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Hydroxyurea (HU) is effective in reducing JAK2V617F mutated clone size in the peripheral blood of essential thrombocythemia (ET) and polycythemia vera (PV) patients

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Abstract Ph-negative chronic myeloproliferative disorders (Ph^{neg}cMPD) are treated according to the estimated vascular risk. The recent discovery of V617F point mutation of the JAK2 kinase, which frequently occurs in these diseases, has not changed their management so far. However, emerging data tend to support a prothrombotic role for the mutation, along with a better response of JAK2V617F mutated patients to hydroxyurea treatment. Our data further support this notion.

Keywords Ph^{neg}cMPD · JAK2V617F · Hydroxyurea

Introduction

The introduction of JAK2V617F point mutation has undoubtedly simplified the diagnostic algorithm of Ph-negative chronic myeloproliferative disorders (Ph^{neg}cMPD). However, no particular therapeutic intervention is at the moment based on JAK2V617F mutational status [1]. Awaiting the effect of JAK2 kinase inhibitors, treatment is mainly

administered to Ph^{neg}cMPD patients predicted to have high vascular risk [2]. Risk factors include advanced age and thrombotic history while leucocytosis has recently been identified as an independent risk factor [3, 4]. Even though it is well established that JAK2V617F positive patients shows enhanced myelopoiesis [5, 6], the effect of JAK2V617F as a new thrombotic risk factor is still under investigation. Hydroxyurea (HU) is currently the cytoreductive treatment of choice, while interferon- α (IFN α) and anagrelide are preferred in selected cases. Accumulating evidence with this agent suggests that HU is an effective, well-tolerated, and probably safe agent for Ph^{neg}cMPD treatment [7, 8].

Interestingly, Ph^{neg}cMPD patients who carry the JAK2V617F mutation are more responsive to HU treatment and their disease can be controlled with lower drug doses [9]. The possibility of increased thrombotic risk in JAK2V617F positive patients in conjunction with the efficacy of HU treatment suggest its use upfront in this selected subgroup of Ph^{neg}cMPD patients. Therefore, the aim of our study was to establish the efficacy of HU treatment in reducing the load of JAK2V617F mutated hematopoiesis.

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Materials and methods

Patient samples

Peripheral blood samples from 12 essential thrombocythemia (ET) and six polycythemia vera (PV) patients, all positive for the JAK2V617F mutation, were obtained at diagnosis and thereafter at different time points, while

Table 1 Patients characteristics

| Patient | Pretreatment | | | HU g/week | Time on HU mo | Posttreatment | | | Jak2 V617F |
|---------|--------------|-------|------------------------|--------------|------------------|---------------|------|------------------------|---------------|
| | Ht % | WBC | Plt 10 ⁹ /L | | | Ht % | WBC | Plt 10 ⁹ /L | |
| ET 1 | 42.3 | 6.8 | 812 | 5 | 23 | 39 | 5.4 | 333 | Neg |
| ET 2 | 41.8 | 6.7 | 691 | 6 | 18 | 41 | 7.2 | 411 | Pos |
| ET 3 | 40 | 8.1 | 752 | 3 | 18 | 36 | 7.0 | 445 | Neg |
| ET 4 | 47.6 | 10.7 | 584 | 3 | 7 | 35 | 10 | 110 | Pos |
| ET 5 | 43.7 | 6.3 | 715 | 5 | 24 | 44 | 4.7 | 293 | Pos |
| ET 6 | 43.5 | 10.6 | 698 | 10.5 | 30 | 41 | 8.0 | 453 | Pos |
| ET 7 | 35 | 13.6 | 523 | 17.5 | 36 | 34 | 12 | 431 | Pos |
| ET 8 | 48.6 | 12 | 594 | 10.5 | 20 | 44 | 5.3 | 361 | Pos |
| ET 9 | 43 | 11.3 | 1.044 | 4.5 | 12 | 4 | 5.7 | 315 | Pos |
| ET 10 | 40.5 | 15.4 | 1.653 | 5 | 24 | 34.6 | 4.3 | 252 | Pos |
| ET 11 | 37.6 | 11 | 995 | 3 | 40 | 33.9 | 4.5 | 353 | Pos |
| ET 12 | 42 | 9 | 785 | 7 | 28 | 44 | 3.7 | 322 | Pos |
| Median | 42.5 | 10.62 | 733.5 | 5 | 23.5 | 39.5 | 5.55 | 343 | |
| PV 1 | 62.2 | 9.4 | 218 | 5 | 8 | 39 | 6.2 | 226 | Neg |
| PV 2 | 48 | 22 | 372 | 3.5 | 38 | 42 | 12.8 | 125 | Pos |
| PV 3 | 48 | 31.6 | 1201 | 10.5 | 23 | 42 | 11 | 217 | Pos |
| PV 4 | 69.2 | 10.2 | 838 | 7 | 18 | 46 | 5.2 | 345 | Pos |
| PV 5 | 54.4 | 9.6 | 583 | 8 | 56 | 45.7 | 5.2 | 201 | Pos |
| PV 6 | 58.4 | 7.6 | 369 | 7 | 56 | 45.7 | 5.2 | 201 | Pos |
| Median | 56.4 | 9.9 | 477.5 | 7 | 30.5 | 42 | 7.1 | 221.5 | |

