of which can occur alone or in combination. We discuss these patients in detail, covering clinical, molecular, and cytogenetic, as well as therapeutic aspects.

The increasing use of NGS has been enabled by a number of commercially available panels that cover the most frequently mutated genes. In contrast to traditional diagnostic tools (i.e., cytomorphology, cytogenetics, qPCR) that provide classification and prognostic information only within certain categories, molecular profiling by NGS enables us to depict a unique genetic make-up for each AML patient. Genetic information provides crucial parameters within the current AML classification systems^{1,2} and has not only an impact on prognosis but also influences treatment options.

However, despite improving remission rates, around 40-60% of AML patients will ultimately relapse³, which remains the major determinant of outcome. At relapse, AML patients can either present with the same genetic

mutation pattern as observed at initial diagnosis (clonal stability), or present with higher complexity, e.g., through the acquisition of additional mutations (clonal evolution), or lose some of the initial mutations at relapse (clonal devolution), or, alternatively, show both gains and losses of mutations.⁴⁻⁶

Concerning the pathophysiologic mechanisms involved during clonal evolution at relapse, our current paradigms suggest that pre-existing clones (or subclones) may gain a survival advantage under the selective chemotherapeutic pressure.⁶ We have recently provided a comprehensive overview of the current knowledge of NGS during relapse of AML.⁷ Here, we present six illustrative patient studies observed during clinical practice to demonstrate characteristic genetic scenarios accompanying hematologic relapse of AML following intensive chemotherapy. All patients were treated and analyzed by cytogenetics and myeloid NGS panels at diagnosis and at relapse at our department according to the methodology described in **Table 1**. The sensitivity of NGS analyses was limited to a 5% variant allele frequency (VAF) at diagnosis, and 1% at follow-up as the exact mutation localizations were known.

Patient #1: Molecular and cytogenetic stability at relapse after intensive chemotherapy.

A 51-year-old male patient presented with *de novo* AML, FAB M1, 46, XY, and bone marrow (BM) blasts >90%. Fragment analysis revealed an *FLT3*-ITD with a mutant/wild-type ratio of >0.5, and PCR detected an *NPM1* (type B) mutation. No additional mutations were detected by NGS. Standard induction chemotherapy (cytarabine/idarubicin followed by cytarabine/daunorubicin) induced complete morphologic remission (CR) with minimal/measurable residual disease (MRD) positivity according to HOVON-132 for the previously known *NPM1* mutation. After high-dose chemotherapy (HDCT) with busulfan/cyclophosphamide and

Table 1: Examples of six AML patients relapsing after intensive chemotherapy; NGS is performed at diagnosis and relapse.

