

Mutational spectrum analysis of chronic myelomonocytic leukemia includes genes associated with epigenetic regulation: *UTX*, *EZH2*, and *DNMT3A*

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Chronic myelomonocytic leukemia (CMML), a myelodysplastic/myeloproliferative neoplasm, is characterized by monocytic proliferation, dysplasia, and progression to acute myeloid leukemia. CMML has been associated with somatic mutations in diverse recently identified genes. We analyzed 72 well-characterized patients with CMML (N = 52) and CMML-derived acute myeloid leukemia (N = 20) for recurrent chromosomal abnormalities with the use

of routine cytogenetics and single nucleotide polymorphism arrays along with comprehensive mutational screening. Cytogenetic aberrations were present in 46% of cases, whereas single nucleotide polymorphism array increased the diagnostic yield to 60%. At least 1 mutation was found in 86% of all cases; novel *UTX*, *DNMT3A*, and *EZH2* mutations were found in 8%, 10%, and 5.5% of patients, respectively. *TET2* mutations were present in

49%, *ASXL1* in 43%, *CBL* in 14%, *IDH1/2* in 4%, *KRAS* in 7%, *NRAS* in 4%, and *JAK2* V617F in 1% of patients. Various mutant genotype combinations were observed, indicating molecular heterogeneity in CMML. Our results suggest that molecular defects affecting distinct pathways can lead to similar clinical phenotypes. (*Blood*. 2011;118(14):3932-3941)

Introduction

Chronic myelomonocytic leukemia (CMML) is a distinct entity, a myelodysplastic/myeloproliferative neoplasm (MDS/MPN) characterized by morphologic dysplasia and monocytosis. Pathomorphologic similarities exist between more advanced forms of CMML, including CMML-2 and CMML-derived secondary acute myeloid leukemia (sAML), and some primary forms of AML with monocytic differentiation.

Unlike chronic myelogenous leukemia, characterized by a *BCR-ABL1* fusion, the molecular pathogenesis of closely related CMML remains unclear.^{1,2} Recurrent reciprocal translocations, involving *PDGFRA* and *PDGFRB*, are rare in CMML; if present, they indicate that myeloblast/monocyte proliferation can be mediated by activation of phospho-tyrosine kinase pathways.³ Nevertheless, large, unbiased tyrosine kinase sequencing projects performed in CMML to identify kinase or regulatory domain mutations have not been revealing.⁴ Recurrent chromosomal abnormalities are mostly unbalanced and are similar to those in typical MDS.⁵⁻⁷ Despite reports of new chromosomal defects and mutations, a significant proportion of patients are found to have normal karyotype by metaphase cytogenetics (MC).

RAS family and *RUNX1* mutations have been found in a proportion of CMML cases.^{8,9} Recently, several genes have been found mutated in myeloid malignancies, including CMML.¹⁰⁻¹³ These discoveries were facilitated by single nucleotide polymorphism array (SNP-A) karyotyping, which enables detection of somatic regions of copy number neutral loss of heterozygosity

(CN-LOH), also called uniparental disomy (UPD). In CMML homozygous mutations in *CBL* and *TET2* are associated with regions of UPD.^{6,14-19} In addition, mutations in the *EZH2* gene have been detected in a substantial fraction of patients with myeloid malignancies characterized by the presence of CN-LOH 7q.²⁰⁻²² In contrast, mutations in *ASXL1* and *IDH1/2* genes are mostly heterozygous.^{11,23-25}

TET2 mediates the hydroxylation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in DNA.²⁶⁻²⁸ *DNMT3A* mutations, recently identified by next-generation sequencing in de novo AML,^{29,30} exemplify another type of mutation affecting epigenetic DNA modification. Similar to DNA modifying genes, mutations of genes regulating histone methylation have been found in myeloid malignancies. For instance, *ASXL1*, a polycomb and mixed lineage leukemia/trithorax chromatin modifier, was found to be mutated in CMML.^{11,23} *ASXL1*, in association with *LSD1*, is involved in H3K4 demethylation and thereby chromatin remodeling.³¹ Although *ASXL1* mutations are ubiquitous among myeloid malignancies, a knockout mouse model displayed mild defects in myelopoiesis and did not develop MDS or AML.³² Trimethylation of H3K27 can be affected by *EZH2*, a H3K27 methyltransferase,³³ also mutated in myeloid malignancies.²⁰⁻²² Alteration of this histone mark may contribute to the pathogenesis of malignant evolution. Indeed, *UTX*, which encodes for a demethylase also specific for H3K27, has been found mutated in some hematopoietic cell lines of myeloid origin.^{34,35}

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It is probable that multiple molecular defects can be present in an individual patient, explaining clinical heterogeneity, or conversely, distinct mutational events and chromosomal aberrations may produce similar clinical/pathomorphologic phenotypes, leading to unifying features. To address these issues, we have performed comprehensive cytogenetic analysis and targeted sequencing of various genes known to be mutated in CMML, including some newer candidate genes, particularly *UTX* (*KDM6A*) and *DNMT3A*, in a cohort of 72 CMML and CMML-derived AML cases. Rational molecular screening may successfully show mutational patterns and may identify surrogate phenotypic markers associated with mutations or their combinations.

Methods

Patients

Informed consent for sample collection was obtained according to protocols approved by the institutional review boards of Cleveland Clinic and Johns Hopkins University, following the Declaration of Helsinki. Diagnosis was assigned according to the World Health Organization (WHO) classification criteria, with all diagnoses confirmed at the 2 participating institutions.^{1,2} SNP-A karyotyping was performed on 70 patients and mutational screening on 72 patients with CMML and AML derived from CMML (Table 1). Clinical parameters examined included survival, blood counts and pathomorphologic criteria. The mutational status of *CBL*, *TET2*, *RAS*, and *EZH2* were previously reported in 40, 28, 1, and 24 patients of 72 present in this study, respectively.^{14,15,19,22}

SNP-A analysis

Genotyping was performed with Affymetrix Gene Chip Human Mapping 250K Array and Genome-Wide Human SNP Array 6.0 and processed according to manufacturer's instructions (Affymetrix). Further details for SNP-A analysis and the bioanalytic algorithm used in this study were previously described.^{7,19}

