Molecular analyses of 15,542 patients with suspected *BCR-ABL1*-negative myeloproliferative disorders allow to develop a stepwise diagnostic workflow

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ABSTRACT

We investigated 15,542 patients with suspected *BCR-ABL1*-negative myeloproliferative or myelodysplastic/myeloproliferative neoplasm (including 359 chronic myelomonocytic leukemia) by a molecular marker set. *JAK2*V617F was detected in the suspected categories as follows: polycythemia vera 88.3%, primary myelofibrosis 53.8%, essential thrombocythemia 50.2%, and not further classifiable myeloproliferative neoplasms 38.0%. *JAK2* exon 12 mutations were detected in 40.0% *JAK2*V617F-negative suspected polycythemia vera, *MPLW515* mutations in 13.2% *JAK2*V617F-negative primary myelofibrosis and 7.1% *JAK2*V617F-negative essential thrombocythemia. *TET2* mutations were distributed across all entities but were most frequent in suspected chronic myelomonocytic leukemia (77.8%). *CBL* mutations were identified in suspected chronic myelomonocytic leukemia (13.9%), primary myelofibrosis (8.0%), and not further classifiable myeloproliferative neoplasm (7.0%). This leads to a stepwise workflow for suspected myeloproliferative neoplasms starting with *JAK2*V617F and investigating *JAK2*V617F-negative patients for *JAK2* exon 12 or *MPL* mutations, respectively. In cases in which a myeloproliferative neoplasm cannot be established, analysis for *TET2*, *CBL* and *EZH2* mutations may be indicated.

Key words: myeloproliferative neoplasms, chronic myelomonocytic leukemia, molecular analyses, mutation screening, diagnostic workflow.

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Introduction

In recent years, the expansion of the molecular marker panel for the myeloproliferative neoplasms (MPNs) has paved the way to new diagnostic strategies for patients with persistent and otherwise unexplained elevated peripheral blood counts or symptoms such as splenomegaly. Following the detection of the *JAK2*V617F as the most frequent mutation in the *BCR-ABL1*-negative MPNs, especially in polycythemia vera (PV), other JAK-STAT pathway activating mutations were detected: mutations in exon 12 of *JAK2* in roughly one-third of V617F-negative PV cases, which sometimes present with an apparently isolated erythrocytosis,[1,2] and mutations of the *MPL* gene in V617F-negative essential thrombocythemia (ET) and primary myelofibrosis (PMF).[3] Grand *et al.* identified mutations of the *CBL* (Casitas B-cell lymphoma) gene in patients with PMF, but not in ET or PV.[4] Mutations of *TET2* (TET oncogene family member 2) were described in PV, ET, PMF, and in post-PV and post-ET myelofibrosis.[5] The *EZH2* (histone methyltransferase; enhancer of zeste homolog 2) gene was found to be mutated in 7-13% of patients with myelofibrosis.[6,7] Patients with chronic myelomonocytic leukemia (CMML) were observed to have a high frequency of *TET2*, *CBL*, and *EZH2* mutations.[8-10]

Design and Methods

To further evaluate the power and applicability of this new molecular marker set in a routine diagnostic setting, we investigated 15,542 patients with suspicion for a myeloproliferative or myelodysplastic/myeloproliferative neoplasm in whom *BCR-ABL1* was ruled out, and developed a stepwise diagnostic workflow based on the results. The study cohort was made up of 7,879 males and 7,663 females, median age 62.9 years (range 0.1-97.5 years). From August 2005 to June 2010, bone marrow as sole sample material (n=5,222), bone marrow in combination with peripheral blood (n=96), or peripheral blood only (n=10,224) were sent from different hematologic centers for diagnosis to the MLL Munich Leukemia Laboratory. In detail, all samples sent with the suspicion for a *BCR-ABL1*-negative MPN, myelodysplastic/myeloproliferative neoplasm, CMML, or with constellations such as "unexplained leukocytosis" or "unexplained thrombocytosis", and in whom a diagnosis of chronic myeloid leukemia (CML) had been excluded by genetic analysis, were included in the

