SPECIAL ARTICLE

Laboratory Practice Guidelines for Detecting and Reporting JAK2 and MPL Mutations in Myeloproliferative Neoplasms

A Report of the Association for Molecular Pathology

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Recurrent mutations in JAK2 and MPL genes are genetic hallmarks of BCR-ABL1—negative myeloproliferative neoplasms. Detection of JAK2 and MPL mutations has been incorporated into routine diagnostic algorithms for these diseases. This Special Article summarizes results from a nationwide laboratory survey of JAK2 and MPL mutation analysis. Based on the current practice pattern and the literature, this Special Article provides recommendations and guidelines for laboratory practice for detection of mutations in the JAK2 and MPL genes, including clinical manifestations for prompting the mutation analysis, current and recommended methodologies for testing the mutations, and standardization for reporting the test results. This Special Article also points to future directions for genomic testing in BCR-ABL1—negative myeloproliferative neoplasms. (J Mol Diagn 2013, 15: 733–744; http://dx.doi.org/10.1016/j.jmoldx.2013.07.002)

In 2005, several groups simultaneously described a point mutation in codon 617 of the protein tyrosine kinase gene Janus kinase 2 (JAK2) in BCR-ABL1—negative myeloproliferative neoplasms (MPN). This mutation was found in the vast majority of patients with polycythemia vera (PV) and in approximately half of patients with essential thrombocythemia (ET) or primary myelofibrosis (PMF).1–4 In 2007, additional mutations in exon 12 of JAK2 gene were found in a small percentage of PV patients.5 The exon 12 mutations and the V617F mutation are mutually exclusive; either V617F or
exon 12 mutations are present in virtually all cases of PV. In 2006, mutations in a second gene, myeloproliferative leukemia virus oncogene (MPL), were identified in both ET and PMF patients.10 In contrast to JAK2 mutations, MPL mutations were found less frequently in PMF and ET, and may be present in either JAK2 mutation-positive or mutation-negative cases.6–8 Laboratory tests for these mutations have become standard in assessing clinically suspected BCR-ABL1—negative MPN.

The diagnostic value of JAK2 and MPL mutations in MPN is well established. The 2008 World Health Organization classification of hematopoietic neoplasms includes JAK2 mutations as diagnostic criteria in PV and JAK2 and MPL mutations in ET and PMF.9–11 A diagnosis of PV can be made when JAK2 V617F or exon 12 mutation is detected, along with increased hemoglobin and low or normal levels of erythropoietin.9 Clinically, patients with exon 12 mutations typically present with isolated erythrocytosis and suppressed erythropoietin, in contrast to the trilineage hyperplasia characteristic of patients with V617F mutation. Bone marrow from patients with exon 12 mutation often exhibits nonspecific morphology, with isolated erythroid proliferation and absence of prominent megakaryocyte atypia and clustering. Demonstration of exon 12 mutation in these patients is particularly helpful for ruling out reactive erythrocytosis.5

In ET and PMF, JAK2 V617F mutations and/or MPL exon 10 mutations are present in approximately 60% to 70% of patients, with no other well-defined, recurrent JAK2 or MPL mutations found in the remaining patients. Although detection of JAK2 V617F and MPL mutations in the appropriate clinical settings confirms the diagnosis of ET or PMF, absence of the mutations does not rule out disease. Rare mutations in TET2, ASXL1, IDH1/IDH2, EZH2, DNMT3A, and CBL have been described, although the precise roles of these mutations in the pathogenesis of ET and PMF are unknown.12–17 Other hematological neoplasms (eg, myelodysplastic syndrome, chronic myelomonocytic leukemia, acute myeloid leukemia, and acute lymphoblastic leukemia) may harbor JAK2 and MPL mutations in low frequencies.18–20 Because JAK2 and MPL mutations are not completely specific for MPN, finding of these mutations in isolation does not warrant a diagnosis of MPN.21

Several methods have been used to detect JAK2 and MPL mutations. Clinical laboratories may choose to use either commercial detection kits or laboratory-developed tests. With the availability of a broad selection of test methods, laboratories may find it challenging to select the methodology and platform most suitable for their practice. Results from different methods may differ with respect to analytical sensitivity, specificity, and clinical relevance.22 There is a lack of well-established consensus concerning common analytical issues, such as what specimen type should be tested, whether it is clinically relevant to differentiate heterozygous from homozygous mutations, whether granulocyte enrichment should be performed, how to interpret a low level of a positive result, and whether quantitative results should be used and reported (and, if yes, in which disease type and in what clinical condition). To address these issues from the laboratory perspective, here we present practice guidelines for JAK2 and MPL mutation testing and reporting. The guidelines were developed by an expert panel from the Association for Molecular Pathology (AMP) based on the current literature and practices in the United States and Canada. The guidelines are intended to establish a helpful reference for laboratory professionals in the selection of methodologies, validation of procedures, and interpretation of results in JAK2 and MPL testing. In addition, the guidelines can serve as a reference guide for clinical practitioners and other health care professionals in interpretation and utilization of the results. Practice guidelines also serve to identify questions and suggest areas for future development.

What Are the Currently Known JAK2 and MPL Mutations?

