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Brief report

Clonal analysis of *TET2* and *JAK2* mutations suggests that *TET2* can be a late event in the progression of myeloproliferative neoplasms

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Somatic mutations in *TET2* occur in patients with myeloproliferative neoplasms and other hematologic malignancies. It has been suggested that *TET2* is a tumor suppressor gene and mutations in *TET2* precede the acquisition of *JAK2*-V617F. To examine the order of events, we performed colony assays and genotyped *TET2* and *JAK2* in individual colonies. In 4 of 8 myeloproliferative neoplasm patients, we found that some colonies with mutated *TET2* carried wild-type *JAK2*, whereas others were *JAK2*-V617F positive, indicating that *TET2* occurred before *JAK2*-V617F. One of these patients carried a germline *TET2* mutation. However, in 2 other patients, we obtained data compatible with the opposite order of events, with *JAK2* exon 12 mutation preceding *TET2* mutation in one case. Finally, in 2 of 8 patients, the *TET2* and *JAK2*-V617F mutations defined 2 separate clones. The lack of a strict temporal order of occurrence makes it unlikely that mutations in *TET2* represent a predisposing event for acquiring mutations in *JAK2*. (Blood. 2010;115:2003-2007)

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Introduction

TET2, a member of the *ten-eleven-translocation* (*TET*) family of genes,[1,2] can be mutated in various hematopoietic disorders, including myeloprolieferative neoplasms (MPNs), myelodysplastic syndromes, acute myeloid leukemia, and chronic myelomonocytic leukemia.[3-11] *TET2* is mutated in 13% of the MPN cases, with the highest frequency occurring in patients with primary myelofibrosis (PMF) and polycythemia vera (PV; 17% and 16%, respectively) and lowest in patients with essential thrombocythemia (ET; 5%).[3] The mutations in *TET2* do not cluster in a particular region and show a very diverse pattern of frame shift, nonsense, and missense mutations. The *TET2* mutations are generally present in a heterozygous state, and only a minority of patients displays mutations in both *TET2* alleles. A recent study showed that loss of heterozygosity in *TET2* might occur through mitotic recombination or gene copy number changes.[9] The loss of both gene copies through mutations and chromosomal aberrations is compatible with a potential tumor suppressor activity of *TET2*. However, the mechanism of how *TET2* is involved in disease initiation and/or progression is unknown.

Methods

Patients

The collection of blood samples was performed at the study center in Basel, Switzerland, and was approved by the Ethik Kommission Beider Basel. Written consent was obtained from all patients in accordance with the Declaration of Helsinki. The diagnosis of MPN was established according to the criteria of the World Health Organization.[12-14]

Cells and DNA analysis

Purification of granulocytes, peripheral mononuclear cells, extraction of DNA, and colony assays were performed as described.[15-17] Methylcellulose-based media (no. 4431 and no. 4434) containing erythropoietin and methylcellulose based media without erythropoietin (no. 4531; StemCell Technologies) were used. The cells were plated at a density of 100 000 cells/mL, which provided an optimal density of colonies for picking, without the danger of contamination by neighboring colonies. Primers used for sequencing, copy number analysis, and X chromosomal inactivation analyses are provided in supplemental Table 1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article) and were performed as described.[17-20]

Results and discussion

To study the order of events in the clonal evolution of *TET2* and *JAK2* mutations, we examined individual colonies derived from peripheral blood of 8 MPN patients with *TET2* mutations (6 PV, 1 ET, and 1 PMF) that were identified by sequencing of DNA from 57 MPN patients. All patients with *TET2* mutation were also positive for *JAK2*-V617F (Table 1).

