Extent of hematopoietic involvement by *TET2* mutations in *JAK2*^{V617F} polycythemia vera

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ABSTRACT

TET2 mutations are found in polycythemia vera and it was initially reported that there is a greater *TET2* mutational burden than *JAK2*^{V617F} in polycythemia vera stem cells and that *TET2* mutations precede *JAK2*^{V617F}. We quantified the proportion of *TET2*, *JAK2*^{V617F} mutations and X-chromosome allelic usage in polycythemia vera cells, BFU-Es and *in vitro* expanded erythroid progenitors and found clonal reticulocytes, granulocytes, platelets and CD34^{+} cells. We found that *TET2* mutations may also follow rather than precede *JAK2*^{V617F} as recently reported by others. Only a fraction of clonal early hematopoietic precursors and largely polyclonal T cells carry the *TET2* mutation. We showed that *in vitro* the concomitant presence of *JAK2*^{V617F} and *TET2* mutations favors clonal polycythemia vera erythroid progenitors in contrast with non-TET2 mutated progenitors. We conclude that loss-of-function *TET2* mutations are not the polycythemia vera initiating events and that the acquisition of *TET2* somatic mutations may increase the aggressivity of the polycythemia vera clone.

Key words: polycythemia vera, *TET2* mutations.

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Introduction

The *TET2* gene mutation was first found in acute myeloid leukemia (AML) and it was suggested that it is a tumor suppressor gene. The diverse loss-of-function mutations, including nonsense, insertion, and deletion of *TET2*, are found in patients with myeloproliferative disorders/neoplasms (MPNs), AML, and myelodysplastic syndromes (MDS) have been reported.[1] Published work has demonstrated that *TET2* loss-of-function mutations originate in pluripotent hematopoietic stem cells and that both alleles were affected in many patients.[1] In 5 patients with MPN who were also *JAK2*^{V617F} positive, authors reported that this mutation preceded the *JAK2*^{V617F} mutation.[1,2] Whether or not *TET2* mutations alter the severity of MPN is controversial.[3,4] While ourselves and others have reported that the *JAK2*^{V617F} mutation is not a disease-initiating mutation,[5,6] the question has arisen whether the *TET2* mutation could be the pre-*JAK2*^{V617F} somatic event responsible for MPN. However, studies of families with multiple MPN members have shown that the *TET2* mutations cannot be a disease-initiating mutation in familial MPN as it differs among affected relatives.[3]

We studied 4 *JAK2*^{V617F}-positive women with PV with diverse *TET2* mutations and 7 additional *JAK2*^{V617F}-positive PV patients without known *TET2* mutations. We determined the mutational burden of *JAK2*^{V617F} and *TET2* in their reticulocytes, granulocytes, platelets, and in CD34^{+} and CD3^{+} cells, as well as in BFU-E colonies.

Design and Methods

Study subjects, separation of cells and preparation of DNA and RNA

This study included the following three groups of prospectively recruited subjects: i) 2 sisters, P1 and P2 (family F2 in (3)) and 2 other unrelated PV females P3 and P4, whose *TET2* mutations were determined in France. Acid citrate dextrose anticoagulated peripheral blood (~28 ml) was obtained from each subject and shipped to Salt Lake City; ii) 7 *JAK2*^{V617F}-positive PV patients without known *TET2* mutations (*TET2* gene sequenced); iii) 47 patients with *JAK2*^{V617F}-positive MPN patients not evaluated for *TET2* mutations, including 31 PV, 12 ET and 4 PMF. All subjects signed the Institutional Approved Informed Consent from University of Utah IRB Board Committee (IRB@hsc.utah.edu. Approved on 6/16/2010. Statement file number: IRB 00035784).

The subjects' platelets, granulocytes, mononuclear cells, T lymphocytes (CD3), pluripotent progenitors (CD34-positive cells) and reticulocytes were isolated, and genomic DNAs and

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total RNAs were obtained, as previously described.[7,8] $CD34^+$ cells and $CD3^+$ were obtained by FACS sorting.

