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REVIEW ARTICLE

Molecular diagnostics of myeloproliferative neoplasms

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Abstract

Since the discovery of the *JAK2* V617F mutation in the majority of the myeloproliferative neoplasms (MPN) of polycythemia vera, essential thrombocythemia and primary myelofibrosis ten years ago, further MPN-specific mutational events, notably in *JAK2* exon 12, *MPL* exon 10 and *CALR* exon 9 have been identified. These discoveries have been rapidly incorporated into evolving molecular diagnostic algorithms. Whilst many of these mutations appear to have prognostic implications, establishing MPN diagnosis is of immediate clinical importance with selection, implementation and the continual evaluation of the appropriate laboratory methodology to achieve this diagnosis similarly vital. The advantages and limitations of these approaches in identifying and quantitating the common MPN-associated mutations are considered herein with particular regard to their clinical utility. The evolution of molecular diagnostic applications and platforms has occurred in parallel with the discovery of MPN-associated mutations, and it therefore appears likely that emerging technologies such as next-generation sequencing and digital PCR will in the future play an increasing role in the molecular diagnosis of MPN.

Key words myeloproliferative neoplasms; polycythemia vera; essential thrombocythemia; primary myelofibrosis; molecular diagnostics; *JAK2*; *MPL*; *CALR*

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The three diseases of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF) are related on the basis of their overlapping laboratory and clinical features. They are commonly referred to as the classical Philadelphia chromosome-negative myeloproliferative neoplasms (MPN), and it is the molecular diagnosis of these diseases which will be considered herein. The landmark discovery of the *JAK2* V617F (c.1849G>T) ten years ago (1–4) not only revolutionised the area of MPN molecular diagnostics but has also prompted disease re-classification, investigation into many new aspects of MPN genetics and biology, and has led to the clinical introduction of novel therapeutic agents (5, 6). The presence of the *JAK2* V617F in most PV patients and in 50–60% of patients with ET and PMF allows this mutation to be utilised as a marker of disease burden with this approach becoming a useful measure of assessing response to various therapeutic interventions. Subsequent identification of further recurring mutations specific to MPN

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subtypes, such as *JAK2* exon 12 (7), *MPL* exon 10 (8), *CALR* exon 9 (9, 10), and several other MPN-associated mutations (Table 1) allows the design of molecular diagnostic algorithms that are able to identify a clonal marker of disease in the vast majority of patients with a classical MPN. Implementation of such algorithms requires consideration of which patients to screen, which type of methodology to employ, technical aspects of the selected assays such as reliability, reproducibility and analytical sensitivity, how results should be reported, and what options are available for both standardisation and prospective proficiency assessment. This review aims to address the laboratory aspects of MPN molecular diagnosis and their clinical utility.

JAK2 V617F mutation

Parallel functional, genetic and genomic approaches lead to the breakthrough identification of the *JAK2* V617F mutation in MPN patients (1–4). Subsequent studies confirmed the presence of this mutation in the majority of PV cases, in up to 60% of ET and PMF patients, and at a lower frequency in other myeloid malignancies such as myelodysplastic syndromes (MDS), particularly refractory anaemia with ringed sideroblasts with marked thrombocytosis (RARS-T), and acute myeloid leukaemia (AML) (11, 12). The obvious benefit of molecular diagnostic markers is the demonstration of primary clonal hematopoietic disorders, and its distinction from a reactive myeloproliferation with the importance of *JAK2* V617F detection acknowledged by its inclusion as a major diagnostic criterion for PV, ET and PMF in the World Health Organization (WHO) classification (5). The presence of the *JAK2* V617F mutation in those cases not fulfilling WHO criteria suggests a latent MPN: such scenarios include splanchnic vein thrombosis or an indolent, subclinical MPN (13, 14).