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study. According to clinical symptoms as reported by the referring hematologists, laboratory parameters, and cytomorphological evaluation, indications for molecular diagnostics were as follows: in 739 cases the suspected diagnosis could be narrowed down to PV, in 1,620 cases to ET, and in 324 patients to PMF. In contrast, a total of 11,461 patients had suspicion of an MPN that could not be further subcategorized based on clinical symptoms or laboratory parameters when samples were taken. Isolated erythrocytosis was present in 753 patients. Thirty-six patients had otherwise unexplained thrombosis. Regarding the category of myelodysplastic/myeloproliferative disorders, 250 patients were suspected for an unclassifiable MDS/MPN (MDS/MPNu) and 359 for CMML.[11] All patients gave their written informed consent to genetic analysis and scientific studies. The study was approved by the Internal Review Board of the MLL Munich Leukemia Laboratory and was carried out in accordance with the Declaration of Helsinki.

Analysis for the *JAK2*V617F (15,363 cases analyzed; sensitivity 1%),[12] *JAK2* exon 12 (n=2,224; sensitivity 5%),[13] and *MPLW515*[14] (n=2678; sensitivity 5%) mutations by different PCR assays followed previous descriptions. The workflow was expanded in the final study period so smaller subsets of patients were investigated for recently detected mutations in genes such as *CBL* (n=480 investigated), *TET2* (n=123), and *EZH2* (n=34), sensitivity 10% for each. *CBL* was analyzed by direct Sanger sequencing of exons 8-9.[4] The whole coding region of *TET2* was covered with 24 amplicons and also analyzed by Sanger sequencing. *EZH2* mutations[9] were investigated by high-throughput sequencing (454 technology, Life Sciences, Branford, CT, USA). All mutations that were not obviously damaging (stop or frameshift) were confirmed by data base research, analysis of remission controls and/or buccal swaps to make sure that they are real mutations. CML was excluded in all cases of this study by multiplex RT (reverse transcription)-PCR for various *BCR-ABL1* transcripts.[15] The diagnosis of CMML was based on cytomorphology. In cases with eosinophilia, the presence of *PDGFR* rearrangements was excluded according to WHO 2008.[11]

Results and Discussion

*JAK2*V617F was detected in 650 of 736 (88.3%) analyzed patients with suspected PV, in 172 of 320 (53.8%) suspected PMF, and in 810 of 1,615 (50.2%) analyzed patients with suspected ET. In addition, 122 of 751 (16.2%) patients with isolated erythrocytosis were *JAK2*V617F-positive leading to a diagnosis of PV. In patients with suspected not further classifiable MPN, the *JAK2*V617F was found in 4,314 of 11,340 (38.0%) cases, confirming a diagnosis of an MPN. The frequency of the *JAK2*V617F was lower in patients with suspected myelodysplastic/myeloproliferative neoplasms (MDS/MPNu 47 of 248, 19.0%; CMML 24 of 317, 7.6%). Patients with unexplained thrombosis were *JAK2*V617F-positive in 10 of 36 (27.8%) patients. *JAK2*V617F-negative cases but suspected PV were analyzed for *JAK2* exon 12 mutations; these were found in 20 of 50 (40.0%) patients. However, *JAK2* exon 12 mutations were rarely detected (5 of 541, 0.9%) in patients with isolated erythrocytosis. *JAK2* exon 12 mutations were also found in 12 of 1,595 (0.8%) patients with suspicion for a not further classifiable MPN. No *JAK2* exon 12 mutation was identified in 32 cases with ET or 6 PMF, respectively. *MPL*W515 mutations were detected in 17 of 129 (13.2%) *JAK2*V617F-negative PMF and in 44 of 622 (7.1%) *JAK2*V617F-negative ET. *JAK2*V617F-negative patients with a suspected not further classifiable MPN but with elevated thrombocyte count showed *MPL*W515 mutations in 80 of 1,927 (4.2%) leading to narrowing down the diagnosis to ET or PMF (Table 1).

