Activating mutation in the tyrosine kinase JAK2 in polycythemia vera, essential thrombocythemia, and myeloid metaplasia with myelofibrosis

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Summary

Polycythemia vera (PV), essential thrombocythemia (ET), and myeloid metaplasia with myelofibrosis (MMM) are clonal disorders arising from hematopoietic progenitors. An internet-based protocol was used to collect clinical information and biological specimens from patients with these diseases. High-throughput DNA resequencing identified a recurrent somatic missense mutation *JAK2V617F* in granulocyte DNA samples of 121 of 164 PV patients, of which 41 had homozygous and 80 had heterozygous mutations. Molecular and cytogenetic analyses demonstrated that homozygous mutations were due to duplication of the mutant allele. *JAK2V617F* was also identified in granulocyte DNA samples from 37 of 115 ET and 16 of 46 MMM patients, but was not observed in 269 normal individuals. In vitro analysis demonstrated that JAK2V617F is a constitutively active tyrosine kinase.

Introduction

Myeloproliferative disorders (MPD) include polycythemia vera (PV), essential thrombocythemia (ET), myeloid metaplasia with myelofibrosis (MMM), chronic myelogenous leukemia (CML), chronic myelomonocytic leukemia (CMML), hypereosinophilic syndrome (HES), and systemic mast cell disease (SMCD). A clonal population of hematopoetic cells has been observed in patients with PV, ET, and MMM, indicating that these diseases are caused by acquired somatic mutation in hematopoietic progenitors, but the genetic basis has not been known (Adamson et al., 1976; Gilliland et al., 1991). Reports of decreased expression of the thrombopoietin receptor in platelets from patients with PV, and of overexpression of the antiapoptotic protein Bcl-x in PV (Moliterno et al., 1998; Silva et al., 1998), suggest that abnormalities in signal transduction and in apoptosis are important in the pathogenesis of PV and other MPD. In addition, activating mutations in the erythropoietin (EPO) re-

SIGNIFICANCE

Although the myeloproliferative disorders (MPD) PV, ET, and MMM are thought to be caused by acquired somatic mutation in hematopoietic progenitors, the genetic basis for these diseases has not been known. We report that hematopoietic cells from a majority of patients with PV and a significant number of patients with ET and MMM possess a recurrent somatic activating mutation in the JAK2 tyrosine kinase. Inhibition of the JAK2V617F kinase with a small molecule inhibitor leads to inhibition of proliferation of hematopoietic cells, suggesting that the JAK2 tyrosine kinase is a potential target for pharmacologic inhibition in patients with PV, ET, and MMM.

ceptor and in the von Hippel-Lindau (VHL) protein have been identified in a subset of patients with familial and congenital polycythemia, but these genes have not been pathogenetically linked to sporadic PV or other MPD (Arcasoy et al., 1997; Kralovics et al., 1997; Pastore et al., 2003; Sokol et al., 1995).

In contrast to PV, ET, and MMM, the molecular pathogenesis of several other MPD has been well characterized, and is frequently attributable to mutations that result in constitutive activation of a protein tyrosine kinase. Furthermore, these mutant kinases have proven to be good candidates for molecularly targeted therapy. The paradigm established by Druker and colleagues is the *BCR-ABL* gene rearrangement associated with CML (Bartram et al., 1983), which is effectively treated with the small molecule tyrosine kinase inhibitor imatinib (Gleevec) (Druker et al., 2001). In addition to ABL, imatinib also inhibits PDGFR family members, which has led to the successful treatment of patients with *PDGFRB* rearrangements and CMML (Golub et al., 1994) and patients with the *FIP1L1-PDGFRA* fusion and HES (Cools et al., 2003). Other examples of mutant tyrosine kinases in MPD include *FGFR1* gene rearrangements in stem cell myeloproliferative disorders (Xiao et al., 1998), and activating mutations in *KIT* in systemic mast cell disease (Furitsu et al., 1993). Thus, tyrosine kinases are often mutated in MPD, and have been demonstrated to be excellent targets for therapeutic intervention.

These observations suggest the hypothesis that activating mutations in tyrosine kinases are important in the pathogenesis of PV, ET, and MMM. We used an internet-based clinical protocol and high-throughput DNA sequence analysis to screen for tyrosine kinase mutations in PV, ET, and MMM.

Results

Myeloproliferative disorders study

586 patients expressed interest in the study, and 345 participants consented to participate and returned the questionnaire and at least one biologic sample (Supplemental Figure S1). The clinical characteristics of the 345 participants are shown in Table 1. The median age of the case series was 59 (range 31–84 years) and the median age at diagnosis was 52 (range 17–78 years). The majority of patients were female (59%), and the majority of patients were Caucasian (94%). By patient self-report, the cross-sectional study included 171 patients with PV (50%), 126 patients with ET (37%), and 46 patients with MMM (13%). 2 patients did not provide a specific diagnosis and were classified as MPD "not otherwise specified." The internet recruitment method selected for a highly educated and affluent population; 60% of participants were college graduates, and 47% reported incomes greater than $75,000/year. All patients provided blood samples, and 338 patients provided buccal swabs. Of 345 blood samples, 327 (95%) yielded sufficient DNA for sequence analysis. We retrospectively abstracted medical records to verify patient self-reported diagnoses. Medical records were available for 304 patients, and we were able to verify self-reported diagnosis for 249/257 (97%) patients who provided sufficient medical records (Supplemental Data, section "MPD patients with confirmed diagnoses"). These results demonstrate the feasibility of using the internet to recruit and enroll patients in a clinical protocol, and that patient self-report correlates with confirmed diagnosis by medical record abstraction.

Table 1. Patient characteristics

Characteristic	
Total, n	345
Male/female, n	142/203
Median age, years (range)	59 (31–84)
Median age at diagnosis, years (range)	52 (17–78)
Race, n (%)	
Caucasian	325 (94)
Hispanic	10 (2.9)
Asian	4 (1.2)
Black/African-American	1 (0.3)
Multiracial or other	5 (1.5)
Self-reported diagnosis, n (%)	
PV	171 (50)
ET	126 (37)
MMM	46 (13)
MPD, NOS	2 (0.6)
Median disease duration, months (range)	
Case series	68 (3–549)
PV	60 (4–433)
ET	79 (9–549)
MMM	64 (14–379)
Prior thrombosis, n (%)	
Case series	76 (22)
PV	31 (18)
ET	40 (32)
MMM	5 (11)

PV, polycythemia vera; ET, essential thrombocythemia; MMM, myeloid metaplasia with myelofibrosis.

