

CORRESPONDENCE

Dominance of mutations in epigenetic regulators and a diversity of signaling alterations in blast-phase *BCR::ABL1*-negative myeloproliferative neoplasms

To the Editor:

BCR::ABL1-negative myeloproliferative neoplasms (MPNs) can evolve to secondary acute myeloid leukemia (sAML) or blast-phase (BP) MPN, a very severe condition with lack of effective therapy.¹ Leukemic transformation (LT) of MPNs displays a variable incidence according to MPN phenotype: 9%–13% in primary myelofibrosis (PMF), 3%–7% in polycythemia vera (PV), and 1%–4% in essential thrombocythemia (ET).¹ Here, we investigated the mutational landscape, copy number variations (CNVs), and uniparental disomy (UPD) events in BP-MPN cases that were diagnosed over a 6-year period of monitoring in three different hematology units (Fundeni Clinical Institute, Coltea Hospital and Emergency University Hospitals, Bucharest, Romania) and the patterns of clonal evolution in a subset of patients with available paired chronic phase (CP)-BP DNA samples.

The study was approved by the local ethics committee (No. 136/06.02.2017 rev. no.131/18.01.2019) and was performed in conformity with the Declaration of Helsinki. A written informed consent was provided by each patient at collection of samples that were referred to Stefan S Nicolau Institute of Virology, Romania, for molecular analysis. Clinical, morphological, and immunophenotypic data were provided from the medical records for all recruited patients. Peripheral blood or bone marrow (BM) mononuclear cells were isolated and processed to obtain various cell fractions. CD3+ T cells were used as reference for germline mutations. Molecular testing for MPN-driver mutations, targeted next-generation sequencing (NGS), whole-exome sequencing (WES), single nucleotide polymorphism (SNP) microarray analysis, and multiplex ligation-dependent probe amplification (MLPA) were performed as described by manufacturers (see Supplemental file; Data S1 for a complete description of methods).

A total of 33 patients (median age, 63 years; 57.6% males) were diagnosed with BP-MPN between 2017 and 2023, in the above-mentioned centers, including 20 post-PMF (60.4%), and 13 post-ET/PV (39.4%) cases (Table S1). A prior stage of secondary myelofibrosis was confirmed by BM biopsy in 8 out of 13 post-ET/PV AML (61.5%) patients. According to morphologic and immunophenotypic data, sAML cases were classified as AML with myelodysplasia-related changes ($n = 4$, 12.1%) and AML, not otherwise specified ($n = 29$, 87.9%), as follows: AML with minimal differentiation ($n = 6$, 18.2%), AML without maturation ($n = 12$, 36.4%), acute myelomonocytic leukemia ($n = 6$, 18.2%), acute monocytic leukemia ($n = 1$, 3%), pure erythroid leukemia ($n = 1$, 3%), and acute megakaryoblastic leukemia ($n = 3$, 9.1%).

Concerning the MPN drivers detected at CP, 60.6% of patients carried *JAK2* V617F mutation, 21.2% harbored calreticulin (*CALR*) mutations (5 type1/type 1-like, 2 type2/type-2 like), and 18.2% were classified as triple-negative (TN-MPNs). We report a median age at BP diagnosis significantly lower in *CALR*-mutated and TN-MPNs compared with *JAK2*-mutated ones (54, 56, vs. 67.5 years, $p = .014$), and a median time from MPN diagnosis to BP conversion significantly shorter in TN-MPNs compared with *CALR* and *JAK2*-mutated ones (1, 3 vs. 7 years, $p = .0476$) (Table S1). Overall, the median survival was 3 months (range, 1–54 months) without significant differences between the three groups of patients (Table S1).

By targeted NGS/WES testing of matched blast and CD3+ DNA samples, a total number of 62 somatic mutations apart from MPN-driver mutations were detected. The most frequent anomalies were represented by epigenetic mutations (72.8%), followed by *TP53* mutations (33.3%), mutations of signaling molecules (24.2%, significantly more frequent in *JAK2* V617F-negative groups, $p = .005$), transcriptional regulators (21.2%), and mRNA splicing factors (15.2%) (Table S1). The frequencies of individual mutations are shown in Figure 1A. Prevalence of prior exposure to ruxolitinib versus hydroxyurea was similar among the *ASXL1*, *EZH2*, and/or *NRAS*-mutated patients (Figure 1B). Importantly, a high occurrence of multiple CNVs (≥ 3) (42.4%) was detected by SNP array and MLPA. *Del(17p)* including *TP53* gene was the most frequent anomaly, followed by *del(5q)*, *del(7q)* including *EZH2* gene, *del(20q)*, gain of chromosome 21, and other less frequent anomalies. Also, UPD events involving *JAK2* (9p), *TP53* (17p), *CBL* (11q), and *NRAS* (1p) were observed in the analyzed cohort (Figure 1C). *TP53* mutations co-occurred with multiple CNVs in 10 cases (30.3%) and with *del(17p)* in 9 cases (27.3%), being absent in *CALR*-mutated patients. All 5 detected *RUNX1* mutations were identified as comutations to *JAK2* V617F. The co-occurrence of *JAK2-TP53* was found in 6 patients at BP (18.2%) (Figure 1D). Detailed information about demographics, genomic aberrations, therapy in CP and BP, clinical outcome, and survival for each BP-MPN case is displayed in Table S2.

