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Analysis of nonleukemic cellular subcompartments reconstructs clonal evolution of acute myeloid leukemia and identifies therapy-resistant preleukemic clones

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Abstract

To acquire a better understanding of clonal evolution of acute myeloid leukemia (AML) and to identify the clone(s) responsible for disease recurrence, we have comparatively studied leukemia-specific mutations by whole-exome-sequencing (WES) of both the leukemia and the nonleukemia compartments derived from the bone marrow of AML patients. The T-lymphocytes, B-lymphocytes and the functionally normal hematopoietic stem cells (HSC), that is, $CD34^+/CD38^-/ALDH^+$ cells for AML with rare-$ALDH^+$ blasts (<1.9% $ALDH^+$ cells) were defined as the nonleukemia compartments. WES identified 62 point-mutations in the leukemia compartment derived from 12 AML-patients at the time of diagnosis and 73 mutations in 3 matched relapse cases. Most patients (8/12) showed 4 to 6 point-mutations per sample at diagnosis. Other than the mutations in the recurrently mutated genes such as *DNMT3A*, *NRAS* and *KIT*, we were able to identify novel point-mutations that have not yet been described in AML. Some leukemia-specific mutations and cytogenetic abnormalities including DNMT3A(R882H), EZH2(I146T) and inversion(16) were also detectable in the respective T-lymphocytes, B-lymphocytes and HSC in 5/12 patients, suggesting that preleukemia HSC might represent the source of leukemogenesis for these cases. The leukemic evolution was reconstructed for five cases with detectable preleukemia clones, which were tracked in follow-up and relapse

Abbreviations: AF, allele frequency; ALDH, aldehyde dehydrogenase; AML, acute myeloid leukemia; BM, bone marrow; CR, complete remission; DS, deep sequencing; HSC, hematopoietic stem cells; HSCT, hematopoietic stem cell transplantation; LSC, leukemia stem cells; MNC, mononuclear cells; NGS, next generation sequencing; nl-HSC, nonleukemic hematopoietic stem cells; pl-HSC, preleukemic HSC; SSP-PCR, sequence-specific primer PCR; WES, whole-exome-sequencing.

samples. Four of the five patients with detectable preleukemic mutations developed relapse. The presence of leukemia-specific mutations in these nonleukemia compartments, especially after chemotherapy or after allogeneic stem cell transplantation, is highly relevant, as these could be responsible for relapse. This discovery may facilitate the identification of novel targets for long-term cure.

KEYWORDS
acute myeloid leukemia (AML), clonal evolution, hematopoietic stem cells (HSC), relapse

1 | INTRODUCTION

Acute myeloid leukemia (AML) is considered a hematopoietic stem cell-derived malignancy which is associated with a number of specific molecular and cytogenetic abnormalities.[1] In the last decades, many AML-associated mutations have been identified in certain genes including the fms-related tyrosine kinase 3 (*FLT3*), CCAAT/enhancer binding protein alpha (*C/EBPa*), runt-related transcription factor 1 (*RUNX1*), myeloid-lymphoid or mixed lineage leukemia (*MLL*), Wilms tumor (*WT1*) and nucleophosmin 1 (*NPM1*).[2] These mutations were formerly classified into two classes according to the two-hit hypothesis of leukemogenesis.[2,3] Present evidence changes our understanding of AML evolution from the simple two-hit model to a multistep process that requires accumulation of several mutations over time.[4,5] Recently, more molecular abnormalities associated with AML have been identified by the use of sequencing approaches like next generation sequencing (NGS).[2,6,7] As many of these mutations have been shown to be associated to good or poor prognosis, the understanding of origin and function of these mutations at an early stage of transformation provides an important knowledge to predict the risk of progression or relapse for individual patients.[5,8]

Data from functional studies suggest that certain mutations may be able to transform normal hematopoietic stem cells (HSC) into leukemic stem cells (LSC).[9,10] The molecular mechanisms of AML evolution; however, are not yet fully understood.[1,11] To define the molecular mechanisms of AML evolution, a precise molecular comparison between HSC and LSC of the same patient is necessary.[12,13] Recent studies suggested that it is feasible to distinguish functionally normal HSC from leukemic cells.[12,14-16] T-cell immunoglobulin mucin-3 (TIM-3) has been reported as LSC marker[12,17] and $CD34^+/CD38^-/TIM\text{-}3^-$ cells isolated from AML patients represent functionally normal HSC.[12] High aldehyde dehydrogenase (ALDH) enzyme activity has also been shown that it can be used to distinguish HSC from leukemic cells.[13] We have previously shown that functionally normal HSC (called also nonleukemic HSC [nl-HSC]) can be separated from most AML patients using the surface marker combination $CD34^+/CD38^-$ and high ALDH enzyme activity ($CD34^+/CD38^-/ALDH^+$).[16,18] These cells were examined in vivo and in vitro to confirm their normal functionality. Moreover, cytogenetic and molecular genetic analysis showed that this population contained no diagnostic molecular and cytogenetic abnormalities (eg, FLT3-ITD, NPM1, inv(16) and trisomy 8).[16]

Comparative analysis of leukemic and nonleukemic cells like HSC and T-cells from patients with AML have shown that these nonleukemic cells also carry leukemia-specific mutations leading to the concept of "preleukemic HSC" (pl-HSC). These pl-HSC are functionally normal and are believed to represent the cells from which AML arises by acquiring additional mutations.[12,14,19,20] Mutations in genes like *DNMT3A*, *TET2*, *IDH1*, *IDH2*, *NPM1*, *ASXL1*, *IKZF1* and *SMC1A* were detectable in residual HSC and progenitor cell (HSPC) populations isolated from de novo AMLs.[12,14,19,20] These mutations by themselves appear to function only as preleukemic mutations unable to induce AML. In this scenario, additional mutations like FLT3-ITD and NRAS mutations are necessary to transform pl-HSC into AML.[12,20] Despite these findings, the role of pl-HSC in AML evolution remains not fully understood and it is unclear whether chemotherapy-resistant pl-HSC play a role in AML recurrence.

In the present study, we examined 12 de novo AML cases in order to identify initiating mutations and to study the role of pl-HSC in AML evolution and relapse.

What's new

Acute myeloid leukemia (AML) develops and progresses through a multistep process, involving the accumulation of multiple molecular abnormalities over time. Little is known, however, about AML-inducing mutations and the role of clones in AML evolution and prognosis. Here, using molecular comparison of leukemic and non-leukemic cells derived from the same bone marrow of AML patients, the authors identified novel point mutations and initiating mutations in AML. Moreover, leukemic evolution was reconstructed from pre-leukemic clones in five patients, four of whom experienced relapse. The findings provide insight into clonal evolution in AML and could aid the identification of novel therapeutic targets.

2 | MATERIALS AND METHODS

2.1 | Sample collection and preparation

Diagnostic, follow-up and relapse bone marrow (BM) aspirates and peripheral blood samples were obtained from 12 patients suffering from de novo AML. Mononuclear cells (MNC) were isolated using Biocoll separation solution (Biochrom, Berlin, Germany) by gradient centrifugation and frozen in liquid nitrogen with FCS/12.5% DMSO for future studies as described previously.[16,18] Patients' characteristics are available in Table 1.