Patient # age, sex	AML subtype	Genetic profile at initial AML diagnosis	Genetic profile at relapse
#1: Molecular and cytogenetic stability at relapse after intensive chemotherapy			
51; M†	<i>de novo</i> , FAB M1	46,XY[20]; FLT3-ITD ((ratio (mut/wt) >0.5%)); NPM1 (type B) [c.863_864insCATG, p.Trp288Cysfs*12].	46,XY[20]; FLT3-ITD (ratio (mut/wt) <0.5%); NPM1 (type B) [c.863_864insCATG, p.Trp288Cysfs*12; VAF: 51%].
#2: Molecular and cytogenetic stability at relapse after allogeneic transplant			
63; M††	sAML (post MDS)	46,XY[20]; ASXL1 [c.1934dup, p.Gly646Trpfs*12; VAF 44%], RUNX1 [c.776_780delTTAAC, p.Phe259Serfs*339; VAF 54%] EZH2 [c.1409A>T, p.Gln470Leu; VAF 100%] CEBPA [c.441C>A, p.(Tyr147*) VAF 31%] &c.678_682dupGCCGC, p.Pro228Argfs*92; VAF 10%] DNMT3A [c.2645G>A, p.Arg882His; VAF 50%] SF3B1 [c.2098A>G, p.Lys700Glu; VAF 50%] NRAS [c.38G>A, p.Gly13Asp; VAF 11%] TET2 [c.1630C>T, p.Arg544*; VAF 5%] & [c.2725C>T, p.Gln909*; VAF 94%] NFI [c.6772C>T, p.Arg2258*; VAF 3.1%].	46,XY[20]; ASXL1 [c.1934dup, p.(Gly646Trpfs*12) VAF 38%] RUNX1 [c.776_780delTTAAC, p.Phe259Serfs*339; VAF 40%] EZH2 [c.1409A>T, p.Gln470Leu; VAF 100%] CEBPA [c.441C>A, p.Tyr147*; VAF 19%] & c.678_682dupGCCGC, p.Pro228Argfs*92; VAF 2.6%] DNMT3A [c.2645G>A, p.Arg882His; VAF 35%] SF3B1 [c.2098A>G, p.Lys700Glu; VAF 34%] NRAS [c.38G>A, p.Gly13Asp; VAF 13%] TET2 [c.1630C>T, p.Arg544*; VAF 43%] & [c.2725C>T, p.Gln909*; VAF 72%] NFI [c.6772C>T, p.Arg2258*; VAF 18%].
#3: Molecular stability with cytogenetic clonal devolution at relapse			
72; F††	<i>de novo</i> ; FAB M2	45, X, -X, der(1)(t(1;13)(q?41;q12),del(5) (q1-14q33),del(8)(q13q22), del(9)(p12p24),-12,-13,- 16,?add(22)(p10),+3mar[3]/45,sl,i(11)(q10)[5]/44,sl, add(3)(p24),-7,-8,add(11)(q?24),-16,- 17,18,+4mar[11]/46,XX[1] TP53 [c.613T>G, p.(Tyr205Asp); VAF 43%] & [c.841G>C, p.Asp281His; VAF 41%].	46,XX[20]; TP53 [c.613T>G, p.Tyr205Asp; VAF 43%] & [c.841G>C, p.Asp281His; VAF 43%].
#4: Simultaneous clonal evolution and devolution at relapse			
41; M†	sAML (post MDS)	46,XY[20] KRAS [c.437C>T, p.Ala146Val; VAF 9%];	46,XY[20] KRAS negative WT1 [c.1105_1106insATCCTGC, p.Arg369Hisfs*18; VAF 14%] & [c.1340G>T, p. Gly447Val, VAF 12%] FLT3-ITD (ratio=0.202).
#5: Both clonal evolution and devolution at relapse			
65; F†	<i>de novo</i> , FAB M1	46,XX,del(9)(q12q32)[15] NPM1 (type D) [c.863_864insCCTG, p.W288fs*12] DNMT3A [c.2644C>T, p.Arg882Cys; VAF 43%] retrospectively;	46,XX[20] NPM1 (type A) [c.860_863dup, p.Trp288Cysfs*12] DNMT3A [c.2644C>T, p.Arg882Cys, VAF 43%] TET2 [c.3302_3303delTC, p.Leu1101Glnfs*2; VAF 34%].
#6: Cytogenetic and molecular evolution at relapse			
62; F††	<i>de novo</i> ; FAB M1	46,XX[20]; RUNX1 [c.485G>A,p.Arg162Lys; VAF 31%];	47,XY,+21[13]/46,XX[7]; RUNX1 [c.485G>A, p.Arg162Lys; VAF 32%] & [c.780_781insAA, p.Pro261Asnfs*5; VAF 8%]* *occurring 5 months after start of relapse therapy.

AML, acute myeloid leukemia; F., female; FAB, French-American-British classification of AML; M., male; MDS, myelodysplastic syndrome; mut/wt., mutated/wild-type; sAML, secondary AML; VAF, variant allele frequency.

