

Research Article

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Abnormal Megakaryopoiesis in Patients With A JAK2 Exon 12 Mutation May Be A Non-Cell-Autonomous Phenomenon

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Abstract

Background: In contrast to patients with JAK2V617F-positive polycythemia vera, those patients with a JAK2 exon 12 mutation present with platelet counts within the normal range. Furthermore, the bone marrow samples from most JAK2 exon 12 mutation-positive patients lack the prominent clusters of large, bizarre-looking megakaryocytes that characterize classic JAK2V617F-positive polycythemia vera. This study examines the effects on megakaryopoiesis associated with the presence of a JAK2 exon 12 mutation.

Results: These mutations were found to be present within the platelet population of affected individuals at levels comparable to those in paired granulocyte samples, suggesting that they do not profoundly affect the viability of platelets or their precursors. Furthermore, *in vitro* assays demonstrate that JAK2K539L is capable of interacting with and mediating intracellular signalling through the thrombopoietin receptor. An interesting phenomenon was identified when the genotype of individual atypical megakaryocytes present in the bone marrow was determined: only a proportion of those that may be observed in JAK2 exon 12 mutation-positive patients were positive for this mutation.

Conclusions: It remains unclear why patients positive for a JAK2 exon 12 mutation do not have the elevated platelet counts typically observed in patients with classic JAK2V617F-positive PV. The analysis of megakaryocytes with atypical nuclear structure suggests that other hematopoietic or non-hematopoietic cells within the bone marrow environment of MPN patients may influence the phenotype of cells that themselves lack a JAK2 mutation.

Keywords: JAK2 exon 12 mutation; Megakaryopoiesis; Bone marrow microenvironment

Introduction

Polycythemia vera (PV), one of the myeloproliferative neoplasms (MPNs), is characterized by the predominant expansion of morphologically normal erythroid cells. Most, if not all, patients with PV have acquired mutations in Janus kinase 2 (JAK2) [1], a cytoplasmic tyrosine kinase that is constitutively associated with cytokine receptors that lack intrinsic tyrosine kinase activity. The majority of patients have a JAK2V617F mutation, the result of a single base change within JAK2 exon 14 [2-5]. This substitution occurs in the pseudokinase (or JH2) domain, results in constitutive JAK2 activation *in vitro* and *in vivo*, and recapitulates many aspects of PV biology in several different transgenic mouse model systems [6-10]. JAK2V617F is also closely associated with the erythropoietin-hypersensitive expansion of erythroid progenitor cells that is a hallmark feature of the MPNs [11,12].

JAK2V617F-negative PV patients are instead positive for one of a series of JAK2 mutations that are collectively referred to as the JAK2 exon 12 mutations by virtue of their genomic location [13,14]. There have been at least 37 exon 12-encoded mutations identified to date; these all affect a stretch of 12 amino acids located immediately adjacent to JH2 domain. Despite having many functional similarities *in vitro*, the JAK2V617F and JAK2 exon 12 mutations are associated with distinct hematologic phenotypes *in vivo*. Whereas patients with JAK2V617F-positive PV characteristically have tri-lineage involvement, JAK2 exon 12 mutation-positive patients tend to present with an isolated erythrocytosis [13,14]; most individuals have normal leukocyte and platelet counts, although approximately a fifth have elevated counts of either lineage (defined here as greater than $10 \times 10^6/L$ and $400 \times 10^6/L$, respectively). Bone marrow samples from JAK2 exon 12 mutation-positive patients typically lack the prominent clusters of large, bizarre-

looking megakaryocytes that characterize JAK2V617F-positive PV, although small clusters of two to four megakaryocytes, some of which may appear atypical, can be identified [15,16]. However, the effects on megakaryopoiesis associated with the presence of a JAK2 exon 12 mutation have not been examined. Here, *in vitro* assays as well as patient samples and haematologic data were used to determine the effects of a JAK2 exon 12 mutation on the viability and proliferation of megakaryocytic cells. Interestingly, evidence is uncovered that suggests that the megakaryocytic atypical observed in a proportion of mutation-positive patients is not cell-autonomous; that is, it is driven by other hematopoietic or non-hematopoietic elements within the bone marrow environment.

