Analysis of the *Ten-Eleven Translocation (TET)*2 gene in familial myeloproliferative neoplasms

Running title: *TET2* in familial myeloproliferative neoplasms

Cécile Saint-Martin 1,2, Gwendoline Leroy 1, François Delhommeau 2,3, Gérard Panelatti 4, Sabrina Dupont 2, Chloé James 5, Isabelle Plo 2, Dominique Bordessoule 6, Christine Chomienne 7, André Delannoy 8, Alain Devidas 9, Martine Gardembas-Pain 10, Françoise Isnard 11, Yves Plumelle 12, Olivier Bernard 13, William Vainchenker 2, Albert Najman 11, Christine Bellanné-Chantelot 1,2 and the French Group of Familial Myeloproliferative Disorders *

1 Department of Genetics, AP-HP Groupe Hospitalier Pitié-Salpêtrière, Université Pierre et Marie Curie-Paris 6, Paris, France
2 INSERM, U790, Institut Gustave Roussy, Villejuif, France
3 Laboratory of Hematology, AP-HP Hôpital Saint Antoine, Paris, France
4 Department of Internal Medicine, CHU de Fort de France, Fort de France, France
5 INSERM, U876, Bordeaux, France
6 Department of Hematology, CHU de Limoges, Limoges, France
7 Department of Cellular Biology, AP-HP Hôpital Saint Louis, Université Denis Diderot-Paris 7, Paris, France
8 Department of Internal Medicine, Université Catholique de Louvain, Leuven, Belgium
9 Department of Hematology, CH Sud-Francilien, Corbeil-Essonnes, France
10 Department of Hematology, CHU d’Angers, Angers, France
11 Department of Hematology, AP-HP Hôpital Saint Antoine, Université Pierre et Marie Curie-Paris 6, Paris, France
12 Laboratory of Hematology, CHU de Fort de France, Fort de France, France
13 INSERM, E210, Université René Descartes–Paris 5, Paris, France
* For a complete list of the French Group of Familial Myeloproliferative Disorders, see the Supplemental Appendix.

**Address for Correspondence:**

Dr Christine Bellanné-Chantelot  
Centre de Génétique Moléculaire et Chromosomique  
Groupe Hospitalier Pitié Salpêtrière, 47-83 bd de l’Hôpital, 75651 PARIS Cedex 13, FRANCE  
Phone: +33 142 17 76 52  
Fax: +33 142 17 76 18  
E-mail: christine.bellanne-chantelot@psl.aphp.fr

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ABSTRACT

The \( JAK2^{V617F} \) mutation does not elucidate the phenotypic variability observed in myeloproliferative neoplasm (MPN) families. A putative tumor suppressor gene, \( TET2 \), was recently implicated in MPN and myelodysplastic syndromes through the identification of acquired mutations affecting hematopoietic stem cells. The present study analyzed the \( TET2 \) gene in 61 MPN cases from 42 families. Fifteen distinct mutations were identified in 12 \( JAK2^{V617F} \) positive or negative patients. In a patient with two TET2 mutations, the analysis of five blood samples at different phases of her disease showed the sequential occurrence of \( JAK2^{V617F} \) and \( TET2 \) mutations concomitantly to the disease evolution. Analysis of familial segregation confirmed that \( TET2 \) mutations were not inherited but somatically acquired. \( TET2 \) mutations were mainly observed (10/12) in patients with primary myelofibrosis (PMF) or patients with polycythemia vera (PV) or essential thrombocythemia (ET) who secondarily evolved towards myelofibrosis or acute myeloid leukemia.
INTRODUCTION

Families of myeloproliferative neoplasms (MPN) are characterized by a clinical and genetic heterogeneity. First, within MPN families, distinct clinical entities are observed, the three main being polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF).\(^1,2\) Second, disease evolution can be highly variable within families presenting with the same type of MPN.\(^2,3\) The acquired \textit{JAK2}^{V617F} mutation present in most PV and half of ET and PMF sporadic or familial cases does not totally explain the phenotypic variability.\(^2-7\) The existence of additional molecular events, either germline or acquired, may explain the MPN predisposition and the distinct phenotypes observed within MPN families.\(^8-10\)

