Evolution of Cytogenetically Normal Acute Myeloid Leukemia During Therapy and Relapse: An Exome Sequencing Study of 50 Patients

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Abstract

Purpose: To study mechanisms of therapy resistance and disease progression, we analyzed the evolution of cytogenetically normal acute myeloid leukemia (CN-AML) based on somatic alterations.

Experimental Design: We performed exome sequencing of matched diagnosis, remission, and relapse samples from 50 CN-AML patients treated with intensive chemotherapy. Mutation patterns were correlated with clinical parameters.

Results: Evolutionary patterns correlated with clinical outcome. Gain of mutations was associated with late relapse. Alterations of epigenetic regulators were frequently gained at relapse with recurring alterations of KDM6A constituting a mechanism of cytarabine resistance. Low KDM6A expression correlated with adverse clinical outcome, particularly in male patients. At complete remission, persistent mutations representing preleukemic lesions were observed in 48% of patients. The persistence of DNMT3A mutations correlated with shorter time to relapse.

Conclusions: Chemotherapy resistance might be acquired through gain of mutations. Insights into the evolution during therapy and disease progression lay the foundation for tailored approaches to treat or prevent relapse of CN-AML. Clin Cancer Res; 24(7); 1716–26. ©2018 AACR.

Introduction

The evolution of acute myeloid leukemia (AML) has been previously described either in studies of large patient cohorts with focus on only a limited set of AML-associated genes (1, 2) or in smaller series of relapsed patients studied by genome or exome sequencing (3–5). The clonal composition of AML may change during the course of disease due to the acquisition of additional mutations or competitive advantage of preexisting subclones during therapy; however, the precise influence of AML therapy as evolutionary pressure is mostly unclear. Especially in cytogenetically normal (CN) AML with intermediate prognosis, which is found in about half of adult AML patients, a better understanding of the molecular complexity and its dynamics is highly warranted. In the vast majority of relapsed AML patients the mutational profile of AML is different from the time point of initial diagnosis. For example, it was observed that lesions affecting certain genes such as DNMT3A or TET2, presumably constituting early founder mutations, tend to be stable from diagnosis to relapse, whereas mutations affecting genes such as NPM1 or FLT3 can be gained or lost during disease progression (1, 2). Recently, it was shown that, even after successful induction therapy, AML founder mutations may persist at complete remission indicating clonal hematopoiesis (6, 7). It was suggested that such clonal hematopoiesis after...
therapy represents a preleukemic stage, but the clinical relevance of preleukemic lesions remains poorly understood. Population-based screens revealed clonal hematopoesis in about 10% of healthy persons older than 65 years and found it to be associated with an approximately 10-fold increased risk for hematologic cancer (6–9). Thus, in AML patients there are at least two sources of leukemia relapse: residual leukemia cells, which have the same mutations as the presenting clone, or a preleukemic or intermediate clone harboring one or several but not all of the original leukemia driver mutations (10). In addition, acquired alterations during therapy potentially mediating resistance are likely to add to the clonal complexity of the disease. We set out to comprehensively characterize clonal evolution in a large CN-AML cohort to learn more about molecular mechanisms of relapse and therapy resistance.

Materials and Methods

Patients and clinical analysis

Patient samples were collected within the German Cancer Consortium (DKTK) at the partner sites Berlin and Munich. The median age at diagnosis was 66y (range: 21–89 years). FLT3 internal tandem duplication (ITD) and NPM1 mutation status at diagnosis was available for all patients (FLT3-ITD”/NPM1”, n = 6; FLT3-ITD+/NPM1”, n = 10; FLT3-ITD+/NPM1”, n = 19; FLT3-ITD+/NPM1”, n = 15). All patients were treated intensively with curative intent according to AMLCG or SAL protocols (10). Patients initially respond to chemotherapy, the majority of patients will eventually develop chemotherapy resistance and relapse. We have analyzed the clonal evolution of the disease and identified mutations in the histone demethylase gene KDM6A as mechanism of chemotherapy resistance in AML. KDM6A expression is gender-specific and of prognostic relevance in male AML patients. The persistence of DNMT3A mutations at remission may serve as marker for therapy response.