JAK2

The JAK2 gene maps to chromosome band 9p24 and encodes a tyrosine kinase protein composed of 1132 amino acids. It contains three critical domains: JH1, JH2, and four-point-one, ezrin, radixin, moesin (FERM) homolog domains. JH1, the catalytic phosphokinase domain, is located at the carboxyl terminus and induces phosphorylation of target proteins. JH2, which resides upstream to JH1, is structurally similar to JH1 but functions as a pseudokinase domain that negatively regulates basal activity of the kinase domain and receptor-induced activation of the catalytic function.23 The FERM domain, which resides at the amino terminus, functions as a binding protein transducing signals from JAK2 to other transmembrane cytokine receptors (Figure 1).24

JAK2 protein kinase activity is activated by phosphorylation of its kinase domain. Activation of JAK2 induces signal transduction from both type 1 and type 2 cytokine receptors. Constitutive activation of JAK2 by either point mutation or fusion protein causes activation of the JAK/STAT pathway. The activated JAK2 causes phosphorylation of STATs, which then dimerize and translocate to the nucleus, where they regulate gene transcription. Somatic mutations, such as V617F mutation in JH2, disrupt the inhibitory function of the pseudokinase and thus lead to constitutive tyrosine phosphorylation activity that promotes cytokine hypersensitivity (UniProt, http://www.uniprot.org/uniprot/O60674, last accessed November 28, 2012).

Although various JAK2 mutations have been reported in exons 12, 13, 14, and 15, in the vast majority of the cases mutations are found in codon 617, resulting in the replacement of the amino acid valine with phenylalanine [V617F, alias JAK2 NM_004972.3:c.1849G>T (p.Val617Phe)]. This mutation is present in approximately 96% of PV cases, 55% of ET cases, and 65% of PMF cases. Exon 12 mutations are far less common, present in only approximately 3% of PV cases. In contrast to the V617F mutation, which involves
one amino acid codon, exon 12 mutations affect a larger region, spanning codons 533 to 547. At least 27 clinically verified exon 12 mutations have been identified to date, including amino acid substitution, deletions, and duplications (Table 1). Additional exon 12 mutations have been reported, but their clinical significance is unknown. The most frequent exon 12 mutation involves an in-frame deletion of six nucleotides at codons 542 and 543 (N542_E543del); it is present in approximately 40% of V617F-negative PV. Other relatively common mutations include R541_E543delinsK (approximately 10%), E543_D544del (approximately 10%), F537_K539delinsL (approximately 10%), K539L (approximately 10%), H538_K539delinsL (approximately 5%), and I540_E543delinsMK (approximately 5%).

**Table 1** JAK2 Mutations in Myeloproliferative Neoplasms

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Exon</th>
<th>Frequency</th>
<th>Nucleotide position†</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>V617F</td>
<td>14</td>
<td>~96% PV, ~55% ET, ~65% PMF</td>
<td>c.1849G&gt;T</td>
<td>1–4</td>
</tr>
<tr>
<td>V617F,C618R</td>
<td>14</td>
<td>&lt;1% PV</td>
<td>c.1849G&gt;T, c.1851C&gt;T, c.1852T&gt;C</td>
<td>25</td>
</tr>
<tr>
<td>V617F,C618F</td>
<td>14</td>
<td>&lt;1% ET</td>
<td>c.1849G&gt;T, c.1853G&gt;T, c.1849G&gt;T</td>
<td>25</td>
</tr>
<tr>
<td>V617F,D620E</td>
<td>14</td>
<td>&lt;1% PV</td>
<td>c.1849G&gt;T, c.1860C&gt;A, c.1831T&gt;G, c.1849G&gt;T</td>
<td>26</td>
</tr>
<tr>
<td>L611V,V617F</td>
<td>14</td>
<td>&lt;1% PV</td>
<td>c.1848T&gt;C, c.1849G&gt;T</td>
<td>28</td>
</tr>
<tr>
<td>C616C,V617F</td>
<td>14</td>
<td>&lt;1% ET</td>
<td>c.1860G&gt;A</td>
<td>29</td>
</tr>
<tr>
<td>D620E</td>
<td>14</td>
<td>&lt;1% MPN</td>
<td>c.1606_1638dup33</td>
<td>30</td>
</tr>
<tr>
<td>V536_I546dup</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>31</td>
</tr>
<tr>
<td>V536_F547dup</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>31</td>
</tr>
<tr>
<td>F537_L539I</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1609T&gt;A, c.1616A&gt;T</td>
<td>32</td>
</tr>
<tr>
<td>F537_K539delinsL</td>
<td>12</td>
<td>~10% V617F-negative PV</td>
<td>c.1609_1616delins</td>
<td>5</td>
</tr>
<tr>
<td>F537_L546dup10,F547L</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1608_1640dup33</td>
<td>30</td>
</tr>
<tr>
<td>F537_L547dup</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1609_1641dup33</td>
<td>33</td>
</tr>
<tr>
<td>H538O,K539L</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1614C&gt;A, c.1615A&gt;T, c.1616A&gt;T</td>
<td>5</td>
</tr>
<tr>
<td>H538O,K539,L540S</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>31</td>
</tr>
<tr>
<td>H538_K539del</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>31</td>
</tr>
<tr>
<td>H538K539delinsF</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1612_1617delins</td>
<td>34</td>
</tr>
<tr>
<td>H538_K539delinsI</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1612_1617delins</td>
<td>35</td>
</tr>
<tr>
<td>H538_K539delinsL</td>
<td>12</td>
<td>~5% V617F-negative PV</td>
<td>c.1612_1617delins</td>
<td>36,37</td>
</tr>
<tr>
<td>K539L</td>
<td>12</td>
<td>~10% V617F-negative PV</td>
<td>c.1615A&gt;T or A&gt;C, c.1616A&gt;T</td>
<td>5,37</td>
</tr>
<tr>
<td>K539L,L545V</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1615A&gt;T, c.1633T&gt;G</td>
<td>31</td>
</tr>
<tr>
<td>I540T</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1619T&gt;C</td>
<td>32</td>
</tr>
<tr>
<td>I540_N542delinsS</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>38</td>
</tr>
<tr>
<td>I540_E543delinsMK</td>
<td>12</td>
<td>~5% V617F-negative PV</td>
<td>c.1620_1627delins</td>
<td>39,40</td>
</tr>
<tr>
<td>I540_E543delinsKK</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1619_1627delins</td>
<td>41</td>
</tr>
<tr>
<td>R541_E543delinsK</td>
<td>12</td>
<td>~10% V617F-negative PV</td>
<td>c.1622_1627delins</td>
<td>36,39</td>
</tr>
<tr>
<td>R541K_A542_G543del</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1622_1627delins</td>
<td>42</td>
</tr>
<tr>
<td>R541_E543delinsK</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1622_1627delins</td>
<td>34</td>
</tr>
<tr>
<td>N542_E543del</td>
<td>12</td>
<td>~40% V617F-negative PV</td>
<td>c.1624_1629del</td>
<td>5</td>
</tr>
<tr>
<td>E543_D544del</td>
<td>12</td>
<td>~10% V617F-negative PV</td>
<td>c.1627_1632del</td>
<td>43</td>
</tr>
<tr>
<td>D544G</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1631A&gt;G</td>
<td>44</td>
</tr>
<tr>
<td>D544,L545del</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1630_1635del</td>
<td>31</td>
</tr>
<tr>
<td>F547_K549delinsL</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>NA</td>
<td>45</td>
</tr>
<tr>
<td>547insLJ540_F547dup8</td>
<td>12</td>
<td>&lt;1% V617F-negative PV</td>
<td>c.1642_1644ins, c.1645_1668dup</td>
<td>46</td>
</tr>
</tbody>
</table>