Recently, acquired mutations of the Ten-Eleven Translocation (\textit{TET})2 gene, were reported in about 12\% of sporadic MPN (PV, ET and PMF) as well as in myelodysplastic syndrome (MDS) and acute myeloid leukemia (AML).\(^11\) By \textit{in vitro} clonal assays, Delhommeau et al showed that \textit{TET}2 defects target both multipotent and committed progenitors and were associated with a selective advantage of early hematopoiesis.\(^11\) These recent findings led us to analyze \textit{TET}2 in familial MPN cases in order to determine whether \textit{TET}2 could be a predisposition factor, to estimate the prevalence of acquired \textit{TET}2 events in MPN cases and to describe the clinical profile of patients with \textit{TET}2 mutations.
METHODS

Families with at least 2 affected patients with MPN were collected through a national network. The diagnoses of MPN were reviewed based on the 2008 WHO criteria. The study was approved by the French Comité de Protection des Personnes. All participants gave their written informed consent in accordance with the Declaration of Helsinki.

We analyzed 61 patients from 42 MPN families (40 European, 2 African: families F3 and F4). Thirty-three patients displayed a simple phenotype consisting of either PV (14), or ET (12) or PMF (7) with no observed hematological evolution of the disease after a mean follow-up period of 12 years. Twenty-eight other patients had experienced an evolution in their MPD phenotype with a mean follow-up of 14.3 years: PV evolving into myelofibrosis (post PV MF, 6) and/or into AML (12); ET evolving into MF (4) and/or AML (5), or PMF turning into AML (1).

TET2 molecular analysis

Amplifications of TET2 (NM_001127208.1) were performed either on genomic DNA extracted from mononuclear blood cells and from buccal swabs or directly on hematopoietic colonies after heating at 95°C for 10 minutes. Purified PCR products were sequenced using the BigDye Terminator chemistry (Applied Biosystems) and run on an ABI3100 capillary sequencer.

The search for large genomic rearrangement was performed by quantitative multiplex PCR of short fluorescent fragments (QMPSF) and applied to patients homozygous for all TET2 polymorphisms to exclude hemizygosity of the region. Primer pairs were designed for exons 3, 6 and 11. PCR products were separated on an ABI3100 capillary sequencer. Analysis using GeneMapper version 4.0 (Applied Biosystems) is based on the comparison of the peak heights generated from the tested DNA sample and the control DNA. A heterozygous exon deletion
will lead to a two-fold reduction of the height of the corresponding peak. Primer sequences for TET2 sequencing and QMPSF are shown in supplemental table1.

**Cell culture**

Peripheral blood granulocytes were isolated using standard protocols. Thawed cells were sorted on the CD34 and CD38 antigens using a FACSDiva cell sorter. CD34⁺CD38⁺ cells were seeded in methylcellulose medium supplemented with EPO, IL3 and SCF. Hematopoietic colonies were picked at day 14.
RESULTS AND DISCUSSION

Sixty-one patients were screened for mutations in the 6009 bp coding sequence of the \textit{TET2} gene, composed of 11 exons spanning 130 kb. Fifteen distinct molecular defects including 11 (73\%) truncating mutations, 3 (20\%) missense mutations and one whole gene deletion were identified (Table 1). They were spread throughout the gene. The 11 truncating mutations consisted of 3 nonsense mutations, 6 out-of-frame insertions/deletions and 2 splice site mutations. The three missense mutations affected amino acids that were conserved in at least one of the TET2 paralogs, TET1 and TET3, and in TET2 orthologs.\textsuperscript{13,14} Two, p.Leu1340Pro and p.His1868Arg, were located in the two highly conserved \textit{TET2} functional domains (1134-1444 and 1842-1921) of the drosophila ortholog.\textsuperscript{13} Furthermore all three missense mutations were absent from 165 control individuals of ethnically matched populations, leading us to consider them as putative mutations. The proportion and the type of \textit{TET2} mutations identified in familial MPNs were similar to the ones found in sporadic MPN cases.\textsuperscript{11,15} TET2 mutations have also been reported in other myeloid malignancies.\textsuperscript{11,15-19}

All mutations identified were each found in a single patient and segregation with the phenotype could never be observed in the families (Figure 1). Moreover, in three families (F2, F3 and F4), different \textit{TET2} mutations were identified in affected members. Sequence analysis of buccal swabs available for patients P1(F1) and P4(F3) showed the absence of the \textit{TET2} mutations. Therefore, the analysis of the 42 families showed that \textit{TET2} is not a major predisposing gene factor in familial MPN and that identified \textit{TET2} defects were acquired events.