Table 1 Commonly mutated genes in the myeloproliferative neoplasms

Gene	Chromosome location	Mutation location	Frequency (%)		
			PV	ET	PMF
JAK2	9p24	exon 14	97	50–60	55–60
JAK2	9p24	exon 12	1–2	rare	rare
MPL	1p34	exon 10	rare	3–5	5–10
CALR	19p13	exon 9	rare	20–30	25–35
TET2	4q24	all coding regions	10–20	5	10–20
IDH1/*IDH2*	2q33/15q26	exons 4	rare	rare	5
DNMT3A	2p23	exons 7–23	5–10	1–5	5–10
ASXL1	20q11	exon 13	2–5	2–5	15–30
EZH2	7q35-q36	all coding regions	1–3	rare	5–10
CBL	11q23	exons 8–9	rare	rare	5–10
SH2B3	12q24	exon 2	rare	rare	rare
SF3B1	2q33	exons 12–16	rare	rare	5–10
SRSF2	17q25	exon 1	rare	rare	10–15
U2AF1	21q22	exons 2–7	rare	rare	5–15

PV, polycythemia vera; ET, essential thrombocythemia; PMF, primary myelofibrosis

Pre-analytical variables that should be considered for the detection of the *JAK2* V617F, and all other MPN-associated mutations, include the type of sample, cellular fraction of the sample and the nucleic acid template (15). Detection of the *JAK2* V617F mutation can be achieved by Sanger sequencing but initial reports immediately demonstrated that this method underestimated the number of patients harbouring the mutation and suggested that an allele-specific PCR (AS-PCR) approach could improve the detection rate (2). Alternative qualitative methods to detect the *JAK2* V617F include high-resolution melting (HRM) analysis and denaturing high-performance liquid chromatography that have the advantage of being able to identify the rare, alternative *JAK2* exon 14 mutations (16, 17). The limitation of qualitative assays is their inability to identify those patients with lower allele burdens (15), and therefore, quantitative, real-time, allele-specific PCR (AS-qPCR) assays have been widely adopted.

JAK2 V617F quantitation

The initial descriptions of the *JAK2* V617F mutation in MPN showed that various profiles existed, with some patients showing a complete disappearance of their wild-type allele by Sanger sequencing whilst others had seemingly heterozygous mutations (1). These homozygous profiles could be explained by mitotic recombination (3). Taken together with the low mutational load which could only be detected by a more sensitive AS-PCR technique (2), these data suggested that, even though 'pure myeloid', that is selected granulocytes were analysed, the mutational load was distinct from the expected 0%, 50% or 100% for wild type, heterozygous or homozygous patients, respectively. Further studies indicated that the different MPN phenotypes had different mutational loads (18, 19), with more than half of PV and fibrotic PMF patients having greater than 50% *JAK2* V617F, whilst most ET and prefibrotic PMF patients had less than 50%. The mutation status of single cells analysed by genotyping colonies clearly demonstrated that PV patients had a mix of homozygous and heterozygous cells, whilst ET patients had nearly exclusively heterozygous cells, explaining that their mutational burden was overall less than 50% (20). However, some PV patients with demonstrable homozygous clones had less than 50% *JAK2* V617F mutational load, indicating that the term 'heterozygous' sometimes used to refer to these patients is inappropriate. Therefore, a *JAK2* V617F burden above 50% indicates that only a proportion of the cells analysed are homozygous for the mutation.

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Evolution of *JAK2* V617F mutated ET to PV is sometimes observed, correlating with an increase in *JAK2* V617F burden. Similarly, the evolution towards myelofibrosis is accompanied by, or possibly due to, an increase in *JAK2* V617F burden. Therefore, at MPN diagnosis measurement of the allele burden may help to discriminate between different MPN phenotypes: when an allele burden is greater than 50%, the likelihood exists of a masked PV (possibly due to iron deficiency) or myelofibrotic evolution. The *JAK2* V617F allelic burden may also have prognostic significance: PV patients with a high *JAK2* V617F burden are more likely to evolve towards myelofibrosis, to have a thrombotic event, and to present with higher haemoglobin levels, higher white blood cell (WBC) counts, lower platelet counts and splenomegaly (21). For ET patients, *JAK2* V617F levels equate with WBC counts but inversely correlate with platelet counts with high allelic burdens also appearing to predict a higher risk of thrombosis (21). Somewhat conversely, independent studies have shown that PMF patients with a *JAK2* V617F mutation but a low burden (<25%) have a poor survival (22). The evolution of the *JAK2* V617F mutated clone is also a prognostic indicator as significant elevations have been correlated with a higher risk of fibrotic evolution (23).

