Review

JAK2 V617F in Myeloid Disorders: Molecular Diagnostic Techniques and Their Clinical Utility

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In early 2005, several groups of investigators studying myeloid malignancies described a novel somatic point mutation (V617F) in the conserved autoinhibitory pseudokinase domain of the Janus kinase 2 (JAK2) protein, which plays an important role in normal hematopoietic growth factor signaling. The V617F mutation is present in blood and marrow from a large proportion of patients with classic BCR/ABL-negative chronic myeloproliferative disorders and of a few patients with other clonal hematological diseases such as myelodysplastic syndrome, atypical myeloproliferative disorders, and acute myeloid leukemia. The JAK2 V617F mutation causes constitutive activation of the kinase, with deregulated intracellular signaling that mimics continuous hematopoietic growth factor stimulation. Within 7 months of the first electronic publication describing this new mutation, clinical molecular diagnostic laboratories in the United States and Europe began offering JAK2 mutation testing on a fee-for-service basis. Here, I review the various techniques used by research groups and clinical laboratories to detect the genetic mutation underlying JAK2 V617F, including fluorescent dye chemistry sequencing, allele-specific polymerase chain reaction (PCR), real-time PCR, DNA-melting curve analysis, pyrosequencing, and others. I also discuss diagnostic sensitivity, performance, and other practical concerns relevant to the clinical laboratory in addition to the potential diagnostic utility of JAK2 mutation tests. (*J Mol Diagn* 2006, 8:397–411; DOI: 10.2353/jmoldx.2006.060007)

In March and April of 2005, multiple research groups with an interest in myeloid neoplasia reported a new and exciting insight into the pathobiology of myeloproliferative disorders (MPDs): the widespread occurrence of a somatic, acquired point mutation in a highly conserved residue of the autoinhibitory domain of the Janus kinase 2 (JAK2) tyrosine kinase.^{1–5} Before this discovery, several other tyrosine kinase mutations had been associated with myeloid malignancies,⁶ but the key signaling abnormalities in most of the BCR/ABL-negative myeloproliferative disorders remained mysterious. The remarkable clinical success with the drug imatinib mesylate, a BCR/ABL kinase inhibitor active in chronic myeloid leukemia and in several rarer forms of myeloid neoplasia, spurred great interest in this line of investigation.^{7,8}

The specific genetic mutation observed, c.1849 G>T (GenBank accession no. NM_004972; mutations are described herein using the proposed standard nomenclature of den Dunnen and Antonarakis⁹), results in substitution of phenylalanine for valine, both hydrophobic nonpolar amino acids, at position 617 of the JAK2 protein (p.V617F, hereafter referred to simply as V617F), within the JH2 pseudokinase domain.¹⁰ Loss of JAK2 autoinhibition results in constitutive activation of the kinase, analogous to other mutations in MPDs and leukemia that aberrantly activate tyrosine kinases.^{1,11,12} Because JAK2 is normally responsible for signaling from various growth

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Figure 1. Schematic of the JAK-STAT signaling pathway. An extracellular ligand initiates the JAK-STAT signaling cascade by binding to type I cell-surface cytokine receptor (ie, a receptor without its own intracytoplasmic kinase domain). This event triggers a conformation change in the receptor that may include dimerization; the erythropoietin receptor is already dimerized, so the two chains merely move closer when erythropoietin binds. Regardless, ligand-receptor binding brings two nonreceptor JAK molecules that are already bound to the receptor into close apposition. Several hematopoietic growth factor receptors, including those for erythropoietin and thrombopoietin, are type I cell-surface receptors. On receptor activation, JAK molecules phosphorylate each other as well as the intracytoplasmic domain of their associated receptor. Phosphorylated cell-surface receptors attract STAT molecules, which also dimerize and are consequently activated. Then the activated STAT dimer translocates to the nucleus and binds to target genes, altering transcription. The process is tightly regulated by various extrinsic proteins that are not shown for clarity. Reprinted from Nelson and Steensma¹⁰ with permission.

factor receptors (Figure 1), including those for erythropoietin and thrombopoietin, this mutation results in deregulated intracellular signaling with cell proliferation that is independent of normal growth factor control (eg, abnormal “endogenous” erythroid colony growth *in vitro* and erythrocytosis in mice transfected with the mutant gene).

In the original patient series that appeared in early 2005, JAK2 V617F was found in 65 to 97% of patients with clonal polycythemia vera (PV) (overall detection rate 74%), in 23 to 57% of samples from patients with essential thrombocythemia (ET) (overall, 36%), and in 35 to 57% of those with chronic idiopathic myelofibrosis (IMF) (overall, 44%) (reviewed in Refs. 10 and 13). Most series used the 2001 World Health Organization clinicopathological diagnostic criteria for hematopoietic neoplasms^{14,15} as the “gold standard” for purposes of determining sensitivity and specificity of the JAK2 mutation. The relationship of the mutation to MPD cases classified using the older Polycythemia Vera Study Group diagnostic criteria¹⁶ was similar.¹⁷

A flurry of papers describing the prevalence of the JAK2 V617F in various disease subsets and clinical correlates of the mutation quickly followed these seminal reports. As a barometer of this activity, it is notable that there were 502 PubMed-accessible articles on JAK2 published in 2004, compared with more than 1200 in 2005 (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?DB=pubmed>; accessed January 9, 2006). The subsequent studies confirmed the strong link between the mutation and classic BCR/ABL-negative MPDs but also detected the JAK2 V617F mutation in rare cases of other clonal myeloid disorders (reviewed in Ref. 18). The latter included subtypes of myelodysplastic syndrome (MDS),¹⁹ chronic myelomonocytic leukemia,^{19–21} acute myeloid leukemia (AML, especially AML arising from MPD^{20–23}), systemic mastocytosis,¹⁹ hypereosinophilic syndrome,^{19,24} chronic neutrophilic leukemia,^{19,25} atypical CML,²¹ juvenile myelomonocytic leukemia,²⁶ MDS with fibrosis,²⁷ the World Health Organization provisional entity refractory anemia with ringed sideroblasts with thrombocytosis (RARS-T),^{28,29} and various unclassi-

fiable MDS/MPD cases.²⁴ Why the same mutation should be associated with such diverse phenotypes remains an unanswered question^{10,18} and is a subject of active investigation.