Clonality studies

All studied females were evaluated for clonality of granulocytes, platelets, $CD34^+$ cells, $CD3^+$ cells and reticulocytes, established by genotyping of 5 X-chromosome exonic polymorphisms followed by determination of the informative heterozygous X-chromosome allelic mRNA usage ratio by quantitative real-time AS-PCR (qAS-PCR), as described.[8] We previously established that exonic SNP polymorphic X-chromosome allelic frequency utilization greater than 90% is an indicator of the clonal phenotype.[9,10]

JAK2 and TET2 mutational analyses

The *JAK2*^{V617F} mutational burden in separated peripheral blood cells was determined by quantitative allele-specific PCR (qAS-PCR).[6] The *TET2* coding sequence and intron/exon boundaries were determined, and the allelic burden of each *TET2* mutation was established by qAS-PCR utilizing mutation-specific primers containing *locked nucleic acid* and a mismatched nucleotide, as used for clonality and *JAK2*^{V617F} assays[6,8] (*Online Supplementary Table S1*). Quantitation of the relative proportion of *TET2* alleles was accomplished by subcloning each of the mutants and determining the relative amount of each mutant by serial dilutions of plasmids containing each of the mutations, using the principles previously published.[6]

Table 1. ***JAK2*V617F and *TET2* mutational burden and X-chromosome allelic usage transcriptional ratios in platelets (PLT), granulocytes (GNC), CD34-positive cells, T lymphocytes ($CD3^+$), and reticulocytes. The X-chromosome allelic usage ratio indicating clonal or predominantly clonal cell population is depicted in italics and is underlined.**

Patients	Type of cells	*JAK2* V617F%	*TET2* MT%	Clonality Allelic frequencies of expressed exonic polymorphism
P1				FHL1 G/A
	GNC	55.3	6.2	*4/96*
	$CD34^+$	10.6	2.8	*3/97*
	CD3	0.46	0	47/53
	PLT			*5/95*
	Reticulocytes			*4/96*
P2				FHL1 G/A
	GNC	89.7	36.4	89/11
	$CD34^+$	94.5	42	90/10
	CD3	4.5	3.6	55/45
	PLT			*90/10*
	Reticulocytes			*95/5*
P3				MPP1 G/T
	GNC	81.5	9	*99/1*
	$CD34^+$	56.9	26.1	*93/7*
	CD3	3	0	58/42
	PLT			*95/5*
	Reticulocytes			*96/4*
P4				IDS C/T
	GNC	3	64.3	*100/0*
	$CD34^+$	15.3	60.5	*99/1*
	CD3	0	3	62/38
	PLT			98/2
	Reticulocytes			99/1

Expressional analyses of TET2 and JAK2 transcripts

Granulocytes, platelets and expanded erythroid progenitor *TET2* and *JAK2* mRNA levels were determined using TaqMan Gene Expression Assays and normalized to an endogenous control HPRT using commercial reagents and following the manufacturer's reaction conditions (Applied Biosystems, CA, USA).

BFU-E analysis

Erythroid colonies (BFU-E) were grown from peripheral blood mononuclear cells in the absence or presence of erythropoietin (EPO) at 3 U/ml.[6,11] Individual BFU-Es were picked, genomic DNA and total RNA isolated, their clonality determined, and their genotype and relative proportions of *JAK2*^{V617F} and *TET2* mutations established. Some colonies were too small for successful analysis or were contaminated by mononuclear cells and/or other colonies, as determined by their clonality assays; approximately 15%-contaminated colonies were excluded from analysis.

In vitro *expansion of synchronized peripheral blood erythroid progenitors*

In vitro expansion of erythroid cells starting from peripheral blood mononuclear cells was performed, as previously published.[12,13] Progenitor cells were harvested at different times from cultures reflecting their progressive erythroid differentiation.

Results and Discussion

TET2 *and* JAK2^{V617F} *mutational burden and clonal analyses in peripheral blood lineages*

Two PV sisters (P1 and P2) and 2 other unrelated PV females (P3 and P4) were *JAK2* positive and had different heterozygous *TET2* mutations: P1, a splice-site defect in intron 7 (c.*3954+2T>A*); P2, deletion of a single nucleotide in exon 3 (*c.3138delT*, p.Thr1047fs) (3); P3, deletion of a single nucleotide in exon 3 (*c.1378delT,p.Ser460fs.*); and P4, duplication of a single nucleotide in exon 3 (*c.2290dupC,*p.Gln764fs.).

