Coexisting driver mutations in MPN: clinical and molecular characteristics of a series of 11 patients

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INTRODUCTION

The four most common myeloproliferative neoplasms (MPN) are chronic myeloid leukemia (CML), polycythemia vera (PV), primary myelofibrosis (PMF) and essential thrombocythemia (ET). A major breakthrough in the understanding of Philadelphia-negative (Ph−) MPN was the discovery of somatic mutations in three driver genes (JAK2, CALR and MPL), now representing a major criterion in the WHO diagnostic criteria [1]. Although driver mutations were thought to be mutually exclusive, several cases of CML coexisting with Ph− MPN have been reported [2–27].

The incidence of coexisting driver mutations in MPN has therefore recently been studied. In 5455 patients with suspected MPN, 1775 (32%) showed mutations of JAK2, CALR and/or MPL. Coexistence of these driver mutations was detected in 1% of Ph− MPN. In addition: JAK2 or CALR mutations were found in 2% of CML patients [28]. In another study, the JAK2 V617F mutation and the BCR-ABL1 fusion gene were combined in 23/10,875 (0.2%) of MPN patients [29]. The finding of two driver mutations in one patient is thus uncommon. We retrospectively examined the characteristics of 11 MPN patients with coexisting driver mutations and explored which clinical or biological features can be distinctive of the coexistence of driver mutations.

Furthermore, Next Generation Sequencing (NGS) has recently been introduced in the diagnostic and prognostic assessment of MPN and identified several mutations in non-driver genes [30,31]. Accordingly, we analysed the mutational status of 50 genes recurrently mutated in myeloid disorders by this sequencing technique in six patients, in order to assess possible associated somatic mutations in our study cohort. Finally, we evaluated the efficacy and toxicity of combination therapy with tyrosine kinase inhibitors (TKI) and hydroxyurea in our study population.

In this paper, we present a detailed description of the clinical, biological and molecular characteristics of a series of 11 MPN patients with coexisting driver mutations. In addition, we show data concerning the...
efficacy and toxicity of combination therapy of TKI with other cytoreductive medication (e.g. hydroxyurea).

**Patients and methods**

Eleven MPN patients with coexisting driver mutations were identified at the University Hospitals of Leuven by cytogenetic and molecular analysis between 1994 and 2017. We retrospectively studied the patient characteristics of these 11 patients including age at diagnosis, disease-related symptoms and clinical characteristics, as well as treatment, treatment response and toxicity. Furthermore, we analysed the cytogenetic abnormalities and molecular analyses of these patients and the evolution during follow-up. The diagnosis of CML was made by chromosome banding analysis, supplemented if needed by interphase fluorescence in situ hybridization (FISH). The JAK2 allele burden (% JAK2 V617F-mutated cells) was assessed by quantitative PCR on DNA enhanced from peripheral non-fractionated leukocytes. The CALR mutation was identified by means of Sanger sequencing. All data were collected from the electronic medical records of our institution and five other hospitals (OLV Aalst, ZOL Genk, Clinique St-Luc Bouge, AZ Damiaan Oostende, AZ Sint-Maarten Duffel, AZ Damiaan Oostende). Three patients died during follow-up.

We analysed the DNA of six MPN patients with coexisting driver mutations for mutations of the following genes: ABL1, ASXL1, ATRX, BCOR, BCORL1, BRAF, CALR, CBL, CBLB, CBLC, CDKN2A, CSF3R, CUX1, DNMT3A, ETV6, EZH2, FBXW7, FLT3, GATA1, GATA2, GNAS, IDH1, IDH2, IKZF1, JAK2, KDM6A, KIT, KRAS, MPL, MYD88, NOTCH1, NPM1, NRAS, PDGFRα, PHF6, PTPN11, RAD21, RUNX1, SETBP1, SF3B1, SMC1A, SMC3, SRSF2, STAG2, TET2, TP53, U2AF1, WT1, ZRSR2 (50 gene TruSight Myeloid Sequencing kit of Illumina, San Diego, CA, USA). The detection limit of the assay was <5% of mutated alleles.

