Clonality Analysis of Hematopoiesis in Essential Thrombocythemia: Advantages of Studying T Lymphocytes and Platelets

By Nahed El-Kassar, Gilles Hetet, Jean Brière, and Bernard Grandchamp

Essential thrombocythemia (ET) is a myeloproliferative disorder characterized by a sustained elevation of the platelet count in the absence of other causes of thrombocytosis. ET is difficult to diagnose, and the demonstration of clonal hematopoiesis may be of value. However, clonality analysis of hematopoietic cells based on the study of the X-chromosome inactivation pattern is complicated by the observation that some normal females present skewed lyonization. Moreover, DNA methylation of X-linked genes in hematopoietic cells may differ from that in other tissues. Appropriate controls for skewed lyonization are therefore critical for the study of clonality. We developed two techniques based on X-chromosome inactivation and polymerase chain reaction (PCR) analysis of polymorphisms, to study clonality in ET patients. Reverse transcriptase-PCR analysis of IDS, P55, and G6PD mRNAs was used to examine the different hematopoietic cell lineages including platelets in patients heterozygous for these polymorphisms and analysis of the HUMARA gene methylation pattern permitted us to study clonality in all nucleated cell fractions of the other patients. Using both types of assay and T lymphocytes as a control tissue for lyonization, clonal hematopoiesis was demonstrated in 28 patients. In 14 patients, the granulocytes were polyclonal; among these patients, platelets were monoclonal in 3 cases, polyclonal in 7 cases, and in the remaining 4 cases this fraction could not be studied because the patients were homoygotes for all RNA markers. No conclusion about clonality could be drawn in 6 cases. Polyclonal hematopoiesis was found in all the cases of reactive thrombocytosis. These findings confirm the high frequency of monoclonal hematopoiesis in ET, the utility of studying platelets, and the possibility of using T lymphocytes as a control tissue for X-chromosome inactivation patterns.

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To extend our initial study and to further evaluate the clonality of platelets in ET, we developed reverse transcriptase-polymerase chain reaction (RT-PCR) assays to determine the X-chromosome inactivation pattern from the allelic expression of three polymorphic genes: idurionate-2-sulfatase (IDS), P55 and G6PD. Clonality results based on the detection of these mRNA transcripts were compared to those deduced from the methylation pattern of the HUMARA gene in nucleated cells. We used the T-lymphocyte fraction as a control tissue for the X-chromosome inactivation patterns, as most previous studies have shown that they are not involved in this disease. 

**MATERIALS AND METHODS**

**Subjects.** Blood samples were obtained from 48 females with ET, 51 normal controls, and 8 patients with reactive thrombocytosis with their informed consent.

ET patients aged from 13 to 80 years (mean, 53 years) at the time of diagnosis were selected according to PVSG criteria and had no known cause for reactive thrombocytosis for at least 6 months after the diagnosis. Clinical symptoms and platelet counts at diagnosis, and the follow-up period at the time of clonality analysis are shown (see Tables 4 and 5).

Normal controls were aged from 22 to 87 years (mean, 36.5 years) and patients with reactive thrombocytosis were aged from 33 to 75 years (mean age: 53 years). The mean platelet count in this latter group was $10^9/L$ (600 to 1,047). One patient had lung cancer, 3 had iron deficiency, and 4 had infectious diseases.

**Cell fractionation.** PB (50 mL) was collected on citrate or EDTA. DNA was extracted from 5 mL with the Genome Kit (Bio 101, Ozyme, France). Forty-five milliliters of blood was used to isolate platelets, granulocytes, and lymphocytes. After centrifugation at 150g, platelets were collected from the upper half of the plasma layer. The remaining blood was mixed with a Dextran solution (Pharmacia Biotech, Uppsala, Sweden) and left for 45 minutes. The upper layer was centrifuged through an Isopaque gradient (GBBCO-BRL, Gaithersburg, MD) at 300g for 20 minutes, then, mononuclear cells and granulocytes (95% by light microscopy after May-Grunwald-Giemsa staining) were obtained from the interphase and pellet, respectively. T lymphocytes were collected from interphase cells using immunomagnetic beads (Dynabeads, Dynal, France), coated with monoclonal antibodies (MoAbs) CD2 and CD4 MoAbs. DNA was extracted from granulocyte and mononuclear cell fractions by using the Isosquick kit (ORCA Research, Bothell, WA).