*Mutation descriptors are not translated into the standard HGVS nomenclature, to avoid misinterpretation.

†Nucleotide positions are determined based on the cited sources.

NA, not available in the original publication.

**MPL**

The **MPL** gene maps to chromosome band 1p34 and encodes the thrombopoietin receptor, which binds to thrombopoietin, the primary cytokine that regulates megakaryocyte development and platelet production, as well as hematopoietic stem cell homeostasis. Binding of thrombopoietin to MPL...
leads to activation of JAK2, which phosphorylates MPL and initiates a cascade of downstream signaling events that regulate cell survival, proliferation, and differentiation.51

Mutations of the MPL gene occur in BCR-ABL1-negative MPN.57 The mutation W515L represents guanosine-to-thymidine substitution in MPL at nucleotide 1544, which leads to replacement of leucine for tryptophan. The mutation results in impaired function of the autoinhibitory region and subsequent ligand-independent thrombopoietin receptor activation.52 This then leads to subsequent activation of downstream tyrosine kinases and activation of transcription factors STAT3 and STAT5, which in turn leads to transformation of hematopoietic cells into cytokine-independent clones, resulting in megakaryocytic hyperplasia and marrow fibrosis. The Y252H mutation, which is located in the extracellular domain of MPL, confers hypersensitivity to thrombopoietin and increases the generation of megakaryocytes in vitro and leads to increased thrombopoietin signaling and cell growth and survival.53

The majority of the MPL mutations are found in exon 10 codon 515; W515L is the most common, followed by W515K. Overall, MPL W515L or W515K mutations are present in patients with PMF and ET at a frequency of approximately 5% and 3%, respectively.6–8 Other codon 515 mutations include W515A and W515R.54 Rarely, double MPL mutations occurring in cis of exon 10 have been identified.54 Mutations in other exons include V501A, Y252H, and S204P (Figure 2).56,58,59 In addition to their association with sporadic MPN, MPL mutations are also associated with familial diseases. S505N mutation has been associated with familial autosomal dominant thrombocytosis.55 MPL and JAK2 mutations are not mutually exclusive, and rare cases of concurrent MPL mutation and JAK2 V617F mutation have been reported.7,8 Clinically verified MPL mutations are listed in Table 2.

When Should JAK2 and MPL Mutational Analysis Be Performed?

