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MYELOID NEOPLASIA

Disease evolution and outcomes in familial AML with germline *CEBPA* mutations

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Key Points

- Germ-line *CEBPA* mutations are highly penetrant, causing early-onset de novo AML associated with favorable survival outcomes.
- Familial *CEBPA*-mutated AML displays a unique model of disease progression, with recurrence caused by novel, independent leukemic episodes.

In-depth molecular investigation of familial leukemia has been limited by the rarity of recognized cases. This study examines the genetic events initiating leukemia and details the clinical progression of disease across multiple families harboring germ-line *CEBPA* mutations. Clinical data were collected from 10 *CEBPA*-mutated families, representing 24 members with acute myeloid leukemia (AML). Whole-exome (WES) and deep sequencing were performed to genetically profile tumors and define patterns of clonal evolution. Germline *CEBPA* mutations clustered within the N-terminal and were highly penetrant, with AML presenting at a median age of 24.5 years (range, 1.75-46 years). In all diagnostic tumors tested (n = 18), double *CEBPA* mutations (*CEBPA*dm) were detected, with acquired (somatic) mutations preferentially targeting the C-terminal. Somatic *CEBPA* mutations were unstable throughout the disease course, with different mutations identified at recurrence. Deep sequencing of diagnostic and relapse paired samples confirmed that relapse-associated *CEBPA* mutations were absent at diagnosis, suggesting recurrence was triggered by novel, independent clones. Integrated WES and deep sequencing subsequently revealed an entirely new complement of mutations at relapse, verifying the presentation of a de novo leukemic episode. The cumulative incidence of relapse in familial AML was 56% at 10 years (n = 11), and 3 patients experienced ≥3 disease episodes over a period of 17 to 20 years. Durable responses to secondary therapies were observed, with prolonged median survival after relapse (8 years) and long-term overall survival (10-year overall survival, 67%). Our data reveal that familial *CEBPA*-mutated AML exhibits a unique model of disease progression, associated with favorable long-term outcomes. (*Blood*. 2015;126(10):1214-1223)

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Introduction

The transcription factor CCAAT/enhancer binding protein-α (*CEBPA*) is encoded on chromosome 19q13.1 and has an essential role in mediating granulocytic differentiation and cellular growth arrest.[1] Somatic *CEBPA* mutations occur in 10% to 15% of sporadic normal karyotype acute myeloid leukemia (NK-AML), with double mutations (*CEBPA*dm) now considered to represent a unique molecular entity associated with favorable clinical outcomes.[2-4] *CEBPA*dm tumors typically harbor N-terminal frameshift mutations (enforcing translation of the shorter p30 isoform) combined with C-terminal in-frame insertions or deletions (disrupting the DNA binding or leucine zipper domains). These lesions are frequently accompanied by mutations in *GATA2* or *WT1*, which appear to be enriched within *CEBPA*dm AML.[5-9]

We initially described the autosomal dominant transmission of AML in a family harboring a germline *CEBPA* mutation in 2004.[10] Following subsequent reports of further *CEBPA*-mutated families, this unique clinical entity was established and has been recently reviewed with other leukemia predisposition syndromes, including germline *RUNX1* and *GATA2* mutations.[11] Although familial leukemia is rare, its true prevalence is likely underestimated due to variations in disease phenotype and latency, compounded by a lack of available family history. Clinical awareness and recognition is not only essential for the appropriate investigation, counseling, and management of families but also provides the basis for further scientific and clinical investigation. This study represents a multicenter collaboration, whereby families with germline *CEBPA* mutations were collectively identified to characterize both the clinical course of disease and the molecular events underlying disease evolution.

Methods

Familial AML patients

This study was performed in accordance with the declaration of Helsinki; ethical approval was obtained locally and within collaborating centers, with informed consent received from all participants or their guardians. Clinical follow-up data were collected for 10 pedigrees (A-J), 9 of which were previously reported.[2,10,12-17] Pedigree C represents a novel AML pedigree and is detailed below. Germline *CEBPA* mutations were verified in affected individuals from pedigrees A to E, using bidirectional sequencing as previously described.[18] The sources of germline (constitutional) DNA included peripheral blood (PB) or bone marrow (BM) during remission (A.II.1, A.II.5, A.III.2, B.II.2, C.III.1, D.II.2, and E.I.1), saliva (C.III.1 and D.II.1), or skin biopsy (B.I.1).

The index case in pedigree C was C.III.1, who presented in 2012 at 18 years of age. BM examination revealed 70% blasts, morphologically classified as French-American-British (FAB) subtype M4, with aberrant CD7 expression; cytogenetic analysis repeatedly failed. Her mother and maternal aunt had also developed AML at 32 and 3 years of age, respectively. A germline *CEBPA* mutation (c.218insC, p.H24AfsX84) was detected in tumor, remission, and salivary DNA from C.III.1. A further C-terminal *CEBPA* mutation (c.1047_1088dup, p.R300_K313dup) was detected in tumor DNA alone, and the nucleotide sequence of this insertion was subsequently confirmed with cloning and Sanger sequencing of individual colonies (Original TA kit; Invitrogen). A second maternal aunt, C.II.3, aged 41 years, was found to be a healthy carrier of the germline mutation (Figure 1).

Whole-exome sequencing of familial AML

Whole-exome profiling of somatic mutations was performed in AML samples from 4 pedigrees (A-D). Primary tumors were investigated in 7 patients (A.II.1, A.II.5, A.III.2, B.I.1, B.II.2, C.III.1, and D.II.2) with 2 episodes of disease recurrence (B.I.1). Matched DNA from the first complete remission (CR1) was used as a germline reference for primary leukemic episodes, whereas skin DNA was used as a germline reference for the 3 sequential tumors (T1-T3) in B.I.1. Capture libraries were constructed from 1.5 to 3 μg DNA using the Agilent SureSelect XT Human All Exon V5 kit. Enriched exome libraries were multiplexed and sequenced on the Illumina 2500 platform to generate 100-bp paired-end reads. Whole-exome sequencing (WES) data have been deposited in the European Genome-phenome Archive under accession no. EGAS00001000949.

Sequence alignment and variant detection

WES reads were first de-multiplexed and aligned to the reference genome hg19 using the Burrows-Wheeler Aligner version 0.6.2.[19] Picard tools software (version 1.86) was used to generate BAM files and remove polymerase chain reaction duplicates, with local realignment around insertions or deletions (indels) and base quality score recalibration performed with the Genome Analysis Toolkit (GATK) v2.5.2.[20] Somatic single nucleotide variants (SNVs) were called using the MuTect algorithm,[21] and short indels were identified using the GATK Somatic Indel Detector module, with a minimum coverage of 8 reads at the identified site. The minimum average mapping and base quality scores for Indel supporting reads were 10 and 20, respectively, in both tumor and matched normal samples. Pindel[22] was used to detect longer known indels, such as *CEBPA* C-terminal insertions (>10 bp). Only somatic variants with homozygous reference alleles in the matched normal samples were considered and annotated with the SNPnexus tool[23]; variants corresponding to dbSNP137 entries were also noted. Mutations were then manually reviewed to remove sequencing and misalignment errors, prior to validation with Sanger and deep sequencing. To ensure that preleukemic mutations (recently described in sporadic AML[24-29]) had not been excluded from our analytical pipeline, variant calling in candidate genes was performed for all tumor and remission samples using the reference genome, hg19, as detailed in the supplemental Data available on the *Blood* Web site. Further genetic profiling of tumors involved the detection of copy number alterations (CNAs) and loss of heterozygosity (LOH) from WES reads, with copy-neutral LOH (also known as acquired uniparental disomy [aUPD]) revealed by intersecting these data (supplemental Data).

