A molecular diagnostic algorithm for JAK2 V617F investigations in suspected myeloproliferative neoplasms

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Abstract

Background

The *JAK2* V617F mutation is well characterised in the pathogenesis of myeloproliferative neoplasms using a sensitive and non-invasive molecular assay. Due to the relative ease of testing this assay may be inappropriately requested as a screening tool, leading to over-testing. The aim of this study was to perform an audit of *JAK2* V617F genotyping referrals to inform implementation of a sensitive and rational myeloproliferative neoplasm (MPN) molecular diagnostic algorithm.

Methods

JAK2 V617F genotype results from 300 patients were audited. Mutant burdens were estimated using fluorescent allele-specific PCR fragment analysis and patients categorised as positive (≥1%) or negative (<1%). Patients were scored for haematological evidence of erythrocytosis and thrombocytosis. Further, a clinical evaluation of the *JAK2* V617F positive patients with normal haematological parameters was carried out.

Results

Analysis showed 16.7% (50/300) of patients were *JAK2* V617F positive and of these, 86% (43/50) exhibited elevated haematological parameters. The remaining positive patients presented with borderline or post-treatment MPN characteristics or atypical *JAK2* V617F mutated neoplasms.

Conclusion

Implementation of a diagnostic algorithm, incorporating haematological parameter acceptance criteria, may detect all classical *JAK2*V617F mutated MPNs

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Introduction

The *BCR-ABL-1* negative myeloproliferative neoplasms (MPNs) represent a phenotypically heterogeneous group of clonal myeloid malignancies originating in haematopoietic stem cells (HSCs). [1] MPNs classically manifest as Polycythaemia Vera (PV); Essential thrombocythemia (ET) or Primary Myelofibrosis (PMF). PV presents with an erythrocytosis (often with granulocytosis and thrombocytosis) and marrow trilineage hypercellularity. ET and PMF pathogenesis predominantly involves the megakaryocyte lineage. ET presents with thrombocytosis and megakaryocytic proliferation. PMF exhibits megakaryocyte proliferation, left-shifted granulocytic proliferation and cytokine-driven fibrosis. Erythrocytosis is reduced, anaemia is progressive and hepatosplenomegaly is often present. Excessive fibrosis induces marrow failure and cytopenias. [2, 3] MPN patient symptom burdens range from asymptomatic to severe constitutional symptoms; thrombotic and haemorrhagic complications and the potential to transform to myelodysplasia or acute leukaemia. [2, 3] Treatment is founded on phlebotomy, cytoreductive chemotherapy and anti-thrombotic prophylaxis. [2]

MPNs are rare neoplasms, occurring with low incidence in a given population. However, both PV and ET have insidious onsets and protracted median survival rates of over 10 years, resulting in a relatively high prevalence. [4] Constitutively active JAK2-STAT signalling in HSCs is the major driver of aberrant MPN myeloid proliferation and is associated with recurrent and largely mutually exclusive mutations in the *JAK2*, *MPL* and *CALR* genes. [1, 5]

The discovery of an activating *JAK2* exon 14 point mutation the pseudokinase domain (c. 1849 G>T, p.Val617Phe) provided the first insight into MPN molecular pathogenesis. [6] This mutation has the potential to activate all three canonical myeloid cytokine receptors (the erythropoietin receptor, granulocyte colony-stimulating factor receptor, and the MPL/thrombopoietin receptor). [7] Most MPNs harbour the *JAK2* V617F mutation: (96% PV; 55% ET and 65% PMF) and its genotyping is the mainstay of any MPN molecular diagnostic algorithm. [8] There is, however, no unequivocal direct correlation between mutant allele burden and prognosis [9].