Two distinct \textit{TET2} mutations were found in three unrelated patients, (P4, P6 and P9, Table 1). We picked three individuals colonies derived from CD34\(^+\)CD38\(^+\) cells from the blast phase of patient P4 and using allele-specific PCR, we found that each TET2 mutation affected a different allele of the same colony (data not shown). In addition, we showed in mononuclear
cells from patient P6 that the two exon 3 mutations were not located in cis. These observations are in favour of a biallelic inactivation of TET2, in agreement with the hypothesis of a tumor suppressor role of TET2.11

In patient P4 for whom five blood samples were available throughout the last three steps of her evolution: PV, MF and AML (Figure 2A), sequence analysis showed that JAK2\textsuperscript{V617F} and the TET2 p.Arg550X mutation were present at the PV stage and later on. The p.Asn857fs mutation only started being detectable in the second sample, 7 years later and 5 months before the diagnosis of MF. The sequential analysis showed that the burden of JAK2 and TET2 mutant alleles grew in time, concomitantly with the development of the disease. Blood progenitor cells were available at the PV and blast phases (Figure 2B). During the PV stage, endogenous erythroid colonies carried the p.Arg550X mutation (5/29) but p.Asn857fs was never observed and 19/29 clones were found JAK2\textsuperscript{V617F} positive in the absence of any TET2 mutation. These results suggest the initial occurrence of JAK2\textsuperscript{V617F} followed by TET2 p.Arg550X and subsequently by the TET2 p.Asn857fs defect. After leukemic transformation, all colonies (29/31) but 2 carried JAK2\textsuperscript{V617F} and both TET2 mutations. We genotyped the rs16922579 SNP located in intron 9 of JAK2 and heterozygous in patient P4. A homozygous G/G genotype was found in JAK2\textsuperscript{V617F} negative colonies whereas G/A and A/A genotypes were observed in JAK2\textsuperscript{V617F} heterozygous and homozygous colonies respectively. These preliminary results suggest a loss of the JAK2\textsuperscript{V617F} allele through mitotic recombination during disease progression. In contrast, in two other studies, mitotic recombination was excluded in two informative patients who transformed to AML and for whom all blast cells were JAK2\textsuperscript{V617F} negative.20,21

The TET2 defects with an allele burden semi-quantitatively estimated by sequencing varied from 20 to 60% and were identified in 12 patients (12/61, 20%) diagnosed with PV (4/32), ET (5/21) and PMF (3/8) (Table 1). All patients but two were positive for the JAK2\textsuperscript{V617F} mutation.
Altogether, 20% of the \( JAK2^{\text{V617F}} \) positive patients were found mutated for \( TET2 \) (10/49) and 17% of the \( JAK2^{\text{V617F}} \) negative patients (2/12). The two negative cases were ET patients who developed very active AML and died rapidly (P5 and P6, Table 1). In contrast to results recently reported, we did not observe an older age for patients with \( TET2 \) mutations (Supplemental table).\(^{15}\)

Twenty-nine percent (10/35) of patients with PMF or with hematological complications (post-PV/ET MF or AML) were found mutated in \( TET2 \) compared to 7.7% (2/26) of patients without any diagnosed hematological complications after a mean disease duration of 12 years. We first performed a univariate analysis to compare the incidence of transformation between the two groups (supplemental table). The results indicated a trend toward hematological complications in \( TET2 \)-mutated patients although this difference was not statistically significant (p=0.055). This was confirmed by a multivariate analysis (supplemental table).

Similarly, a trend toward malignant proliferation was recently reported in MDS/MPN syndromes.\(^{18}\) Studies on larger cohorts of MPN cases would be required to estimate the prevalence of \( TET2 \) events in patients with more aggressive outcome.

Recent data showed that TET1 catalyzes conversion of 5-methylcytosine to 5-hydroxymethylcytosine.\(^{22}\) TET gene family may play an important role in epigenetic regulation of stem cell functions. In addition it was shown that deletion of \( TET2 \) induced a clonal advantage at the level of early hematopoietic progenitors.\(^{11}\) This may explain that patients with a defect in \( TET2 \) are prone to progress to MF. Moreover \( TET2 \) defects may be a primary or secondary event, preceding or following \( JAK2^{\text{V617F}} \) as reported for the 20q- deletion.\(^{21}\) In future studies it will be important to understand whether different kinetics of occurrence of these two independent genetic defects have the same impact on the clinical course of the disease and are both associated with a clonal dominance at early hematopoietic stages.
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AUTHORSHIP