Tumor subpopulation identification and clonality analysis

To analyze the clonal architecture and population dynamics in tumor samples, all somatic SNVs, indels, LOH variants, and CNAs were combined to estimate clonal expansions and the cellular frequency of each subpopulation (SP) using EXPANDS.[30] Somatic LOH variants were identified according to the method described by Murtaza et al.[31] All somatic variants were assigned to nested populations, with the largest SP forming the dominant clone and any SPs with lower cellular frequencies regarded as subclonal populations.

Integrated whole-exome and deep sequencing analysis of clonal evolution

Somatic *CEBPA* mutations were tested at diagnosis and relapse in 6 patients (B.I.1, B.II.2, E.I.1, E.II.1, F.IV.2, and G.II.2) using Sanger sequencing. Targeted deep sequencing was subsequently performed in tumor pairs from B.I.1 and E.I.1 to determine whether relapse-associated *CEBPA* mutations preexisted at diagnosis or represented a de novo event. To comprehensively characterize disease progression, we performed deep sequencing of 28 selected variants across 3 sequential tumors (T1-T3) in patient B.I.1, enabling us to decipher the pattern of clonal evolution over a disease course of 17 years. For detailed methods, see supplemental Data.

Clinical profiling of an extended familial AML cohort

Clinical demographic and survival data were collected across 10 pedigrees including 24 members with AML. The median duration of follow-up in surviving patients was 7.5 years (range, 2-46 years); 4 patients died within a year of diagnosis, and 1 (F.III.2) was lost to follow-up. Overall, 96% of patients (n = 23) were treated with induction chemotherapy comprising an anthracycline and/or cytarabine regimen. Patient A.I.1 presented in 1963 and achieved a 16-month

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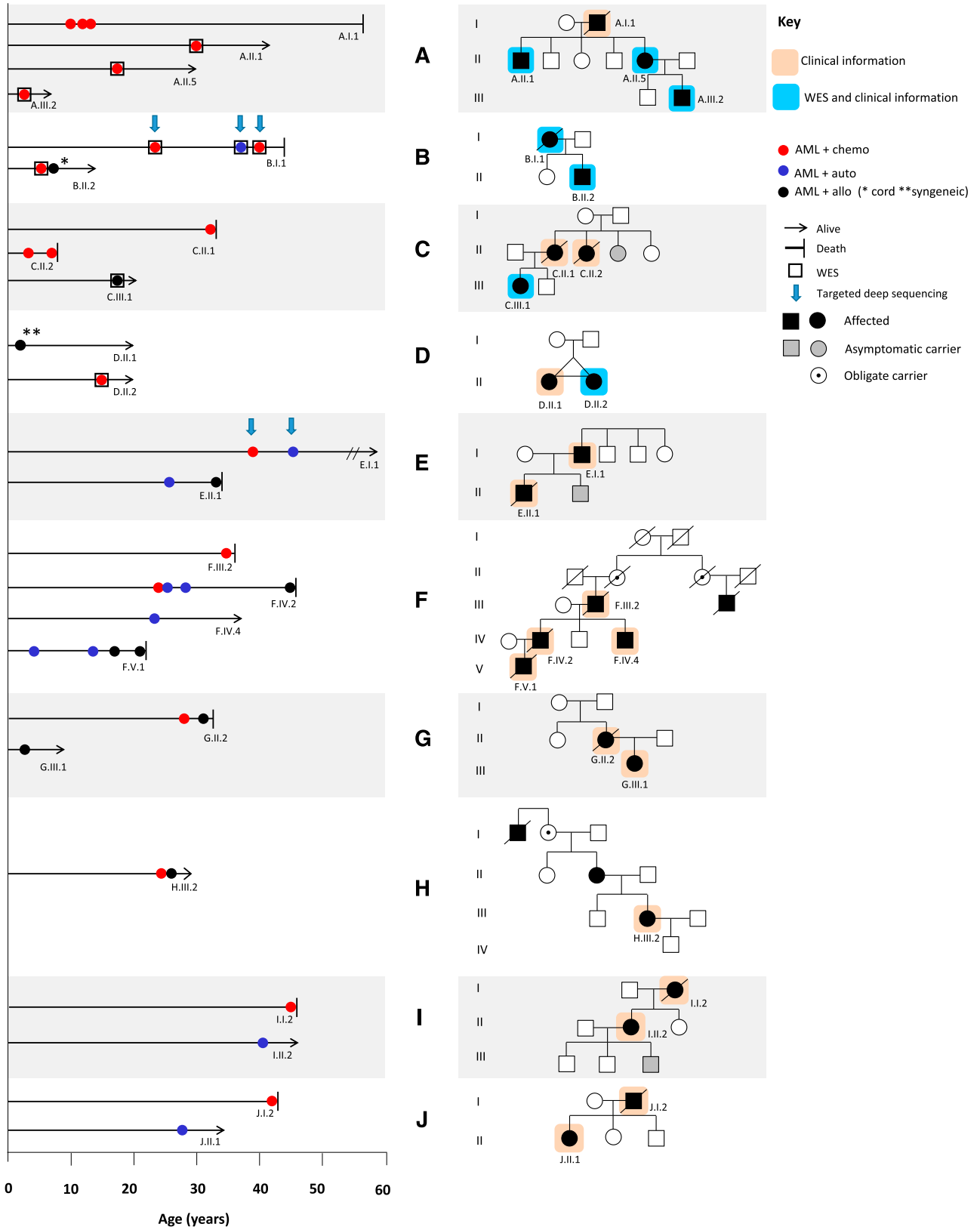

Figure 1. Clinical timeline and pedigrees of the familial AML study cohort. Illustrated is a timeline of disease events and family trees for pedigrees A to J. Clinical follow-up data were collected for 24 patients from 10 pedigrees (highlighted orange): families A,[10] B,[14] C (unpublished), D,[17] E,[15] F,[12] G,[2,16] H,[2] I,[13] and J.[13] Tumor DNA samples were genetically profiled in 7 patients (highlighted blue), including 7 primary tumors and 2 recurrent disease episodes (as shown on the timeline). The clinical timeline identifies disease events in each individual, with the main modality of treatment received: conventional chemotherapy (red), autologous transplant (blue), and allogeneic transplant (black). More than 50% of patients experienced recurrent disease, often with prolonged intervening periods of remission (>5 years), as seen in B.I.1, E.I.1, E.II.1, F.IV.2, and F.V.1. In addition to the confirmed asymptomatic carriers (C.II.3, E.II.2 and I.III.3), 3 individuals appear as obligate carriers (H.I.2, F.II.2 and F.II.3), although these were not tested in the study.

