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Invariant phenotype and molecular association of biallelic *TET2* mutant myeloid neoplasia

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Key Points

- Biallelic *TET2* gene inactivation is frequently observed in myeloid neoplasia.
- It might represent an auxiliary assessment tool to identify specific morphologic subentities of myeloid neoplasia.

Somatic *TET2* mutations (*TET2*^{MT}) are frequent in myeloid neoplasia (MN), particularly chronic myelomonocytic leukemia (CMML). *TET2*^{MT} includes mostly loss-of-function/hypomorphic hits. Impaired TET2 activity skews differentiation of hematopoietic stem cells toward proliferating myeloid precursors. This study was prompted by the observation of frequent biallelic *TET2* gene inactivations (*biTET2*^{i}) in CMML. We speculated that *biTET2*^{i} might be associated with distinct clinicohematological features. We analyzed *TET2*^{MT} in 1045 patients with MN. Of 82 *biTET2*^{i} cases, 66 were *biTET2*^{MT}, 13 were hemizygous *TET2*^{MT}, and 3 were homozygous *TET2*^{MT} (uniparental disomy); the remaining patients (denoted *biTET2*$^{-}$ hereafter) were either monoallelic *TET2*^{MT} (n = 96) or wild-type *TET2* (n = 823). Truncation mutations were found in 83% of *biTET2*^{i} vs 65% of *biTET2*$^{-}$ cases ($P = .02$). *TET2* hits were founder lesions in 72% of *biTET2*^{i} vs 38% of *biTET2*$^{-}$ cases ($P < .0001$). In *biTET2*^{i}, significantly concurrent hits included *SRSF2*^{MT} (33%; $P < .0001$) and *KRAS/NRAS*^{MT} (16%; $P = .03$) as compared with *biTET2*$^{-}$. When the first *TET2* hit was ancestral in *biTET2*^{i}, the most common subsequent hits affected a second *TET2*^{MT}, followed by *SRSF2*^{MT}, *ASXL1*^{MT}, *RAS*^{MT}, and *DNMT3A*^{MT}. *BiTET2*^{i} patients without any monocytosis showed an absence of *SRSF2*^{MT}. *BiTET2*^{i} patients were older and had monocytosis, CMML, normal karyotypes, and lower-risk disease compared with *biTET2*$^{-}$ patients. Hence, while a second *TET2* hit occurred frequently, *biTET2*^{i} did not portend faster progression but rather determined monocytic differentiation, consistent with its prevalence in CMML. Additionally, *biTET2*^{i} showed lower odds of cytopenias and marrow blasts (≥5%) and higher odds of myeloid dysplasia and marrow hypercellularity. Thus, *biTET2*^{i} might represent an auxiliary assessment tool in MN.

Introduction

Increasingly, genomic data are being used to classify myeloid neoplasia (MN). Examples include BCR/ABL in chronic myeloid leukemia (CML)[1]; t(8;21), inv(16), t(15;17), or MLL in acute myeloid leukemia (AML)[2-4]; *PDGFRA/B* translocations in chronic myelomonocytic leukemia (CMML), and, in hereditary cases, germline mutations in *CEBPA*,[5] *RUNX1*,[6,7] *ETV6*,[8,9] *DDX41*,[10] *GATA2*,[11] *TP53*,[12] etc. Such genetic alterations are beginning to supersede the use of morphologies in diagnoses, particularly when the pathomorphologies are less pronounced. For instance, ring sideroblasts are linked to *SF3B1*

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mutations[13,14] or refractory anemia with ring sideroblasts and thrombocytosis with a combination of *SF3B1* with either *CALR*, *JAK2*, or *cMPL* mutations.[13,15,16]

Some mutations are common and thus unlikely to be molecular markers of specific morphologic subentities. Examples include mutations in *TET2*, *ASXL1*, and *DNMT3A*.[17,18] *TET2* is located on the long arm of chromosome 4 (4q24), a region susceptible to microdeletions, copy-neutral losses of heterozygosity, and rare translocations that also result in protein loss of function, producing TET2. TET2 is an Fe^{2+}-dependent dioxygenase that converts 5-methylcytosine to 5-hydroxymethylcytosine (5hmC) to derepress silenced genes. *TET2* mutations (*TET2*^{MT}) impair *TET2*'s ability to carry out this reaction. This decreases 5hmC DNA levels, which in turn skews differentiation toward monocytic progenitors. Indeed, engineered *tet2*$^{-/-}$ mice have earlier onsets of myeloproliferative neoplasm (MPN) disease than *tet2*$^{+/-}$ mice.[19,20] The role and function of *TET2* has been studied in normal and malignant hematopoiesis.[21-24] The contribution of *TET2*^{MT} to clinicohematological features has, however, been controversial, possibly due to small-scale studies and combinatorial diversity of cooccurring lesions. Large studies accounting for clonal architecture and association between molecular and clinical features will be helpful to clarify the consequences of *TET2*^{MT} on disease phenotypes.

Here, we report the clinical course of patients with biallelic *TET2* inactivation (*biTET2*^{i}) in the context of MN. Compared with corresponding monoallelic mutations, these events might be associated with gene-dose–dependent greater intensity phenotypes and clinical outcomes and perhaps exaggerated morphologic features associated with increased risk to leukemia progression. We have comprehensively dissected the clonal nature of *TET2*^{MT} in 4930 patients with MN, of whom 40% harbored *biTET2*^{MT}.[25] We thus investigated possible associations between such abnormalities and clinical features and outcomes. We provide evidence supportive of the notion that *biTET2*^{i} cases belong to a qualitatively distinct morphologic subentity of MN.

Material and methods

Patients

Peripheral blood and bone marrow (BM) samples from patients with MN were collected after receiving written informed consent according to the protocols approved by the Institutional Review Board of Cleveland Clinic in accordance with the Declaration of Helsinki. A total of 1045 patients were initially screened and enrolled in this study. Clinical parameters (age, sex, peripheral blood, BM counts, diagnosis, and overall survival) were obtained from medical records. Diagnosis was assigned based on the 2008 World Health Organization (WHO) classification criteria.[26] Genomic and germline DNA obtained from $CD3^{+}$ lymphocytes was subjected to molecular screening for the coding regions of *TET2* and other gene mutations. Samples that yielded low sequencing quality due to low depth were excluded from the study. Cases in which no *TET2*^{MT} was found by gene sequencing were investigated for possible deletions and microdeletions at chromosome 4q/24 by reviewing metaphase cytogenetics and results of single-nucleotide polymorphism (SNP) array, respectively. The ones with 4q deletion/microdeletion involving *TET2* locus were considered monoallelic *TET2*^{MT} (*monoTET2*^{MT}), while those with absent aberrations were considered wild-type (*TET2*^{WT}) (supplemental Figure 1A). All *TET2*^{MT} were also screened for uniparental disomy (UPD) at chromosome 4q/24 by the SNP-array (SNP-A) method.