JAK2 mutations in myeloproliferative disorders

93 granulocyte DNA samples from PV patients were initially evaluated for sequence alterations in the activation loops and known autoinhibitory domains of 85 tyrosine kinases, and we identified a recurrent guanine to thymine substitution in *JAK2* exon 14 that results in a valine to phenylalanine substitution at codon 617 within the JH2 autoinhibitory domain (Figures 1A–1C). Sequence analysis of the entire open reading frame of *JAK2* in these 93 PV patients identified novel germline nonsynonymous substitutions (R1063H in 3 patients, R340Q in 1 patient, R564L in one patient), but we did not identify additional somatic mutations in *JAK2.* We did not identify somatic mutations in the other tyrosine kinases analyzed. We subsequently sequenced exon 14 of *JAK2* in all patients in the case series with self-reported PV, ET, and MMM (Table 2). Analysis of granulocyte DNA from 164 PV patients identified heterozygous *JAK2V617F* mutations in 80 (49%) patients and homozygous mutations in 41 (25%) patients. Of 115 patients with ET, 34 (30%) were heterozygous and 3 (3%) were homozygous for the *JAK2V617F* allele. Of 46 patients with MMM 12 (26%) were heterozygous and 4 (9%) were homozygous for the *JAK2V617F* allele. Sequence analysis of cDNA from selected PV patients confirmed expression of both mutant and wild-type alleles in heterozygous patients.

Analysis of DNA derived from buccal mucosa

Buccal swabs were provided by 338 patients, and 313 buccal DNA samples were evaluable for the *JAK2V617F* mutation. 301/313 (96%) buccal DNA samples did not contain the *JAK2V617F* allele, but heterozygous *JAK2V617F* alleles were identified in buccal DNA samples from 11 patients with PV and one patient with MMM. In 7 cases, PV patients had a heterozy-

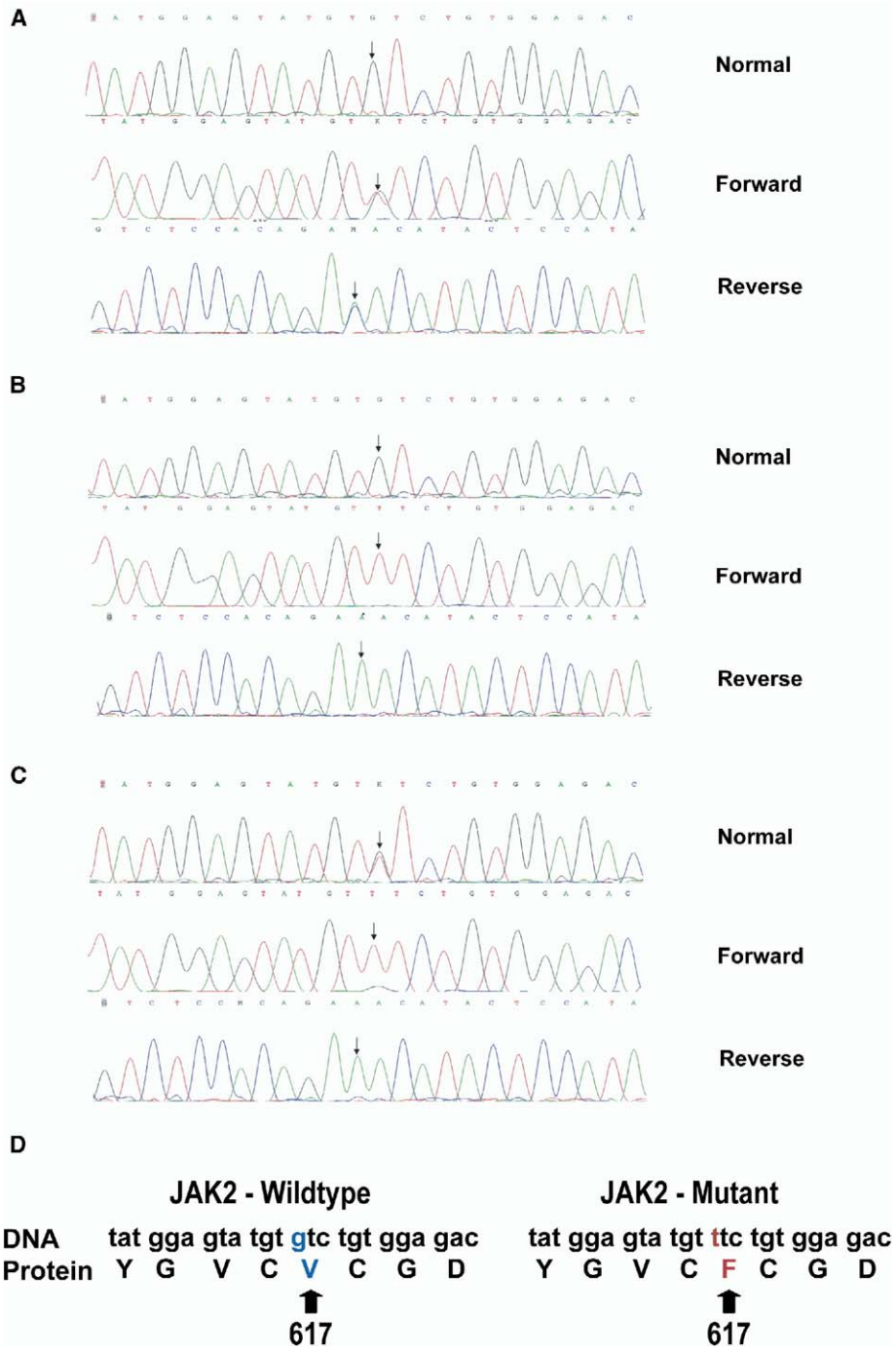

Figure 1. *JAK2V617F* mutations in polycythemia vera samples

A: Forward (middle trace) and reverse (lower trace) sequence demonstrating a heterozygous guanine to thymine substitution (arrows) present in granulocyte DNA from a patient with PV that is not present in matched normal DNA (upper trace).

B: Forward (middle trace) and reverse (lower trace) sequence demonstrating a homozygous guanine to thymine substitution (arrows) present in granulocyte DNA from a patient with PV that is not present in matched normal DNA (upper trace).

C: Forward (middle trace) and reverse (lower trace) sequence demonstrating a homozygous guanine to thymine substitution (arrows) present in granulocyte DNA from a patient with PV that is present as a heterozygous allele in matched normal DNA (upper trace).

D: DNA sequence and protein translation for both the wild-type and mutant *JAK2* alleles. The guanine to thymine substitution results in a valine to phenylalanine substitution at codon 617.

gous allele in their buccal DNA and a homozygous allele in granulocyte DNA, consistent with loss of heterozygosity at this locus (Figure 1C). In the remaining cases, buccal and granulocyte DNA were heterozygous. The *JAK2V617F* allele was not identified in buccal DNA from any patients with ET. These data indicate the *JAK2V617F* is an acquired somatic mutation in the majority of patients with MPD.