Mutational analysis

Screening of selected genes at known mutational hotspot regions and consensus splicing sites, for example, *CBL* (exons 8-9), *KRAS* and *NRAS* (exons 1-2), *IDH1* and *IDH2* (exons 4), *DNMT3A* (exons 18-23), *TET2*, *UTX*, and *EZH2* (all coding exons) was performed with direct genomic sequencing by standard techniques on the ABI 3730×1 DNA analyzer (Applied Biosystems) as described.^{14,15,19,22} *UTX* mutations were detected with either exon-specific primers or cDNA primers as previously described and were scored as pathogenic on the basis of their absence in 400 male controls.³⁵ All mutations were detected by bidirectional sequencing and were scored as pathogenic if not detected in normal or available nonclonal CD3 samples and absent in published SNP databases³⁶ (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Furthermore, canonical mutations (ie, described as somatic in the literature and those associated with somatic CN-LOH) were not further confirmed. Frameshift mutations were validated by cloning and sequencing individual colonies (TOPO TA cloning; Invitrogen). Novel missense mutations were confirmed when possible; for germ line confirmation (when constitutional DNA available), only exons containing mutations were tested. Screening for *JAK2* V617F mutation was performed as previously described.³⁷

Measurement of 5hmC levels

The 5hmC levels in genomic DNA from patients (N = 36) and healthy controls (N = 17) were measured by bisulfite conversion and dot blot with anti-CMS antiserum as described.²⁸ Results were normalized, and patients were divided into groups that were based on high or low 5hmC level as previously described.²⁸

Table 1. Baseline characteristics of patients with CMML and with CMML-derived AML

Characteristic	No.
No. of patients	72
Sex	
Male	48
Female	24
Age, y	
Median	70
Range	38-89
Metaphase cytogenetics (MC)	
Normal	39
Abnormal	30
No growth by MC	3
SNP-A karyotyping	70
Clinical data not available	2
WHO classification	
CMML-1	36
CMML-2	16
sAML to CMML-1/2	20

WHO indicates World Health Organization.

Immunohistochemical detection of pSTAT5

Staining was performed on a Benchmark XT platform (Ventana Medical Systems) according to the manufacturer's instructions, using mouse monoclonal anti-phospho-STAT5a/b (Y694/99; Advantex BioReagents LLP) at 1:500 dilution. All stains were scored without knowledge of the clinical diagnosis or mutational status. Phospho-STAT5-positive staining (nMEG pSTAT5) was defined as previously reported.³⁷

Statistical analysis

When appropriate, Kaplan-Meier statistics were applied to assess overall survival (OS) and compared with the log-rank test. For comparison of the frequency of mutation or other clinical features between diseases groups, categorical variables were analyzed with the Fisher exact test. A *P* value < .05 was set as the threshold of clinical significance.

Results

Characteristics and genetics of patients with CMML

A total of 72 patients were studied, 36 with CMML-1, 16 with CMML-2, and 20 with AML derived from the above conditions (Table 1). Among the patients with CMML, 46% cases had myelodysplastic CMML and 44% had proliferative CMML, as classified by the French-American-British group.³⁸ With the use of conventional MC, 30 patients (43%) showed an abnormal karyotype; the most common shared defects included monosomy 7 (9%), trisomy 8 (6%), and losses of the Y or X chromosomes (7%). Results were noninformative in 3 patients. SNP-A karyotyping confirmed defects identified by MC (except for some balanced translocations that are undetectable by SNP-A). New lesions were found in 60% of patients (42 of 70); previously cryptic somatic areas of CN-LOH were found in 30 of 42 patients (71%) and microdeletions in 19 of 42 patients (45%; supplemental Figure 1). The presence of multiple chromosomal defects as detected by SNP-A had a significant effect on patients' OS compared with patients without novel SNP lesions (16 vs 21 months; *P* = .04) by Kaplan-Meier analysis and univariate analysis (odds ratio [OR], 1.81; 95% confidence interval [CI], 1.02-3.35; Figure 1).

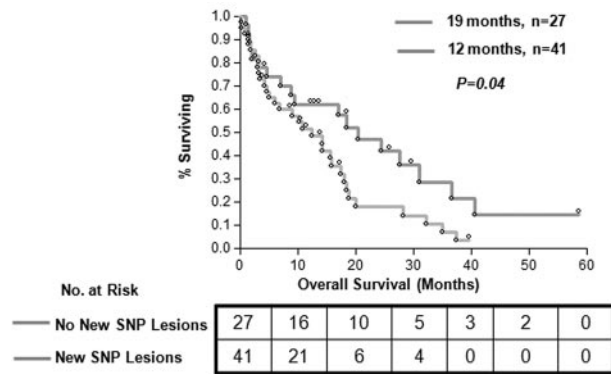


Figure 1. Clinical effect of new lesions detected by SNP-A karyotyping analysis. Patients with novel lesions detected by SNP-A and normal karyotype (MC) showed worse OS (median, 12 months; OR, 1.81; 95% CI, 1.02-3.35) versus patients without novel SNP lesions (median, 19 months; OR, 0.55; 95% CI, 0.29-0.98; $P = .04$). Patients with new SNP lesions and those without are represented by gray line ($N = 41$) and orange line ($N = 27$), respectively.

Identification of novel mutations in *UTX*, *EZH2*, and *DNMT3A*

When all 72 patients were screened, novel *UTX* variants were found in 4 patients (of 52; 8%) with CMML-1/2 and 2 patients with sAML (of 20; 10%); these mutations were spread over several exons (Figure 2A; Table 2). One case showed a homozygous missense *UTX* mutation associated with CN-LOH of chromosome

X. Another female patient with CMML-1 was found to harbor a somatic frameshift mutation, and a male patient had a nonsense mutation. Moreover, we detected 3 novel missense variants for which no constitutional DNA was available. However, these alterations have not been seen in controls (with a frequency of $< 1/400$ of male controls³⁵) or described as polymorphisms in available databases. In addition to *UTX* variants, we have identified a female patient with a hemizygous deletion involving the entire *UTX* gene on one X chromosome homologue. Collectively, deletions and mutations of *UTX* were present in 10% of the affected persons.

EZH2 mutations were identified in 3 of 52 patients (6%) with CMML (all with CMML-1; $N = 36$; 8%) and 1 patient with AML antecedent to CMML ($N = 20$; 5%). In 3 patients mutations were associated with LOH7q encompassing *EZH2*, whereas in 4 patients bearing CN-LOH7q *EZH2* mutations were not found. No mutations have been detected in patients with loss of chromosome 7 and/or 7q ($-7/7q$; $N = 6$). In 1 patient, a heterozygous nonsense mutation was encountered. All detected mutations disrupted highly conserved amino acids of EZH2. Notably, the SET domain, essential for the methyltransferase activity of EZH2, was either altered or truncated in ≥ 1 allele in all mutant cases (Figure 2B; Table 2).

