Clonal evolution in relapsed NPM1 mutated acute myeloid leukemia

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Key Points

- Relapsed AML with NPM1 mutation is genetically related to the primary leukemia and characterized by an increase in high risk aberrations.

- DNMT3A mutations show highest stability and thus may precede NPM1 mutations.

Abstract

Mutations in the nucleophosmin 1 (NPM1) gene are considered as a founder event in the pathogenesis of acute myeloid leukemia (AML). To address the role of clonal evolution in relapsed NPM1 mutated (NPM1mut) AML, we applied high-resolution genome-wide single-nucleotide polymorphism (SNP) array profiling to detect copy number alterations (CNA) and uniparental disomies (UPD) and performed comprehensive gene mutation screening in 53 paired bone marrow/peripheral blood samples obtained at diagnosis and relapse. At diagnosis, 15 aberrations (CNAs, n=10; UPDs, n=5) were identified in 13 patients (25%), whereas at relapse 56 genomic alterations (CNAs, n=46; UPDs, n=10) were detected in 29 patients (55%) indicating an increase in genomic complexity. Recurrent aberrations acquired at relapse included deletions affecting tumor suppressor genes [ETV6 (n=3), TP53 (n=2), NF1 (n=2), WT1 (n=3), FHIT (n=2)] and homozygous FLT3 mutations acquired via UPD13q (n=7). DNMT3A mutations (DNMT3A\textsuperscript{mut}) showed the highest stability (97%). Persistence of DNMT3A\textsuperscript{mut} in 5 patients who lost NPM1\textsuperscript{mut} at relapse suggests that DNMT3A\textsuperscript{mut} may precede NPM1\textsuperscript{mut} in AML pathogenesis. Of note, all relapse samples shared at least one genetic aberration with the matched primary AML sample implying common ancestral clones. In conclusion, our study reveals novel insights into clonal evolution in NPM1\textsuperscript{mut} AML.
Introduction

Acute myeloid leukemia (AML) is a clonal disease that is characterized by the presence of a variety of genetic alterations. Mutations in the nucleophosmin (NPM1) gene represent one of the most common gene mutations (25% to 30%) in AML.1 Approximately 85% of AML with NPM1 mutation (NPM1mut) display a cytogenetically normal (CN) karyotype if determined by chromosome banding analysis. High-resolution techniques like single-nucleotide polymorphism (SNP) arrays revealed only a few submicroscopic copy-number alterations (CNA) in NPM1mut AML, whereas uniparental disomies (UPD) that result in loss of heterozygosity (LOH) were detected in 10% to 20% of the patients.2,3 NPM1mut co-occurs with known AML-associated mutations like FLT3, DNMT3A, IDH1, IDH2 and NRAS.1,4-7 These mutations seem not to accumulate in a random order but instead can be allocated to early and late events in the transformation process from a normal hematopoietic stem (HSC) or progenitor cell to leukemia.8,9 A high proportion of NPM1mut AML patients achieve complete remission (CR) with intensive chemotherapy; however, approximately 50% of these patients experience relapse during the first 3 years after diagnosis, in particular patients with concurrent internal tandem duplications (ITD) of the FLT3 gene.1,10-12 Relapse is generally less responsive to chemotherapy and a major cause of death in patients with AML. Other characteristics of NPM1mut AML include low expression or absence of the hematopoietic cell surface marker CD34, multilineage involvement as well as a unique gene-expression and miRNA signature.13 Based on these clinical and biological characteristics, NPM1mut AML was included as a provisional entity in the current WHO classification.14 Besides the distinct properties of NPM1mut AML, NPM1mut is considered as a founder event in the pathogenesis of AML, mainly based on the observation that the mutation is stable over time and in general maintained at relapse, even occurring after a long latency.15,16 However, we and other groups observed loss of NPM1mut in ~10% of relapsed patients and this loss was generally accompanied by further chromosomal and molecular changes.17-19 To date, it is still unclear whether these cases can be explained by clonal evolution from a common pre-leukemic ancestor or if these patients developed therapy-related AML (t-AML) that evolved from an independent hematopoietic HSC or progenitor cell.
Clonal evolution is a hallmark of cancer. This process is highly affected by cytotoxic therapy as it selects for resistant clones that are the basis for re-occurrence of the disease. Previous studies on relapsed AML estimated clonal evolution in ~40% of the cases based on chromosomal banding analysis. In a study of Raghavan and colleagues, SNP genotyping in 27 patients revealed the acquisition of UPDs, mainly UPD13q, in ~40% of relapses.\textsuperscript{25} In addition, sequential analysis for AML-associated gene mutations such as FLT3, TP53, NRAS, DNMT3A, IDH1 and IDH2 showed a high variance of their stability.\textsuperscript{26-29} So far, most studies were restricted to small cohorts of AML patients and low-resolution chromosome banding analysis. Kühn et al. published a SNP profiling study in adult and pediatric core-binding factor AML that included 39 paired samples obtained at the time of diagnosis and at relapse.\textsuperscript{30} While two-thirds of the relapses were identical to diagnosis or simply acquired additional aberrations, one-third arose from an ancestral clone as they lost one or more aberrations present at diagnosis. In a recent study Parkin et al. integrated cytogenetics, high-resolution SNP profiling and mutational screening to assess clonal evolution in diagnosis and relapse samples from 28 unselected AML cases corroborating a genetic relationship in all pairs as well as a high stability of somatic CNAs and UPDs.\textsuperscript{31} The most comprehensive approach to assess clonal evolution in relapsed AML was performed by Ding et al. who applied whole genome sequencing in 8 primary/relapse AML pairs. Besides identification of novel gene mutations in AML, this study unequivocally demonstrated that relapse either emerged from the predominant founding clone or a smaller subclone that shared some but not all mutations of the predominant clone.\textsuperscript{32} Furthermore, their data suggest that chemotherapy directly causes new gene mutations.