Materials and Methods

Mutation screening in platelets

Granulocyte and platelet cell populations were prepared from individual MPN patients on the same day. First, platelet-rich plasma was isolated from fresh whole blood samples by three cycles of mild centrifugation (150 g for 20 minutes). Platelets were then pelleted by centrifugation at 1500 g for 10 minutes. After the removal of

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platelet-rich plasma, blood samples were further fractionated using a Histopaque-1077 density gradient (Sigma-Aldrich; St Louis, MO). The mononuclear cell population was discarded, and red cells removed by lysis with ammonium chloride. Cell purities were confirmed using a Woodley ABC automated cell counter; only those samples with > 95% purity were used for subsequent analysis. Granulocyte and platelet RNAs were prepared immediately using Triazol reagent (Sigma-Aldrich) according to the manufacturer's recommendations. RNA samples were then used as the template for first-strand DNA synthesis using Moloney murine leukemia virus (M-MLV) reverse transcriptase (Invitrogen; Grand Island, NY). Mutation screening was performed using primers complementary to sequences in *JAK2* exons 11 and 13 (5'-CAACCTCAGTGGGACAAAGAA-3' and 5'-TGTTTCATGCAGTTGACCGTA-3', respectively). The resulting 343 base pair PCR product was directly sequenced.

Mutation screening in megakaryocytes

Deparaffinization of archived bone marrow trephine biopsy sections was performed by sequential 15 minute washes with xylene, followed by sample rehydration with decreasing concentrations of ethanol (95%, 85%, and 70%). Immediately after deparaffinization, slides were stained with hematoxylin and eosin using standard methodologies. To further facilitate their identification, megakaryocytes were highlighted by immuno-histochemical staining with an antibody against factor VIII (MS-722-P0, LabVision; Fremont, CA). Microdissection was performed using a P.A.L.M. laser capture microscope (P.A.L.M. Microlaser Technologies AG; Bernried, Germany). Fourteen atypical megakaryocytes were removed for genotype analysis. Genomic DNA was extracted from individual laser-captured cells using the QIAamp DNA micro kit (Qiagen; Valencia, CA) according to the manufacturer's instructions. Purified DNA was first amplified using primers that flank *JAK2* exon 12 (forward: 5'-CTCCTCTTTGGAGCAATTCA-3'; reverse: 5'-GAGAACTTGGGAGTTGCGATA-3'), and then with nested primers (forward: 5'-AATGGTGGTTTCTGATGTACC-3'; reverse: 5'-AGACA-GTAATGAGTATCTAATGAC-3'), as described previously [17]. The resulting 126 base pair amplicon was directly sequenced.

Retroviral production and 32D cl3 transduction

32D cl3 cells were maintained in RPMI-1640 containing 10% fetal calf serum (FCS) and 1 ng/mL murine interleukin-3 (IL3). The *Jak2* mutations were introduced into an MSCV-*Jak2*-iresGFP vector by site-directed mutagenesis [17], transfected into 293-T kidney epithelial cells, and culture supernatants containing recombinant retroviral particles were produced as described previously [14,18]. 32D cl3 cells were first incubated with MSCV-PGKneo-*EpoR* or MSCV-PGKneo-*TpoR* retroviral supernatant, and then expanded in geneticin for a week. 32D/*EpoR* and 32D/*TpoR* cells were then each transduced with retroviruses encoding either wildtype or mutant *Jak2*, and the resulting green fluorescent protein (GFP)-positive populations purified by fluorescence-activated cell sorting (FACS) four days later [14,18]. Assays of cytokine-independence were performed essentially as previously described for BaF3 cells [14,18].

