JAK2V617F mutation and endogenous erythroid colony formation in patients with polycythaemia vera

Milica Strnad1, Biljana Todoric Zivanovic1, Zeljka Tatomirovic1, Olivera Tarabar2, Marija Elez2, Olga Radic1, Dragana Stamatovic2

1Institute of Pathology, 2Clinic of Hematology, Military Medical Academy, Belgrade, Serbia

Summary

Purpose: The purpose of this study was to investigate the frequency of JAK2V617F gene mutation in patients with polycythemia vera (PV) and to compare the results with the presence of endogenous erythroid colony (EEC) formation.

Methods: Peripheral blood and bone marrow samples of 28 patients with PV were analyzed. The diagnosis of PV was established according to the bone marrow criteria of the World Health Organization (WHO). Mutation of JAK2V617F was determined by allele-specific PCR (AS-PCR) analysis. For detection of EEC formation we used assays of human clonogenic heamatopoietic progenitor cells with agar-leukocyte conditioned medium (Agar-LCM) without recombinant human erythropoietin (EPO).

Results: Mutation was found in the samples of the peripheral blood in 26/28 (92.9%) PV patients. EEC formation was obtained in the sample of bone marrow in 27/28 (96.4%) PV patients. In 25/28 (89.2%) patients we detected presence of EEC formation and mutation of JAK2V617F at the same time.

Conclusion: Considering these results, we hypothesized that the EEC formation observed in myeloproliferative disorders could be partially due to the JAK2-dependent activation signaling pathway.

Key words: endogenous erythroid colony, JAK2V617F, polycythemia vera

Introduction

PV is a chronic myeloproliferative neoplasm (MPN) characterized by increased red blood cell production independent of the mechanisms that normally regulate erythropoiesis. Erythropoiesis is a tightly regulated process which becomes decoupled from its normal differentiation program in patients with PV [1]. The reported annual incidence of PV increases with advanced age and varies from 0.7 to 2.6 per 100,000 inhabitants in Europe and North America. The median age at diagnosis is 60 years, and patients younger than 20 years are only rarely reported. The underlying cause is unknown in most cases. Ionizing radiation and occupational exposure to toxins have been suggested as possible causes in occasional patients. A genetic predisposition has been reported in some families [2]. The most frequent genetic abnormality is the somatic mutation of Janus kinase 2 gene (JAK2V617F) that results in proliferation not only of the erythroid lineage but of the granulocytes and megakaryocytes as well. Although it occurs in more than 90% of the patients with PV, it is not specific and it is found in other MPNs as well but in lower frequency [3-7]. The mutation occurs in a haemapoietic stem cell, and it is found in all of the myeloid lineages. JAK2V617F is a constitutively active tyrosine kinase that can transform factor-dependent hematopoietic cell lines into cytokine-independent cells. Expression of the JAK2V617F mutant activates multiple downstream signaling pathways, such as Stat, Erk, and PI3K/Akt pathways [8-11].

The diagnosis of PV requires integration of clinical, laboratory and bone marrow histological features. Clinical laboratory studies that aid confirmation of the diagnosis of PV include some evidence like increased red cell volume or haemoglobin, subnormal EPO levels, EEC formation and...
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detection of the JAK2V617F or functionally similar mutation [12,13]. In contrast with secondary erythrocytosis, progenitor cells from PV patients can undergo in vitro erythroid differentiation despite the absence of EPO, and presence of such endogenous erythroid colonies is routinely used as a diagnostic assay [14,15]. To date, the mechanisms implicated in EEC formation are poorly understood. An acquired mutation in the JAK2 kinase leading to its constitutive phosphorylation has been described recently in PV patients. This mutation leads to a constitutive activation of JAK2 that seems to play a crucial role in the onset of the disease. During erythropoiesis, JAK2 activates many transduction pathways, which can be implicated in the terminal maturation [16,17].

The purpose of this study was to investigate the frequency of JAK2V617F mutation in patients with PV and to compare the results with presence EEC formation.

Methods

Patients

We analyzed the peripheral blood and bone marrow samples of 28 patients with PV, diagnosed in the Clinic of Hematology, Military Medical Academy, Belgrade, Serbia. There were 15 men and 13 women aged 28-83 years (median 50.3). The diagnosis of PV was established according to the bone marrow criteria of the World Health Organization [2].