TABLE 1 Patient's characteristics

Patient Nr.	Classifica-tion	Sex	Age at diagnosis (years)	Karyotype	Allogeneic SCT in months (M)	Relapse in months (M)	Survival in months (M)	Diagnostic clonal markers (FLT3-ITD, NPM1, MLL-PTD, CEBPA and inv(16)
AML 1	AML	Female	56	Normal	No	4 M	4 M	FLT3-ITD, NPM1
AML 2	AML	Male	71	Normal	No	No	4 M	FLT3-ITD, NPM1
AML 3	AML	Male	58	Inversion(16)	No	No	>60 M[a]	Inv(16)
AML 4	AML	Male	52	Normal	4 M	7 M	11 M	FLT3-ITD, MLL-PTD
AML 5	AML	Female	62	Normal	No	No	26 M	CEBPA
AML 6	AML	Female	55	Complex aberrant	3 M	43 M	47 M	All negative
AML 7	AML	Male	72	Normal	No	10 M	13 M	FLT3-ITD
AML 8	AML	Male	54	Complex aberrant	No	1.5 M	2 M	All negative
AML 9	AML	Male	61	Trisomy 8	4 M	7 M	8 M	All negative
AML 10	AML	Male	45	Inversion(16)	No	No	>60 M[a]	Inv(16)
AML 11	AML	Male	52	Normal	4 M	6 M	7 M	MLL-PTD
AML 12	AML	Male	53	Add (19)(p13.3)	No	No	>60 M[a]	Inv(16)

[a]Patients AML3, AML10 and AML12 are still alive until the date of data collection.

2.2 | Isolation of nonleukemic cellular subcompartments using FACS

Cell populations were sorted using BD FACS-Aria II Cell Sorter (BD Bioscience, Heidelberg, Germany).

Functionally normal HSC were sorted using the surface marker combination $CD34^+/CD38^-/ALDH^+$ (using Aldefluor FITC, 34 APC and 38 PE antibodies) as described previously.[18] The purity and normal functionality analysis of this population was described in our previous work.[16,18] T- and B-cells were sorted as $CD45^+/CD3^+$ and $CD45^+/CD19^+$, respectively. Whereas leukemia cells (Blasts) were sorted as CD45dim ($CD45^+/CD3^-/CD19^-$). The purity of all sorted populations was >95%. Additionally a threshold of 5% for allele frequencies of mutations to be counted as positive was applied in order to avoid wrong results due to low level contamination with leukemic cells.

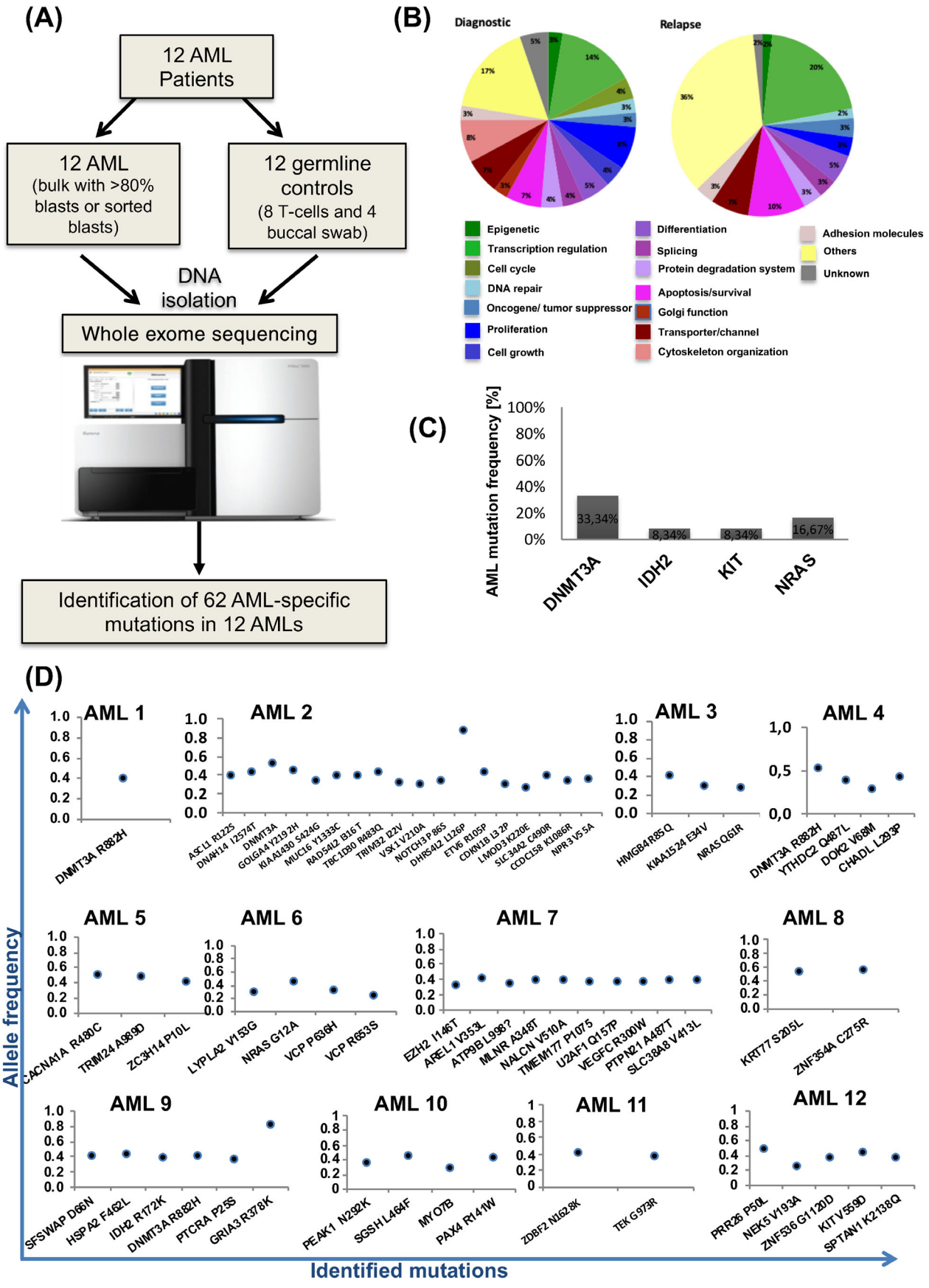
2.3 | Mutation detection and validation

Genomic DNA was extracted from either bulk cells with >80% blasts or sorted blasts (purity >95%) of 12 AML samples at diagnosis and for three cases additionally at relapse. For eight patients isolated T-cells were used as germ-line controls. For the remaining four patients, buccal swab samples were used (more details about sequenced sample's properties are shown in Supplementary Information [Table S1]).

Whole-exome-sequencing (WES) was performed according to the manufacturer's instructions (for more details about WES and library preparation, see Supplementary Methods). Single nucleotide variants calling was done according to specific filtration criteria (see Supplementary Methods section). Leukemia-specific mutations were selected according to selection criteria including (a) global minor allele frequency < 5% to exclude single nucleotide polymorphisms, (b) MaxAF <1%, (c) sort intolerance from tolerance score <0.05, (d) allele frequency (AF) >20% (>15% for relapse samples). Schematic illustration of filtration and selection criteria is shown in Supplementary Information (Figure S1). This analysis identified finally 62 point-mutations in 12 diagnostic AML samples and 73 point-mutations in three matched relapse samples. Moreover, analysis of insertions and deletions (InDels) identified 10 small deletions in the diagnostic samples (Table S2B). Sanger sequencing was used to confirm detection of all these mutations (Sanger sequencing details and primers used are available in the Supplementary Information [Table S7]).

2.4 | Tracking of identified point-mutations using targeted deep sequencing (DS)

A targeted resequencing panel was designed after WES analysis to track those identified mutations in different cellular subcompartments from diagnostic, follow-up and relapse samples (see panel designing in Supplementary Methods). Library preparation was performed according to the manufacturer's protocol (TruSeq^R Custom Amplicon Library preparation Guide, Illumina, San Diego, CA) using 10 μL



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genomic DNA samples with final concentration of 100 ng. Targeted re-sequencing was performed using the MiSeq platform (Illumina) and AmpliSeq application. The data were then filtered according to cutoff criteria (see Supplementary Methods section). All deep seq results were considered if the AF >5% (to avoid any contamination according to sort purity of >95%) and coverage >200 reads (to avoid sequencing errors). All mutations that appeared with AF ≤5% with coverage >200 reads were considered negative. Results appeared with coverage <200 reads were indicated as not available result (n.a.).