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remission following prednisolone and mercaptopurine therapy. He experienced 2 episodes of disease recurrence: the first was treated with prednisolone and methotrexate and the second with cyclophosphamide, resulting in a long-term remission of 45 years.[10] Consolidation strategies are summarized in supplemental Table 1 and comprised chemotherapy alone (n = 11), autologous stem cell transplantation (n = 5), or allogeneic stem cell transplantation (n = 3). Due to treatment-related complications, 2 patients did not receive consolidation therapy after remission (A.I.1 and I.I.2), whereas J.I.2 and C.II.1 died due to hemorrhage and chemotherapy-resistant disease, respectively.

Clinical end point definitions follow revised International Working Group Criteria,[32] except that count recovery was not required for complete remission. Survival percentages were calculated using Kaplan-Meier methodology and cumulative incidence of relapse using death as a competing risk. Univariate analyses were performed using the log-rank test. Clinical outcomes in familial AML were compared with sporadic AML in younger adults, harboring presumed somatic single (n = 48, *CEBPA*sm) and double (n = 59, *CEBPA*dm) *CEBPA* mutations. All sporadic cases were treated within the UK Medical Research Council AML 10 and 12 trials, as previously described.[3] (supplemental Table 1).

Results

Characteristics of disease presentation

Familial AML presented early, at a median age of 24.5 years (range, 1.75-46 years; Figure 1). Three asymptomatic carriers were identified (C.II.3, E.II.2, and I.III.3) aged 41, 24, and 19 years, respectively; they continue to receive annual follow-up with normal PB counts and BM morphology. All episodes of familial AML presented de novo and were predominantly characterized by an NK or FAB M1 or M2 morphology and aberrant expression of the T-cell antigen CD7. Germline *CEBPA* mutations were universally located in the N-terminal, each causing a frameshift preceding the internal p30 start codon. Somatic C-terminal *CEBPA* mutations were detected in all diagnostic tumors tested (n = 18), with the majority representing in-frame insertions or deletions within the DNA-binding and leucine zipper domains (Figure 2A; supplemental Table 2).

Profiling of somatic mutations within familial AML

WES of 9 familial AML samples yielded a mean exonic coverage of 89×, with 94% of bases covered >10-fold (supplemental Table 3). A total of 300 tumor-specific mutations were detected within 252 genes, including 160 nonsynonymous/splice site (protein-altering), 57 synonymous, and 83 untranslated region variants (supplemental Figure 1 and supplemental Tables 4-6). We verified 107 of 118 variants with Sanger and deep sequencing, achieving concordance of 83% for insertions or deletions (indels) and 93% for SNVs, as detailed in supplemental Table 7.

We then focused our analysis on somatic nonsynonymous mutations and compared these with recurrently mutated genes (n ≥ 2 nonsynonymous mutations) identified from 200 sporadic AML exomes, sequenced by the Cancer Genome Atlas Research Network (TCGARN).[33] C-terminal *CEBPA* mutations were acquired in all 9 exome-sequenced tumors, with further mutations observed in *GATA2* (n = 5), *WT1* (n = 3), *EZH2* (n = 2), *SMC3* (n = 1), *TET2* (n = 1), *NRAS* (n = 1), *DDX41* (n = 1), and *CSF3R* (n = 1). These genes were also found to be mutated in sporadic *CEBPA*-mutated tumors, with the exception of *EZH2* and *DDX41* (Figure 2B).

In pedigrees A and B, we performed WES on 2 or more affected members, enabling comparison of somatic leukemic variants. Three members of pedigree A acquired 1 or more *GATA2* mutations, all of which localized to the first zinc finger domain (A.II.1, p.L321F; A.II.5, p.L321F and p.R330Q; A.III.1, p.N317I). Furthermore, mother (B.I.1) and son (B.II.2) in pedigree B both acquired aUPD of chromosome 11p, with an underlying *WT1* mutation detected in the mother's leukemic DNA (*WT1* p.D223-S233dup; supplemental Figure 2). This apparent intrafamilial convergence of tumors suggests that genetic lesions are acquired in a nonrandom manner that may be predetermined by features within the host genotype.

Instability of somatic *CEBPA* mutations at disease recurrence

Sanger sequencing of *CEBPA* at diagnosis and recurrence revealed discordant somatic mutations in 83% of tumor pairs (n = 5), suggesting the presentation of new leukemic episodes (Table 1). Novel mutations were predominantly acquired within the C-terminal (B.I.1, E.I.1, and F.IV.2), although 1 patient (B.II.2) acquired a somatic N-terminal mutation, and another reverted to a wild-type sequence at relapse (E.II.1). To verify that these mutations were unrelated to preexisting leukemic clones, we performed targeted deep sequencing of somatic *CEBPA* mutations at diagnosis and relapse in patients B.I.1 and E.I.1. Relapse-associated mutations were absent from preceding diagnostic tumors at sequencing depths exceeding 16 000× (B.I.1) and 143 000× (E.I.1), signifying that each mutation had developed de novo within an independent clonal expansion. Furthermore, somatic *CEBPA* mutations from the diagnostic tumor were not detected at relapse with sequencing depths exceeding 11 000× (B.I.1) and 212 000× (E.I.1), suggesting the primary episode had been cured. In a third patient, B.II.2, who presented with recurrence 15 months after diagnosis, we investigated the relapse status of 10 variants identified from WES of the primary tumor (supplemental Table 6). Notably, all 10 variants (7 clonal and 3 subclonal) were absent at disease recurrence, with detection of a novel somatic *CEBPA* mutation, further indicating a new clonal expansion. These data highlight the tumor specificity of somatic *CEBPA* mutations and signify that disease recurrence may be initiated by new leukemic episodes that are independent of the preceding tumor.

Molecular profiling reveals 2 distinct models of AML recurrence

Integrated analysis of WES and deep sequencing data across 3 consecutive tumor episodes in B.I.1 identified 2 distinct models of AML recurrence. B.I.1 first presented at 23 years (T1), achieving a 14-year remission following conventional chemotherapy. She developed AML recurrence at 37 years (T2) and underwent autologous stem cell transplantation (harvested during CR1), culminating in a third presentation at 40 years of age (T3). WES data revealed a high degree of genetic semblance between T1 and T3, with both tumors sharing clonal loss of chromosome 7q (~50-Mb deletion, encompassing *EZH2*) and 12 identical mutations, including 10 coding variants (Figure 3A; supplemental Figure 2; supplemental Table 6). Subclonal mutations from T1 were absent in T3, most likely reflecting eradication by chemotherapy or clonal regression. In contrast, T2 was molecularly and structurally distinct from T1 and T3, with no shared somatic mutations or LOH. This tumor was characterized by 11p aUPD with 16 novel nonsynonymous mutations (including *CEBPA* p.K313del and *WT1* p.D223-S233dup), consistent with the emergence of a novel leukemia.

