The JAK2 V617F mutation involves B- and T-lymphocyte lineages in a subgroup of patients with Philadelphia-chromosome negative chronic myeloproliferative disorders

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Summary

The JAK2 V617F mutation is a frequent genetic event in the three classical Philadelphia-chromosome negative chronic myeloproliferative disorders (Phneg.-CMPD), polycythemia vera (PV), essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF). Its occurrence varies in frequency in regards to phenotype. The mutation is found in the majority of patients with PV and about half of the patients with ET and IMF. These diseases are clonal stem cell disorders arising in an early stem cell progenitor. The level in the stem cell hierarchy on which the initiating genetic events and the JAK2 V617F mutation occurs is not known. The mutation has so far been detected in all cells of the myeloid lineage, whereas the potential clonal involvement of the lymphoid lineage is controversial. In this study, we detected the JAK2 V617F mutation by real-time quantitative PCR (qPCR) in both B-lymphocytes and T-lymphocytes in a subgroup of patients with Phneg.-CMPDs. These results demonstrate the origin of the JAK2 V617F positive disorders in an early stem cell with both lymphoid and myeloid differentiation potential.

Keywords: myeloproliferative, clonality, mutation analysis, real-time quantitative PCR, fluorescence-activated cell sorted.

Most recently a major breakthrough in the understanding of molecular pathogenesis of the Philadelphia chromosome negative chronic myeloproliferative disorders (Phneg.-CMPD) has been achieved with the identification of the V617F mutation in the Janus Kinase 2 gene (JAK2). The majority of patients with polycythemia vera (PV) (65–97%) harbor the mutation in their clonal haematopoietic cells, as do approximately half of those with essential thrombocythemia (ET) and idiopathic myelofibrosis (IMF) (Baxter et al, 2005; James et al, 2005; Kralovics et al, 2005; Levine et al, 2005). The V617F mutation occurs in the autoregulatory JH2 domain of the tyrosine kinase, leading to loss of the inhibitory effect of the JH2 domain on the kinase activity, and thereby constitutive activation of JAK2 and downstream signalling molecules, among these STAT5 (signal transducer and activator of transcription 5) and ERK (extracellular signal-regulated kinase) (James et al, 2005). In vitro studies have provided evidence of the capability of erythroid progenitors harbouring the JAK2 V617F to form erythropoietin (EPO)-independent erythroid colonies (EECs), which is a well established hallmark of PV (Casadevall et al, 1982; Baxter et al, 2005; James et al, 2005). Furthermore in vivo studies have demonstrated that mice transplanted with murine bone-marrow cells retrovirally transfected with the JAK2 V617F encoding gene develop a PV-like disease that tends to terminate in a myelofibrosis-like phaenotype (James et al, 2005; Lacout et al, 2006; Wernig et al, 2006). In most studies on the JAK2 V617F in Phneg.-CMPD, the mutational analysis was performed on peripheral blood granulocytes and, to some extent, mononuclear cells and unfractionated leucocytes after red cell lysis. The JAK2 V617F mutation is detectable in granulocytes and cells of the myeloid lineage in general and hence originates in a myeloid lineage precursor, which is in line with earlier studies demonstrating the clonality by glucose-6-phosphate dehydrogenase isoenzyme (G6PD) analyses of different myeloid cell types (granulocytes, monocytes, platelets and erythrocytes) (Adamson et al, 1976; Jacobson et al, 1978; Fialkow et al, 1981). However, controversy exists whether the JAK2 V617F clone involves the B- and T-cells of the lymphoid lineage and hence arises in a pluripotent non-committed stem cell. Clonality studies based
on the same method (G6PD) and later X-linked DNA-analysis have provided evidence of clonal involvement of B-cells in Ph\textsuperscript{neg}.-CMPDs, as also shown in chronic myeloid leukaemia (CML) (Fialkow et al, 1978; Martin et al, 1980; Raskind et al, 1985; Anger et al, 1990; Gilliland et al, 1991; Tsukamoto et al, 1994;el-Kassar et al, 1997). T-cells have been reported as non-clonal, although a few studies on IMF using RAS mutations and karyotypic abnormalities as clonal markers have found T-cell clonal involvement (Buschle et al, 1988; Reeder et al, 2003). Likewise, in a subgroup of PV patients evidence of possible clonal T-cells have been demonstrated (Kralovics et al, 2002). These studies were all published in the pre-JAK2 era. The identification of the JAK2 V617F mutation has provided a novel tool to be used in studies of clonal involvement in different haematopoietic cell types. The data so far published using the JAK2 V617F mutation as a clonal marker are conflicting in regard to the involvement of lymphoid B- and T-cells in the JAK2 V617F clone (Baxter et al, 2005; James et al, 2005; Lasho et al, 2005; Delhommeau et al, 2006; Ishii et al, 2006).