2.5 | Tracking of identified point-mutations in single cells obtained from functionally normal HSC

Single cells obtained from functionally normal HSC ($CD34^+/CD38^-/ALDH^+$) were seeded in 96-well plates and cultured for 5 weeks to prepare single cell colonies (see single cell culturing details in Supplementary Information and summary of analyzed colonies in Table S6). Sequence-specific primer PCR (SSP-PCR) was used to screen the identified point-mutations in single cell colonies, as this kind of analysis on the single cell level does not require determination of quantity (details of SSP-PCR method and primers are available in Supplementary Information [Table S7], sensitivity test experiment shows in Figure S8). All results were also confirmed by Sanger sequencing.

2.6 | Tracking of molecular and cytogenetic diagnostic markers in different cell populations

Diagnostic clonal markers used in routine diagnosis including FLT3-ITD, NPM1, MLL-PTD, inv(16) and trisomy 8 were confirmed in leukemic cells and tracked in different cellular subcompartments. This analysis was performed using the same routine methods used in routine diagnostic lab including routine PCR and interphase FISH analysis (see methodology details in Supplementary Information).

For additional methods, see the Supplementary Methods section.

3 | RESULTS

3.1 | WES identified novel and known leukemia-specific mutations

To identify AML-specific mutations, WES was performed for 12 AML patients (Table 1) using DNA from leukemic cells and germ-line controls (T-cells or buccal swabs [Table S1]). Variants' filtration and selection criteria identified 62 point-mutations and 10 small deletions in 12 diagnostic AML samples (Figure 1A) and 73 relapse specific mutations in 3 relapsed samples (filtration steps are shown in Figure S1, lists of all identified mutations are available in Supplementary Tables S2A,B and S3). Categorization of the mutated genes to their biological functions showed that most identified point-mutations are associated with cancer and occur in genes regulating important processes such as epigenetic mechanisms (2/62 mutations = 3%), transcription regulation (9/62 mutations = 15%), cell cycle (3/62 mutations = 5%), DNA repair (2/62 mutations = 5%), proliferation (5/62 mutations = 8%) and cell growth (3/62 mutations = 5%). These percentages were in the same range within the relapse-specific mutations without big differences; however, the number of mutations associated with transcription regulation slightly increased at relapse (15/73 mutations = 21%) (Figure 1B). WES analysis identified not only new, but also mutations in genes that are recurrently mutated in AML like *DNMT3A*, *NRAS* and *KIT*,[2,3] indicating our analysis accuracy (Figure 1C). Most cases (8/12) showed 4-6 point-mutations per sample at diagnosis stage (range 1-18 mutations/AML) (Figure 1D).

3.2 | Identification of preleukemic mutations by analysis of nonleukemic cellular subcompartments

Our analysis focused on nonleukemic cell populations including functionally normal HSC ($CD34^+/38^-/ALDH^+$), T-cells ($CD45^+/CD3^+$) and B-cells ($CD45^+/CD19^+$). Additionally, we also analyzed engrafted human nonleukemic B-cells ($CD45^+/CD19^+$) in immunodeficient NOD/SCID/IL2Rγ (NSG) mice (engraftment of nl-HSC failed due to low cell number). All mutations that were detectable in leukemic cells were also tracked in those nonleukemic populations in order to identify preleukemic hits.

Analysis of ALDH status of 12 sequenced AMLs showed that 8/12 cases were ALDH-rare AML ($ALDH^+$ cells <1.9% of all MNC), from which nl-HSC were sorted using the surface marker combination $CD34^+/38^-/ALDH^+$ as we previously reported[16,18] (see ALDH status of the 12 sequenced AMLs in Supplementary Table S4). Single cells were cultured for 5 to 6 weeks to confirm normal functionality of sorted single cells. Single cell colonies derived from nl-HSC of diagnostic and follow-up samples were tested by Sanger sequencing and SSP-PCR for the corresponding identified driver mutations (Figure 2). The experiments also included analysis of all mentioned nonleukemic cells for the presences of the known diagnostic clonal markers

FIGURE 1 Identification of AML driver mutations in 12 diagnostic AMLs. A, Workflow to identify AML specific mutations by WES. B, Mutated genes at diagnosis (left) and relapse (right) were categorized according to their biological functions. C, WES identified not only new mutations but also known AML specific mutations like DNMT3A mutation, which was detectable in 4/12 cases and KIT and NRAS mutations that each were detectable in 2/12 cases. D, 62 AML-specific mutations were identified by WES and selected according to the described criteria. Point mutations are shown with their respective allele frequency (black dots). AML, acute myeloid leukemia; WES, whole-exome-sequencing [Color figure can be viewed at wileyonlinelibrary.com]