Contribution: The original design of the study was drawn by C. Bellanné-Chantelot, F. Delhommeau, W. Vainchenker and A. Najman. D. Bordessoule, N. Chomienne, A. Delannoy, A. Devidas, M. Gardembas-Pain, F. Isnard, A. Najman, G. Panelatti, Y. Plumelle, W. Vainchenker and the French Group of Familial Myeloproliferative Disorders recruited the patients. A. Najman recorded all clinical and hematologic data. Molecular analyses were performed by C. Saint-Martin, G. Leroy and C. James. F. Delhommeau, S. Dupont, I. Plo. and O. Bernard assisted with interpretation of research. C. Bellanné-Chantelot and C. Saint-Martin analyzed data and wrote the manuscript. A. Najman, F. Delhommeau, S. Dupont and W. Vainchenker critically reviewed the manuscript. All authors contributed to the amendment of the manuscript.

Conflict-of-interest disclosure: The authors declare no competing financial interests.
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Table 1. Clinical profile of the 12 patients with TET2 mutations

<table>
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<tr>
<th></th>
<th>P1 (F1)</th>
<th>P2 (F2)</th>
<th>P3 (F2)</th>
<th>P4 (F3)</th>
<th>P5 (F3)</th>
<th>P6 (F4)</th>
<th>P7 (F4)</th>
<th>P8</th>
<th>P9</th>
<th>P10</th>
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<td>F</td>
<td>F</td>
<td>F</td>
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<td>F</td>
<td>M</td>
<td>F</td>
<td>M</td>
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<td>PV</td>
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<td>ET</td>
<td>ET</td>
<td>ET</td>
<td>ET</td>
<td>PMF</td>
<td>PMF</td>
<td>PMF</td>
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<td>16.4</td>
<td>18.1</td>
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<td>1.2</td>
<td>6.2</td>
<td>29.2</td>
<td>3.5</td>
<td>4.3</td>
<td>3.6</td>
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<td>15.7</td>
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<tr>
<td><strong>JAK2 V617F</strong></td>
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<tr>
<td>Disease duration at 1st observance, y</td>
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<td>14.4</td>
<td>14.5</td>
<td>8.4</td>
<td>3.6</td>
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<tr>
<td>Disease duration at 1st observance, y</td>
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<td>14.4</td>
<td>14.5</td>
<td>8.4/15.1*</td>
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<td>1.0</td>
<td>5.7</td>
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<td>2.7</td>
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<td>20</td>
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<td>50 / 35</td>
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<td>c.1648C&gt;T</td>
<td>c.2058A&gt;T</td>
<td>c.1955delA</td>
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<td>c.1_6009del</td>
<td>c.1720C&gt;T /c.4999_5014del16</td>
<td>c.694C&gt;T</td>
<td>c.4019T&gt;C</td>
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*Disease duration at first observance for each mutation
**TET2 allele burden at first observance for each mutation.
LEGENDS FOR FIGURES

Figure 1

Pedigrees of 4 MPN families showing that \textit{TET2} mutations do not segregate with the MPN phenotype. Patients are depicted as filled symbols, their clinical phenotype being indicated below. The first line indicates the phenotype at the time of diagnosis; the second gives the evolution and is blank when there is none. The third line accounts for the \textit{JAK2}^{V617F} status (\textit{V617F} when the mutation was found, - otherwise) and the fourth line for any \textit{TET2} mutation. Mutations are annotated in amino acid one-letter code. nd: not done.

Figure 2

Sequential study of \textit{TET2} mutations and \textit{JAK2}^{V617F} in patient P4 (F3) in mononuclear cells and committed progenitors. A. Sequence electrophoregrams are shown for each \textit{TET2} mutation and for \textit{JAK2}^{V617F}. The diagram indicates on the left, the different phases of the disease with their time lapse from diagnosis and on the right, the disease duration at each sample date (in years). Allele burdens of \textit{TET2} mutations and \textit{JAK2}^{V617F}, semi-quantitatively estimated by sequencing as in reference 2, are indicated. B. Histograms show for PV and AL phases the three distinct TET2 genotypes (each bar corresponding to a specific genotype, wt/wt, 550X/wt, 550X/857fs); The \textit{JAK2}^{V617F} mutation was either absent (white), heterozygous (hatched) or homozygous (black). The number of genotyped clones is indicated for each group.
Figure 1
Analysis of the *Ten-Eleven Translocation (TET)2* gene in familial myeloproliferative neoplasms

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