Sequencing and variant calling

Genomic DNA (gDNA) was extracted from archived bone marrow or peripheral blood samples using a Qiagen instrument (Qiagen). For exome sequencing, protein-coding regions of fragmented gDNA were captured using SureSelect Human All Exome target enrichment version 5 (Agilent) followed by paired-end sequencing on a Genome Analyzer Ix or HiSeq 2500 instrument (Illumina). Read mapping and variant calling were performed using BWA (11) and VarScan2 (12) software as described previously (13). On average, 94.9% (range: 75.1%–96.8%) of the target sequence was covered at least 10-fold (minimum coverage defined for variant calling). The following criteria were applied for identification of somatic mutations: number of variant supporting reads ≥3, VAF ≥20% either at diagnosis or at relapse and VAF < 5% at remission. We filtered for mutations with translational consequences based on Ensembl annotation, excluded known error-prone genes and dismissed common germline polymorphisms (dbSNP 138; MAF ≥1%). Candidate variants from exome sequencing were validated using HaloPlex targeted amplicon sequencing (Agilent). A custom amplicon library was designed covering the protein-coding regions of genes with somatic mutations detected by exome analysis and known mutational targets in AML. On average, 95.7% of the target region was covered at least 30-fold (range 92.5–97.1). Diagnostic samples were sequenced at a mean coverage of 568.1 (range 435.5–933.9), relapse samples were sequenced at a mean coverage of 603.2 (range 364–816.1), remission samples were sequenced at a mean coverage of 370.3 (range 170–592.4).

HaloPlex-specific primer sequences were trimmed, and variants were detected with the following cutoffs: minimum coverage 30, minimum number of variant supporting reads ≥3. The minimum VAF was lowered to 10%. Variants detected in exome data but not validated by amplicon sequencing were excluded from further analysis. Given the higher sequence coverage, VAFs from amplicon sequencing were used for further analysis. Mutation gain was defined as VAF < 5% at diagnosis and VAF ≥10% at relapse. Mutation loss was defined as VAF ≥10% at diagnosis and VAF < 5% at relapse. FLT3-ITD and NPM1 mutations status was obtained from routine diagnostic tests as the detection of these markers by NGS is not reliable.

For comparative analysis of mutation types (transitions vs. transversions) between diagnosis and relapse, we used MuTect (14) software enabling detection of low-coverage SNVs with high sensitivity (15).

Cell culture

Low-passage cell lines Mono-Mac-1 (MM-1, DMSZ no.: ACC-252) and Mono-Mac-6 (MM-6, DMSZ no.: ACC-124) were obtained in 2016 (MM1) and 2013 (MM6) from the German Collection of Microorganisms and Cell Culture (DSMZ) and cultured according to the manufacturer’s recommendation. Authentication of cell lines was performed by DSMZ using short tandem repeat (STR) typing. In addition, mutation analysis was performed by targeted, multiplexed amplicon sequencing as described previously (16). Testing for Mycoplasma contamination was done using the MycoAlert Mycoplasma detection kit (Lonza), on a weekly basis and before the start of experiments. After thawing, cells were kept in culture for a maximum period of 5 to 6 weeks.

Western blotting

Whole-cell lysates were extracted with lysis buffer containing 50 mmol/L HEPES pH 8.0, 150 mmol/L NaCl, 100 mmol/L NaF, 10 mmol/L Na4P2O7, 1 mmol/L EDTA, 10% (v/v) glycerol, 1% (v/v) TritonX-100, 5 μg/ml aprotonin, and 1 mmol/L phenylmethylsulfonylfluoride. Nuclear proteins were extracted with the Oproteome nuclear protein kit (Qiagen). Denatured samples were loaded on an 8% or 15% gel for SDS-PAGE, followed by immunoblotting on nitrocellulose membrane (Amersham Protran, GE Healthcare) using a wet blotting system (Bio-Rad Mini Protein Tetra system, Bio-Rad) at 4°C overnight. Immunoblots