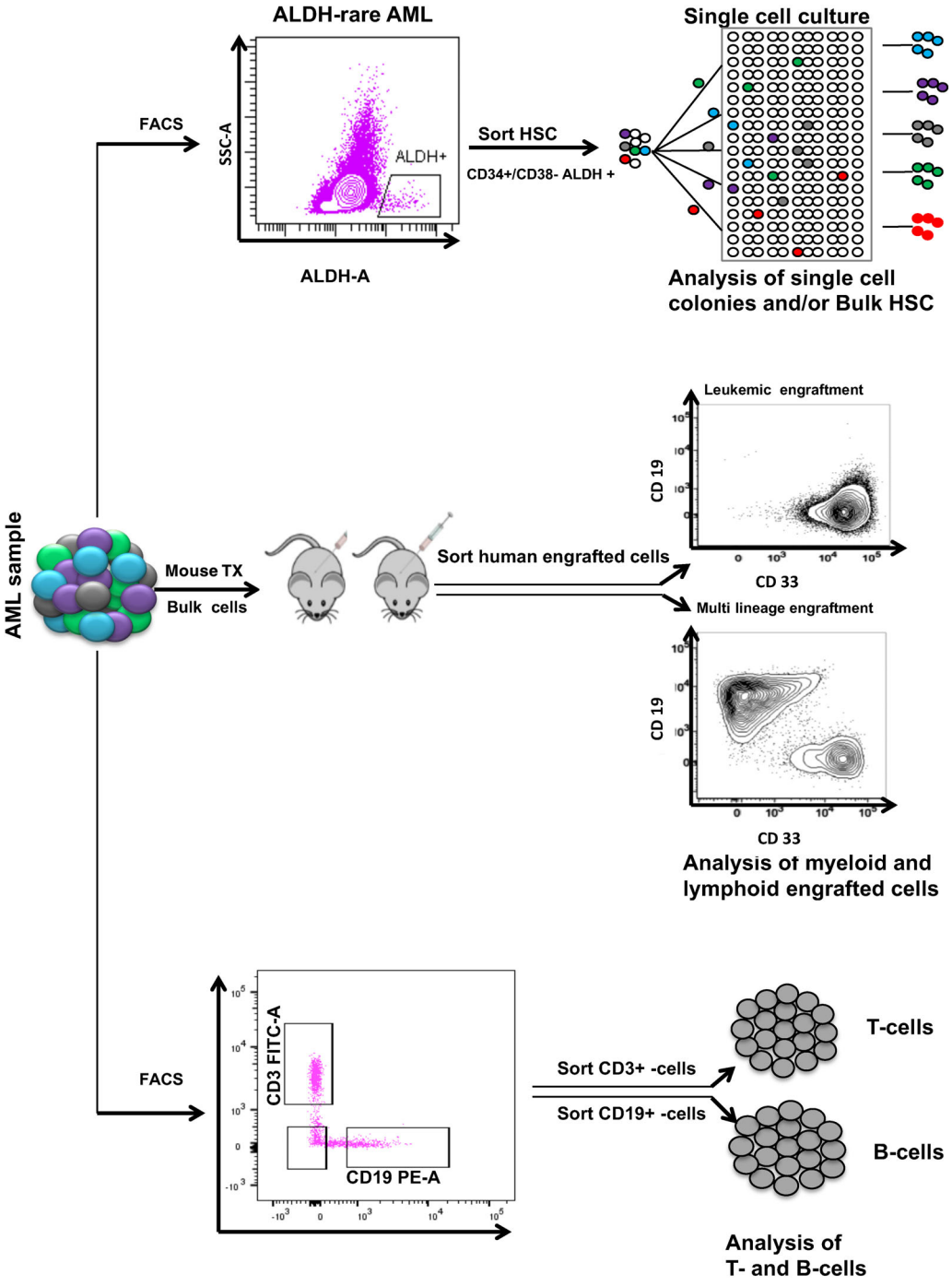


FIGURE 2 Analysis strategy to track acute myeloid leukemia (AML)-specific mutations in different nonleukemic cellular subcompartments. Bulk cells from each patient were used to sort nonleukemic cells including functionally normal hematopoietic stem cell (nl-HSC), T-cells and B- cells. Nl-HSC were used to prepare single cell colonies. Bulk cells were also transplanted into NSG mice. Identified AML-driver mutations were tracked in sorted nonleukemic cells, in single cell colonies and in leukemic and multilineage engrafted cells [Color figure can be viewed at wileyonlinelibrary.com]

including FLT3-ITD, NPM1, MLL-PTD, CEBPA mutations and cytogenetic abnormalities like inv(16) and trisomy 8. These aberrations were detected at the time of diagnosis by routine analysis. These mutations were then screened for in nonleukemic cells using the same screening methods used at diagnosis, that is, PCR and FISH.

Our analysis of $CD34^+/CD38^-/ALDH^+$ cells (nl-HSC) isolated from eight ALDH-rare AMLs identified AML-specific alterations in four out of eight cases including inv(16) in AML3, EZH2(I146T) in AML7 and DNMT3A(R882H) in AML4 and AML9. Detection of these mutations in nl-HSC indicates that these represent founder events and the $CD34^+/CD38^-/ALDH^+$ cells represent already preleukemia HSC.

T- and B-cells were isolated from all 12 cases and screened for the identified mutations in each case. This analysis identified a

DNMT3A(R882H) mutation in 3/12 AML cases including AML1, AML 4 and AML 9, also suggesting a preleukemic status of these mutations.

In summary, our analysis identified 3 preleukemic mutations in 5/12 AMLs, which include EZH2(I146T) in AML7, inv(16) in AML3, and DNMT3A(R882H) in AML1, AML4 and AML9. In the remaining 7/12 cases no mutations in their nonleukemic subcompartments were detectable. FACS sort strategies for this analysis are shown in Supplementary Figure S2A,B.

3.3 | Reconstruction of AML clonal evolution from pl-HSC to fully transformed disease

To reconstruct the hierarchy of leukemia development, leukemia-specific mutations were tracked in different cellular subcompartments of AML samples. For example, in patient AML1, who was diagnosed with FLT3-ITD and NPM1 mutations, WES also identified a DNMT3A(R882H) mutation. Tracking of these mutations in both leukemic myeloid and nonleukemic lymphoid cells exhibited that the FLT3-ITD and the NPM1 mutations were detectable only in leukemic myeloid cells, whereas the DNMT3A mutation was found in the leukemic compartment and the nonleukemic lymphoid T- and B-cells (Figure 3A). Isolation of HSC was not possible in this specific case of ALDH-numerous AML, as we have previously demonstrated.[16,18] These data suggest that the DNMT3A mutation represents an early (preleukemic) event that occurs in a primitive hematopoietic cell and both FLT3-ITD and NPM1 mutations are acquired at a later stage leading to AML (Figure 3B, details of this analysis are available in Supplementary Figure S3A-F), although our analysis cannot exclude the presence of NPM1 mutations with AF lower than our detection limitation (2%) in nonleukemic cells. This finding is in line with published data that DNMT3A represents a founder mutation in NPM1 positive AMLs.[20]

In another patient (AML3), WES identified three point-mutations including KIAA1524(E34V), HMGB4(R85Q) and NRAS(Q61R). Moreover, this patient was also diagnosed with inv (16) by routine diagnostic tests. Tracking these mutations in nonleukemic subcompartments isolated from this patient, including nl-HSC, T-cells, B-cells and engrafted $CD45^+CD19^+$ cells in NSG mice showed that only inv(16) was detectable in 2 of 21 single cell colonies seeded from nl-HSC isolated from this patient (Figure 4A,B). Analysis of the lymphoid lineages for inv(16) showed that inv (16) was detectable in T-cells but not in B-cells. Detection of inv (16) in nl-HSC colonies and in T-cells confirms their preleukemic status (Figure 4B). The absence of inv(16) in B-cells was confirmed by analysis of engrafted B-cells in NSG mouse (Figure 4C). This analysis suggests that inv(16) is a preleukemic mutation that occurs early in

(A)

Mutations AML 1	Blasts	T cells	B cells	Mouse CD45+/CD 33+	Mouse CD45+/CD 19+
DNMT3A	X	X	X	X	n.a.
FLT3-ITD	X	O	O	X	n.a.
NPM1	X	O	O	X	n.a.

X = detectable mutation, O = undetectable mutation, n.a. = not available analysis

(B)