In order to assess clonal evolution in the genetically defined subgroup “AML with \(NPM1^{mut}\)”, we analyzed paired leukemia samples obtained at the time of diagnosis and relapse from 53 \(NPM1^{mut}\) AML patients by using high-resolution SNP array profiling and mutation analysis of 10 AML-associated genes.
Methods

Patients
Patient characteristics for the fifty-three adult AML patients (age 24 to 66 years) are given in Table 1. All patients gave informed consent for both treatment and genetic analysis according to the Declaration of Helsinki. Approval was obtained from the Institutional Review Boards of the participating AMLSG institutions.

Single-nucleotide polymorphism (SNP) microarray-based genotyping analysis
SNP profiling was performed as described recently\(^3\),\(^30\); details are provided in the Supplement. Microarray raw data were made publicly available at NCBI’s Gene Expression Omnibus (GEO-accession number: GSE46951).

Further methods are described in the Supplement.

Results

Cytogenetics, copy number alterations and uniparental disomies at diagnosis and relapse
At the time of diagnosis: Data from chromosome banding analysis were available in 48 patients. Among the 43 patients (90%) with normal karyotype, SNP profiling identified 4 CNAs in 3 patients (UPN #8, #11 and #17) including 3 deletions [13q21.1 (size, 0.02 Mb), 16p13.3-p13.13 (10.8 Mb), 9q21.32 (0.89 Mb)] and one gain [17q (11.1 Mb)]; in 4 patients (UPN #5, #13, #21, #47) cytogenetic analysis revealed a 9q deletion which was confirmed by SNP array profiling. One patient (UPN #4) had a 47,XYY karyotype that was also present in the remission and relapse sample confirming the constitutional nature (XYY syndrome). In this patient a deletion at 9q21.32-q21.33 (2.4 Mb) was identified by SNP-array genotyping. Among the 5 patients without available cytogenetics, SNP profiling detected one gain at 4p16.3 (0.17 Mb). In total, 10 somatic CNAs (gain, n=2, deletions n=8) were identified by SNP profiling at the time of diagnosis. Moreover, SNP profiling allowed the detection of uniparental disomies (UPDs) in 5 patients (as a sole abnormality in 4 patients, in combination with a CNA in 1 patient);
in 4 cases UPD affected chromosomal arm 13q (UPN #10, #11, #23, #24), all resulting in homozygous FLT3-ITD mutations, and in one case chromosomal band 1p (UPN#1) was affected. (Supplementary Tables 1 and 2)

Taken together, we identified one CNA or UPD in 11 patients (21%) and 2 aberrations in 2 patients (4%) at diagnosis (Figure 1, Table 2) with del(9q) and UPD13q being the only recurrent aberrations.

**At the time of relapse:** At relapse, cytogenetic analysis was available for 14 patients and revealed a normal karyotype in 6 patients (all with a normal karyotype at diagnosis) and an aberrant karyotype in 8 patients (6 with normal karyotype, 2 with no available metaphases at diagnosis, respectively) (Supplementary Table 3). The only recurrent abnormality detected by banding analysis in 2 cases (UPN#10 and #26) was an unbalanced translocation der(12;17)(q10;q10) with loss of 17p comprising TP53 and 12p comprising ETV6 that were both confirmed by SNP profiling (Supplementary Figure 1 A-D). One case (UPN#22) had a balanced t(2;17)(q35;q11) and SNP profiling detected a 1.6 Mb deletion at the 17q11 breakpoint comprising the tumor suppressor NF1 (Supplementary Figure 1 E-F).