**Genotyping of G6PD, IDS, and P55 polymorphisms.** The IDS gene contains a silent polymorphism at nucleotide 438 (C/T). We found that this gene is transcribed in different blood fractions, including platelets. The G6PD gene shows a C/T polymorphism at position 1311, and the P55 gene shows a G/T polymorphism at position 358 (Table 1).

Genomic DNA extracted from whole blood was used for genotyping. DNA fragments containing the polymorphic bases were amplified by PCR. Primers and PCR conditions are shown in Table 1. In each case, the sequence of one primer introduced a mismatch to create a restriction site specifically in PCR products from one of the two alleles (Table 1). Restriction fragments were separated by gel electrophoresis and visualized by UV illumination after ethidium bromide staining. In heterozygous females, 2 bands were observed: 151 and 126 for IDS; 183 and 162 pb for G6PD; whereas only one band was found in homozygous females. For P55, bands of 80, 78, and 34 bp were found in cases with homozygous G; 80, 57, 34, and 21 in cases with homozygous T and 80, 78, 57, 34, and 21 in heterozygotes.

**Clonality study by mRNA transcript analysis.** RNA was extracted from cell fractions, including platelets, according to Chomzinski et al. After reverse-transcription from 1 μg of RNA using random priming, cDNAs were amplified and the resulting fragments were digested and analyzed on agarose gel as described above. Fragment lengths, polymerase chain reaction (PCR) conditions, primer sequences, and enzymes are shown in Table 2. After migration, two bands were seen in polyclonal patients: 158 and 133; 79 and 58; 138 and 122; for IDS, G6PD, and P55, respectively, whereas one single band was present in monoclonal patients.

**RESULTS**

**Patterns of X-chromosome inactivation from total blood using DNA and RNA-based clonality assays.** The overall heterozygosity of the markers was 81%, 51%, 37%, and 17%, for HUMARA, IDS, P55, and G6PD, respectively. At least one mRNA marker was informative in 72% of the cases. Thirty-four controls, 28 ET patients and 4 patients with reactive thrombocytosis were informative for both DNA and RNA markers. In normal controls, nonrandom lyonization was found in 9% of cases by DNA analysis (4 out of 44 informative patients) and in 2.4% of cases by mRNA analysis (1 out of 41 informative patients) (see Table 4). The patients with reactive thrombocytosis showed a polyclonal pattern of X-chromosome inactivation as determined by DNA analysis (in 2 patients), RNA analysis (in 2 patients), or both (in 4 patients). Among ET patients, 74% and 68% showed a nonrandom inactivation pattern by DNA and mRNA analysis, respectively. Results were usually concordant between both types of markers. However, some discrepancies were observed: in normal controls, a nonrandom methylation pattern at the HUMARA locus was observed in 3 cases, whereas the expression of X-linked genes was balanced (Table 3); in the patient group, the opposite was observed in 2 cases (Table 3).

**Clonality analysis on cell fractions.** In the ET group, cell fractions containing T lymphocytes, granulocytes, and platelets were studied using RNA markers when at least one of these markers was informative. When no marker was informative, the same fractions but platelets were studied using the HUMARA DNA polymorphism.

