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ARTICLE

Myelodysplastic syndrome

Consequences of mutant *TET2* on clonality and subclonal hierarchy

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Abstract

Somatic mutations in *TET2* are common in myelodysplastic syndromes (MDS), myeloproliferative, and overlap syndromes. *TET2* mutant ($TET2^{MT}$) clones are also found in asymptomatic elderly individuals, a condition referred to as clonal hematopoiesis of indeterminate potential (CHIP). In various entities of $TET2^{MT}$ neoplasia, we examined the phenotype in relation to the strata of *TET2* hits within the clonal hierarchy. Using deep sequencing, 1781 mutations were found in 1205 of 4930 patients; 40% of mutant cases were biallelic. Hierarchical analysis revealed that of $TET2^{MT}$ cases >40% were ancestral, e.g., representing 8% of MDS. Higher (earlier) *TET2* lesion rank within the clonal hierarchy (greater clonal burden) was associated with impaired survival. Moreover, MDS driven by ancestral $TET2^{MT}$ is likely derived from $TET2^{MT}$ CHIP with a penetrance of ~1%. Following ancestral *TET2* mutations, individual disease course is determined by secondary hits. Using multidimensional analyses, we demonstrate how hits following the *TET2* founder defect induces phenotypic shifts toward dysplasia, myeloproliferation, or progression to AML. In summary, $TET2^{MT}$ CHIP-derived MDS is a subclass of MDS that is distinct from de novo disease.

Introduction

TET2 is one of the most commonly mutated genes in myeloid neoplasia [1–3] and also occurs at lower frequencies in some forms of T cell lymphoma [4]. *TET2* mutations ($TET2^{MT}$) have been detected in seemingly asymptomatic older controls, also referred as to having clonal hematopoiesis of indeterminate potential (CHIP). Their presence in these individuals is associated with a higher risk of developing a hematologic neoplasm [5–8]. $TET2^{MT}$ are also encountered in clonal cytopenias of undetermined significance (CCUS), a proportion of these cases likely representing early, subclinical MDS [9].

The *TET2* gene product is a Fe^{2+}-dependent dioxygenase, which uses electrons gained from vitamin C and α-ketoglutarate (aKG) decarboxylation to split O_2 to hydroxylate 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC). This reaction may lead to both passive indirect demethylation (whereby 5hmC prevents methylation of the newly synthesized strand during replication) and active demethylation via further *TET2*-mediated 5hmC oxidation followed by removal of the oxidized base by base excision repair [10, 11]. $TET2^{MT}$ are mainly loss-of-function

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mutations. The loss of hydroxymethylation skews myeloid differentiation toward monocytes, and, as expected, increases 5mC levels [12–15]. *Tet2*$^{-/-}$ mice develop fatal myeloproliferative disease and lymphomas. These conditions arise later in *Tet2*$^{+/-}$ mice suggesting a delayed impact of haploinsufficiency leading to acquisition of secondary hits [16, 17].

Prognostic impact of *TET2*^{MT} has not been reproducibly demonstrated. Various groups have reported no impact on OS in MDS [18–20], while others have found *TET2*^{MT} to be associated with an unfavorable prognosis [21–24]. An earlier study focused on coexisting *JAK2*^{MT} and *TET2*^{MT} in MPN has shown that the acquisition order of mutations influences not only the clinical phenotype, but also response to targeted therapy [25]. It is possible that the heterogeneity of *TET2*^{MT}, their configuration, subclonal context, and co-associated variables result in biological heterogeneity that precludes the ability to establish precise phenotype–genotype associations.

We hypothesized that some of these aforementioned relationships can be clarified by studying cases in which *TET2* hits are founder lesions. These cases appear to be derived from CHIP with *TET2*^{MT} and differ from other CHIP-derived entities and de novo MDS. Specific mutations preceding or following *TET2* in the clonal hierarchy can create signature phenotypic shifts that drive disease. Ancestral *TET2* hits appear to predispose to a certain spectrum of secondary hits, which in turn imprint further clinical features, including myeloproliferative vs. dysplastic features or rates of progression. To address these questions, we analyzed a cohort of patients with myeloid neoplasms using whole exome and targeted deep sequencing of a panel of 22 genes most frequently affected by somatic mutations in myeloid neoplasms [26–28].

Methods

Patient samples

Blood and bone marrow samples were collected from patients at the Cleveland Clinic and the Munich Leukemia Laboratory with myeloid neoplasms (MN) according to protocols approved by the Cleveland Clinic IRB, and the Declaration of Helsinki. Germ line DNA was obtained from CD3^{+} lymphocytes. Sample which yielded low sequencing quality due to low depth were excluded from the study.

Next-generation sequencing (NGS)

Whole-exome libraries were prepared according to the Nextera Rapid Capture Exome protocol (Illumina, San Diego, CA) and subjected to massive parallel sequencing using the HiSeq 2000. Average coverage was ×115 and only variants with variant allele frequency (VAF) >5% were used. Multi-amplicon targeted deep sequencing was performed for a panel of 22 genes most commonly somatically mutated in MDS [26–28] (Supplementary Table 1). Paired-end libraries were generated and deep sequenced on MiSeq (Illumina, San Diego, CA) sequencers according to Illumina protocols. Average coverage was ×250. Variants were extracted using the GATK3.3 pipeline and best practices. *TET2*^{MT} found in the CD3^{-} fraction but not (or highly diminished frequencies) in the germ line CD3^{+} fraction were deemed somatic mutations and included in our analysis. We expect sequence alterations found in both the myeloid and lymphoid cells with equal VAF to be germ line and excluded from our study. Previously, usage of T cells as germ line [13, 29] resulted in similar frequencies of *TET2*^{MT} compared to skin or buccal swabs [27, 30].