ET essential thrombocythemia, PV polycythemia vera, HU hydroxyurea, mo months on treatment, Neg negative for JAK2V617F in peripheral blood, Pos positive for JAK2V617F in the peripheral blood

receiving HU treatment. All of them were considered good responders to HU treatment based on clinical criteria (Ht < 45% for PV and Plts < 400 × 10⁹/L for ET patients) [10]. Individual patient's pre- and posttreatment peripheral blood counts, along with HU dose administered and time on treatment, are presented in Table 1. This study was approved by the local ethics committee.

Allele-specific polymerase chain reaction (ASO-PCR) method for JAK2V617F mutation detection

For JAKV617F detection, the method described by Baxter et al. was used. Briefly, genomic DNA was extracted from 350 µl of whole blood (QIAGEN EZ1 Biorobot). ASO-PCR was performed by using 100 ng of genomic DNA and the

| H2O | 100% JAK2 V617F | 0% JAK2 V617F | 75% JAK2 V617F | 50% JAK2 V617F | 25% JAK2 V617F | 10% JAK2 V617F | 5% JAK2 V617F | 2.5% JAK2 V617F |
|-----|-----------------------|---------------------|----------------------|----------------------|----------------------|----------------------|---------------------|-----------------------|
|-----|-----------------------|---------------------|----------------------|----------------------|----------------------|----------------------|---------------------|-----------------------|