SNP array analysis of the 53 relapse samples revealed that the total number of CNAs increased from 10 at diagnosis to 46 (35 deletions and 11 gains), and the total number of UPDs from 5 to 10 (Figure 1). In 29 patients (55%) we identified at least one CNA or UPD: 17 patients (32%) had one, 6 patients (11%) two, and 6 patients (11%) three or more CNAs and/or UPDs. The mean number of aberrations (CNAs and UPDs) increased from 0.28 per case at diagnosis to 1.06 per case at relapse (Paired t test: p=0.002).

Five of 10 CNAs detected by SNP profiling at diagnosis were also present at the time of relapse [del(9q), (3 of 6); gain(4p16.3); del(13q12.1)], whereas 5 [del(9q) (3 of 6); del(16q); gain(17q)] were not detectable any more (Figure 2A).

Newly acquired recurrent alterations comprised deletions of tumor suppressor genes at 12p13 (ETV6, n=3), 11p13 (WT1, n=3), 17p (TP53, n=2), 17q11.2 (NF1, n=2), 3p14.2 (FHIT, n=2), as well as gain of 11q23.3 (MLL, n=3). Non-recurrent aberrations comprised trisomy 5 and monosomy 7 (Table 2, Supplementary Table 1+2).

The majority of the altered genomic regions at relapse (76%) were exclusively affected in relapse samples. CNAs in these regions could neither be detected in the 53
diagnostic samples nor in an independent cohort of 140 newly diagnosed $NPM1^{\text{mut}}$ AML analyzed on the same platform (data not shown).

Two of the 5 patients with UPD at diagnosis lost UPD at relapse (UPD13q in UPN#23 and UPD1p in UPN#1). Conversely, 7 patients acquired a new UPD13q with homozygous $FLT3$-ITD mutation at relapse (5 with heterozygous and 2 without $FLT3$-ITD mutation at diagnosis)(Figure 2B).

**Gene mutation pattern at diagnosis and relapse**

**At the time of diagnosis,** we observed a typical pattern for concurrent gene mutations in $NPM1^{\text{mut}}$ AML (Figure 3). Heterozygous $DNMT3A$ mutations ($DNMT3A^{\text{mut}}$) were detected in 32 patients (60%). The majority of mutations ($n=19, 59\%$) resulted in an amino acid substitution altering the arginine residue at codon 882. Twenty-four patients (45\%) had $FLT3$-ITD mutations of which 5 were homozygous defined by an $FLT3$-ITD to $FLT3$-wildtype ratio $>1$; four of these cases had UPD13q. The tyrosine kinase domain (TKD) of $FLT3$ was mutated in 11 cases (21\%). Fourteen patients (26\%) harbored $NRAS$ mutations affecting amino acid positions at G12 ($n=7$), G13 ($n=6$), and G175 ($n=1$). Heterozygous $IDH1/IDH2$ mutations (all affecting R132 and R140, respectively) were detected in 8 (15\%) and 12 (25\%) patients, respectively. No $MLL$-PTD, $TP53$, and $ASXL1$ mutations were detected in the samples obtained at diagnosis.

**At the time of relapse,** the gene mutation pattern changed in 34 patients (64\%): five patients (9\%) lost $NPM1^{\text{mut}}$ at relapse, even when applying a highly sensitive RNA-based $NPM1^{\text{mut}}$ specific RQ-PCR\textsuperscript{18} (see next section).