To further characterize the ancestral relationship between T1, T2, and T3, we used deep sequencing to trace a selection of 28 variants across each disease episode (supplemental Figure 3A). Sequencing depths exceeded 70 000× for all samples, providing a variant detection sensitivity of 0.02% (15-20 supporting reads for 70 000-100 000× depth), with variant allele frequency (VAF) measurements supporting

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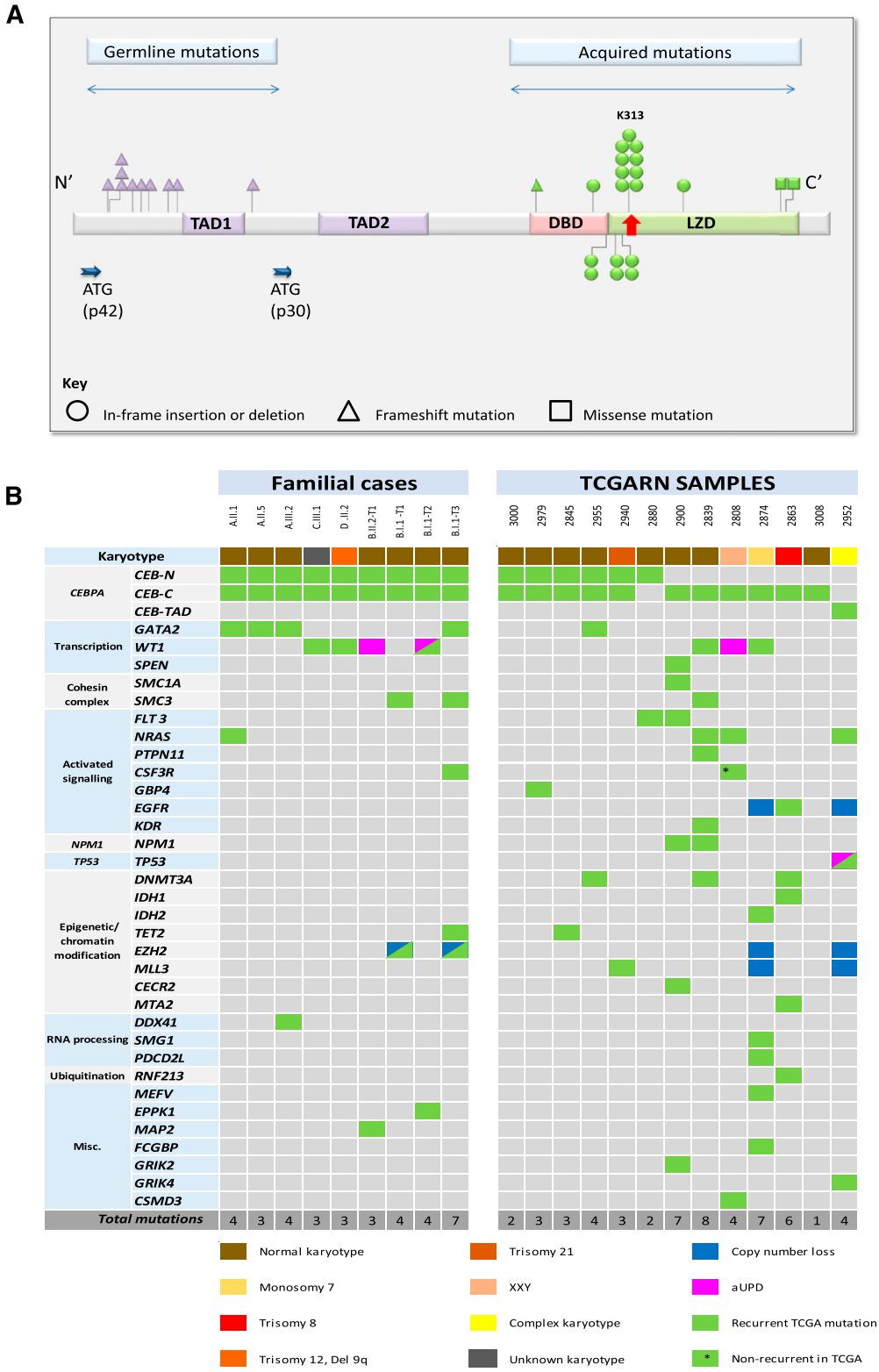

Figure 2. Genetic profiling of familial *CEBPA*-mutated AML. (A) Distribution of germline and acquired *CEBPA* mutations in familial AML. Transactivation domain (TAD) 1 amino acids (AA) 70 to 97; p30 start codon (ATG), AA 120; TAD2, AA 126 to 200; DNA-binding domain (DBD), AA 278 to 306; leucine zipper domain (LZD), AA 307 to 358. All germline mutations were localized to the N-terminal, causing a frameshift preceding the internal *CEBPA*-p30 start codon. Somatic mutations (detailed in supplemental Table 2) clustered in the C-terminal of the gene, with a hotspot located at p.K313. (B) Comparison of mutation profiles between familial and sporadic *CEBPA*-mutant AML. Mutation profiles are shown for familial and sporadic *CEBPA*-mutated AML samples (the latter analyzed within TCGA consortium[33]). All coding genes harboring ≥2 mutations across the 200 sporadic AML exomes and ≥1 mutation in familial or sporadic *CEBPA*-mutated AML are shown. In general, sporadic *CEBPA*sm tumors (predominantly with a lone C-terminal mutation) harbored more mutations than primary familial or sporadic *CEBPA*dm AML. *GATA2* and *WT1* mutations were most frequently observed in familial AML and were mutually exclusive. In familial AML, *WT1* mutations localized to the transcription regulatory N-terminal, contrasting with sporadic *CEBPA*-mutated samples and previous reports of NK-AML, where *WT1* mutations target the C-terminal exons 7 and 9.[43] Identical *CSF3R* (p.T618I) mutations were detected in both familial and sporadic *CEBPA*-mutated AML, as previously reported in chronic neutrophilic leukemia.[44,45] Regions of chromosomal loss and aUPD were identified using WES data in familial AML, and with high-resolution SNP array in TCGA cohort.

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Table 1. Discordant somatic *CEBPA* mutations at AML diagnosis and relapse

Patient	*CEBPA* somatic mutation-diagnosis		*CEBPA* somatic mutation-relapse		Time to relapse, years
	Amino acid	Mutation	Amino acid	Mutation	
B.I.1	p.N281RfsX38	c.991_992insGA	p.K313del	c.1087_1089del	14
B.II.2	p.R306dup	c.1067_1068insGCG	p.L146PfsX24	c.586_587insC	1.3
E.I.1	p.R306_N307insALAPPR	c.1067_1068ins18bp	p.Q312dup	c.1084_1086dup	6.7
E.II.1	p.K313dup	c.1087_1089dup	Wild type	Wild type	6.6
F.IV.2	p.E309_Q311delinsLEAK	c.1075_1081delinsCTGGAGGCCA	p.N307_T318dup	c.1070_1105dup	19.9*
G.II.2	p.K313dup	c.1087_1089dup	p.K313dup	c.1087_1089dup	2.3

*Third relapse.