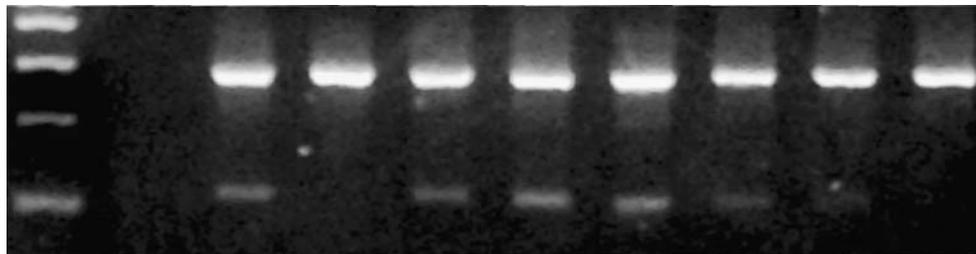
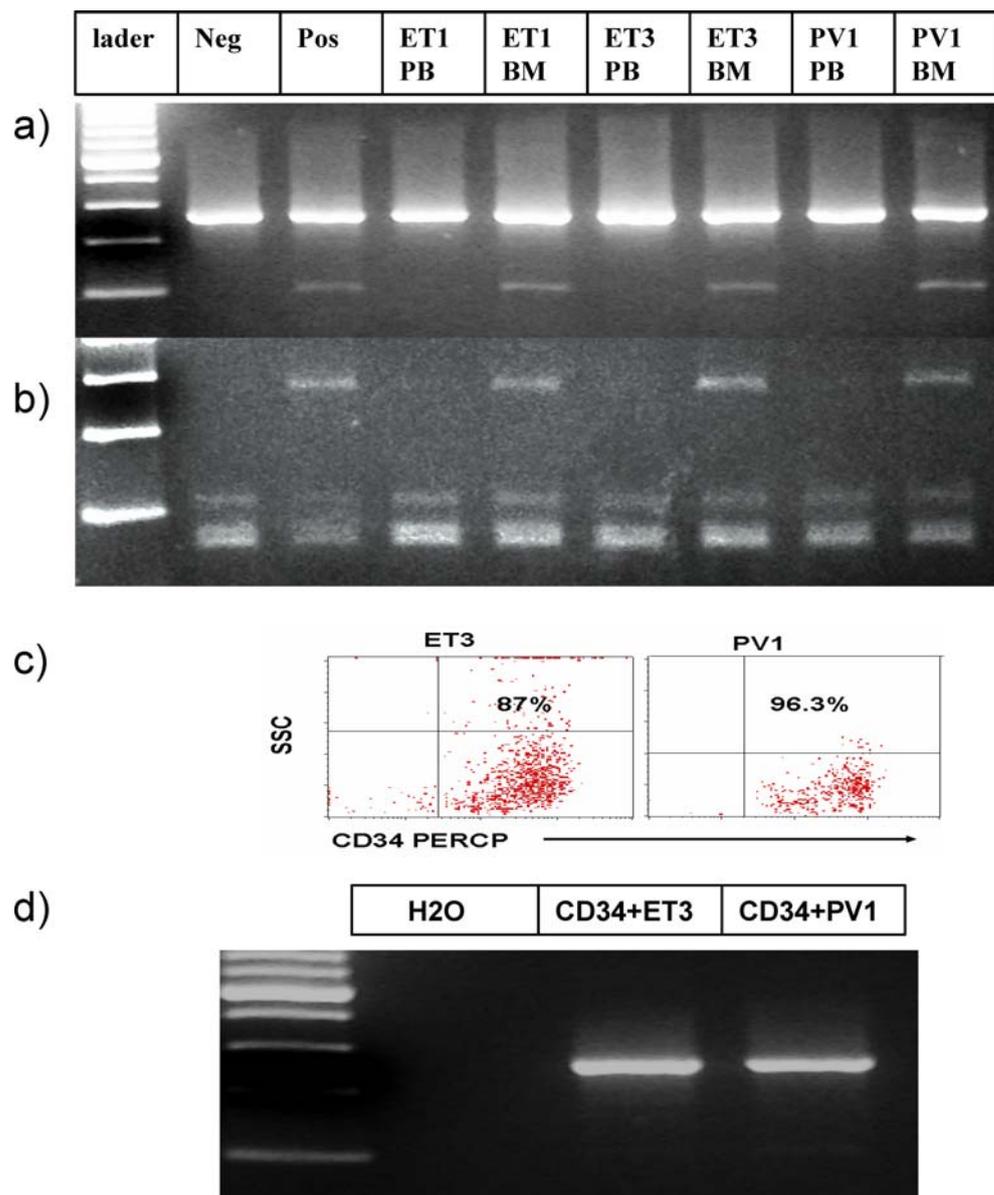


Fig. 1 Sensitivity of ASO-PCR method to detect JAK2V617F mutated clones in peripheral blood samples: A peripheral blood sample from a homozygous for JAK2V617F mutation polycythemia vera patient was obtained and subjected to double gradient Ficoll–Histopaque separation in order to select granulocytes. The same procedure was followed and in a peripheral blood sample from a normal donor (ND). Cells were counted and resuspended in medium to the final concentration of 1 million cells per ml. Then we mixed

proper amounts of PV- and ND-derived cell suspensions in order to have mixed samples with controlled percentages of JAK2V617F mutated clones ranging from 100% to 2.5%. Cells were then spun down, supernatant was removed, and genomic DNA was extracted. Following the method we describe at the “Materials and method” section, we performed ASO-PCR for JAK2V617F mutation detection and the method was proved to have a sensitivity between 2.5% and 5%