In this study, we have performed JAK2 mutation analysis by a highly sensitive real-time quantitative PCR (qPCR) method on fluorescence-activated cell sorted (FACS) granulocytes, monocytes, B-lymphocytes and T-lymphocytes from patients with Ph\textsuperscript{neg}.-CMPDs with a known JAK2 V617F mutation to further elucidate this issue.

Materials and methods

Ethylenediaminetetraacetic acid anticoagulated blood (20 ml) was collected from seven healthy controls and 13 patients with a known Ph\textsuperscript{neg}.-CMPD (PV = 10, IMF = 2, ET = 1). All patients were known to have a JAK2 V617F mutation identified by the three-primer allele-specific method published by Baxter et al (2005). All samples were collected after informed consent according to the Helsinki Declaration and guidelines of the Danish Regional Science Ethics Committee. Patient characteristics are summarised in Table I.

Purification of granulocytes, monocytes, B-lymphocytes and T-lymphocytes

An initial red cell lysis was performed using an NH\textsubscript{4}Cl lysis buffer. After centrifugation and resuspension the cells were labelled with CD3-allophycocyanin (APC), CD19-phycoerythrin (PE), CD14-PE-Cy7 (BD Biosciences, Erembodegem, Belgium) and CD66-flurescein isothiocyanate (FITC) (Dako A/S, Glostrup, Denmark) fluorochrome-conjugated monoclonal antibodies according to manufacturers protocols. The cells were sorted using a FACSVantage (BD Biosciences) in a high purity mode, aborting droplets containing doublet cells or more than one cell because of \textit{in-vitro} aggregation, resulting in 99% purity for CD3\textsuperscript{+} T-cells, CD19\textsuperscript{+} B-cells, CD14\textsuperscript{+} monocytes and CD66\textsuperscript{+} granulocytes. Peripheral blood from one out of the seven healthy controls was sorted as described, while unfraccionised leucocytes were used for analysis in the remaining six healthy controls. The FACS gating strategy is depicted in Fig 1.

DNA purification

DNA was extracted from the four different cell isolates using a MagnaPure robot (Roche Diagnostics, Mannheim, Germany) according to the manufacturers protocol.

Real-time quantitative PCR (qPCR)

We designed two qPCR assays with a common forward primer 5'-CTTTCTTTGAAGCAGCAAGTATGA-3' and a common forward probe 6-FAM-TGAGCAAGCTTCACAAGCATTTGGTTT-TAMRA. The reverse primers were designed as a wildtype-specific primer 5'-GTAGTTTTACTTACTCTCGTCTCA-3' or

Table I. Patient characteristics.

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age (years)</th>
<th>Gender</th>
<th>Disease duration (months)</th>
<th>Cytoreductive treatment at time of present analysis</th>
<th>Previous cytoreductive treatment</th>
<th>Prior thrombosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>PV1</td>
<td>72</td>
<td>Female</td>
<td>17</td>
<td>None</td>
<td>None</td>
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</tr>
<tr>
<td>PV2</td>
<td>66</td>
<td>Female</td>
<td>38</td>
<td>HU and ANA (comb.)</td>
<td>HU, IFN, IMA</td>
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<tr>
<td>PV3</td>
<td>53</td>
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<td>22</td>
<td>IFN</td>
<td>HU</td>
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<tr>
<td>PV4</td>
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<td>Male</td>
<td>17</td>
<td>IFN</td>
<td>HU, ANA</td>
<td>Yes</td>
</tr>
<tr>
<td>PV5</td>
<td>58</td>
<td>Female</td>
<td>21</td>
<td>ANA</td>
<td>IFN</td>
<td>Yes</td>
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<tr>
<td>PV6</td>
<td>70</td>
<td>Female</td>
<td>17</td>
<td>HU</td>
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<td>Yes</td>
</tr>
<tr>
<td>PV7</td>
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<td>Male</td>
<td>14</td>
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<tr>
<td>PV8</td>
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<tr>
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<td>78</td>
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<td>157</td>
<td>None</td>
<td>BU</td>
<td>No</td>
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<td>Male</td>
<td>118</td>
<td>IFN and HU (comb.)</td>
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<tr>
<td>IMF1</td>
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<td>16</td>
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<tr>
<td>IMF2</td>
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<td>12</td>
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<td>None</td>
<td>No</td>
</tr>
<tr>
<td>ET</td>
<td>57</td>
<td>Female</td>
<td>10</td>
<td>ANA</td>
<td>None</td>
<td>Yes</td>
</tr>
</tbody>
</table>