To date, the V617F mutation has not been found in any of more than 700 healthy persons tested^{10,30} nor in any patients with secondary (reactive) blood count abnormalities mimicking clonal myeloproliferation. This specificity is of great importance because there is considerable clinical overlap between reactive cellular proliferations and clonal myeloproliferative disorders, making a definitive diagnosis challenging in some cases; a 100% specific test of even moderate sensitivity would be of marked help to clinicians. Likewise, there have been no convincing descriptions of germline JAK2 mutations in patients with hematopoietic malignancies, including families with multiple members with MPDs.³¹ In the few instances in which the V617F mutation was present in buccal cells in a MPD patient, the mutation was not present in hair follicles,^{1,3} suggesting contamination of the buccal cell collection by blood cells. In addition, JAK2 V617F has not been detected in patients with acute lymphoblastic leukemia,^{20,32} B-cell lymphoproliferative disorders,^{20,33,34} multiple myeloma,³⁴ or various solid tumors.³⁴ It has also not been observed in patients with myeloproliferation who have other activating tyrosine kinase mutations underlying the cell growth (eg, BCR/ABL fusion, FLT3 p.D835 mutations or juxtamembrane-domain internal tandem duplication, or c-KIT p.D816V mutation).³⁵ These observations suggest that JAK2 V617F is specific for myeloid lineage proliferation³⁶ and that it may be mutually exclusive of most other activating tyrosine kinase mutations.

In most cases of ET and IMF, patient samples contain both wild-type and mutant *JAK2* genomic DNA and transcripts. This may be due to mixed clonality, with residual normal hematopoietic elements and contaminating non-clonal tissues such as lymphocytes admixed with mutant clones, or it may be due to heterozygosity for the autosomal mutation (encoded on chromosome 9p24).¹⁰ In contrast, rare patients with ET (ie, <10% of patients) and with IMF and approximately one-third of PV patients have samples containing only the mutant allele and have been referred to as homozygous for the mutation.^{1,2,4,35} Several analyses, including microsatellite analysis and fluorescent *in situ* hybridization, revealed that homozygosity for the *JAK2* mutation arises by mitotic recombination, resulting in uniparental disomy.^{1,2,4,35,37}

Clinical Relevance of JAK2 V617F

The clinical significance of JAK2 V617F in patients with MPDs remains uncertain, at least in terms of influence of the mutation on the disease course, ie, on the incidence of clinically relevant complications and the time to disease progression or death. Because almost all patients with PV have JAK2 V617F (97% in one series⁴), it has been difficult to generate a well-defined comparison group of patients who have wild-type JAK2 exclusively yet can be convincingly diagnosed with bona fide PV. For other MPDs such as ET and IMF, in which the JAK2

mutation incidence is closer to 50%, comparisons can be made more easily. Several groups have described features including an increased hemoglobin level or neutrophil count,^{38–40} higher rate of thrombotic or hemorrhagic events,^{2,36,38,39} increased fibrosis² (see also²⁷), or more frequent pruritus³⁶ in patients who have the JAK2 mutation when compared with those who do not. However, because the consequence of fibrosis itself on the disease course is uncertain in ET, and thrombotic events in MPDs are usually preventable with adequate cytoreductive and antiplatelet therapy,⁴¹ the true clinical relevance of JAK2 mutation status in ET and IMF remains somewhat unclear. In addition, there is disagreement about whether the thrombotic risk is truly elevated with the mutation^{42,43}; in any case, overall survival does not appear to be altered.^{39,42,43}

At least one report suggested that MPD patients with JAK2 V617F required more cytoreductive therapy than those without V617F,² which makes sense in light of the relative elevation of white blood cell count and hemoglobin. Along these lines, Campell et al³⁹ reported that ET patients with the mutation were more sensitive to hydroxyurea but not to anagrelide when treated in the context of the United Kingdom Medical Research Council's Primary Thrombocythaemia trial (MRC PT-1).

Because the relevance of JAK2 mutation status on clinical outcomes once the diagnosis of MPD has been made is unclear, the most important role of JAK2 mutation testing at present seems to be during the initial evaluation of patients with myeloproliferation. Given the high specificity of the mutation for clonal myeloid disease, JAK2 V617F, when present, can definitively confirm the diagnosis. This fact was recognized quickly after initial publication of the mutation's prevalence.¹³ By April 2005, several research laboratories were already offering no-charge JAK2 mutation testing to community hematologist-oncologists, highlighting the great interest and perceived need for such testing among clinicians caring for patients with suspected MPDs. By October 2005, several clinical diagnostic laboratories had validated methods of JAK2 mutation detection and began to offer this test to clinicians and referring pathologists on a fee-for-service basis. Currently, more than a dozen molecular diagnostic laboratories in the United States and several in Europe are offering clinical JAK2 V617F mutation testing, with many others planning to bring tests on-line in the near future. Of interest, the assays offered by these laboratories vary, and the techniques used differ considerably in their performance characteristics. Below, some considerations for JAK2 diagnostic testing relevant to the clinical molecular diagnostic laboratory are reviewed, and then where JAK2 mutation testing might have a role in diagnostic algorithms for specific patient scenarios is discussed.