TET2 mutations were distributed across all cohorts with the highest frequency in CMML (14 of 18, 77.8%) that was higher than in suspected PMF (3 of 11, 27.3%), ET (8 of 30, 26.7%), or suspected not further classifiable MPN (16 of 64, 25.0%). *CBL* mutations showed the highest frequency in suspected CMML (23 of 165, 13.9%). Slightly lower frequencies of *CBL* mutations were found in suspected PMF (2 of 25, 8.0%) or suspected not further classifiable MPN (14 of 201, 7.0%). No case with *CBL* mutation was identified in suspected PV (n=32) or ET (n=57). *EZH2* mutations were detected in 2 of 9 (22.2%) of patients with suspected PMF and in one of 7 (14.3%) suspected CMML cases, whereas all suspected ET analyzed (n=18) were *EZH2* wild-type. Although we investigated only a subset of patients for these novel markers, these results further confirm previous observations that *CBL* mutations can be found in PMF but do not occur in ET.[4] Overall, this study reflects the experience with the above molecular marker panel for a restricted subset of patients rather than providing a complete characterization of molecular mutation profiles of the entities investigated.

Recently, Tefferi *et al.* stated that upfront *en bloc* screening for *JAK2*V617F, *JAK2* exon 12, and *MPL* mutations was scientifically irrational and economically irresponsible.[16] In accordance with this perspective, the results of molecular analysis in our cohort as measured in a routine diagnostic setting emphasize a stepwise molecular diagnostic workflow for patients with suspected myeloproliferative disorders (Figure 1). After exclusion of CML by investigation for the *BCR-ABL1* gene fusion, the algorithm starts with *JAK2*V617F mutation screening. Subsequently, *JAK2*V617F-negative patients with suspected PV (e.g. polyglobulia, bone marrow hypercellularity with increase of erythropoiesis, granulopoiesis, and megakaryopoiesis, or reduced erythropoietin levels) or isolated erythrocytosis should be analyzed for a *JAK2* exon 12 mutation. *LNK* mutations were recently suggested as a further marker in PV;[17] however, further studies showed that this is not restricted to PV[18] and the value of this marker for diagnostics in MPN has to be further evaluated.

When there is suspicion for a *JAK2*V617F-negative PMF or ET, patients should be investigated for an *MPLW*515

Table 1. Results of analyses for *JAK2*V617F, *JAK2* exon 12, and *MPL*W515 mutations in the different categories of suspicion.

Suspected diagnosis	*JAK2*V617F	*JAK2*V617F-negative patients	
		JAK2 exon 12	*MPL*W515
Polycythemia vera	650/736 (88.3%)	20/50 (40.0%)	-
Isolated erythrocytosis	122/751 (16.2%)	5/541 (0.9%)	0/20 (0.0%)
Essential thrombocythemia	810/1,615 (50.2%)	0/32 (0.0%)	44/622 (7.1%)
Primary myelofibrosis	172/320 (53.8%)	0/6 (0.0%)	17/129 (13.2%)
Thrombosis	10/36 (27.8%)	-	-
MDS/MPNu	47/248 (19.0%)	-	-
CMML	24/317 (7.6%)	-	-
Suspected MPN by hematologist sending the sample	4,314/11,340 (38.0%)	12/1,595 (0.8%)	80/1,927 (4.2%)

N. pts: number of patients; MDS/MPNu: myelodysplastic/myeloproliferative neoplasm, unclassifiable; CMML: chronic myelomonocytic leukemia.