Fig. 2 Hydroxyurea treatment reduce to undetectable levels the JAK2V617F clone size in the peripheral blood (PB) of two essential thrombocythemia patients (ET1 and ET3) and a polycythemia vera patient (PV1) but not in bone marrow (BM) samples from the same patients. **a** Allele-specific PCR. **b** BsaXI enzyme restriction analysis. **c** Purity of CD34+ cells after positive selection with immunomagnetic beads (MiniMACS, Miltenyi Biotec) from the bone marrow of patients ET3 and PV1 that both show sustained peripheral blood negativity for JAK2V617F mutated clones. **d** Results of JAK2V617F detection on CD34+ selected hematopoietic progenitors by using the ASO-PCR method. In both patients, mutated clones were detectable



following set of primers were used (OPERON, Cologne, Germany):

R JAK2: 5'CTGAATAGTCCTACAGTGTTTCA
GTTTCA3

F WT: 5'AGCATTGGTTTAAATTATGGAGTA
TATT3'

F V617 F: 5'ATCTATAGTCATGCTGAAAGTAGGA
GAAAG3'

PCR conditions were denaturation 95°C for 5 min followed by 35 cycles of amplification with denaturation 95°C for 30 s, annealing 58°C for 30 s, elongation 72°C for 1 min, and finally 5 min of elongation at 72°C. Then 10 µl of PCR product were subjected to 1.5% agarose gel electrophoresis and the bands were detected under UV

light using ethidium bromide as detection agent. Unmutated JAK2 samples produce a band of 364 bp and the mutated samples an extra band of 203 bps. In our hands, the sensitivity of the method to detect JAK2V617F mutated clones in peripheral blood samples is between 2.5% and 5% as described in Fig. 1 and linearity between JAK2V617F concentration and band width is better at low concentration levels.

BsaXI restriction analysis

A PCR reaction by using R JAK2 and F WT primes was performed as described above. Twenty microliters of PCR product were then subjected to digestion with 1 IU of BsaXI in a water bath settled at 37°C after overnight

incubation. Afterwards, samples were subjected to agarose gel electrophoresis.

Results and discussion

Regarding ET patients, median duration of HU treatment was 23.5 months (range 7–40 months), median HU dose was 5 g/week (3–17.5), and median platelet count at time of JAK2V617F detection was $343 \times 10^9/L$ (110–445). PV patients received 7 g/week HU (3.5–10.5) for a median period of 30.5 months (8–56) and the median Ht values were 42% (36–46%).

In this cohort where all patients approach the therapeutic goal of HU treatment, JAK2V617F mutation status and allele burden were assessed as described above. In two of twelve ET patients and in one of six PV patients, JAK2V617F mutated clones could not be detected on peripheral blood samples by both ASO-PCR and BsaXI enzyme restriction analysis (Fig. 2a, b). All three negative samples came from patients heterozygous for the JAK2V617F mutation. In order to enforce our data, we obtained a second sample from these three patients, 3 months after the detection of the first negative for the JAK2V617F mutation sample. We found out that in two out of three patients, the response was sustained (Supplementary Figure 1A). In contrast, the third patient lost JAK2V617F negativity in the peripheral blood, presumably because of transient HU discontinuation (2 weeks) after prolonged hospitalization due to a major surgery, thus suggesting the need for continuous HU administration. However, bone marrow samples obtained after the peripheral blood became negative for the JAK2V617 mutation were still positive for the mutation (Fig. 2a, b). In addition, we found that even after 3 months of sustained peripheral blood negativity, we were still able to detect JAK2V617 positive clones in the bone marrow (Supplementary Figure 1B). Interestingly, the fraction of highly purified CD34+ bone marrow derived hematopoietic progenitors also contained mutated clones, but they represented a small subset of these progenitors (Figs. 2c, d).

In conclusion, HU can induce a sustained reduction or even eradication of the JAK2V617F clone size in the peripheral blood of Ph^{neg}cMPD patients. However, this effect should not be considered as an evidence of a major molecular response, since the clone exists in the bone marrow and in selected CD34+ hematopoietic progenitor cells. Nevertheless, it is suggestive of an effective treatment that might be proved of clinical benefit, especially considering the correlation between JAK2V617F allele burden and overall vascular risk [11]. Our results are in accord with two recent studies that proved the efficacy of HU treatment in reducing the copies of JAK2V617 in the peripheral blood of Ph^{neg}cMPD patients [12, 13]. Thus,

although not as effective as IFN α [14], HU has considerable efficacy against JAK2 mutated hematopoietic clones.

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