In 2 out of 48 patients studied by DNA and RNA analysis, discordant results obtained from unfraccionated blood (Table 3) were confirmed on cell fractions, running out conclusions about clonality. A nonrandom X-chromosome inactivation pattern was observed in all fractions using both techniques in 2 patients (patients 1 and 2; Tables 4 and 5), and using DNA analysis only in 2 other patients (patients 32 and 33; Table 5). In 28 patients, clonality of the granulocyte fraction (patients 34 through 42; Table 4 and patients 33 through 41; Table 5) was observed in the presence of polyclonality of T lymphocytes. Among these patients, the clonality of platelets was shown in all cases informative for RNA markers (patients 3 through 21; Table 4) (Fig 1). Interestingly, in 3
Table 1. DNA Genotyping: Primer Sequences, PCR Conditions, and Sizes of PCR Products

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
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<th>Exon Hybridization T°</th>
<th>Cycles No.</th>
<th>[MgCl₂] mmol/L</th>
<th>Size of PCR Product</th>
<th>Enzymes</th>
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<td>183</td>
<td>Pvu I</td>
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<tr>
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</table>

* Indicate the mismatched nucleotides introduced by the primer to create restriction sites.

Additional patients, platelets were clonal despite polyclonality of all other hematopoietic cell fractions (patients 22 through 24; Table 4). Therefore, 31 patients had evidence of clonality in at least one hematopoietic lineage. In contrast, 11 patients had polyclonality of all studied lineages (patients 25 through 31; Table 4 and 43 through 46; Table 5), including platelets in 7 cases where RNA markers could be studied (patients 25 through 31; Table 5). An example of each clonality pattern is presented in Fig 2.

Normal controls with a nonrandom pattern of X-chromosome inactivation in total blood displayed the same pattern in all fractions (data not shown).

Comparison of clinical and biological features of ET patients with monoclonal and polyclonal hematopoiesis. We observed no correlation between the severity of the disease, as assessed by the prevalence of ischemic and hemorrhagic signs (Fisher’s test, \( P = .33 \)), the number of treated patients (\( P = .28 \)), or the duration of the disease (\( P = .09 \)) on the one hand, and the pattern of clonality on the other hand. The mean platelet count at diagnosis was higher in the group of patients with monoclonal hematopoiesis (mean \( 1,070 \times 10^9/L \); range 631 to 2, 160 \( 10^9/L \)) than in the group of patients with polyclonal hematopoiesis (mean \( 845 \times 10^9/L \); range 600 to 1,098 \( 10^9/L \)) (\( P = .04 \)). We also found a significant difference in mean age between the patients with monoclonal hematopoiesis (56 years; range 16 to 80),

Table 2. RT-PCR Analysis of RNA Transcripts: Primer Sequences, PCR Conditions, and Sizes of PCR Products

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<th>Gene</th>
<th>Primers</th>
<th>Sequence 5’−3′</th>
<th>Exon Hybridization T°</th>
<th>Cycles No.</th>
<th>[MgCl₂] mmol/L</th>
<th>Size of PCR Product</th>
<th>Enzymes</th>
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</thead>
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</table>

* Indicate the mismatched nucleotides introduced by the primer to create restriction sites.
and the patients with polyclonal hematopoiesis (37 years; range 24 to 76) \((P = .01)\).

Interestingly, 3 ET patients with polyclonal hematopoiesis had clinical signs of MPD: splenomegaly had been present in patient 25 since disease onset. Severe peripheral vascular ischemia was present in patients 29 and 31 (aged 30 and 76 years, respectively). The other 4 patients in this group had no clinical signs of MPD. To search for familial thrombocytosis, the platelet counts of the parents, the 2 children of patients 29 and the 3 children of patient 30 were determined and were found to be normal.

**DISCUSSION**

Like other MPD, ET is thought to be a clonal disorder. In a previous study, we described a technique based on HUMARA CAG polymorphism. This technique permitted clonality analysis in 81% of females, but did not allow us to study the clonality of hematopoiesis in platelets. This was overcome by making use of techniques based on the detection of RNA transcripts. In addition to G6PD and P55 transcript analysis described by Prchal,\(^21\),\(^22\) the study of IDS

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**Table 3. Comparison of the Results of X-Chromosome Inactivation Pattern Between RNA and DNA Markers in Total Blood**

<table>
<thead>
<tr>
<th>Normal Controls ((n = 34))</th>
<th>RNA</th>
<th>DNA</th>
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<td>3</td>
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<tr>
<td>Nonrandom</td>
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**Patients \((n = 28)\)**

<table>
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<th>RNA</th>
<th>DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Random</td>
<td>5</td>
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<tr>
<td>Nonrandom</td>
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</table>

Thirty-four normal controls and 28 patients were informative for the HUMARA polymorphism and at least one of the three RNA markers. The X-chromosome inactivation pattern was scored as random or nonrandom and the results obtained using RNA and DNA markers are compared.