Results

One possible explanation for the normal or near-normal platelet counts at presentation seen in most patients with a *JAK2* exon 12 mutation could be that these confer a slight proliferative advantage to affected megakaryocytes or their precursors that only manifests over a long period. Those patients with elevated platelet counts at diagnosis would therefore have a more advanced disease than those with normal

platelet counts. To address this possibility, two *JAK2* exon 12 mutation-positive PV patients, for whom cell counts over a long period of time were available, were identified: Patient A had been treated for a decade, and Patient B for just over eight years (Figure 1). At diagnosis, each patient had a hemoglobin (Hb) concentration sufficient to meet the WHO 2008 major diagnostic criteria [19]: that is, 201g/L and 174 g/L, respectively. Hb levels decreased relatively rapidly after venesection was initiated. Over the treatment period, leukocyte numbers remained elevated in Patient B, whereas they rose gradually over time in Patient A; a molecular explanation for this increase was not readily apparent. However, platelet numbers remained static in both cases, not rising above a threshold of $400 \times 10^6/L$. Platelet counts did not increase appreciably in a third *JAK2* exon 12 mutation-positive patient over a contiguous 15-year period; however, the data from this patient is not included in Figure 1, as his platelet counts for the first 40 months following diagnosis were not available. Taken together, these clinical data suggest that, in contrast to *JAK2V617F*, *JAK2* exon 12 mutations may not confer a proliferative advantage to affected megakaryocytic cells.

Another explanation for the lack of an apparent platelet phenotype may be that the *JAK2* exon 12 mutant proteins are unable to interact with and/or mediate intracellular signalling through the *TpoR*. To investigate this possibility *in vitro*, the *Jak2K539L* mutation was expressed in the 32D cl3 murine myeloid cell-line, and tested for its ability to confer IL3-independent proliferation in the presence of the thrombopoietin or erythropoietin receptors (*TpoR* and *EpoR*, respectively). This mutant *Jak2* was previously shown to enable cytokine-independence to murine lymphoid cells (BaF3) expressing *EpoR* [14]. In the absence of exogenous IL3, 32D/*TpoR* cells expressing *Jak2K539L* proliferated at a comparable rate to *Jak2K539L*-positive 32D/*EpoR* cells, and to either cell-line expressing *Jak2V617F* (Figure 2A). Cells that expressed wild type *Jak2* failed to proliferate significantly, as shown previously