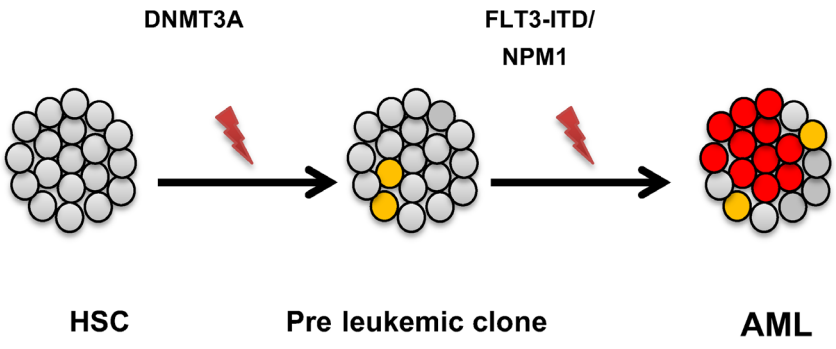
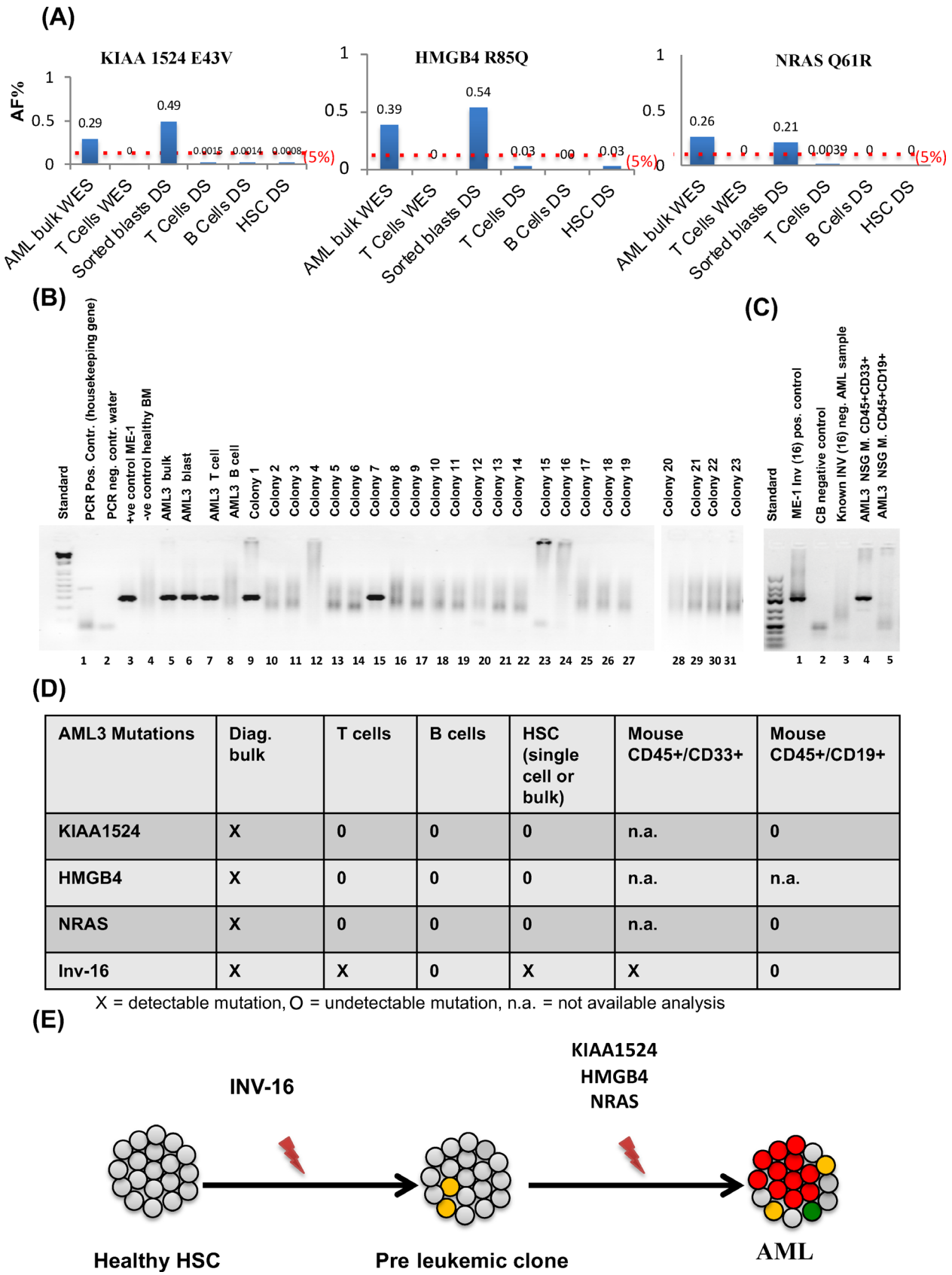


FIGURE 3 Clonal evolution reconstruction of acute myeloid leukemia (AML) 1. A, Summary of AML1 analysis. The DNMT3A mutation was detectable in blasts, T-cells, B-cells and in the myeloid lineage of leukemic engrafted NSG mouse ($CD45^+$/$CD33^+$). FLT3-ITD and NPM1 mutations were only detectable in blasts and in the matched leukemic engrafted mouse. B, Results of whole-exome-sequencing (WES) and PCR showed that DNMT3A(R882H) mutation represents preleukemic founder mutation then FLT3-ITD and NPM1 accumulate later, leading to full leukemic transformation. The DNMT3A mutation was detectable in blasts, T-cells, B-cells and in the myeloid lineage of leukemic engrafted NSG mouse ($CD45^+$/$CD33^+$). FLT3-ITD and NPM1 mutations were only detectable in blasts and in the matched leukemic engrafted mouse [Color figure can be viewed at wileyonlinelibrary.com]



AML3 Mutations	Diag. bulk	T cells	B cells	HSC (single cell or bulk)	Mouse CD45+/CD33+	Mouse CD45+/CD19+
KIAA1524	X	0	0	0	n.a.	0
HMGB4	X	0	0	0	n.a.	n.a.
NRAS	X	0	0	0	n.a.	0
Inv-16	X	X	0	X	X	0

X = detectable mutation, O = undetectable mutation, n.a. = not available analysis

FIGURE 4 Legend on next page.

the primitive cells whereas the other identified mutations including KIAA1524, HMGB4 and NRAS mutations accumulate at a later stage (Figure 4D,E).

3.4 | Identification of a therapy-resistant preleukemic clone

The presence of preleukemic mutations was associated with clinical recurrence, that is, in 4/5 patients in this series. In one case, (AML4) WES detected 4 AML-specific mutations including DNMT3A(R882H), YTHDC2(Q487L), DOK2(V68M) and CHADL(L293P). Only the DNMT3A (R882H) mutation seems to be preleukemic as it was detectable in 2/2 single cell colonies (Figure S4A) and in the T-cell control with low AF (5%) (Figure 5A; Figure S4B). The diagnostic clonal marker, FLT3-ITD, of this case was only positive in bulk leukemic cells at diagnosis and relapse and in engrafted leukemic blasts in NSG mice but negative in single cell colonies confirming that there was no contamination with leukemic cells (Figure 5B; Figure S4C). The results indicate that the DNMT3A mutation in this patient functions as a preleukemic hit with subsequent acquisition of the other point mutations (YTHDC2, DOK2 and CHADL), which together with the FLT3-ITD mutation has led to leukemic transformation (Figure 5C). Targeted DS of the matched early relapse sample, which was collected 7 months after initial diagnosis and after first complete remission (CR) and allogeneic hematopoietic stem cell transplantation (HSCT) showed that the same diagnostic mutations including the preleukemic DNMT3A mutation were detectable at relapse. This result suggests that a therapy-resistant diagnostic clone caused relapse in this case.

In patient AML7, 10 point-mutations were identified including EZH2(I146T) mutation, which was found in nl-HSC at the time of diagnosis. At early relapse 10 months after diagnosis and initial achievement of CR, the same mutations were found. This result suggests that relapse in this case was driven by a diagnostic clone that probably has survived chemotherapy (Figures S5).