In 31 (97\%) relapse samples, we detected the same $DNMT3A^{\text{mut}}$ type as determined at diagnosis while in 1 patient the $DNMT3A^{\text{mut}}$ was lost. Only one (5\%) of 22 patients with $DNMT3A$ wildtype at diagnosis acquired a $DNMT3A^{\text{mut}}$ at relapse; two patients with heterozygous $DNMT3A^{\text{mut}}$ at diagnosis acquired a second $DNMT3A^{\text{mut}}$ at relapse. $FLT3$-ITD persisted in 18 of 24 patients (75\%), 2 patients (8\%) lost a heterozygous mutation and one patient (4\%) a homozygous mutation together with loss of UPD13q; in 6 cases heterozygous $FLT3$-ITD evolved to a homozygous state, 5 of them by UPD13q. Additionally, 3 of 24 patients (13\%) with $FLT3$-ITD mutation at diagnosis had a different ITD length at relapse and 7 patients lost one or more of the ITD-clones present at diagnosis while maintaining at least one of the original ITD-clones (Supplementary figure
Conversely, 9 out of 29 (31%) \(FLT3\)-ITD negative patients acquired \(FLT3\)-ITD mutations (heterozygous, \(n=7\); homozygous, \(n=2\)) at relapse. \(FLT3\)-TKD mutation was maintained at relapse in only 1 out of 11 patients (9%) and none of the 42 patients without \(FLT3\)-TKD mutation at diagnosis acquired this mutation at relapse. Only 5 of 14 (36%) patients maintained the same \(NRAS\) mutation type while 4 of 39 (10%) previously \(NRAS\) wildtype cases acquired an \(NRAS\) mutation at relapse; one patient showed a different mutation type at relapse. Two of 8 patients lost \(IDH1\) and 1 of 12 cases \(IDH2\) mutation at relapse while the remaining 6 \(IDH1^{\text{mut}}\) and 11 \(IDH2^{\text{mut}}\) patients presented with the identical mutation types at relapse. Four (9%) of 45 \(IDH1\) wildtype patients acquired a new \(IDH1^{\text{mut}}\). One (2%) \(TP53\) mutation (G245S) was detected in a relapse sample with del(17p) on cytogenetic analysis. Of note, three of five patients that lost \(NPM1^{\text{mut}}\) at relapse acquired an \(MLL\)-PTD mutation that corresponded to a small gain at 11q23.3 detected by SNP array analysis. Two patients acquired an \(ASXL1\) mutation at relapse.

**Loss of \(NPM1\) mutation at relapse**

Five patients lost \(NPM1^{\text{mut}}\) at relapse; at diagnosis we detected genomic aberrations in 3 of these patients by SNP analysis [UPN#5, del(9q21.1-q31.1); UPN#1, UPD 1pter-p32.2; UPN#4, del(9q21.32-q21.33]) (Figure 4A), while no aberrations were identified in the two other patients (UPN#2 and UPN#3). At relapse, none of these genomic lesions was detectable anymore while new aberrations occurred: patients UPN #5, UPN#1, and UPN#4 all acquired a small gain at chromosomal band 11q23 that corresponded to an \(MLL\)-PTD mutation; in addition, patient UPN#4 acquired trisomy 8, and patient UPN#1 a del(12p13.2) comprising the \(ETV6\) gene. UPN#2 maintained a normal SNP-profile at relapse, while patient UPN#3 acquired a monosomy 7 at relapse.

Characterization of concurrent gene mutation patterns revealed further insights into the clonal evolution of these 5 AML: UPN#5 lost the \(FLT3\)-TKD mutation and acquired an \(IDH1\) and \(NRAS\) mutation; UPN#4 changed its \(NRAS\) mutation type (G13V to G13D) at relapse and UPN#3 still showed the same \(IDH1\) mutation and acquired an \(ASXL1\) mutation; UPN#1 had a heterozygous \(FLT3\)-ITD mutation at diagnosis with an ITD size of 15 bp. This ITD clone was lost at relapse and two new \(FLT3\)-ITD clones with a longer size (36 and 54 bp) were acquired. The only stable marker that was consistently present
at both time points in all 5 cases was $DNMT3A^{mut}$ without any changes in mutation type. The median time interval between achievement of CR and relapse in these 5 AML cases was 33.7 months (range, 5.0 to 110.5 months) and thus significantly longer than in the 48 patients maintaining the $NPM1^{mut}$ at relapse (median: 8.6 months, range 1.3 to 57.4 months, p=0.03) (Figure 4B). UPN#3 developed peripheral blood cytopenia 87.8 months after AML diagnosis and a bone marrow smear revealed dysplasias with 5% myeloid blasts consistent with the diagnosis of MDS. At that time, cytogenetic analysis revealed monosomy 7 that was also detected after transformation in AML 22.7 months later. No history of MDS preceding AML relapse was reported in the remaining 4 patients.

None of the 5 patients with $NPM1^{mut}$ loss at relapse responded to intensive salvage therapy, while 66% of patients that maintained $NPM1^{mut}$ achieved a 2nd CR (p=0.009).

There was no striking difference with regard to clinical characteristics at diagnosis between patients that lost $NPM1^{mut}$ and patients that maintained $NPM1^{mut}$ (Supplementary table 4).