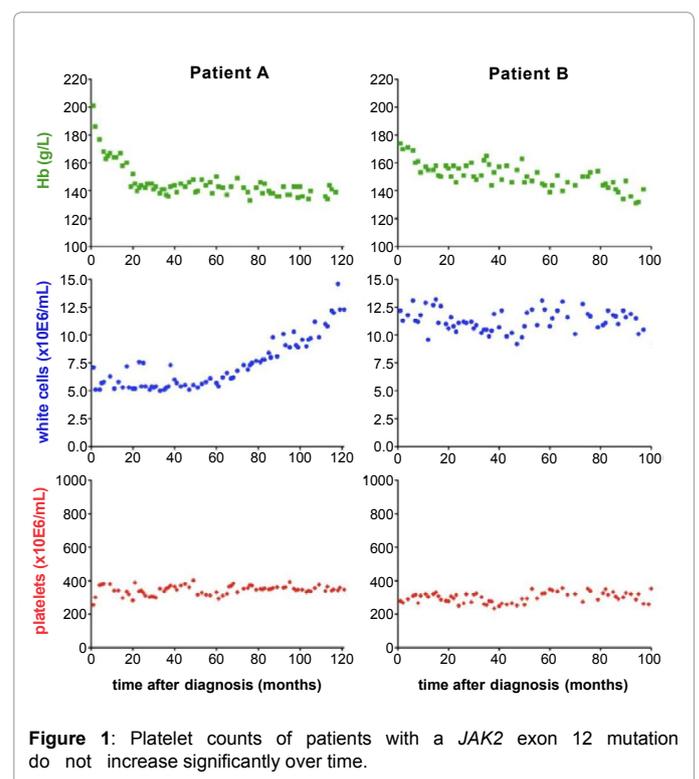
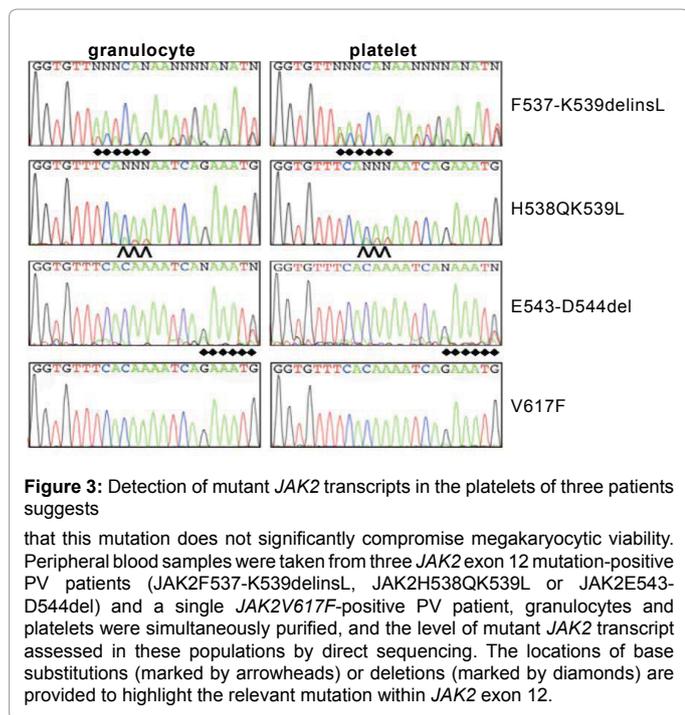
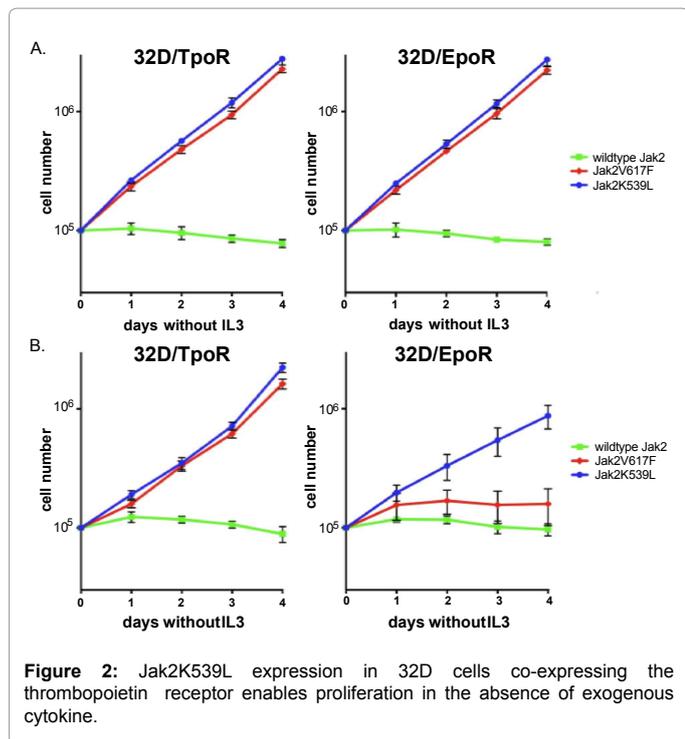


Figure 1: Platelet counts of patients with a *JAK2* exon 12 mutation do not increase significantly over time.