V617F genotyping in the general population

To assess the prevalence of the *V617F* allele in normal individuals of Caucasian descent, and in the general population, we analyzed the *JAK2* genotype in a standard panel of 270 samples collected by the International HapMap Consortium (The International HapMap Consortium, 2003). The samples were collected from four populations: CEPH (Centre d'Etude du Polymorphism Humain)—Utah residents with Caucasian ancestry from Northern and Western Europe (30 parent-offspring trios, or 120 independent chromosomes); Yoruba, Nigeria (30 parent-offspring trios, or 120 independent chromosomes); Han Chinese (45 unrelated individuals, or 90 independent chromosomes); and Japanese (45 unrelated individuals, or 90 independent chromosomes). Pilot matrix-assisted laser desorption/

Table 2. Mutational status by disease

Mutation	Total (n = 345[1])	PV (n = 164[2])	ET (n = 115[3])	MMM (n = 46)
None	151 (44)	43 (26)	78 (68)	30 (65)
Heterozygous	126 (36)	80 (49)	34 (29)	12 (26)
Homozygous	48 (14)	41 (25)	3 (3)	4 (9)
Unknown	20 (6)			

Number and (percent) are indicated.
[1] Two subjects have MPD, not otherwise specified.
[2] 7 additional patients were not evaluable for *JAK2V617F* mutations.
[3] 11 additional patients were not evaluable for *JAK2V617F* mutations.

ionization time-of-flight mass spectrometry (MALDI-TOF MS) genotyping of 95 granulocyte and buccal DNA samples from our case series correctly identified all *JAK2V617F* alleles identified by conventional DNA sequencing. High-confidence genotypes were obtained using the MALDI-TOF MS assay for 269 of the 270 HapMap samples (Figure 2A). All 269 samples were homozygous for the wild-type V617 allele. Comparing the frequency of the *JAK2V617F* allele in buccal DNA from patients with PV, 94% of whom are Caucasian, with unaffected individuals in the CEPH panel, the difference was significant ($p = 0.021$ by Fisher's exact test).

Loss of heterozygosity at the *JAK2* locus is due to mitotic recombination

We amplified and sequenced a synonymous single nucleotide polymorphism in *JAK2* exon 6 (refSNP ID: rs2230722) 22.8 kb upstream of *JAK2V617F* in buccal DNA from 17 patients with homozygous *JAK2V617F* mutations. Seven patients were het-

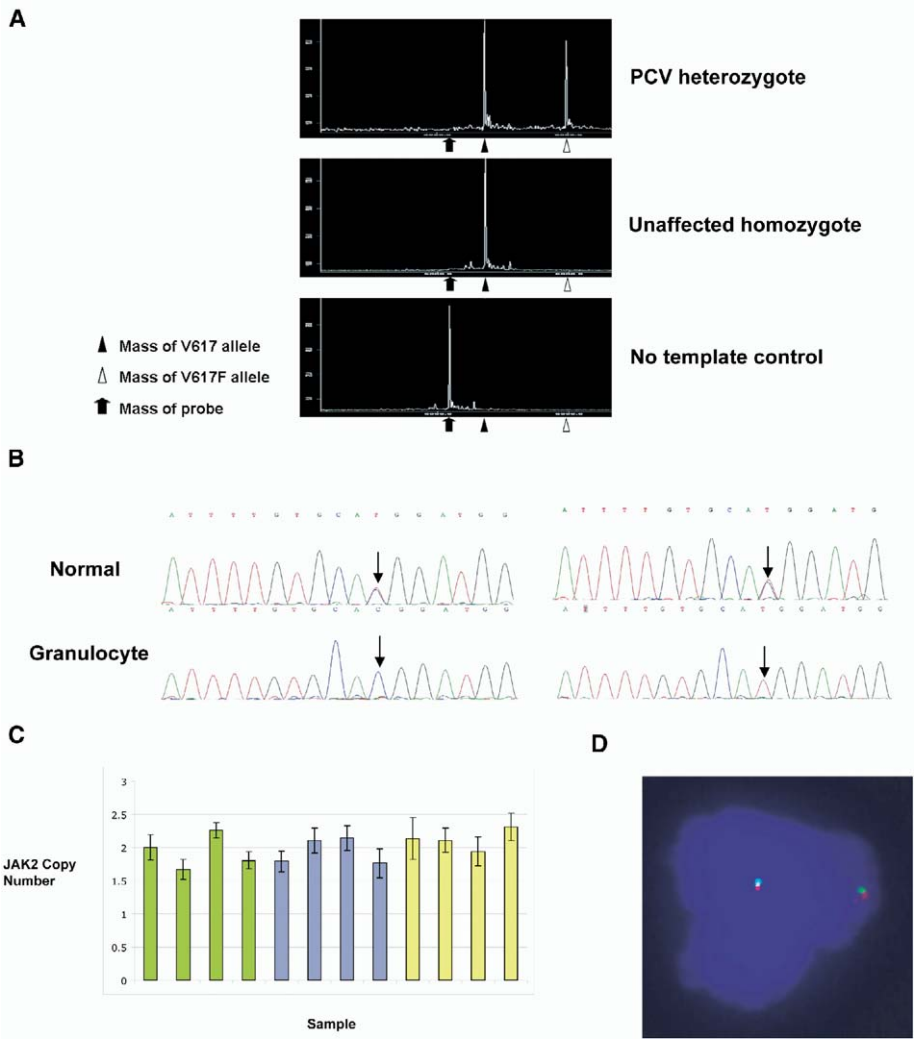

Figure 2. Genotypic analysis of the *JAK2V617F* allele

A: MALDI-TOF MS genotypic analysis for the *JAK2V617F* allele in a PV patient with a heterozygous *JAK2V617F* mutation (upper trace) and for a normal individual from the HapMap Panel (middle trace). The arrows denote the mass of the genotyping probe, the wild-type V617 allele, and the *JAK2V617F* mutant allele, respectively.

B: Sequence traces of *JAK2* exon 6 from two patients with homozygous *JAK2V617F* mutations demonstrating loss of heterozygosity at the *JAK2* locus. The arrows show heterozygous alleles (upper traces) at a known polymorphism in the buccal DNA of both patients with loss of heterozygosity in the granulocyte DNA (bottom traces) in both cases.

C: Quantitative PCR for the *JAK2* locus relative to *Factor VIII* for 4 patients, each with wild-type *JAK2* (green), heterozygous *JAK2V617F* mutations (blue), or homozygous *JAK2V617F* mutations (yellow). Error bars denote the standard deviation for each sample measured in triplicate. All samples tested showed two copies of the *JAK2* locus, consistent with mitotic recombination in homozygotes.