Distinction of ancestral and subclonal mutations

To identify the ancestral and secondary mutations for each patient, VAFs were serially analyzed in a subcohort of patients ($N = 40$; Supplementary Fig. 1). Mutations appearing during clinical course but absent at initial presentation were deemed subclonal, while ancestral mutations were detected at all time points. Evolution followed the expected order (including contractions or expansions) with the exception of ambivalent results for VAFs within ±5%. When serial samples were not available, VAFs of mutations (adjusted for copy number and zygosity) were ranked and assigned as ancestral for first or dominant hits, and secondary for any subsequent subclonal hit. Acknowledging resolution limitations we used a cutoff of at least a 5% difference between VAFs to identify ancestral mutations. If the difference in VAFs between two mutations was <5%, for the purpose of this study, we referred to them as co-dominant.

In cases with multiple *TET2*^{MT}, *x*–*y* plots of VAFs were generated to assess the probability that they are strictly biallelic or may also be biclonal or subclonal (Supplementary Fig. 2). In either case, higher VAFs indicate earlier events (including possibly an ancestral hit, if other higher hierarchal events are absent).

Calculation of TET2 mutant CHIP penetrance and TET2-derived CHIP fraction

A meta-analysis within large CHIP cohorts was performed to determine the prevalence of *TET2*^{MT} CHIP [5, 6, 31–34]. For meta-analysis, cases were excluded when hematologic neoplasms were present before sampling, or clinical and molecular data were unavailable. The penetrance of *TET2*^{MT} MDS derived from *TET2*^{MT} CHIP was recapitulated

Table 1 Distribution of TET2 mutations in myeloid neoplasms.

	TET2 MT	TET2 WT	
Total	1205	3725	
DX			
MDS	247	1192	$p < .001$
MDS/MPN	432	511	$p < .001$
MPN	75	333	$p = .004$
pAML	377	1436	$p < .001$
sAML	58	188	
t-MN	16	65	
Gender = M	589	782	
Age			
Under 30	5	43	$p < .001$
30–39	13	50	$p < .001$
40–49	25	110	$p < .001$
50–59	72	203	$p < .001$
60–69	243	354	
70–79	409	377	$p < .001$
80–89	156	133	$p < .001$
90+	10	1	$p < .001$
Cytogenetics			
Normal	643	579	$p < .001$
Complex	29	159	$p = .004$
5q−	26	70	
Del 20	12	46	
Del 7	22	45	
Del Y	31	21	$p < .001$
Trisomy 8	43	48	$p < .001$
Other	123	229	$p < .001$

according to the frequencies of ancestral *TET2* hits in the MDS cohort. Supplementary Table 2 summarizes the meta-analysis. For the purpose of this study, CHIP was defined as the presence a somatic mutation with VAF ≥2% in an otherwise asymptomatic individual. Patients with unexplained persistent cytopenias, lack of dysplasia, and the absence of an MDS-associated somatic mutation are considered to be idiopathic cytopenias of undetermined significance (ICUS). Clonal cytopenias of undetermined significance (CCUS) describes a condition where somatic mutations are associated with cytopenias, but lack dysplasia [35].

Analysis of genotype/phenotype relationships

Wilcoxon tests were performed for pairwise continuous variable comparisons, Fisher's exact test was used to compare proportions, and log-rank tests were used to compare survival times. All p values were two-sided and values less than 0.05 were considered statistically significant. All analyses were performed using the statistical computing environment R (3.4.3).

Three-dimensional plots

Odds ratios (OR) of phenotypes based on mutational status were calculated using 2×2 contingency tables, and plotted on an x–y–z plane to illustrate the trifold pheno–morphologic relationships. Confidence intervals of OR vs. the OR of patients with only a *TET2* and no other mutations were deemed separate if they were non-overlapping. Phenotype definitions for the classification of dysplasia and cytopenia can be found in Supplementary Table 3.

Results

Patient characteristics

A total of 4930 patients with myeloid neoplasia were included (Supplementary Table 4) and underwent sequencing. *TET2* was the most commonly mutated gene. Following sequencing of all-coding regions, we mapped and classified all somatic $TET2^{MT}$ according to their position and type. A total of 1781 somatic $TET2^{MT}$ were identified in 1205 patients (Table 1). Most were truncating: 47% were frame shifts, 34% were nonsense, and 19% were missense. Although the truncating mutations were widely dispersed across the gene, 89% of the missense mutations were located in the catalytic domain, which spans base pairs (bp) 1129–1936.

The prevalence of $TET2^{MT}$ increased with patient age (Fig. 1a, $R = 0.878$, $p < 0.0001$), irrespective of histologic subtype and mutation type (missense, frame shift, and nonsense; Supplementary Fig. 3A). Among co-occurring mutations, JAK2 had the strongest correlation with increasing age ($R^2 = 0.924$), followed by SRSF2 ($R^2 = 0.906$) and ASXL1 ($R^2 = 0.867$). Secondary ($R^2 = 0.876$) and ancestral ($R^2 = 0.726$) $TET2^{MT}$ correlated with an increase in age (Supplementary Fig. 3B). Of $TET2^{MT}$ patients, 43% harbored more than one $TET2^{MT}$ and 12% were either homo- or hemizygous (Fig. 1b). Of these, 65% had two truncating mutations, while the remaining cases showed a missense and truncation combination. Those with >1 $TET2^{MT}$ and low VAF could be either biallelic or biclonal, a distinction that is difficult to make using VAF. Plotting the VAFs of each $TET2^{MT}$ in patients with multiple $TET2^{MT}$, on an x–y plot (individual VAF on each axis; Supplementary Fig. 2) yielded two populations, one clearly biallelic $TET2^{MT}$ (75% of double mutants) and the other either biallelic or biclonal $TET2^{MT}$ (25% of double mutants). In either case, the higher-ranked $TET2^{MT}$ may represent a