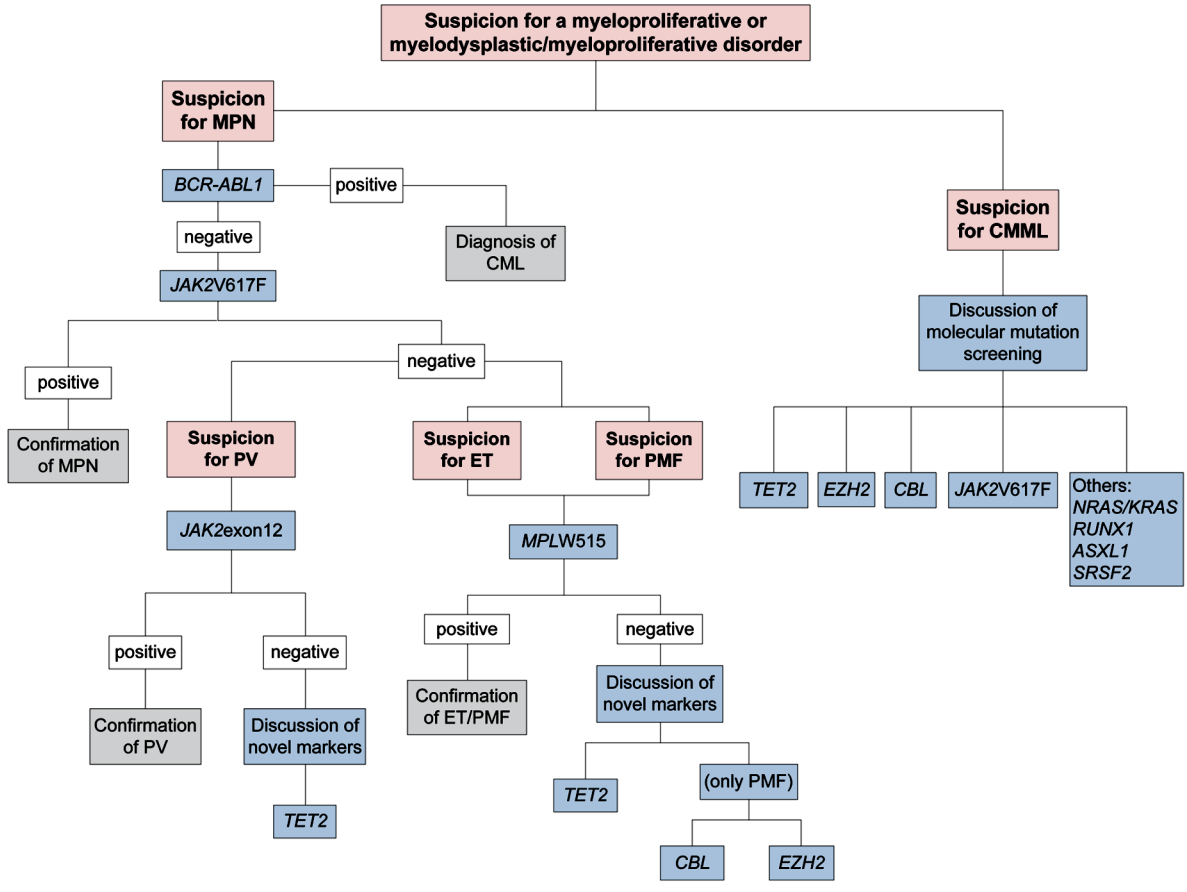

Figure 1. Proposal of a workflow for molecular diagnostics in patients with the suspicion for myeloproliferative or myelodysplastic/myeloproliferative disorders. MPN: myeloproliferative neoplasm; CML: chronic myeloid leukemia; PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; CMML: chronic myelomonocytic leukemia.

mutation. In case the diagnosis of an MPN cannot be established with any of these three markers, screening for a *TET2* mutation may be discussed for all still unexplained symptomatic patients. Samples with suspected PMF could be investigated for *CBL* or *EZH2* mutations in cases in which none of the above markers is positive. In cases of suspicion for CMML, a comprehensive molecular analysis including *JAK2*V617F, *NRAS*, *RUNX1*, *KRAS*, *TET2*, *CBL*, *EZH2*, *ASXL1*, and *SRSF2*[19] should be performed, as this is a genetically very complex disease with significant frequencies of mutations in the abovementioned genes.[9,20] The exact sequence of the respective analyses should be defined in future studies. These step-wise procedures can guide the workflow in the laboratory and establish the correct diagnosis in patients with suspected myeloproliferative disorders or CMML in a more efficient and economic way.

Authorship and Disclosures

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