DNMT3A mutations were detected in 2 of 52 patients with CMML (4%), all with CMML-2 (2 of 16; 12%). In addition, 5 mutant cases were identified in patients with CMML-derived

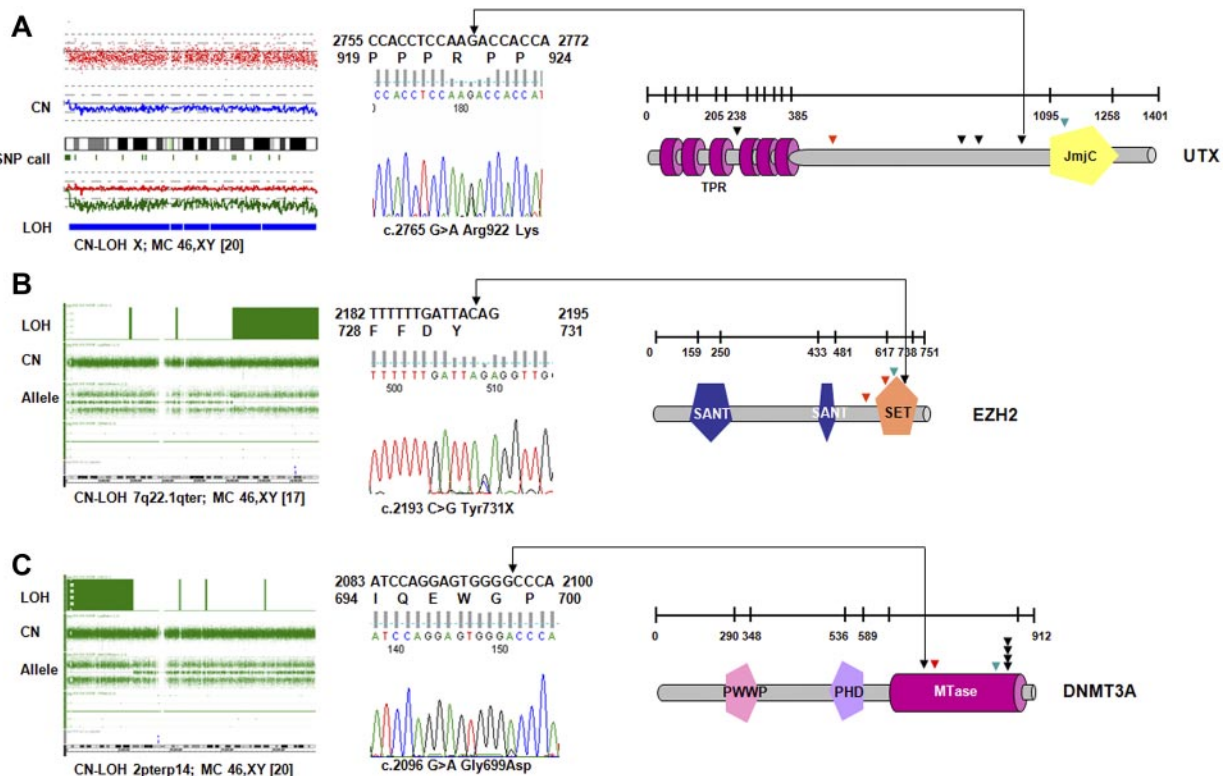


Figure 2. *UTX*, *EZH2*, and *DNMT3A* mutations identified in CMML. (A) In a cohort of 72 patients with CMML and AML-derived CMML, 2 somatic, inactivating *UTX* mutations were detected by sequencing. Four additional missense variants that could correspond to rare polymorphisms were identified. Profile of CN-LOH of X chromosome by SNP-A in one male patient with a *UTX* missense variant. Mutations/variants are denoted and relative to the *UTX* sequence NM_021140.1. The location of the TRP (tetratricopeptide repeat region) and JmjC (Jumonji C) domains is shown. (B) Identification of variations in *EZH2*-domain structure and positions of mutations in patients. All mutations were found in the SET domain or in close proximity. In 3 subjects mutations were identified along with CN-LOH7q encompassing *EZH2* as shown for representative patient (SET indicates suppressor of variegation 3-9, enhancer of zeste and trithorax domain; SANT-DNA-binding domain in the SWI-SNF and ADA complexes, the transcriptional co-repressor N-CoR and TFIIIB). (C) Mutations identified in methyltransferase domain of *DNMT3A* in 7 patients with CMML and AML-derived CMML. Homozygous *DNMT3A* mutations were associated with CN-LOH2p involving *DNMT3A*. The location of the PWWP (a highly conserved proline-tryptophan-tryptophan-proline motif), PHD (plant homeodomain finger, a zinc-fingerlike motif), and MTase (methyltransferase) domains is shown. Missense mutations are indicated by black; frameshift by red, and nonsense mutations by green arrows.

Table 2. Summary of identified mutations in *UTX*, *EZH2*, and *DNMT3A* genes in patients with CMML and with CMML-derived AML

Sex	Age, y	WHO Dx	Metaphase cytogenetics	SNP-A lesions			UTX mutation	<i>EZH2</i> mutation	<i>DNMT3A</i> mutation
				Gain	Loss	CN-LOH			
M	69	sAML	46,XX[20]	22q11.1	7q22.1	4q21.21qter 11q13.5qter	c.1324 C > T p.Gln442X*	Negative	Negative
M	78	CMML-1	46,XY,inv(3)(q21;q26)[20]	N	N	N	c.2373 A > G p.Asn791Ser (homozygous)*	Negative	Negative
F	53	CMML-2	47,XX,+21[19]/46,XX[1]	N	N	11q22.3q25	c.2502 G > T p.Glu834Asp*	Negative	Negative
M	78	sAML	46,XY[20]	N	N	X	c.2765 G > A p.Arg922Lys*	Negative	c.2645 G > A p.Arg882His†
F	65	CMML-2	46,XX[20]	N	12q24.31	7q11.23qter 14q23.2qter	c.3551 delA p.Asn1184fsX2*	Negative	Negative
F	60	CMML-1	46,XX,der(16)(t1;16)(q12;q11.2)[17]/46,XX[3]	1p11.2qter8q12.1	16p11.1qter	N	c.808 A > G p.Ile270Val*	Negative	Negative
F	76	CMML-1	46,XX[20]	8p23.2	N	N	Negative	c.1747 C > T p.Arg583X†	Negative
M	60	sAML	45,X,-Y[20]	N	N	4q23qter 7q11.23qter	Negative	c.2006-2063 (dup57nt) p.Ser669fs (homozygous)	Negative
F	78	CMML-1	47,XX,+8	8p23.2-q24.3	7q34	7q11.21-q36.3	Negative	c.2069 G > A p.Arg690His (homozygous)†	Negative
M	69	CMML-1	46,XY[17]	N	N	7q22.1qter	Negative	c.2193 C > G p.Tyr731X (homozygous)†	Negative
M	72	sAML	46,XY[20]	Yq11.23	N	2pterp14	Negative	Negative	c.2096 G > A p. Gly699Asp (homozygous)
F	69	CMML-2	46,XX[20]	N	N	2p11.2p25.3	Negative	Negative	c.2446 C > T p. Gln816X (homozygous)
M	68	sAML	46,XY[20]	N	N	13	Negative	Negative	c.2644 C > T p.Arg882Cys†
M	72	sAML	46,XY[20]	N	N	3p21.31p21.1	Negative	Negative	c.2645 G > A p.Arg882His†
F	74	sAML	46,XX,-7[3]/46,XX,add(12)(p13)[4]/46,XX,-7,add(12)(p13)[2]/46,XX[11]	Yp11.2	7	N	Negative	Negative	c.2191 del(TTC) p.Phe731
F	58	CMML-2	46,XX,del(17)(q24)[6]/46,XX[24]	21q22.13q22.2	2q24.3q32.1	1p36.13pter	Negative	Negative	c.2645 G > C p.Arg882Pro†