Translational Relevance

Even though two-thirds of acute myeloid leukemia (AML) patients initially respond to chemotherapy, the majority of these patients will eventually develop chemotherapy resistance and relapse. We have analyzed the clonal evolution of the disease and identified mutations in the histone demethylase gene KDM6A as mechanism of chemotherapy resistance in AML. KDM6A expression is gender-specific and of prognostic relevance in male AML patients. The persistence of DNMT3A mutations at remission may serve as marker for therapy response.
were blocked with 5% nonfat dried milk/Tris-buffered saline with 0.1% (v/v) Tween, incubated with primary and secondary antibody dilutions, and developed using enhanced chemiluminescence (ECL; Pierce; Thermo Fisher Scientific). Proteins were visualized on Fusion SL imaging system (Vilber Lourmat). The following antibodies were used: anti-KDM6A (ab91231, Abcam) and anti-tubulin (TG199, Sigma-Aldrich), anti-H3 (ab1791, Abcam), anti-H3K27me1 (07-448, Merck Millipore), anti-H3K27me2 (07-452, Merck Millipore), and anti-H3K27me3 (07-449, Merck Millipore). Western blot signals were quantified using ImageJ version 1.50d and relative protein levels were normalized to tubulin or histone H3 levels.

Proliferation assay
MM-1 and MM-6 cells were treated with cytarabine (Sigma-Aldrich), daunorubicin (Selleck Chemicals) or AC220 (quizartem) using dilution series ranging from 100 nmol/L to 5 nmol/L; Selleck Chemicals) using dilution series ranging from 100 nmol/L to 5 nmol/L for cytarabine, 5–640 nmol/L for daunorubicin and 2–128 nmol/L for AC220. After 72 or 96 hours, viable cells were counted on Vi-Cell Cell Viability Analyzer (Beckman Coulter) with Trypan Blue exclusion. Unpaired two-tailed Student t test and calculation of half-maximal inhibitory (IC50) concentration values were performed using GraphPad Prism version 6.07 (GraphPad Software).

Apoptosis analysis
After treatment with cytarabine (0.01–0.8 µmol/L) for 48 hours, amount of apoptotic cells were measured by Annexin V-PE and 7-amino-actinomycin D (7-AAD) staining (Annexin V-PE Apoptosis Detection Kit, BD Pharmingen) as recommended by the manufacturer using a FACSCanto II flow cytometer (BD Biosciences). Briefly, cells were washed twice with ice-cold PBS and then incubated with 100-µL binding buffer, 5 µL Annexin-V-PE, and 5 µL 7-AAD for 15 minutes at room temperature in the dark. Next, 400 µL of binding buffer was added and apoptotic cells, early (Annexin-V-PE positive) and late (Annexin-V-PE and 7-AAD positive) apoptotic, were determined by flow cytometry.

Multiplex ligation-dependent probe amplification
MLPA analysis was carried out according to the manufacturer’s recommendations (MRC Holland). Briefly, 50 ng of DNA purified from MM-1 and MM-6 cells was used as starting material with the SALSA MLPA P445 KDM6A probe mix. After hybridization, ligation, and amplification steps, products were run on an ABI Prism 3500 XL Genetic Analyzer (Applied Biosystems) and analyzed using the CoffalyserNet software version v.140721.1958 (MRC Holland). The ratio of each relative probe signal from cell lines compared with control samples was calculated. The following human genomic DNA was used as control samples: female G01 (Biochain). Gene expression levels (GC0XP044732_at) was evaluated using the Kaplan–Meier method and the log-rank test. High KDM6A expression was defined as >75th percentile, whereas low expression was defined as ≤25th percentile of expression values. All patients included in this analysis were treated on clinical trial (NCT002666136).

For validation, we evaluated KDM6A expression levels (203990_s_at) in publicly available from the HOVON study group (GSE6891). In RNA-Seq data from TCGA (www.cbioportal.org), the analysis was limited to intensively treated patients and, due to the smaller sample size, high KDM6A expression was defined as ≥50th percentile and low expression was defined as <50th percentile.