Diagnostic Techniques and Test Characteristics

Direct DNA Sequencing

Most of the original reports of JAK2 V617F studied the prevalence of the encoding mutation by means of direct

Figure 2. Fluorescent dye sequencing chromatographs from patients with *JAK2* c.1849G>T point mutations of varying clonality (mixed wild-type with mutant DNA in the **middle** and exclusively mutant DNA on **top**). Wild-type sequence is shown at the **bottom** for comparison. The patients in this series had atypical myeloproliferative disorders, and detection of the mutation facilitated diagnosis. Reprinted from Steensma et al¹⁹ with permission.

sequencing of polymerase chain reaction (PCR)-amplified genomic DNA template^{1,3,19,24} or PCR-amplified complementary DNA template generated from mRNA^{2,5,44} or both using the fluorescent dye-terminator variant (eg, BigDye) of the Sanger sequencing method (Figure 2).⁴⁵ In this method, a primer-extension reaction is performed with an amplified DNA template using mixture of dye-labeled dNTPs, and the product is detected by a standard multiwave fluorescence detector (eg, ABI Prism 3100-Avant four capillary system; Applied Biosystems, Foster City, CA) after capillary gel electrophoresis. Although this method provides detailed information and is considered a method of “direct visualization” of sequence information, it has limited sensitivity because of background noise in the generated chromatograms.⁴⁶

DNA mixing experiments have demonstrated that for most point mutations, automated sequencing is only sensitive down to about 20% of mutant DNA in a wild-type background.^{4,47} Sanger sequencing techniques may be even less sensitive if autoradiography is used for product detection, as was formerly common, rather than fluorescence. This issue is quite relevant to chronic myeloid disorders, where blood and marrow are often composed of a mixture of neoplastic and residual normal hematopoietic elements. Especially in the case of ET, in which “homozygosity” for *JAK2* V617F is rare, and MDS, in which phenotypically apparent gene mutations may be present in tiny clones comprising less than <10% of the total marrow cell population (compare with the *ATRX* gene mutations in acquired thalassemia in MDS⁴⁸), direct sequencing simply may not offer the desired or required sensitivity for diagnostic purposes. James et al¹⁷ explored this issue specifically with respect to *JAK2* c.1849 G>T by performing a series of mixing experiments with HEL erythroleukemia cells, which bear the *JAK2* mutation, admixed with TF-1 erythroleukemia cells, which do not. They failed to detect the mutated allele when it was present in <5% of the total DNA. With homozygous mutant patient DNA diluted in DNA from a healthy person, sequencing was even less sensitive (10%) than it was with the cell lines.¹⁷

Various groups have used differing oligonucleotide primers and PCR amplification protocols for *JAK2* sequencing. Our laboratory uses the following protocol, which we have found to be quite robust and which has

been successfully ported to several other laboratories.^{19,36,49} For a 50- μ l PCR reaction, we use 25 ng of DNA template, 5 μ l of 10 $\times$ Roche buffer (Roche Diagnostics, Mannheim, Germany) with a final concentration of MgCl₂ of 1.5 mmol/L, 1.5 U of *Taq* polymerase (Roche), 0.8 mmol/L dNTPs (Roche), and 20 pmol/L each of sense and antisense primers: 5'-TGCTGAAAGTAGGAGAAAGTGCAT-3' and 5'-TCCTACAGTGTTCCTCAGTTTCAA-3', respectively. PCR cycling parameters are as follows: an initial denaturing cycle of 94°C for 2 minutes; then 35 cycles of 94°C for 30 seconds, 52°C for 40 seconds, and 72°C for 40 seconds; followed by a final extension cycle of 72°C for 2 minutes. The amplicons are then verified by agarose gel electrophoresis and sequenced using the same primers used for PCR amplification.

Direct DNA sequencing is valuable as a research technique but faces challenges when used in the clinical laboratory setting. Sequencing is relatively labor intensive, time consuming, and expensive and requires specialized equipment that may not always be readily available. In some cases, the extra effort of sequencing may be worthwhile, such as when there is considerable allelic heterogeneity in a gene that underlies a clinical phenotype. When there is little or no allelic heterogeneity, as is the case with *JAK2* V617F, however, then other techniques are often more suitable for a diagnostic laboratory. Other than the c.1849 G>T mutation that results in V617F transversion, only two other point mutations have been described to date in the *JAK2* gene in association with hematological disease: p.L611S in a patient with acute lymphoblastic leukemia³² and p.K607N in a patient with AML.²² The L611S and K607N mutations were also clonally restricted, probably constitutively activating *JAK2* in the same fashion as V617F.³² However, in more than 1000 patients with nonleukemic MPDs studied by sequencing, no other *JAK2* mutations besides V617F have yet been described. Thus, the V617F mutation apparently represents the overwhelming majority of hematological neoplasia-associated *JAK2* mutations, yet testing for this specific allele is not perfectly sensitive (discussed further below).

Allele-Specific PCR (Amplification Refractory Mutation System [ARMS])

The ARMS exploits the fact that oligonucleotide primers must be perfectly annealed at their 3' ends for a DNA polymerase to extend these primers during PCR.⁵⁰ By designing oligonucleotide primers that match only a specific DNA point mutation, such as that encoding *JAK2* V617F—primers that do not bind the wild-type allele—ARMS can distinguish between polymorphic alleles. Therefore, these techniques go by the alternative names of “allele-specific PCR” (AS-PCR) or “sequence-specific primer PCR.” It is necessary to set up a control reaction in the same tube as the ARMS reaction to ensure that lack of product generation from a given sample is not simply due to failure of the PCR reaction rather than absence of the mutation that the assay is probing for.

Using established principles³⁰ or a bespoke computer program,³⁵ several groups have generated ARMS/AS-PCR primer sets for detection of the *JAK2* c.1849 G>T mutation. Oligonucleotides used by Jones et al³⁵ for this purpose included forward outer, 5'-TCCTCAGAACGTTGATGGCAG-3'; reverse outer, 5'-ATTGCTTCCTTTTCACAAGAT-3'; forward wild-type-specific, 5'-GCATTTGGTTTAAATTATGGAGTATaTG-3'; and reverse mutant-specific, 5'-GTTTTACTTACTCTCGTCTCCACAaAA-3', where the underlined final base in the latter two primers anneals at the site of the G>T mutation, whereas the 3rd bp from the 3' end (lowercase) is intentionally mismatched to maximize allelic specificity. When amplicons generated in this way are resolved in an agarose gel, the wild-type primers can be shown to have generated a 229-bp product, whereas the mutant-specific primers give a 279-bp product.³⁵ Baxter et al⁴ used a different set of allele-specific primers with a mismatch at the 3rd bp from the 3' end for AS-PCR, whereas McClure et al³⁰ validated yet another set of AS-PCR primers for clinical diagnostic laboratory purposes that included a control reaction plus the following oligonucleotides: forward mutant-specific, 5'-GGTTTAAATTATGGAGTATGTI-3'; and reverse, 5'-5-carboxyfluorescein-TACACTGACACCTAGCTGTGA-3', where the 5-carboxyfluorescein dye was used to facilitate later PCR product resolution by chromatography.