Thirteen paired CP-BP DNA samples were available for combined genomic analysis. In CP-MPN patients, the median number of aberrations was 3 (maximum 8). Eleven patients carried MPN-driver mutations: seven *JAK2* V617F, three *CALR* type1/type 1-like, and one with both *CALR* type 2 and low-burden *JAK2* V617F. All of them had at least one additional genetic lesion, the most frequent being *TP53* heterozygous mutations, *del(5q)*, and *ASXL1* mutations. In BP-MPN, the median

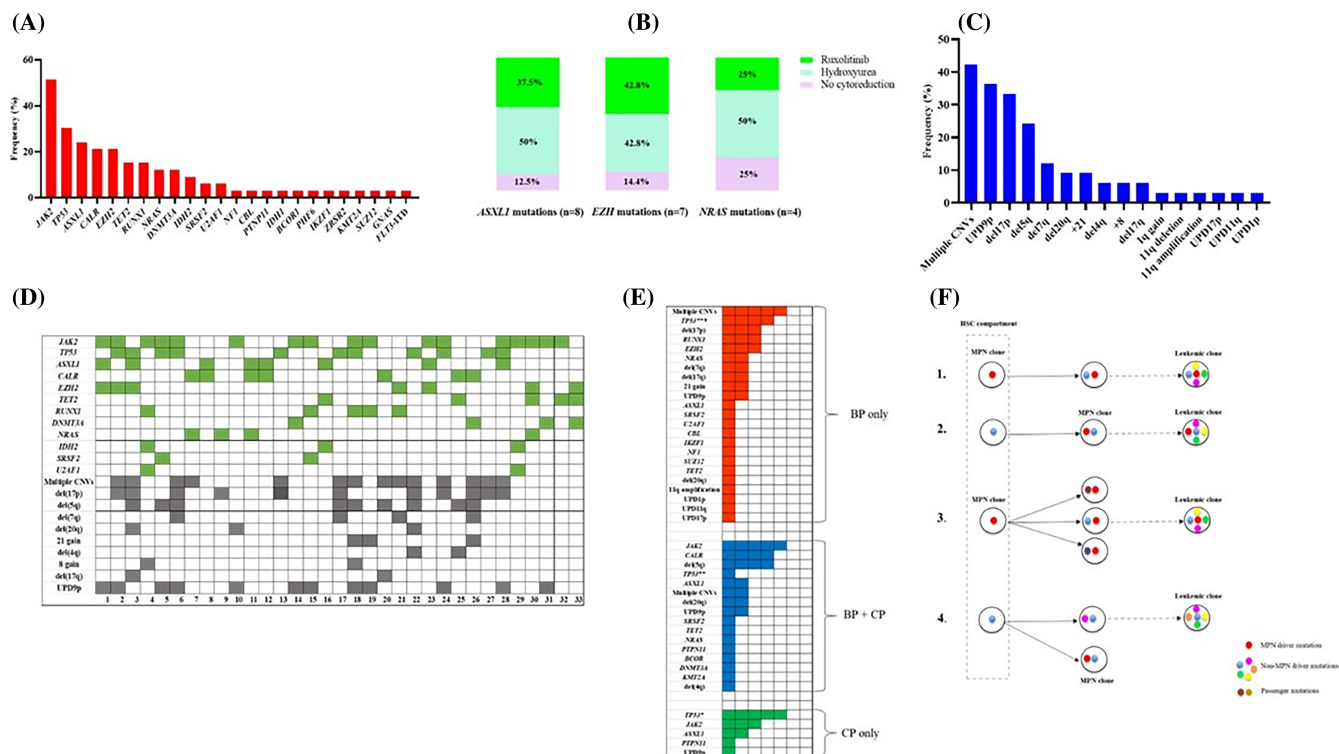


FIGURE 1 (A) Frequency of somatic mutations displayed by genes involved. (B) Prevalence of prior exposure to ruxolitinib or hydroxyurea in BP-MPN patients carrying ASXL1, EZH2, and/or NRAS mutations. (C) Frequency of CNVs and UPD events. (D) Co-occurrence of genomic alterations. The columns represent samples (33), and the rows represent mutated genes and chromosomal aberrations. Only anomalies identified in at least 2 patients were included. Somatic mutations are depicted in green, while CNVs and UPD events in gray. (E) Profiles of genomic alterations found exclusively in BP or CP or in both CP-BP. ** multi-hit TP53 with LOH at 17p TP53 locus, ** multi-hit TP53 with 2 TP53 mutations, * single-hit TP53 (F). Schematic representation of four clonal evolution patterns in MPNs. CP—chronic phase; BP—blast phase; MPN—myeloproliferative neoplasms; HSC—hematopoietic stem cell; CNVs—copy number variations; UPD—uniparental disomy. Frequency and distribution of genomic aberrations in BP-MPN patients and temporal changes in distribution of genomic aberrations on MPN progression to sAML.

number of aberrations increased to 6 (maximum 9). JAK2 V617F was lost in three BP-MPNs, while CALR mutations were present at both MPN and LT in all cases. Loss of heterozygosity (LOH) events affecting TP53, NF1 and SUZ12 (17q), and CBL, as well as RUNX1 mutations, EZH2 mutations, del(7q), and 21q gain were detected exclusively in BP (Figure 1E).

Based on the VAFs of the somatic mutations identified by targeted NGS/WES and also on the information provided by SNP array/MLPA in paired CP-BP DNA samples, four patterns of clonal evolution were inferred of which three involved the MPN phenotypic driver mutation clone(s), while one involved the non-MPN-mutated clone (Figure 1F): (i) a linear pattern, in which the leukemic clone developed from the driver mutation clone through acquisition of one or more genomic alterations; (ii) acquisition of a MPN-driver mutation in HSC carrying a pre-existing mutation that was also present in the leukemic clone; (iii) a