HU, hydroxyurea; IFN, interferon-alpha; ANA, anagrelide; BU, busulfan; IMA, imatinib.
a JAK2 V617F mutation-specific primer 5'-GTAGTTTTACTAAGCTGTCCTCCACATAA-3', both with an intended mismatch at the 3'-minus 2-position. The qPCR reaction volume was 25 μl and primer-concentrations were 300 nmol/l, whereas the concentration of the probe was 200 nmol/l. The PCR amplification conditions were: An initial enzyme activation step of 10 min at 95°C, followed by 50 cycles of 15 s at 95°C and 60 s at 60°C. All qPCR reactions were performed in triplicates on an ABI Prism7900HT (Applied Biosystems, Foster City, CA, USA).

qPCR Data analysis

In order to determine the sensitivity of the mutation-specific primer set, a standard curve was created by fivefold dilution series of homozygous JAK2 V617F mutated DNA into donor wildtype DNA. From standard curves the slope was calculated for both the wildtype-specific (3-6) and mutation-specific (3-7) primer-probe sets. The assay sensitivity was calculated to 1:10 000 (Fig 2). However we defined a 10-fold higher cut-off limit, corresponding to 1:1000, to be significant regarding detection of JAK2 V617F mutated alleles. The Y-intercept [cycle threshold (Ct) value] corresponding to one copy of the target gene in the sample, was calculated from limiting twofold dilution series of both primer sets. The copy-numberJAK2V617F was calculated as 10((Y-interceptJAK2V617F – mean CtJAK2V617F)/slopeJAK2V617F), and the copy-numberJAK2Wildtype as 10((Y-interceptJAK2Wildtype – mean CtJAK2Wildtype)/slopeJAK2Wildtype). Finally the percentage of JAK2 V617F mutated alleles were calculated as [copy-numberJAK2V617F/(copy-numberJAK2V617F + copy-numberJAK2Wildtype)] × 100.

Results

FACS sorting

Fluorescence-activated cell-sorting was performed in a high purity mode, aborting droplets containing more than one cell leading to a decrease in the sorting efficiency. This was most prominent for the monocytes and granulocytes, median 55% (range 34–78%) and 67% (range 47–88%) respectively, whereas the sorting efficiency was markedly higher in the lymphoid compartments, median 94% (range 90–97%) and 93% (range 89–97%) for the B-cells and T-cells respectively. This decrease in sorting efficiency for the myeloid cells was probably caused by a tendency of in vivo and in vitro aggregation of monocytes, granulocytes and platelets in the Phneg-CMPD.

qPCR

As described the assay sensitivity was calculated to 1:10 000, however we defined an assay cut-off limit of 1:1000. The sensitivity of the individual qPCR reactions was solely dependent on the assay sensitivity (1:1000) in reactions with no limitations in the amount of DNA. In qPCR reactions with limited amount of DNA, as it was the case in especially the CD19+ B-lymphocyte and CD14+ monocyte compartment, the sensitivity was calculated as 1/(copy number JAK2 wildtype + copy number JAK2 V617F) × 100. The individual qPCR reaction sensitivities are given in Fig 3A. As a result of the FACS, efficiency of 99% a lower limit of 1% mutated alleles must be present for a cell compartment to be interpreted as ‘JAK2 V617F positive’.