One advantage of ARMS is its apparent high sensitivity to small amounts of mutant DNA in a wild-type background, at least when hot start *Taq*DNA polymerases are used to diminish the likelihood of nonspecific primer annealing. Baxter et al⁴ found 73% of PV samples to be mutant by conventional fluorescent dye chemistry sequencing, but this proportion increased to 97% when using ARMS. For ET, the difference was even greater—12% shown to be mutant by sequencing increased to 57% with ARMS—whereas for IMF, only one additional patient (of 16) was identified as mutant by ARMS (44 to 50% proportion increment). Baxter et al⁴ also claimed that their ARMS primers could detect a mixed-clonality V617F mutation if it was present in only 3% of cells, whereas Jones et al³⁵ demonstrated that they could detect the mutation at 1 to 2% admixture levels. McClure et al³⁰ reported 0.01% mutant detection sensitivity with their ARMS primers, but they compared this in clinical samples with a less demanding melting curve assay (discussed further below) and found complete concordance for results, ie, ARMS did not offer any diagnostic advantage. Although the clinical relevance of identifying tiny mutant clones comprising less than 10% of the total DNA is uncertain, detection of any neoplastic clone may be of diagnostic help in some situations, and the possible development of *JAK2* pathway inhibiting drugs in the future may make the level of detection offered by ARMS quite important. At present, several laboratories (eg, ARUP, Salt Lake City, UT; Oregon Medical Laboratories, Eugene, OR; and Rush Medical Laboratories, Chicago, IL) offer AS-PCR-based assays for clinical testing purposes.

Real-Time PCR and DNA-Melting Curve Analysis

Real-time monitoring of PCR product accumulation during thermocycling can be of value as a semiquantitative method of detecting a previously described gene mutation,⁵¹ such as *BCR/ABL* fusion or the missense mutation underlying *JAK2* V617F. In a real-time PCR experiment, fluorescent molecules (either nonspecific DNA-binding dyes such as SYBR Green I or specially labeled oligonucleotide probes) are incorporated into DNA during the exponential phase of PCR and are monitored as they accumulate. The assumption underlying real-time PCR is that the amount of fluorescence in the reaction chamber is directly proportional to the amount of amplicon present in the tube, which in turn is dependent on the amount of target template present at the beginning of the reaction.⁵²

DNA-melting curve assays can be used in conjunction with real-time PCR. Such assays are based on the principle that complementary-strand DNA hybridization is much stronger in the presence of a perfect primer-template match, compared with primer-template pairs that have a degree of base mismatching. To exploit this property, a short fragment of genomic DNA spanning a mutation site can be amplified in a real-time PCR machine—basically a thermocycler equipped with a fluorescence detector—and then analyzed through a temperature gradient to see at what temperature the two-strand interaction is disrupted.

In addition to unlabeled sense and antisense oligonucleotide primers, two fluorescent hybridization probes are also included in the PCR reaction, one with a donor fluorophore (such as a 3'-fluorescein tag) and the other with an acceptor fluorophore (eg, 5'-LC Red 640 tag). These probes bind within 1 bp of each other, and during the post-PCR-melting curve evaluation, this proximity allows fluorescence resonance energy transfer⁵³ with light emission that can be detected by the instrument. One fluorescent probe is designed to span the mutation site, and the probe itself contains sequence that is complementary to the wild-type sequence. This probe binds more tightly to wild-type sequence rather than sequence-containing point mutation.

Because the temperature of the reaction tube is continuously raised in the melting step, the probes dissociate from the accumulated PCR product as a function of their binding avidity. When dissociation from the PCR product occurs, fluorescence decreases. In the *JAK2* mutation detection assay designed by McClure et al,³⁰ the probe that binds to mutant DNA and has a 1-bp mismatch dissociates at 55°C, whereas the probe bound across wild-type sequence dissociates at 65°C, allowing the mutated and wild-type amplified DNA to be distinguished (Figure 3). This assay was sensitive to approximately 1 to 10% mutant DNA in a wild-type background.³⁰

Likewise, James et al¹⁷ compared fluorescent dye chemistry sequencing with two different real-time PCR-based mutation detection systems, one using a LightCycler instrument (Roche Diagnostics) and the other using a Taqman ABI Prism 7500 machine (Applied Biosystems). These real-time PCR techniques were each much more

Figure 3. Melting curve analysis for detection of *JAK2* c. 1849 G>T mutation. The PCR primer-probe setup is shown at **top**, and typical results for wild-type, mixed clonality, and exclusively mutant patient samples are at **bottom**. When the fluorescein and LC Red 640 tagged-probes are bound adjacently on the DNA template, this allows fluorescent resonance electron transfer, which is later disrupted by the DNA melting, facilitating product detection. The method is that described by McClure et al.³⁰

sensitive than sequencing and detected 0.5 to 1% of HEL cell line DNA diluted in TF-1 cell line DNA and 2 to 4% of homozygously mutated patient DNA diluted in DNA from a healthy person. Primer sequences were not provided in the report by James and colleagues, but the DNA-melting curves are distinct from those reported by McClure and colleagues, suggesting that the primer sets used were different. James and colleagues pointed out that if *JAK2* V617F mutation analysis by real-time PCR were used as the first step to investigate erythrocytosis (defined as a hematocrit >51%), 58 of 81 patients examined might have been spared other investigations because the positive predictive value of *JAK2* V617F for clonal myeloid disease appears to be 100%. A DNA-melting curve assay is the basis of the current orderable clinical assay provided by Mayo Medical Laboratories (Rochester, MN). The primers are as follows, with the site of the G>T mutation bound by the sensor probe underlined: forward primer, 5'-AAGCAGCAAGTATGATGAG-3'; reverse primer, 5'-CCTAGCTGTGATCCTGAA-3'; sensor probe, 5'-ATGTGTCTGTGGAGACGAGAG-fluorescein-3'; anchor probe, 5'-AGAAAGGCATTAGAAA-GCCTGTAGTTTACTT-LC640-3'.