We then examined the relative proportions of the above-described *TET2* mutants in circulating cells and their clonality. The mutational burden of *JAK2*^{V617F} and *TET2* and clonality were determined in reticulocytes, granulocytes, platelets, $CD34^+$ and $CD3^+$ cells (Table 1). In polyclonal $CD3^+$ cells, we detected no granulocyte contamination by analytical FACS. We found that a small proportion of largely polyclonal T cells also carry *TET2* mutation(s). This indicates that loss-of-function *TET2* somatic mutations favor myeloid differentiation but do not preclude T-lymphocyte differentiation.

Moreover, CD34-positive cells from 4 analyzed subjects were largely clonal, yet only a proportion carried the *TET2* mutation (Table 1) demonstrating that *TET2* mutation-bearing progenitors constitute a subclone of PV.

BFU-E analysis

Individual erythroid BFU-E colonies were genotyped. Results of analyses of the individual BFU-E colonies are shown in Figure 1. Patients P1, P2, P3 and P4 had 28, 14, 20 and 16 EPO-independent BFU-Es, respectively, that have either approximately 50% or 100% of *JAK2*^{V617F} (*Online Supplementary Figure S1*). In each patient, all colonies grown with or without EPO expressed the same single X-chromosome allele (*data not shown*). However, several colonies (P1: 61%; P2: 14%; P3: 45%; and P4: 12%) of the total number of BFU-Es had *JAK2*^{V617F} but not *TET2* mutations, demonstrating the primordial origin of the *JAK2*^{V617F} somatic genetic event in these PV patients. The majority of the analyzed colonies for patients P2, P3 and P4 had heterozygous *TET2* mutations. Three BFU-E colonies (two in P1 and one in P3) were homozygous for the *TET2* mutation, indicating their genetic instability and formation of a new subclone with loss-of-heterozygosity; unfortunately, a paucity of available DNA precluded determining whether the loss-of-heterozygosity was due to a loss of the wild-type *TET2* allele or from uniparenteral disomy.[7]

In vitro expansions of erythroid progenitors and their analyses are shown in Figure 1A and B and Figure 2A and C. In our 7 analyzed *JAK2* V617F-positive PV patients (P5-P11) without known *TET2* mutations, we observed decreased or unchanged *JAK2*^{V617F} mutational burdens with preferential expansion of polyclonal erythroid progenitors. These data are similar to what we have reported previously; i.e. that in most PV subjects, the erythroid progenitors' *JAK2*^{V617F} allelic burden decreased with ongoing erythroid maturation,[13] possibly and in contrast to *in vivo* conditions,