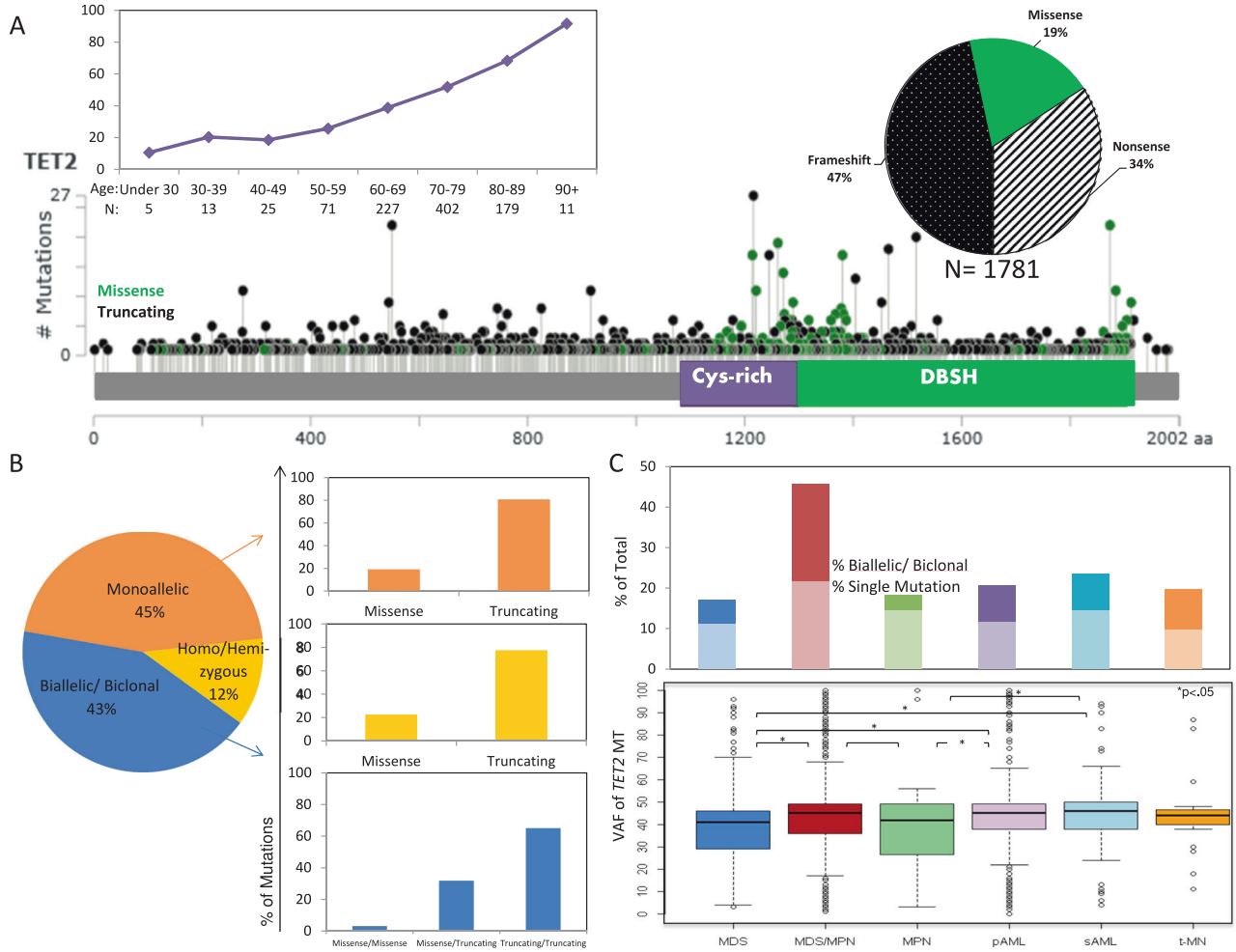


Fig. 1 Topology and demographics of *TET2* mutations in myeloid neoplasms. **a** Schematic drawing of *TET2* gene showing location, distribution, types of mutations, and age-related increases in the number of mutations. For details of mutation and disease subtypes, see Supplementary Fig. 1. **b** Distribution of number and type of $TET2^{MT}$ across the spectrum $TET2^{MT}$. **c** The frequencies of single and multiple mutations in each disease subtype and the distribution of mutant VAF by MDS subtype

founder event assuming that no earlier events exist in other genes.

$TET2^{MT}$ were found in 17% of patients with MDS, 46% of MDS/myeloproliferative neoplasms (MDS/MPN), 19% of MPN, 21% of primary acute myeloid leukemia (pAML), 24% of secondary AML (sAML), and 20% of treatment-related MN (t-MN) patients. In general, single mutations were more common than multiple mutations, except in MDS/MPN, where there was a similar proportion of single and multiple mutant cases. No differences in $TET2^{MT}$ clonal burden were found among disease subtypes (Fig. 1c).

Genotypic context of TET2 mutations

$TET2^{MT}$ may occur in association with distinct mutational spectra (Fig. 2a). Overall, $TET2^{MT}$ most often co-occurred with another mutation in *TET2* (43%) and with mutations in *ASXL1* (21%), *SRSF2* (18%), and *NPM1* (13%, Fig. 2b). Patients with multiple $TET2^{MT}$ harbored, on average, more alterations than those with one or *TET2* wild type ($TET2^{WT}$; Fig. 2c). By disease subtype, in MDS patients, $TET2^{MT}$ most often co-occurred with another $TET2^{MT}$, *ASXL1*, *SF3B1*, and *SRSF2*. MDS patients with $TET2^{MT}$ more frequently had mutations in *ASXL1* (22% vs. 10%, $p < .001$), *SRSF2* (20% vs. 9%, $p < .001$), and *RUNX1* (14%, vs. 6%, $p = .003$), compared to those with $TET2^{WT}$ (Fig. 2d). In MDS/MPN, $TET2^{MT}$ most often coincided with another *TET2*, or *SRSF2*, *ASXL1*, *RUNX1*, and *CBL*. *SRSF2* mutations were more common in $TET2^{MT}$ vs. $TET2^{WT}$ MDS/MPN (52% vs. 14%, $p < .001$) as were *ASXL1* mutations (36% vs. 14%, $p < .001$). In MPN, the most common coexisting mutations were *JAK2*, *ASXL1*, and *SRSF2*; while double $TET2^{MT}$ were less frequent. Subclonal lesions of *NPM1* and *DNMT3A* were predominant in pAML, while the lesions seen in sAML were more similar to that of MDS. $TET2^{MT}$ were significantly associated with normal