**Clonal evolution in $NPM1^{mut}$ AML**

We integrated our findings from high-resolution SNP profiling and mutation screening to assess the clonal relationship of the diagnosis/relapse pairs. Twenty-six relapses (49%) maintained all mutations, CNAs and UPDs at relapse with 10 relapses (19% of total cohort) being genetically identical and 16 (30%) acquiring additional alterations to the ones present at diagnosis. The remaining 27 relapse samples (51%) lost (with or without acquisition of a new aberration) one or more aberrations that were present in their matched diagnosis sample. Ten (19% of total cohort) of these cases simply lost aberrations or mutations present at diagnosis while in 17 (32%) we found one or more newly acquired somatic genetic lesions. None of the 53 AML diagnosis/relapse pairs showed a completely unrelated aberration pattern as all diagnosis/relapse pairs shared at least one genetic lesion (Figure 5). We used the number of identical genetic aberrations (gene mutations, CNAs, UPDs) at diagnosis and relapse in an individual patient as a quantitative estimate for genetic relationship between both AML samples. This number correlated inversely with the time to relapse with primary/relapse pairs showing a high degree of genetic relationship relapsing earlier than pairs that are less genetically related (Supplementary figure 3).
Discussion

High-resolution SNP-array profiling of 53 relapsed NPM1\textsuperscript{mut} AML patients revealed only few genomic aberrations in the primary AML samples (mean: 0.28/case) corroborating the chromosomal stability of leukemic cells in newly diagnosed NPM1\textsuperscript{mut} AML. At the time of relapse, we observed a significant increase (mean: 1.06/case) in the number of genomic alterations identified by SNP profiling. This change to a higher genetic complexity at relapse is consistent with previous studies that compared diagnosis and relapse samples in unselected AML cohorts by chromosomal banding analysis.\textsuperscript{21-24}

CNAs and UPDs detected at diagnosis were not necessarily present in the matched relapse sample as exemplified by the recurrent del(9q) that was lost in 3 of 6 (50%) cases and therefore represent late events in NPM1\textsuperscript{mut} AML. This in contrast to the study by Parkin et al.\textsuperscript{31} where all CNAs detected at diagnosis persisted at relapse in 28 cases of different cytogenetic and genetic AML subgroups and were therefore interpreted as early events.

Distinct chromosomal regions were recurrently affected by CNAs at relapse like gain of 11q23 comprising MLL (n=3) and deletions on 17p (n=2), 17q (n=2), 12p (n=3), 11p (n=3) and 3p14.2 (n=2) affecting TP53, NF1, ETV6, WT1, and FHIT, respectively. Parallel cytogenetic and SNP-array based analysis in 14 relapse samples revealed that deletions in part resulted from unbalanced translocations. The observation that most aberrations (76%) were exclusively found in relapse samples as opposed to NPM1\textsuperscript{mut} AML at diagnosis suggests that they were induced by chemotherapy or present in small subclones that were selected by therapy. Most of these aberrations found at the time of relapse are associated with high-risk AML and therefore might in part explain the poor outcome of relapsed AML.\textsuperscript{33,34}