branched subclonal evolution in which several subclones derived from the driver mutation clone coexisted and one of them, by acquiring novel genomic aberrations, gained growth advantage and promoted leukemogenesis; and (iv) acquisition of genomic aberrations leading to proliferation advantage and transformation in a non-MPN clone in patients where two independent clones or subclones existed at CP, one of them carrying the MPN-driver mutation. Additionally, a linear pattern was observed in TN-MPNs that progressed to BP, in

which the leukemic clone developed from a TP53 clone or subclone present at CP that predominantly gained del(17p) among multiple CNVs. While these patterns have been described before, the precise acquired mutations in the patterns are of interest and show a diversity of pathways leading to LT. For example, signaling mutations in NRAS, CBL, NF1, and PTPN11 occurred at BP. It is also interesting that competition between mutated JAK2 and CALR clones has been detected, with one leading to LT, as well as competition between different TP53-mutated clones. Individual profiles of CP-BP genomic aberrations in 12 patients are described in Figure S1. Clonal evolution of patient 13# was not presented as it was previously published.²

Our results point to a dominance of epigenetic mutations in BP-MPN patients (72.8% of cases). When considering also CNVs that affect chromatin modifiers and DNA methylation, 84.8% of patients exhibited epigenetics alterations. Particularly, anomalies of EZH2 gene were relatively frequent in JAK2 V617F-mutated and TN-MPN patients. In serial DNA samples, EZH2 anomalies were detected exclusively in BP. In agreement, in a single-cell analysis of clonal evolution in paired CP-BP samples,³ EZH2 was recurrently mutated or affected by CNVs in transition to BP.

In BP-MPN patients carrying TP53 alterations, epigenetic modifications were observed in 75% of cases. In paired-sample analysis, the same

TP53 mutations found in BP were already present in CP-MPNs in a heterozygous state, as previously reported.⁴ Chronic inflammation was recently demonstrated to play an important role in clonal evolution of *TP53*-mutant MPNs by suppressing unmutated HSCs, and therefore conferring a fitness advantage to *TP53*-positive HSCs and progenitors.⁵ As expected, in our BP-MPN patients, multi-hit *TP53* were dominant, mostly represented by a single *TP53* missense mutations accompanied by del(17p) (Supplemental file, Figure S2). Among *TP53* missense mutations, structural mutants were more frequent than contact ones (Table S3).