Dx indicates diagnosis.

*Variants not identified in 400 male controls.³⁵

†Variants of somatic origin by analyses of nonclonal CD3 cells or previously described in the literature as somatic and collected in COSMIC.³⁶

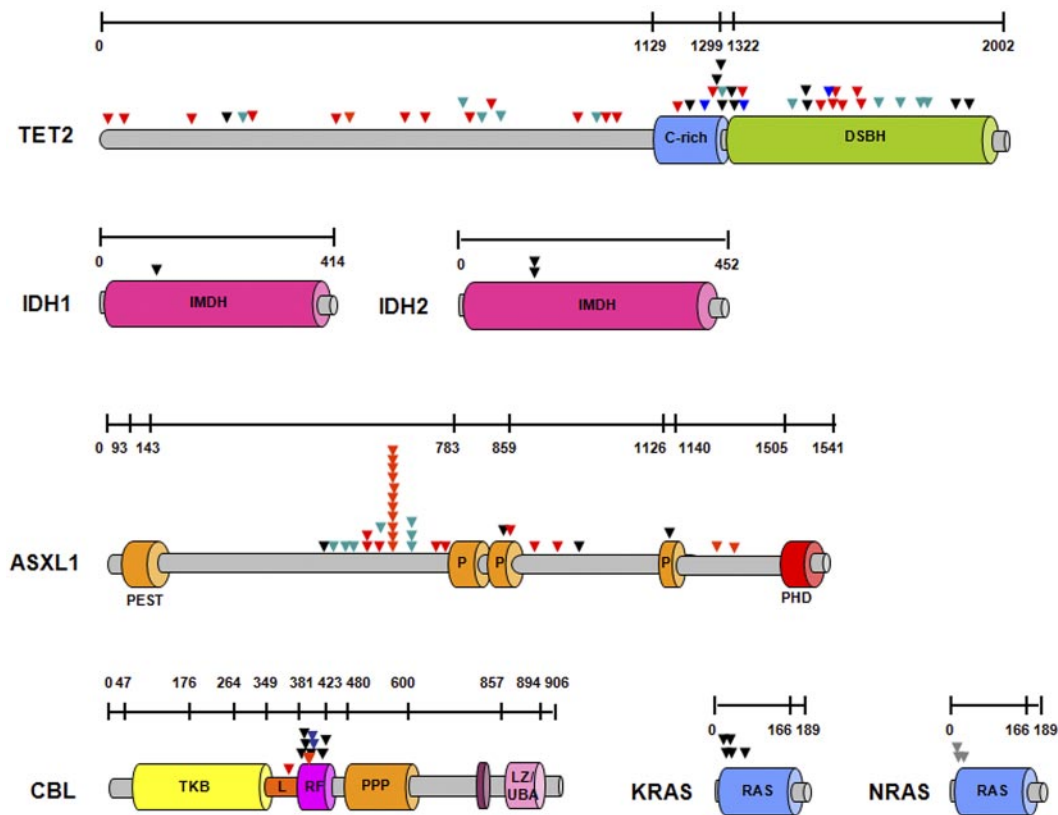


Figure 3. Schematic representation of the localization of molecular mutations at the protein level. Localization of all mutations detected in 72 persons with CMML and CMML-derived AML within the protein. Genomic sequencing of the protein-coding region and splice donor/acceptor sites showed missense (black triangles), nonsense (green triangles), and frameshift mutations (red triangles). Mutations found in *CBL* and *TET2* genes resulted in new splice variants (blue triangles). Most mutations were found in conserved domains, and specific known conserved motifs and domains are shown for each protein. DSBH indicates 2OG-dependent dioxygenase domain; IMDH, isocitrate/isopropylmalate dehydrogenase, conserved sites; PEST sequence, P indicates proline (P), glutamic acid (E), serine (S), and threonine (T); PHD, the plant homeodomain; TKB, tyrosine kinase binding domain; RF, ring finger; L, linker sequence; PPP, proline-rich region; LZ/UBA, leucine zipper/ubiquitin-associated domain; and RAS, subfamily of RAS small GTPases binding domain.

AML (6 of 20; 25%). The *DNMT3A* mutations were mostly heterozygous and affected the R882 position (Figure 2C; Table 2), a mutational hotspot previously reported to be of somatic origin.^{29,30} This mutation is predicted to act in a dominant-negative fashion and to reduce methyltransferase activity by > 50% in vitro.²⁹ Most mutant cases displayed a normal karyotype by MC (5 of 7; 71%). CN-LOH encompassing *DNMT3A* was found in 3 patients; 2 of these patients harbored novel homozygous *DNMT3A* mutations.

Frequency and distribution of other mutations

To gain potential insights into pathway abnormalities and relationships, we performed a targeted mutational screen of 11 candidate genes in our cohort. In patients with CMML-1/2 (N = 52), *TET2* mutations were identified in 44% (N = 23), *ASXL1* in 46% (N = 24), *CBL* in 13% (N = 7), *IDH1/2* in 4% (N = 2), *KRAS* in 8% (N = 4), and *NRAS* in 2% (N = 1). In patients with CMML-derived AML (N = 20), *TET2* mutations were detected in 60% (N = 12), *ASXL1* in 35% (N = 7), *CBL* in 15% (N = 3), *IDH1/2* in 5% (N = 1), *KRAS* in 5% (N = 1), *NRAS* in 10% (N = 2). *JAK2* V617F was found in 1% of patients (Figure 3; supplemental Table 1).