Restriction Fragment Length Polymorphism (RFLP)

Unfortunately, the *JAK2* c.1849 G>T mutation does not result in creation of a new restriction site that is recognized by current commercially available restriction endonucleases. This mutation does, however, abolish a motif in the wild-type *JAK2* sequence that is recognized by the restriction enzyme *BsaXI*, derived from the *Bacillus stearothermophilus* 25B (Z. Chen) strain. The mutation cleavage and recognition sites are depicted in Figure 4. Although

abolition of a restriction site is not as satisfying as creation of a new site, because a negative enzymatic cleavage reaction could be due either to absence of the mutation or to failure of the digestion procedure, it can be useful as a first pass analysis. Reported proportional sensitivity depends in part on the method used to detect the fragments and is approximately 20% mutant DNA in wild-type background.

Despite the relative lack of sensitivity, RFLP analysis is simple to perform and quite inexpensive and therefore useful as a screening technique. Abnormal results generally require confirmation by another technique. Several commercial and academic laboratories (eg, Oregon Health Sciences University, Portland, OR) currently use RFLP techniques as an initial screening assay for *JAK2* V617F, which is then followed by fluorescent dye chemistry sequencing for confirmation. Restriction enzyme digestion is the basis of the *JAK2* V617F Activating Mutation Assay kit recently marketed "for research use only" by InVivoScribe Technologies (San Diego, CA). Additionally, both Baxter et al⁴ and Antonioli et al⁴² successfully used *BsaXI* in research studies of *JAK2* V617F.

Pyrosequencing

Pyrosequencing is a method of rapid genotyping that depends on the liberation of pyrophosphate (PPI) whenever a dNTP is incorporated into a growing DNA chain during template-driven DNA polymerization (Figure 5). During pyrosequencing, PPI is generated when the correct complementary dNTP is added sequentially to a reaction mix that includes patient-derived DNA template, DNA polymerase, and a sequencing primer. PPI then serves as the substrate for a subsequent detection reaction. In the presence of adenosine 5' phosphosulfate, PPI

BsaXI Restriction Endonuclease Recognition and Cleavage Sites

Relevant JAK2 Genomic DNA Sequence:

Wild-Type:

AAATTATGGAGTATGT**G**TCTGTGGAGACGA (sense)

Mutant:

AAATTATGGAGTATGT**T**TCTGTGGAGACGA (sense)

Figure 4. Recognition site for the *BsaXI* restriction endonuclease in the *JAK2* gene, used by several laboratories as the basis of a restriction length fragment polymorphism screening assay for the genetic mutation underlying JAK2 V617F. The mutation results in loss of enzyme recognition of the usual cleavage site. Undigested PCR amplicons after *BsaXI* incubation can be detected in various ways, including gel electrophoresis, electropherography, and chromatography.

is quantitatively converted to ATP by ATP sulfurylase. The ATP drives luciferase-mediated conversion of luciferin to oxyluciferin, generating visible light that can be detected with a charge-coupled device camera. Unincorporated dNTPs and excess ATP are degraded continuously by another enzyme, apyrase. The ATP sulfurylase, adenosine 5' phosphosulfate, luciferase, luciferin, and apyrase are all part of the pyrosequencing reaction mix.

Pyrosequencing has the advantage of presenting sequence data "in real time," without an additional post-PCR analysis procedure, and it is also semiquantitative. The latter property allows estimation of allelic ratio in hematopoietic chimerism (eg, in the post-stem cell transplant setting) or in mixed clonality/heterogeneous tissue samples, which is characteristic of MPDs. Pyrosequencing of *JAK2* using the automated PSQ HS 96 system (Biotage, Uppsala, Sweden) has been attempted by several groups,^{21,35} with dilution experiments similar to those described above showing a reported assay sensitivity of 5 to 10% mutant allele in a wild-type background.^{21,35} These same groups used the pyrosequencing technique to assess whether the *JAK2* mutation was "homozygous" or mixed clonality/"heterozygous." To my knowledge, at present, pyrosequencing is not the basis of clinical molecular testing in any laboratory. This is likely because of the relative expense of the proprietary systems that perform pyrosequencing and the limited numbers of personnel who are familiar with these devices.

Other Mutation Detection Techniques

Several other mutation detection techniques lend themselves to analysis of a known point mutation. At the nucleic acid level, these other techniques include single-stranded conformational polymorphism (SSCP) analysis, denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), single-nucleotide primer extension assays (Pronto), and others. In fact, DHPLC can detect the genomic DNA mutation underlying JAK2 V617F reliably (D.P. Steensma, unpublished data), and it can detect mutations at a proportionality of <1 to 2%. However,

DHPLC and the other techniques are either technically challenging or labor-intensive or both. They either do not allow high throughput at a cost suitable for a clinical laboratory (SSCP and DGGE) or require a considerable initial investment for equipment (DHPLC), or else they are proprietary technologies with appropriate kits available only for certain mutations that do not yet include JAK2 alleles (Pronto). In addition, mutations detected by DHPLC, DGGE, and SSCP usually must be confirmed with another technique. Like sequencing analysis, DHPLC, DGGE, and SSCP may be sensitive to other non-V617F mutations such as L611S and K607N, whereas techniques like ARMS, DNA-melting curve, and RFLP analysis would miss these, unless the assay were designed to detect them specifically.

Theoretically, protein-based techniques could also be used to detect the JAK2 V617F mutation, but these are generally cumbersome, and access to such resources is limited. Therefore, protein-based assays are usually not preferred if DNA- or RNA-based tests are feasible.