Results
Mutation profile and clonal evolution
We sequenced exomes of matched bone marrow or peripheral blood samples taken at diagnosis, complete remission and relapse of 50 CN-AML patients. Patient characteristics are provided in Supplementary Table S1 and Supplementary Table S2. Candidate mutations were validated by targeted amplicon sequencing. In total, we identified 432 genes to be affected by somatic mutations with translational consequences, of which 38 were recurrently mutated in our cohort (Fig. 1; Supplementary Table S3). Mutation frequencies in our cohort compared with 86 CN-AML patients at diagnosis reported by The Cancer Genome Atlas (18) are shown in Supplementary Fig. S1. In comparison with the clinically nonselected TCGA patients, we found higher frequencies of mutations in genes associated with poor prognosis (FLT3, RUNX1) in our cohort of relapsed CN-AML patients at diagnosis. NPM1 and DNMT3A were also more frequently mutated in our cohort likely due to their association with FLT3–internal tandem duplication (ITD). Two genes were recurrently altered only at diagnosis (CBL and TP53N11) and 8 genes were recurrently altered only at relapse in our cohort (e.g., KDM6A, DDR1, and NFE2). At diagnosis, the number of somatic mutations per patient varied between 2 and 28 (median: 10). The number of mutations did not differ significantly between patients with ELN Favorable or Intermediate I risk classification (ref. 19; P = 0.81, unpaired, two-tailed Student t test). At relapse, the number of mutations increased ranging from 1 to 47 (median: 11.5, P = 0.05, two-tailed Wilcoxon signed-rank test). Mutations in several AML-associated genes (e.g., DNMT3A, RUNX1, IDH1, and IDH2) showed similar variant allele frequencies (VAF) at diagnosis and relapse in the vast majority of cases. In contrast, WT1 mutations were gained at relapse in 5 of 50 (10%) patients and FLT3 point mutations were lost at relapse in 4 of 7 (57%) patients initially positive for these variants. In total, 67 mutations present at diagnosis were lost at relapse (VAF<5%) while 104 mutations were acquired during disease progression. Mutations of WT1, IDH1, KDM6A, and KPNB1 were recurrently gained at relapse.

We found 19 genes mutated in at least two patients and not previously reported as mutational targets in AML. Several of these genes have been reported to be recurrently mutated in other hematologic malignancies (i.e. MYD88, STAT5A, KMT2D, NFE2, refs. 20–23), while both ATP2B2 and DRD5 are downstream targets of the leukemic KMT2A/MLLT3 fusion gene (24). On the basis of cytogenetics and copy number alteration (CNA) analysis of exome sequencing data (25), we detected partial or complete gain/loss of chromosomes (Fig. 1B; Supplementary Table S2). Five patients (10%) acquired chromosomal alterations during disease progression. Trisomy 8 was the only recurrent chromosomal abnormality gained in 3 patients (6%) at relapse.
In summary, clonal evolution, that is, gain or loss of sequence variants, cytogenetic, or copy-number changes at the time point of relapse, was observed in 47 of 50 (94%) of patients.

Evolutionary patterns are associated with time to relapse

From diagnosis to relapse we observed distinct mutational patterns in the individual patients defined by overall stability, gain, loss or both gain and loss of genetic lesions (Fig. 2A–C; Supplementary Figs. S2 and S3). This analysis included sequence variants as well as cytogenetic changes or CNAs (hereafter referred to as "mutations"). On the basis of these mutational patterns, patients were classified into the following subgroups: (i) patients with an identical mutation profile at diagnosis and at relapse ("stable", n = 3, 6%), (ii) patients who gained mutations at relapse ("stable + gain", n = 24, 48%), (iii) patients that lost mutations at relapse ("stable + loss", n = 8, 16%), and (iv) patients with both gain and loss of mutations at relapse ("mixed", n = 15, 30%). Individual genes altered in several patients also tended to follow a particular pattern regarding the stability of their somatic lesions over time (Fig. 1B; Supplementary Fig. S4). The time from remission to relapse is an important clinical indicator of disease aggressiveness. Patients relapsing within the first 6 months have a poor prognosis in terms of response to salvage therapy and overall survival compared to patients with a later relapse (26).