TET2 was the most frequently mutated gene in our study. *TET2* mutations were found throughout the entire coding region, including the N-terminus and the conserved Cys-rich and DSBH 2OG-Fe(II)-dependent dioxygenase domains²⁶ (Figure 3). In 13 patients, compound heterozygous (biallelic) *TET2* mutations were found; in 7 patients mutations were associated with LOH spanning the entire

coding region of *TET2*. Among all mutations, 11 (26%) were single-base substitutions, 22 (52%) were frameshift, 11 (26%) were nonsense mutations, and 4 mutations (9%) targeted acceptor/donor splice sites (N = 48). Except for 4 patients, missense mutations were associated with homozygosity or compound heterozygosity. In contrast to a diversity of *TET2* lesions, 31 *ASXL1* mutations were all heterozygous, including 19 frameshift and 7 nonsense mutations presumed to truncate the PHD. In addition, the controversial *ASXL1* variant, c.1934dupG p.Gly646TrpfsX12, was found in 15% of all patients.³⁹ Missense mutations in *ASXL1* were found only in 5 patients, and no LOH encompassing *ASXL1* gene was detected. Both homozygous and hemizygous mutations in *CBL* were identified (60% of mutant cases showed CN-LOH11q or loss of 11q23.3), as well as heterozygous mutations. All *RAS* mutations were heterozygous, except for one *NRAS* homozygous variant found in a patient with UPD1p. No LOH involving 2q (*IDH1* gene) and 15q (*IDH2* gene) was found, confirming the heterozygous nature of the corresponding mutations (Figure 3; supplemental Table 1).

Pathogenetic relationships

Comprehensive mutational analysis of the CMML-1/2 and CMML-derived AML cohorts resulted in the identification of patients with multiple mutational events and defined mutational patterns (Figure 4). Most of the patients with normal cytogenetics (by MC) showed the presence of ≥ 1 of the mutations studied (35 of 39; 90%). Among those patients with chromosomal defects, more mutations

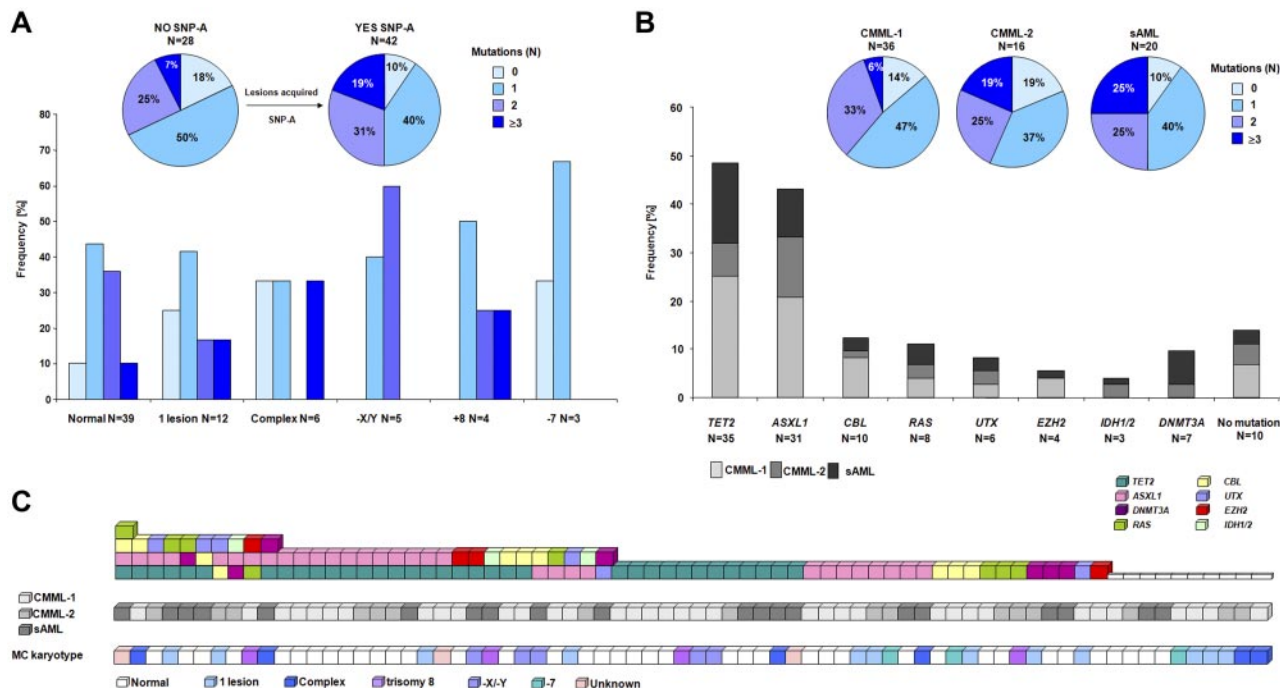


Figure 4. Frequencies and distributions of molecular mutations. Identified mutations and their frequencies in patients annotated according to karyotype by MC and SNP-A (A) and diagnostic criteria of CMML-1, CMML-2, and sAML (B). Most identified mutations were detected in patients with a normal karyotype by MC. SNP-A improved the detection rate, and only 10% of patients with uncovered SNP-A lesions did not carry any mutation. Among these patients an accumulation of mutations was observed (≥ 3 mutations in 19% vs 7% in those without new SNP-A lesions). Additional accumulation of mutations was found in AML derived from CMML compared with CMML-1/2. (C) Systematic mutational sequencing shows almost all possible combinations of genetic states for studied genes in patients with CMML and AML derived from CMML. Of 72 patients, only 14% of patients did not display mutations in any of the studied genes.

were detected (≥ 3 mutations in 19% vs 7% in those without chromosomal defects by SNP array; $P = .29$; Figure 4A). Furthermore, as expected patients with more advanced disease accumulated more mutations (≥ 3 mutations in 25% vs 19% and 6% in CMML-derived AML vs CMML-2 and CMML-1; $P = .70$ and $P = .08$, respectively; Figure 4B).

Novel *UTX* mutations were present as a sole event (of those tested) in one patient, whereas 5 of 6 cases harbored *UTX* mutations associated with other mutations (*ASXL1*, *TET2*, or *CBL*). Although the sample size is a limitation, *UTX* mutations were more frequent in advanced CMML (CMML-2, CMML-AML). *EZH2* variants were also accompanied by other molecular mutations affecting *TET2* and *ASXL1* or *RAS* genes. Intriguingly, *UTX* and *EZH2* mutations were not identified simultaneously in any of the patients studied. *DNMT3A* mutations were detected as the sole abnormality in 3 patients and in 4 cases were associated with other mutational events. Among *IDH1/2* mutant cases, one patient harbored a nonsense *TET2* mutation (Figure 4C).