Next-generation sequencing

Whole-exome sequencing libraries were prepared according to the Nextera Rapid Capture Exome protocol (Illumina, San Diego, CA) and subjected to massive parallel sequencing using HiSeq 2000. Average coverage of samples subjected to whole-exome sequencing and targeted deep sequencing was 115× and 250×, respectively. Variants with a variant allele frequency (VAF) >5% were included. Multiamplicon targeted deep sequencing included a panel of 36 genes commonly mutated in myelodysplastic syndrome (MDS)[18,27,28] and other myeloid malignancies (supplemental Table 1). Paired-end libraries were subjected to deep sequencing on MiSeq sequencers according to Illumina protocols. Variants were extracted using the GATK3.3 pipeline. *TET2*^{MT} were called somatic when absent or at very low frequencies in germline $CD3^{+}$ lymphocytes. Alterations found in both the myeloid and lymphoid cells with an equal VAF were considered germline and excluded from the study. Previously, usage of T cells as germline[29,30] resulted in similar frequencies of *TET2*^{MT} compared with skin or buccal swab specimens.[18,31] For original data, please contact H.A. (awadah@ccf.org).

SNP-A–based karyotyping

SNP-A karyotyping for confirming metaphase cytogenetics and detecting copy-number–neutral loss of heterozygosity was performed as previously described.[32,33] Briefly, Affymetrix 250K and 6.0 SNP-As were used to evaluate cop-number alterations and copy-number natural loss of heterozygosity. Using our internal database and a publicly available database (http://dgv.tcag.ca/dgv/app/home), the screening algorithm validated each lesion as somatic. Nonsomatic lesions were excluded from further analysis. Affected genomic positions in each lesion were visualized and extracted using CNAG (v3.0) or Genotyping Console (Affymetrix, Santa Clara, CA).[34,35] Metaphase cytogenetic requires cellular proliferation, and its sensitivity and resolution depend on the proportion of clonal cells in the sample and size of the lesion, respectively. SNP-A does not depend on the presence of dividing cells and is able to detect copy-number variations and UPD with a high resolution. For this purpose, we included this method to further investigate for 4q/24 cryptic chromosomal lesions not identified by metaphase cytogenetic in our cohort.[36]

Conventional cytogenetics

Metaphase cytogenetics was performed on BM aspirates. The median number of metaphases analyzed was 20. Chromosomal preparation was performed on G-banded metaphase cells using standard techniques, and karyotypes were described in 86% (862/1001) of patients according to the International System for Human Cytogenetic Nomenclature.[37]

Ancestral/dominant-codominant and secondary mutation estimation

VAFs were used to categorize first-hit *TET2*^{MT} into ancestral (dominant or codominant) mutations vs subclonal secondary mutations. A mutation with the highest VAF that is at least 5% more than the second highest VAF in each sample was defined as an ancestral/dominant mutation; those with <5% difference from

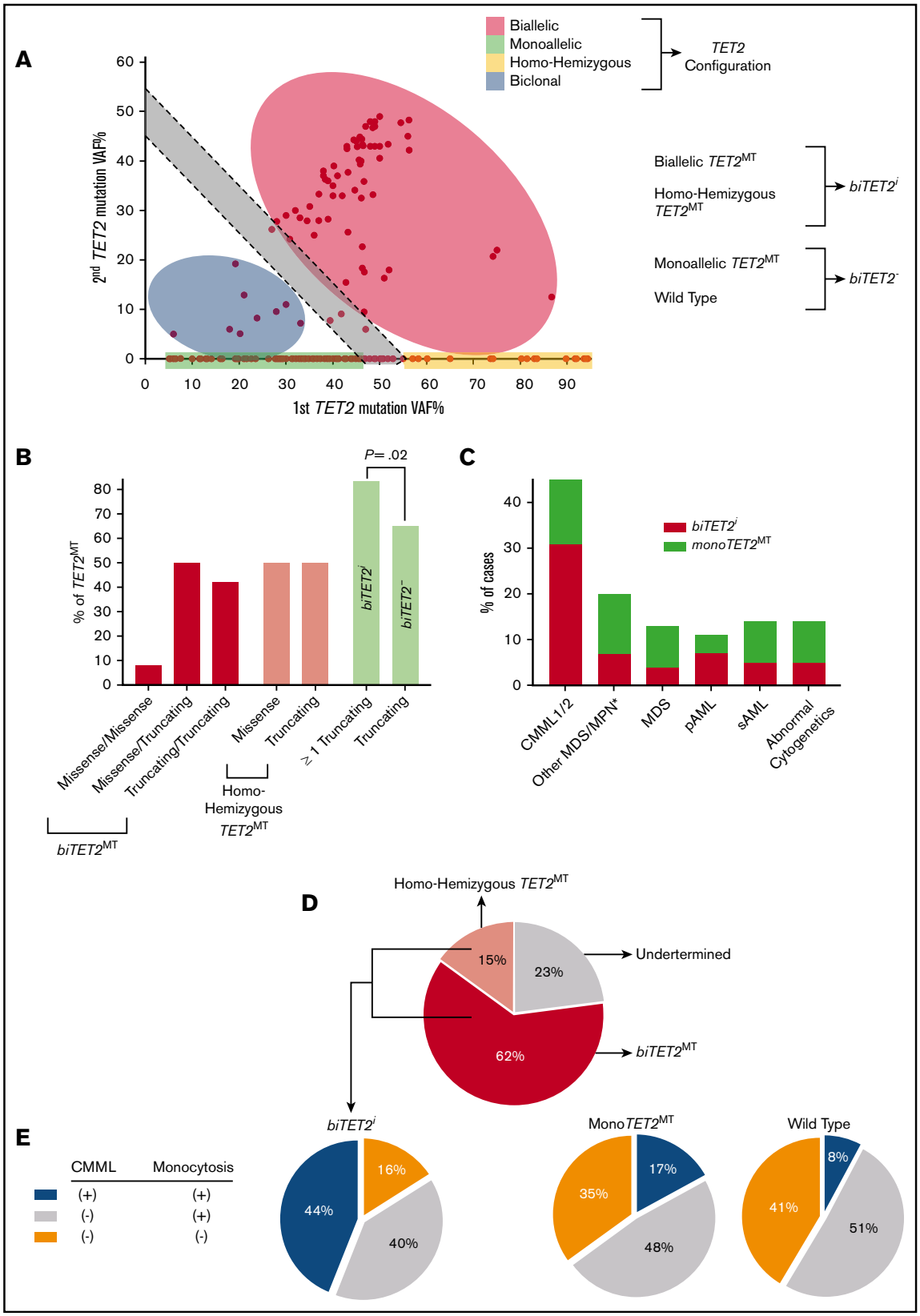

Figure 1. *TET2* gene mutation classification, type, and clinical characteristics. (A) Scatterplot of the VAFs of patients with *TET2*^{MT}. The VAF of first-hit *TET2*^{MT} was plotted on the x-axis and that of the second-hit *TET2*^{MT}, if present, on the y-axis. Patients were categorized into 5 groups as explained in the text. The red oval corresponds to *biTET2*^{MT} cases, the gray bar to undetermined cases, the blue oval to biclonal *TET2*^{MT} cases, the light green oval to *monoTET2*^{MT} cases, and the yellow oval to

the highest VAF were defined as ancestral/codominant, while VAFs with a >5% difference from the highest VAF were considered subclonal/secondary mutations (supplemental Figure 2A-F). Mutations in selected genes (commonly mutated in myeloid neoplasms) were assessed for differences in *biTET2*^{i} vs *biTET2*$^{-}$ cases.

