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

Running title: Molecular diagnostics in patients with MPNs

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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. JAK2V617F 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% JAK2V617F-negative suspected polycythemia vera, MPLW515 mutations in 13.2% JAK2V617F-negative primary myelofibrosis and 7.1% JAK2V617F-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 JAK2V617F and investigating JAK2V617F-negative patients for JAK2 exon 12 or MPL mutations, respectively. In case a myeloproliferative neoplasm cannot be established, analysis for TET2, CBL, and EZH2 mutations may be indicated.

Key words: Myeloproliferative neoplasms (MPN); chronic myelomonocytic leukemia (CMML); molecular analyses; mutation screening; diagnostic workflow.
Introduction

In recent years, the expansion of the molecular marker panel for the myeloproliferative neoplasms (MPNs) 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 JAK2V617F 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 with 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 for a routine diagnostic setting, we investigated 15,542 patients with the 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 in this cohort (7,879 males; 7,663 females; median age, 62.9 years; range, 0.1 – 97.5 years). 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 hematological centers for diagnosis to the MLL Munich Leukemia Laboratory from 08/2005 to 06/2010. In more 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 study. According to clinical symptoms as reported by the referring hematologists, laboratory parameters, and cytomorphologic 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 which 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 followed the Declaration of Helsinki.
Analysis for the JAK2V617F (15,363 cases analyzed; sensitivity 1%), JAK2 exon 12 (n=2,224; sensitivity 5%), and MPLW515 (n=2678; sensitivity 5%) mutations by different PCR assays followed previous descriptions. The work-flow 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. The whole coding region of TET2 was covered with 24 amplicons and also analyzed by Sanger sequencing. EZH2 mutations 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 researches, 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. The diagnosis of CMML was based on cytomorphology. In cases with eosinophilia the presence of PDGFR rearrangements was excluded (according to WHO 2008).

Results and Discussion

The JAK2V617F was detected in 650/736 (88.3%) of patients with suspected PV analyzed, in 172/320 (53.8%) suspected PMF, and in 810/1,615 (50.2%) of patients analyzed for suspected ET. In addition, 122/751 (16.2%) patients with isolated erythrocytosis were JAK2V617F-positive, leading to a diagnosis of PV. In patients with suspected not further classifiable MPN, the JAK2V617F was found in 4,314/11,340 (38.0%) cases confirming a diagnosis of an MPN. The frequency of the JAK2V617F was lower in patients with suspected myelodysplastic/myeloproliferative neoplasms (MDS/MPNu: 47/248; 19.0%; CMML: 24/317; 7.6%). Patients with unexplained thrombosis were JAK2V617F-positive in 10/36 (27.8%). JAK2V617F-negative cases but suspected PV were analyzed for JAK2 exon 12 mutations. These were found in 20/50 (40.0%). However, in patients with isolated erythrocytosis JAK2 exon 12 mutations were rarely detected (5/541; 0.9%). JAK2 exon 12 mutations were also found in 12/1,595 (0.8%) of 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. MPLW515 mutations were detected in 17/129 (13.2%) JAK2V617F-negative PMF and in 44/622 (7.1%) JAK2V617F-negative ET. JAK2V617F-negative patients with a suspected not further classifiable MPN but with elevated thrombocyte count showed MPLW515 mutations in 80/1,927 (4.2%) leading to a narrow down of the diagnosis to ET or PMF (Table 1).