Allele-specific PCR (AS-PCR) analysis

Mutation of JAK2V617F was determined according to a previously described protocol [18]. DNA was extracted by PureLink Genomic DNA MiniKit (Invitrogen, Karlsbad, USA) after isolation of mononuclear cells (MNCs) by lymphocyte separation medium (StemCell Technologies, Vancouver, Canada) from blood samples according to the manufacturer’s instructions. PCR primers were: forward outer (FO)-5’TCCTCAGAAACGTGGATG6CGAG3’, reverse outer (RO)-5’ATTGCTTTCCTTTTCTACAAGT3’ and reverse-mutant-specific (Rmt)-5’GTTTACTTACTCTGCTCCACAAA3’. FO and RO primers generate a product of 463 bp which is obtained from the normal and the mutated gene, and serves as an internal PCR control. FO and Rmt primers generate a 279 bp mutant specific product. Amplification was performed with 55 cycles with Qiagen Taq PCR Core Kit (Crawley, UK) an annealing temperature of 60°C, 80 ng genomic DNA, and standard amplification conditions, except that the final concentrations of the outer primers and the mutant-specific inner primers were 1µM and 0.5 µM, respectively. Products were resolved on 2% agarose gels and visualised after staining with ethidium bromide.

Results

Results of AS-PCR analysis

All samples of PV were examined for JAK2V617F mutation using the AS-PCR method. Mutation was found in the samples of MNCs of the peripheral blood in 26/28 (92.9%) PV patients (Figure 1).

Results of haematopoietic colony assays

BFU-E produced 3 or more clusters of erythroblasts or an equivalent number of erythroblasts. Normally, BFU-E require the presence of EPO in the medium to stimulate division and differentiation, but in PV patients they can arise spontaneously. The colony assay was performed on 28

![Figure 1. Percent distribution of patients with and without Jak2V617F mutation.](image-url)
PV samples. EEC formation was observed in the sample of bone marrow MNCs in 27/28 (96.4%) PV patients (Figure 2).

In 25/28 (89.2%) of the samples we detected presence of EEC formation and mutation of JAK2V617F at the same time (Figure 3).

**Discussion**

MPNs are a group of haematological conditions where there is a primary disorder at the level of the multi-potent haematopoietic stem cell, leading to increased production of one or more blood cell types. The three main disorders are PV, essential thrombocythaemia (ET) and idiopathic myelofibrosis (IMF). PV is characterised by increase in red cells, white cells and platelets and clinically by a plethoric appearance, itch and splenomegaly. The disease can be complicated by thromboembolic episodes and haemorrhage and in the terminal stages it can progress to myelofibrosis and acute leukaemia [20,21].

A number of different biological phenomena has been described in haematopoietic cells of PV patients and other MPDs, the majority of which involve dysregulation of key signalling mediators. The key molecular events in the pathogenesis of these disorders have been poorly defined up to date, except in the case of chronic myeloid leukaemia (CML) with the associated characteristic chromosomal translocation (the Philadelphia/Ph chromosome) and the associated rearranged gene BCR–ABL [22,23].