Besides characterisation of MPN patients at diagnosis, *JAK2* V617F quantitation can be useful to assess the efficacy of therapy. Non-specific cytoreductive treatments such as hydroxycarbamide have been shown to decrease *JAK2* V617F burdens in some patients, but with overall limited efficiency (24). The specific JAK2 inhibitors, although more efficient in controlling spleen size or general symptoms, show a modest effect on the reduction of the mutant burden (25). The most efficient drug to date is pegylated interferon-alpha-2a which has been reported to induce molecular remissions with techniques able to detect less than 0.1% *JAK2* V617F (26). Allogeneic stem cell transplantation (ASCT) is the only treatment potentially able to cure MPN but there remain considerable procedure-related morbidity and mortality issues. Patients for whom the *JAK2* V617F level remains >1% one month post-ASCT may possess a higher risk of relapse than those patients with deeper responses with close monitoring able to guide intervention with donor lymphocyte infusion (27, 28).

A plethora of techniques have been described to quantify *JAK2* V617F allelic burdens, based upon sequencing (Sanger or pyrosequencing), allelic discrimination or more commonly, quantitative AS-qPCR. Assay performance may vary in terms of limit of detection and specificity as demonstrated by an international comparison of these techniques (29). Suitable techniques should possess the following attributes: a low limit of detection (at least 1% for diagnosis and at least 0.1% for residual disease monitoring), a close to 100% specificity (no false positive at the minimal detection level), a high reproducibility and should be easily transferable between laboratories. Two major studies comparing *JAK2* V617F quantitation techniques have shown the most reliable and sensitive to be AS-qPCR with specificity based on primers (29, 30). One such assay using a specific reverse primer had an excellent detection level and specificity and is recommended by the European LeukemiaNet (30, 31). Selection of a PCR primer that anneals 5′ of the mutation may be beneficial to avoid false negatives caused by the rare, alternative *JAK2* exon 14 mutations (32).

JAK2 exon 12 mutations

Although the *JAK2* V617F mutation is present in approximately 97% of PV cases, a minority of patients that fulfil the criteria for PV diagnosis have no evidence of the *JAK2* V617F mutation. Scrutiny of these patients revealed the presence of several different mutations in exon 12 of the *JAK2* gene (7). Mutations were detected in individuals who presented with erythroid hyperplasia and reduced serum erythropoietin levels without concurrent expansion of the megakaryocytic or granulocytic lineages. Furthermore, endogenous erythroid colonies (EEC) could be grown from patient's blood samples in the absence of erythropoietin. *JAK2* exon 12 mutations were absent in ET and PMF patients (7).

A myriad of *JAK2* exon 12 mutations have been reported which can be substitutions, deletions, insertions and duplications, and all occur within a 44 nucleotide region in the *JAK2* gene which encompasses amino acids 533–547 at the protein level. The most commonly reported mutations are small in-frame deletions of 3–12 nucleotides with a six nucleotide deletion being the most frequent. Complex mutations are present in one-third of cases with some mutations occurring outside this hotspot region. The N542-E543del is the most common mutation (23–30%), the K537L, E543-D544del and F537-K39delinsL represent 10–14%, and R541-E543delinsK comprise less than 10% of these mutations (33). *JAK2* exon 12 mutations are located in a region close to the pseudo-kinase domain which acts as a linker between this domain and the Src homology 2 domain of JAK2. Functional studies have shown that *JAK2* exon 12 mutations cause factor independent growth similar to *JAK2* V617F that can be blocked by JAK inhibitors with phenotypic differences between the V617F and exon 12 resulting in different signalling activation downstream of JAK2. Clinically, patients with a *JAK2* exon 12 mutation present at a younger age with approximately 40% of cases being reported at, or below 50 yr of age. Although predominantly associated with a pure erythroid phenotype with haemoglobin levels and haematocrit higher compared to *JAK2* V617F positive cases, there is tri-lineage involvement in a small minority of cases (33).

The WHO diagnostic algorithm for suspected PV indicates screening for *JAK2* exon 12 mutations in those individuals

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without the *JAK2* V617F (5) making identification increasingly important in the differential diagnosis of PV. As *JAK2* exon 12 mutations are often present at low levels, Sanger sequencing is suboptimal therefore requiring alternative methods to comprehensively detect all possible mutations. The initial four *JAK2* exon 12 mutations were confirmed by AS-PCR (7) but as the number of mutations reported increased, it became apparent that AS-PCR has limited applicability in the routine diagnostic setting. Subsequently, HRM techniques, based upon the kinetics of DNA strand denaturation, have been evaluated by several groups. HRM is able to detect previously described and unknown mutations; however, sensitivity of these individual assays can vary and is dependent on the mutation detected and template quality (34, 35). Locked nucleic acid (LNA)-clamped fragment analysis exhibits superior sensitivity compared to HRM but is unable to detect substitution mutations and large duplications (36). A fragment analysis-based assay with a 2% limit of detection has been developed which can detect insertions or deletions based on amplicon size (37). These techniques are useful for discriminating between mutated and non-mutated patients with characterisation of specific mutations requiring a more sensitive AS-qPCR approach.