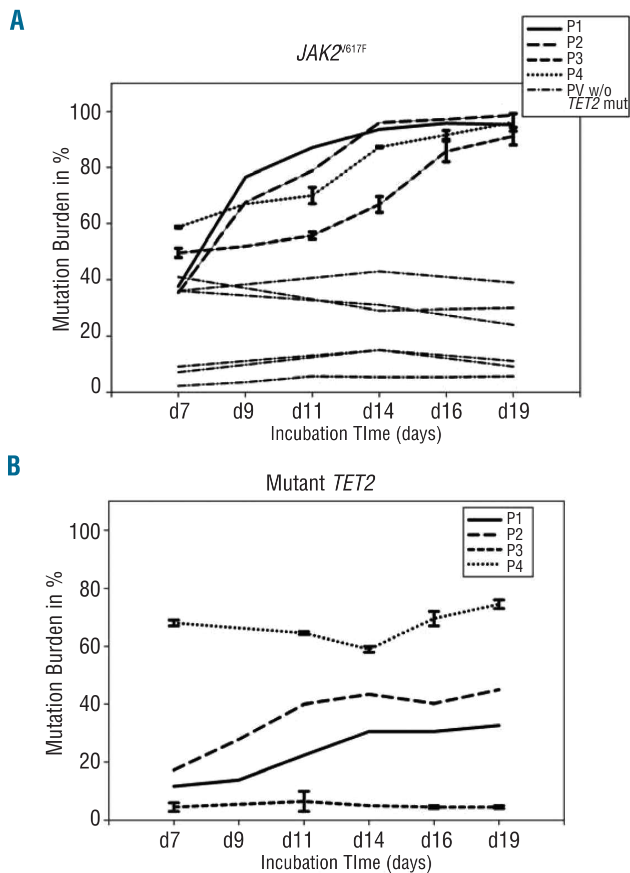

Figure 1. *JAK2*V617F and *TET2* mutational burden during *in vitro* erythroid expansion. Peripheral blood-derived erythroid cells were expanded *in vitro*. At each time point, erythroid progenitors (0.5x10^6 cells) were harvested and their genomic DNAs isolated and *JAK2*V617F and *TET2* mutational burden established. (A) *JAK2*V617F mutational burden during *in vitro* erythroid expansion with Standard Error. PV subjects depicted by solid lines represent *JAK2*V617F-positive PV patients with *TET2* mutations; dashed lines represent a group of *JAK2*V617F-positive PV patients without known *TET2* mutations. (B) *TET2* mutational burden during *in vitro* erythroid expansion for a group of *JAK2*V617F PV patients with *TET2* mutations with Standard Error.

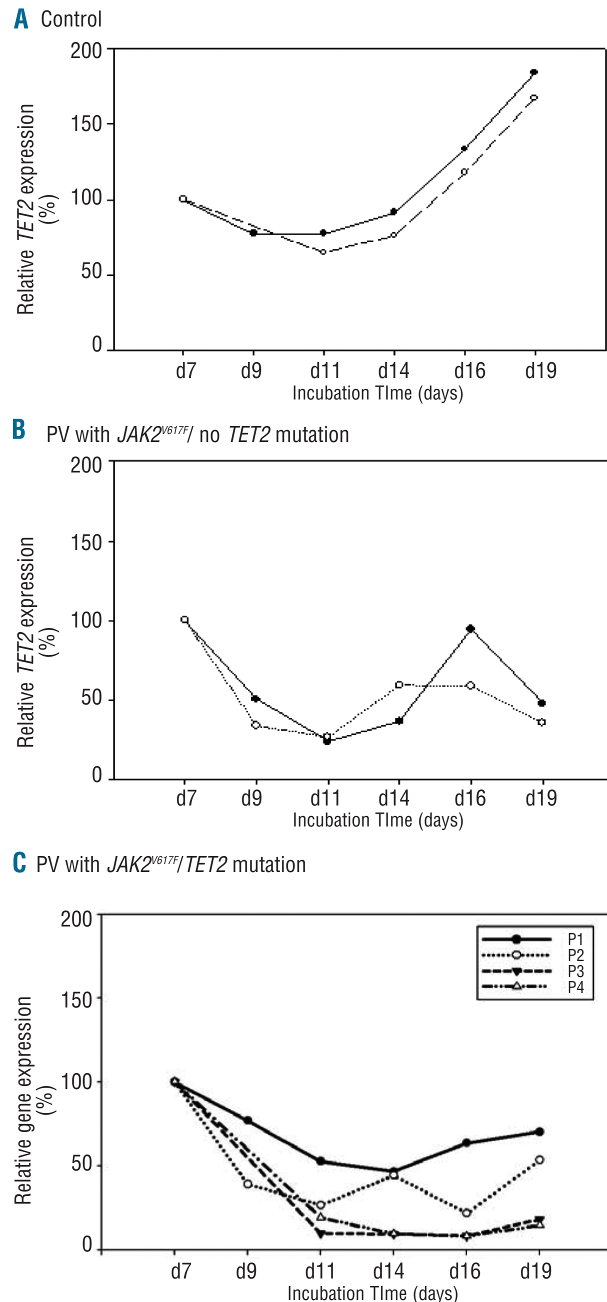

Figure 2. Expression of *TET2* in controls and PV patients during *in vitro* maturation of erythroid progenitors. (A) Controls; unaffected subjects. (B) Sporadic PV subjects with *JAK2*V617F mutation without known *TET2* mutations. (C) P1-P4 PV subjects with *TET2* and *JAK2*V617F mutations.