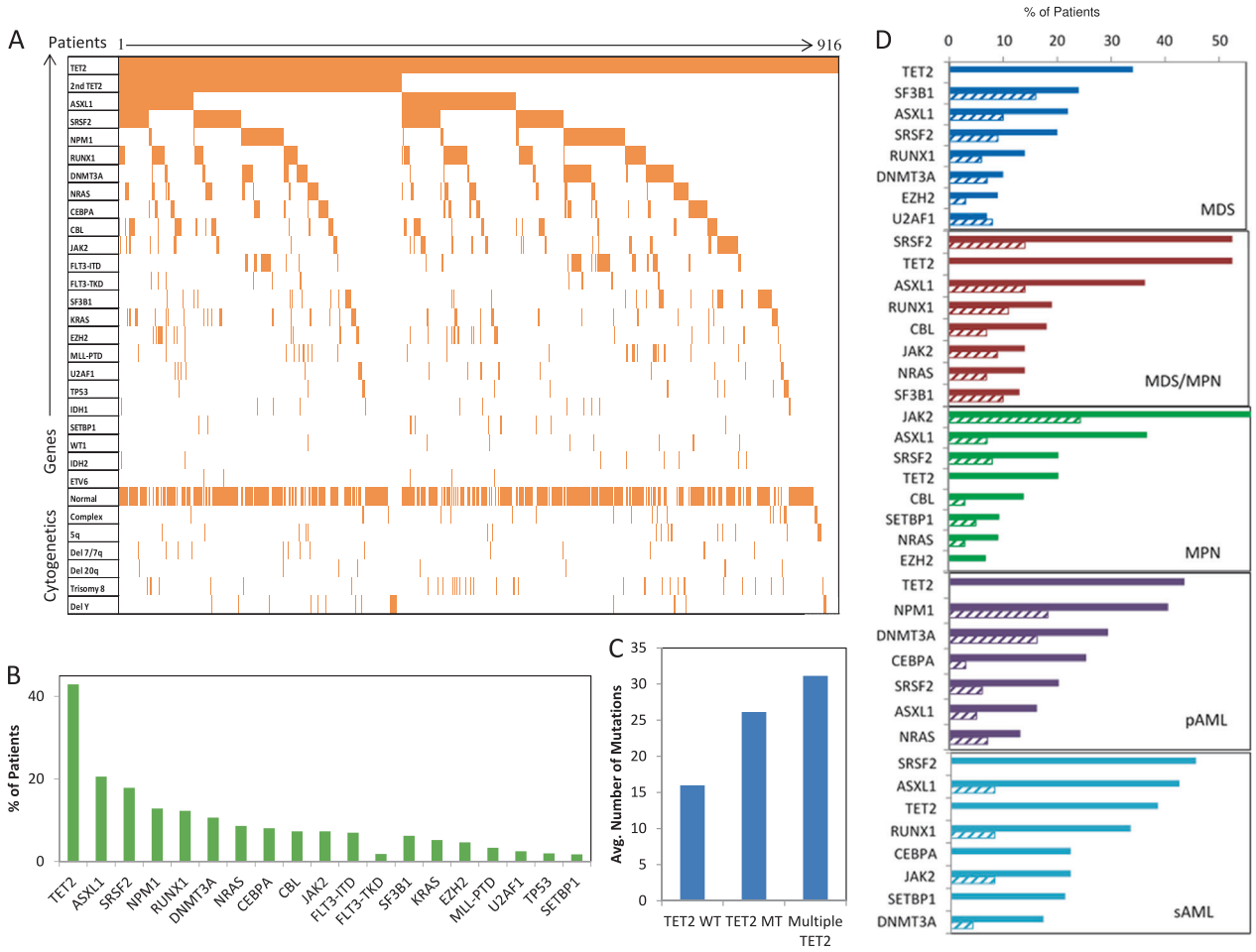


Fig. 2 Clonal architecture of TET2 mutants. **a** Co-occurring mutations in $TET2^{MT}$ patients. **b** Frequency of somatic mutations co-occurring with $TET2^{MT}$. **c** The average number of mutations of patients without a $TET2^{MT}$, a single $TET2^{MT}$, or double $TET2^{MT}$. **d** Mutational profiles of $TET2^{MT}$ (solid bars) and $TET2^{WT}$ (hashed bars) within disease subtype

cytogenetics, deletion Y, and trisomy 8, while TET^{WT} were associated with more complex karyotypes (Table 1).