In 86% of all patients, > 1 mutation was found (62 of 72). One mutation was found in 31 patients (43%); concomitant second or

≥ 3 mutations were found in 21 (29%) and 11 (14%) patients, respectively (Figure 4). In the CMML cohort, > 1 mutation was found in 85% (44 of 52 patients), whereas in the CMML-derived AML cohort, multiple lesions were detected in 90% of patients (18 of 20). Concomitant second or ≥ 3 mutations in CMML-1/2 were found in 16 (31%) and 11 (21%) patients, respectively, and in AML antecedent CMML was found in 5 (25%) patients each. The most frequently observed mutation combinations included *TET2* and *ASXL1*, as well as *TET2* and *CBL*, in 22% and 7% of all patients, respectively. *TET2/ASXL1* combinations were present in 46% of all *TET2* mutants (16 of 35), whereas *TET2/CBL* were present in 14% of all *TET2* mutant cases (5 of 35).

Combined analysis of clinical effect of mutations

We analyzed the association of specific mutations within the whole cohort and within individual risk groups of CMML (CMML-1/2 and AML-derived CMML; supplemental Table 2A-C). Although few mutant cases within each group were found, patients with CMML-derived AML harbored *DNMT3A* mutations more

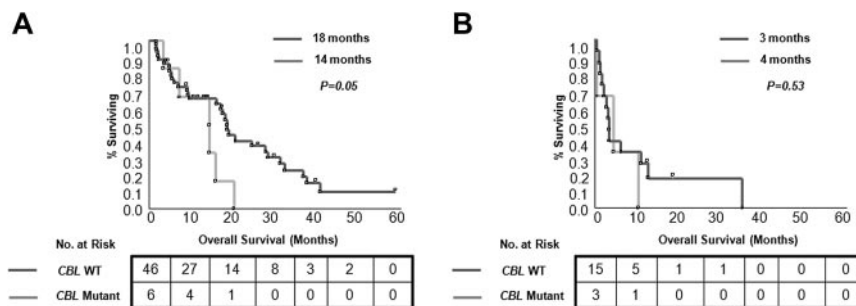


Figure 5. Kaplan-Meier survival curves estimated according to presence of *CBL* mutations. Differences in OS for patients with CMML (A) and with CMML-derived AML (B) are shown. For each group median months and number of analyzed cases are presented.

frequently than patients with other subtypes (25% [5 of 20] vs 4% [2 of 52]; $P = .015$). We have also compared patients carrying a specific mutation (eg, in *ASXL1* or *TET2* gene, etc) to the remaining patients without mutation of the corresponding gene with respect to a number of patient factors (supplemental Table 3A-C). Because of a significant overlap in the distribution of the 11 gene mutations tested, specific mutations cannot be compared with each other, and analysis would only be able to detect very pervasive features. Nevertheless, we were able to note certain biologic trends. For instance, there was a significant association of *TET2* and *CBL* mutations with more advanced age ($P = .007$ and $P = .04$, respectively). These associations remain significant even after patients with CMML-1/2 were analyzed separately ($P = .013$ and $P = .043$, respectively). In the whole cohort, *CBL* mutant cases more frequently displayed splenomegaly (67% vs 36% in patients without these mutations, respectively; $P = .14$) as well as in separate analyses of CMML-1/2 cases (83% vs 37%, $P = .075$) (supplemental Table 3). In general, increased marrow blasts were seen in *RAS* and *DNMT3A* mutants ($P = .019$ and $P = .019$, respectively), whereas higher total leukocytes were seen in *DNMT3A*-positive cases only ($P = .016$) (supplemental Table 3). When CMML-1/2 cases were analyzed separately ($N = 52$), the association with increased marrow blasts was seen only in *RAS* mutant cases ($P = .035$; supplemental Table 3). We also observed that in the CMML-1/2 group, patients with *TET2* mutations were more probable to have normal karyotype by MC compared with patients without mutations (32% vs 61%, respectively; $P = .048$).

Survival analyses

Univariate analyses of clinical and laboratory factors for OS were performed. Patients with CMML with cytogenetic abnormalities detected by SNP-A karyotyping show a shorter OS (Figure 1). In the whole cohort, blast count and disease stage as well as *DNMT3A* mutational status had a significant effect on patient survival ($P = .01$, $P = .001$, and $P = .04$, respectively). However, in multivariate analyses, which included age and other conventional prognostic factors, only disease stage (CMML-1/2 vs sAML) remained an independent prognostic factor ($P = .05$). When CMML cases were analyzed separately (without sAML derived from CMML), disease category (CMML-1 vs CMML-2) and the presence of *CBL* mutations were independent prognostic factors ($P = .04$ and $P = .03$, respectively; supplemental Table 4). In general, no differences in OS were observed for patients divided into subgroups on the basis of the presence of individual mutations, except for *CBL* mutants (Figure 5; supplemental Figure 2). Inferior outcomes were observed for *CBL* mutant cases compared with patients without mutations (OS, 9 months vs 16 months; $P = .04$), and this relationship was preserved within the CMML-1/2 subgroup (OS, 14 months vs 18 months; $P = .05$). In addition, patients with an accumulation of several mutations showed a trend toward inferior outcomes versus patients with single mutations and patients without mutations (in the CMML-1/2 subgroup ≥ 3 mutations vs 1 mutation vs none: OS, 14 months vs 20 months vs 18 months; and in the CMML-derived AML subgroup: OS, 1 month vs 3 months vs 7 months, respectively; supplemental Figure 3).

Although no significant difference was observed in OS between patients with or without *TET2* mutations, the analysis of 5hmC levels (which could be affected by various pathogenic processes, including *TET2*, *IDH1/2*,^{28,40,41} and possibly other undiscovered mutations) showed a difference in OS. When patients were divided into groups on the basis of high or low levels of 5hmC as previously described,²⁸ a trend toward a better OS was noticed in

cases with lower levels of 5hmC (OS, 19 months vs 7 months, respectively; $P = .06$), with significant differences for cases with normal karyotype compared with others (OS, 26 months vs 3 months, respectively; $P < .0001$). In the high-risk CMML group, patients with lower 5hmC levels showed better OS than patients with higher levels (OS, 20 months vs 4 months, respectively; $P = .03$).