The Role of JAK2 Testing in Evaluating Patients with Suspected Myeloid Disorders

Cell of Detection

The JAK2 V617F mutation appears to arise in a common myeloid progenitor cell, and T lymphocytes are generally not part of the neoplastic clone bearing the mutation.¹⁹ In the one analysis that specifically studied CD19+ B cells from IMF patients who had the V617F mutation detectable in granulocytes, the JAK2 mutation was not found in the lymphocytes.⁵⁴ In contrast, the same group had previously detected B-lymphocyte involvement as part of the neoplastic clone in a subset of IMF patients with other mutations, suggesting that there is variability in the cell of origin in MPD.⁵⁵

Because Ficoll-Hypaque-separated mononuclear cell preparations from MPD patients contain both monocytes (clonal cells) and lymphocytes (usually not clonal), the proportion of JAK2-mutant cells is somewhat lower in such preparations than when granulocytes or buffy coat

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Figure 5. Pyrosequencing, as applied to *JAK2* mutation analysis. Pyrosequencing allows rapid generation of allele-specific information. **A:** Principles of pyrosequencing. dNTPs are added sequentially to primed DNA template, and cleaved ATP is generated from cleaved pyrophosphate. This ATP drives a luciferase reaction that is detected by a camera on the instrument, generating a histogram called a "pyrogram." **B:** Representative results in *JAK2* wild-type (top), mixed clonality mutant (middle), and homozygous mutant (bottom) patient samples. Nucleotides are designated below the diagrams; E and S stand for enzyme and substrate, respectively, and are used in pyrosequencing as controls. The rightmost G-peak is taller than the others because the wild-type sequence is GpG at this locus. Gray box denotes the site of the mutation. Pyrograms were generated by the method of Jelinek et al,²¹ which proceeds in the sense direction of the gene.

preparations are analyzed. For maximal diagnostic sensitivity, therefore, granulocytes purified by density centrifugation or flow cytometry should be used as the nucleic acid source for mutation analysis. Nevertheless, the *JAK2* V617F mutation is routinely detectable in monocyte preparations by any of the above techniques (T.L. Lasho,

personal communication) or in whole blood or marrow.³ Isolated CD34+ cells are of mixed clonality, even if granulocytes appear homozygous for the mutation.⁵⁴

To date, there have been no formal direct comparisons of *JAK2* mutation status between bone marrow and blood samples from the same patients, but anecdotal evidence

Table 1. Clinical Situations in Which JAK2 Mutation Testing May Be Useful

Erythrocytosis
If there is no obvious cause for secondary erythrocytosis (eg, hypoxia) or pseudo-erythrocytosis (eg, plasma volume contraction) or familial erythrocytosis
With low or normal serum erythropoietin level
With elevated serum erythropoietin level in the setting of hepatic injury (eg, Budd-Chiari syndrome)
If the patient refuses a bone marrow examination
Thrombocytosis
If BCR/ABL1 mutation analysis is negative
If there is no obvious cause of reactive thrombocytosis
If a cause of reactive thrombocytosis is present but the possibility of coexisting ET is suspected
If the patient refuses a bone marrow examination
Suspected "cellular phase" IMF
Marrow fibrosis where IMF is possible but karyotype is normal
Idiopathic neutrophilia where chronic neutrophilic leukemia is possible
Erythroid-only dysplasia with normal cytogenetics
Hypereosinophilia or mastocytosis where other mutation tests (c-KIT, PDGFR) are normal
Atypical conditions where a clonal myeloid disorder is suspected but marrow cytogenetics are normal
Familial myeloproliferative disorder of unknown etiology
Any myeloid disorder, when an effective JAK2 pathway inhibitor becomes available

(R.F. McClure, personal communication) suggests that there is complete concordance between these results.

General Diagnostic Considerations

The relatively high frequency with which the JAK2 V617F allele is found in MPD, especially PV, makes it an especially attractive target for diagnostic investigation. Currently, the evaluation of patients with cellular excess in one or more lineages is imperfect, relying heavily on exclusion criteria.⁵⁶ The first molecular test that offered an element of diagnostic certainty in MPD was the detection of *BCR/ABL1* fusion, and in view of the striking success with imatinib therapy and other tyrosine kinase inhibitors in CML,^{7,57} fluorescent *in situ* hybridization or reverse transcription-PCR assays for this rearrangement are still critical whenever a patient presents with leukocytosis or thrombocytosis and CML is a possibility. Additional molecular tests such as *FLT3* mutation analysis have been available for some years and offered modest prognostic value but were rarely of use in initial diagnosis.⁵⁸ Several laboratories offer testing for rearrangements of the subunits of the platelet-derived growth factor receptor,⁵⁹ c-KIT mutations, or other rarely found acquired genetic lesions, but these are only useful in certain narrow diagnostic niches such as hypereosinophilia⁶⁰ or mastocytosis.⁶¹ In contrast, the discovery of the JAK2 V617F mutation gives clinicians a second broadly applicable molecular test for MPD.⁶² Situations in which JAK2 mutation testing can be considered to prove clonality or offer diagnostic support are summarized in Table 1.

Because detection of JAK2 V617F so convincingly establishes the presence of a clonal disorder, I believe JAK2 mutation testing should be considered a front-line screening test for suspected MPD in the situations listed in Table 1, and its use as a first-intention diagnostic test may spare some patients further investigations.^{12,62,63} Which diagnostic investigations are still strictly necessary is an issue that needs to be studied formally. JAK2 mutation analysis is not a highly sensitive assay, especially in myeloid disorders other than PV. ("Sensitivity" is used

here in terms of the test's negative predictive value—used hereafter in the sense of "diagnostic sensitivity," which is distinct from the "laboratory detection sensitivity" of the various techniques for resolving small proportions of mutant DNA that is discussed above.) However, detection of acquired V617F appears extremely specific for a clonal myeloid disorder. In addition, the assays described above are relatively simple to perform and are easier for a clinical laboratory to set up and to validate than measurements such as endogenous *in vitro* erythroid colony formation, alterations in c-MPL and granulocyte *PRV-1* expression, or other markers of reported diagnostic utility in MPD.^{44,64} Furthermore, V617F has better test characteristics than these other assays, especially with respect to specificity, because c-MPL and *PRV-1* expression changes overlap between PV and secondary erythrocytosis.⁶⁵

Role of JAK2 V617F in Erythrocytosis Evaluation

A simple diagnostic algorithm for polycythemic conditions is presented in Figure 6. It should be noted that this proposed algorithm, although based on extensive clinical experience, has not been formally validated, and there is room for disagreement.