Screening

The usual indications for JAK2 mutation testing include unexplained polycythemia, neutrophilia, or thrombocytosis (when secondary causes have been ruled out), because each may represent a MPN57 (Table 3). Other indications for JAK2 mutation analysis include splanchnic vein thrombosis and the provisional entity refractory anemia with ring sideroblasts and thrombocytosis (RARS-T). Of patients with splanchic vein thrombosis, 31% were found to have associated JAK2 mutations and often had a latent MPN, with the most common site of thrombosis occurring in the portal venous system.59 Similarly, nearly one third of patients with RARS-T were found to have a JAK2 mutation.60

After secondary causes of polycythemia have been ruled out, JAK2 analysis (first for exon 14 V617F, and then, if findings are negative, for exon 12) is indicated to support the diagnosis of PV. Ideally, JAK2 analysis for investigating the cause of neutrophilia and thrombocytosis would follow a determination of the absence of the BCR-ABL1 translocation t(9;22). In practice, however, clinicians find it easier to order both the BCR-ABL1 and the JAK2 assays at the same time in screening peripheral blood. There is no difference in analytical performance using peripheral blood or bone marrow samples for testing for JAK2 mutations, because granulocytes constitute the predominant population in both specimens.61

MPL mutation analysis is not indicated in the consideration of PV, because no MPL mutations have been described for this entity. The use of MPL analysis is best considered after pathology review of a bone marrow biopsy and aspirate. If a diagnosis of PMF or ET is entertained, then MPL analysis should be performed in the setting of a negative JAK2 V617F assay, if required to meet World Health Organization diagnostic criteria.10,11

Follow-Up Testing

The role of follow-up testing for JAK2 and MPL mutations after therapy is not clearly established. Quantitative real-

Table 2 MPL Mutations in Myeloproliferative Neoplasms

<table>
<thead>
<tr>
<th>Mutation*</th>
<th>Exon</th>
<th>Frequency</th>
<th>Nucleotide position1</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>W515L or W515K</td>
<td>10</td>
<td>~5% PMF, ~3% ET</td>
<td>c.1544G&gt;T</td>
<td>6–8</td>
</tr>
<tr>
<td>V501A, W515L</td>
<td>9, 10</td>
<td>&lt;1% PMF</td>
<td>c.1543T&gt;A</td>
<td>6</td>
</tr>
<tr>
<td>W501A, W515L</td>
<td>9, 10</td>
<td>&lt;1% PMF, ET</td>
<td>c.1502T&gt;C</td>
<td>54</td>
</tr>
<tr>
<td>S505C, W515L</td>
<td>10</td>
<td>&lt;1% ET</td>
<td>c.1514G&gt;A</td>
<td>54</td>
</tr>
<tr>
<td>S505N</td>
<td>10</td>
<td>&lt;1% PMF, ET</td>
<td>c.1514G&gt;A</td>
<td>55,56</td>
</tr>
<tr>
<td>A506T</td>
<td>10</td>
<td>&lt;1% PMF</td>
<td>c.1516G&gt;A</td>
<td>56</td>
</tr>
<tr>
<td>L510P</td>
<td>10</td>
<td>&lt;1% PMF</td>
<td>c.1529T&gt;C</td>
<td>56</td>
</tr>
<tr>
<td>W515A</td>
<td>10</td>
<td>&lt;1% ET</td>
<td>c.1543G&gt;T</td>
<td>54,56</td>
</tr>
<tr>
<td>A519T</td>
<td>10</td>
<td>&lt;1% PMF</td>
<td>c.1555G&gt;A</td>
<td>56</td>
</tr>
<tr>
<td>S204P</td>
<td>4</td>
<td>&lt;1% PMF</td>
<td>c.610T&gt;C</td>
<td>36</td>
</tr>
<tr>
<td>Y252H</td>
<td>5</td>
<td>&lt;1% ET</td>
<td>c.754T&gt;C</td>
<td>53</td>
</tr>
</tbody>
</table>

*Mutation descriptors are not translated into the standard Human Genome Variation Society (HGVS) nomenclature, to avoid misinterpretation.

1Nucleotide positions are determined based on the cited sources.

Gong et al
time PCR (qPCR) testing for monitoring JAK2 V617F allele burden has been limited to patients enrolled in clinical trials such as the peg-IFN-α-2a and MF-TG101348-001 trials. Although there are small-molecule inhibitors for JAK2, such as ruxolitinib [approved by the U.S. Food and Drug Administration (FDA) for myelofibrosis], these target the JAK2 and often the JAK1 proteins, and thus are not specific for the V617F mutation. Although allele burden has been shown to decrease after use of some inhibitors, there is no one-to-one correlation of allele burden with clinical response (eg, reduction in splenomegaly). Highly sensitive qPCR for JAK2 V617F may have value in follow-up of patients with primary and secondary myelofibrosis after allogeneic stem cell transplantation. Quantitative tests for JAK2 V617F allele have been used to determine the depth of clinical remission and to guide adoptive immunotherapy, such as donor lymphocyte infusion.

Thus, for routine clinical practice, there is no recommended schedule for follow-up JAK2 testing, whether qualitative or quantitative. There is a dearth of follow-up information for MPL mutation analysis, likely because of the low number of cases identified to date.

### Which Methodologies Are Used to Detect JAK2 and MPL Mutations?

Although there are no FDA-cleared tests, a number of methods have been developed for detecting JAK2 V617F mutation. Traditional Sanger sequencing has been used extensively, but it has relatively poor sensitivity. Other methods used to detect V617F include restriction fragment length polymorphism, denaturing high-performance liquid chromatography, high-resolution melting-curve analysis, pyrosequencing, and various allele-specific PCR systems with electrophoretic analysis of the products. Most of these methods typically do not achieve sensitivities of less than 5% of alleles (Table 4).