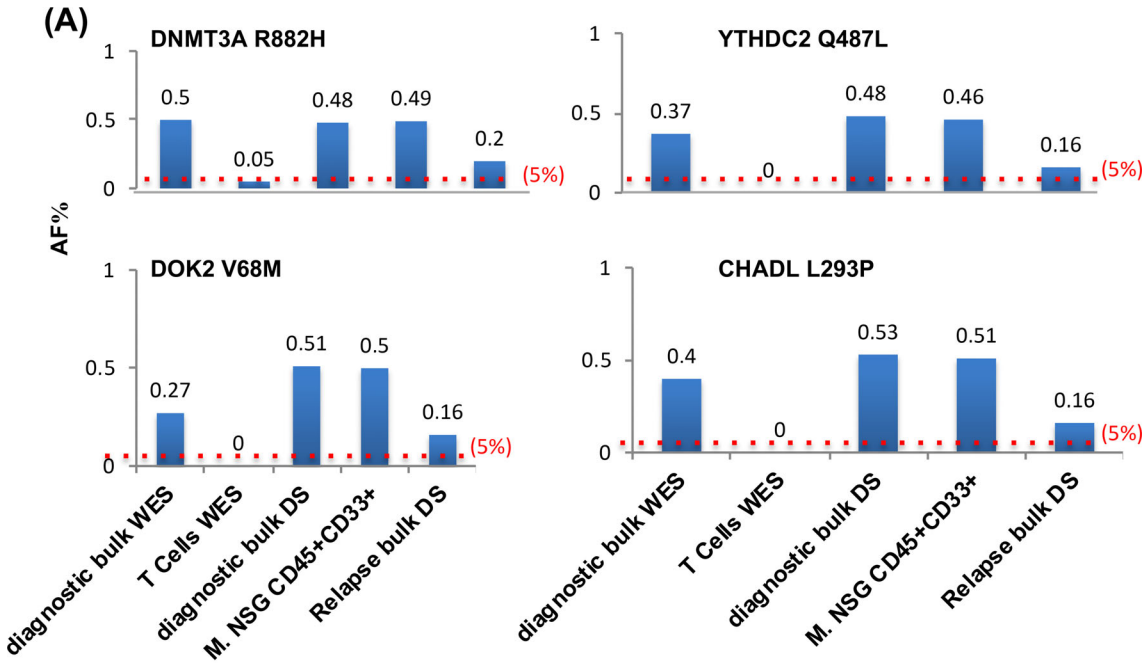
Patient AML9 was diagnosed with trisomy 8 and BM samples were available at diagnosis, follow-up and relapse. WES analysis of diagnostic bulk with 90% blasts identified six point-mutations including SFSWAP(D66N), HSPA2(F462L), IDH2(R172K), DNMT3A (R882H), PTCRA(P25S) and GRIA3(R378K). Targeted DS analysis of the leukemia blasts and B-cells of a follow-up BM sample collected at day 100 showed that only the DNMT3A(R882H) mutation appeared with an AF of 27% in sorted blasts and 12% in B-cells. All other mutations appeared with lower AF than the suggested negativity-threshold of 5% (see deep seq data filtration in the methodology section) or borderline frequency (Figure 6A,B). These data indicate the existence of a chemotherapy resistant subclone carrying DNMT3A(R882H) mutation. Sequencing analysis of bulk nl-HSC ($CD34^+/38^-/ALDH^+$) isolated from another follow-up sample collected 30 days after allogeneic HSCT showed none of the previous identified mutations, whereas single cell colony analysis from this time-point was able to detect the DNMT3A(R882H) mutation (-Figure S6) indicating that DNMT3A-positive HSC persisted even after allogeneic HSCT.

In patient AML9, analysis of insertions and deletions identified a STAG2 deletion, which has already been reported in many cancers and which plays a functional role in chromosome segregation.[21] The STAG2 deletion was subsequently analyzed in different cellular subcompartments. Sanger sequencing revealed that this deletion was detected in diagnostic blasts, day 100 follow-up sample with 15% blasts and in the relapse sample (40% blasts), whereas T- and B-cells at the time of diagnosis, as well as in the follow-up sample collected at day 30 after allogeneic HSCT, were all negative for this STAG2-deletion (Figure S7A). Trisomy 8 was only detectable at diagnosis and relapse. Although we were not able to isolate pure nl-HSC from the diagnostic BM of patient AML9 as the $CD34^+/38^-/ALDH^+$ sorted cells also contained leukemic cells, analysis of this population showed the presence of the STAG2 deletion but not that of trisomy 8. This result suggests that trisomy 8 is a subclonal event, which occurs last and only in a fraction of leukemic cells (Figure S7B). However, the trisomy 8 subclone must be one of the surviving clones giving rise to relapse.

The patient relapsed at day 90 after allogeneic HSCT, 7 months after initial diagnosis, with 40% blasts in his BM. In the BM sample, all diagnostic mutations were found again together with 16 new variants. Nine out of 16 new variants were detectable in donor cells, whereas 7 variants were identified as new mutations at relapse. Targeted sequencing; however, was not able to detect any of these 7 relapse-specific mutations in the patient initial diagnostic sample, nor in various follow-up samples. These results have provided evidence for ongoing clonal evolution between diagnosis and relapse.

Taken together, analysis of AML9 at different time-points demonstrated a DNMT3A(R882H)-positive clone which represents a preleukemic and therapy-resistant clone that survived chemotherapy and even allogeneic HSCT. The fact that the dominant clone at relapse also carried all detected diagnostic mutations suggests that the diagnostic clone survived chemotherapy and allogeneic HSCT but subsequently evolved in the relapse clone (Figure 6C).

FIGURE 4 Clonal evolution reconstruction of acute myeloid leukemia (AML) 3. A, Targeted deep seq (DS) analysis for the identified three point mutations showed that no any of this three mutations was detectable in T-cells, B-cells or nonleukemic hematopoietic stem cell (nl-HSC). B, Seminested PCR result shows that AML3 bulk, blasts and T-cells are inv(16) positive (reactions 5, 6 and 7) but B-cells PCR reaction is inv(16)-negative (reaction8). Single cell colony 1 and colony 7 appear inv(16) positive (reactions 9 and 15) but colony 2 through 23 appear negative for inv(16). Colonies 20 to 23 were prepared on a separate gel (reactions 28, 29, 30 and 31). C, Nested PCR showed that inv(16) is positive in $CD45^+CD33^+$ cells (reaction 4) and negative in $CD45^+CD19^+$ engrafted cells (reaction 5). D, Summary of AML3 analysis. E, Inv(16) appeared as a preleukemic event that is detectable in blasts, T cells, 2/23 single cell colonies derived from nl-HSC and in the myeloid lineage of leukemic engrafted NSG mouse. Other identified mutations including KIAA1524(E34V), HMGB4(R85Q) and NRAS(Q61R) seems to accumulate over time after inv(16) leading to expansion of the leukemic clone [Color figure can be viewed at wileyonlinelibrary.com]



(B)

AML 4 Mutations	Bulk	T cells	HSC Single cell colonies	Mouse CD45+/CD33+	Relapse bulk
DNMT3A	X	X	X	X	X
YTHDC2	X	0	0	X	X
DOK2	X	0	0	X	X
CHADL	X	0	n.a.	X	X
FLT3-ITD	X	0	n.a.	X	X

X = detectable mutation, O = undetectable mutation, n.a. = not available analysis

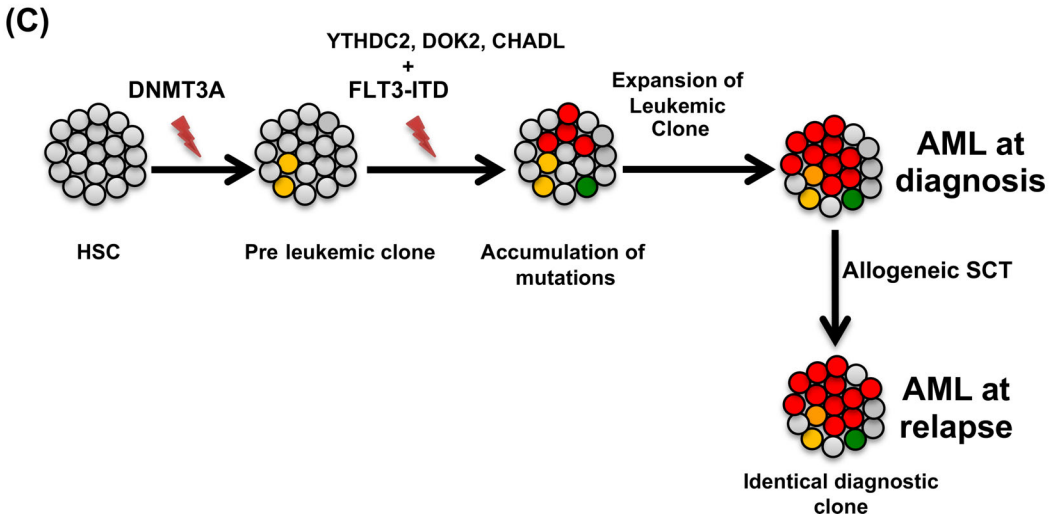
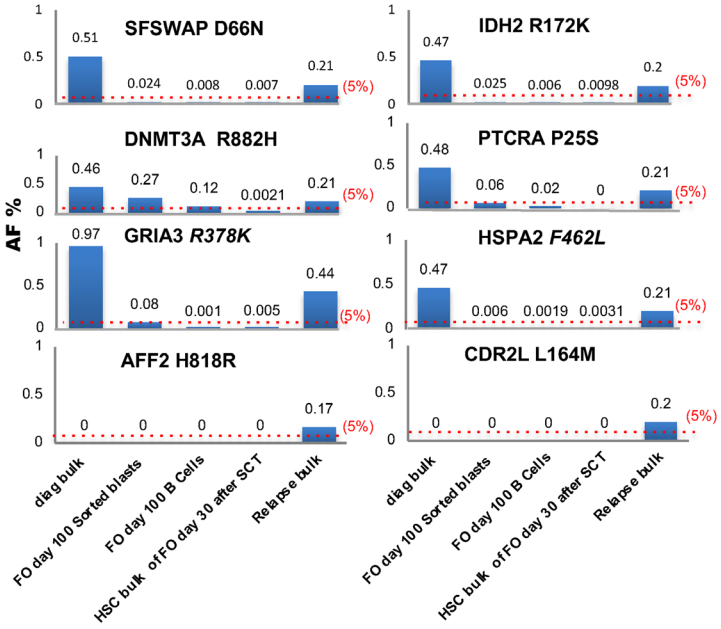