Mutational analysis of single colonies allowed us to distinguish 3 different patterns of mutation accumulation (Figure 1): In 4 of 8 MPN patients, we found that some colonies with mutated *TET2* carried wild-type *JAK2*, whereas others were *JAK2*-V617F$^+$, indicating that *TET2* occurred before the acquisition of *JAK2*-V617F (Figure 1A). These 4 patients confirm previous data by Delhommeau et al,[3] who showed that *TET2* occurs before *JAK2*-V617F. A second group showed a pattern compatible with the inverse order of events, ie, *JAK2* mutations occurred before the acquisition of *TET2* mutations (Figure 1B):

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Table 1. Characteristics of MPN patients with mutations in *TET2*

UPN	Sex	Diagnosis	Age at diagnosis, y	Disease duration, mo	*TET2* mutation	*JAK2*-V617F, percentage T	*JAK2*-exon 12, percentage mutation	Complications	Treatment
p021	Male	PV	55	107	p.Thr1372Ile	3	21	None	Aspirin, phlebotomies
p101	Male	ET	61	38	p.Ser1285del	19	NA	Bleeding	Aspirin, hydroxyurea
p136	Male	PV	75	38	p.Gln891X	40	NA	Died, cause of death unknown	NA
p191	Male	PMF	66	59	p.Leu1210Pro	100	NA	Intracranial bleeding, pulmonary Embolism	Oral anticoagulants
p209	Male	PV	64	30	p.Arg544X	88	NA	Post-PV myelofibrosis	Aspirin, hydroxyurea
p225	Female	PV	75	1	splice donor c.3980 + 2G→T	82	NA	Cerebrovascular insult, arterial occlusion	Aspirin, hydroxyurea
p226	Female	PV	49	28	p.Asp1858fs	82	NA	None	Aspirin, hydroxyurea
p234	Male	PV	71	1	p.Gly1275Glu	20	NA	Transient ischemic attack	Clopidogrel, hydroxyurea

UPN indicates unique patient number; PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis; and NA, not applicable.

patient p191 displayed colonies positive for *JAK2*-V617F without the *TET2* mutation, and all colonies with mutated *TET2* were also positive for *JAK2*-V617F. Unfortunately, 9 single nucleotide polymorphisms (SNPs) in the vicinity of *JAK2*-V617F were noninformative to provide evidence for a possible biclonal acquisition of *JAK2*-V617F. The transition from heterozygous to homozygous *JAK2*-V617F appears to have independently occurred twice. Indeed, the analysis of individual colonies homozygous for *JAK2*-V617F revealed the presence of 2 subclones with different sizes of the 9p uniparental disomy (UPD) region (supplemental Figure 1A). By analyzing an informative SNP in exon 11 of *TET2* (Figure 1B), we can exclude the possibility that the *JAK2*-V617F positive and *TET2*-L1210P-negative colonies arose through mitotic recombination, in which the *TET2*-L1210P was lost. In patient p021, 2 independent clones were present: a smaller clone positive for *JAK2*-V617F and a larger clone positive for a *JAK2* exon 12 mutation (*JAK2*-N542-E543del).[21,22] Interestingly, the *TET2* mutation in this patient occurs only in combination with the *JAK2* exon 12 mutation, and the *TET2* mutation in this patient was acquired after the *JAK2* exon 12 mutation. A third pattern consisted of biclonal disease, as illustrated in patient p101 with colonies positive either for *JAK2*-V617F or *TET2*, but absence of double-positive colonies (Figure 1C). A similar biclonal pattern was previously described in one patient with del20q and *JAK2*-V617F, consistent with the presence of a predisposition to independently acquire 2 otherwise rare somatic events.[23] Patient p234 also shows a pattern compatible with biclonal disease.