From estimates of mutant allele burden obtained by Sanger sequencing and fragment analysis, patients almost exclusively display low *JAK2* exon 12 mutant allele burdens (7, 38) necessitating quantitative assays that possess a high degree of sensitivity for monitoring disease. A sensitivity of 0.01% *JAK2* exon 12 mutant alleles has been achieved using AS-qPCR assays for ten mutations that represent 80% of all mutations (39) whereas an alternative, quantitative, bead-based assay has shown a sensitivity of approximately 2.5% and covers 56% of known exon 12 mutations (40). The rarity of patients with *JAK2* exon 12 mutations and the variation of the mutant allele burden make selection of an appropriate assay for the diagnostic setting challenging.

MPL exon 10 mutations

One year after the discovery of the *JAK2* V617F mutation, somatic activating mutations of the myeloproliferative leukaemia virus oncogene (*MPL*) were identified in patients with *JAK2* V617F negative ET and PMF but are absent in PV (8, 41). The *MPL* gene is located on chromosome 1p34, encodes the thrombopoietin receptor and is a key factor for growth and survival of megakaryocytes. Acquired mutations at codon W515 constitutively activate the thrombopoietin receptor by cytokine-independent activation of the downstream JAK-STAT pathway. *MPL* 515 somatic mutations are stem cell-derived events that involve both myeloid and lymphoid progenitors. The two most recurrent mutations W515L and W515K are found in approximately 15% of *JAK2* V617F-negative MPN, that is 5% of ET and up to 10% of PMF (41). *MPL* mutations may occur concurrently with the *JAK2* V617F mutation (42, 43), and concomitant *MPL* exon 10 mutations have been reported (42, 44, 45).

Mutations in *MPL* cluster in exon 10 around amino acid 515 which is located in a stretch of five amino acids (K/RWQFP) found in the cytoplasmic section of the trans-membrane domain. Recurrent pathogenic mutations include the common W515L and W515K and the rare W515A, W515R and W515S mutations. The S505N mutation was first described in familial thrombocythemia but has subsequently been identified as a somatic mutation in ET and PMF (46, 47). Alternative mutations have also reported in rare cases including V501A, S505C, A506T, V507I, G509C, L510P, R514K and R519T, although the pathogenic significance of some of these mutations is not clear (48–50).

Patients with *MPL* exon 10 mutations have a distinct clinical phenotype tending to have lower haemoglobin levels. ET patients with *MPL* mutations are also reported to have higher platelet counts, isolated megakaryocytic proliferation and higher serum erythropoietin levels compared to *JAK2* V617F positive ET patients. There seems to be no prognostic significance with respect to thrombosis, haemorrhage, myelofibrotic transformation, leukaemic transformation or survival. Patients carrying the W515K mutation have higher allelic burdens than those with the W515L allele suggesting a functional difference between these variants (41, 47). The mutant allele burden is often greater than 50% and homozygosity has been described, suggesting biallelic mutation or loss of heterozygosity (47, 51). Low-level mutations in the range of 3–15% are usually of the W515L type (45, 47).

Screening for *MPL* mutations is recommended in cases of suspected ET or PMF with unmutated *JAK2* and *CALR*. Different methods have been applied for detection of *MPL* mutations of which the two main strategies are specific detection of each known mutation separately or scanning the entire exon 10. The first strategy includes AS-PCR, AS-qPCR, pyrosequencing, deep sequencing, bead-based assay with LNA-probes and amplification refractory mutation system-PCR (43, 47, 52–55). Sensitivities are approximately 5% for all approaches with AS-qPCR achieving a sensitivity of 0.1–0.5%. However, several assays are required to detect all mutations. Recently, a multiplexed AS-PCR assay that detects the four most frequent *MPL* exon 10 mutations (W515L, W515K, W515A and S505N) with 100% specificity and a sensitivity of 2.5% has been developed (45). This multiplex PCR is performed in a two-tube format with detection achieved by fragment analysis. Alternatively, HRM and melting curve analysis (42, 50) allow the detection of all W515, S505 and unknown exon 10 mutations in one reaction with sensitivities of 2–5%. Quantitation of mutant allele burden can be used for monitoring response to therapy as demonstrated in the post-ASCT setting (56). AS-qPCR is the most sensitive method, but exhibits lower specificity due to frequent non-specific binding of the primers. *MPL* mutation detection is therefore a helpful tool to