Statistical analyses

Fisher's exact test was used to compare proportions. All *P* values were 2 sided; those <.05 were considered statistically significant. Kaplan-Meier methods were used to plot survival probabilities, and log-rank tests were used to compare such curves. Univariate and multivariate Cox model analyses were also performed. All statistical computations were performed using R 3.5.1 (www.r-project.org).

Results

Identification of *biTET2*^{i} in myeloid neoplasms

We analyzed configurations of *TET2*^{MT} using VAFs (see Materials and methods) in 1045 patients with MN. Patients with *TET2*^{MT} (n = 200) were classified into heterozygous biallelic *TET2*^{MT} (*biTET2*^{MT}; ≥2 *TET2*^{MT} with VAF sum of >55%; n = 66), biclonal *TET2*^{MT} (≥2 *TET2*^{MT} with VAF sum of <45%; n = 11), undetermined (either biallelic or biclonal *TET2*^{MT}, as their VAF sum lays between 45% and 55%; n = 33), hemizygous *TET2*^{MT} (single *TET2*^{MT} with VAF >55% in the presence *TET2* locus alteration on chromosome 4q24; n = 13), homozygous *TET2*^{MT} (homozygous mutation at 4q24 with UPD detected by SNP analysis; n = 3), and mono*TET2*^{MT} (single *TET2*^{MT} with VAF <45% and normal cytogenetics; n = 74; Figure 1A). Biclonal *TET2*^{MT} and undetermined cases were ambivalent and thus filtered out of the study (n = 44), resulting in a cohort of 1001 MN patients. *BiTET2*^{MT}, hemizygous *TET2*^{MT}, and homozygous *TET2*^{MT} have all inactivation (impairment) of both parental copies of *TET2* and therefore were grouped in as *biTET2*^{i} cases (n = 82) (Figure 1D). The remainder of the population, with either *monoTET2*^{MT} (n = 96; 74 cases with single *TET2*^{MT} and normal metaphase cytogenetic/SNP-A screening; 22 cases with 4q/TET2 locus deletion in absence of *TET2*^{MT}) or wild-type configuration (*TET2*^{WT}, n = 823; normal metaphase cytogenetic/SNP-A screening and absent *TET2*^{MT}), were considered negative for *biTET2*^{i} (*biTET2*$^{-}$; n = 919). Patients were also divided into those with CMML and without CMML (−) according to the presence of WHO-defined CMML hallmark clinical features.[1] The CMML (−) cohort (n = 885) was then subgrouped according to the presence of monocytosis. Monocytosis was present in 56% (n = 497) of these cases. *TET2*^{MT} configuration and respective number of patients is summarized in (supplemental Figure 1A).

Somatic *TET2*^{MT} was found in 156 out of 1001 of cases (16%), of which 53% were *biTET2*^{i}. A total of 83% of *biTET2*^{i} cases were truncating (frameshift deletion/insertion and nonsense), while 27% of somatic alterations were missense (Figure 1B). A comparison of the VAF ratio of the first and second *TET2*^{MT} per type of mutation and succession in *biTET2*^{MT} is presented in supplemental Figure 1D. The majority of *monoTET2*^{MT} patients (77%) harbored a *TET2*^{MT} with a VAF <45%; the minority had 4q/24 aberrations (locus deletion) (23%) (supplemental Figure 1B). *MonoTET2*^{MT} cases included 65% truncating and 35% missense mutations (supplemental Figure 1C). Truncating mutations were significantly more common in *biTET2*^{i} cases than *monoTET2*^{MT} cases (odds ratio [OR], 2.7; *P* = .02; Figure 1B).

Table 1. Comparison of baseline characteristics in *biTET2*^{i} vs *biTET2*$^{-}$ cases

Variables	Biallelic *TET2* inactivation		*P*
	Yes	No	
Age, median (range), y	72 (13-100)	67 (16-91)	
Age ≥60 y	76 (91)	684 (74)	**.0004**
Sex			
Male	55 (70)	575 (63)	
Female	27 (30)	344 (37)	
Risk stratification by WHO*			
Low	53 (62)	433 (47)	**.003**
High	29 (38)	486 (53)	
WHO classification			
MDS	22 (27)	476 (52)	**<.0001**
RA/RCUD/RCMD/isolated del(5q)/MDS-U	11 (13)	251 (27)	
RARS/RCMD-RS	4 (5)	61 (7)	
RAEB-1/2	7 (9)	164 (18)	
MDS/MPN overlap	41 (50)	145 (16)	**<.0001**
CMML-1/2	36 (44)	80 (9)	**<.0001**
MDS/MPN-U/RARS-T/aCML	5 (6)	65 (7)	
AML	19 (23)	298 (32)	
pAML	8 (10)	107 (11)	
sAML	11 (13)	191 (21)	
Metaphase cytogenetics†			
Normal	53 (65)	417 (45)	**.0007**
Abnormal	28 (35)	502 (55)	

Values represent n (%) of patients, unless otherwise indicated. Significantly different values are in bold text. Fisher's exact test was used for statistical analysis. Please refer to supplemental Table 2 for comparison between mono- and biallelic *TET2*^{MT}groups.

aCML, atypical chronic myeloid leukemia; del; deletion; MDS/MPN-U, MDS/MPN unclassifiable; MDS-U, MDS unclassified; pAML, primary acute myeloid leukemia; RA, refractory anemia; RAEB, refractory anemia with excess blasts; RARS, refractory anemia with ring sideroblasts; RARS-T, refractory anemia with ring sideroblasts associated with marked thrombocytosis; RCMD, refractory cytopenia with multilineage dysplasia; RCMD-RS, refractory cytopenia with multilineage dysplasia and ring sideroblasts; RCUD, refractory cytopenia with unilineage dysplasia; sAML, secondary acute myeloid leukemia.

*Lower risk includes RA, RARS, RCMD, RCMD-RS, CMML-1, MDS/MPN-U ≤5% blast, RARS-T, MDS-U, and isolated del(5q). Higher risk includes RAEB-1/2, CMML-2, MDS/MPN-U ≥5% blasts, and AML.

†Some data could not be assessed.