WES data (supplemental Figure 3B). All T1 and T3 somatic variants were absent from T2, confirming that the latter represented an independent leukemic episode (Figure 3B). WES data suggested that T1 and T3 had evolved from an identical ancestral clone, but deep sequencing more accurately defined the T3 founding population as a minor divergent subclone of T1, which harbored the *TET2* p.R571X mutation in an estimated 3% of cells (supplemental Figure 3C). This nascent subpopulation of T1 appeared to survive chemotherapy and markedly expanded 3 years after autologous transplantation, to reemerge as the T3 founding clone (Figure 3C). T3 was characterized by chemotherapy resistance, indicating that this tumor was biologically distinct from T1 and T2. Although convergent evolution was observed between T1 and T3, with each acquiring different *EZH2* mutations, the gain of novel subclonal *GATA2* (p.L321P) and *CSF3R* (p.T618I) mutations led to further clonal heterogeneity in the final disease episode.

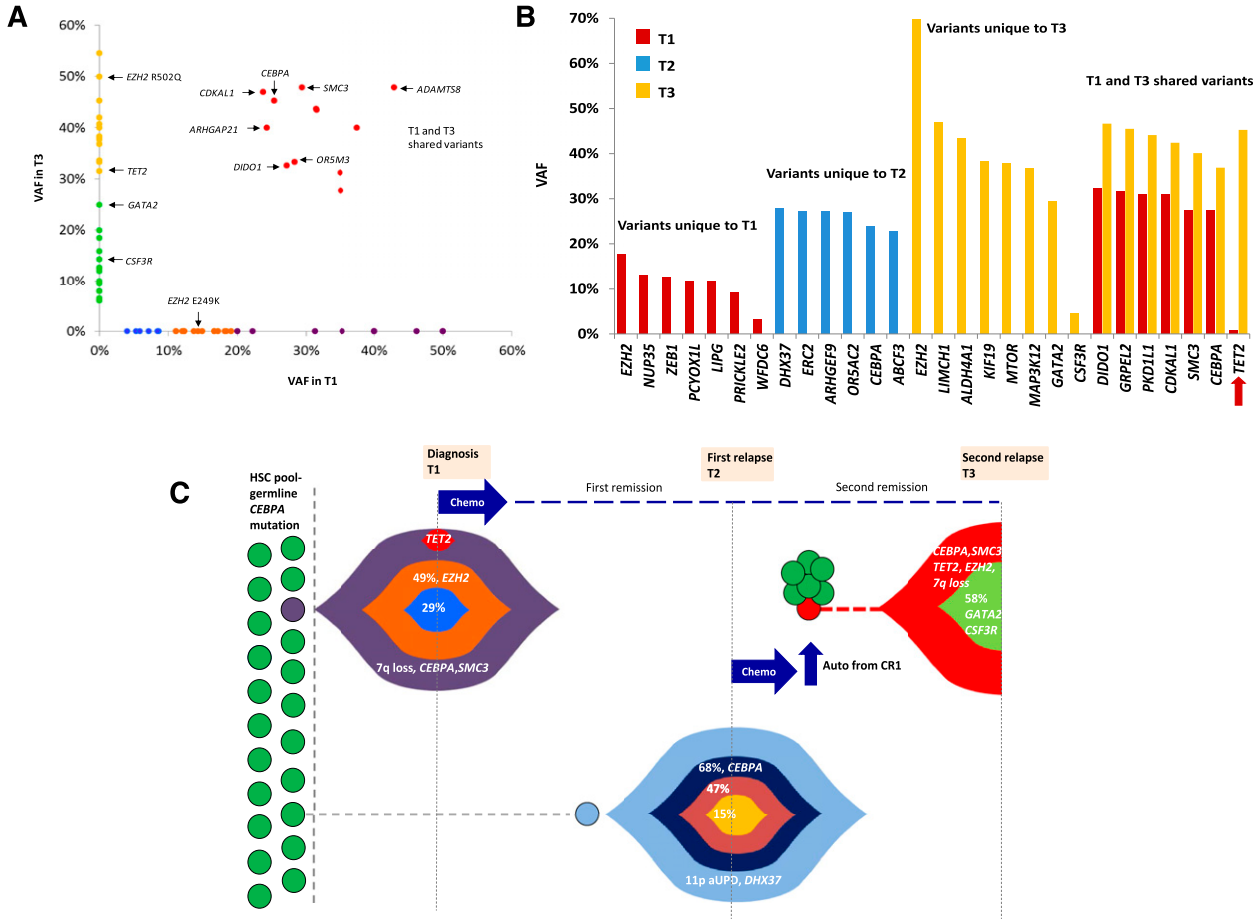

Figure 3. Clonal architecture and evolution of sequential disease episodes. Individual B.I.1 presented with 3 disease episodes (T1-T3), analyzed with WES and deep sequencing. (A) The scatter plot shows WES somatic VAF measurements for T1 and T3. Variants shown in red were shared by T1 and T3, with further evolution of T3 represented by novel clonal (yellow) and subclonal (green) mutations on the vertical axis. Clonal (purple) and subclonal variants (orange and blue) unique to T1 are represented on the horizontal axis. (B) Histogram showing deep sequencing VAF measurements of 28 variants across T1, T2, and T3. T2 was genetically distinct, with no shared variants observed in T1 or T3. In contrast, T1 and T3 shared multiple clonal mutations, with *TET2* (p.R571X) representing the only subclonal variant from T1 that was present in T3 (red arrow). (C) The T1 founding clone was characterized by 7q loss with 8 nonsynonymous mutations including *CEBPA* (p.N281RfsX38) and *SMC3* (p.R381Q). The patient received conventional chemotherapy and stem cells were harvested in CR1 (for future use). T3 occurred 3 years after autologous transplantation of these cells, demonstrating expansion of a latent T1-derived subclone, sharing 10 somatic coding variants and the subclonal *TET2* mutation (shown in red). Novel clonal variants in T3 included *EZH2* (p.R502Q) with a new subpopulation harboring *GATA2* and *CSF3R* mutations. The intervening tumor, T2, was distinct from T1 and T3, characterized by 11p aUPD and 16 novel nonsynonymous variants including the *WT1* mutation, p.D223-S233dup. The percentage of cells in each tumor subpopulation is shown relative to the dominant tumor clone.

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Clinical outcomes in familial AML

Survival probabilities were calculated at 10 years, revealing significant differences between *CEBPA*-mutated subgroups (Figure 4A-B). Overall survival (OS) in familial AML was superior to sporadic *CEBPA*sm ($P = .003$ for all ages and $P = .03$ for ≤45 years), with no significant difference observed between familial and sporadic *CEBPA*dm patients ($P = .4$ for all ages, $P = .6$ for ≤45 years). The CR1 rate of familial AML was 91% (n = 21/23), although disease recurrence was frequent, and often a late event, as shown in the clinical timeline (Figure 1). The cumulative incidence of relapse in familial AML was 56% at 10 years, with the first recurrence presenting at a median of 27 months (patients with relapse, n = 11; range, 4-167 months). The cumulative incidence of relapse rates in sporadic *CEBPA*sm and *CEBPA*dm were 62% and 44%, respectively. Relapse occurred earlier in sporadic AML (median time to relapse *CEBPA*sm, 11.8 months; *CEBPA*dm, 11.2 months), but this did not differ significantly from familial AML ($P = .07$, familial vs sporadic AML).