While genomic aberrations as detected by chromosome banding analysis or SNP profiling are rare in newly diagnosed NPM1\textsuperscript{mut} AML, most cases harbor concurrent gene mutations. Comparative mutation analysis of primary and relapse samples revealed a high stability for mutations in DNMT3A (97%), IDH2 (92%) and NPM1 (91%) while FLT3-ITD (75%) and IDH1 (75%) were less stable what is mostly in line with previous studies.\textsuperscript{16,27,28,35-38} Conversely, the majority of FLT3-TKD (91%) and NRAS mutations (64%) present in the primary leukemia were not retained at relapse. The loss of a
somatic mutation or aberration at relapse implies that it is i) a late event in the
development of AML and ii) cells with these mutations are more sensitive to
chemotherapy. In this respect, previous studies did not find an impact of either FLT3-
TKD or NRAS mutations on outcome. FLT3-ITD mutations are acquired in one third
of FLT3-ITD negative patients at the time of relapse, highlighting that FLT3-ITD
mutations occur late in the pathogenesis of AML as corroborated by other studies, but
in contrast to FLT3-TKD and NRAS mutations represent important driver mutations for
relapsed AML. Furthermore, the incidence of UPD13q with homozygous FLT3-ITD
mutations increased from diagnosis (8%, n=4) to relapse (19%, n=10), similar to the
study of Raghavan et al that observed an acquisition of UPD13q at the time of relapse
in 22% of patients. In our cohort IDH1 mutations were less stable and more often
acquired than in previous studies. Therefore, IDH1 mutations might represent later
events in NPM1mut AML than in other AML subgroups. In relapsed NPM1mut AML, TP53
and ASXL1 mutations do not seem to play a major role since only one TP53 and two
ASXL1 mutations were detected at this time point.
The limited sensitivity (~5%) of the techniques used in this study did not allow us to
determine whether newly acquired mutations or genomic alterations at relapse already
pre-existed in small subclones at the time of diagnosis or whether they were acquired
during or after chemotherapy. Deep sequencing of 8 paired diagnosis/relapse AML
samples by Ding et al. could not detect the majority of the relapse-specific mutations in
the primary AML sample suggesting that these mutations were acquired after therapy.
However, even high-coverage targeted sequencing has a detection limit of ~2% and
therefore will not allow to detect very small subclones that expand under selection
pressure with chemotherapy. We also cannot rule out the possibility that distinct
mutations, in particular frequent ones like NPM1 mutation type A, were lost and re-
acquired at relapse. Within this context, one patient showed different NRAS mutation
types at diagnosis and relapse, indicating that this is a possible mechanism of clonal
evolution. Similarly, 3 of 24 patients showed different FLT3-ITD clones at relapse.
However, this was not the case for DNMT3Amut where all patients maintained the same
nucleotide changes.
Loss of NPM1mut has been described to occur in ~10% of relapsed AML. Because of
the long latency between primary leukemia and relapse, together with a diverging
pattern of genomic alterations and gene mutations, these cases were initially interpreted as therapy-related AML (t-AML) rather than relapse.\textsuperscript{17,18} Extending the number of analyzed genes in these patients, we consistently found $\text{DNMT3A}^{\text{mut}}$ in the primary and relapse sample in 5 out of 5 $\text{NPM1}^{\text{mut}}$ loss cases. For each patient, we found the same nucleotide changes in $\text{DNMT3A}$, making it more likely that a common ancestral $\text{DNMT3A}^{\text{mut}}$ but $\text{NPM1}$ wildtype clone gave rise to both primary and relapsed AML as opposed to a scenario where two hematopoietic clones have independently acquired the same $\text{DNMT3A}^{\text{mut}}$. The observation that at least in these 5 cases $\text{DNMT3A}^{\text{mut}}$ most likely preceded $\text{NPM1}^{\text{mut}}$ controverts the current concept that $\text{NPM1}^{\text{mut}}$ is generally the founder event in $\text{NPM1}^{\text{mut}}$ AML.\textsuperscript{13} However, one case in our cohort lost $\text{DNMT3A}^{\text{mut}}$ at relapse and maintained $\text{NPM1}^{\text{mut}}$ highlighting that the sequential order in which they occur is not strictly determined. $\text{DNMT3A}$ knockout in mouse hematopoietic stem cells strongly induces self-renewal, supporting its role in malignant transformation.\textsuperscript{41} However, even after several rounds of transplantation these mice do not develop AML or other myeloid malignancies, suggesting the need for secondary genetic hits. This might explain the long latency between the primary leukemia and relapse observed in the 5 patients with $\text{NPM1}^{\text{mut}}$ loss. While results are based on a very small patient number, we nevertheless find the observation intriguing as it supports the hypothesis that the persisting $\text{DNMT3A}^{\text{mut}}/\text{NPM1}^{\text{wt}}$ clone again has to acquire additional mutations to transform to AML. Further studies including patients in continuous complete remission are warranted to assess the likelihood of persisting $\text{DNMT3A}^{\text{mut}}$ clones to evolve to MDS or AML.

In our study, combined SNP-array profiling and mutational analysis revealed that all 53 relapses shared at least one mutation or aberration with their matched primary sample, implicating a common cell of origin. Based on our findings, there is no evidence for t-AML that derived from a genetically unrelated hematopoietic clone. Similarly, previous combined genomic and molecular studies in AML\textsuperscript{31,32} and acute lymphoblastic leukemia\textsuperscript{42,43} have demonstrated that the primary leukemia and relapse in general are genetically related and derive directly from the dominant leukemic clone or from a common ancestral clone. In addition, the increase of specific genomic aberrations that we found in our $\text{NPM1}^{\text{mut}}$ cohort at the time of relapse suggests that DNA-damaging chemotherapy causes secondary genetic changes in persisting (pre-)leukemic clones. In this respect, some of the specific alterations we found exclusively at relapse, such as -
7/del(7q) and der(12;17)(q10;q10), are frequent in patients that were previously treated with chemotherapy.\textsuperscript{44} This hypothesis is further supported by the whole-genome sequencing study that revealed a marked increase in the absolute number of a specific type of mutation (transversions) in the genomes of relapsed AML compared to de novo AML.\textsuperscript{32}