Figure 1. (continued) hemihomozygous *TET2*^{MT} (UPD) cases. (B) Percentages of different types of *TET2*^{MT} in *biTET2*^{i} cases and significance of truncating mutations vs *monoTET2*^{MT}. Fisher's exact test was used for analysis. (C) Bar graphs showing the distribution of *biTET2*^{i} and *monoTET2*^{MT} cases per diagnosis and cytogenetics. The bar columns indicate percentages. (D) Pie chart showing the percentage of cases per configuration. (E) Pie charts of *biTET2*^{i}, *monoTET2*^{MT}, and *TET2*^{WT} respectively representing the percentage of cases per classification. CMML (+) indicates cases with CMML at the time of presentation, CMML (−) indicates no CMML diagnosis, monocytosis (+) indicates the presence of monocytosis, and monocytosis (−) indicates the absence of monocytosis.

Clinical phenotypes of cases with *biTET2*^{i}

Clinical analysis revealed that *biTET2*^{i}, in comparison with *biTET2*$^{-}$, was associated with older age (91% ≥60 years vs 74%, $P = .0004$; Table 1). Among *biTET2*^{i} cases, 27% were classified as MDS, 50% as MDS/MPN, and 23% as AML (10% pAML and 13% sAML). *BiTET2*^{i} was enriched in patients with CMML1/2 (44%; $P < .0001$), predominantly in lower-risk cases (62% vs 47% in *biTET2*$^{-}$; $P = .003$) and more commonly had normal metaphase cytogenetics (65%; $P = .0007$; Figure 1C). We also assessed phenotype/genotype association of *biTET2*^{i} (Table 2). In *biTET2*$^{-}$ cases, leukopenia (81%; $P < .0001$), neutropenia (52%; $P = .008$), pancytopenia (27%; $P = .008$), and increased marrow blast percentages (blasts ≥5% in 33%; $P = .01$) were more prevalent than in *biTET2*^{i} cases, which in return cosegregated with monocytosis (84%; $P < .0001$; Table 2; Figure 1E), marrow hypercellularity (cellularity ≥70% in 67%; $P < .0001$), and more pronounced myeloid dysplasia (68%; $P = .0003$).

We next compared *biTET2*^{i} cases to those with *monoTET2*^{MT} to evaluate the consequence of a second *TET2* inactivation on disease features (supplemental Tables 2 and 3). Biallelic inactivation of *TET2* was more likely to occur with MDS/MPN ($P = .001$), particularly the CMML1/2 subtype ($P < .0001$; Figure 1C,E), and with normal cytogenetics ($P = .003$). In addition, it was correlated with a lower odds of leukopenia ($P = .002$) and ring sideroblasts (≥15%; $P = .02$) and a higher likelihood of monocytosis ($P = .003$) and marrow hypercellularity ($P = .02$) (Figure 1E).

Because we observed a highly significant ($P < .0001$) relationship between *biTET2*^{i} and CMML diagnosis and/or monocytosis, we focused on patients without obvious CMML (monocytosis; absence of *BCR/ABL1*, *PDGFRA/B*, or 11q23; presence of <20% blasts; and myeloid dysplasia) and compared *biTET2*^{i} and *biTET2*$^{-}$ for the association with monocytosis and myeloid dysplasia (supplemental Figure 1E). Increased monocyte counts among CMML (−) was also significantly overrepresented in *biTET2*^{i} cases (72%; $P = .03$) compared with *biTET2*$^{-}$ cases (55%), as was myeloid dysplasia (72% vs 46%; $P = .0001$).

Clonal substructure of cases with *biTET2*^{i}

The rank of *TET2*^{MT} within the clonal hierarchy can be determined according to VAF methodology (see Materials and methods). We first defined each *TET2*^{MT} as ancestral vs secondary and then identified other hits in relation to *TET2* status. Due to resolution limits of the VAF approach and the difficulty of distinguishing subclonal from ancestral hits, we applied an arbitrary cutoff of 5% between VAFs to discriminate ancestral first hits (dominant and codominant) from subsequent "secondary" hits (supplemental Figure 2A-F). A summary of clonal hierarchy of somatic mutations, cytogenetic findings, and diagnoses in all *biTET2*^{i} cases is presented in Figure 2A. Seventy-two percent of first *TET2* hits in *biTET2*^{i} were founder (dominant/codominant) lesions ($P < .0001$), while only 28% were secondary to another antecedent somatic mutations (Figure 2B). In mono*TET2*^{MT} cases, only 38% *TET2* hits were dominant. In *biTET2*^{i}, when the first *TET2*^{MT} was subclonal, the preceding founder clone was most likely characterized by *BCOR/BCORL1*^{MT}, *PRC2-family*^{MT}, *ZRSR2*^{MT}, *ASXL1*^{MT}, and others (Figure 2C). When the first *TET2* hit was ancestral, the most common secondary mutation affected *TET2*, followed in frequency by *SRSF2*^{MT}, *ASXL1*^{MT}, *RAS*^{MT}, and *DNMT3A*^{MT} (Figure 2D-E).

Table 2. Comparison of clinical characteristics in *biTET2*^{i} vs *biTET2*$^{-}$ cases

Variables	Biallelic *TET2* inactivation		
	Yes	No	*P*
Hematological parameters*			
WBC, median (range), $\times 10^9$/L	5.9 (1.1-109)	4.3 (0.3-228.3)	
$<4 \times 10^9$/L	57	81	**<.0001**
Hemoglobin, median (range), g/dL	9.8 (3.6-14.5)	9.6 (5.1-15.1)	
<10 g/dL	55	62	.2
Platelets, median (range), $\times 10^9$/L	75 (4-558)	82 (2-1116)	
$<100 \times 10^9$/L	65	64	.9
ANC, median (range), $\times 10^9$/L	3.1 (0.1-56.7)	1.9 (0-145.6)	
$<1.5 \times 10^9$/L	37	52	**.008**
Monocytes			
$\geq 1 \times 10^9$/L†	84	59	**<.0001**
Ring sideroblast (≥15%)	11	19	.1
Cytopenias*			
Monocytopenia	38	29	.1
Bicytopenia	39	33	.3
Pancytopenia‡	13	27	**.008**
BM morphology*			
Blasts, median (range), %	3 (0-95)	4 (0-89)	
≥5%	33	48	**.01**
Cellularity, median (range), %§	80 (10-100)	70 (5-100)	
Hypercellular	67	40	**<.0001**
Normocellular	28	44	
Hypocellular	5	16	
M/E ratio, median (range)	3.9 (0.4-49)	2.1 (0.2-45)	
Myelofibrosis	20	18	.8
Dysplastic lineages*			
Myeloid	68	46	**.0003**
Erythroid	60	62	.9
Megakaryocytic	58	60	.8
Number of dysplastic BM lineages*			
Unilineage	21	26	.4
Bilineage	31	30	.9
Trilineage	34	28	.2

Values represent n (%) of patients, unless otherwise indicated. Significantly different values are in bold text. Fisher's exact test was used for statistical analysis. Please refer to supplemental Table 3 for comparison between mono- and biallelic *TET2*^{MT}groups.