D: FISH analysis of one patient with a known homozygous *JAK2V617F* mutation with probes upstream (red) and downstream (green) of the *JAK2V617F* mutation, demonstrating two copies of the *JAK2* locus, also consistent with mitotic recombination.

erozygous for the polymorphism in their buccal DNA, and all 7 demonstrated loss of heterozygosity in their granulocyte DNA (Figure 2B). To distinguish between deletion of the wild-type allele and mitotic recombination, we performed quantitative genomic polymerase chain reaction (PCR) on 32 granulocyte DNA samples, including 16 samples from patients with homozygous *JAK2V617F* mutations (Figure 2C). In all cases analyzed there were two copies of the *JAK2* locus, consistent with reduplication of the mutant allele. Fluorescent in situ hybridization (FISH) analysis confirmed the presence of two copies of the *JAK2* locus in 35 patients, including in 12 patients with homozygous *JAK2V617F* mutations (Figure 2D).

Comparison of genotype and clinical outcome

Considering the case series as a whole, no association was observed between mutational status and current age, age at diagnosis, gender, race, comorbidity, physical functioning, prior thrombosis, or self-reported family history (data not shown). However, subjects with PV were more likely to have a detectable mutation in their granulocyte DNA (74% versus 33%, $p < 0.0001$) or buccal DNA (7% versus 1%, $p = 0.003$) than subjects with either ET or MMM. In addition, within the PV subset, women were more likely than men to have either a heterozygous or homozygous mutation (83% versus 64%, $p = 0.004$). A homozygous mutation was associated with a longer duration of disease than either heterozygous or no mutation among patients with PV or ET (median 89 versus 61 months, $p = 0.008$), but not MMM. No association was seen between the presence of the *JAK2V617F* allele in buccal DNA and self-reported family history of MPD.

Structural and functional analysis of JAK2V617F

The JH2 (pseudokinase) domain lacks several residues that are critical for phosphotransferase activity, but is expected to adopt the overall protein architecture that is characteristic of bona fide tyrosine and serine/threonine kinases. Using the SwissModel automated comparative protein modeling server, we constructed a homology model of the JAK2 JH2 domain using the structure of Bruton's tyrosine kinase (Mao et al., 2001) (PDB accession code 1K2P), which has a 26% identity to the JAK2 JH2 domain. Val617 is expected to lie on the upper surface of the N-terminal lobe of the JH2 domain, at the C-terminal end of strand β4 (Figure 3A). Modeling suggests that this residue is largely solvent-exposed, and that substitution with phenylalanine is unlikely to significantly destabilize the fold of the domain. This residue is a component of a surface patch that is conserved among JAK pseudokinase domains.

In functional assays, JAK2V617F, but not wild-type JAK2, was a constitutively activated tyrosine kinase as assessed by autophosphorylation (Figure 3B) when expressed in 293T cells. When increasing amounts of wild-type JAK2 were coexpressed with JAK2V617F, there was no change in the level of JAKV617F tyrosine phosphorylation (Figure 3C), suggesting that the wild-type allele does not interfere with constitutive activation of JAK2V617F. Expression of JAK2V617F, but not wild-type JAK2, in Ba/F3-EPOR cells conferred erythropoietin hypersensitivity and erythropoietin-independent survival, consistent with in vitro erythroid colony formation in patients with PV (Figure 3D) (Prchal and Axelrad, 1974; Zanjani et al., 1977).

Sequence analysis of the HEL human erythroleukemia cell line identified a homozygous *JAK2V617F* mutation in this cell line (Figure 4A), but not in K562, MOLT16, or RPMI-8402 cell lines. JAK2V617F was constitutively tyrosine-phosphorylated in HEL cells, as were downstream effectors STAT5 and ERK (Figure 4B). Furthermore, treatment with a small molecule inhibitor of JAK2 (Thompson et al., 2002) showed a dose-dependent inhibition of proliferation of HEL cells, but not leukemia cell lines K562 (Figure 4C), MOLT16, or RPMI-8402 (data not shown). Inhibition of proliferation corresponded with reduced phosphorylation of JAK2 and STAT5 (Figure 4D). Moreover, treatment of HEL cells with the JAK2 inhibitor resulted in induction of apoptosis, which was not observed in the control cell line K562 that is known to express the constitutively active tyrosine kinase BCR-ABL (Figure 4E). These findings indicate that JAK2V617F is a constitutively activated tyrosine kinase that enhances hematopoietic cell proliferation and survival, and is sensitive to inhibition with small molecule inhibitors.

Discussion

We identified *JAK2V617F* mutations in granulocyte DNA from ~3/4 of PV patients, as well as in a substantial fraction of ET and MMM patients. Sequence analysis showed wild-type *JAK2* sequence in DNA from buccal mucosa of 301/313 (96%) evaluable patients, providing strong genetic evidence that *JAK2V617F* is an acquired somatic disease allele that arises in hematopoietic progenitors and confers a selective growth advantage. In addition, a significant proportion of patients had homozygous mutations due to mitotic recombination, as has been described in patients with homozygous *FLT3* mutations with retention of two copies of the *FLT3* locus (Frohling et al., 2002). Since *JAK2V617F* is an acquired mutation, duplication of the mutant allele is most likely due to mitotic recombination. This provides genetic evidence that *JAK2V617F* confers a further selective advantage when two mutant alleles of *JAK2* are present.

The *JAK2V617F* mutation constitutively activates the JAK2 tyrosine kinase as assessed by anti-phosphotyrosine blotting. The increase in phosphotyrosine content cannot be explained as a consequence of overexpression alone, in that wild-type JAK2 is not tyrosine-phosphorylated when expressed at comparable levels. Selection for cells with homozygous *JAK2V617F* alleles suggests either that the native JAK2 has dominant interfering activity for the mutant JAK2V617F, or that duplication of the allele confers a selective advantage compared to cells with only one mutant allele. The observation that expression of increasing levels of wild-type JAK2 has no effect on phosphotyrosine content of JAKV617F in 293T cells favors the latter hypothesis, but should also be evaluated in hematopoietic contexts in which the erythropoietin receptor (EPOR) is expressed as well.