Serial studies

Sequential samples of 6 patients with mutated CMML before AML transformation, at the time of AML transformation, or at the time of AML relapse were also studied (supplemental Table 5). Patient no. 12 was initially found to have a *TET2* mutation at the time of initial diagnosis of CMML-1, but, on remission induction therapy for AML, the *TET2* mutation was not detected. This mutation reappeared when therapy was discontinued because the patient progressed. The patient subsequently underwent a matched related donor nonmyeloablative allogeneic peripheral blood stem cell transplantation that resulted in a complete remission, and no *TET2* mutation was detected at that time and for years after. Patient no. 28 was initially classified as having refractory cytopenia with unilineage dysplasia (RCUD). No molecular mutations were noted, but his disease progressed and a diagnosis of CMML-1 was established, at which point a homozygous *CBL* mutation was detected. Subsequently, the patient transformed to AML, but no new mutations were found. Patient no. 41 was originally diagnosed with CMML-1 and was found to have a *DNMT3A* mutation. Soon after, the patient progressed to CMML-2, and the same type of mutation was present during disease evolution. Patient no. 65 was found to have *TET2*, *ASXL1*, and *DNMT3A* mutations at the time of initial diagnosis of therapy-related MDS. The same clones persisted at the time of CMML diagnosis and at subsequent disease transformation to AML several years later. Patient no. 49 had a BM examination that did not support the diagnosis of MDS and had normal cytogenetics but was found to have heterozygous *TET2* and *ASXL1* mutations. Subsequent follow-up showed the diagnosis of CMML-2 with CN-LOH4q spanning region of *TET2* gene and *TET2* and *ASXL1* mutations. Patient no. 2 was found to have homozygous *TET2* and *EZH2* mutations at initial diagnosis of CMML-1; after allogeneic hematopoietic stem cell transplantation, molecular remission was achieved. On relapse, the original homozygous *TET2* and heterozygous *EZH2* mutations were found.

Discussion

The prognosis of CMML is generally poor, and effective therapies are limited. The identification of the underlying pathogenesis, including recurrent molecular lesions, is of the utmost importance in developing disease-modifying treatment strategies.

On routine analysis of CMML with MC, clonal chromosomal abnormalities were identified in $< 50\%$ of cases, whereas SNP-A increased the potential identification of clonal markers in $\leq 70\%$ of cases; patients with previously cryptic lesions had decreased survival. This finding is consistent with results obtained in MDS.⁷ Here, we show that targeted mutational analysis of patients with CMML can identify a mutation and clonal marker in $\sim 86\%$ of patients, including those with an otherwise normal karyotype as assessed by routine and array-based karyotyping.

Recently, somatic mutations in genes involved in epigenetic regulation have been described in myeloid malignancies, including *ASXL1*, *EZH2*, *UTX*, and *DNMT3A*.^{11,20-22,29,30,35} On the basis of the

functional involvement in epigenetic regulation, our study focused on the potential role of *UTX*, *EZH2*, and *DNMT3A* alterations in CMML. We report for the first time *UTX* mutations in patients with CMML. *UTX* mutations were present at the C-terminus and the N-terminus of the *UTX* protein but mostly in the region adjacent to the JmjC domain required for *UTX* activity. In addition, among identified germ line *UTX* variants, 4 of 6 were missense substitutions (with a frequency of < 1/400 in male controls³⁵), but the functional consequences of these variants remain unclear. Overall, *UTX* mutations were detected less often than mutations in *TET2* or *ASXL1* and in some instances were concomitantly present. Consequently, inactivation of *UTX* because of mutation or haploinsufficiency through LOH may also impair differentiation programs in affected progenitor cells. Recurrent homozygous *EZH2* mutations have been previously identified in patients with MDS, MPN, and MDS/MPN,²⁰⁻²² implicating loss of *EZH2* function in the pathogenesis of these conditions. In our cohort, recurrent *EZH2* mutations were identified in 5.5% of all patients. Notably, all mutations except one missense variant were predicted to result in premature termination as a consequence of nonsense or frameshift mutations. These mutations were often associated with the presence of CN-LOH encompassing *EZH2* but not del(7) or del(7q). The mutations targeted evolutionary highly conserved residues in the SET and adjacent CXC domains, required for histone methyltransferase activity. We have not identified simultaneous occurrence of both *EZH2* and *UTX* mutations, although this observation is based on the small number of identified mutants. With respect to the function of these genes in the regulation of H3K27me3 levels, one could stipulate that corresponding mutations will be associated with alterations in H3K27 methylation patterns. Indeed, in all studied acquired *EZH2* mutations, significantly decreased levels of H3K27 trimethylation were found as shown by our previous studies and other investigators.^{21,22} Most probable, similar, although opposite in action, consequences might occur with the inactivation of *UTX*. Nevertheless, no significant changes on trimethylation status of H3K27 were found so far in 2 patients with mutated *UTX*,⁴² a finding consistent with another report.³⁵

The foregoing mutations in CMML will help to reconcile the existence of a presumptive reduction of H3K27me3 levels because of *EZH2* heterozygosity or homozygosity and loss-of-function of the histone H3K27 demethylase *UTX*. In addition, overexpression of *EZH2* has been reported in patients with AML and with MDS,⁴³ most probably resulting in silencing of genes involved in differentiation. Loss of *UTX* activity would be enzymatically equivalent to a gain of function for *EZH2* or simply overexpression of this enzyme. In either situation greater steady state levels of H3K27me3 are induced in malignant cells. H3K27me3 is associated with transcriptionally repressed genes, whereas H3K4me3 turns transcription on.⁴⁴ A rapid decrease of H3K27me3 occurs during stem cell differentiation.⁴⁵ Thus, inactivation of *UTX* in stem cells should lead to the maintenance of H3K27me3 marks on target genes and should prevent differentiation. Indeed, this is parallel to our observations whereby *UTX* mutations were detected in more-aggressive forms of CMML and AML derived from CMML. Conversely, *EZH2* mutations were detected among patients with CMML-1, suggesting an early event in malignant evolution.