We therefore correlated the four different evolutionary patterns with time to relapse (Fig. 2D and E). Patients with "stable + loss" relapsed earlier (median 4.1 months) than patients with gain of mutation at relapse (groups "stable + gain" and "mixed", median 12.2 months). All patients in the category "stable + loss" developed relapse within the first year after complete remission. The "stable" group of three patients showed an intermediate time to relapse (median 9.6 months), but was too small for a statistically valid comparison. Hence, we simplified the stratification and compared the outcome of patients with and without evidence...
Figure 2. Patterns of clonal evolution. A, Exemplary VAF plots for the different evolutionary patterns observed from diagnosis to relapse. Each line represents one mutation. B, VAF plot of all identified mutations. C, Proportion of the observed patterns. Time to relapse according to evolutionary patterns (D) and gain of mutations (E) during disease course.
for mutation gain at relapse (Fig. 2E). Ultimately, the clinical course of CN-AML patients without gain of new mutations at relapse (categories "stable" and "stable + loss") was associated with significantly earlier relapse compared with patients who gained mutations at relapse (categories "stable + gain" and "mixed"; \( P = 0.001 \), two-tailed log-rank test). The number of patients without any evidence for gain of genetic lesions during the disease course is significantly higher among patients relapsing within 12 months after CR (15/28, 54% vs. 4/22, 18%; \( P = 0.02 \), two-tailed Fisher exact test, Supplementary Fig. S5). In 21 of 39 (54%) patients with gained mutations at relapse, at least one of the gained variants was detectable already at initial diagnosis with VAF ranging from >0% to <5%.

Distinct predominant patterns of clonal evolution were observed associated with the ELN groups, as only one patient of the "stable + loss" group was initially classified as Favorable. Interestingly, applying the ELN classification to relapse samples revealed a switch from Favorable to Intermediate-I in 6 patients, all with gain of mutations at relapse. This points toward more aggressive genetic profiles at relapse in these patients.

**Mutations in genes linked to epigenetic regulation are frequently gained at relapse**

Recent sequencing studies have categorized the growing number of recurrently mutated genes into different functional groups, for example, myeloid transcription factors, tumor suppressors, signal transducers, chromatin modifiers, cohesin-complex, and spliceosome-complex (Supplementary Fig. S6). With regards to the evolutionary patterns observed in our patients, we found that mutations in genes linked to epigenetic regulation are frequently gained during the progression of CN-AML. Besides genes directly involved in chromatin modification (i.e., DNMT3A, TET2, ASXL1, KDM6A, KDM2A, NSD1, UTX, and EZH2), we also considered WT1 and IDH1/2 as "epigenetic regulators" as they are known to modify expression (27, 28).

At diagnosis, 38 of 50 patients (76%) carried a total of 58 mutations in epigenetic regulators (Fig. 3A). Many patients (\( n = 16 \)) harbored two mutations affecting this functional group, while one such mutation was observed in 22 patients. The median VAF of these mutations was 44% (range: 6%–95%), indicating that mutations in epigenetic regulators are early events and are present in the founding clone. Remarkably, of the 58 mutations detected at diagnosis, none was lost at relapse, highlighting the stability of these lesions during disease progression. Moreover, in 10 of 50 patients (20%) a total of 14 mutations in epigenetic regulators were acquired at relapse (Fig. 3A). All but one of these patients already had a mutation in another epigenetic regulator at diagnosis. We did not identify patients who acquired DNMT3A, TET2 or ASXL1 mutations during disease progression. However, mutations in WT1, IDH1, and KDM6A were gained in several patients at relapse. Seven of the 14 gained mutations were already detectable at low levels at diagnosis (median VAF: 0.6%; range: 0.06%–4.8%; mean coverage at the investigated sites: 509, range: 124–1,688).