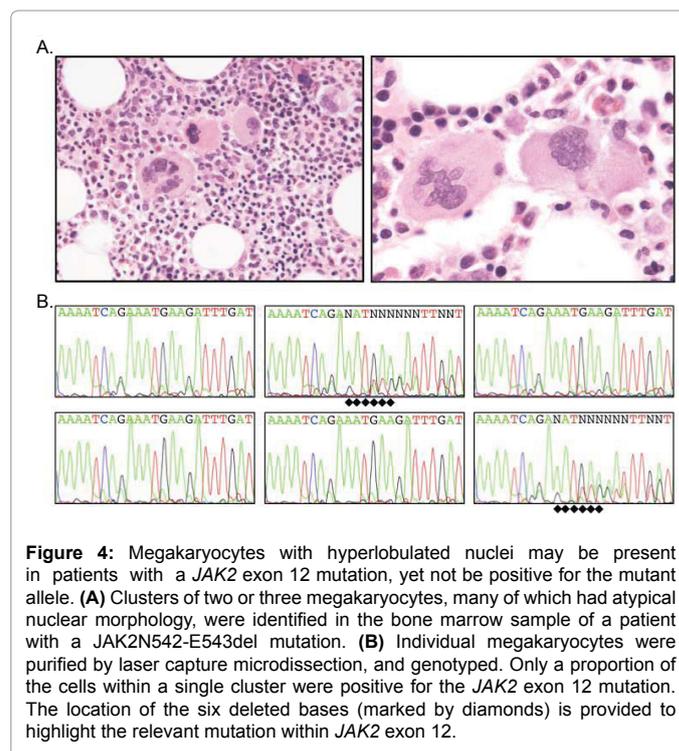
[3-5,14]. To alleviate any concerns that the relatively abundant level of Jak2 in these cell-lines (indicated by the level of co-expressed GFP) was saturating, 32D/EpoR and 32D/TpoR cells that express lower Jak2 levels were engineered, using less retroviral supernatant to transduce cells and selecting those with low GFP expression. Again, 32D/TpoR cells expressing Jak2K539L or Jak2V617F continued to proliferate robustly after cytokine withdrawal (Figure 2B). Interestingly, the reduction in Jak2 expression levels had a more profound effect in 32D/EpoR cells

than in 32D/TpoR cells, with net proliferation rates being lower in the former. Taken together, our *in vitro* studies suggest that Jak2K539L is able to associate with the TpoR *in vivo*, and that the normal platelet counts in patients with a JAK2 exon 12 mutation might not reflect an absence of JAK2-mediated signalling in megakaryocytic cells.

A third potential explanation for the lack of thrombocytosis in JAK2 exon 12 mutation-positive patients may be that the mutant protein has deleterious effects on megakaryocyte viability or differentiation, or on pro-platelet formation. To address this possibility, the level of mutant JAK2 transcript was analyzed in the granulocyte and platelet cell populations present within single individuals. Peripheral blood samples were taken from three JAK2 exon 12 mutation-positive PV patients and a single JAK2V617F-positive PV patient, and these populations were purified and analyzed immediately; the results obtained are depicted in figure 3. Irrespective of the nature of the JAK2 exon 12 mutation, a significant proportion of platelets in each individual expressed the mutant JAK2 transcript. Whilst Sanger sequencing may not be generally considered to be an accurate method for measuring mutation burdens, comparison to the level of mutant transcript in granulocytes from the same patient strongly suggests that platelet viability is not significantly impacted by the presence of a JAK2 exon 12 mutation.

The JAK2 exon 12 mutation status of bone marrow-derived megakaryocytes was also assessed. Examination of trephine sections taken from five patients with a JAK2 exon 12 mutation revealed clusters of two to four megakaryocytes, many of which had atypical nuclear morphology, could be identified in one case (Figure 4A). Individual megakaryocytes were purified by laser capture microdissection, and their JAK2 mutation status determined. Surprisingly, the genotyping of ten individual megakaryocytes from three separate clusters revealed that only four of these cells were positive for the JAK2N542-E543del mutation; typical DNA sequences are shown in figure 4B.