Molecular implications from clonal architecture

The position of $TET2^{MT}$ in the clonal hierarchy can be inferred by ranking heterogeneous somatic events. Where serial samples are not available, the position can be inferred from a cross-sectional analysis based on VAF (Fig. 3a). The results indicate that $TET2^{MT}$ are first hits (dominant clones) in 40% of $TET2^{MT}$ cases and later hits (subclonal events) in other cases. Furthermore, a subclonal $TET2^{MT}$ can follow an ancestral *TET2* hit or be subclonal to other mutations in a linear or branching fashion. When *TET2* is the first hit, the most common second mutation is another *TET2* lesion, followed by *SRSF2*, *ASXL1*, *DNMT3A*, and *SF3B1* mutations. When *TET2* is subclonal, the dominant antecedent clone is defined by the presence of *SRSF2*, *EZH2*, *ASXL1*, *DNMT3A*, or *CEBPA* mutations (Fig. 3b). A significantly greater number of pAML patients had ancestral *TET2* lesions than sAML and were also associated with abnormal cytogenetics and *NPM1* mutations (Supplementary Fig. 4). This observation could be explained by the older age of our cohort as compared to TCGA AML patients (69 vs. 55 years in TCGA). There was no significant difference in the frequency of $FLT3^{ITD}$ between primary and secondary AML. Secondary *TET2* hits coincided with *ASXL1, DNMT3A, EZH2, JAK2*, and *RUNX1* mutations. When subgrouped according to "class-defining" ancestral hits (*TET2, SRSF2*, *SF3B1*, etc.), patients with ancestral frame shift $TET2^{MT}$ harbored the highest numbers of additional subclonal alterations (Fig. 3c).

Demographic and pathogenic relationship between $TET2^{MT}$ CHIP and MDS

Meta-analysis of six major CHIP studies revealed that 9% of healthy individuals have CHIP (4470/49290). It was found that 11–15% of CHIP is due to *TET2* hits (513/4470, Fig. 3d and Supplementary Table 2). We estimate that in

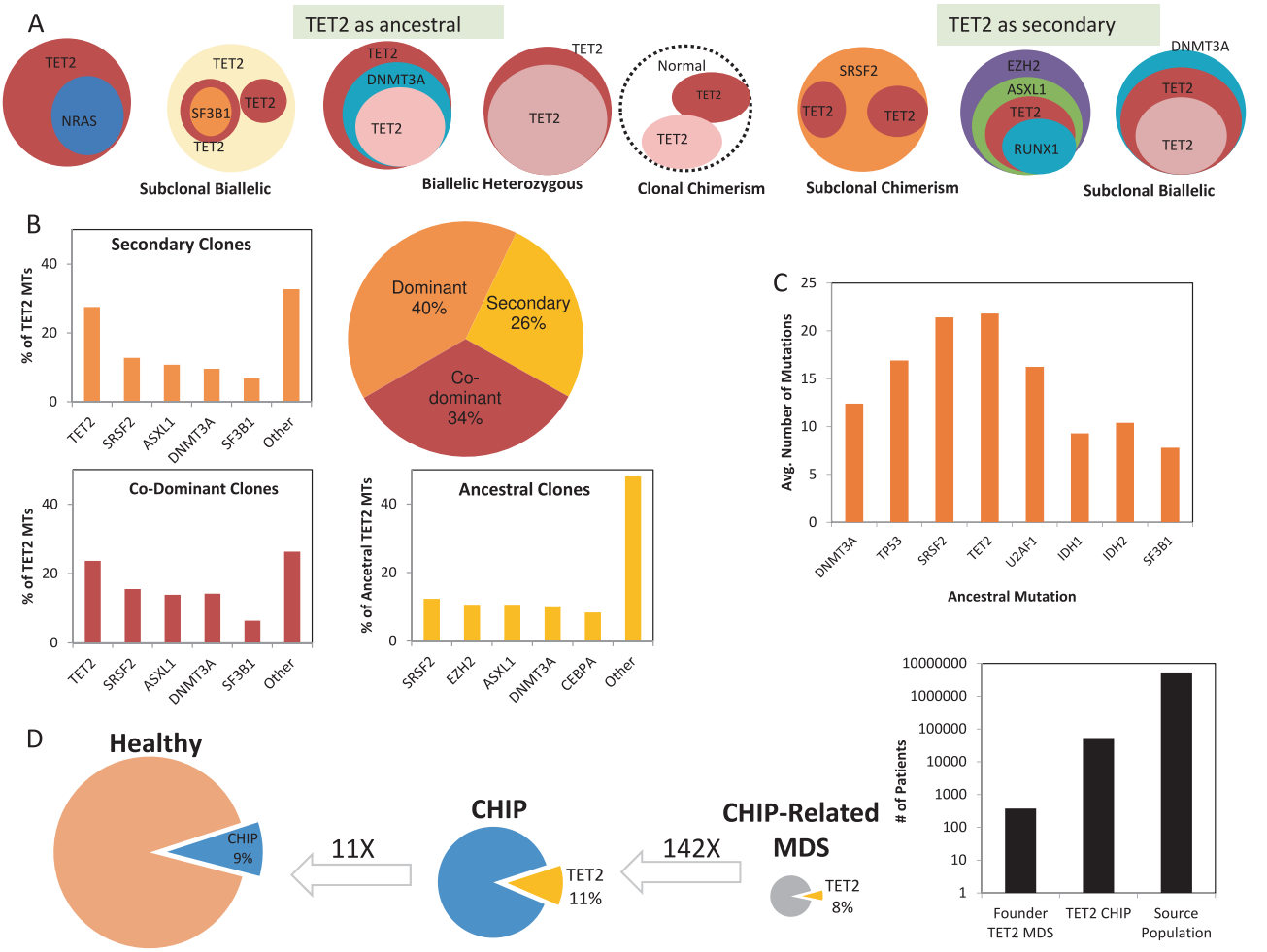
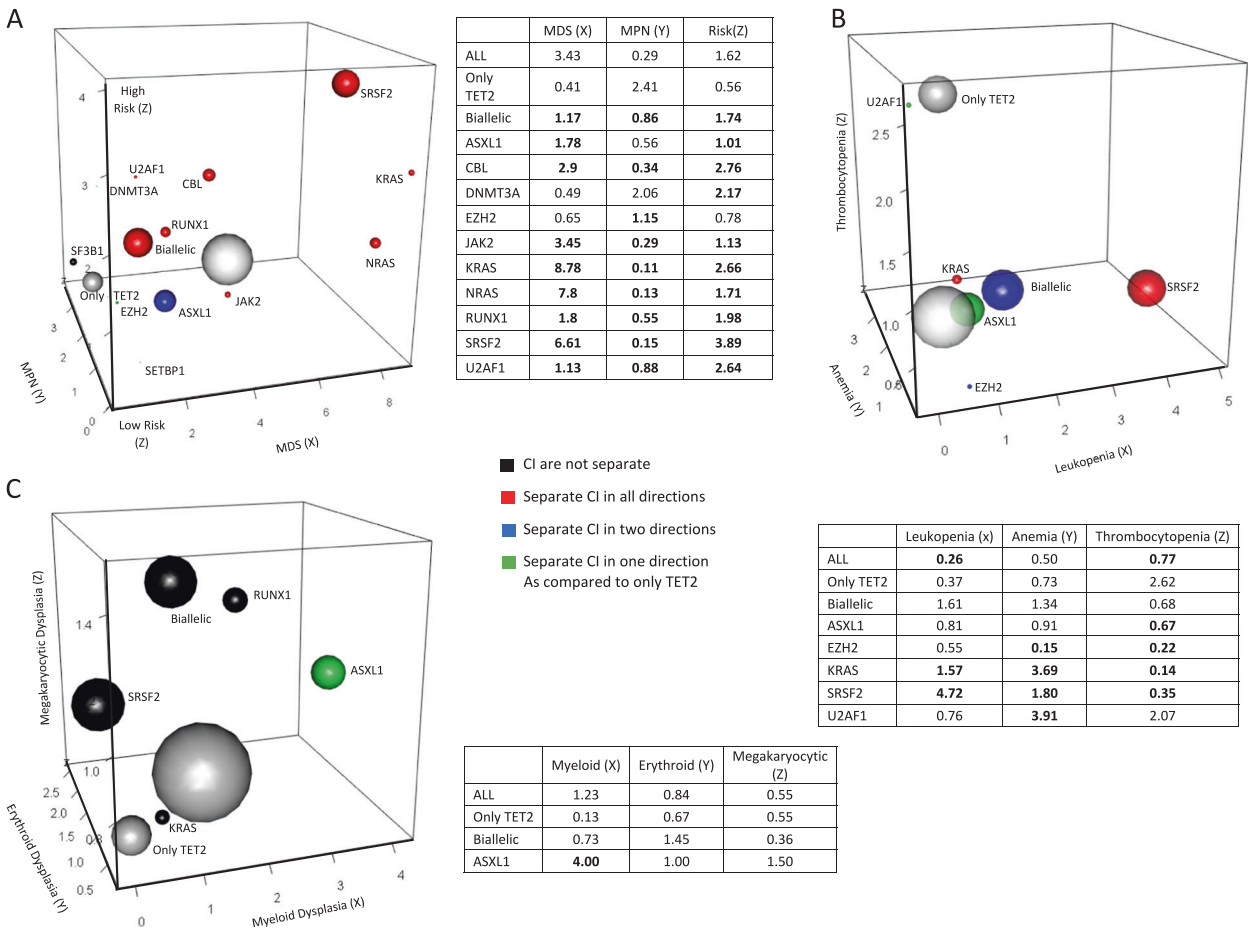