**Loss of KDM6A increases resistance toward cytarabine treatment**

Four patients gained mutations in histone demethylase genes. Besides gain of KDM6A mutations in two patients, additional histone demethylase genes (KDM2A and UTY also known as KDM6L) were altered in a relapse-specific manner, each in one patient (Fig. 3B). In two patients, relapse-specific mutations in KDM6A targeted a C-terminal hotspot within the JmjC (Jumonji C) domain, responsible for histone demethylation (Fig. 3C). On the basis of a large-scale drug screen in cancer cell lines (29), we hypothesized that loss of KDM6A function might render leukemia cells more resistant to treatment with cytarabine, a drug commonly used in AML induction therapy. MM-1 and MM-6 are sister cell lines, which have been established from the same refractory AML patient (30). Targeted sequencing of AML driver genes in MM-1 and MM-6 showed an identical mutation profile (Supplementary Table S4). Interestingly, while the two cell lines share most of their driver mutations (e.g., KMT2A/MLLT3 fusion, FLT3 592A), only MM-6 cells were reported to harbor a homozygous deletion (exon 3-10) in the KDM6A gene (31). Indeed, we confirmed the KDM6A deletion in MM-6 by multiplex ligation-dependent probe amplification (Supplementary Fig. S7). On the protein level, we did not detect any KDM6A expression in the MM-6 cells, in contrast to MM-1 cells with detectable KDM6A protein (Fig. 3D). Absence of KDM6A in MM-6 was associated with a trend for decreased mono- or di-methylation and increased tri-methylation of histone H3 K27 (Supplementary Fig. S8). Upon cytarabine treatment, the KDM6A-mutant cell line MM-6 showed enhanced resistance and a lower apoptosis rate, as compared with the KDM6A-wild-type MM-1 cell line (Fig. 3E and F). Determination of the IC\(_{50}\) concentration value of cytarabine revealed an IC\(_{50}\) about 4-fold higher in the MM-6 cells (852 nmol/L) than in the MM-1 cell line (199 nmol/L). Hence, loss of KDM6A expression appears to be linked to an increased resistance to cytarabine treatment. In contrast, both MM-1 and MM-6 cells responded in a similar manner to treatment with daunorubicin or with the tyrosine kinase inhibitor AC220 underscoring the specific nature of cytarabine resistance in MM-6 (Supplementary Fig. S9). Consistent with a role in treatment failure, low KDM6A expression at diagnosis correlated with poor clinical outcome in a cohort of CN-AML patients treated within the AMLCG-99 clinical trial (Fig. 3G). Interestingly, the clinical relevance of KDM6A expression was most obvious in male patients (Supplementary Fig. S10A) pointing toward a gene dosage effect (KDM6A is located on the X-chromosome). The correlation of KDM6A expression with clinical outcome and gender was confirmed in publicly available data from the HOVON study group (32) and TCGA (18) (Supplementary Fig. S10B and S10C). Overall, KDM6A expression was lower in male than in female patients (Supplementary Fig. S10D).

Other recurrently mutated pathways and gene families

Facing the genetic complexity from diagnosis to relapse, we looked for additional recurrently altered gene families or pathways with a potential role in malignancy. In contrast to epigenetic regulators, mutations of genes involved in signaling pathways (e.g., receptor tyrosine kinase genes, RAS signaling: hereafter named signaling genes) were rather unstable, with frequent gains and losses from diagnosis to relapse (Supplementary Fig. S6). Of note, in 6 of 50 patients (12%) mutations in signaling genes (e.g., in FLT3 or NRAS) were lost and other mutations in signaling genes were acquired either in a different signaling gene or at a different position in the same gene (e.g., loss of FLT3-ITD and gain of FLT3-point mutation). Moreover,
the kinesin gene family (KIF1C, KIF25, KIF26B, KIF2B, KIF2C, KIF5A) was recurrently altered with a stable or relapse-specific pattern in 5 patients (10%). In a similar fashion, dopamine receptors 1 and 5 (DRD1/5) were altered each in two patients and DRD3 in one patient (in total 10%). Another gene family targeted by mutations in a total of 3 patients (6%) were the ABCA transporters (ABCA2/4/12).

A chemotherapy scar reflects treatment-induced mutagenesis
As intensive chemotherapy has mutagenic potential, we compared the different types of mutations found at diagnosis and at relapse (Supplementary Fig. S11). Single-nucleotide variants (SNV) specific to the relapse sample showed an increased prevalence of transversions (i.e., changes from purine to pyrimidine bases and vice versa) compared with diagnosis-specific SNVs (P =

Figure 3.
Mutations in genes linked to epigenetic regulation. A, Distribution of mutations in 11 selected genes. Each column represents one patient. Each line represents one gene. B, VAF plots for mutations in histone demethylase genes. Each line represents one patient. C, Protein structure of KDM6A (NP_066963.2). The identified mutations (red) affect the jumonji (JmjC) domain. D, Western blot analysis of KDM6A expression in the sister cell lines MM-1 and MM-6. Tubulin serves as a loading control. E, Cytarabine (Ara-C) treatment of MM-1 and MM-6 cells. F, Analysis of apoptosis rate after Ara-C treatment of MM-1 and MM-6 cells. The dotted line indicates the amount of early apoptotic cells without treatment. G, Overall survival of CN-AML patients treated within the AMLCG-99 trial (NCT00266136) stratified according to KDM6A expression. *, P < 0.05; **, P < 0.01; ***, P < 0.001; unpaired, two-tailed Student t test; In growth curves, error bars indicate mean ± SD of at least three independent experiments.
Figure 4.
Persistent mutations at remission. A, Distribution of identified persistent mutations. VAF plots of persistent DNA\(_{M3A}\) mutations (B), other epigenetic regulators (C), and spliceosome components (D). E, Persistence of DNA\(_{M3A}\) mutation at remission is associated with shorter time to relapse.
Persistence of AML driver mutations in remission