FIGURE 5 Therapy-resistant preleukemic clone appears at relapse of acute myeloid leukemia (AML) 4. A, WES and deep seq (DS) analysis of AML4 show that only the DNMT3A appears in T-cell with AF of 5%. All mutations appear in the leukemia engrafted mice. B, Summary of AML4 analysis. C, DNMT3A mutation represents a preleukemic founder mutation. Additional mutations including the FLT3-ITD mutation are required for full leukemic transformation. In this case, it seems that relapse evolved from the same diagnostic leukemia clone [Color figure can be viewed at wileyonlinelibrary.com]

(A)



(B)

Mutation AML 9	Diag bulk	FO blast at day 100 (before SCT)	FO B cells at day 100 (before SCT)	FO HSCs at day 30after SCT	Mouse diag. CD45+/CD33+	Relapse bulk
SFSWAP D66N	X	0	0	0	X	X
IDH2 R172K	X	0	0	0	X	X
DNMT3A R882H	X	X (27%)	X (12%)	X (on single cell level)	X	X
PTCRA P25S	X	X (6%)	0	0	X	X
GRIA3 R378K	X	X (8%)	0	0	X	X
HSPA2 F462L	X	0	0	0	X	X
AFF2 *	0	0	0	0	NA	X
CDR2L *	0	0	0	0	NA	X
STAG2 del	X	X	NA	0	NA	X
Trisomy 8	X	X	NA	0	NA	X

(C)

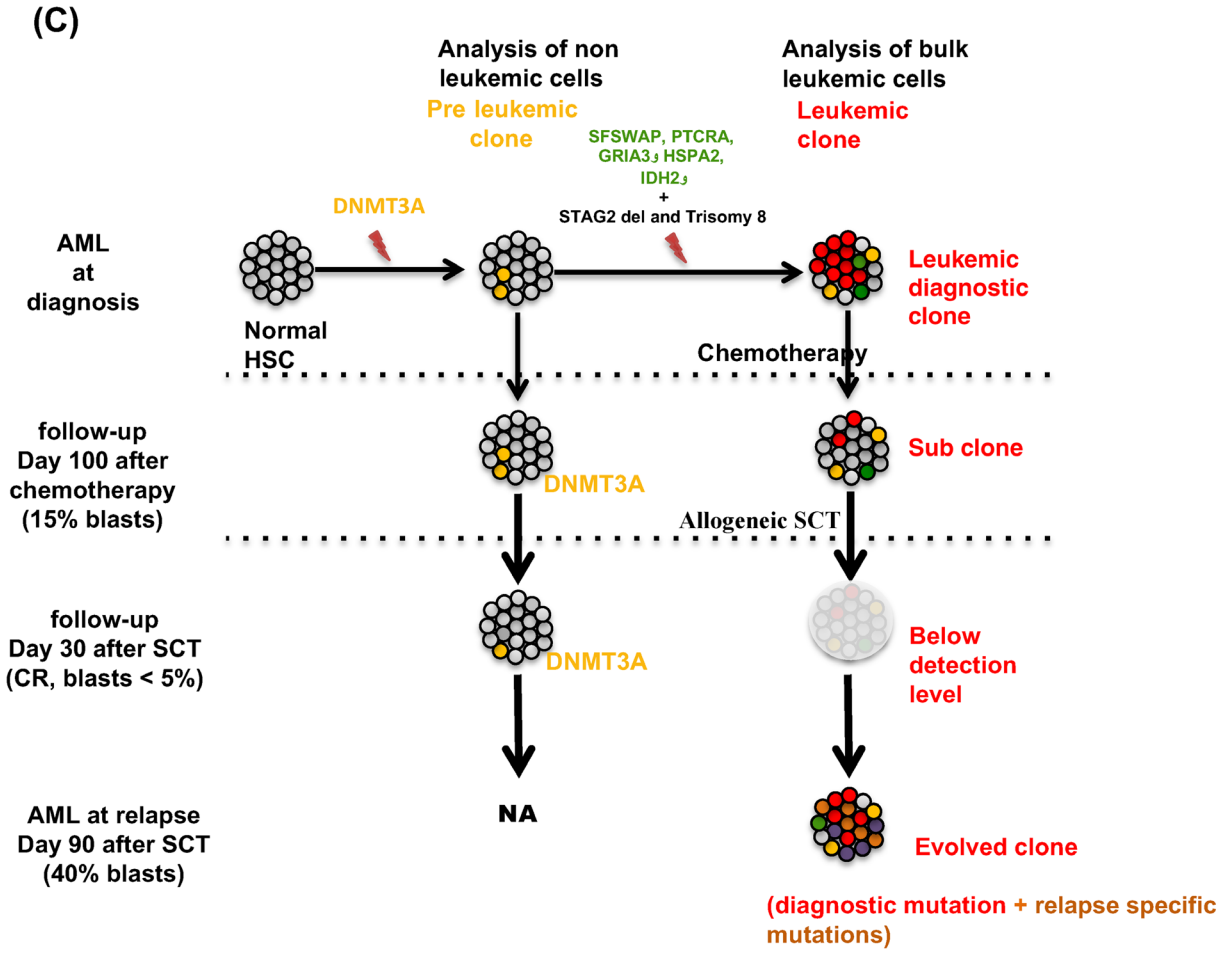


FIGURE 6 Legend on next page.

4 | DISCUSSION

Recently sequencing studies have identified recurrently mutated genes in AML.[2,12,20,22] Applying NGS analysis we were able to identify leukemia-specific mutations from 12 AML patients. Functionally normal HSC were isolated using ALDH enzyme level and cell surface marker combination ($CD34^+/CD38^-/ALDH^+$). Functional analysis confirmed normal functionality of this subpopulation; however, some identified AML-specific mutations were detectable suggesting their preleukemic status. Some identified preleukemic mutations were also detectable in both T- and B-cells or only in T- but not in B-cells. This work identified not only known preleukemic mutations such as DNMT3A(R882H) and Inv(16), but also novel preleukemic mutation like EZH2(1146T).

Analysis of nonleukemic subpopulations enabled us to reconstruct the clonal evolution from the first hit in nonleukemic cells to fully transformed AML in five patients and to identify therapy-resistant preleukemic clones. Moreover, the present work showed persistence of preleukemic clone after allogeneic SCT suggesting an important role of preleukemic mutations in disease recurrence.