Recurrence of familial AML appeared to retain sensitivity to chemotherapy; the median survival after relapse was 8 years (familial) vs 16 months (*CEBPA*dm) vs 3.5 months (*CEBPA*sm) ($P = .01$; $P = .006$ for familial vs sporadic *CEBPA*sm and $P = .03$ for familial vs all sporadic; Figure 4C). This ongoing sensitivity to treatment was further illustrated by the clinical course of several patients including A.I.1 and F.IV.2, who achieved a CR3 of 45 and 17 years, respectively (Figure 1). Of 7 familial patients who died following treatment of disease recurrence, 71% (n = 5) were in remission, including 3 in CR3 or CR4. These patients had treatment (cardiotoxicity, G.II.2) or transplant-related complications (graft vs host disease [E.II.1], posttransplant lymphoma [F.V.1], and infection [F.IV.2]), with 1 developing esophageal cancer several decades later (A.I.1). Such observations of late chemotherapy-sensitive recurrence support the hypothesis of novel, independent clones initiating new leukemic episodes. More importantly for clinicians, they reveal important biological and prognostic insights into the clinical disease course of familial AML.

Discussion

This multicenter study documents our collective experience of managing this rare subgroup of patients, providing extended clinical follow-up and molecular profiling of familial AML at diagnosis and relapse. Germline *CEBPA* mutations appear highly penetrant, although the identification of 3 unrelated healthy adult carriers suggests the possibility of inter- and intrafamilial variations in mutation penetrance. The majority of AML cases presented at an early age, and, in contrast to other leukemia predisposition syndromes, germline *CEBPA* mutations were associated with the occurrence of de novo disease, without a preceding dysplastic or cytopenic phase. Both the molecular and clinical profiles of familial tumors resembled those of sporadic *CEBPA*dm AML.

For the first time, we describe the molecular events underlying disease progression and evolution in familial AML. In contrast to multiple reports[34-36] describing the stability of *CEBPA* mutations in sporadic AML, the opposite was observed in familial AML, with the majority of cases demonstrating variation of somatic *CEBPA* mutations at disease recurrence. Deep sequencing confirmed that these mutations were not derived from preexisting subclones but had developed de novo, suggesting the occurrence of novel, independent leukemic episodes arising from highly penetrant germline mutations.

This unique model of disease recurrence was distinguished from conventional patterns of AML relapse[37] by in-depth molecular profiling of consecutive leukemic episodes within a single patient. We identified the expansion of a novel leukemic clone initiating the first episode of disease recurrence, whereas the second evolved from a subclone of the primary tumor (as observed in sporadic AML). Although the latter 2 episodes represented independent clonal expansions, their respective *WT1* and *TET2* mutations both targeted a common pathway of DNA hydroxymethylation.[38]

Residual leukemic variants from the primary episode were not detected by deep sequencing, suggesting that leukemia-initiating cells may have been reintroduced following autologous transplantation. Because nascent primary leukemic populations cannot be fully excluded, the expansion of an independent leukemic episode may also be explained by novel clones outcompeting preexisting clones. Further analysis of leukemia predisposition syndromes will elucidate whether this phenomenon is unique to germline *CEBPA* mutations or whether it universally applies to all familial leukemia subtypes. It seems likely that the variable penetrance of *RUNX1* and *GATA2* mutations (40-80%) may favor conventional models of relapse, with expansion of preexisting clones.

The acquisition of shared genetic lesions among family members suggests intrafamilial convergence of tumors. Despite limited studies to date, this phenomenon does not appear to be restricted to germline *CEBPA* mutations. An analogous observation was reported within a *GATA2*-mutated pedigree, where 2 cousins developed myelodysplasia (MDS)/AML, both acquiring identical somatic *ASXL1* mutations (c.1934dupG, p.G646WfsX12) and monosomy 7.[39] Enrichment of somatic *ASXL1* mutations was later reported in 30% of germline *GATA2* -mutated patients, coinciding with progression to a proliferative MDS phenotype.[40] More recently, Yoshimi et al[41] reported the inter- and intrafamilial acquisition of somatic *CDC25C* mutations in families with germline *RUNX1* mutations. These observations suggest it is possible that germline mutations may influence the acquisition or selection of specific cooperating mutations and that the susceptibility to mutation acquisition may be governed by inherited factors, often shared within families.

From a clinical perspective, our data reinforce the need for long-term surveillance of families with germline *CEBPA* mutations and, crucially, highlight the need for reassessment of somatic mutations at relapse. Although recurrent episodes arising from novel leukemic clones may retain chemotherapy sensitivity, allogeneic hematopoietic stem cell transplantation remains an important consideration for the prevention of future disease episodes. Comprehensive genetic counseling and screening is essential prior to the selection of related stem cell donors, with the possibility of identifying asymptomatic mutation carriers often weighing significant anxiety on families.

The accurate identification of familial leukemia requires clinical vigilance and appropriate investigation. Earlier reports suggest that 5% to 10% of all *CEBPA*-mutated leukemias harbor germline mutations.[2,13] Evaluation of family history is of paramount importance, although, in practice, this information may not be readily available. As germline *CEBPA* mutations are unique among leukemia predisposition syndromes, presenting with de novo AML in the absence of prodromal cytopenias, it is more likely that they may escape clinical recognition. We therefore advocate a thorough investigation of family history and germline DNA assessment of patients <50 years, presenting with *CEBPA*dm AML. Importantly, the distinction of pathogenic *CEBPA* mutations has been facilitated by the creation of extensive databases from large sequencing consortia, minimizing the potential for confusion with common germline polymorphisms.[42]