Research on the molecular pathogenesis of PV suggested a defect in the signalling pathways that lead to autonomous erythrocytosis. In 1974, Prchal and Axelrad observed that primary PV progenitors form erythroid colonies in vitro in the absence of exogenous EPO [24]. In contrast, bone marrow cells from healthy individuals do not form these EECs. EEC can be isolated in 97-100% of PV cases who have not been exposed to cytoreductive therapy [25], and may also be cultured from a subset of cases with ET and IMF [19]. In our research we detected EEC in 27/28 (96.4%) PV patients in the sample of bone marrow. PV progenitor cells are highly sensitive to stimulation with cytokines, e.g. granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte colony stimulating factor (G-CSF), interleukin-3 (IL-3), insulin growth factor-1 (IGF-1), and thrombopoietin (TPO), but are surprisingly unresponsive to stimulation with EPO [26,27]. Since the principal feature of PV is the autonomous formation of EEC without exogenous stimulation with EPO, it was logical to infer a defect in the normal erythropoietic pathways in the pathogenesis of PV. Based on this and findings of EPOR (EPO receptor) mutations in familial erythrocytosis, the EPOR and components of its downstream signalling pathway were investigated for abnormalities in primary PV, but the receptor was found to be structurally and functionally intact [28]. Similarly, no mutations were identified in another candidate, SHP-1 (Src homology region 2 domain-containing phosphatase-1), a tyrosine phosphatase previously shown to interact with the EPOR [29] and be downregulated in progenitor cells isolated from PV patients [30]. Erythrocytes from most PV patients have elevated levels of the signalling intermediates STAT3 and PKC [31-34], plus upregulation of the PRV-1 gene [35,36] and an abundance of anti-apoptotic proteins like BCL-XL [16]. Unlike other malignancies where the INK4a locus is inactivated, PV erythroid progenitors exhibit increased expression of this gene [37]. It was only relatively recently that understanding of the molecular pathogenesis behind PV and other classic MPNs was furthered by the finding of a single mutation targeting the JAK2 gene [38].

Janus kinase 2 is a cytoplasmic tyrosine kinase that mediates growth factor receptor signalling. The first evidence that JAK2 could contribute
to the pathogenesis of leukaemia came from the characterisation of various chromosomal translocations, all involving the fusion of JAK2 to different transcription factors. Nevertheless, JAK2 translocations remain rare in MPN, especially when compared with the incidence of JAK2V617F. In 2005 it emerged that JAK2 plays a very important role in the molecular pathogenesis of MPN, after the finding of a consistent, acquired point mutation, isolated in 97% of patients with PV, 51% with ET and 50% with IMF [39,40]. The V617F mutation is a G>T transversion at nucleotide 2543, resulting in substitution of valine to phenylalanine (V617F) in the JAK2 protein. This point mutation at 617 amino acid residue appears to cause constitutive activation of the JAK2 tyrosine kinase owing to loss of autoinhibitory control. The mutant has enhanced kinase activity, and when overexpressed together with the EPOR in cells, it causes hyperactivation of EPO-induced cell signaling. This gain-of-function mutation of JAK2 may explain the hypersensitivity of PV progenitor cells to growth factors and cytokines [41]. A point mutation in the Janus kinase 2 gene was identified in several MPNs, most frequently in PV (65–97%), ET (23–57%), and IMF (35–57%). It is also infrequently present (3–5%) in myelodysplastic syndrome, chronic myelomonocytic leukemia, and other atypical chronic myeloid disorders. The common occurrence of this mutation in BCR-ABL- MPNs can be used as a unique molecular marker for distinguishing PV, ET, and IMF from reactive hematopoietic disorders. Also, JAK2 mutation has not been detected in Ph chromosome positive CML, and these mutations are currently considered exclusive [9,41,42]. In our study JAK2V617F mutation was found in the samples of MNCs of the peripheral blood in 26/28 (92.9%) PV patients. Recent focus on the JAK2 mutation in PV patients argues for a direct implication of JAK2-dependent signaling pathways in EEC formation [14,17,43]. In our study we detected the presence of EEC and mutation of JAK2V617F in 25/28 (89.2%) patients at the same time. The observation that the spontaneous formation of EEC grown from PV progenitors could be blocked by JAK inhibitors led one group of researchers to sequence the entire JAK2 gene in a small group of PV cases, revealing V617F [18]. EEC formation was inhibited by short-interfering RNA (siRNA) directed against JAK2, but not against other various candidate genes [44]. James et al. also performed several functional studies to define the role JAK2V617F plays in the pathogenesis of PV. When expressed in vitro, JAK2V617F is capable of inducing constitutive STAT5-mediated signalling independently of any stimulation by EPO. Furthermore, expression of the mutant JAK2 (but not the wild-type) induced EPO hypersensitivity and EPO-independent survival of cultured cell lines in vitro [59]. When expressed in mice, JAK2V617F induced a PV-like phenotype with splenomegaly and marked erythrocytosis [45,46]. A similar approach was undertaken by Zhao et al. who isolated JAK2V617F in 20/24 cases with PV, and confirmed that the mutant JAK2 protein had heightened kinase activity. Cells transfected with JAK2V617F displayed hyper-activated signalling after stimulation with EPO [9]. This mutation leads to a constitutive activation of JAK2 that seems to play a crucial role in the onset of the disease. During erythropoiesis, JAK2 activates many transduction pathways, which can be implicated in terminal maturation. For example, recent data showed that activation of AKT was sufficient for EPO-independent colony-forming unit-erythroid formation in mice [16].