ANC, absolute neutrophil count; M/E, myeloid to erythroid ratio; WBC, white blood cell count.

*Some data could not be assessed.

†Criteria used for monocytosis was a reported absolute monocyte count $\geq 1 \times 10^9$/L.

‡Deficiency of all 3 cellular components of the blood: red blood cells, white blood cells, and platelets.

§Hypercellular (>70%), normocellular (30-70%), and hypocellular (<30%) BM.

When we investigated associations between concurrent mutations and *TET2*^{MT} configuration, *ASXL1*^{MT} (25%), *TP53*^{MT} (16%), and *CBL*^{MT} (7%) were more frequent in *monoTET2*^{MT} cases; *SRSF2*^{MT} (33%), *KRAS/NRAS*^{MT} (16%), *RUNX1*^{MT} (16%), and *ZRSR2*^{MT} (6%) were more frequent in *biTET2*^{i} cases; and *DNMT3A*^{MT} (13%) and *U2AF1*^{MT} (9%) were more common in the *TET2*^{WT} population

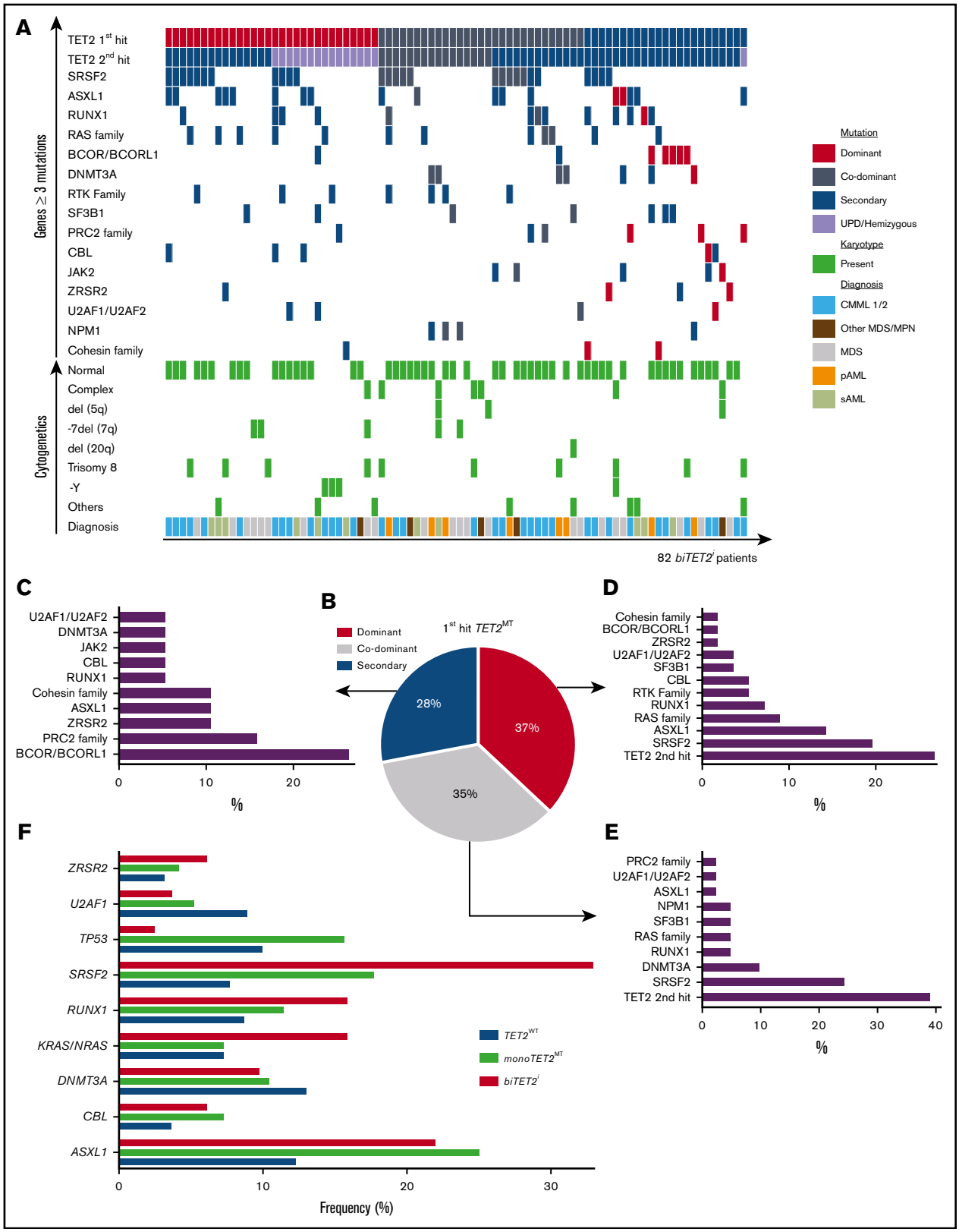

Figure 2. Clonal architecture and hierarchy of *TET2*^{MT} in *biTET2*^{i}. (A) Plot showing dominant, codominant, and secondary mutations in the 82 *biTET2*^{i} patients. Mutated gene names, cytogenetics, and diagnosis are color coded as indicated. For this presentation, only genes mutated ≥3 times among the *biTET2*^{i} population are represented. Each column represents 1 patient, and each row corresponds to 1 gene or family of genes. (B) Pie chart displaying the percentage of first-hit *TET2* occurring as dominant (ancestral), codominant (ancestral), and subclonal (secondary) in the *biTET2*^{i} population. (C-E) The bar graphs show the percentages of the corresponding dominant genes to