Fig. 3 Clonal hierarchy of *TET2* mutations. **a** Cross-sectional analysis of patient samples to identify clonal hierarchy of *TET2* (second sphere to the left top row represents a patient with three *TET2* mutations, including an ancestral and two different subclonal hits). **b** Distribution of $TET2^{MT}$ patients based on the position of $TET2^{MT}$ within the clonal hierarchy, and the frequency of other mutations throughout the clonal hierarchy. **c** The average number of mutations of MDS, MPN, and AML patients with ancestral hits of various genes. **d** Meta-analysis to show the frequency of $TET2^{MT}$ CHIP and CHIP-related MDS. Values above arrows are reverse direction multipliers of percentages of individuals

turn <1% of $TET2^{MT}$ CHIP evolves to MDS (0.7%, 2/277), but the low number of events available precludes precise estimates. Conversely, we show 8% of MDS are initiated by *TET2* hits and thus likely derive from antecedent *TET2*-mutated CHIP. From the 373 ancestral $TET2^{MT}$ MDS cases in our cohort, we can estimate that we "need" 53,285 individuals with $TET2^{MT}$ CHIP to account for the cases diagnosed and calculate the evolution rate. These calculations allowed us to conclude that while most of $TET2^{MT}$ CHIP is not "productive," all of the ancestral $TET2^{MT}$ MDS cases are likely CHIP derived rather than de novo cases. The mean age of our cohort was 67, while it was found to be 59 for the individuals of the meta-analysis. Consequently, it is more likely that we are underestimating the number of CHIP cases because the population is younger than ours, providing further evidence that nearly all MDS with ancestral $TET2^{MT}$ are CHIP derived. Of note, is that our study primarily focused on $TET2^{MT}$ CHIP and other CHIP-associated mutations were not further investigated.

Impact of subclonal lesions on phenotype and disease progression in $TET2^{MT}$-initiated cases

The odds of a molecular lesion being associated with proliferation vs. dysplasia, and low vs. high-risk disease (based on blasts >5% and IPSS) can be used to separate secondary mutations acquired after a founder $TET2^{MT}$ in MDS and MDS/MPN patients (Fig. 4a). Such three-dimensional plots illustrate how hits secondary to those in *TET2* bias the rate of progression (high- vs. low-risk disease) and phenotype (dysplastic vs. proliferative features). Patients with only an ancestral $TET2^{MT}$ tend to be low-risk MPN. Secondary hits