Figure 1. Analysis of single colonies for mutations in *TET2* and *JAK2*. Mononuclear cells from peripheral blood were grown in methylcellulose in the presence or absence of erythropoietin. Single burst-forming units erythroid (BFU-E), endogenous erythroid colonies (EEC), and colony-forming units granulocyte (CFU-G) were picked and analyzed individually for the presence of *TET2* and *JAK2*-V617F mutations by DNA sequencing and allele-specific polymerase chain reaction (PCR), respectively. Each colony is represented by a dot that is placed into one of 6 quadrangles representing the 6 possible genotypes: wild-type (wt), heterozygous (het), and homozygous (hom) for *JAK2*-V617F on the vertical axis, and for *TET2* mutations on the horizontal axis. The unique patient numbers, the diagnoses (PMF, ET, and PV) and the allelic ratio of *JAK2*-V617F in purified granulocytes (%T) are shown above the corresponding boxes. Light blue arrows indicate the suggested order of mutation events. (A) Patterns compatible with *TET2* mutations occurring before *JAK2*-V617F. The sequencing chromatograms for patient p226 show the presence of *TET2* mutation in DNA from hair follicles, T cells, and granulocytes, demonstrating the germline origin of the mutation. Allele-specific PCR assay for the X-chromosomal gene *IDS* is shown for p226. The genomic DNA from patient p226 was heterozygous for a C/T single nucleotide polymorphism (not shown). The relative expression of the 2 *IDS* alleles was determined by comparing the C and T peak intensities obtained by the allele-specific reverse-transcribed PCR assay in T cells and granulocytes. The skewing of expression toward the C-allele is shown for 10 individual colonies (gray area). The inactivated *IDS* allele is marked with an arrow. (B) Patterns compatible with *JAK2* mutations occurring before *TET2* was mutated. Patient p021 carries 2 *JAK2* mutations, *JAK2*-V617F and *JAK2* N542-E543del, but the *TET2* mutation can be only found together with the deletion in exon 12 of *JAK2*. In patient p191, the sequencing chromatogram for the SNP rs34402524 located in *TET2* is shown for one BFU-E colony marked in red. The presence of a heterozygous SNP excludes the possibility that this colony is the product of a mitotic recombination event. (C) Patterns compatible with a biclonal state of the disease. In patient p234, the sequencing chromatogram for the SNP rs6843141 located in *TET2* is shown for one BFU-E and one CFU-G colony marked in red. Again, the presence of a heterozygous SNP excludes the possibility that these colonies are the product of a mitotic recombination event. (D) Location of mutations in the Tet2 protein in patients from whom data on single colonies are available. Mutations from this study are shown above the protein strand, and mutations analyzed in previous publications[3,26] are shown below. The gray boxes represent regions conserved between the different *TET* family members; blue boxes, *TET2* mutations that occur before *JAK2* mutations; yellow boxes, *TET2* mutations that occurred after *JAK2*; white boxes, *TET2* and *JAK2* mutations compatible with biclonal disease; and red box, germline *TET2* mutation.

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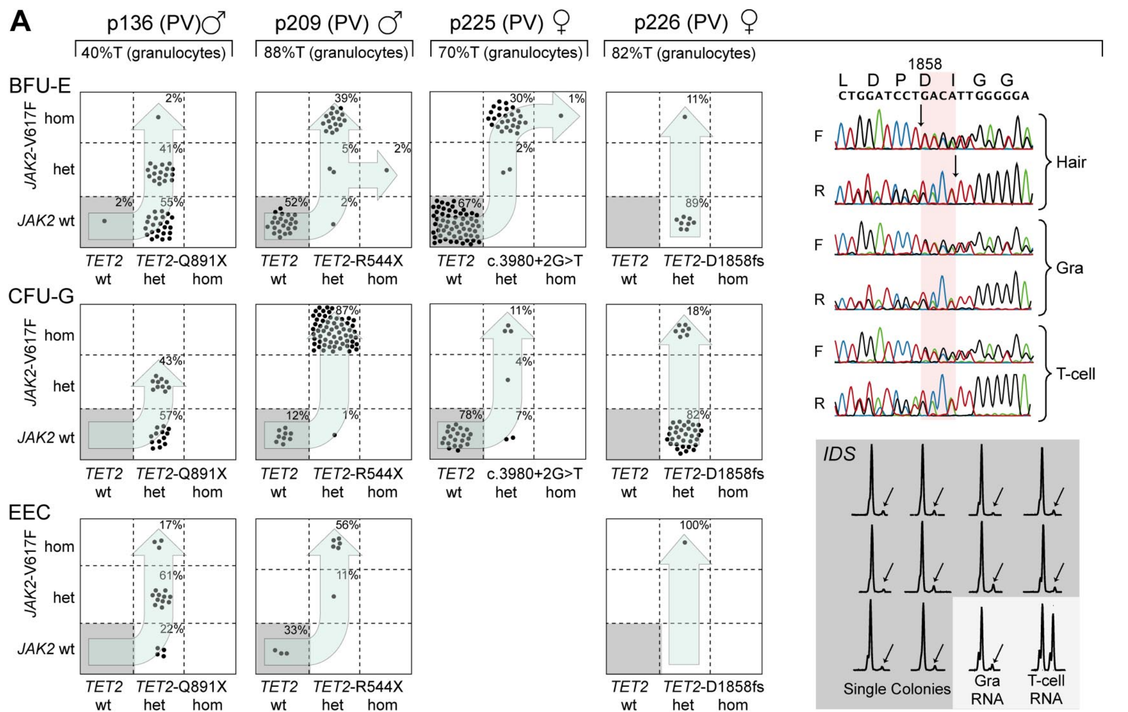