JAK2V617F, but not JAK2, confers factor-independent growth to Ba/F3 cells stably transduced with the EPOR, as well as hypersensitivity to erythropoietin. These data are in consonance with observations in PV patients in which erythropoietin-independent BFU-E may be generated, and which show hypersensitivity to erythropoietin (Prchal and Axelrad, 1974; Zanjani et al., 1977). In addition, we report that the HEL cell line contains a homozygous *JAK2V617F* mutation. HEL cells, but not cells with wild-type JAK2, undergo apoptotic cell death in response to a JAK inhibitor. Furthermore, activity of JAK2V617F and the downstream effector STAT5, as assessed by phospho-

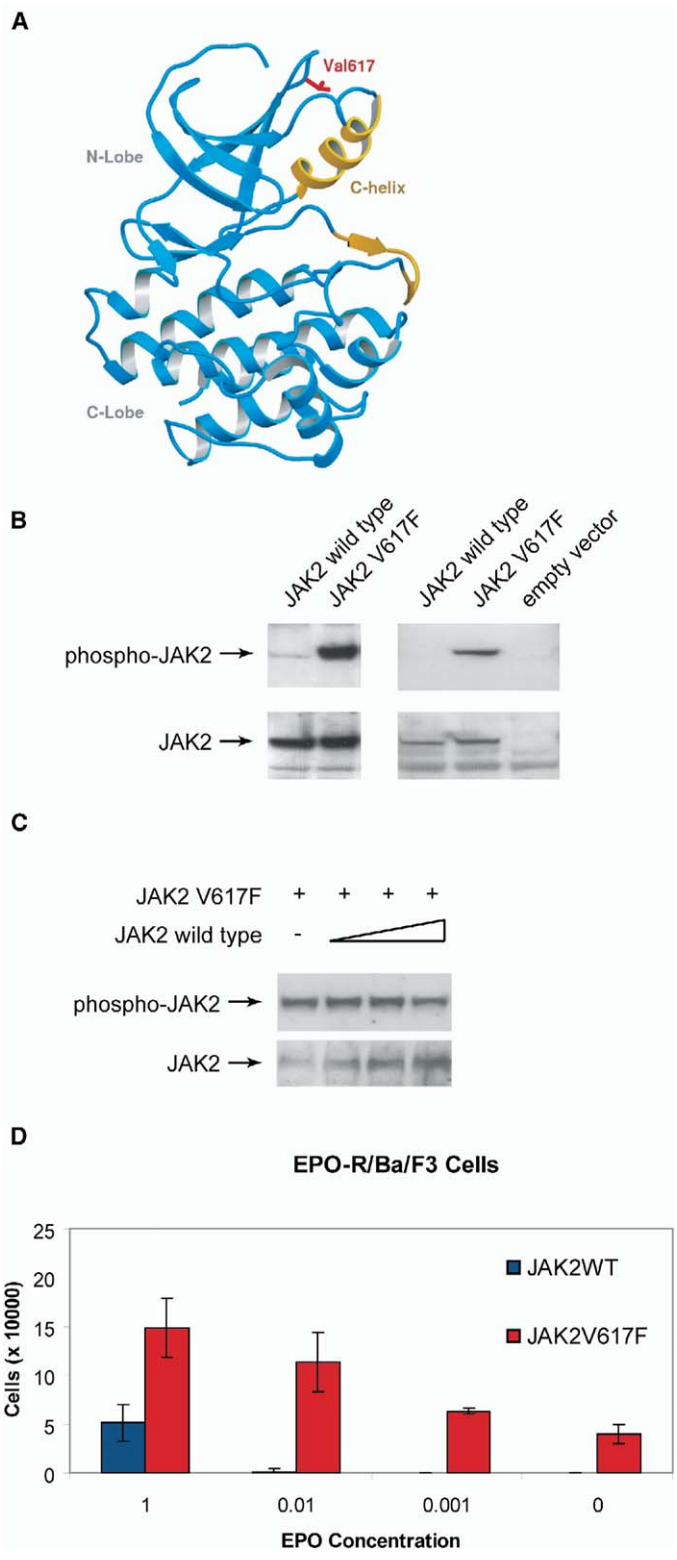

Figure 3. Structure and function of the JAK2V617F protein

A: Homology model of the JH2 (pseudokinase) domain of human JAK2. The model was constructed using the SwissModel automated comparative protein modeling server using the JAK2 pseudokinase domain sequence and the crystallographic structure of Bruton's tyrosine kinase (PDB accession code 1K2P) (Mao et al., 2001). Although several residues critical for kinase activity are not conserved, the pseudokinase domain is expected to retain the essential features of the protein kinase fold, including the smaller β sheet-rich N-lobe and the larger, mostly helical C-lobe. Val 617 (indicated in red) is expected to lie exposed on the upper surface of the N-lobe at the C-terminal end of strand β4. Note that Val617 lies at the edge of a conserved patch that includes residues of the C-helix and the N-terminal portion of the region corresponding to the activation loop (colored orange). We hypothesize that this residue participates in interdomain interactions that inhibit JAK catalytic activity. The figure was made using the program SETOR (Evans, 1993).

B: Western blot experiments with 293T cells. The upper blots show phosphorylation status of JAK2 in 293T cells overexpressing wild-type JAK2 or the JAK2V617F protein (left panel), or in 293T cells with lower level expression of wild-type JAK2, JAK2V617F, or empty vector (right panel). These blots demonstrate a marked increase in JAK2 autophosphorylation in cells expressing the JAK2V617F mutant. The lower panels show similar levels of expression of JAK2 in the cells transfected with wild-type or mutant JAK2, respectively.

C: Expression of increasing amounts of wild-type JAK2 does not influence tyrosine phosphorylation of JAK2V617F, indicating that the wild-type allele does not have dominant negative inhibitory activity. From left to right, 0, 300, 600, and 900 ng of the wild-type JAK2 expression vector was cotransfected with 300 ng of the JAK2V617F expression vector. The upper blot shows no change in JAK2 phosphorylation, while the lower blot shows increasing expression of JAK2.

D: Erythropoietin hypersensitivity in Ba/F3-EPOR cells expressing JAK2V617F. 2.5×10^5 Ba/F3-EPOR cells stably expressing wild-type JAK2 or JAK2V617F were cultured in triplicate in RPMI/10% FCS containing EPO 1 U/ml, 0.01 U/ml, 0.001 U/ml, or in the absence of EPO for 7 days. Error bars denote the standard deviation for each sample measured in triplicate. There are an increased number of viable cells at EPO 1 U/ml in the Ba/F3-EPOR cells transfected with JAK2V617F relative to wild-type JAK2. At limiting (0.01 and 0.001 U/ml) concentrations of EPO and in cytokine-free media, there were no viable Ba/F3-EPOR cells expressing wild-type JAK2, whereas Ba/F3-EPOR cells expressing JAK2V617F were viable at all concentrations of EPO tested and in the absence of EPO.

tyrosine content, are also inhibited. These data collectively indicate that *JAK2V617F* is a gain-of-function allele that results in expression of a constitutively activated tyrosine kinase that confers predicted growth properties to hematopoietic cells, and is sensitive to small molecule inhibition.