All 13 patients had JAK2 V617F clonal granulocytes and monocytes with a median proportion of mutated alleles of 60%, ranging from 8–96% in the granulocyte compartment, and 35%, range (2–84%) in the monocyte compartment. Eight patients (PV1-PV2, PV6, PV8-PV10 and IMF1-IMF2) had more than 50% mutated alleles in their granulocytes, indicating
homozygocity, whereas five patients (PV2, PV8, PV10 and IMF1-IMF2) had JAK2 V617F homozygous monocytes. The median proportion of mutated alleles in T-lymphocytes was 3%, range (0.2–83%). Four patients (PV3, PV4, PV5 and PV7) had 1% or less mutated alleles and were therefore interpreted as ‘JAK2-negative’ in their T-cell compartment. Nine patients (PV1, PV2, PV6, PV8, PV9, PV10, IMF1, IMF2 and ET) had 3–83% mutated alleles in their T-cells with a reasonably high sensitivity (0.1–0.04). Two of these patients (PV8 and IMF1) were remarkably homozygous with 65% and 83% JAK2 V617F mutated alleles respectively. Six patients (PV2, PV3, PV7, PV8, PV10 and IMF1) had JAK2 V617F mutated alleles in their B-cells, but in all but one patient (PV2 with 46% mutated alleles) at relatively low levels (2–6%). None had JAK2 V617F homozygous B-lymphocytes. The median proportion of JAK2 V617F mutated alleles in B-cells was 3.5%, range (1–46%). The qPCR data are summarised in Fig 3.

Discussion

Two of the first published papers on the JAK2 V617F mutation described the absence of the mutation in T-cells within all three Ph⁻CMPDs (Baxter et al, 2005; James et al, 2005), which has later been confirmed. In addition, the absence of JAK2 V617F mutation in the B-cell compartment in one PV patient and two myelofibrosis patients with previous ET and PV respectively, was demonstrated (Lasho et al, 2005). Accordingly, it was concluded, that the JAK2 V617F mutation is restricted to a myeloid precursor cell. The same group has previously shown that both B- and T-cells may be of clonal origin in IMF (Reeder et al, 2003). This observation does not exclude the possibility that the JAK2 V617F mutation is a secondary event in an already existing clone with a possible other genetic marker e.g. the del 20q, as it has been proposed by others (Kralovics et al, 2006). All these data were based on sequencing methods, which are known to have a limited sensitivity (Campbell et al, 2005). Small B- and T-cell JAK2 V617F clones could have been undetectable because of the limited sensitivity. Most recently, strict myeloid lineage involvement of the JAK2 V617F mutation has been challenged by the findings of the JAK2 V617F mutation in both CD19⁺ B- and CD3⁺ T cells in one patient and JAK2 V617F clonal B-cells but not T-cells in another patient by qPCR on FACS sorted cells in a group of ten patients with PV (Ishii et al, 2006). A similar pattern of heterogeneous lympho-myeloid JAK2 V617F clonal involvement in addition to involvement of NK-cells in both PV and the majority of IMF patients has been confirmed in a most recent study (Delhommeau et al, 2006).
In the present study we designed a qPCR assay with a very high sensitivity of at least 1:1000. Because of limitations in cell numbers, and hence DNA amount in the CD19⁺ B-cell and CD14⁺ monocyte compartments, this high sensitivity could not be reached in all qPCR reactions, and thus was calculated for each qPCR reaction. In addition to the expected findings of the JAK2 V617F mutation in granulocytes and monocytes, the results clearly demonstrated detectable levels of JAK2 V617F alleles in both CD19⁺ B-lymphocytes and CD3⁺ T-lymphocytes in a subgroup of patients, in total six (PV2, PV3, PV7, PV8, PV10, IMF1 and IMF2) and nine (PV1, PV2, PV6, PV8, PV9, PV10, IMF1, IMF2 and ET) respectively. Both IMF patients had JAK2 V617F clonal T-cells, one of them (IMF1) had a majority (83%) of mutated alleles, which definitely should be detectable with sequencing techniques, and this is also likely to be the case for patient PV8. In the other patients (PV1, PV2, PV6, PV9, PV10, IMF2 and ET) the T-cell clone would probably have been missed by sequencing because less than 13% mutated alleles were detected. One of the patients (PV2) with B-lymphocyte clonal involvement had 46% JAK2 V617F mutated alleles and should therefore be detectable by sequencing. In the present study, four (PV2, PV8, PV10 and IMF1) out of the 13 patients had JAK2 V617F clonal involvement of all four cell compartments analysed (granulocytes, monocytes, B-lymphocytes and T-lymphocytes). JAK2 V617F clonal involvement of all four cell compartments has previously been demonstrated in IMF patients (Delhommeau et al, 2006). Interestingly the three PV patients (PV2, PV8 and PV10) with detectable JAK2 V617F levels in all four cell compartments had very high proportions of mutated alleles in their monocytes and granulocytes as well as a significantly longer disease duration than the other PV patients in this study. The results of the present study are comparable with previously published data (Delhommeau et al, 2006; Ishii et al, 2006), and provide further evidence that the JAK2 V617F mutation occurs in a lympho-myeloid progenitor within the PhNEG-CMPhDs. In the present study though, JAK2 V617F clonal involvement of T-cells were detected in nine out of 13 patients, which is a considerably higher frequency than reported by Delhommeau et al (2006), whereas the proportion of patients with JAK2 V617F clonal B-cells were comparable. The reason for this finding is not clear, but in the present study the detection of potential JAK2 V617F ‘positive’ B-cells could have been missed regardless of the use of a highly sensitive