Mutations in *JAK2* exon 12 mutations were later identified in the majority of PV cases that did not harbour *JAK2* V617F. The most common result in two residue deletions adjacent to the pseudo-kinase domain and induce inappropriate JAK2-STAT signalling to different extents. [10]

Additional mutations were identified in two other signal transduction components that promote hyperactivity of the MPL-restricted JAK2-STAT pathway. *MPL* exon 10 point mutations (W515X) promote receptor spontaneous activation [3,11] and *CALR* indels all cause +1 frame-shifts in this multifunctional chaperone. [3,12] Positively charged C-terminal mutant CALR proteins bind to their client MPL, inducing constitutive receptor activity. [13]. *MPL* and *CALR* mutations are restricted to ET and PMF and their genotyping supports MPN diagnostic stratification. [3]

Accessory mutations in epigenetic (e.g. *ASXL1, TET2, IKZF1 , EZH2*) and splicing (e.g. *SRSF2 , SF3B1*) regulators are postulated to influence the specific MPN phenotype [14], promote progression and induce phenotype switching.

Most MPNs now have a recognised genetic basis and sequential testing for *JAK2, MPL* and *CALR* mutations is an essential component of diagnostic pathways. [8,15,16] Accurate MPN diagnosis is crucial for optimal patient management. The World Health Organisation (WHO) [15] and British Society of Haematology (BSH) [17] have published updated MPN diagnostic best practice standards

integrating haematological, histological and clinical features with molecular testing.
Genotyping provides a differentiation between primary and secondary/ reactive erythrocytosis and thrombocytosis. It may also be indicated in: splanchnic vein thrombosis; unexplained *BCR-ABL1* negative granulocytosis; unexplained aquagenic pruritus or splenomegaly and with medullary fibrotic histology. [8] However, referrals are increasing for isolated thrombotic events and leukocytosis. [8, 18] Low detection rates (around 20%) have been reported for *JAK2* V617F genotyping referrals, with rates varying widely between referring centres [18] highlighting the need for molecular diagnostic streamlining, to reduce insidious and inappropriate over-testing. [2,8]

The aim of this study was to perform an audit of *JAK2* V617F genotyping referrals to leading to the implementation of a sensitive and rational myeloproliferative neoplasm (MPN) molecular diagnostic algorithm based on haematological parameters.

Methods

JAK2 V617F genotyping

JAK2 V617F allele-specific fluorescent PCR with fragment analysis by capillary electrophoresis was used to genotype patients. Briefly, genomic DNA was extracted from whole peripheral blood. A fluorescent *JAK2* V617F-specific and *JAK2* wild-type amplicon, produced by allele-specific PCR, [6] were subsequently resolved by capillary electrophoresis. GeneMapper® v4.0 software was used to manually estimate fragment peak heights, relative to LIZ 500 fluorescent standards (Applied Biosystems™). *JAK2* V617F was calculated as: [mutation/ mutation = wildtype) x 100. All samples were analysed in duplicate. The assay has a sensitivity of 1% and this threshold was used for genotype status dichotomisation: negative: *JAK2* V617F <1%; positive: *JAK2* V617F ≥1%.

Audit of patient *JAK2* V617F genotype and haematological parameters

Genotype status and contemporaneous CBC haematological parameters were manually extracted from electronic healthcare records of a retrospective cohort of 300 consecutive patients referred to a tertiary testing centre (from January-June 2018).
Haemoglobin concentration (Hb g/dL); haematocrit (Hct); red blood cell count (Rbc 10^{12} /L) and platelet count (Plt 10^{9}/L) were extracted. Elevation of any parameter above the sex-matched reference range upper limit of normal (ULN)* conferred an “elevated haematological parameter” status (otherwise “normal”). Erythrocytosis was designated if Hb, Hct or Rbc was elevated. Thrombocytosis was designated if Plts were elevated.

**Hb ULN (male 180, female 165 g/L); Hct ULN (male 0.54, female 0.47); Rbc ULN (male 6.2, female 5.8 10^{12}/L); Plt ULN (male and female 450 10^{9}/L)*

Clinical evaluation of *JAK2* V617F positive patients with normal haematological parameters

For each *JAK2* V617F positive patient with normal haematological parameters the following information was manually extracted from electronic healthcare records: specific diagnosis; evidence of splenomegaly; evidence of bone marrow histopathology and/or blood film abnormalities; history of haematological abnormalities and a summary of patient management.