The mechanism of activation of JAK2 by the V617F substitution is not known, but the JH2 pseudokinase domain is thought to inhibit the activity of the JAK2 kinase (JH1) domain, in that deletion of the JH2 domain leads to a marked increase in JAK2 kinase activity (Saharinen et al., 2000). Autoinhibition of receptor tyrosine kinases is often accomplished by residues within the kinase domain or by immediately adjacent juxtamembrane or C-terminal sequences, with activating mutations in these regions identified in Kit, Flt-3, and EGFR (Furitsu et al., 1993; Lynch et al., 2004; Nakao et al., 1996; Paez et al., 2004). In contrast, autoinhibition of nonreceptor tyrosine kinases and phosphatases, such as JAK2, results from interdomain interactions with activating mutations often arising in interdomain interfaces. For example, mutations in the SH2 and SH3 domain interfaces in BCR-ABL relieve autoinhibition of the kinase domain (Smith et al., 2003), and activating mutations in the SHP-2 phosphatase seen in Noonan's syndrome and in a subset of juvenile myelomonocytic leukemias disrupt the autoinhibitory SH2 domain/phosphatase domain interface (Tartaglia et al., 2001; Tartaglia et al., 2003). We propose that the V617F substitution in the pseudokinase domain of JAK2 releases autoinhibitory interdomain interactions, leading to constitutive kinase activation. Structural modeling of the JH1-JH2 domains of JAK2 is consistent with this possibility (see Supplemental

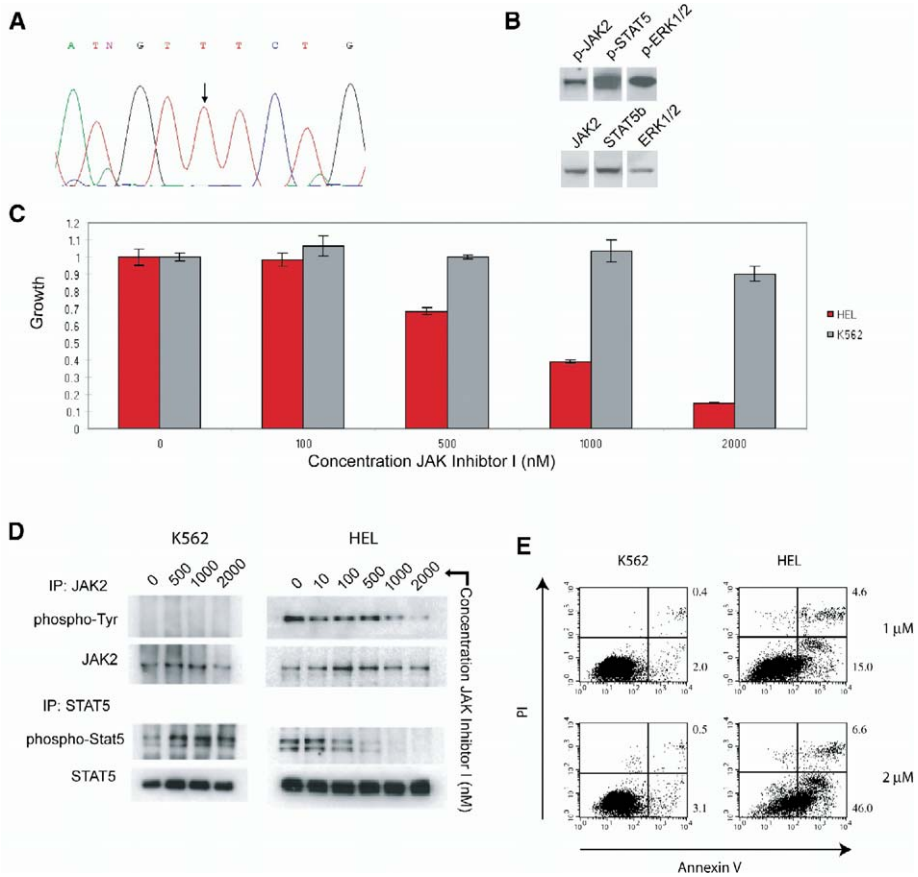

Figure 4. Functional activity of JAK2V617F in the HEL cell line

A: The JAK2V617F homozygous mutation in HEL cells.

B: Phosphorylation of JAK2, STAT5, and ERK1/2 kinases in the HEL cell line.

C: Dose-dependent inhibition of growth of the HEL cell line but not the K562 cell line with increasing concentrations of JAK inhibitor I. Error bars denote the standard deviation for each sample measured in triplicate.

D: Reduction in JAK2 phosphorylation and inhibition of STAT5 phosphorylation in HEL cells treated with increasing doses of JAK inhibitor I. Phospho-JAK2 was not expressed in K562 cells, nor was any effect observed on STAT5 phosphorylation in K562 cells.

E: Induction of apoptosis by JAK inhibitor I in HEL cells, but not K562 cells. The percentage of apoptotic cells (lower right quadrant) after treatment with 1–2 μM JAK inhibitor I was higher in HEL cells (15%–46%) versus K562 cells (2%–3.1%).

Data, section "Predicted model of the JAK2V617F allele.") (Lindauer et al., 2001). Solution of the JAK2 JH1-JH2 structure will be required to confirm this possibility, and may also provide insight into the selection for the *JAK2V617F* mutation, as opposed to other activating alleles, in all patients.

The observation that a single mutant allele is present in a significant proportion of PV, ET, and MMM is also of interest in that these are distinct clinical entities. This is similar to the association of a single disease allele, the BCR-ABL gene rearrangement, with a spectrum of hematologic malignancies that includes CML, CML in blast crisis, de novo AML, and B cell ALL. The basis for phenotypic pleiotropy in patients with the same disease allele could include host modifiers, differences in the hematopoietic progenitor in which the mutation occurs, or as yet unidentified second mutations that contribute to phenotype. However, there is also significant clinical overlap between the MPD syndromes. For example, patients with ET may progress to PV, patients with PV who are iron-deficient may present with signs and symptoms of ET, and patients with ET or PV may progress to develop severe myelofibrosis. It will also be of interest to determine whether there are substantive differences in patterns of global gene expression between MMM, PV, and ET patients, but these genotypic data suggest these diseases may be more alike than disparate.

A significant proportion of PV, ET, or MMM patients (26%, 68%, and 65%, respectively) do not have detectable *JAK2V617F* mutations in granulocyte DNA. This observation may be explained by disease alleles other than *JAK2V617F*. We did not observe additional *JAK2* mutations in PV, nor were mutations detected in the activation loop or in the JH2 domain of *JAK1*, *JAK3*, or *TYK2*, respectively (data not shown). High-throughput sequencing combined with other genome-wide screening methods may eventually identify other mutations. Alternatively, it is possible that patients lacking the *JAK2V617F* mutation may be false negatives due to sequencing granulocyte DNA in patients with erythroid (PV) or megakaryocytic (ET) lineage diseases. Mutational analysis of purified hematopoietic progenitors from patients with each of these diseases should resolve this question and may also identify the target cell for transformation.