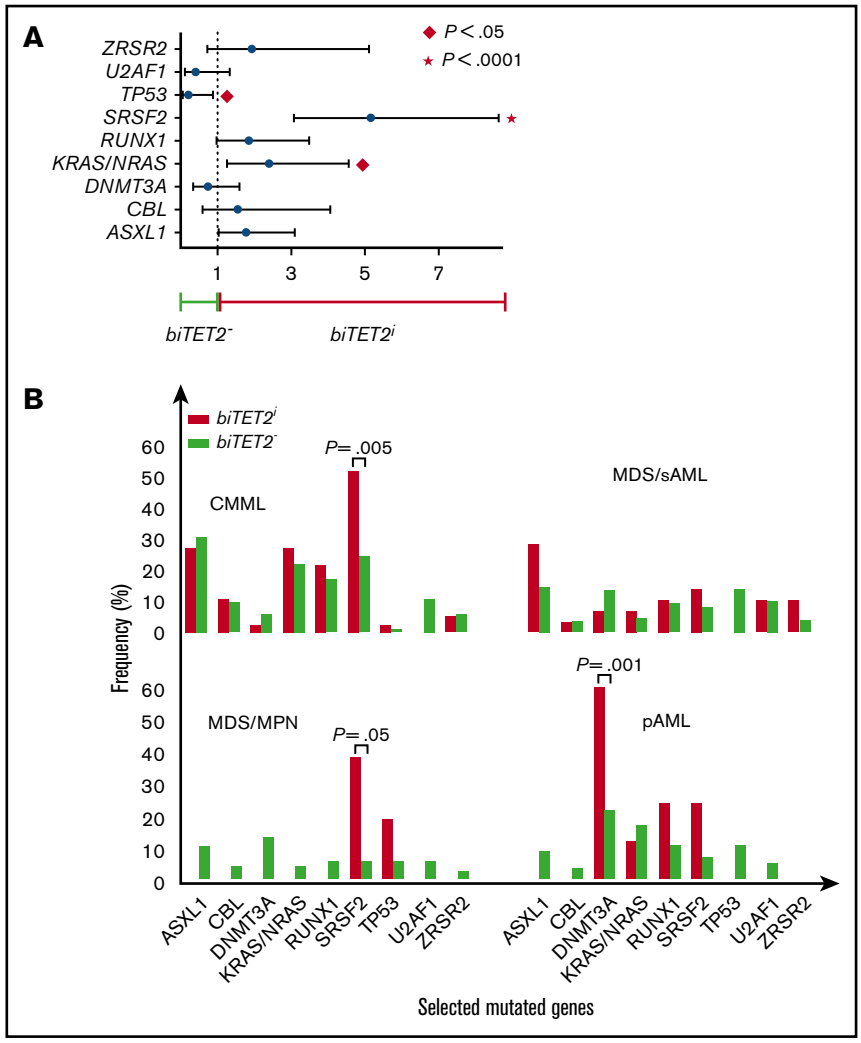

Figure 3. Significance of concurrent gene mutations in *biTET2*^{i} and correlation per disease subtype. (A) Forest plot showing the OR of associated gene mutations in *biTET2*^{i} vs *biTET2*$^{-}$ cases. As indicated, red squares correspond to significant cases, while red stars correspond to highly significant cases. Fisher's exact test was used to test significance. (B) Frequency (in percentage) of mutations in selected genes per disease subtype in *biTET2*^{i} vs *biTET2*$^{-}$. Significance was tested via Fisher's exact test.

(Figure 2F). When compared with patients with and without *biTET2*^{i} (Figure 3A), a significant co-occurrence with *SRSF2*^{MT} ($P < .0001$) and *KRAS/NRAS*^{MT} ($P = .03$) in *biTET2*^{i} and *TP53*^{MT} ($P = .03$) in *biTET2*$^{-}$ was noted. *SRSF2*^{MT} was also found to be significantly associated with *biTET2*^{i} when compared with *monoTET2*^{MT} ($P = .02$) (supplemental Figure 3). In contrast, in *biTET2*^{i} without monocytosis (16%; n = 13), *SRSF2*^{MT} was absent and *KRAS/NRAS*^{MT} was only detected in 8% (n = 1) of the cases.

In CMML, among *biTET2*^{i} cases, *SRSF2*^{MT} was the most commonly found co-occurring lesion (53%; $P = .005$), followed by *KRAS/NRAS*^{MT} (28%), *ASXL1*^{MT} (28%), and *RUNX1*^{MT} (22%). Investigation for the incidence of a secondary/subclonal *ASXL1*^{MT} among CMML with preexisting *biTET2*^{i} (20%) vs CMML with *monoTET2*^{MT} (72%) tended to be significant ($P = .05$). In *biTET2*$^{-}$ cases, *ASXL1*^{MT} (31%), *SRSF2*^{MT} (25%), *KRAS/NRAS*^{MT} (23%), and *RUNX1*^{MT} (18%) were seen in high frequencies. In MDS/MPN (excluding CMML), *SRSF2*^{MT} was present in 40% and *TP53*^{MT} in 20% of *biTET2*^{i} cases, respectively, while *DNMT3A*^{MT} occurred in 14% and *ASXL1*^{MT} in 11% of *biTET2*$^{-}$ cases. Similar to what we observed in CMML, patients with MDS/MPN carrying *biTET2*^{i} showed a striking concordance with *SRSF2*^{MT} ($P = .05$). In MDS/sAML, *ASXL1*^{MT} (24%) and *SRSF2*^{MT} (12%) were most commonly found together with *biTET2*^{i}, while *ASXL1*^{MT} (13%), *DNMT3A*^{MT} (12%), and *TP53*^{MT} (12%) clustered with *biTET2*$^{-}$ cases. Finally, in pAML, *DNMT3A*^{MT} (63%), *SRSF2*^{MT} (25%), and *RUNX1*^{MT} (25%) were the most frequent molecular events in *biTET2*^{i} cases, while *DNMT3A*^{MT} (22%) and *KRAS/NRAS*^{MT} (18%) were seen in *biTET2*$^{-}$ cases (Figure 3B).

We then grouped *biTET2*^{i} cases into those with and without diagnosis of CMML (supplemental Figure 4A) and found that *biTET2*^{i} cases were strongly associated with *SRSF2*^{MT} ($P = .0009$) and *KRAS/NRAS*^{MT} ($P = .01$) in CMML. Among *biTET2*^{i} cases with *SRSF2*^{MT} and those with *KRAS/NRAS*^{MT}, CMML was diagnosed in 70% ($P = .001$) and 77% ($P = .01$), respectively (supplemental

Figure 2. (continued) the secondary/subclonal first-hit *TET2* gene (C), secondary clones to the dominant first-hit *TET2* gene (D), and codominant genes to the codominant first-hit *TET2* gene (E). (F) Frequency (in percentage) of mutations in selected genes in the population. Ten genes that are frequently mutated in myeloid neoplasms were selected. Columns are color coded per *TET2*^{MT} configuration (*TET2*^{WT}, mono*TET2*^{MT}, and *biTET2*^{i}).