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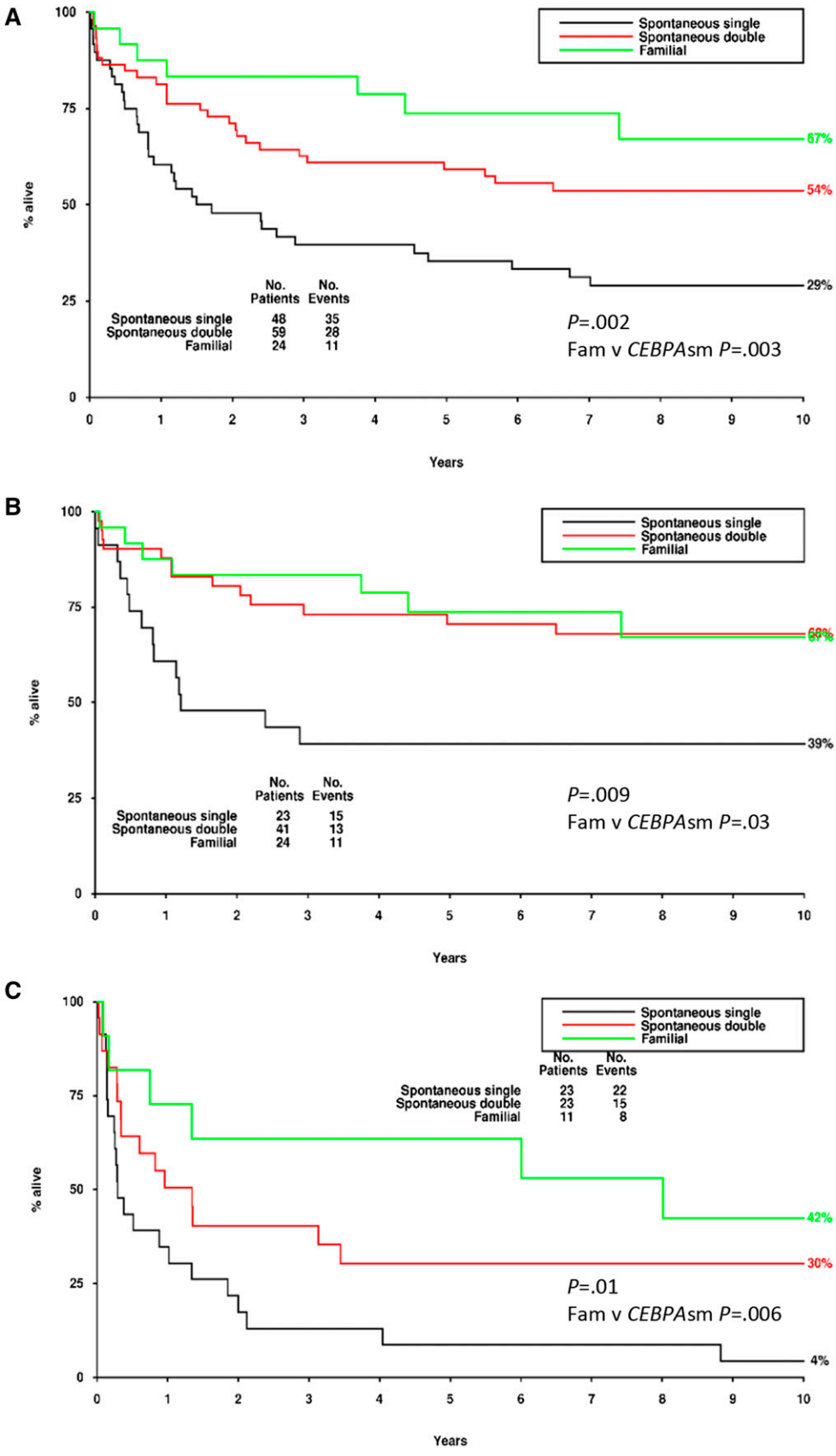

Figure 4. Survival outcomes in familial and sporadic *CEBPA*-mutated AML. Kaplan-Meier curves are stratified by *CEBPA* mutation status: familial (green); sporadic *CEBPA*sm (black), and sporadic CEBPA*dm* (red), with *P* values representing 3-way comparison of curves and direct comparison of familial (Fam) vs sporadic *CEBPA*sm patients. (A) OS in all sporadic and familial patients. (B) OS in sporadic and familial patients ≤45 years. (C) Survival after relapse for all patients with relapse. Familial patients demonstrated a median survival of 8 years, whereas sporadic AML patients, in particular *CEBPA*sm, displayed a rapid decline in survival within 2 years of relapse.

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By demonstrating a unique model of disease evolution, we reveal important differences underlying the biology of sporadic and familial leukemias. With improved clinical awareness and ongoing research efforts, we hope to provide a foundation for evidence-based genetic counseling and tailored management of this unique patient population.

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Authorship

Contribution: K.T., J.W., C.B., R.H., and J.F. wrote the paper; K.T., A.R., A.S., F.W.V.D., J.T., N.A., N.U., M.D., J.J., P.A., X.T., V.M., M. Smith, J.C., B.P., C.N., I.D., B.S., C.O., J.G., C.P., and J.F. designed research and contributed vital materials; K.T., C.L., P.G., E.U., E.W., S.I., A.-K.F., and F.B.L. performed research; and J.W., R.H., K.T., C.B., F.W.V.D., V.M., X.T., K.D., L.B., B.M., T.P., M. Stelljes, S.A., J.O., R.G., C.O., and C.C. performed data analysis.

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References

1. Nerlov C. The C/EBP family of transcription factors: a paradigm for interaction between gene expression and proliferation control. *Trends Cell Biol.* 2007;17(7):318-324.
2. Taskesen E, Bullinger L, Corbacioglu A, et al. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. *Blood.* 2011;117(8): 2469-2475.
3. Green CL, Koo KK, Hills RK, Burnett AK, Linch DC, Gale RE. Prognostic significance of CEBPA mutations in a large cohort of younger adult patients with acute myeloid leukemia: impact of double CEBPA mutations and the interaction with FLT3 and NPM1 mutations. *J Clin Oncol.* 2010; 28(16):2739-2747.
4. Dufour A, Schneider F, Metzeler KH, et al. Acute myeloid leukemia with biallelic CEBPA gene mutations and normal karyotype represents a distinct genetic entity associated with a favorable clinical outcome. *J Clin Oncol.* 2010; 28(4):570-577.
5. Greif PA, Dufour A, Konstandin NP, et al. GATA2 zinc finger 1 mutations associated with biallelic CEBPA mutations define a unique genetic entity of acute myeloid leukemia. *Blood.* 2012;120(2): 395-403.
6. Fasan A, Eder C, Haferlach C, et al. GATA2 mutations are frequent in intermediate-risk karyotype AML with biallelic CEBPA mutations and are associated with favorable prognosis. *Leukemia.* 2013;27(2):482-485.
7. Green CL, Tawana K, Hills RK, et al. GATA2 mutations in sporadic and familial acute myeloid leukaemia patients with CEBPA mutations. *Br J Haematol.* 2013;161(5):701-705.
8. Grossmann V, Haferlach C, Nadarajah N, et al. CEBPA double-mutated acute myeloid leukaemia harbours concomitant molecular mutations in 76·8% of cases with TET2 and GATA2 alterations impacting prognosis. *Br J Haematol.* 2013;161(5): 649-658.
9. Fasan A, Haferlach C, Alpermann T, et al. The role of different genetic subtypes of CEBPA mutated AML. *Leukemia.* 2014;28(4):794-803.
10. Smith ML, Cavenagh JD, Lister TA, Fitzgibbon J. Mutation of CEBPA in familial acute myeloid leukemia. *N Engl J Med.* 2004;351(23): 2403-2407.
11. Nickels EM, Soodalter J, Churpek JE, Godley LA. Recognizing familial myeloid leukemia in adults. *Ther Adv Hematol.* 2013;4(4):254-269.
12. Sellick GS, Spendlove HE, Catovsky D, Pritchard-Jones K, Houlston RS. Further evidence that germline CEBPA mutations cause dominant inheritance of acute myeloid leukaemia. *Leukemia.* 2005;19(7):1276-1278.
13. Pabst T, Eyholzer M, Haefliger S, Schardt J, Mueller BU. Somatic CEBPA mutations are a frequent second event in families with germline CEBPA mutations and familial acute myeloid leukemia. *J Clin Oncol.* 2008;26(31):5088-5093.
14. Renneville A, Mialou V, Philippe N, et al. Another pedigree with familial acute myeloid leukemia and germline CEBPA mutation. *Leukemia.* 2009; 23(4):804-806.
15. Nanri T, Uike N, Kawakita T, Iwanaga E, Mitsuya H, Asou N. A family harboring a germ-line N-terminal C/EBPalpha mutation and development of acute myeloid leukemia with an additional somatic C-terminal C/EBPalpha mutation. *Genes Chromosomes Cancer.* 2010; 49(3):237-241.
16. Stelljes M, Corbacioglu A, Schlenk RF, et al. Allogeneic stem cell transplant to eliminate germ-line mutations in the gene for CCAAT-enhancer-binding protein α from hematopoietic cells in a family with AML. *Leukemia.* 2011;25(7): 1209-1210.
17. Debeljak M, Kitanovski L, Pajič T, Jazbec J. Concordant acute myeloblastic leukemia in monozygotic twins with germline and shared somatic mutations in the gene for CCAAT-enhancer-binding protein α with 13 years difference at onset. *Haematologica.* 2013;98(7): e73-e74.
18. Burnett AK, Hills RK, Green C, et al. The impact on outcome of the addition of all-trans retinoic acid to intensive chemotherapy in younger patients with nonacute promyelocytic acute myeloid leukemia: overall results and results in genotypic subgroups defined by mutations in NPM1, FLT3, and CEBPA. *Blood.* 2010;115(5): 948-956.
19. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics.* 2009;25(14):1754-1760.
20. DePristo MA, Banks E, Poplin R, et al. A framework for variation discovery and genotyping using next-generation DNA sequencing data. *Nat Genet.* 2011;43(5):491-498.
21. Cibulskis K, Lawrence MS, Carter SL, et al. Sensitive detection of somatic point mutations in impure and heterogeneous cancer samples. *Nat Biotechnol.* 2013;31(3):213-219.
22. Ye K, Schulz MH, Long Q, Apweiler R, Ning Z. Pindel: a pattern growth approach to detect break points of large deletions and medium sized insertions from paired-end short reads. *Bioinformatics.* 2009;25(21):2865-2871.
23. Dayem Ullah AZ, Lemoine NR, Chelala C. SNPnexus: a web server for functional annotation of novel and publicly known genetic variants (2012 update). *Nucleic Acids Res.* 2012; 40(Web Server issue):W65-W70.
24. Busque L, Patel JP, Figueroa ME, et al. Recurrent somatic TET2 mutations in normal elderly individuals with clonal hematopoiesis. *Nat Genet.* 2012;44(11):1179-1181.
25. Corces-Zimmerman MR, Hong WJ, Weissman IL, Medeiros BC, Majeti R. Preleukemic mutations in human acute myeloid leukemia affect epigenetic regulators and persist in remission. *Proc Natl Acad Sci USA.* 2014;111(7):2548-2553.
26. Shlush LI, Zandi S, Mitchell A, et al; HALT Pan-Leukemia Gene Panel Consortium. Identification of pre-leukaemic haematopoietic stem cells in acute leukaemia. *Nature.* 2014;506(7488): 328-333.
27. Xie M, Lu C, Wang J, et al. Age-related mutations associated with clonal hematopoietic expansion and malignancies. *Nat Med.* 2014;20(12): 1472-1478.
28. Klco JM, Spencer DH, Miller CA, et al. Functional heterogeneity of genetically defined subclones in acute myeloid leukemia. *Cancer Cell.* 2014;25(3): 379-392.
29. Pløen GG, Nederby L, Guldberg P, et al. Persistence of DNMT3A mutations at long-term