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assess clonality in *JAK2* V617F-negative MPN and establish a diagnosis. For detection of low-level mutations and quantification of mutant allele burden, highly specific and sensitive AS-qPCR methods are warranted.

CALR exon 9 mutations

The application of whole exome sequencing to MPN patients led to the recent discovery of somatic mutations in *CALR*, the gene encoding calreticulin, a calcium binding chaperone associated with the endoplasmic reticulum (9, 10). MPN-associated *CALR* mutations are insertions, deletions or insertions and deletions (indels) and occur in exon 9 resulting in a +1 base pair frame-shift of the coding sequence and generation of a translated protein with a novel C-terminus that lacks a retrieval sequence (KDEL) typical of normal *CALR* and other endoplasmic reticulum resident proteins. *CALR* exon 9 mutations are predominantly heterozygous with the majority of cases (80–90%) carrying a 52-bp deletion: (*CALR*del52/type I; c.1092_1143del; L367 fs*46) or a 5-bp insertion *CALR*ins5/type II; c1154_1155insTTGTC; K385 fs*47). Of the remaining cases, more than 50 different variations have been identified.

Extended screening in large cohorts has confirmed the presence of *CALR* mutations in 60–80% of patients with ET and PMF that are *JAK2* and *MPL* mutation negative (57–59). *CALR* mutations are occasionally present in RARS-T (60), rarely observed in PV (61) and very rarely may coexist with the *JAK2* V617F or *BCR-ABL1* (62, 63).

CALR mutation status may also provide information on the clinical phenotype and prognosis. In retrospective analyses of ET and PMF, *CALR*-mutated patients were found to present with higher platelet count, lower haemoglobin and improved overall survival when compared to *JAK2* mutated, *MPL* mutated or triple negative patients (9). In ET, *CALR*-mutated cases are associated with a reduced cumulative incidence of thrombosis, reduced major cardiovascular events and a higher incidence of myelofibrotic transformation versus *JAK2*-mutated patients (9, 57–59). PMF patients with *CALR* mutations have a reduced rate of red-cell transfusion dependency with an improved survival as compared to *JAK2* mutated patients (64). The high prevalence of type 1 and type 2 *CALR* mutations in ET and PMF has prompted investigators to assess the impact of mutation type on prognosis and phenotype, with a higher frequency of type 1 *CALR* mutations noted in PMF compared to ET, an association in PMF of type 2 *CALR* mutations with higher circulating blast count, leucocyte count and inferior survival compared to type 1, and a potential link between *CALR* mutation type and megakaryocyte function (65–67). *CALR* mutations are thought to arise in multipotent progenitors, often as initiating events (9, 10) with mutational load increasing with myelofibrotic transformation (68).

The incidence and specificity of *CALR* mutations in ET and PMF require analytical methods which can determine *CALR* mutation status accurately and rapidly to aid the diagnostic workup of MPN. Given the multitude and complexity of *CALR* exon 9 mutations, methodologies that can accommodate this variability will provide most utility in a diagnostic setting. Whilst initial reports employed PCR of *CALR* exon 9 followed by fragment analysis or Sanger sequencing to determine *CALR* mutation status (9, 10), subsequent studies have explored the utility of alternative HRM techniques (69). A comparison of the limits of detection for HRM, next-generation sequencing (NGS), and fragment analysis techniques to detect *CALR* mutations found a bespoke NGS approach capable of detecting mutations when present at a level of 1.25%, an improvement in sensitivity over HRM and fragment analysis (70). The majority of *CALR* positive samples tested by NGS had greater than 15% mutant alleles suggesting the more readily deployed fragment analysis may be sufficient for routine molecular diagnosis in the majority of clinical laboratories (70). Further investigation by Sanger sequencing would be required to determine the exact nature of the *CALR* mutation detected by screening approaches.