To detect preleukemic lesions (6, 7), we evaluated our sequencing data for the persistence of mutations at the time point of remission. To avoid confounding with private germline variants, we limited our analysis to genes that are known to be AML associated. We included only mutations that were (i) previously reported in cancer, or (ii) resulted in a truncated protein sequence, or (iii) showed changes in VAF, and (iv) were not reported as common SNPs. Strikingly, 24 of 50 (48%) of patients carried 39 nonsilent mutations with VAF > 5% in remission (median: 21%, range: 6%-73%). Recurring persistent mutations were observed in DNMT3A, TET2, SRSF2, ASXL1, IDH2, and SF3B1 accounting for 38%, 8%, 6%, and 4% each of our patients, respectively (Fig. 4A). In addition to DNMT3A and other epigenetic regulators, spliceosome components constituted the second group of genes recurrently affected by persistent mutations. Of note, 9 patients had more than one persistent mutation in remission. On the basis of VAF, 35 of 39 (90%) of the persistent mutations showed a dynamic pattern over the course of disease with a relative change of >20%, likely due to partial eradication and expansion of leukemic and/or preleukemic clones (Fig. 4B–D). In contrast, other mutations (e.g., in FLT3 or Nras) found in these patients could not be detected in remission or only at a VAF <1%, consistent with therapy response.

Longer time to relapse was observed in patients with DNMT3A mutations that did not persist in remission (np-DNMT3A, n = 8) in comparison with patients with persisting DNMT3A mutations (p-DNMT3A, n = 19; Fig. 4E). There was no significant difference in the sequence coverage of the relevant DNMT3A positions between the two groups of patients (Supplementary Fig. S12). Remarkably, the latter group showed a trend for enrichment of patients that also harbored FLT3-ITDs at diagnosis (12/19 vs. 2/8; Fisher exact test, \( P = 0.10 \)). The vast majority of p-DNMT3A mutations affected R882, whereas mutations at other positions in DNMT3A tended to be cleared in remission more often. When considering the NPM1 and FLT3-ITD status as well, only 1 of 8 patients with np-DNMT3A originally showed NPM1c and FLT3-ITD mutations as well (triple mutated), compared with 10 of 19 patients with p-DNMT3A, suggesting that cooccurrence of DNMT3A, FLT3-ITD and NPM1c is associated with p-DNMT3A (Fisher exact test, \( P = 0.08 \)). The nonclearance of DNMT3A mutations in remission did not correlate with age in our study (median age: 65 vs. 64, persistent vs. nonpersistent; \( P = 0.48 \); two-tailed, unpaired Student t test). When comparing patients with persistent mutations in any gene to patients without any evidence for persistent mutations in remission, there was no significant difference regarding time to relapse (log-rank-test, \( P = 0.34 \); Supplementary Fig. S13).

**Discussion**

Our analysis of the mutational landscape of relapsed CN-AML has provided us with several important insights. In some patients, the AML cells may require additional genetic alterations to become chemotherapy resistant, whereas in other patients the selective eradication of a sensitive clone is a potential mechanism underlying disease progression. The acquisition of additional mutations and/or the outgrowth of a resistant clone during and after chemotherapy appear to require a longer time or is per se associated with a longer time to relapse and a more favorable prognosis. Loss of mutations at relapse suggests the presence of two clones at diagnosis, with a chemotherapy resistant clone expanding after the eradication of the sensitive one. As both clones share mutations and only the sensitive clone contains specific alterations, the resistant clone might be an ancestor of the sensitive clone. Thus, it will be challenging to predict clinical outcome and evolutionary trajectories based on the clonal architecture at diagnosis. However, our findings suggest that relapse-associated clones may preexist before therapy and the detection of relapse-associated mutations at low levels in the diagnostic sample may facilitate the anticipation of an early relapse.