The most widely used methods for the detection of V617F involve allele-specific qPCR. These methods easily achieve analytical sensitivities of ≤1% mutant alleles. Allele-specific qPCR also allows for quantification of the mutant as a percentage of all of the JAK2 alleles, as an estimate of disease burden. Using standard curves for both the wild-type and mutant forms, it is possible to perform this calculation; however, there is no standardization for measuring quantity. The clinical utility of quantification of V617F has not yet been established. A limitation of allele-specific qPCR is that, in many cases, signal is generated in the mutant PCR reaction at high C_T counts even for known negative samples. In this situation, it is necessary for the laboratory to establish the minimal amount of signal needed to confidently call a sample positive.

There are no standard methods for detecting the rare JAK2 mutations, nor for MPL mutations. Direct Sanger sequencing or pyrosequencing has been used for assessment of JAK2 and MPL mutations. Some published methods screen for mutations using denaturing high-performance liquid chromatography or high-resolution melting-curve analysis, followed by

### Table 4 Methods in Detecting JAK2 Mutations

<table>
<thead>
<tr>
<th>Method</th>
<th>Sensitivity (%)</th>
<th>Advantage</th>
<th>Disadvantage</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>qPCR (AS, LNA)</td>
<td>0.1–0.01</td>
<td>High sensitivity; quantitative</td>
<td>Detects only target mutations</td>
<td>66–69</td>
</tr>
<tr>
<td>PCR (AS)</td>
<td>0.1–1</td>
<td>High sensitivity; simple to perform</td>
<td>Detects only target mutations; not quantitative</td>
<td>4,70,71</td>
</tr>
<tr>
<td>Melting curve analysis</td>
<td>5–10</td>
<td>Simple to perform; semiquantitative; low cost</td>
<td>Detects target mutation only; moderate to low sensitivity; poor reproducibility in low+ samples</td>
<td>22,32,70,72</td>
</tr>
<tr>
<td>Pyrosequencing</td>
<td>5–10</td>
<td>Simple to perform; quantitative; low cost</td>
<td>Detects target mutation only; relatively low sensitivity</td>
<td>73</td>
</tr>
<tr>
<td>RFLP</td>
<td>1–10</td>
<td>Low cost</td>
<td>Relatively low sensitivity; requires post-PCR manipulation; unreliable in low+ samples; not quantitative.</td>
<td>22,74</td>
</tr>
<tr>
<td>Sanger sequencing</td>
<td>20</td>
<td>Detects all mutations; bidirectional confirmation</td>
<td>Low sensitivity; time-consuming; not quantitative</td>
<td>1–4</td>
</tr>
</tbody>
</table>

AS, allele-specific; LNA, locked nucleic acid; RFLP, restriction fragment length polymorphism.
direct sequencing to confirm that a mutation is present.\textsuperscript{75,76} Higher analytical sensitivity may be achieved by using clamped PCR, followed by nucleotide sequencing.\textsuperscript{77} Traditional allele-specific PCR and allele-specific qPCR assays have also been used, but these assays are limited to assessing specific mutations. With less common mutations, it is not clear what level of analytical sensitivity is needed.

Intellectual property restrictions may influence the choice of a JAK2 V617F assay, given that Ipsogen (Marseille, France) acquired a worldwide license on testing for this mutation in 2006. (Ipsogen became Qiagen Marseille in 2013.) Limitations of the Ipsogen qualitative assay (MutaScreen assay) were highlighted in a report that described two MPNs containing variant JAK2 mutations, each with the typical codon 617 (1849G>T) mutation, and in one case an additional codon 617 (1851C>T) substitution and a codon 618 (1852T>C) mutation, and in the other case an additional codon 618 (1853G>T) mutation.\textsuperscript{25} These two cases gave no signal and a wild-type result, respectively, by the Ipsogen assay, but were detected as abnormal by melting curve analysis and subsequent Sanger sequencing. In principle, the presence of additional exon 14 mutations can interfere with binding of primers and probes in allele-specific assays, but such mutations are exceptionally rare (<1% of all JAK2 mutations).\textsuperscript{78} Assays that are not allele specific, such as melting curve analysis, offer the potential to detect rare variants, but have a significantly lower sensitivity (typically 5% to 10% mutant alleles). The advantages and disadvantages of each technique, including financial considerations associated with intellectual property rights, must therefore be carefully weighed before deciding on the type of assay to implement (Table 4).

What Are the Technical Issues Associated with JAK2 and MPL Mutation Testing?

Blood versus Bone Marrow

Initial testing of JAK2 and MPL mutations is most commonly performed on peripheral blood samples. There are several reasons why blood is the preferred type of specimen, including convenience and the fact that the diagnosis of PV can in most cases be made without a bone marrow evaluation.\textsuperscript{79} Total white blood cells from the peripheral blood are the preferred type of cell population for these analyses. White blood cells can be obtained by simply performing a red blood cell lysis procedure. Ficoll density centrifugation should be avoided, because it depletes the granulocytic population (which carries the mutations). Granulocyte isolation is not necessary because granulocytes comprise approximately 45% to 75% of total white blood cells in normal individuals, and this proportion is even higher in peripheral blood of MPN patients. It has been shown that granulocytic isolation achieves, on average, only 15% higher JAK2 allele burden compared with that of whole white blood cells.\textsuperscript{80} In comparison, a 10-fold to 100-fold increase in assay sensitivity can be easily achieved by designing a sensitive PCR method.\textsuperscript{81,82} Unlike granulocyte isolation, optimization in a PCR procedure does not increase time, labor, or cost on a routine basis.