	MDS (X)	MPN (Y)	Risk(Z)
ALL	3.43	0.29	1.62
Only TET2	0.41	2.41	0.56
Biallelic	**1.17**	**0.86**	**1.74**
ASXL1	**1.78**	0.56	**1.01**
CBL	**2.9**	**0.34**	**2.76**
DNMT3A	0.49	2.06	**2.17**
EZH2	0.65	**1.15**	0.78
JAK2	**3.45**	**0.29**	**1.13**
KRAS	**8.78**	**0.11**	**2.66**
NRAS	**7.8**	**0.13**	**1.71**
RUNX1	**1.8**	**0.55**	**1.98**
SRSF2	**6.61**	**0.15**	**3.89**
U2AF1	**1.13**	**0.88**	**2.64**

	Leukopenia (x)	Anemia (Y)	Thrombocytopenia (Z)
ALL	**0.26**	0.50	**0.77**
Only TET2	0.37	0.73	2.62
Biallelic	1.61	1.34	0.68
ASXL1	0.81	0.91	**0.67**
EZH2	0.55	**0.15**	**0.22**
KRAS	**1.57**	**3.69**	**0.14**
SRSF2	**4.72**	**1.80**	**0.35**
U2AF1	0.76	**3.91**	2.07

	Myeloid (X)	Erythroid (Y)	Megakaryocytic (Z)
ALL	1.23	0.84	0.55
Only TET2	0.13	0.67	0.55
Biallelic	0.73	1.45	0.36
ASXL1	**4.00**	1.00	1.50

Fig. 4 Secondary hits of *TET2* mutants. **a** Associations between disease phenotypes and mutation rates are quantified by the odds ratios, MDS (X) vs. MPN (Y), and high risk vs. low risk (Z). Mutations showing significant enrichment in comparison to patients with only a $TET2^{MT}$ (shown in smaller gray ball) are indicated by color according to OR 95% CI limits. Red color indicates separation of CI in all directions, blue indicates separation in two of the three directions, green indicates separation in a single direction, black indicates no separation. The sizes of the spheres are proportional to the frequency of the mutation in our cohort. The largest white ball is the total cohort of all ancestral $TET2^{MT}$ carriers combined. Tables shown provide odds ratio point estimates for each associated plot. b. Associations between phenotypes and mutation rates are quantified by odds ratios, leukopenia (X), anemia (Y), and thrombocytopenia (Z). c. Associations between phenotypes and mutation rates are quantified by odds ratios, myeloid dysplasia (X), erythroid dysplasia (Y), and megakaryoctye dysplasia (Z). Definitions for classification of dysplasia types and cytopenias are provided in Supplementary Table 2

following an ancestral *TET2* lesion alter disease trajectories in distinct manners, pushing them toward different phenotypes and progression rates; e.g., a second (biallelic) *TET2* hit increases the "MDS-like" character of the disease. A secondary *SRSF2* mutation greatly increases a patient's progression risk, and retained its impact in a multivariate analysis that adjusted for IPSS (Supplementary Table 5). When mutational profiles of $TET2^{MT}$ were correlated with cytopenias, patients with exclusive $TET2^{MT}$ tended to be thrombocytopenic, while the addition of a *U2AF1* mutation increased odds of the anemia phenotype (Fig. 4b). Anemia and leukopenia were associated with secondary *SRSF2* and *KRAS* mutations. A similar analysis was done for lineage dysplasia; we found subclonal *ASXL1* mutations to be associated with myeloid, rather than erythroid or megakaryocytic dysplasia (Fig. 4c). We also investigated how phenotypic features change when *TET2* is secondary to ancestral hits affecting other genes. The latter originated from different starting points within the phenotypic continuum, and subclonal *TET2* mutations may further redirect the founder phenotype. For example, when we examined this scenario with ancestral *ASXL1* and secondary *TET2* hit (Supplementary Fig. 5A vs. Fig. 4a), we found that subclonal *TET2* hits increase the propensity to progression. The opposite succession (*TET2* preceding *ASXL1*) was not associated with the risk of evolution. Similarly, when we studied the effects of subclonal *TET2* hits on *SRSF2*-initiated disease, we found a shift toward anemia and less prominent leukopenia more than in *TET2*-initated disease with a secondary *SRSF2* mutation (Supplementary Fig. 5B vs. Fig. 4b). *SRSF2*-initiated disease with a secondary $TET2^{MT}$ had greater myeloid and less erythroid dysplasia

than the reciprocal scenario (Supplementary Fig. 5C vs. Fig. 4c).

Overall, *TET2*^{MT} had no impact on survival (Supplementary Fig. 6A–D). When the size of the *TET2*^{MT} clone was considered, survival was worse in patients with larger clones ($p = .014$; Supplementary Fig. 7A), yet there was no statistical survival difference between patients with ancestral vs. secondary *TET2*^{MT}. Focusing on disease subgroups, MPN and sAML patients with ancestral *TET2* hits showed a trend toward worse survival (not shown). When patients were grouped by genetic configuration of *TET2*^{MT}, those with hemizygous and homozygous mutations had significantly poorer survival ($p = .008$; Supplementary Fig. 7B).

We also examined the impact of additional mutations on survival in *TET2*^{MT} cases (Supplementary Fig. 8). *U2AF1*, *TP53*, and *SRSF2* mutants have significantly higher hazard ratios, but in combinations with a *TET2*^{MT}, their hazard ratio decreases. *TET2*^{MT} with *EZH2* mutations had a significantly higher hazard ratio; the same was seen in *TET2*^{WT} patients.

Discussion

While the immediate biochemical consequences of *TET2*^{MT} are known [4, 10, 12, 36–39] their downstream pro-leukemogenic impact on disease evolution remains elusive. Apart from its biochemical function and role in passive demethylation, our speculative view is that other *TET2* functions (and their deficiency) may be important, such as its oxygen-sensing function, modification of double-stranded RNA, and DNA repair. Irrespective of these activities, *TET2* is a tumor suppressor gene because it limits both the number of hematopoietic stem cells (HSC, i.e., target cells of MDS and MDS/MPN) and also likely the rate at which they mutate per cell [17]. Nevertheless, the shared prevalence of *TET2* defects implies their "general" pathogenic importance and propensity for leukemogenesis. The lack of mutations in highly homologous *TET1/TET3* indicates a distinct pathophysiologic role of this specific gene likely due to differences in tissue-specific expression [2, 40].