The value of quantitative assessment of *CALR* mutations remains to be fully elucidated, yet preliminary data have shown a reduction in *CALR* allele burden in patients treated with interferon-alpha therapy and a potential role for monitoring disease burden post-ASCT (71, 72).

Other MPN-associated mutations

In addition to the phenotypic driver mutations of *JAK2*, *MPL* and *CALR*, many MPN patients also harbour mutations present in patients with other myeloid malignancies such as MDS and AML. Although not entirely diagnostic of an MPN, mutations in these genes that affect epigenetic regulation, cytokine signalling and mRNA splicing are of increased interest in the biology of MPN but incorporation into a molecular diagnostic algorithm is not currently advocated.

Epigenetics concerns the changes in DNA structure that result in alterations of gene expression with DNA methylation and chromatin remodelling the two main mechanisms of epigenetic regulation. Mutations in genes involved in this process such as *TET2*, *DNMT3A* or *IDH1* and *IDH2* have been described in MPN patients in variable frequencies. Two other genes, *EZH2* and *ASXL1*, which are part of the polycomb repressor complex that regulates chromatin remodelling by methylating histones, are also frequently altered in MPN (Table 1).

The *TET2* gene encodes a protein that belongs to a family of methylcytosine dioxygenases that are involved in the DNA demethylation process. *TET2* mutations may occur in the entire coding sequence and can be of various types: small deletions, insertions, substitutions leading to stop co-

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dons or substitutions affecting amino acid located in conserved positions that result in a loss of catalytic function (73). Mutations of *TET2* can occur in both *JAK2* V617F-positive and *JAK2* V617F-negative MPN but have minimal prognostic impact. Alterations of *DNMT3A* have been described over the entire coding sequence of the gene, although most mutations occur in exon 23. *DNMT3A* mutations are found in less than 10% of chronic phase MPN cases (74). *IDH1* and *IDH2* genes encode the isocitrate dehydrogenases 1 and 2, respectively. Mutations in these genes, *IDH1* (R132) or *IDH2* (R140, R172), generate metabolites that prevent histone demethylation and block hematopoietic differentiation. *IDH1/2* mutations have been detected at a very low frequency in patients with chronic phase MPN but possibly play a pathogenic role in acute transformation (75). Mutations of *EZH2* can occur throughout the entire *EZH2* coding sequence resulting in protein truncation or alteration of amino acids essential for the methyltransferase function. *EZH2* mutations are detected in a small proportion of PV patients but in a greater number of PMF patients where they appear to be associated with an adverse prognosis (76). The *ASXL1* gene encodes one of a three-member family of proteins likely to be involved in the control of chromatin structure. *ASXL1* mutations are mainly frame shift or nonsense mutations that primarily affect exon 13, but can also be detected throughout the coding sequence. *ASXL1* mutations are uncommon in chronic phase ET and PV but are more prevalent in PMF where they appear to impart a significant prognostic impact (77).

Activating mutations of *CBL* which encodes a regulator of kinase signalling are rare in the classical MPN with exon 8 and exon 9 mutations occurring most commonly in the MDS/MPN syndromes (78). SH2B3 (LNK) is a negative regulator of hematopoietic cytokine signalling with mutations in the coding gene present rarely in chronic phase MPN (79).

In addition to epigenetic regulators, mutations in genes that encode proteins which participate in the process of splicing immature mRNA have also been described in MPN, of which the most widely studied are *SF3B1*, *SRSF2* and *U2AF1*. Somatic mutations of *SF3B1* were initially detected in MDS patients and are especially prevalent in RARS-t where their presence predicts a favourable prognosis (80). In MPN, *SF3B1* mutations are mostly found in PMF where they cluster within exons 12–16 (81). The *SRSF2* and *U2AF1* genes belong to the family of regulatory factors that can activate or repress mRNA splicing. Alterations in *SRSF2* are the most frequent in splicing genes and are mostly found in PMF and MPN evolved to AML (19%). Mutations in *SRSF2* confer a poor prognosis and may coexist with other mutations as *TET2*, *RUNX1* or *ASXL1*. *U2AF1* mutations are detected in up to 15% of PMF cases, but are not present in ET or PV patients (82). All these mutations appear to accumulate during progression and indicate and advanced state of the disease.