due to the high concentrations of EPO and other cytokines that are present in this *in vitro* system (Figure 1A). In contrast, all 4 PV subjects with *TET2* mutations (P1-P4) had a progressive increase of *JAK2*^{V617F} (Figure 1A) and *TET2* mutational burden (Figure 1B) with ongoing maturation of clonal erythroid progenitors; however, the TET2 increase was not noted in patient P3 (Figure 1B) suggesting that JAK2^{V617F} and TET-wild type may preferentially expand as a consequence of the presence of their JAK2^{V617F} and TET-mutated counterparts. These results suggested that the progenitors in the 4 studied patients with *TET2* mutations had a selective proliferative advantage under the employed conditions (*Online Supplementary Table S2*).

TET2 *and* JAK2 *mRNAs transcripts during* in vitro *expansion of normal and* JAK2^{V617F} *erythroid progenitors*

As shown in Figure 2A, expression of the TET2 tumor suppressor gene increases in normal erythroid progenitors with ongoing maturation. In contrast, in 7 PV *JAK2*^{V617F}-positive progenitors, expression of *TET2* decreases until day 11, when expanded cells still contain non-myeloid cells, and then oscillates around mean from the mid- to late stages of erythroid differentiation (days 14-19)[12] concomitant with an increase in differentiated erythroid progenitor cells (*TET2*-negative, Figure 2B and *TET2*-positive, Figure 2C; please note that P1 has a *TET2* mutation resulting in aberrant splicing that is expected to reduce its mRNA content).

We found no meaningful difference in *JAK2* mRNA transcript levels in these conditions in PV, other MPNs, or normal controls (*data not shown*).

TET2 mRNA in the peripheral blood non-erythroid cells

In contrast to data from PV erythroid progenitors, *TET2* mRNA in PV granulocytes and platelets was significantly increased regardless of *TET2* mutation, compared with normal controls (*Online Supplementary Figure S2*), except in patient P1 wherein an intronic *TET2* mutation causes aberrant splicing and predictably, based on observations,[14] decreased *TET2* mRNA (*Online Supplementary Figure S2*).

We provide further evidence that *TET2* in PV is not a disease-initiating mutation. While Delhommeau[1] found that the *TET2* somatic mutation preceded the *JAK2* mutation in several PV patients, three different chronological orders of accumulation of *JAK2*^{V617F} and *TET2* mutations were observed by others.[2] The first pattern was compatible with *TET2* mutations occurring before JAK2^{V617F}; the second was compatible with *JAK2*^{V617F} accruing before *TET2* mutations; and, in the third, *TET2* mutations and *JAK2*^{V617F} defined two separate clones. This lack of strict order of occurrence suggests that mutations in *TET2* are unlikely to represent a predisposition for accruing mutations in *JAK2*. In our study of 4 *JAK2*^{V617F}-positive PV patients with *TET2* mutations, we also demonstrate that *TET2* mutated progenitors constitute a subclone of PV pluripotent and committed hematopoietic progenitors, and that the *TET2* somatic mutation often follows the *JAK2* somatic mutation. The latter conclusion is further strengthened by the relative proportions of *JAK2*^{V617F} mutational burdens that were higher than *TET2* mutational burdens in clonal hematopoietic progenitors and cells of different lineages. That the *TET2* mutation in PV is not a disease-initiating mutation has also been validated by analysis of BFU-E individual colonies in these subjects, which showed several EPO independent colonies that were *JAK2*^{V617F}-positive and *TET2*-negative; similar to the conclusions of a study of familial PV.[3]

Our data provide additional evidence that loss-of-function *TET2* mutations are not the PV-initiating events and that, in some PV patients, *TET2* mutations follow rather than precede *JAK2*^{V617F} mutations. Our data also suggest that the concomitant *JAK2*^{V617F} and *TET2* mutations increase proliferation and provide a competitive advantage for the *TET2* mutant PV subclone, supporting the notion that *TET2* mutations may contribute to the aggressivity of *TET2* mutation-positive PV.

Authorship and Disclosures

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