Allele Burden

The allele burden varies greatly (between 1% and 100%) from patient to patient at the time of first diagnosis, and low levels of JAK2 V617F are not uncommon. With qualitative assessment, it has been reported that approximately 20% to 30% of PV patients have less than 25% JAK2 V617F alleles.\textsuperscript{83–85} Insensitive molecular assays would cause an even bigger problem in ET, in which up to 75% of cases have a JAK2 V617F level of less than 25% and a considerable number (<40% of cases) have a JAK2 V617F level of less than 10%.\textsuperscript{83} A quantitative assay or a reasonably sensitive qualitative assay is therefore essential for capturing the cases with low levels of the mutation. An allele burden of greater than 50% suggests homozygosity, which is most likely to occur in PV; it is uncommon in ET.\textsuperscript{58} A high allele burden in PV and ET is associated with progression to myelofibrosis.\textsuperscript{86}

Analytical Sensitivity

Assays with adequate analytic sensitivity are required for the sensitive detection of JAK2 V617F, because the mutation may be present in a small fraction of the neoplastic clonal cell population. In a study using direct sequencing, lower frequencies of the JAK2 V617F were found in all three classical MPN entities: 65% in PV, 23% in ET, and 30% in PMF.\textsuperscript{4} Re-evaluation using more sensitive techniques revealed higher frequencies: 95% to 97% in PV, approximately 55% in ET, and approximately 65% in PMF, suggesting that insensitive assay methods (eg, Sanger sequencing, which has an analytic sensitivity of 20%) generated a significant number of false-negative results.\textsuperscript{87,88}

To ensure that more than 90% of cases are detected, the adequate analytical sensitivity of a clinical JAK2 assay should be at least 1%.\textsuperscript{22,82,88,89} When the JAK2 V617F level of a particular patient falls below 1%, however, caution must be exercised, because very low levels of JAK2 V617F mutations (usually <0.1%) have been described in peripheral blood of unaffected individuals.\textsuperscript{80,89} An assay that detects less than 0.1% of JAK2 allele, therefore, is more likely to produce false-positive results in the diagnostic setting.\textsuperscript{90} On the other hand, low levels (<1%) of the mutant JAK2 have been reported in ET patients (as determined using allele-specific loop-mediated amplification assay).\textsuperscript{92} Therefore, if other World Health Organization major and minor diagnostic criteria are met, finding such a low-positive JAK2 mutation may still be clinically relevant. If there is doubt, a peripheral blood or a bone marrow specimen collected at a later time point may be used to confirm the finding.

How Should the Results of JAK2 and MPL Mutation Testing Be Reported?

All final reports must address preanalytic, analytic, and postanalytic components.\textsuperscript{93} Preanalytic components should
include the tissue source (eg, peripheral blood, bone marrow, paraffin-embedded tissue) and, if available, the clinical indication for testing. The analytic components include the analytic result (mutation detected or not detected) and a brief description of the methodology used. The post-analytic components include interpretive comments, if needed. A sample report for a quantitative JAK2 V617F assay is presented as Table 5.

For qualitative JAK2 V617F testing, the report should clearly indicate a final analytic result (eg, JAK2 V617F mutation DETECTED or JAK2 V617F mutation NOT DETECTED). The methodology summary should include source information regarding any commercial primers and probes used, and it must include a statement of assay sensitivity. JAK2 Sanger sequencing assays, such as those for exon 12 mutations, should report any detected mutations using standard Human Genome Variation Society (HGVS) nucleotide and amino acid nomenclature (HGVS, http://www.hgvs.org/mutnomen, last accessed February 19, 2013). The methodology summary of sequencing assays should clearly state the regions covered by the assay. An interpretive comment should be included, indicating whether the observed mutation has been previously reported in MPNs or other malignancies or whether the mutation appears to be novel. Online databases, such as the Catalogue of Somatic Mutations in Cancer (COSMIC; Catalogue of Somatic Mutations in Cancer, http://cancer.sanger.ac.uk/cancergenome/projects/cosmic, last accessed February 19, 2013) and the Database of Single Nucleotide Polymorphisms (dbSNP; National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/snp, last accessed February 19, 2013), are particularly useful for determining whether an uncommon abnormality has been previously reported. An estimate of the relative abundance of mutant to wild-type alleles may be provided; this is especially important for novel mutations, which could represent previously unreported germline SNPs.

Because most laboratories offer V617F and exon 12 mutations as separate tests, exon 12 mutation may be mistakenly requested as the initial screening test instead of V617F mutation. This error can be easily avoided by restricting exon 12 testing only to a reflex test. The results can be better clarified by incorporating a disclaimer in the exon 12 test report: This test does not include testing for the more common JAK2 V617F (exon 14, c.1849G>T) mutation.