Although nearly all patients had wild-type *JAK2* alleles in DNA derived from buccal swabs, 12 patients (4%) had V617F substitutions. Further study will be required to determine whether the *JAK2V617F* allele is a heritable disease allele in families at increased risk for PV and other MPD. Our study protocol did not permit for followup with family members for patients who reported family histories of MPD, but ultimately it should be possible to determine whether *JAKV617F* can be transmitted in the germline. Familial predisposition to PV and other MPD has been described, although the genetic basis for predisposition is not known (Kralovics et al., 2003). Inherited mutant alleles that activate kinases and other signaling intermediates in cancer have been described, including *PTPN11* in Noonan's syndrome associated with juvenile myelomonocytic

leukemia and *RET* mutations in multiple endocrine neoplasia type II (Mulligan et al., 1993; Tartaglia et al., 2001). The observation that patients may carry heterozygous *JAK2V617F* alleles in their germline but not develop clinical disease until later in life suggests that the *JAK2V617F* allele may be a less potent disease allele than other inherited disease alleles; this has been observed with specific *RET* mutations in multiple endocrine neoplasia type II which often do not manifest until later in life (Gimm et al., 2002).

The identification of *JAK2* mutations in MPD is consistent with previous genetic and functional data. Loss of heterozygosity due to mitotic recombination at chromosome 9p24, the genomic locus of *JAK2*, has been identified in ~30% of patients with PV (Kralovics et al., 2002), which is similar to the frequency of *JAK2V617F* homozygous mutations in PV patients (~25%). *TEL/JAK2* fusion genes have been cloned from patients with atypical CML and ALL (Lacronique et al., 1997; Peeters et al., 1997), and expression of the TEL/JAK2 fusion protein in a retroviral murine bone marrow transplant model results in a high penetrance and short latency myeloproliferative and lymphoproliferative disease (Schwaller et al., 1998). Activating EPOR mutations that result in activation of JAK2 and its downstream effectors have been identified in patients with familial and congenital polycythemia (Arcasoy et al., 1997; Sokol et al., 1995; Watowich et al., 1999). In addition, JAK2-deficient mice die in utero due to defective definitive erythropoiesis, and JAK2-deficient myeloid progenitors do not respond to erythropoietin, thrombopoietin, or interleukin-3 (Parganas et al., 1998). Finally, several indirect lines of evidence have suggested involvement of the JAK-STAT pathway in PV (Moliterno et al., 1998; Schwartz, 1998). Taken together, these data provide additional support for the *JAK2V617F* mutation as a disease allele in MPD.

The *JAK2V617F* allele may have diagnostic and therapeutic value in myeloproliferative diseases. For example, it may be of value as a complement to diagnostic tests, such as red blood cell mass, in delineating PV from secondary erythrocytosis. In addition, comprehensive genotypic analysis should ultimately allow for the development of molecularly annotated diagnostic approaches to PV, ET, and MMM, in which there is considerable phenotypic overlap. Perhaps most importantly, the *JAK2V617F* mutation may represent a therapeutic target in patients with MPD. Pharmacologic inhibition of activated tyrosine kinases is effective in treatment of several cancers (Apperley et al., 2002; Cools et al., 2003; Demetri et al., 2002; Druker et al., 2001; Lynch et al., 2004; Paez et al., 2004). Our data indicate that JAK2V617F is a constitutively activated tyrosine kinase that can also be inhibited with small molecule inhibitors in vitro, and may be a good target for therapeutic intervention in a subset of PV, ET, or MMM. Finally, activation of the JAK-STAT pathway has also been reported in a spectrum of solid tumors, and thus genotypic analysis of JAK family members in solid tumors may also be warranted.

Experimental procedures

Harvard myeloproliferative disorders study

From January 2004 through January 2005, biologic samples and clinical data were collected through a protocol that was approved by the Dana-Farber Cancer Institute Institutional Review Board, and all subjects provided written informed consent. The study was advertised through the website http://www.mpdinfo.org. All patients 18 years or older with ET, PV, or MMM residing in the United States who had not undergone stem cell transplantation were eligible for the study. Potential participants e-mailed investigators indicating their initial interest, their names, and their mailing addresses. Information packets including a cover letter detailing study goals and requirements, a consent form, a release of medical records form, a self-administered questionnaire, and a self-addressed, stamped envelope were sent via U.S. mail to potential participants. The self-administered questionnaire consisted of 46 items, including the Medical Outcomes Study Short Form 12 (SF12) (Ware et al., 1996) and the Katz modification of the Charlson comorbidity index (Katz et al., 1996). Additional constructed questions collected information regarding diagnosis, symptoms at presentation, and treatment.

After signed consent forms and questionnaires were returned to investigators and eligibility verified, medical records were requested for abstraction and biologic sample kits were mailed to participants. In this study design, diagnosis relied upon patient self-report. In cases where patients indicated they had two or more diagnoses, first diagnosis was taken as the patient's diagnosis. If a blood sample did not yield adequate DNA, or if self-reported diagnosis was other than PV, ET, or MMM, the patient was excluded from the genetic analysis. In order to verify validity of self-reported diagnosis, medical records including pathology samples, laboratory values, and physician notes were reviewed to confirm if records supported the patient's self-reported diagnosis.

Sample collection and processing

Biological sample kits contained two 10 ml sodium heparin tubes and a buccal swab (MasterAmp, Epicentre). Patients were asked to have two tubes of blood drawn at the time of their next visit to their local hematologist, and sample kits were returned by overnight delivery. Blood samples were subjected to density centrifugation with Ficoll Histopaque 1077 (Sigma Chemical). The granulocyte layer was collected and DNA was extracted using the QIAamp DNA Blood Maxi Kit (Qiagen). RNA was isolated using Trizol (Invitrogen). DNA was isolated from buccal swabs using the QIAamp DNA Mini Kit (Qiagen).

Tyrosine kinase exon sequence analysis

Primers were designed to amplify and sequence exons encoding the activation loops and autoinhibitory domains of 85 of the 90 tyrosine kinases (Manning et al., 2002), with the exception of *AATK*, *DKFZp434C1418, STYK1, LMTK2*, and *LMTK3.* External gene-specific primers and internal M13-appended PCR primers for the activation loops of receptor tyrosine kinases were identical to those previously described (Paez et al., 2004), and additional primers are listed in Supplemental Table S3. PCR products were purified (SPRI, Agencourt Bioscience Corporation), followed by bidirectional sequencing and sequence detection on an ABI Prism 3730 DNA Analyzer (Applied Biosystems) using conditions previously described (Paez et al., 2004). Sequence analysis of *JAK2* exon 14 was performed by single round of PCR amplification using primers Exon 14F (5′ GTAAAACGACGGCCAG TTGCTGAAAGTAGGAGAAAGTGCAT') and Exon 14R (5′CAGGAAACAGCT ATGACCTCCTACAGTGTTTTCAGTTTCAAAAA3′). Sequencing used a specific forward sequencing primer (5′AGTCTTTCTTTGAAGCAGCAA3′) and M13 reverse primer. Sequence analysis of bidirectional sequence traces was performed using Mutation Surveyor version 2.28 (SoftGenetics). Candidate mutations were reamplified and sequenced from the original DNA sample for independent verification.