Results

Cohort summary statistics

The patient cohort comprised 146 males 48.7% (146/300) and 51.3% (154/300) females. The median age was 59 years. *JAK2* V617F positivity was demonstrated in 16.7% (50/300) of patients and 83.3% (250/300) were *JAK2* V617F negative.

Elevated haematological parameters per *JAK2* V617F genotype status
Of the negative patients: 41.2% (103/250) had elevated haematological parameters at the time of genotyping referral and 58.8% (147/250) had normal parameters. Of the positive patients: 86% (43/50) had elevated parameters and 14% (7/50) had normal parameters (Fig 1a).

Haematological abnormality per *JAK2* V617F genotype status
Of the negative patients with elevated parameters: 34% (35/103) displayed haematological evidence of erythrocytosis (>ULN Hb, Hct or Rbc); 64.1% (66/103) of thrombocytosis (>ULN Plt) or 1.9% (2/103) of both at the time of genotyping referral. For positive patients with elevated parameters: 20.9% (9/43) displayed haematological evidence of erythrocytosis (>ULN Hb, Hct or Rbc); 65.1% (28/43) of thrombocytosis (>ULN Plt) or 14% 6/43) of both at the time of genotyping referral (Fig 1b).

Clinical overview of *JAK2* V617F patients with normal haematological parameters
This sub-group of patients presented with advanced PMF; borderline ET and atypical *JAK2* V617F mutated neoplasms (chronic phase BCR-ABL positive CML and CMML) (Table 1).

Conclusion
Suspicion of a classical MPN occurs in the presence of a sustained erythrocytosis and/or thrombocytosis. Haematological evidence of such cytoses therefore represent rational parameters for molecular diagnostic algorithm acceptance criteria, to gate first-line *JAK2* V617F genotyping. Such a rationalised algorithm would continue to support differential diagnoses of secondary erythrocytosis and thrombocytosis. The ubiquity of CBC analysis, undertaken routinely or during an initial diagnostic work-up, would promote straightforward implementation. Preservation of detection sensitivity constitutes the major hurdle to overcome and we have assessed this here.

A retrospective audit was carried out of 300 consecutive *JAK2* V617F genotyping referrals. Patients were dichotomised according to genotype status (negative: *JAK2* V617F <1%; positive: *JAK2* V617F ≥1%) and scored for haematological evidence of erythrocytosis (>ULN Hb, Hct or Rbc) and/or thrombocytosis (>ULN Plt). The positive detection rate was 16.7% (50/300). This reflects published rates [18, 19] and underscores the need for improved efficiency. Abnormal (elevated) parameters were found in 48.7% (146/300) of the total cohort: 41.2% (103/250) of negative patients and 86% (43/50) of positive patients. (Figure 1.a.) Therefore, the majority of *JAK2* V617F positive patients would be accepted if these minimal criteria were applied (parameters in excess of the respective ULNs); whilst achieving a substantial reduction in testing burden with 51.3% (154/300) of this cohort would have been rejected.

As previously noted 147/250 (58.8%) of our cohort had normal parameters at the time of testing. To investigate this category further we assessed the clinical referral pattern. The majority had an isolated elevated haematological parameter 34% (thrombocytosis/erythrocytosis), venous thrombosis (17%), leucocytosis (17%), thrombocytopenia (9%), splenomegaly (8%) or no available clinical information (15%).
With a median follow up of 18 months we have reassessed our cohort of 147 patients with a normal haematological parameters. We have found 4 patients with a *CALR* positive ET that have been on cytoreductive therapy and have not been assessed previously in our unit. We have also found 2 cases of triple-negative ET that have additional mutations in keeping with this diagnosis. In the other 141 cases no evidence of a MPN was demonstrated.