On the basis of the recent discovery of *DNMT3A* mutations, we have also investigated their presence in CMML, detecting mutations in 10% of patients. *DNMT3A* mutations were present in the methyltransferase domain and mostly affected R882 amino acid, possibly causing changes in the DNA-binding groove or its interaction with DNMT3L.^{29,30} All of these mutations were heterozy-

gous, except for 2 homozygous variants associated with CN-LOH in the region containing the gene. *DNMT3A*-recurrent mutations at a single amino acid position suggest a gain-of-function mechanism, although very heterogeneous mutations at different positions in this gene generally might suggest loss of function. *DNMT3A* mutations were enriched in patients with normal karyotype, similarly to those reported in de novo AML.³⁰

Similar to *DNMT3A*, *TET2* and *IDH1/2* may affect DNA methylation and thereby convey a clonally restricted epigenetic instability phenotype. Like *TET1*, *TET2* is an α -ketoglutarate-dependent enzyme that converts 5mC to 5hmC,^{27,28} and loss of *TET2* function is associated with altered DNA methylation.²⁸ Neomorphic *IDH* mutations may indirectly affect the activity of α -ketoglutarate-dependent enzymes, including *TET* proteins and *UTX*, through the generation of high levels of 2-hydroxyglutarate and may therefore have consequences analogous to mutations of these genes (eg, decreased 5hmC levels or alteration of H3K27 methylation status).^{40,41,46}

In our study, mutations of *TET2* were found at a high frequency in patients with CMML, comparable to frequencies reported by other groups.^{12,13,23} A similar observation was found for *ASXL1*; 43% of patients harbored a mutation, including 15% of cases that carried the controversial variant c.1934dupG p.Gly646TrpfsX12.³⁹ *CBL* mutations were identified in 14%, *RAS* mutations in 11%, and the *JAK2* V617F in only 1% of patients with otherwise typical CMML. *IDH1/2* mutations were identified in 4% of patients at more advanced stage of disease, probably contributing as secondary events to their progression.⁴⁷ Only in 14% of patients did we not find any mutation in all genes analyzed. Most probably, apart from new mutations yet to be identified, they could carry *RUNX1* mutations found in patients with CMML.⁸

Combined analyses of mutational spectrums in CMML shows the molecular heterogeneity of the disease, allows for subclassification of patients on the basis of mutations or the combination of mutations present, and facilitates analyses of outcome studies on the basis of the presence of objective mutational biomarkers. Our study clearly shows the molecular heterogeneity of CMML and that the disease can be associated with multiple mutations that probably accumulated in the course of disease. It is not clear which of these mutations are of ancestral type and which clearly herald progression. Clinical analysis of patients with *UTX* or *EZH2* mutations did not show any distinct features, probably because of the small number of identified cases and overlap with other mutations. With regard to *DNMT3A* mutations, clinical association has been enriched, and correlation with higher marrow blasts and with blood cells counts was observed, but, again, this preliminary observation has to be confirmed in a larger cohort. Similarly, no specific phenotype associated with *ASXL1* or *TET2* mutations was identified, with the exception for the association of *TET2* and *CBL* mutations with older age and increased marrow blasts in *RAS*-mutant cases.

The analysis of clinical outcomes did not show a significant effect of specific mutations; however, there are a number of methodologic issues that may explain this finding. In general, the diagnosis of CMML carries a poor prognosis, with a median survival of 12 months,⁴⁸ precluding detection of clinical differences within the current cohort size. Nevertheless, *CBL* mutant cases showed less favorable survival, and a similar trend was seen for *DNMT3A* mutant cases. Moreover, patients who have accumulated more mutations had inferior outcomes compared with those with single mutations or those without any mutations.

A similar lack of distinctive features has been reported for *ASXL1*, *IDH1/2*, and *TET2* in CMML.^{10-13,49} However, a worse prognosis and acute transformation has been linked to *ASXL1* mutations.²³ In contrast, the reports on the prognostic effect of *TET2* mutations were controversial,^{10,12,13} but our study suggests that they do not convey additional negative effects on prognosis, at least in this very homogenous and well-defined CMML cohort. It is also possible that other mutations may be present in some patients with known lesions, such as those with *TET2*, indicating that analyzed cohorts are not sufficiently homogeneous. For instance, decreased levels of 5hmC were found in patients without *TET2* or *IDH1/2* mutations, implying the presence of other functional related molecular defects.²⁸ We therefore also investigated global 5hmC levels as a possible prognostic marker in CMML that would unify patients with a defective common molecular pathway. When we correlated this surrogate marker with survival, a trend toward better outcomes was seen for patients with low 5hmC levels. Although this has to be clarified in a larger study, similar outcomes were reported for *TET2* mutants in other studies.^{12,13}

Screening of sequential samples suggests that some of the molecular mutations may precede disease evolution. In particular, *ASXL1* and *TET2* mutations are detectable early in the clinical course. It is also probable that multiple mutations detected in one patient are a result of a stepwise evolution process. However, only deep sequencing and calculation of clonal sizes could, albeit indirectly, prove this point. Additional mechanisms, such as epigenetics and altered signaling pathways, may also be responsible for disease progression in CMML. The persistence of the same clone at the time of relapse and disease progression also provide helpful insight, because it suggests that future therapies aimed at the original molecular defects may result in disease eradication. Following molecular markers during the disease course may also be helpful in determining the likelihood of successful therapy and, conversely, the possibility of impending disease relapse.

In summary, our study shows that, despite a relatively uniform clinical phenotype and globally poor prognosis, CMML shows tremendous underlying molecular heterogeneity, with some mutations affecting convergent pathways or compensating for each other. For example, *CBL* mutations, via activation of RTK receptors, could result in activation of the RAS pathway, producing similar effects to RAS mutations and aberrant pSTAT5 activation. Similarly, *TET2* and *IDH-1/2* mutations may lead to a decrease in 5mC hydroxylation or *EZH2* and *UTX* mutations to altered H3K27me3 and be functionally similar to mutations of *ASXL1*

which also affect histone methylation. Above and beyond these findings our study shows, for the first time, the detection of *UTX* and *DNMT3A* mutations in patients with CMML. In general, we propose that epigenetic machinery may be frequently affected by mutations in myeloid malignancies in particular, as shown in this CMML cohort. However, larger patient cohorts will be needed to determine whether any of these gene mutations are critical in the pathogenesis of CMML. These diverse mutations can be associated with a uniform phenotype not distinguishable on clinical grounds, further showing that molecular diagnostics is needed for selection of targeted therapies for individual patients.

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Authorship

Contribution: A.M.J., H.M., and H.S. designed, performed, and analyzed most of the experimental work; Y.S., F.T., V.V., and C.P. helped with patient genotyping; A.M.J., M.A.M., and J.P.M. compiled the manuscript; C.O. performed SNP-A and cytogenetic analyses; R.V.T. performed classification of patients and database and performed clinical analyses; Y.H., and A.R. generated the anti-CMS antiserum, developed the quantitative assay for genomic 5-hmC,²⁸ and quantified the levels of 5-hydroxymethylcytosine in genomic DNA; E.D.H. reviewed pathologic specimens, interpreted results, and edited the manuscript; M.A.S. helped in classification and identification of patients and edited the manuscript; A.R. provided vital reagents and contributed to the design and analysis of the study; M.A.M. and A.L. selected some of the patients and contributed to the design and analysis of the study; and J.P.M. conceptualized the project, supervised the experimentation, analyzed the results, and wrote the manuscript.

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