To date, only a modest impact of *TET2*^{MT} on clinical outcomes has been described, and its effect on AML progression in MDS has been found to be rather neutral [18–23, 40, 41]. We and others have demonstrated a favorable association of *TET2*^{MT} with responsiveness to hypomethylating agents; however, this finding has not been uniformly reproduced, likely due to the diverse impact of co-associated events [22, 42, 43]. Larger treatment groups will be needed to appropriately taxonomize such heterogeneity.

The diversity of *TET2*-associated phenotypes is high. This might be due to the heterogeneity of *TET2* lesions, including their intragenic topology, allelic configuration, and position within the clonal hierarchy and combinations of other mutations with which they tend to coincide. For instance, it might reflect a generic role of *TET2* loss in the expansion of HSC, perhaps enabling them to exist in a more oxic/DNA-damaging environment prone to acquisition of secondary hits.

Our study of *TET2*^{MT} included enough patients to perform subset analyses and therefore answers some outstanding questions regarding the role of *TET2* in correlation to morphologic phenotypes. Although using VAF rankings to decipher clonal architecture is not ideal, we have various lines of computational and analytic confirmation of our method allowing it to apply in principle to the large number of samples included in our study. Our results indicate that most *TET2*^{MT} represent phenotype-neutral ubiquitous ancestral hits, which seem to create a leukemogenic predisposition (mutator phenotype) rather than leukemic drive as evidenced by the lack of impact on progression and a higher total number of subclonal mutational events. The presumed mutator phenotype is consistent with results of murine studies showing accumulation of secondary hits during evolution of *Tet2*^{KD}-mediated disease [44]. We also show that phenotypic features in mutant cases are determined by pairings with secondary events that are not entirely random. Analysis of mutational events in cases with ancestral *TET2*^{MT} indicate a higher number of subsequent subclonal events than with other ancestral events, including hits in *SF3B1*, *SRSF2*, *DNMT3A*, *TP53*, *U2AF1*, or *IDH1/2* as previously described [18, 45]. This effect might arise by increases of absolute numbers of target cells, by increases in mutation rates per cell, or both. In *Tet2*$^{KD/KO}$ mouse models, increases in numbers of HSC (target cells) [14] and elevated mutation rates have been described [17]; the latter was confirmed in human samples using whole-exome sequencing [44].

TET2^{MT} also increase with age in CHIP [5] and CHIP is associated with an increased risk of developing hematologic neoplasms [6]. To that end myeloid neoplasia, especially MDS that are characterized by ancestral *TET2* hits are likely to have developed from previous CHIP. A higher frequency of CHIP mutations, such as *TET2*, are seen in people over the age of 60 years old explaining that more ancestral *TET2*^{MT} were found in pAML than sAML likely due to underrepresentation of CBF AML occurring in younger age [34]. Indeed, our pAML cohort is older than previously reported (69 vs. 55 years, $p < 0.001$) [46].

We demonstrated that *TET2*^{MT} can also arise, albeit less frequently, as secondary events. *TET2* hits tend to accumulate, with second hits resulting in biallelic mutations, hemizygous deletions, or uniparental disomy with

homozygous mutations. Patients with secondary $TET2^{MT}$, with the exception of those with ancestral events observed in CHIP (chiefly, *DNMT3A* or *ASXL1*), are not CHIP-related and have founder hits with higher pathogenicity and likely faster progression.

The secondary hits following ancestral $TET2^{MT}$ are not entirely random; the probabilities of different secondary hits differ following *TET2* vs. other founder mutations and they result in differences in the phenotype they generate (dysplastic vs. proliferative disease phenotypes) and their propensity to progress. For instance, $TET2^{MT}$ show predilection for biallelic mutations, hemizygous deletions involving the *TET2* locus, or somatic uniparental disomy [47]. Other secondary mutations also show a pattern with certain secondary hits overrepresented compared to neoplasia that was initiated by other founder mutations. Such secondary hits affect phenotypic features. For example, *SRSF2* and *K/NRAS* secondary hits are common in CMML and thus lead to development of MDS/MPN overlap syndromes. Similarly, *ASXL1, EZH2*, and *SF3B1* secondary hits are common in MDS, and *JAK2* V617F secondary hits are common in MPN. Other $TET2^{MT}$ associations include *RUNX1* and *EZH2* and its mutual exclusivity with del5q in MDS or *DNMT3A* and *NPM1* mutations negatively correlating with *IDH1/2* mutations in AML. Some secondary hits (e.g., in *SRSF2* and *CBL*) impact the pace of progression and therefore survival [48, 49]. Some secondary hits such as *ASXL1*, *EZH2*, and *TP53* following ancestral $TET2^{MT}$ were surprisingly not associated with advanced disease, but, as expected showed a negative impact on survival [27, 50–56]. This finding indicates that high-risk lesions can be found early in the disease course.

In summary, our results demonstrate how originally phenotype-neutral founder $TET2^{MT}$ are followed by hits resulting in modifications of clone morphology, type of lineage production defect, myeloproliferative features, or rates of progression to AML.

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Author contributions CMH performed DNA-sequencing experiments, collected/analyzed data, and wrote the manuscript. AN and KK collected data. BPP, MM, YG, and NN performed DNA-sequencing experiments, and analyzed DNA-sequencing data. MAS and TH provided patient samples and clinical data. AG, HA, and MFA provided clinical data. VA, VV, and YN edited the manuscript and advised on experiments. MEA, BKJ, TL, TR, RO, and JPM designed and conceptualized the overall research and wrote the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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