Erythrocytosis is defined as a hemoglobin or hematocrit level above the normal reference for the testing laboratory or a level within the normal reference range but still markedly (>2 g/dL) above the patient's own mean chronic normal value.⁶⁶ Like anemia, erythrocytosis should prompt further evaluation aimed at defining a specific diagnosis.

The first step in the evaluation of erythrocytosis is to establish clinically whether there is likely to be a genuine elevation in the patient's total body red cell mass.^{66,67} Relative erythrocytosis due to plasma volume changes causing elevated hematocrit is usually apparent from a history and physical examination, as when it is associated with marked edema or severe diarrhea. An elevated hemoglobin or hematocrit level on repeat testing usually represents a bona fide elevation in red cell mass. Mea-

Figure 6. Proposed diagnostic evaluation for erythrocytosis, incorporating JAK2 mutation analysis. Details are discussed in the text. Modified from Nelson and Steensma¹⁰ and Tefferi and Gilliland.⁶²

asuring the red cell mass in the nuclear medicine laboratory is primarily of historical interest,⁶⁸ except in rare situations, because this test does not distinguish non-clonal from clonal erythrocytosis and has poor test characteristics.⁶⁹ In addition, relative erythrocytosis of the degree that is most reliably detectable with red cell mass should already have been excluded in the initial clinical assessment of the patient.⁷⁰ Some still argue that a red cell mass measurement is important in all patients with suspect MPD, even if the hemoglobin and hematocrit are normal because the plasma volume may actually be expanded proportionate to the elevated red cell mass, leading to a pseudo-normalization of hematocrit and “inapparent PV.” Although a patient with PV could be labeled as having ET or IMF if a red cell mass measurement were not obtained, this is unlikely to be clinically relevant. There is no specific treatment for PV at present that is ineffective for ET or for cellular-phase IMF; phlebotomy, the mainstay of PV therapy, is directed toward lowering the hematocrit to <42 to 45%, which may correlate better with thromboembolic risk than does the total body red cell mass.⁷¹

Secondary erythrocytosis is most often due to hypoxia. A noninvasive *trans*-cutaneous measurement of blood oxygen

saturation is important, and overnight oximetry, echocardiography, or other tests may be needed to rule this out definitively. A family history of elevated hemoglobin or testing of first-degree relatives can be important. Erythrocytosis due to a familial defect in hypoxia sensing (eg, erythropoietin receptor mutations or the *VHL* mutation seen in Chuvas-type polycythemia) or a high-affinity hemoglobin (eg, Hb Yakima⁷²) is also not difficult to exclude, as long as it is suspected. Evaluation in this case would include measurement of hemoglobin oxygen-dissociation curves if a familial hemoglobin variant is possible. Several laboratories offer analysis of the erythropoietin receptor or *VHL* gene on a research basis,⁷³ if a mutation is suspected.

Measurement of the endogenous serum erythropoietin (EPO) level can provide appropriate “triage” of patients with erythrocytosis and directs further evaluation (Figure 6).^{62,74,75} Some clinicians obtain leukocyte alkaline phosphatase scores (usually high in MPD and low in CML) and vitamin B12 levels (often elevated in PV) as well, providing supplemental information, although these tests—minor criteria of the Polycythemia Vera Study Group’s algorithm—may no longer be necessary with JAK2 mutation testing.

High EPO

Patients with high endogenous EPO levels rarely have PV, except perhaps in the setting of acute Budd-Chiari syndrome with liver necrosis^{76,77} (see below), and such individuals should be asked about use of erythropoietic supplements (eg, clandestine use of darbepoetin alfa or epoetin alfa for augmenting athletic prowess) and investigated for conditions associated with hypoxia and for various erythropoietin-producing growths, including renal tumors, hepatomas, or cerebellar hemangiomas.

Low EPO

A low endogenous EPO level in a patient with erythrocytosis makes the diagnosis of PV likely,⁷⁸ although rare inherited polycythemic states can also be associated with low EPO levels.^{74,75,79} (Many patients with familial polycythemias also have an EPO level within the normal range.) Some argue that documentation of JAK2 V617F in peripheral blood is enough to secure the diagnosis of PV, regardless of the EPO level.⁶³ Certainly, in the setting of erythrocytosis with both JAK2 V617F and low EPO, there can be little doubt about this diagnosis. Others argue for more caution and strongly suggest proceeding with bone marrow examination regardless, because the discovery of JAK2 V617F is relatively new, the suspected 100% specificity of the test is not completely established, and there is much yet to learn about MPD.⁶² However, marrow examination in PV is not an exact science. Sometimes other possibly prognostically useful information can be obtained from the marrow, including cytogenetics⁸⁰ and the degree of reticulin and collagen fibrosis; the presence of another co-existent disorder, such as a plasma cell dyscrasia or mastocytosis, may be detected. There seems to be little role for testing for JAK2 V617F in both marrow and blood, given the concordance for the mutation mentioned above.

Normal EPO

Patients with erythrocytosis and normal EPO levels have historically presented the greatest diagnostic challenge. A normal EPO level may be physiologically appropriate or may be inappropriately normal. Here, JAK2 V617F mutation analysis may be particularly valuable. In the absence of PV-associated clinical features, JAK2 V617F blood testing may be a useful first screen for a clonal disorder. Patients with no JAK2 mutation and no worrisome clinical findings can be monitored over time with periodic blood counts. However, if PV-associated features are present—these include aquagenic pruritus, erythromelalgia, thrombosis, hepatosplenomegaly, abnormalities in other cell lineages, an elevated leukocyte alkaline phosphatase score, and others—the pre-test probability of PV is elevated, and bone marrow examination may be diagnostic. Here, documentation of the JAK2 V617F mutation would provide an extra measure of confidence.⁶²

Role of JAK2 V617F in Evaluation of Thrombocytosis

Because JAK2 V617F is less common in ET (30 to 50% of cases) than it is in PV, and because mutant clones in ET generally represent a smaller proportion of the total cellularity than for other MPD, defining the role of JAK2 mutation testing in the diagnostic evaluation of unexplained thrombocytosis is more challenging than for erythrocytosis.⁸¹ Exclusion of reactive thrombocytosis, iron deficiency, and functional hyposplenism accounting for an elevated platelet count remains important (Figure 7). Unless these causes for thrombocytosis are obviously present or are detected with simple blood tests (eg, C-reactive protein, iron studies, and a peripheral smear looking for Howell Jolly bodies), it seems prudent to obtain a bone marrow examination with cytogenetic studies and *BCR/ABL1* fluorescence in situ hybridization or reverse transcription-PCR testing (because CML can present with isolated thrombocytosis), as previously recommended.⁸² Even if a potential cause of secondary thrombocytosis is present, ET may still co-exist, although there are few data on the prevalence of this finding.