Quality assurance of MPN molecular diagnostics

To ensure continued high performance of molecular diagnostics and clinical utility, external quality assurance (EQA) programmes are vital. These are especially important when sensitivity of the assay is critical or when quantitative results are requested, known to vary significantly due to different methodologies and assay designs. However, even among laboratories using qPCR with the same experimental protocol variation can be substantial. To overcome this variation, different approaches for standardisation of qPCR have been assessed. For quantification of *BCR-ABL1* fusion gene expression in response to tyrosine kinase inhibition in chronic myeloid leukaemia patients, results may be aligned to an international scale using a laboratory-specific conversion factor enabling consistent interpretation of individual patient response and comparison of response rates in clinical trials. This approach to establish and maintain a valid conversion factor is however, both expensive and time-consuming and has led to the development of an internationally recognised *BCR-ABL1* primary reference panel from which commercially available, secondary standards can be calibrated.

Although EQA for *JAK2* V617F detection is available, a program dedicated to assays based on AS-qPCR as a single technology is advantageous as quantitative levels can be directly compared without bias introduced by different technologies. In an attempt to standardise the quantitative measurement of the *JAK2* V617F, the European LeukemiaNet and MPN&MPNr-EuroNet have evaluated different AS-qPCR assays, including assays in widespread clinical use (30). A series of quality control rounds, involving 12 laboratories from seven countries, were undertaken to evaluate performance and finally recommend a single assay with consistent performance across different qPCR platforms for sensitive and quantitative mutation detection demonstrated. In a further MPN&MPNr-EuroNet EQA study involving 24 European laboratories, it was shown that there was relative consistency in quantitation of *JAK2* V617F allelic burdens above 1% but for values below this level, variation in the specificity and therefore sensitivity exists and is likely due to the assay employed (83).

Proficiency analysis of *MPL* mutation detection has also been addressed by the MPN&MPNr-EuroNet by quality control rounds utilising serial dilutions of plasmids containing the common exon 10 mutations. Whilst there was comparable specificity, a significant variation in sensitivity was noted and was dependent upon assay used (84). These findings suggest caution when interpreting *MPL* mutation allele

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burdens and call for the establishment of standardised protocols and reference materials.

Future directions

The ability of NGS technologies, platforms and associated bioinformatics pipelines to simultaneously sequence multiple genes and identify mutations within these genes with high specificity and comparable sensitivity lends itself to the molecular diagnosis of MPN in which a high degree of genomic complexity exists. In recent years, NGS has become more widely available and although these platforms may not currently be suited for the detection and/or quantitation of MPN-associated mutations in all diagnostic laboratories, the feasibility and accuracy of these methods have already been established in MPN molecular diagnostics: targeted exome sequencing allows the identification of several mutations that have prognostic significance with identification of these mutations demonstrated to be of clinical value in predicting relapse in the post-ASCT setting (85–87). With improvements in sensitivity, targeted NGS of specific MPN-associated mutations may also afford the detection of residual disease and leukaemic transformation (88, 89). Development and implementation of both commercial and in-house NGS-based assays for the detection of MPN-associated mutations will require both inter- and intralaboratory evaluation of ongoing performance with preliminary studies addressing these issues achieving high concordance (90).

Another emerging approach for the detection and quantitation of MPN-associated mutations is digital PCR which can achieve absolute quantitation of the target allele without the requirement for standard curve construction or comparison to a reference gene. This is achieved by partition of the template DNA into multiple PCR reactions by either droplet formation or nano-fluidics, resulting in improved sensitivity and precision with minimal requirements for validation and standardisation. Such technical advantages of digital PCR have been validated for detection of the *JAK2* V617F (91).

Continual appraisal and adaptation will be required by the MPN community to harness the multitude of molecular diagnostic approaches to maximise the efficiency of detection and quantitation of MPN-associated mutations, which in turn should allow improved clinical outcomes for patients. Whilst the diagnosis of MPN remains multidisciplinary, requiring consideration of clinical features, histo-morphological appearances and other laboratory parameters, molecular diagnostics has become, and will continue to be, an increasingly integral part of this process.

Conflict of interest

SEL, HA, JA, BB, SC, KH, LK, EL, OM, EOL, MJP, NPorret, LP, JS, MFM, NPallisgaard & SH have no conflict of interests to declare. SS is part owner of the MLL Munich Leukemia Laboratory.

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