Genotyping of *JAK2V617F* allele

Genotyping was performed by MALDI-TOF MS analysis. Forward (5′-ACGTTGGATGAGGCATTAGAAAGCCTGTAG-3′) and reverse (5′ACGTTG GATGGCTTTCTCACAAGACATTTGG-3′) PCR primers and the primer extension probe (5′-ACTCTCGTCTCCACAGA-3′) were designed using SpectroDESIGNER software (Sequenom). Following PCR amplification, unincorporated nucleotides were dephosphorylated using shrimp alkaline phosphatase (Amersham). Primer extension was carried out in the presence of dATP, ddCTP, ddGTP, and ddTTP using ThermoSequence polymerase (Amersham). Primer extension reaction products were loaded onto a SpectroChip (Sequenom) and analyzed by MALDI-TOF (SpectroREADER, Sequenom). SpectroTYPER (Sequenom) was used to analyze mass spectra and determine genotypes based on peak intensities of the predicted primer

extension products. A detailed protocol is available at http://www.hapmap.org/downloads/genotyping_protocols.html.

Measurement of gene copy number by quantitative PCR

Quantitative PCR was performed using the *Factor VIII* locus as a reference locus as previously described, except that an ABI 7700 sequence detector was used (Wilke et al., 2000). A probe was designed for *JAK2* exon 22 (5′TCCAATTTTAACTCTGTTCTCGTTCTCCACCA3′) using Primer Express software (Applied Biosystems). Priming sequences were JAK2 F (5′-CAC AGGGATCTGGCAACGA-3′), and JAK2 R (5′-TTGTGGCAAGACTTTGGT TAACC-3′).

Amplification of sample DNA and of male reference DNA was prepared in parallel and amplified on the same run for each sample in triplicate. A single copy control was not available, so all samples were compared to male reference DNA with 2 copies of the *JAK2* locus. Copy number of the *JAK2* locus per haploid genome for each sample was determined by the ratio of the means of relative copy number of the *JAK2* locus and the *Factor VIII* locus in males, and by multiplying this ratio by a factor of 2 in females.

Fluorescence in situ hybridization

Bone marrow fixed cells from patients with verified diagnosis of PV were retrospectively selected from the collection at the Department of Human Genetics (Leuven) for FISH. FISH was performed as described (Martin-Subero et al., 2002). Two probes spanning the *JAK2* locus, 197L5 and 725M10, were used (RPCI6 library, available at http://www.chori.org/BACPAC). These patients were also genotyped for the presence of the *JAK2V617F* mutation.

Expression vectors

The murine and human *JAK2* cDNAs, respectively, were cloned into the retroviral vector MSCV-Neo/Puro. The *JAK2V617F* mutation was generated using site directed mutagenesis (Quikchange-XL, Stratagene) and confirmed by full-length DNA sequencing.

Cell culture

293T cells were grown in Dulbecco's modified Eagle's medium with 10% fetal calf serum. Transient cotransfection of 293T cells and generation of retroviral supernatant were performed as previously described (Schwaller et al., 1998). Ba/F3 cells expressing the murine EPOR (BaF3-EPOR) (D'Andrea et al., 1991) were grown in RPMI medium with 10% fetal calf serum and 1 unit of EPO per milliliter. Ba/F3-EPOR cells were transduced with retroviral supernatant containing either the MSCV-*JAK2*-Neo or MSCV-*JAK2V617F*-Neo vectors, respectively, and then selected in G418 (1 mg/ml). To assess for EPO sensitivity, the transduced Ba/F3-EPOR cells were washed three times in phosphate-buffered saline (PBS) and cultured at concentrations of EPO 1 U/ml, 0.01 U/ml, 0.001 U/ml, or without EPO for 7 days, respectively. The number of viable cells was determined by trypan blue exclusion. Leukemia cell lines HEL, K562, RPMI-8402, and MOLT16 were grown in RPMI medium with 10% fetal calf serum without cytokines. For Western blotting, leukemia cell lines were incubated in the presence of JAK inhibitor I (Calbiochem) for 4 hr. For dose response curves, leukemia cell lines were incubated for 72 hr in the presence of JAK inhibitor I and the number of viable cells was determined with the CellTiter 96 Aqueous One Cell Proliferation Assay (Promega). The percentage of apoptotic cells at 72 hr was determined by flow cytometric analysis of annexin V-fluorescein and propidium iodide (Roche) as previously described (Cools et al., 2004).

Western blotting

Cell lines were collected and lysed in lysis buffer (Invitrogen) and separated by electrophoresis as previously described (Cools et al., 2003). The following antibodies were used for Western blot analysis: anti-phospho-JAK2 (Cell Signaling), anti-JAK2 (polyclonal antibody raised against the JH1 domain of JAK2), antiphosphotyrosine (4G10) (Upstate), anti-phospho-STAT5 (Cell Signaling), anti-STAT5α (Cell Signaling), anti-phospho-ERK1/2 (Cell Signaling), peroxidase-conjugated antimouse immunoglobulin (Amersham Pharmacia Biotech), and peroxidase-conjugated antirabbit immunoglobulin (Amersham Pharmacia Biotech).

Model of JAK2 JH2 domain structure

The SwissModel automated comparative protein modeling server (http://swissmodel.expasy.org) was used to create an energy-minimized model of the JH2 domain of JAK2 (Schwede et al., 2003). The sequence for residues Ser550 to Leu808 of JAK2 was submitted to SwissModel for a first approach mode search using the Protein Data Bank (http://www.pdb.org) deposited structure for Bruton's tyrosine kinase (PDB accession code 1K2P) as a three-dimensional template (sequence identity 26%) (Mao et al., 2001). An energy-minimized model of the JH2 domain of JAK2 was determined and manually checked for global reasonableness. The location of Val 617 in this model is in a similar conformation to the analogous valine residue in other tyrosine kinase crystal structures that have been solved, e.g., Val256 in CSK (PDB accession code 1BYG) (Lamers et al., 1999).

Statistical methods

The association between mutational status, either heterozygous, homozygous, or germline, and subject characteristics such as current age, age at diagnosis, gender, race, comorbidity, physical functioning, prior thrombosis, self-reported family history, and disease duration was tested by Chi square, Fisher's exact, or Wilcoxon rank-sum statistics as appropriate. Only p values less than 0.01 are reported because of multiple testing.

Supplemental data

Supplemental Data for this article can be found at http://www.cancercell.org/cgi/content/full/7/4/387/DC1/.

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