In both genotype subgroups, single lineage elevations were most prevalent (Figure 1.b). In negative patients with elevated parameters, 64.1% (66/103) presented with thrombocytosis; 34% (35/103) with erythrocytosis and 1.9% (2/103) with both. Data from this genotype group in particular are useful predictors for *JAK2* exon12 and *CALR*/*MPL* reflexive testing rates and in service provision planning.

A minority (14%; 7/50) of positive patients exhibited no haematological evidence of erythrocytosis and/or thrombocytosis at the time of referral, motivating a thorough evaluation of their clinical histories. These patients were all diagnosed with advanced PMF; borderline or pre-treated ET or atypical *JAK2* V617F mutated neoplasms. (Table 1) Patient PN1 was diagnosed with PMF and presented with splenomegaly, anaemia and thrombocytopenia. Medullary reticulin fibrosis, as well as hyperplastic and dysplastic features, consistent with PMF were demonstrated and such histopathology is *an absolute* requirement for definitive PMF diagnosis. In the context of these data, *JAK2* V617F testing would be warranted; although the minimal haematological acceptance criteria were not met at referral (parameters were low, rather than elevated). Ideally, this should encourage provision of brief clinical and histopathology details on genetic testing requests.

Patients PN3, 4, 6 had overt ET diagnoses and were receiving hydroxycarbamide (HC) cytoreductive or anti-platelet (anagrelide) therapy to normalise their platelet counts. At genetic testing referral their haematological parameters were not elevated, indicative of successful management. Patient PN5 presented with borderline features of ET, where their platelet counts regularly approached the ULN. Of note, 5% of healthy patients have haematological parameters outside laboratory reference ranges. Conversely, deviations in red blood cell mass and platelet count can occur within the normal limits. These cases illustrate well the need to establish (and to provide) patient baseline parameters and to document the chronology of any departures from this. Cross-sectional values at the time of genetic testing referral may therefore fail to meet the acceptance criteria.

Patient PN2 harboured *JAK2* V617F positive MPN clones coincident with *BCR-ABL1* (p210) positive CP-CML. The patient has commenced treatment for CP-CML and the *JAK2* V617F clone remains stable. Rarely, such clones have been reported to be coincident in these patients and their relevance remains uncharacterised. Patient PN7 had a diagnosis of CMML, that features both myeloproliferative and myelodysplastic characteristics.

Genetic testing for mutant *JAK2* and *CALR*/*MPL* comprises a major facet of classical MPN diagnostics. However, such investigations are also amenable to inappropriate referrals, putting strain on diagnostic service resources. Here, we propose a streamlined MPN molecular diagnostic algorithm (Figure 2), incorporating WHO/BSH guideline compliant acceptance criteria and demonstrate its clinical validity, without concomitant loss of sensitivity. This approach has been attempted with other studies to address the issue of over-testing. [19,20] We plan to implement a re-evaluation of our proposed algorithm following an appropriate period of use with the aim of reducing the *JAK2* testing burden for service providers, whilst maintaining robust diagnosis for patients.

Statements:

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Figure legends:

Figure 1. Haematological abnormalities per JAK2 V617F genotype. a. Elevated haematological parameters per genotype. For each genotype (negative/positive, threshold 1%) the number of patients with either normal or elevated haematological parameters was scored. **b. Haematological abnormality per genotype.** For each genotype with elevated parameters, the number of patients with evidence of erythrocytosis, thrombocytosis or both was scored.

Figure 2. MPN molecular diagnostic algorithm incorporating haematological parameter acceptance criteria.

Table legends

Table 1. Clinical overview of *JAK2* V617F positive patients with normal haematological parameters.
Summary of clinical, morphological, haematological and management details for *JAK2* V617F positive patients with normal haematological parameters. Patient ID: (PN1-7); Sex: (M/F); Age in years; final diagnosis; clinical presentation; bone marrow histopathology and blood film morphology; haematological parameter values at time of genetic testing referral; management. PMF: primary myelofibrosis; CP-CML: chronic phase chronic myeloid leukaemia; ET: essential thrombocythemia; CMML: chronic myelomonocytic leukaemia; HC: hydroxycarbamide. NA Not available

Authorship Form

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