JAK2 mutation analysis can provide useful supplemental information in the work-up of thrombocytosis, especially when JAK2 mutation analysis results are abnormal.^{10,81,83} JAK2 V617F in the presence of isolated thrombocytosis (ie, where there is no erythrocytosis) and negative *BCR/ABL1* testing strongly support a diagnosis of ET, even if the marrow findings are ambiguous.⁶² JAK2 wild-type patients with thrombocytosis may need to be observed over time with serial blood counts or perhaps analyzed with supplemental techniques such as X-chromosome inactivation patterns. The latter—only relevant for younger women, because males have X hemizygosity and healthy women can develop age-relating X-inactivation skewing—can be problematic, because chromosomal skewing does not always correlate with clonally restricted hematopoiesis or with an ET phenotype with a risk of thrombohemorrhagic complications.^{81,84}

Occasionally, patients who are elderly or frail may not wish to undergo bone marrow examination under any circumstances. JAK2 V617F, if present in peripheral blood, may provide valuable diagnostic information in this setting, allowing more confident initiation of appropriate cytoreductive therapy with hydroxyurea or anagrelide. However, if the JAK2 mutation test is negative, one is no further along the road away from agnosticism.

Diagnostic Role of JAK2 V617F in Evaluating Other Suspected Myeloid Disorders

When marrow fibrosis and peripheral blood cytopenias are present but the marrow karyotype is normal, JAK2 V617F may confirm the presence of clonal IMF as opposed to a reactive marrow fibrotic process (eg, due to metastatic carcinoma, infection, or a connective tissue disease). However, as discussed above, the clinical rel-

Figure 7. Proposed diagnostic evaluation for thrombocytosis, incorporating JAK2 mutation analysis. Details are discussed in the text.

evance of the JAK2 V617F mutation in IMF is uncertain, and in the fibrotic phase of typical IMF, there is usually little diagnostic challenge. Thus, in practice, JAK2 molecular testing may not often be necessary for diagnosing IMF. A diagnosis of “cellular phase” IMF is more difficult to be certain about than typical fibrotic IMF, and there may be a role for JAK2 testing here if the morphological pattern is unclear and the marrow or blood karyotype is normal.

Atypical MPD cases (chronic myelomonocytic leukemia, chronic neutrophilic leukemia, idiopathic hypereosinophilia, etc.) can present significant diagnostic and management challenges. Therefore, even though JAK2 V617F appears to be uncommon in these patient groups,^{19,24} clinicians and pathologists may be looking for any available help, and JAK2 mutation testing is reasonable. If abnormal, the finding proves that the disorder is a clonal myeloid disease; if JAK2 results are negative, then nothing is lost.

The prevalence of V617F seems low enough in MDS (about 7%)^{19,24,85} and in AML (about 4%),^{24,34,86,87} and morphological and cytogenetic diagnosis of these conditions is solid enough that there is no compelling reason to test routinely for JAK2 mutations in these settings. However, a subset of patients with early MDS—espe-

cially those with pure erythroid dysplasia and normal cytogenetics—are difficult to distinguish from individuals with nonclonal disordered hematopoiesis; in such settings, JAK2 mutation assays could be useful, although this has not been tested. The JAK2 mutation status of the rare World Health Organization provisional MDS/MPD overlap syndrome known as refractory anemia with RARS-T²⁹ is not yet known, but a recent abstract suggested that a subset of RARS-T patients also have the JAK2 V617F mutation, as do some patients with unclassifiable MPD/MDS overlap conditions.²⁸

Special Situations: Budd-Chiari Syndrome and Familial MPD

Hepatic vein thrombosis (Budd-Chiari syndrome) is a well-recognized complication of MPD, especially PV, and of other hypercoagulable states.^{76,88} Occasionally, Budd-Chiari syndrome may be a presenting sign of MPD, and JAK2 V617F may be detectable at that time, helping to confirm the underlying etiology of the thrombosis.^{77,89} In one analysis, bone marrow examination in patients presenting with Budd-Chiari syndrome and an elevated platelet count or hemoglobin level was nondiagnostic in

12 of 25 cases that later turned out to be JAK2 V617F-positive, confirming an underlying clonal myeloid disorder.⁸⁹ Although most physiological EPO protein is synthesized by the kidney, a small amount is manufactured in hepatocytes, and hepatic necrosis can cause a transient release of EPO; therefore, an elevated serum EPO should not rule out PV in this setting.^{76,77} JAK2 mutation testing may be useful in this circumstance.

Although many familial erythrocytoses and thrombocytoses have now been molecularly defined, there are still some hereditary myeloproliferation susceptibility syndromes that remain mysterious.^{72,90} Thus far, there are no convincing reports of germline JAK2 mutations, but clinicians should remain open to this possibility and should consider JAK2 testing if there is a history of familial MPD. To this end, a testing method that assays for other JAK2 mutations besides JAK2 V617F (eg, direct sequencing instead of allele-specific PCR) could be used to broaden the approach.

Conclusions

JAK2 mutation testing provides clinicians and pathologists with valuable diagnostic information in many situations in which a patient is suspected of harboring a clonal myeloid disorder. Allele-specific PCR, DNA-melting curve analysis, and RFLP screening supplemented by direct fluorescent dye chemistry sequencing are currently the most popular techniques used in clinical laboratories for JAK2 V617F mutation detection.

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