† NGS with Ampliseq EA myeloid panel (Thermo Fisher Scientific, Reinach, Switzerland; 30 genes/hotspots) at diagnosis and relapse. †† NGS with Ampliseq EA myeloid panel at diagnosis and Oncomine myeloid panel (Thermo Fisher Scientific, Reinach, Switzerland; 40 genes/hotspots) at relapse.

autologous stem cell transplantation (ASCT), the patient achieved molecular MRD-negative CR1 as assessed by PCR/fragment analysis. 4.5 months after ASCT, both the *NPM1* and *FLT3-ITD* mutations re-emerged, followed by an overt hematologic relapse one month later. NGS and cytogenetics revealed no additional

changes. Salvage therapy (cladribine, cytarabine, and idarubicin; CLA-Ida) plus sorafenib resulted in morphologic CR2, albeit with the persistence of the *NPM1* and *FLT3-ITD* mutations. Matched-related myeloablative allogeneic HSCT (11/2017) was performed, but molecular MRD persisted at 3 months

post-transplant, and sorafenib was once more initiated and combined with azacitidine (AZA). At the time of this report, the patient remained in molecular CR2, meanwhile under continued sorafenib monotherapy.

Patient #2: Molecular and cytogenetic stability at relapse after allogeneic transplant. A 63 years old male patient developed s-AML following 4 years of MDS with a “wait and watch” approach. At the time of s-AML, the karyotype was normal, and NGS revealed nine mutations in *ASXL1*, *RUNX1*, *EZH2*, *CEBPA*, *DNMT3A*, *SF3B1*, *NRAS*, *TET2*, and *NFI*. The patient underwent cytarabine/idarubicin induction followed by myeloablative allogeneic HSCT in CR1 with the molecular persistence of all mutations. Subsequently, the patient developed hematologic relapse (d+78 after HSCT), demonstrating a normal karyotype and the identical mutations seen before. The patient was resistant to AZA, donor lymphocyte infusion, and gemtuzumab ozogamicin and died 4 months after HSCT due to progressive disease.

Patient #3: Molecular stability with cytogenetic clonal devolution at relapse. A 72 years old female patient was diagnosed with high-risk *de novo* AML M2 with a complex multi-clonal karyotype. NGS revealed two different mutations in *TP53*. The patient was refractory to standard induction chemotherapy, but second-line decitabine (10 cycles) resulted in CR1, and NGS documented clearance of the *TP53* mutations. Due to poor veins, decitabine therapy was switched to AZA. Relapse occurred 11.5 months after the achievement of CR1; the karyotype was normal, whereas both *TP53* mutations remained detectable. Decitabine treatment combined with sorafenib failed to induce any response. The patient died one month after relapse detection due to progressive disease.

Patient #4: Simultaneous clonal evolution and devolution at relapse. Forty-one years old male patient presented with s-AML transformed from untreated high-risk MDS diagnosed three months earlier. Genetic analysis revealed a normal karyotype and an isolated *KRAS* mutation by NGS. Induction with two cycles of cytarabine and idarubicin resulted in MRD-negative CR1. ASCT was performed after melphalan/cyclophosphamide HDCT, but hematologic relapse occurred 6.4 months following ASCT. In contrast to the results obtained in the clone at initial diagnosis, the BM at relapse was negative for the *KRAS* mutation but presented with two *WT1* mutations and *FLT3-ITD* (ratio=0.202). The patient received CLA-Ida salvage therapy and allogeneic HSCT from his HLA-identical sister. He has been in hematologic and molecular CR2 at last follow-up 19 months after relapse.