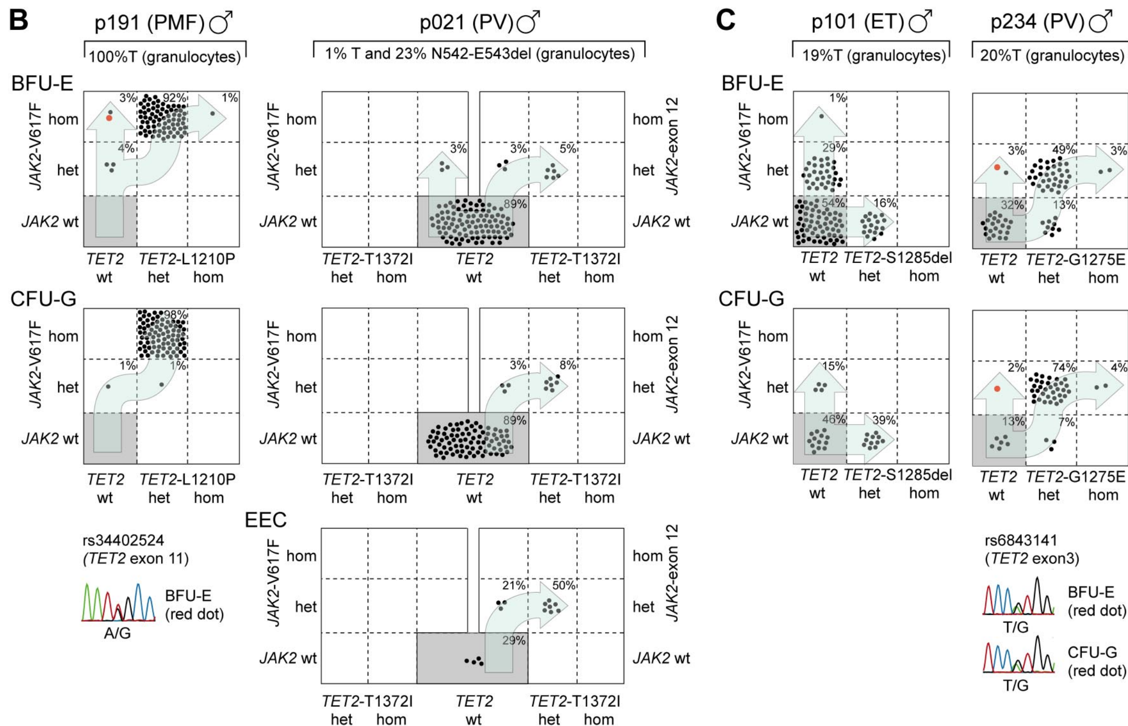

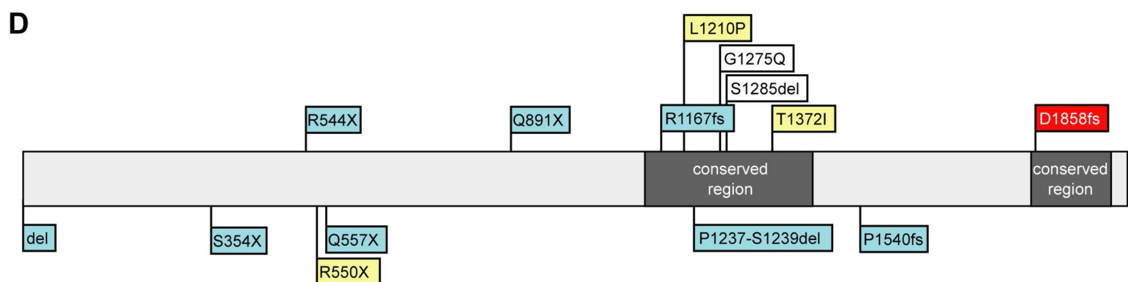

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However, to progress to the double-positive stage, either *JAK2* or *TET2* must have mutated twice independently. Again, SNP analysis excluded the possibility that the *JAK2*-V617F$^+$ and *TET2*-G1275E$^-$ colonies arose through mitotic recombination (Figure 1C). Because it is unlikely that *TET2* independently mutated twice at the exact same position (*TET2*-G1275E), we conclude that the majority of colonies first acquired *TET2*-G1275E followed by *JAK2*-V617F. A second independent event produced the subclone that is positive for *JAK2*-V617F only. The SNPs in close proximity (rs10974944 and rs12343867) were noninformative and were not part of the recently described GGCC or 46/1 *JAK2* haplotype (data not shown).[24,25]

In 4 of 8 patients, we observed a small number of colonies that were homozygous for the *TET2* mutations. Gene copy number analysis revealed that p209 and p191 retained 2 copies of the *TET2* gene, whereas in p225 loss of one copy of the *TET2* gene was found, indicating that the normal *TET2* allele was lost through a deletion (supplemental Figure 1B). In patient p234, homozygosity was achieved through UPD in some colonies and deletion in other colonies. Colonies homozygous for the TET2 mutations from all 4 patients displayed loss of heterozygosity of SNPs or microsatellite markers in the *TET2* locus, as expected for a deletion or UPD at chromosome 4q (supplemental Figure 1B).

The heterozygous *TET2*-D1858fs mutation in patient p226 was also present in DNA from hair roots, indicating that the mutation was germline. This 4-bp deletion, located in the C-terminal conserved domain of *TET2*, results in a frame shift and premature stop and is probably functionally relevant. The same mutation was also found in buccal DNA from an asymptomatic sister of p226 (data not shown). This appears to be the first report