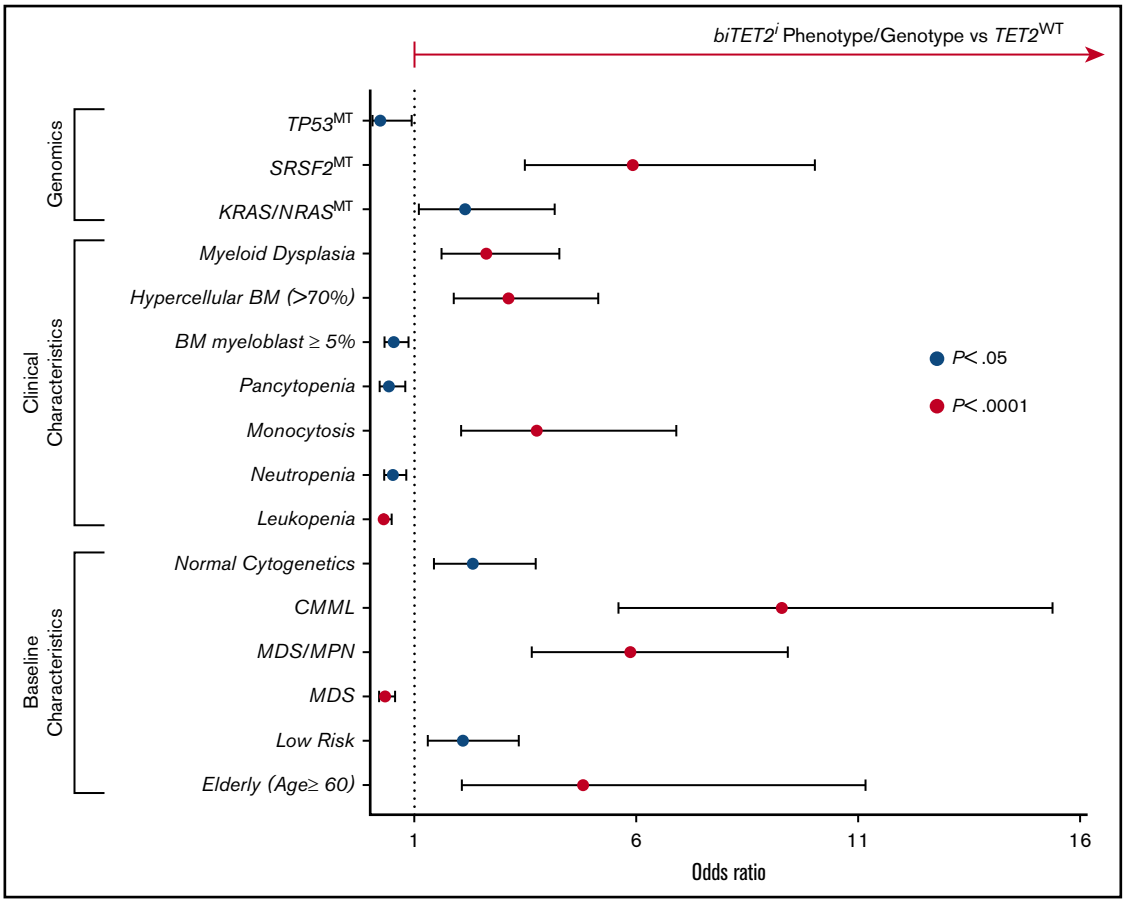

Figure 4. Univariate analysis for baseline, clinical, and genomic features in *biTET2*^{i} vs wild-type. Univariate analysis showing the significant results for baseline (older age, lower risk, MDS, MDS/MPN, CMML, and normal cytogenetics), clinical (leukopenia, neutropenia, monocytosis, pancytopenia, BM blasts, BM hypercellularity, and myeloid dysplasia), and genomic (*KRAS/NRAS*^{MT}, *SRSF2*^{MT}, and *TP53*^{MT}) features in *biTET2*^{i} vs *TET2*^{WT}.

Figure 4B), which is higher than what was seen in the *biTET2*^{i} population (44%; Figure 1E). We then investigated the overall impact of *biTET2*^{i} by comparing the effect of *biTET2*^{i} vs *biTET2*$^{-}$ in relation to CMML diagnosis, the presence and absence of monocytosis, and the concomitant presence of *SRSF2*^{MT} or *KRAS/NRAS*^{MT}. No differences in survival outcomes could be attributed to the presence of *biTET2*^{i} as a sole factor or in combination with others (supplemental Figure 5A-J).

Independent features associated with *biTET2*^{i}

When univariate analyses were conducted in the *biTET2*^{i} vs *TET2*^{WT} population (Figure 4A), *biTET2*^{i} was associated with older age, lower risk, MDS/MPN, CMML, normal cytogenetics, monocytosis, marrow hypercellularity, myeloid dysplasia, and the presence of *SRSF2*^{MT} and *NRAS/KRAS*^{MT}. In contrast, *TET2*^{WT} status correlated with MDS, leukopenia, neutropenia, pancytopenia, elevated BM blasts, and *TP53*^{MT}. We then conducted a multivariate Cox regression analysis (Table 3). For *biTET2*^{i} vs *TET2*^{WT}, older age (≥60 years; OR, 4.2; P = .002), CMML (OR, 3.4; P = .03), monocytosis (OR, 2.1; P = .05), myeloid dysplasia (OR, 1.8; P = .04), marrow hypercellularity (OR, 2.4; P = .005), and *SRSF2*^{MT} (OR, 2.2; P = .02) were independent features associated with *biTET2*^{i}. In *biTET2*^{i} vs *monoTET2*^{MT}, CMML (OR, 6.7; P = .02), truncating *TET2*^{MT} (OR, 3.5; P = .02), and ancestral *TET2*^{MT} (OR, 5.5; P = .0002) were independently associated with *biTET2*^{i}. When we compared the cohort of *biTET2*^{i} vs *biTET2*$^{-}$, CMML (OR, 6.9; P = .02), truncating *TET2*^{MT} (OR, 3.5; P = .02) and ancestral *TET2*^{MT} (OR, 5.5; P = .0002) were found to be independent prognostic factors in *biTET2*^{i}, while elevated marrow blasts (OR, 0.2; P = .02) were more common in *biTET2*$^{-}$. Finally, for *monoTET2*^{MT} vs *TET2*^{WT}, elderly (age ≥60 years; OR, 3.3; P = .002), *TP53*^{MT} (OR, 2.5; P = .01), and *SRSF2*^{MT} (OR, 2.1; P = .03) were found to be distinct for *monoTET2*^{MT}.

Discussion

Objective molecular tools are complementing morphological methods in clinical practice. Morphology remains, however, the golden standard for identifying strong associations between phenotype and genotype in MN. We report here on phenotypical and morphological characteristics of cases harboring biallelic *TET2* defects.

To date, only a few studies have investigated the clinical consequences of *biTET2*^{i} in MN. We used a well-characterized cohort of patients with *biTET2*^{i}. Our hypothesis was that *biTET2*^{i} associates with a group of pathomorphological features that independently define a distinct MN subtype. To test our idea, we

Table 3. Multivariate analysis showing significant independent results in *biTET2*^{i} vs *TET2*^{WT}, *biTET2*^{i} vs *monoTET2*^{MT}, *biTET2*^{i} vs *biTET2*$^{-}$, and *monoTET2*^{MT} vs *TET2*^{WT}

	biTET2^{i} vs *TET2*^{WT}		*biTET2*^{i} vs *monoTET2*^{MT}		*biTET2*^{i} vs *biTET2*$^{-}$		*MonoTET2*^{MT} vs *TET2*^{WT}	
Variables	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***
Elderly	4.2 (1.7-10.3)	.002					3.3 (1.5-7.1)	.002
CMML	3.4 (1.1-10.7)	.03	6.7 (1.4-33.5)	.02	6.9 (1.4-34.0)	.02		
Monocytosis	2.1 (1.0-4.6)	.05						
Myeloid dysplasia	1.8 (1.0-3.3)	.04						
BM blast ≥5%					0.2 (0.1-2.8)	.02		
Hypercellular BM	2.4 (1.3-4.4)	.005						
TP53 mutation							2.5 (1.2-4.8)	.01
SRSF2 mutation	2.2 (1.1-4.2)	.02					2.1 (1.1-4.0)	.03
Truncating *TET2*			3.5 (1.2-10.4), *P* = .02					
Ancestral *TET2*			5.5 (2.3-13.5), *P* = .0002					

biTET2^{i}, biallelic *TET2* inactivation (n = 82); *biTET2*$^{-}$, "negative" *biTET2*^{i} (n = 919); CI, confidence interval; *monoTET2*^{MT}, monoallelic *TET2* mutant (n = 96); *TET2*^{WT}, wild-type *TET2* (n = 823).

first identified *biTET2*^{i} cases among a cohort of 1045 patients with MN and then studied correlations between mutational configuration and clinicohematological morphology in comparison with *biTET2*$^{-}$ cases.