Reports of quantitative JAK2 V617F assays should include a statement of qualitative results (eg, JAK2 V617F mutation DETECTED), as well as a statement of quantitative allele burden. The allele burden may be expressed as a ratio of mutant (mut) to wild-type (wt) alleles, or more commonly, as a percentage of mutated JAK2 alleles, calculated as \( \frac{\text{JAK2}_{\text{mut}}/(\text{JAK2}_{\text{mut}} + \text{JAK2}_{\text{wt}})}{100}. \)

If available, results of prior serial testing may be provided. If a quantitative cutoff is used to define a positive result, this value should be stated as part of the methodology summary, and a limit of sensitivity must also be included. Allele burden may also be reported in semiquantitative fashion (eg, >50% to 75% mutant alleles). The terms heterozygous and homozygous have been used in the literature to describe cases with allele burdens of <50% and ≥50%, respectively, but this terminology is potentially misleading. For example, in a specimen with 40% mutant alleles, it is unclear whether the sample is actually composed of 40% cells with a monoallelic mutation or 20% cells with a biallelic mutation. For this reason, the terms heterozygous and homozygous should generally be avoided in clinical reports.

Results of MPL assays should be reported in a similar fashion as those described above for JAK2. Given the diversity of known MPL mutations, the precise mutation detected should be reported using HGVS-standard nucleotide and amino acid nomenclature.

### What Are the Needs for Standardization and Proficiency Testing?

#### Standardization

There is a lack of standardization among the variety of qualitative and quantitative methods available to detect the range of molecular alterations in JAK2 and MPL. As of writing, there were no kits cleared by the FDA for clinical use in testing for JAK2 or MPL mutations; however, it is possible that a companion diagnostic for the JAK2 V617F mutation will be developed to detect the JAK2 mutation and

<table>
<thead>
<tr>
<th>Heading</th>
<th>Report</th>
</tr>
</thead>
<tbody>
<tr>
<td>Specimen type</td>
<td>Peripheral blood</td>
</tr>
<tr>
<td>Clinical indication</td>
<td>Erythrocytosis, rule out myeloproliferative neoplasm</td>
</tr>
<tr>
<td>Qualitative result</td>
<td>JAK2 V617F mutation detected</td>
</tr>
<tr>
<td>Quantitative result</td>
<td>72% mutant alleles</td>
</tr>
<tr>
<td>Interpretation</td>
<td>Positive</td>
</tr>
<tr>
<td>Methodology</td>
<td>Genomic DNA was extracted and assayed for the V617F mutation using a TaqMan allelic discrimination assay with a FAM-labeled mutant-specific probe and a VIC-labeled wild-type specific probe. The sensitivity of this assay is 1% JAK2 V617F mutant in a wild-type background.</td>
</tr>
</tbody>
</table>
to quantify the response to targeted therapies. If a quantitative assay is clinically indicated to achieve precise mutant levels or to monitor allele burden, there will be a need for quantitative standards to accurately quantify the allele burden and to assess the sensitivity and dynamic range of the assay. A number of AML cell lines are positive for JAK2 V617F, including HEL, MB-02, MUTZ-8, SET-2, UKE-1. All except SET-2 are homozygous mutant, but some (e.g., HEL) have more than two copies of JAK2 and as such are not ideal as standards. Quantitative controls for the JAK2 V617F mutation are commercially available (Horizon Discovery, Cambridge, UK; Qiagen Marseille, Marseille, France) to serve as a quantitative or low-positive control. Positive controls for MPL mutations are more difficult to obtain. As reference standards for quantitative JAK2 testing are made available, comparable results across laboratories can be achieved in monitoring treatment response and minimal residual disease.

Test Validation and Proficiency Testing

The approach to validation differs between qualitative and quantitative assays. For a qualitative assay, the sensitivity must be defined. For a qPCR assay, the threshold for a positive result must be determined, because of cross-amplification of the wild-type allele. A series of normal DNA samples should be analyzed to derive the threshold for a positive result. Because the allele burden varies between MPN entities or between patients at diagnosis and after treatment, samples with a low allelic frequency should be included. A low-positive control should be included in each run, to demonstrate that the assay is performing at the level of the measured sensitivity. It may be difficult to accumulate JAK2 exon 12 or MPL mutant cases for validation, given the low frequency of these variants.

Proficiency testing for JAK2 V617F is now standard, and it is likely that MPL codon 515 mutations will be added in the future. A proficiency test for JAK2 V617F is available from the College of American Pathologists (2013 Surveys and Anatomic Pathology Education Programs, http://www.cap.org/apps/docs/proficiency_testing/2013_surveys_catalog.pdf, last accessed November 28, 2012). Currently, testing is based only on qualitative results, but the accuracy and sensitivity of quantitative assays may be assessed in future iterations.

What Is the Current State of Practice in Clinical Laboratories for JAK2 and MPL Mutation Testing?

In late 2010, a sample exchange for interlaboratory comparison of assays for MPNs was organized by the AMP. A total of 66 specimens were shared among the 22 laboratories that participated (18 in the United States, 2 in Canada, and 2 in Asia). One laboratory tested only a subset of the specimens. The participating laboratories tested the samples using a combination of quantitative and qualitative assays for JAK2 mutations, and several of the laboratories also performed MPL mutation assays.

Each laboratory was asked to provide three DNA specimens: one known to be JAK2 V617F positive, one weakly positive, and one from a patient suspected to have a MPN but that was found to be JAK2 V617F negative. In addition, one laboratory provided formalin-fixed, paraffin-embedded sections of spleen from a patient with polycythemia vera. Of the 22 laboratories, 13 used in-house tests for JAK2 V617F and the remaining 9 used commercial assays; 14 laboratories performed only qualitative assays, 5 performed only quantitative assays, and 3 performed both quantitative and qualitative assays. Five laboratories also offered JAK2 exon 12 assays, two laboratories offered MPL mutation assays, and another two provided MPL mutation results (although their assays were still being validated). Most laboratories reported analytical sensitivities in the 1% to 5% range; only two laboratories indicated analytical sensitivities of less than 1%. Approximately half of the laboratories performed single replicates, and half performed assays in duplicate.