In our study, 26 of the 53 relapses (49%) evolved directly from the dominant primary clone as it maintained all the mutations and genomic aberrations present in the primary AML sample. The other group of 27 (51%) relapse samples lost some of the genetic changes of the initial clone but shared at least one common genetic alteration implying that they derived from an ancestral clone. Although limited by the markers studied, our data is in line with data from a whole-genome sequencing study in relapsed AML where 3 out of 8 relapses evolved from the dominant primary clone and the remaining 5 from a minor, genetically related subclone.\textsuperscript{32} Genetic characterization of FACS-sorted HSCs and leukemic stem cells of AML patient samples directly demonstrated that these (pre-)leukemic subclones that comprise some but not all mutations (mostly FLT3-ITD) co-exist with the dominant leukemic clone at diagnosis.\textsuperscript{40}

In conclusion, while NPM1\textsuperscript{mut} is generally considered as a primary event in NPM1\textsuperscript{mut} AML our data suggest that mutations in DNMT3A can precede NPM1 mutations. Even after a long latency of up to 110 months, all relapses were genetically related to the initial AML suggesting that persisting subclones or preleukemic HSCs acquire new secondary aberrations rather than being genetically unrelated t-AML. In order to achieve long-term leukemia-free survival, future therapies in AML need to eradicate all (pre-)leukemic clones present at diagnosis that persist during therapy and contribute to relapse.
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Contributions

The authors declare no competing financial interests.
References


Tables

**Table 1:** Clinical characteristics of 53 $NPM1^{mut}$ patients.

**Table 2:** Copy number alteration (CNAs) and uniparental disomies (UPDs) present at diagnosis and relapse that are recurrent or common abnormalities in AML. For the complete list see Supplementary Tables 1 to 3.
### Table 1. Patients characteristics at diagnosis (n=53)

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<tr>
<td>B</td>
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<td>9</td>
</tr>
<tr>
<td>D</td>
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<td>8</td>
</tr>
<tr>
<td>Other</td>
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<tr>
<td><strong>WBC count, x 10^9/L</strong></td>
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<tr>
<td>Median</td>
<td>23</td>
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<tr>
<td>Range</td>
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<td><strong>BM blasts, %</strong></td>
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<td>Range</td>
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<td><strong>Induction cycles, no</strong></td>
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<td><strong>Remission status after 1st induction cycle (n=53)</strong></td>
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<td>CR</td>
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<td>PR</td>
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<td>RD</td>
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<td><strong>Remission status after 2nd induction cycle (n=41)</strong></td>
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<td>CR</td>
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<tr>
<td>Relapse</td>
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<td><strong>Consolidation therapy (n=52)</strong></td>
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<td>High dose Cytarabin</td>
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<td>Allogeneic SCT</td>
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<td>Autologous SCT</td>
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<td><strong>CIR, months</strong></td>
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<td>Range</td>
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<td><strong>Overall survival after diagnosis, months</strong></td>
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<tr>
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<tr>
<td><strong>Overall survival after relapse, months</strong></td>
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<tr>
<td>Median</td>
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Abbreviations: WBC, white blood cell; BM, bone marrow; SCT, stem cell transplantation; CR, complete remission; PR, partial remission; RD, refractory disease; CIR, cumulative incidence of relapse
<table>
<thead>
<tr>
<th>Aberration</th>
<th>Candidate Genes in CDR</th>
<th>Diagnosis</th>
<th>Lost</th>
<th>Acquired</th>
<th>Relapse</th>
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<tr>
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<td>TP53</td>
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<td>Non-recurrent CNAs at Relapse</td>
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<td></td>
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<tr>
<td>Total</td>
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<td>15</td>
<td>-7</td>
<td>+48</td>
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*CDR, commonly deleted region
Figure legends

Figure 1: Frequencies of somatic copy number alterations (CNA) and uniparental disomies (UPD) identified by single nucleotide polymorphism (SNP) array. (A) Number of SNP findings (combined CNA and UPD) per patient at diagnosis and relapse. (B) Absolute number of gains (bright grey), deletions (white) and UPDs (dark grey) at diagnosis and relapse

Figure 2: Visualization of recurrent deletions on 9q and UPD on 13q that were present at diagnosis (indicated D) and/or relapse (indicated R) by dCHIP: (A) Log2 ratios of 6 patients with del(9q) at the time of diagnosis. Patients #8, #13, and #47 maintained the same deletions at both time points, while in patients #4, #5, and #21 del(9q) was lost. Blue indicates deleted and red gained chromosomal segments. (B) Cases with UPD13q at diagnosis and relapse (n=3), at diagnosis only (n=1), or at relapse only (n=7). Below the corresponding FLT3-ITD mutation status is indicated: -/- no mutation; -/+ heterozygous mutation; +/+ homozygous mutation. Blue indicates homozygous (=loss of heterozygosity), and yellow heterozygous regions of chromosome 13 as determined by SNP profiling.