TET2 mutations were distributed across all cohorts with the highest frequency in CMML (14/18; 77.8%) which was higher than in suspected PMF (3/11; 27.3%), ET (8/30; 26.7%), or suspected not further classifiable MPN (16/64; 25.0%). CBL mutations showed the highest frequency in suspected CMML (23/165; 13.9%). Slightly lower frequencies of CBL mutations were found in suspected PMF (2/25; 8.0%) or suspected not further classifiable MPN (14/201; 7.0%). No case with CBL mutation was identified in suspected PV (n=32) or ET (n=57). EZH2 mutations were detected in 2/9 (22.2%) of patients with suspected PMF and in 1/7 (14.3%) of 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 give further confirm to previous observations that CBL mutations can be
found in PMF but do not occur in ET.\textsuperscript{5} 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\textsuperscript{V617F}, JAK2 exon 12, and MPL mutations was scientifically irrational and economically irresponsible.\textsuperscript{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 by JAK2\textsuperscript{V617F} mutation screening. Subsequently, JAK2\textsuperscript{V617F}-negative patients with suspected PV (e.g. polycythemia, 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\textsuperscript{17}, however further studies showed that this is not restricted to PV\textsuperscript{18} and the value of this marker for diagnostics in MPN has to be further evaluated. When there is suspicion for a JAK2\textsuperscript{V617F}-negative PMF or ET, patients should be investigated for an MPL\textsubscript{W515} 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 in all still unexplained symptomatic patients. Samples with suspected PMF could be investigated for CBL or EZH2 mutations in case none of the above markers is positive. In case of suspicion for CMML, a comprehensive molecular analysis including JAK2\textsuperscript{V617F}, NRAS, RUNX1, KRAS, TET2, CBL, EZH2, ASXL1, and SRSF2\textsuperscript{19} should be performed as this is a genetically very complex disease with significant frequencies of mutations in the before mentioned genes.\textsuperscript{6,20} The exact sequence of the respective analyses should be defined in future studies. These step-wise procedures allow to guide the workflow in the laboratory and to establish the correct diagnosis in patients with suspected myeloproliferative disorders or CMML in a most efficient and economic way.

**AUTHORSHIP AND DISCLOSURES**

SS and TH performed design of study. SS, UB, TA, and WK performed data analysis. UB, SS, and TH wrote manuscript. SS, CE, FD, VG, and AK did molecular analyses. CH was responsible for cytogenetics, TH performed cytomorphology. All authors contributed to writing of the paper and reviewed and approved the final version.

SS, WK, CH, and TH declare part ownership of the MLL Munich Leukemia Laboratory GmbH. CE, FD, TA, VG, and AK are employed by the MLL Munich Leukemia Laboratory GmbH. UB has nothing to disclose.
References


# TABLES

**Table 1.** Results of analyses for JAK2V617F, JAK2 exon 12, and MPLW515 mutations in the different suspicion categories.

<table>
<thead>
<tr>
<th>Suspected diagnosis</th>
<th>JAK2V617F</th>
<th>JAK2V617F-negative patients</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>JAK2 exon 12</td>
</tr>
<tr>
<td>PV</td>
<td>650/736 (88.3%)</td>
<td>20/50 (40.0%)</td>
</tr>
<tr>
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<td>122/751 (16.2%)</td>
<td>5/541 (0.9%)</td>
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<tr>
<td>ET</td>
<td>810/1,615 (50.2%)</td>
<td>0/32 (0.0%)</td>
</tr>
<tr>
<td>PMF</td>
<td>172/320 (53.8%)</td>
<td>0/6 (0.0%)</td>
</tr>
<tr>
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<td>10/36 (27.8%)</td>
<td>-</td>
</tr>
<tr>
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<td>47/248 (19.0%)</td>
<td>-</td>
</tr>
<tr>
<td>CMML</td>
<td>24/317 (7.6%)</td>
<td>-</td>
</tr>
<tr>
<td>Suspected MPN by hematologist sending the sample</td>
<td>4,314/11,340 (38.0%)</td>
<td>12/1,595 (0.8%)</td>
</tr>
</tbody>
</table>

no of pts: number of patients; PV: polycythemia vera; ET: essential thrombocythemia; PMF: primary myelofibrosis; MDS/MPNu: myelodysplastic/myeloproliferative neoplasm, unclassifiable; CMML: chronic myelomonocytic leukemia.
FIGURE LEGENDS

**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).