The reported DNA concentrations for the 66 specimens ranged from 0.3 to 318 ng/µL. Five of the samples resulted in failed amplifications in more than one laboratory, and these had very low DNA concentrations. Three other samples appeared to have adequate DNA but still resulted in failed amplification in more than one laboratory.

Results were concordant for 26 of the 66 specimens (ie, the same results were obtained by all participating laboratories), with 21 positive and 5 negative. An additional five specimens were positive in all laboratories where results were obtained; some of the laboratories did not obtain a result with these specimens, most likely because of low DNA concentration.

 Discordant results were categorized as false negative if more than 15 laboratories obtained a positive result and as false positive if more than 15 laboratories obtained negative results for a given specimen. The 16 specimens having a high rate of false-negative results tended to have lower JAK2 allele burden in those laboratories doing quantitative assays, suggesting that the false-negative results were mostly due to assays with lower analytical sensitivity. Five laboratories had two or more false-negative results, suggesting that the assays of these laboratories may have been of lower sensitivity than those of most participating laboratories. Another 16 specimens yielded false-positive results. These tended to be low positive when tested in laboratories using quantitative assays, so it is likely that these specimens had JAK2 V617F levels that were below the limit of detection for most laboratories. However, there is a concern that some laboratories may have had a high true false-positive rate, because one laboratory had a positive result that was not detected by any other laboratory. In a second example, one assay reported a specimen positive for JAK2 V617F that was reported negative in all other laboratories; however, that specimen was found to have a JAK2 exon 12 mutation, which is mutually exclusive with the V617F mutation.
No consensus result was obtained with four of the specimens. All of these were found to have low JAK2 V617F allele burden when tested by quantitative assays. Interestingly, some laboratories obtained positive results with all four specimens, whereas others obtained negative results with all four. Presumably, these results reflect differences in analytical sensitivity among the participating laboratories. For the false-negative, false-positive, and no-consensus results, there appeared to be no correlation between the discordant results and assay methodology.

Comparison among the 12 laboratories performing quantitative JAK2 V617F assays was complicated by the various ways in which the laboratories reported their results. Most laboratories reported their results as a JAK2 V617F percentage of unmutated alleles. Two laboratories reported results as one of six categories (≥78%, 50% to 78%, ..., <5%), and one laboratory reported results in three categories (homozygous, heterozygous, and low-level heterozygous). For the purposes of comparison, results were grouped as high (>50%), moderate (5% to 50%), low (2% to 5%), and negative (<2%). One laboratory had a higher rate of discordance than the others; it is possible that this laboratory was truly an outlier, although it is also possible that there were clerical errors in reporting the results to the study. When this laboratory was excluded, there was complete concordance for 29 specimens, 18 of which were in the high category, 9 were in the moderate category, and 2 were in the low category. There was less reproducibility between laboratories in the lower ranges, presumably because of greater variance of the method in the low range. No specimen was negative for all 12 laboratories, again presumably reflecting poorer reproducibility at the low end and possibly indicating high sensitivity (which could result in false-positive results).

MPL mutation detection was compared among the four participating laboratories. Two specimens were found positive for the W515L mutation in all four laboratories. One laboratory found a third specimen positive for W515L, but the mutation was not detected in the other two laboratories that tested this specimen. One laboratory found an S505N mutation that was not detected by the other laboratories. Interestingly, these last two specimens were also found to be positive for JAK2 V617F by many of the participating laboratories.

A number of conclusions can be reached, taking these data together. First, overall JAK2 V617F mutation testing was reproducible among the laboratories performing this testing. Because there was incomplete concordance, it is important that laboratories participate in proficiency testing programs that include a wide spectrum of clinical laboratories. There is also reasonably good agreement among laboratories for quantitative testing. Although such testing is still not standard for diagnosing MPNs, the findings from the sample exchange study provide hope that quantitative testing will be reliable when such testing becomes standard practice.

Future Directions for JAK2 and MPL Mutation Testing

Targeted gene panels assessed by next-generation sequencing are increasingly being used in clinical laboratories. This allows for broad multigene coverage in a single assay, thereby interrogating all of the targets deemed relevant for a particular clinical entity. Full sequencing of JAK2 and MPL, as well as other genes relevant for MPNs, may be performed in this manner. If the clinical utility of mutations in other genes (eg, TET2, ASXL1, and EZH2) is established for MPNs, next-generation sequencing provides a way to expand the scope of the assay without significantly increasing the work required to perform the test. Although there is substantial interest in these technologies, nonetheless there are limitations, especially regarding sensitivity. Estimates for general mutation detection indicate that approximately 1000-fold coverage is needed for a given base to achieve a 99% confidence of detecting more than 5% heterozygous mutant alleles.

It may also be possible to estimate mutant burden based on the allele frequency calculated from next-generation sequencing data; however, there has been only limited validation of the ability of next-generation sequencing to make this type of estimation.

Disclaimer

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