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**Table 4. Clinical and Laboratory Findings in ET Patients and Results of Clonality Analysis Using mRNA Genes**

<table>
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<tr>
<th>No.</th>
<th>Age at Diagnosis</th>
<th>Follow-up Time</th>
<th>Treatment</th>
<th>C.S</th>
<th>Unfractionated Blood</th>
<th>T. Lymphocyte Fraction</th>
<th>Granulocyte Fraction</th>
<th>Platelets</th>
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<td>50</td>
<td>Hydrea</td>
<td>+</td>
<td>PO</td>
<td>PO</td>
<td>PO</td>
<td>MO</td>
<td>660</td>
</tr>
</tbody>
</table>

Follow-up (months) at the time of clonality analysis.
Abbreviations: C.S, ischemic or hemorrhagic symptoms; MO, monoclonal; PO, polyclonal; RNA genes, G6PD or IDS or P55.
* Patients reported in our previous study.\(^14\)
Table 5. Clinical and Laboratory Findings in ET Patients and Results of Clonality Analysis Using the AR Gene

<table>
<thead>
<tr>
<th>No.</th>
<th>Age at Diagnosis</th>
<th>Follow-up Time</th>
<th>Treatment</th>
<th>C.S</th>
<th>Unfractionated Blood</th>
<th>T-Lymphocyte Fraction</th>
<th>Granulocyte Fraction</th>
<th>Platelet Counts at Diagnosis x 10^9/L</th>
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<tbody>
<tr>
<td>1*</td>
<td>36</td>
<td>53</td>
<td>Hydrea</td>
<td>+</td>
<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>1,100</td>
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<tr>
<td>2*</td>
<td>34</td>
<td>148</td>
<td>Hydrea</td>
<td>+</td>
<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>889</td>
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<tr>
<td>32</td>
<td>57</td>
<td>7</td>
<td>Hydrea</td>
<td>–</td>
<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>1,300</td>
</tr>
<tr>
<td>33*</td>
<td>68</td>
<td>56</td>
<td>Hydrea</td>
<td>–</td>
<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>962</td>
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<tr>
<td>34</td>
<td>70</td>
<td>48</td>
<td></td>
<td>0</td>
<td>+</td>
<td>MO</td>
<td>PO</td>
<td>693</td>
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<td>12</td>
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<td>+</td>
<td>MO</td>
<td>PO</td>
<td>MO</td>
<td>1,060</td>
</tr>
<tr>
<td>36</td>
<td>16</td>
<td>70</td>
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<td>–</td>
<td>MO</td>
<td>PO</td>
<td>PO</td>
<td>907</td>
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<td>19</td>
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<td>+</td>
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<td>PO</td>
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<td></td>
<td>0</td>
<td>–</td>
<td>MO</td>
<td>PO</td>
<td>843</td>
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<tr>
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<td>2</td>
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<td>+</td>
<td>MO</td>
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<td>990</td>
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<td>40*</td>
<td>63</td>
<td>36</td>
<td>Hydrea</td>
<td>–</td>
<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>1,025</td>
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<tr>
<td>41*</td>
<td>47</td>
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<td>Hydrea</td>
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<td>MO</td>
<td>MO</td>
<td>MO</td>
<td>1,067</td>
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<tr>
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<td>–</td>
<td>MO</td>
<td>PO</td>
<td>MO</td>
<td>631</td>
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<tr>
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<td>37</td>
<td>1</td>
<td></td>
<td>0</td>
<td>–</td>
<td>PO</td>
<td>PO</td>
<td>880</td>
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<tr>
<td>44</td>
<td>16</td>
<td>2</td>
<td></td>
<td>0</td>
<td>–</td>
<td>PO</td>
<td>PO</td>
<td>900</td>
</tr>
<tr>
<td>45</td>
<td>13</td>
<td>14</td>
<td>Interferon</td>
<td>–</td>
<td>PO</td>
<td>PO</td>
<td>PO</td>
<td>1,400</td>
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<td>65</td>
<td>94</td>
<td>Hydrea</td>
<td>–</td>
<td>PO</td>
<td>PO</td>
<td>PO</td>
<td>624</td>
</tr>
</tbody>
</table>

Follow-up (months) at the time of clonality analysis.