We found that WT1, IDH1, KDM6A, and KPNB1 tend to gain mutations at relapse suggesting that mutations in these genes are drivers of disease progression and might play a role in therapy resistance. Of note, mutations of both WT1 and IDH1 impair TET2-mediated conversion of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (27, 28), whereas KDM6A encodes a histone H3K27 demethylase involved in HOX gene regulation (33, 34). Indeed, two additional histone demethylase genes, namely KDM2A and UTY, acquired mutations at relapse each in one patient, suggesting a recurrently altered gene family (Fig. 3A and B). KPNB1 (karyopherin beta 1) mediates nucleocytoplasmic transport and acts downstream of the histone methyl transferase EZH2 (35). In total, 22% of our patients showed relapse-specific lesions of WT1, IDH1, KDM6A, KDM2A, UTY, or KPNB1. These findings highlight the importance of epigenetic mechanisms for the progression of AML. In particular, inactivation or loss of KDM6A plays a role in cytarabine resistance based on our findings in the KDM6A-mutant cell line MM-6 (Fig. 3E and F). In line with these results, we observed a positive correlation between KDM6A expression and overall survival in CN-AML patients (Fig. 3G), suggesting KDM6A as potential biomarker for treatment response. KDM6A is located on the X-chromosome and was reported as gender-specific tumor suppressor in male patients with T-cell acute lymphoblastic leukemia (36). Thus, female cells with two KDM6A gene copies likely have more functional reserves to compensate for downregulation or inactivation of KDM6A compared with male cells carrying only a single copy. Consistent with the adverse prognostic relevance of low KDM6A expression affecting predominantly male AML patients in the current study, it was recently shown that in females KDM6A and other tumor suppressor genes may escape from X-inactivation (37). Of note, both our AML patients with relapse-specific KDM6A mutations were females, however, one of the two lost an X-chromosome at relapse based on CNA analysis, consistent with a VAF for the KDM6A mutation of 91.4% (Fig. 3B). Inactivating KDM6A mutations have been reported in a variety of cancer types and KDM6A reintroduction into cancer cells with inactivating KDM6A mutations reduced proliferation (31). Deletions of KDM6A are associated with disease progression in a mouse model of acute promyelocytic leukemia (38). Alterations of KDM6A were also reported in therapy-resistant metastatic prostate cancer (39).

Other recurrently altered pathways or gene families with implications in cancer include kinesin and dopamine receptor (DRD) genes. Kinesins are involved in mitosis and have emerged as
potential targets for cancer drug development (40), whereas dopaminergic receptor expression was shown to correlate with engraftment of patient samples in xenotransplants and prognosis in AML (41). The dopamine receptor antagonist thiordazine is currently being evaluated for the treatment of relapsed or refractory AML in an early-stage clinical trial (NCT02096289). Remarkably, DRD1 and DRD5, each mutated in two of our AML patients, are both small genes each encoded by a single exon. Given that the probability to acquire mutations randomly increases with gene size, it seems rather unlikely that recurring DRD1 and DRD5 mutations are bystanders.

The higher frequency of transversion-type mutations found in post-chemotherapy relapse samples is consistent with previous reports (3, 42), demonstrating that cytarabine treatment increases the mutational load of AML cells. However, it remains unclear to which extent this effect contributes to the evolution of relapse as there is no control group of relapsed patients, who had not received induction therapy including high-dose cytarabine.

A very important finding is that DNMT3A and TET2 mutations are the most stable genetic lesions representing not only initiating events, but also being indispensable drivers during the course of disease. It is therefore quite obvious that failure of DNMT3A clearance at remission has adverse prognostic effects. Considering that our detection of persistent mutations was limited to known AML driver genes (to avoid confounding with germline SNPs) the persistence of mutations in unknown AML drivers might have been overlooked.

In summary, our findings provide insights into the genetic evolution during the course of disease in a large uniformly treated cohort of relapsed CN-AML. Better knowledge of the dynamics of genetic lesions (e.g., persistent or relapse-specific mutations) will improve our prognostic and disease monitoring capabilities and allow for tailored approaches to treat or to prevent relapse in AML.

References


Disclosures of Potential Conflicts of Interest

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Greif et al.

Evolution of Cytogenetically Normal Acute Myeloid Leukemia During Therapy and Relapse: An Exome Sequencing Study of 50 Patients

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