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remission in adult patients with AML. *Br J Haematol.* 2014;167(4):478-486.

30. Andor N, Harness JV, Müller S, Mewes HW, Petritsch C. EXPANDS: expanding ploidy and allele frequency on nested subpopulations. *Bioinformatics.* 2014;30(1):50-60.
31. Murtaza M, Dawson SJ, Tsui DW, et al. Non-invasive analysis of acquired resistance to cancer therapy by sequencing of plasma DNA. *Nature.* 2013;497(7447):108-112.
32. Cheson BD, Bennett JM, Kopecky KJ, et al; International Working Group for Diagnosis, Standardization of Response Criteria, Treatment Outcomes, and Reporting Standards for Therapeutic Trials in Acute Myeloid Leukemia. Revised recommendations of the International Working Group for Diagnosis, standardization of response criteria, treatment outcomes, and reporting standards for therapeutic trials in acute myeloid leukemia. *J Clin Oncol.* 2003;21(24): 4642-4649.
33. Network CGAR; Cancer Genome Atlas Research Network. Genomic and epigenomic landscapes of adult de novo acute myeloid leukemia. *N Engl J Med.* 2013;368(22):2059-2074.
34. Shih LY, Liang DC, Huang CF, et al. AML patients with CEBPalpha mutations mostly retain identical mutant patterns but frequently change in allelic distribution at relapse: a comparative analysis on paired diagnosis and relapse samples. *Leukemia.* 2006;20(4):604-609.
35. Tiesmeier J, Czwalinna A, Müller-Tidow C, et al. Evidence for allelic evolution of C/EBPalpha mutations in acute myeloid leukaemia. *Br J Haematol.* 2003;123(3):413-419.
36. Hollink IH, van den Heuvel-Eibrink MM, Arentsen-Peters ST, et al. Characterization of CEBPA mutations and promoter hypermethylation in pediatric acute myeloid leukemia. *Haematologica.* 2011;96(3):384-392.
37. Ding L, Ley TJ, Larson DE, et al. Clonal evolution in relapsed acute myeloid leukaemia revealed by whole-genome sequencing. *Nature.* 2012; 481(7382):506-510.
38. Rampal R, Alkalin A, Madzo J, et al. DNA hydroxymethylation profiling reveals that WT1 mutations result in loss of TET2 function in acute myeloid leukemia. *Cell Reports.* 2014;9(5): 1841-1855.
39. Bödör C, Renneville A, Smith M, et al. Germ-line GATA2 p.THR354MET mutation in familial myelodysplastic syndrome with acquired monosomy 7 and ASXL1 mutation demonstrating rapid onset and poor survival. *Haematologica.* 2012;97(6):890-894.
40. West RR, Hsu AP, Holland SM, Cuellar-Rodriguez J, Hickstein DD. Acquired ASXL1 mutations are common in patients with inherited GATA2 mutations and correlate with myeloid transformation. *Haematologica.* 2014;99(2): 276-281.
41. Yoshimi A, Toya T, Kawazu M, et al. Recurrent CDC25C mutations drive malignant transformation in FPD/AML. *Nat Commun.* 2014;5:4770.
42. Resende C, Regalo G, Durães C, Carneiro F, Machado JC. Genetic changes of CEBPA in cancer: mutations or polymorphisms? *J Clin Oncol.* 2007;25(17):2493-2494, author reply 2494-2495.
43. Gaidzik VI, Schlenk RF, Moschny S, et al; German-Austrian AML Study Group. Prognostic impact of WT1 mutations in cytogenetically normal acute myeloid leukemia: a study of the German-Austrian AML Study Group. *Blood.* 2009; 113(19):4505-4511.
44. Maxson JE, Gotlib J, Pollyea DA, et al. Oncogenic CSF3R mutations in chronic neutrophilic leukemia and atypical CML. *N Engl J Med.* 2013;368(19): 1781-1790.
45. Pardanani A, Lasho TL, Laborde RR, et al. CSF3R T618I is a highly prevalent and specific mutation in chronic neutrophilic leukemia. *Leukemia.* 2013;27(9):1870-1873.

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