Abbreviations: C.S, ischemic or hemorrhagic symptoms; MO, monoclonal; PO, polyclonal; AR, androgen receptor gene.

* Patients reported in our previous study.

Polymorphic mRNAs permitted clonality analysis of platelets in 72% of females.

Because the X-chromosome inactivation phenomenon occurs in different tissues at different times during the embryonic period, the clonality of hematopoiesis cannot be confirmed without finding polyclonality in one or more hematopoietic lineage, thus ruling out a skewed lyonization. T cells appear to be the best choice as a control tissue in myeloid malignancies like acute myeloid leukemia, and we tested for their absence of involvement in ET. Our present results confirm that T lymphocytes derive from polyclonal hematopoiesis in most ET patients. Although 88% of patients with a nonrandom X-chromosome inactivation pattern in granulocytes and/or platelets showed a random pattern in T lymphocytes, only 12% of them probably had skewed lyonization, as the same nonrandom pattern was observed in all hematopoietic lineages. However, as T cells and myeloid cells are derived from a common stem cell, we cannot formally exclude the involvement of pluripotent stem cells in these patients.

This study shows that the majority of patients with ET have monoclonal hematopoiesis (74%) detectable at least in platelets. The present data confirm and extend previous observations indicating a variable contribution of monoclonal hematopoiesis to different lineages. Interestingly, in some patients, monoclonality of hematopoiesis is restricted to platelets, despite polyclonality in the other lineages. Surprisingly, a minority of patients with clinical and biological features of ET had polyclonal hematopoiesis in all cell lineages. Reactive thrombocytosis in these patients was formally excluded by the long clinical history of the disease (see Results). Although we cannot formally exclude the possibility of a monoclonal cellular fraction mixed with cells from persistent normal hematopoiesis in these patients, visual inspection of two bands corresponding to amplified fragments derived from both alleles shows whether or not the allelic ratio differs between lineages (Fig 1).

The presence of an allelic ratio variation between T lymphocytes and other lineages excludes the possibility that a significant proportion of clonal cells exists in the granulocyte or platelet fractions in these patients.

The variable involvement of different lineages in patients with monoclonal hematopoiesis could result from two possi-

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**Fig 1.** RT-PCR analysis expression of P55, IDS, and G6PD genes in patients with monoclonal hematopoiesis. RNA of heterozygous patients was submitted to RT-PCR. Amplified products were digested with *Hha I*, *Hpa I* and *Pvu I*, respectively, and analyzed by gel electrophoresis and ethidium bromide staining. Three to 24 correspond to the patient numbers in Table 4. U, undigested PCR products; P, platelets; T, T lymphocytes. Arrows indicate the size of the fragments in base pairs.
Fig 2. RT-PCR analysis of allelic expression of the P55 gene. RNA of heterozygous patients was submitted to RT-PCR. Amplified products were digested with Hha I and analyzed by gel electrophoresis and ethidium bromide staining. (A) Illustrates the results obtained from a patient with monoclonal hematopoiesis in platelets and granulocytes; (B) patient with monoclonal hematopoiesis only in platelets; (C) patient with polyclonal hematopoiesis; (D) patient with a pseudonormal X-chromosome inactivation pattern. U, undigested PCR products; 1, platelets; 2, T lymphocytes; 3, granulocytes. Arrows indicate the size of the fragments in base pairs.


Clonality Analysis of Hematopoiesis in Essential Thrombocythemia: Advantages of Studying T Lymphocytes and Platelets

Nahed El-Kassar, Gilles Hetet, Jean Brière and Bernard Grandchamp

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