![Fig 3. (A) Real-time quantitative PCR (qPCR) data: the percentage of JAK2 V617F alleles and the corresponding specific sensitivity in each qPCR reaction calculated as: \[1/(\text{copy-number}_{\text{V617F}} + \text{copy-number}_{\text{Wildtype}})\] × 100. (B) qPCR data: Histogram plot showing the percentages of JAK2 V617F alleles in CD66⁺ granulocytes, CD14⁺ monocytes, CD3⁺ T-cells and CD19⁺ B-cells, respectively, in patients PV1-PV10, IMF1-IMF2 and ET.](image-url)
qPCR assay (>0.1%), because of limitations in DNA amount. On the other hand, both Delhommeau et al. (2006) and Ishii et al. (2006) used another qPCR method with competitive mutation-specific probes and reported a sensitivity of 2% (Delhommeau et al., 2006). Some of the discrepancies between the studies could therefore be related to technical differences. Nevertheless, the proportion of JAK2 V617F alleles seems to be at much lower levels in lymphoid cells than in myeloid cells in the majority of patients. T-cells are long-living cells, but they are produced throughout adult life by periodically importation of haematopoietic stem cells from the bone marrow to the thymus (Schwarz & Bhandoola, 2006). There is some evidence that the haematopoietic stem cells trafficking from the bone marrow to the thymus are progenitor cells with both myeloid and lymphoid differentiation potential (Katsura, 2002), which could explain the more scarce occurrence of T-cell JAK2 V617F clonal involvement as recorded in the present and previous studies (Delhommeau et al., 2006; Ishii et al., 2006). The identification of progenitors capable of producing B-cells and differentiating into cells of the myeloid lineage has further challenged our classical understanding of lineage-specific progenitors (Hou et al., 2005). It is intriguing to consider the possibility that the JAK2 V617F clone has a proliferative advantage in the myeloid lineage. During progression of the disease the percentage of JAK2 V617F cells increases, myeloid cells become homozygous for the JAK2 V617F mutation and in parallel, the possibility of the JAK2 V617F clone to proliferate in lymphoid lineage may increase. The percentage of JAK2 V617F alleles in progenitors seems to increase from ET to PV (Scott et al., 2006), and it is likely that JAK2 V617F positive CMPDs could be considered as a biological continuum from ET over PV to myelofibrosis (Campbell et al., 2006; Wolanskyj et al., 2005). Although the numbers are too small to draw firm conclusions, a trend towards increasing lymphoid involvement in late PV and IMF was recorded in the present study. A similar association has been described most recently (Delhommeau et al., 2006). It is important to notice that the JAK2 V617F clonal involvement is very heterogenous, probably partly reflecting biological variation among patients, although previous and current cytoreductive therapy might contribute. Moreover, it is of interest to note that T-cell involvement was actually observed in all disease entities (ET, PV and IMF), supporting the model of a biological continuum within the JAK2 V617F-CMPDs. In conclusion, this study provides further evidence that the JAK2 V617F mutation in the Ph+©-CMPDs occurs in a lympho-myeloid progenitor cell. Future studies in a large series of untreated patients are needed to further delineate precisely the level in the stem cell hierarchy at which the JAK2 V617F mutation occurs.

Authors’ contributions

Thomas Stauffer Larsen organised the studies, performed the FACS sort and JAK2 analysis and wrote the manuscript. Niels Pallisgaard designed the qPCR assay and contributed to the study design and the final manuscript. Jacob Haaber Christensen contributed with his expertise on FACS sorting and in reviewing the manuscript. Hans Carl Hasselbalch followed the patients, contributed to the study design, organised the sample collection and reviewed the manuscript.

References

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