of a germline mutation in *TET2*. Accordingly, all colonies in this patient were positive for the *TET2* mutation. The X-chromosome inactivation pattern in individual colonies from p226 with wild-type *JAK2*, as determined by scoring a C/T polymorphism in the 3′-untranslated region of the *IDS* mRNA,[18-20] revealed a strong skewing (10 of 10 expressed the C allele of *IDS*), indicating that these progenitors were of clonal origin (Figure 1A). The finding of clonality suggests that this patient has a second significant disease clone, which does not carry a mutation in *JAK2*.

We showed that a *JAK2* exon 12 mutation preceded the *TET2* mutation in p021. In addition, the data in p191 are compatible with *JAK2*-V617F preceding TET2. A similar conclusion was reached in one patient with familial MPN positive for *TET2* and *JAK2*-V617F mutations.[26] In 4 of 8 patients, some colonies carried a homozygous *TET2* mutation that was the result of the loss of the wild-type allele through deletion or UPD. The percentage of such homozygous colonies in all 4 patients was very low ($< 5\%$), opening the possibility that the loss of the wild-type *TET2* may not provide an additional competitive advantage. The location of the *TET2* mutation, which has been analyzed using single colony assays, is summarized in Figure 1D. Most of the *TET2* frame shift and nonsense mutations occurred in patients in whom *TET2* preceded the acquisition of *JAK2*. The lack of a strict temporal order of occurrence resembles the findings obtained for del20q and makes it unlikely that mutations in *TET2* represent a predisposing event for acquiring *JAK2*.

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Authorship

Contribution: F.X.S. performed research, analyzed data, and wrote the paper; R.L., S.L., and H.H.-S. performed research; T.L. and A.T. provided clinical data; and R.C.S. designed research, analyzed data, and wrote the paper.

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