**Results**

**Patient characteristics**

The characteristics of 11 patients diagnosed with an MPN and coexisting driver mutations are listed in Table 1. A brief overview is described in the next section. Median age at first diagnosis in our series was 71 years (range 27–81 years). Six (55%) patients were male and all patients were Caucasian. The most common combination (n = 8) in our study population was the combination of the JAK2 V617F mutation and the BCR-ABL1 fusion gene (in patients with an initial diagnosis of ET (n = 4), PV (n = 2), PMF (n = 1) and myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) (n = 1)). In five out of nine patients, the diagnosis of MPN preceded the diagnosis of CML. In three out of nine patients, both mutations were detected concurrently and one patient was diagnosed with JAK2 V617F+ post-polycythemia vera myelofibrosis (PPV-MF) after the diagnosis of CML.

All CML patients were diagnosed in chronic phase. Three out of five patients diagnosed with Ph− MPN before CML were asymptomatic at the time of this first diagnosis. Yet, a shift to the clinical phenotype of a BCR-ABL1+CML, including splenomegaly and leukocytosis, occurred in most patients at the time of this second diagnosis. However some typical features of the initial MPN, e.g. a high haematocrit in PV, remained. This observation is consistent with previous reports [6,11–15]. Laboratory findings at diagnosis of first and second MPN are shown in Table S1. The molecular features at diagnosis of first and second MPN are shown in Table 2.

In four patients follow-up data of the BCR-ABL1 transcript and JAK2/CALR allele burden over time were available. These are illustrated in Figure 1. As shown, in these four patients, after start of imatinib, the BCR-ABL1 transcript level decreases followed by an increase in JAK2/CALR allele burden.

**Brief overview of case reports**

Patient 1 was diagnosed with CML 13 years after the diagnosis of ET. Major molecular response (MMR) was achieved after 23 months of imatinib and persists to the present day.

Three years after diagnosis of PMF, patient 2 was diagnosed with CML. Splenectomy was performed because of abdominal discomfort. Cytogenetic analysis of the splenic cells showed presence of the Philadelphia chromosome, which was subsequently confirmed in bone marrow (BM) cells. He was started on imatinib but switched to nilotinib after nine months due to intolerance, reaching MMR seven months later.

Patient 3 was started on imatinib 400 mg/d after diagnosis of ET and CML simultaneously. He achieved complete cytogenic response (CCyR) after 17 months but never achieved MMR. Dose of imatinib could not be adjusted because of intolerance. ABL1 kinase domain mutations (c.1195G > A; p.A399T) were found by cytogenetic analysis in 25% of the BCR-ABL1 transcripts but this clone could not be detected in later analyses despite continuation of imatinib.

Patient 4 was diagnosed with a p190 BCR-ABL1+CML (high Sokal score: 1.74) unlike other patients who expressed a p210 BCR-ABL1 fusion protein. He reached CCyR after 6 months of imatinib, but never achieved MMR. Cytogenetic analysis showed no ABL1 kinase domain mutations. One year later, he acquired a JAK2 V617F+ PPV-MF for which hydroxyurea was associated. 38 months after diagnosis of CML, he
progressed to CML in blast phase and died one month later.

Patient 5 presented with a Budd-Chiari syndrome one year after the diagnosis of PV. Vitamin K antagonist and hydroxyurea were added to the therapy with intermittent phlebotomies. 61 months later, she presented with progressive splenomegaly and leukocytosis with thrombocytosis. Karyotype showed a t(9;22)(q34;q11), addition to t(9;22), a del(11)(q13q24) in 18/20 mitoses (different clones). This deletion is a common cytogenetic finding in myeloid malignancies and is, according to the IPSS-R model, a prognostically favourable finding in MDS [32]. However, this abnormality had not been observed at initial diagnosis.

In patient 6, cytogenetic analysis one year after the diagnosis of a JAK2 V617F" MDS/MPN-RS-T showed in 18/20 mitoses (different clones). This deletion is a common cytogenetic finding in myeloid malignancies and is, according to the IPSS-R model, a prognostically favourable finding in MDS [32]. However, this abnormality had not been observed at initial diagnosis.