While our finding that most *TET2*^{MT} cases are truncating (frameshift or nonsense changes) agrees with previous studies,[38-40] we further show that a second *TET2* hit in *biTET2*^{MT} cases significantly increases the chances of accumulating more truncating changes in those already harboring a *TET2*^{MT}. The prevalence of *biTET2*^{i} among older patients demonstrated in this investigation is also in agreement with patterns observed in other studies.[25] This can be explained by the effect of aging on the accumulation of *TET2*^{MT} in clonal hematopoiesis of indeterminate potential and subsequent subclonal hits over time.[41,42]

TET2^{MT} is detected in a large fraction of myeloid disorders[38,43] but predominantly in CMML.[44] Here, we show that the MDS/MPN CMML subtype significantly correlated with *biTET2*^{i} events (Figures 1C,E and 4). This relationship is modified by mutations in additional genes, such as *SRSF2*^{MT} in cases of CMML,[44,45] that are found to correlate strongly with *biTET2*^{i} even when CMML is absent. This observation was further demonstrated by comparing *biTET2*^{i} with *monoTET2*^{MT}, which showed a significant accumulation of MDS/MPN, CMML subtype, monocytosis, and *SRSF2*^{MT} solely resulting from the clonal succession following the second *TET2* hit. When criteria for CMML diagnosis[46] were not clinically fulfilled, *biTET2*^{i} remained invariably associated with monocytosis and myeloid dysplasia, both hallmarks of CMML proliferative and dysplastic features.[43,47]

Along with studies suggesting that *TET2*^{MT} has a neutral impact on the rate of progression to AML,[25,48] we also identified that *biTET2*^{i} tended to occur in lower-risk patients with a lower likelihood of leukemic transformation, unless additional deleterious events co-occur. Given that most *TET2* hits were ancestral to subsequent secondary mutations (second *TET2*^{MT}, *SRSF2*^{MT}, *ASXL1*^{MT}, *RAS*^{MT}, and *DNMT3A*^{MT}), we concluded that these founder lesions represent a leukemogenic predisposition (mutator phenotype) rather than driving leukemia. *BiTET2*^{i} correlated with normal karyotype, as did the second *TET2* hit in those harboring *TET2*^{MT}, implying a significantly lower likelihood of cytogenetic abnormalities and an association with lower-risk disease.

Other notable features of *biTET2*^{i} included, in addition to monocytosis and *SRSF2*^{MT}, significantly higher leukocyte and neutrophil counts and less pancytopenia. When reviewing BM morphology, in addition to myeloid dysplasia, we observed low percentages of marrow blasts despite prominent hypercellularity. Moreover, lower odds of leukopenia and ring sideroblasts and an increased marrow cellularity were strongly correlated with a second hit in *TET2* in comparisons of *biTET2*^{i} and *monoTET2*^{MT}.

Inactivating *TET2*^{MT} leads to low 5hmC levels. 5hmC is the first oxidative product of the *TET* demethylation pathway marking tissue and cell-specific genes. A decrease in 5hmC levels is associated with malignant phenotypes and poor survival outcomes.[49] 5hmC is highly localized at binding sites of the p300/CREB binding protein, and in vitro studies have shown that upon *TET2* acetylation, 5hmC levels increase significantly. As previously reported, *TET2*^{MT} displayed low levels of 5hmC showing hypomethylation compared with healthy subjects at the majority of differentially methylated CpG sites. The greater degree of deficiency by the impairment of both *TET2* alleles, the more the hydroxylation function is affected.[22] Consequently, more methylation of CpG sites would be expected. As a result, the previously described expansion of the stem cell compartment and differentiation that skewed toward monocytic differentiation should be expected to be more pronounced. In our study, *biTET2*^{i} did not lead to a strong deleterious phenotype, suggesting that the acetylation mechanism might have been stabilized TET2 protein.

Given a more pronounced phenotype and genotype, it is important to speculate why *biTET2*^{i} does not result in more serious clinical consequences. It is possible that the impairment of *TET2* via inactivating mutations might be compensated by other TET enzymes (eg, TET1) or by decreased posttranslational modification (eg, acetylation) leading to a higher fraction of catalytically active protein. TET2 activity is regulated by acetylation, which increases TET2 stability (by protecting TET2 from ubiquitination and proteasome degradation) and promotes the cooperation with DNA

methyltransferase 1 (DNMT1).[44] Acetylation might represent a regulatory mechanism of TET2 protein. TET2 is acetylated by p300/CREB binding protein at key lysine residues (K110 and K111) located in the N terminus of the protein, and this acetylation can be switched by HDAC1/2 and SIRT1/2 deacetylases. The N terminus of TET2 seems to contain high levels of enzymatic activity and positive regulatory feedback, possibly because the acetylation of lysine residues is a natural mechanism increasing TET2 activity.[50]

In conclusion, our collective observations demonstrate that *biTET2*^{i} is a frequent event in MN. Furthermore, the presence of *biTET2*^{i} contributes additional information to the genetic complexity of MN and thus might represent a putative assessment tool of certain morphologic MN subentities associated with invariant phenotypic and molecular characteristics. The increasing collection of omics data and their correlation with clinical factors will further shed the light on the nature of *biTET2*^{i} in MN.

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Authorship

Contribution: H.A. designed the study, collected, analyzed, and interpreted clinical and molecular data, and wrote the manuscript; Y.N. collected data and provided important feedback to the manuscript; A.G., M.F.A., B.P., and Y.G. collected and analyzed data; C.M.H. and B.P.P., performed and analyzed DNA sequencing data and edited the manuscript; T.K., M.A., V.A., W.S., and L.W. collected clinical data and edited the manuscript; A.N., M.E.A., M.A.S., T.H., and B.K.J. provided clinical specimens and important insights on the manuscript; T.R. provided statistical advice and important editing in the manuscript; V.V. provided important editing and wrote the manuscript; J.P.M. designed the study, conceptualized and sponsored the overall research, and wrote the manuscript; and all authors read and approved the final manuscript.

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