This observation suggests that megakaryocyte atypia may not be a



cell-intrinsic phenomenon in MPN patients, but rather a response to other factors within the bone marrow microenvironment.

Discussion

In this study, we sought to more fully understand the effects of a *JAK2* exon 12 mutation on megakaryopoiesis. These mutations were found to be present within the platelet population of affected individuals at levels comparable to those in paired granulocyte samples, suggesting that they do not profoundly affect the viability of platelets or their precursors. Nevertheless, most mutation-positive patients present with platelet counts in the normal range [13,14,16], and numbers fail to rise substantially over time. Cytokine withdrawal assays also demonstrated that Jak2K539L is able to interact with, and mediate intracellular signalling through, the TpoR *in vitro*. It therefore remains unclear why patients with a *JAK2* exon 12 mutation do not have the elevated platelet counts typically observed in patients with *JAK2V617F*-positive PV. However, in performing these analyses, an interesting phenomenon was identified: only a subset of those megakaryocytes with an atypical nuclear structure were positive for a *JAK2* exon 12 mutation. This observation suggests that cell types within the bone marrow environment of MPN patients may influence the phenotype of cells that lack a *JAK2* mutation.

Several different mouse models of human hematologic malignancies suggest that changes within the bone marrow microenvironment may impact significantly upon myelopoiesis [20-22]. In one instance, deletion of the *Dicer* microRNA processing enzyme specifically within osteogenic cells resulted in myelodysplasia, with mice having anemia, leukopenia and thrombocytopenia, along with marked dysplasia in the granulocytic and megakaryocytic lineages [20]. The cell-extrinsic nature of this defect was confirmed when wildtype bone marrow cells were transplanted into *Dicer*-deficient recipients, recapitulating the myelodysplastic phenotype. Further insights into the underlying mechanism behind this phenotype were obtained when gene set enrichment analysis revealed significant downregulation of the Shwachman-Diamond-Bodian Syndrome gene (*Sbds*). In humans, inactivating *SBDS* mutations are associated with a bone marrow failure syndrome that includes myelodysplastic changes [23]. Targeted *Sbds* deletion in murine osteoprogenitor cells also resulted in leukopenia and dysplastic changes in granulocytic and megakaryocytic cells [20].

Although more frequently associated with cell-intrinsic defects, myeloproliferation can also arise as a consequence of changes within the bone marrow environment. Modulation of the number or function of osteoblastic cells extrinsically influences hematopoiesis by altering the hematopoietic stem cell (HSC) niche [24-26]. In other instances, the cell type within the microenvironment that drives myeloproliferation is not entirely clear, but likely includes both stromal and endothelial elements and even other hematopoietic cells [21,27]. Individual proteins can also positively or negatively affect the number of HSCs present within an organism: the loss of angiopoietin, osteopontin or retinoic acid receptor- γ expression results in marked myeloproliferation *in vivo* [28-30], whereas over-expression of Jagged-1 or HoxB4 has a similar effect *in vitro* [31,32].

To date, there has been little evidence of a non-cell-autonomous effect in patients with an MPN. Of particular note, however, is a study in which immunocompromised mice were transplanted with human cord blood cells expressing TEL-JAK2 [22]. This fusion protein is the consequence of a translocation between chromosomes 9 and 12 that can be observed in rare leukemic patients [33,34], confers cytokine independence to BaF3 cells *in vitro*, and produces an acute leukemia

in transgenic animals [35]. Mice xenotransplanted with TEL-JAK2-positive human cells rapidly developed myelofibrosis, with their bone marrow containing increased numbers of atypical megakaryocytes. These had hyperlobulated nuclei, were distributed in clusters and, surprisingly, were murine in origin. This finding demonstrates that the formation of megakaryocyte atypia may be a reactive event, and lends support to the hypothesis that the megakaryocytic hyperplasia seen in MPN patients is not cell-intrinsic. Further studies will be required to more clearly investigate this possibility.

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