In patient 7, karyotype at diagnosis of ET was: 46,XX, del(20)(q12). 18 years later, a new bone marrow biopsy was performed because of unexpected leuko- and thrombocytosis. Karyotype showed a t(9;22)(q13;q24), but the del(20)(q12) could no longer be retrieved. This deletion is a recurrent finding in PV and PMF and may also occur in ET, MDS and acute myeloid leukemia (AML). In MPN, the presence of del(20)(q12) does not appear to adversely affect survival [33]. At present, both CML and PET MF are well controlled, respectively with imatinib and ruxolitinib which was associated for persisting splenomegaly.

Patient 8 was diagnosed with a (KIT/PDGFRA-negative) GIST tumour. After surgery and six months adjuvant therapy with imatinib a bone marrow biopsy was performed because of persistent thrombocytosis. Molecular and cytogenetic analysis showed a JAK2 V617F mutation (allele burden: 54%) and a low BCR-ABL fusion gene (no MMR), probably due to the previous TKI exposure. In retrospect, leukocytosis and thrombocytosis had been present already before surgery but leukocytosis disappeared during adjuvant treatment. Treatment with imatinib was resumed and MMR was achieved 6 months later.

Patient 9 and 11 were diagnosed with Ph- MPN with driver mutations in two distinct genes at the time of diagnosis. Patient 9 (ET) developed hydroxyurea-induced ILD after 8 months and was switched to anagrelide.

Patient 10 was diagnosed with ET and CML, treated with nilotinib at diagnosis and reached a documented MMR after four months, but was switched to dasatinib, reaching MMR eight months later.
**Table 2.** Molecular characteristics of MPN patients with concurrent driver mutations.

| Patient | MPN | Driver mutations | Cytogenetic abnormalities at 1st diagnosis | Cytogenetic abnormalities at 2nd diagnosis |
|---------|-----|------------------|------------------------------------------|------------------------------------------|
| CML after Ph− MPN | | | | |
| 1 | ET/CML | JAK2 V617F/BCR-ABL1 (p210) – | t(9;22)(q34q11) |
| 2 | PMF/CML | JAK2 V617F/BCR-ABL1 (p210) – | t(9;22)(q34q11)* |
| 3 | PV/PMF | JAK2 V617F/BCR-ABL1 (p210) – | t(9;22)(q34q11) |
| 4 | MDS/MPN-RS-T | JAK2 V617F/BCR-ABL1 (p210) – | t(9;22)(del[11](q13q24);add[21](q11)) |
| 5 | ET/ET-PET/MF/CML | CALR type 2/BCR-ABL1 (p210) del(20)(q12) | t(1;9;22)(p35;q34q11q28) |
| MPN after CML | | | | |
| 6 | PPV-MF/CML | JAK2 V617F/BCR-ABL1 (p190) | t(9;22)(q34q11) |
| Concomitantly | | | | |
| 7 | ET/CML | JAK2 V617F/BCR-ABL1 (p210) t(9;22)(q34q11p12) | t(9;22)(q34q11p12) |
| 8 | ET/CML | JAK2 V617F/BCR-ABL1 (p210) Normal* | Normal* |
| 9 | ET/CML | JAK2 V617F/BCR-ABL1 (p210) t(9;22)(q34q11) | t(9;22)(q34q11) |
| One MPN with two driver mutations | | | | |
| 10 | ET | JAK2 V617F/MPM W515R – | – |
| 11 | PMF | JAK2 V617F/CALR type 1 – | – |

*aKaryotype on splenic cells.

*bDiagnosis of CML after 6 months adjuvant treatment with imatinib for GIST tumour.

Abbreviations: BM: bone marrow cells; ET: essential thrombocythemia; PV: polycythemia vera; PPV-MF: post-polycythemia vera myelofibrosis; CML: chronic myeloid leukemia; PMF: primary myelofibrosis; PET-MF: post-essential thrombocythemia myelofibrosis; MDS/MPN-RS-T: Myelodysplastic/MPN overlap syndrome with ring sideroblasts and thrombocytosis.