Figure 3: Mutation pattern in paired (diagnosis (D) / relapse (R)) samples of 53 NPM1mut patients. Each column represents an individual patient (UPN numbers are indicated in the top row). Colored bars indicate the presence of a mutation, blank bars represent wildtype for the respective gene, data not available are indicated by a grey bar. Bright and dark green bars represent heterozygous and homozygous FLT3-ITD mutations, respectively. Bars marked by “X” represent different mutation types found at diagnosis and relapse. “Stability” was calculated by the number of mutations that persisted in relapse divided by all mutations present at diagnosis. “Acquired at Relapse” was calculated by the number of cases that acquired a new mutation at relapse and not detected at diagnosis divided by the number of cases not having the same mutation at diagnosis. Mutations in the same gene but of different type (FLT3-ITD, n=3, NRAS, n=1) in a diagnosis/relapse pair are considered as loss and new acquisition of a mutation. For
**MLL-PTD, TP53, and ASXL1** stability could not be determined (na, not applicable) as no mutations were detected at diagnosis.

**Figure 4:** (A) Genetic profiling by single-nucleotide polymorphism (SNP) array and gene mutation analyses of 5 patients with loss of $NPM1^{mut}$ at relapse (UPD, uniparental disomy; CNA, copy number alteration). Bars marked by “X” represent different mutation types found at diagnosis and relapse. (B) Cumulative incidence of relapse (CIR) in patients with loss of $NPM1^{mut}$ and patients who retained $NPM1^{mut}$.

**Figure 5:** A model of clonal evolution in $NPM1^{mut}$ AML based on the stability of each genetic marker evaluated in our study.
Figure 1

A

Aberrations per case

Diagnosis

- 4% (0)
- 21% (1)
- 75% (≥2)

Relapse

- 11% (0)
- 11% (1)
- 32% (2)
- 45% (≥3)

B

Total number of aberrations

<table>
<thead>
<tr>
<th># aberrations</th>
<th>Diagnosis</th>
<th>Relapse</th>
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<td>0 aberrations</td>
<td>10</td>
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<td>11</td>
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- UPDs
- Gains
- Deletions
Figure 2

A

B
### Figure 4

#### A

<table>
<thead>
<tr>
<th>UPN#</th>
<th>UPDs and CNAs by SNP array</th>
<th>Diagnosis</th>
<th>Time to relapse (months)</th>
<th>Relapse</th>
<th>UPDs and CNAs by SNP array</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>UPD1pter-p32.3</td>
<td></td>
<td>26.2</td>
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<td>gain(11q23.3), del(12p13.2)</td>
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<td>2</td>
<td>-</td>
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<td>5.0</td>
<td></td>
<td>x</td>
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<td>3</td>
<td>-</td>
<td>MDS 87.8</td>
<td>AML 110.5</td>
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<tr>
<td>4</td>
<td>del(9q21.32-q21.33)</td>
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<td>98.1</td>
<td>Trisomy 8, gain(11q23.3)</td>
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<tr>
<td>5</td>
<td>del(9q21.1-q31.1)</td>
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<td>33.7</td>
<td></td>
<td>gain(11q23.3)</td>
</tr>
</tbody>
</table>

#### B

- **NPM1**<sup>mut</sup> maintained
- **NPM1**<sup>mut</sup> lost

![Graph showing CIR% vs. Time (months)](image)
Figure 5

Early events
- DNMT3A
- NPM1
- IDH2

Late events
- NRAS
- FLT3-TKD
- FLT3-ITD
- UPD13q
- del(9q)
- IDH1

Events acquired at relapse
- del(17p) (TP53)
- del(12p) (ETV6)
- del(11p) (WT1)
- del(17q) (NF1)
- FLT3-ITD / UPD13q
- IDH1; NRAS

Therapy

Transformation

AML

Relapsed AML

Identical clone
n=10 (19%)

Initial clone with evolution
n=16 (30%) 49%

Ancestral clone
n=10 (19%) 51%

Ancestral clone with evolution
n=17 (32%)

Unrelated clone
0%
Clonal evolution in relapsed NPM1 mutated acute myeloid leukemia

Jan Krönke, Lars Bullinger, Veronica Teleanu, Florian Tschürtz, Verena I. Gaidzik, Michael W.M. Kühn, Frank G. Rücker, Karlheinz Holzmann, Peter Paschka, Silke Kapp-Schwörer, Daniela Späth, Thomas Kindler, Marcus Schittenhelm, Jürgen Krauter, Arnold Ganser, Gudrun Göhring, Brigitte Schlegelberger, Richard F. Schlenk, Hartmut Döhner and Konstanze Döhner

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