Our analysis detected not only novel leukemia-specific mutations but also a number of known mutations in recurrent mutated genes such as *DNMT3A* in 4/12 (33%) patients, *IDH2* in 1/12 (7%), *KIT* in 2/12 (16%) and *NRAS* in 2/12 (16%) patients with percentages in agreement with the published data.[23] Detection of these known AML mutations served as quality control for our analysis. The specific roles of the newly identified mutations need to be further studied in vitro and in vivo in order to reveal their exact contributions to leukemogenesis.

Every individual patient showed different mutated genes indicating interindividual AML heterogeneity on the molecular level. Our longitudinal study has shown that the $CD34^+/CD38^-/ALDH^+$ cells contain AML-driver mutations indicating their preleukemic status (pl-HSC). In one patient, an EZH2(I146T) mutation was already found in the pl-HSC, whereas additional mutations such as FLT3-ITD were only found in overt leukemia blasts. Although EZH2 mutations are known in leukemia, this result represents the first report that the EZH2 (I146T) mutation might be an initiating event in AML. In three other patients, DNMT3A(R882H) mutations were detected in pl-HSC and in two out of these three cases FLT3-ITD evolved as a late event. This result confirms published data by Shlush et al that the DNMT3A (R882H) mutation represents a preleukemic mutation in 25% of AML patient.[20]

In another case, inv(16) was detected as an early event with the NRAS(Q61R) mutation occurring at a later stage. This observation is in agreement with Corces-Zimmerman et al that inv(16) represents an early event[14] and Miyamoto et al who demonstrated a preleukemic role of AML1-ETO, another AML associated core binding factor mutation, in a single AML case after long-term remission.[24]

Our present study indicates that each AML case might undergo a unique preleukemic process, albeit many patients share similar characteristics. We have demonstrated that preleukemic events such as DNMT3A(R882H), EZH2(I146T) and inv(16) were detectable in $CD34^+/CD38^-/ALDH^+$ cells of 5/12 cases. This finding is in agreement with published data by Jan and colleagues who showed that initiating events accumulate in residual HSC ($CD34^+/CD38^-/TIM\text{-}3^-$ cells).[12]

It has been reported that the DNMT3A(R882H) mutation represents an initial event that precedes a NPM1A mutation.[20] Jan et al showed also that TET2 mutations represent preleukemic hits that precede FLT3-ITD.[12] In our study, two out of three DNMT3A mutated cases showed cooccurrence of FLT3-ITD and DNMT3A, which is also in line with other reports.[25] These reports showed that FLT3-ITD evolution represents a late event after TET2 or DNMT3A mutations. This result is in line with published data that preleukemic mutations affect epigenetic genes and late mutations accumulate in proliferative genes involved in signaling like FLT3-ITD.[14] Furthermore, our data showed that not only the FLT3-ITD mutation represents a secondary hit but also NPM1 mutations which are in agreement with other published reports.[26,27]

Our study has demonstrated that T-cells might not be an ideal control for WES analysis since T-cells also carry AML-specific preleukemic mutations that may be filtered out as germ-line variants. This finding confirms published data that T-cells may contain preleukemic mutations with low AF.[20,28]

Accumulation of mutations over time leads to transformation of pl-HSC into overt LSC.[9,10] Therefore, pl-HSC may represent a reservoir for recurrent disease.[27,29,30] In our analysis, one patient (AML9) showed persistence of DNMT3A(R882H) mutation at various time-points of disease and even after allogeneic HSCT. Although routine diagnostic and monitoring methods showed a CR after HSCT and the chimera study at this time-point showed 98% donor cells (Table S5), the patient relapsed at Day 90 after allogeneic HSCT.

Other authors have also reported that preleukemic mutations have been detected in first remission samples suggesting the persistence of pl-HSC after standard chemotherapy.[20,31] Moreover, our data of single-cell analysis suggest that pl-HSC could even persist after allogeneic HSCT with very low frequency. They were detected only on the single cell level that could not be detected by common

FIGURE 6 Preleukemic therapy-resistant clone survive chemotherapy or even allogeneic hematopoietic stem cell transplantation (HSCT). A, Tracking of diagnostic mutations in different subpopulations by targeted deep sequencing showed that DNMT3A represents a preleukemic mutation, as it was detectable in B cells. It represents also a subclone that survives chemotherapy, as it was detectable with an AF of 27% in a follow-up sample at Day 100 after initial diagnosis and chemotherapy. Threshold of 5% appears as red line (see cutoff criteria in methodology section). B, Summary of AML9 analysis. C, Preleukemic mutation (DNMT3A mut) initiates the disease and survives chemotherapy and allogeneic HSCT. Other mutations accumulate over time and finally trisomy 8 may lead to leukemic clone expansion. A leukemic subclone that contains DNMT3A, which seems to be chemotherapy resistant, may initiate relapse after chemotherapy and allogeneic HSCT. Relapse-specific mutations, which appeared only at relapse and represent relapse-specific clone, indicate to ongoing clonal evolution at this stage [Color figure can be viewed at wileyonlinelibrary.com]

screening methods. In this patient, longitudinal studies showed that the DNMT3A(R882H) mutation that was present at diagnosis was found in all samples during the course of disease. Mutations that were present at diagnosis were found again at relapse in addition to new relapse-specific mutations. The persistence of preleukemic clones after allogeneic HSCT in this patient highlights the evolution of additional mutations in the pl-HSC upon relapse. Analysis of 3 matched relapse cases showed a general increase in numbers of mutations at relapse (73 relapse-specific mutations). Although this finding indicates ongoing clonal evolution at relapse, some relapse-specific variants may also be caused by chemotherapy.

Four out of five cases with detectable preleukemic mutations showed poor prognosis with development of relapse. The only exception was AML3 that carried an inv(16) as preleukemic event. These clinical and experimental data are in line with data that AML with inv (16) shows favorable prognosis.[32] Our data indicate a correlation between existence of preleukemic mutations like DNMT3A and EZH2 and poor clinical outcome; however, we only observe this result in a small series. More research using larger patient series is necessary to confirm this observation.

Shlush et al showed that pl-HSC could give rise to multilineage engraftment.[20] In our analysis, bulk transplantation showed mostly leukemic engraftment probably due to the presence of the aggressive leukemic clones within bulk cells. Transplantation using purified pl-HSC represents a challenge due to the low number of purified cells and the technical difficulties in transplanting these rare cells.

In patient AML3, which appeared with inv(16) as preleukemic event, inv(16) was only detectable in 2/23 single-cell colonies indicating the low rate of occurrence of this mutation in HSC. Analysis of lymphoid cells (T- and B-cells) for inv(16) showed that inv(16) was detectable in T-cells but B-cells were negative. Analysis of engrafted B-cells confirmed also the absence of inv(16). In this scenario, the B-cells were either derived from healthy HSC or only a few B-cells below our detection sensitivity carried inv(16).

In summary, our study has demonstrated the importance of analyzing various nonleukemic cell subcompartments (pl-HSC, T-cells, B-cells, engrafted B-cells in corresponding mice) of the same patient. With this strategy, we have identified preleukemic mutations and were able to reconstruct the leukemic hierarchy from preleukemic to fully transformed disease. The identification of initial mutations in pl-HSC or other nonleukemia subcompartments is highly relevant, as these discoveries improve our understanding of the origin and evolution of AML and help to identify potential novel targets for long-term cure.

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CONFLICT OF INTEREST

Eike C. Buss as coauthor declares the following conflict of interest: Current employment at AstraZeneca. Prior employment and share ownership at/of AbbVie. Prior travel sponsorships by Novartis and BMS. All the other authors declared no potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data for this study have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB41177 (https://www.ebi.ac.uk/ena/browser/view/PRJEB41177).

ETHICS STATEMENT

All samples were collected at Heidelberg University Hospital after written informed consent according to the Ethics Committee of the Medical Faculty of the University of Heidelberg.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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