**Treatment and response**

Eight out of nine patients with a coexisting BCR-ABL1 translocation were treated with imatinib at diagnosis and monitored by BM karyotyping and quantitative RT–PCR on peripheral blood leukocytes. Five out of eight patients reached MMR after a median treatment time of 14 months (range 4–23 months). Four out of eight patients failed to reach MMR after 18 months (Table S2). In patients with good treatment response, a reconversion to the initial MPN phenotype was observed.

In this limited series, only four out of eight MPN cases with coexisting BCR-ABL1 fusion gene and JAK2 V617F/CALR mutation achieved an optimal response with treatment of imatinib, while three patients developed resistance to TKI, in two of whom BCR-ABL1 kinase domain mutations could be detected.

**Toxicity**

Table S2 lists all treatment strategies and corresponding toxicities in chronological order. In 6 out of 6 MPN patients treated with hydroxyurea alone (ET: n = 3, PV: n = 1, PMF, n = 1, MDS/MPN-RS-T: n = 1), this cytoreductive therapy had to be discontinued or switched to another cytoreductive drug. Median treatment time before occurrence of significant toxicity was 15 months (range 1–61 months). Furthermore, 4 out of 4 patients treated with combination therapy of hydroxyurea and imatinib (ET + CML: n = 2, PMF + CML: n = 1, PPV-MF + CML: n = 1) developed major intolerance after an median treatment time of 2 months (range 1–9 months). The most common side effect was haematological toxicity, particularly anaemia, thrombopenia and pancytopenia. Thus, we observe a lower than expected overall tolerance and higher than expected toxicity of hydroxyurea and/or imatinib.

**Next Generation Sequencing**

We analysed the DNA of six MPN patients with coexisting driver mutations (JAK2 V617F + BCR-ABL1: n = 3; CALR type 2 (p.K385Fs*47) + BCR-ABL1: n = 1; JAK2 V617F + MPL W515R: n = 1; JAK2 V617F + CALR type 1 (p.Leu367Thrfs*46): n = 1) for mutations in 50 myeloid related genes. NGS revealed five (83%) additional somatic mutations in four non-driver genes, i.e. in FBXW7, TET2, ASXL1 and CUX1. Two patients presented with CUX1 mutation. Variants were classified according to the classification system of Sukhai et al, whereas class 1 and 2 variants are the most clinically relevant [34] (Table S3).

**Discussion**

The coexistence of driver mutations in MPN patients is a rare event and has intrigued several authors in the last few years. We here present a detailed clinical, biological and molecular description of 11 MPN patients with coexisting driver mutations. These data demonstrate that MPN patients with coexisting driver mutations are heterogeneous in both clinical and molecular presentation, which is also supported by NGS. Intriguingly, imatinib only induced a suboptimal response in 4 out of 8 patients with BCR-ABL1 coexisting with another driver mutation. The overall tolerance of treatment with hydroxyurea and/or imatinib in our series was poor with a higher toxicity than expected.

The finding of coexisting mutations raises the question whether both mutations are acquired by one malignant clone or whether they are each acquired within a separate clone. In the latter case, two coexisting clones and MPN have to be postulated [2,3,7,11]. In four patients, we observed decreasing BCR-ABL1 transcript levels and increasing JAK2 V617F/CALR allele burden under TKI (Figure 1), in line with several other case reports [5,7,14,22,24] and a recent analysis of 23
MPN patients [29]. This increase of the JAK2 V617F/CALR allele burden despite a decreasing BCR-ABL1 transcript level strongly suggests the coexistence of two different clones or subclones growing independently. However, the presence of both mutations in the same clone has also been demonstrated in a patient with CALR mutation and BCR-ABL1 rearrangement [35]. It is well known that acute leukemias arising in JAK2 V617F positive MPN can either be JAK2 V617F positive or negative, supporting the notion of a distinct subclonal origin of AML versus AML from a JAK2 V617F-negative MPN progenitor. To study the origin of the JAK2 V617F negative leukemic clone, Theocharides and colleagues performed additional molecular analyses on two samples. In the second case, the JAK2 V617F allele comprised 51% of JAK2 alleles in unfractionated leukocytes, but was absent in sorted leukemic blasts. In contrast, a del(11q) was identified in all metaphases and 99% of interphase nuclei, highly suggestive of a common parental clone [36]. The divergent evolution of BCR-ABL1 transcript level and JAK2 V617F/CALR allele burden that we observed, supports the notion that the Philadelphia translocation and the JAK2 V617F mutation are present in distinct (sub)clones. The molecular mechanism that gives rise to these multiple mutations/clones remains unclear. Although the acquisition of two MPN driver mutations could be a mere coincidence, some authors claim that the mutated clones underlies the acquisition of multiple mutations/different clones [29,37].