Patient #5: Both clonal evolution and devolution at

relapse. A 65 years old female patient presented with *de novo* AML, FAB M1, with a 9q deletion. PCR revealed *NPM1* mutation type D. The patient received induction consisting of cytarabine/idarubicin/laromustin followed by busulfan/cyclophosphamide HDCT/ASCT. Following a period of long-lasting remission with MRD negativity for the *NPM1* type D mutation over 8 years, the patient showed a 4-log increase of the *NPM1* mutation load detected by qPCR. Still being at hematologic CR1, *NPM1* re-appearance was detected by qPCR as well as NGS but surprisingly identified type A instead of type D. Yet, the latter was also confirmed retrospectively by NGS at initial diagnosis. In addition, a *DNMT3A* mutation was discovered in the relapse sample. Subsequent qPCR assay with a primer designed for type A revealed a ratio of 6.285. Six weeks later, BM cytomorphology and immunophenotyping revealed up to 35% myeloid blasts corresponding to overt AML M2. Cytogenetics revealed a normal karyotype, with a lack of the 9q deletion documented at first diagnosis. We have previously described this switch of *NPM1* types (from type D to type A).⁹ The *DNMT3A* mutation identified at relapse was retrospectively detected in stored material from initial diagnosis. The patient underwent salvage chemotherapy, followed by busulfan/melphalan HDCT/ASCT. Subsequently, maintenance therapy with AZA was started resulting in *NPM1* MRD-negative CR2 ongoing 22 months after diagnosis of relapse. Finally, a *TET2* mutation was also found retrospectively with a variant allele frequency (VAF) of 43% in addition to *DNMT3A* (47% VAF) in the autologous stem cell harvest preceding the 2nd ASCT. *TET2* and *DNMT3A* mutations persisted at last follow-up (34%/43% VAF) despite *NPM1* type A mutation clearance suggesting an interpretation of clonal hematopoiesis of indeterminate potential (CHIP).

Patient #6: Cytogenetic and molecular evolution at relapse. A 62-year-old female patient came to observation with *de novo* AML, FAB M1, and a peripheral blast count of 55%. The NGS panel revealed a *RUNX1* mutation with a VAF of 31%, and cytogenetics showed a normal karyotype. The patient received two cycles of induction (daunorubicin, cytarabine), resulting in CR1 and MRD-negativity for the *RUNX1* mutation by NGS. This was followed by HDCT consisting of busulfan/melphalan and ASCT since the patient declined allogeneic transplantation. Hematological regeneration was heavily delayed. Five months after ASCT, the patient developed hematologic relapse. At this stage, BM presented a clonal cytogenetic evolution with a novel gain of chromosome 21 in addition to the re-emergence of the *RUNX1* mutation that had already been present at the initial diagnosis. Relapse therapy was initiated by decitabine after the patient declined intensive re-induction treatment. Blasts, however, persisted, which was 5 months later

accompanied by an additional, i.e., second, *RUNX1* mutation suggesting further molecular clonal evolution. Palliative therapy was administered by decitabine (9 cycles), then with low-dose cytarabine with glasdegib (hedgehog pathway inhibitor), and finally, sorafenib. The patient succumbed to refractory disease one month after the sorafenib start and 15 months after the first relapse.

Conclusions. As demonstrated by our case series, each patient may present with an individual genetic composition at relapse of AML. Compared to the initial diagnosis, this may comprise clonal stability, evolution, or devolution alone or in combination both at the molecular and/or cytogenetic level. Consequently, the genetic characterization during relapse may identify novel lesions treatable by targeted therapies or may open new pathways for bridging strategies towards allogeneic HSCT. This can be illustrated, for example, by *FLT3* mutations, which are emerging in around 10% of AML patients at relapse¹⁰, and may provide an option for specific *FLT3* inhibitor treatment, such as midostaurin, gilteritinib or others.^{11,12} Similarly, both *IDH1*

(ivosidenib) and *IDH2* (enasidenib) inhibitors were recently approved in AML with the respective mutations.^{13,14} Knowledge of relapse genetics may imply consequences also for prognosis. Adverse prognostic markers, such as *TP53* mutations emerging at relapse, may allow timely initiation of donor search for subsequent allogeneic HSCT. Accordingly, the development of distinct diagnostics and therapeutic algorithms for clonal stability, evolution, and devolution, as well as defining of “founder” mutations in relapsed AML settings may further ease the management of such patients. Anticipating anti-relapse treatment with targeted agents, the determination of mutant allele frequencies is of high importance as these provide a sensitive diagnostic tool to assess response on the molecular level and predict progression over time. In conclusion, NGS may be discussed for all patients at AML relapse. Due to recent improvements in treatment options and an increasing understanding of the molecular drivers of AML, therapy in the relapse situation becomes more and more individualized, and, consequently, NGS will gain increasing importance in this scenario.

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