Introduction of V617F into mice gives rise to a PV-like phenotype but there are several lines of evidence to suggest that the mutation might be a secondary event, at least in some cases. These include the transformation of JAK2V617F-positive MPN to V617F negative leukaemia [47-49], growth of JAK2V617F negative EEC from V617F-positive patients [50,51] and an apparent familial tendency to develop an MPN [52], all providing evidence that support this notion. On the other hand, introduction of V617F into mice reproducibly results in a PV-like disease, suggesting that a single hit may be sufficient [59]. The JAK2V617F mutation may also be prevalent in individuals without overt signs of myeloproliferative malignancies. However, the significance of the mutation in this setting is unknown, leading us to examine whether the presence of the JAK2V617F mutation in individuals from the general population is associated with poor health. For this purpose, Nielesen et al. screened DNA isolated from whole blood samples of 10,507 participants from the Copenhagen City Heart Study and JAK2V617F was present only in 18 of 10,507 [53].

In PV, the JAK2V617F mutation was included as major criterion in a revised WHO classification. However, considering the ≥ 5% of PV cases with an unmutated JAK2V617F codon, the recent description of mutations in exon 12 of JAK2 contributes to the genetic background of this disorder [54]. MPN patients without the mutation should
be in the early stages of disease or maybe present some other genetic change like exon 12 mutation of the JAK2 gene. On the other hand, in some patients with exon 12 mutations or JAK2V617F, a portion of endogenous erythroid colonies were negative for both JAK2 mutations. One patient carried 2 independent clones: one with an exon 12 mutation and a second with JAK2V617F. The finding of clonal heterogeneity is compatible with the hypothesis that additional clonal events are involved in the pathogenesis of PV [50]. Dupont et al. suggested that, for PV, erythrocytosis can occur through two mechanisms: terminal erythroid amplification triggered by JAK2 617V>F homozygosity, and a 2-step process including the upstream amplification of heterozygous cells that may involve additional molecular events [55]. Results of Gaikvad et al. showed that JAK2V617F mutation does not provide a proliferative/survival advantage to the PV clone during in vitro expansion [56]. These data suggest that the JAK2V617F mutation plays an important role in the biology of PV, yet it may not be the PV-initiating event. It has been suggested that more than one mutation is required for an MPD clone to develop. The second mutation may be heterogeneous among MPD patients. Alternatively, the MPD clone may arise in different progenitor cells, resulting in phenotypic variability. Finally, individual genetic backgrounds may modulate the effect of the JAK2V617F mutation, resulting in the heterogeneous clinical presentation [14].

Many changes have occurred in recent years in the field of myeloproliferative disorders, including a new WHO classification changing their name to “myeloproliferative neoplasms” (MPNs). Most of these significant changes resulted from the discovery of the JAK2V617F mutation, which clearly opened a new era in the understanding of MPN biology, clinical evolution, and therapy [57-61]. Particularly important benefits connected with the discovery of JAK2V617F mutation in most patients with Ph-negative myeloproliferative neoplasms, is the development of JAK2 kinase inhibitors. However, JAK2 inhibitor therapy has shown limited efficacy and dose-limiting haematopoietic toxicities in clinical trials. Recent results have suggested that JAK2 inhibitor therapy can reduce splenomegaly and constitutional symptoms, but can cause significant haematologic toxicities in MPN patients. Complete remissions similar to those seen in CML with the BCR-ABL inhibitor imatinib cannot be achieved with the JAK2 inhibitors. Therefore, it is very likely that JAK2 inhibitor alone would not be sufficient to effectively treat MPN patients [8] and there is a clear need for further research on the molecular basis of disease, identification and development of alternative therapeutic approaches for the treatment of these patients. Also, continuing studies on the molecular alterations may significantly improve our understanding of PV pathogenesis and facilitate its medical management.

References

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