The incidence of suboptimal responses to TKI in CML with coexisting mutations was higher than expected for CML in first chronic phase. This was not due to toxicity of high dose TKI or more aggressive disease; most patients were treated with imatinib 400 mg daily and EUTOS scores in these patients were mostly low (Table S2). If successful, TKI treatment led to the restoration of the initial MPN phenotype.

We also observed a rather poor tolerance of hydroxyurea, necessitating discontinuation of treatment in all. In randomized trials, the discontinuation rates of hydroxyurea in Ph⁻ MPN patients varied from 5% to 10% [38]. Combination of imatinib with hydroxyurea was even tolerated more poorly. We observed significant toxicity in all patients after a median treatment time of merely 2 months. Dasatinib or combination of hydroxyurea with nilotinib or imatinib with ruxolitinib seemed to be better tolerated. The latter combination therapy has already proven to be effective in two patients with diagnosis of PPV-MF and coexisting CML [39]. To the best of our knowledge, data concerning the efficacy and toxicity of TKI combined with other cytoreductive medication were never reported previously.

For many years, authors have suggested a hypermutable state in MPN [40–42]. A recent study however shows a low mutation rate, i.e. 0.2 mutations per Mb
(0.37 mutations per Mb for AML and 1 mutation per Mb for multiple myeloma) [30]. We therefore analysed six patients for mutations in 50 genes, recurrently mutated in myeloid malignancies (Table S3). This revealed five additional somatic mutations in four different non-driver genes, i.e. in FBXW7, TET2, ASXL1 and CUX1. Interestingly, in two patients a CUX1 mutation was found. These 2 patients corresponded to the only non-CML patients included in the present series with coexisting driver mutations in JAK2/MPL and JAK2/CALR, respectively.

Overall, NGS in these MPN patients with coexisting driver mutations revealed four different additional somatic mutations in five out of six patients (83%). However, four out of six patients harboured mutations in genes who are believed to precede or follow the acquisition of driver mutations in MPN, i.e. TET2, ASXL1 and CUX1 [43].

Moreover, several studies on the prognostic relevance of these somatic mutations suggest that the presence of two or more somatic mutations in MPN patients represents a negative prognostic factor [30,31]. Other recent evidence shows that not only the number but the specific type of somatic mutation is prognostically relevant [44–46]. For example, both number and type of mutation are interrogated in the MIPSS570 and the MIPSS70-plus score, which are prognostic systems for transplantation-age patients with PMF that integrate clinical, cytogenetic, and mutation data [47]. In order to establish whether the prognosis of MPN patients with coexisting mutations differs from other MPN patients, further studies with longer follow-up are needed.

Conclusion

From this retrospective analysis of 11 cases of MPN with coexisting driver mutations, it comes forward that these patients are heterogeneous in both clinical and molecular presentation, a finding also supported by NGS. Our observations of the evolution of BCR-ABL1 transcript level and JAK2 V617F/CALR allele burden under TKI support that the coexistence of driver mutations likely originates from two independent clones, rather than a compound mutation arising in a subclone of the ancestral clone.

Despite the extreme rarity of coexisting mutations in MPN patients, this phenomenon should be sought in MPN patients who present or develop an atypical clinical phenotype or clinical course. The results of the present study suggest that these patients are more likely to respond suboptimally to TKI, and are more susceptible to therapy-related toxicities and that treatment of these patients should be focused on CML with additional measures for controlling hematocrit, platelets and symptomatic splenomegaly in an individualized approach.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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