Gain of chromosome 21 was observed in one type-2 *CALR* and two *JAK2* V617F-positive patients (trisomy 21 in one patient and a duplication of 21q11.2-q22.3 in two cases, both associated with multiple CNVs and one with *TP53* mutations). This is in agreement with a recent study⁶ that identified an amplified region of chromosome 21 as a recurrent event in BP-MPN patients, being accompanied by chromothripsis and displaying a very aggressive phenotype.⁶ This genetic alteration leads to an upregulation of *DYRK1A* gene (21q22) that in functional studies promoted genomic instability and increased JAK/STAT signaling.⁶

Compared with *JAK2*-mutated MPNs that exhibited complex patterns of clonal evolution, giving rise to either *JAK2*-mutated AML or *JAK2* wild-type AML, *CALR*-mutated PMF patients evolved to BP by acquiring novel genomic alterations on the top of MPN-driver mutation. Paired-sample analysis in two PMF patients carrying type 1 *CALR* mutation that developed sAML with hyperleukocytosis resembling BP chronic myeloid leukemia detected the presence of LOH events leading to a hyperactive RAS/tyrosine kinase receptor signaling, respectively. At time of LT, one patient displayed *NF1* biallelic inactivation, while another carried a homozygous *CBL* mutation due to an UPD11q. Regarding mutated *CALR* proteins and granulocytosis, it has been shown that besides activating thrombopoietin receptor, which explains the ET and PMF phenotypes, these mutated proteins can also activate granulocyte colony-stimulating factor receptor (GCSFR).⁷ Further studies are required to assess if extreme granulocytosis was related to activation of GCSFR or to other cytokines associated with inflammation in PMF, for example, granulocyte-macrophage colony-stimulating factor.

The profile of genomic aberrations in AML post-TN-PMF was characterized by a high-incidence of biallelic *TP53* alterations (66.6%) and multiple CNVs (66.6%). The spectrum of anomalies together with a shorter median time from diagnosis to BP revealed common features with fibrotic myelodysplastic neoplasms (f-MDS). The distinction between TN-PMF and f-MDS is often challenging, both entities being associated with a higher risk of LT at 3 years from diagnosis.⁸

As a limitation of our study, inherently related to the small number of patients included in the study, we could not assess the impact on survival of the detected genomic alterations in a multivariable analysis.

To conclude, we highlight the overwhelming prominence of epigenetic alterations and importance of serial evaluation of clonal evolution in MPNs for early prediction of disease transformation. We also describe a diversity of signaling mutations contributing to transformation.

AUTHOR CONTRIBUTIONS

Petruta Gurban involved in conceptualization, data curation, formal analysis, investigation, methodology, and writing/review and editing. Cristina Mambet involved in conceptualization, data curation, formal analysis, investigation, methodology, and writing/review and editing. Anca Botezatu involved in data curation, formal analysis, investigation, methodology, and writing/review and editing. Laura G. Necula involved in formal analysis, investigation, methodology, and writing/review and editing. Lilia Matei involved in formal analysis, investigation, methodology, and writing/review and editing. Ana Iulia Neagu involved in data curation, investigation, methodology, and writing/review and editing. Ioana Pitica involved in formal analysis, investigation, methodology, and writing/review and editing. Marius Ataman involved in investigation, methodology, and writing/review and editing. Aurelia Tatic involved in investigation, methodology, and writing/review and editing. Alexandru Bardas involved in investigation, methodology, and writing/review and editing. Mihnea A. Gaman involved in investigation, methodology, and writing/review and editing. Camelia Dobrea involved in investigation, methodology, and writing/review and editing. Mihaela Dragomir involved in investigation, methodology, and writing/review and editing. Cecilia Ghimici involved in investigation, methodology, and writing/review and editing. Silvana Angelescu involved in investigation, methodology, and writing/review and editing. Doina Barbu involved in investigation, methodology, and writing/review and editing. Oana Stanca involved in investigation, methodology, and writing/review and editing. Marina Danila involved in investigation, methodology, and writing/review and editing. Nicoleta Berbec involved in investigation, methodology, and writing/review and editing. Andrei Colita involved in investigation, methodology, and writing/review and editing. Ana Maria Vladareanu involved in investigation, methodology, and writing/review and editing. Saviana Nedeianu involved in data curation, formal analysis, investigation, methodology, and writing/review and editing. Mihaela Chivu-Economescu involved in data curation, formal analysis, investigation, methodology, and writing/review and editing. Coralia Bleotu involved in data curation, formal analysis, investigation, methodology, and writing/review and editing. Daniel Coriu involved in formal analysis, investigation, and writing/review and editing. Elise Sepulchre involved in data curation, formal analysis, investigation, methodology, and writing/review and editing. Gabriela Anton involved in conceptualization, validation, and writing/review and editing. Carmen Cristina Diaconu, senior author, involved in conceptualization, funding acquisition, methodology, project administration, resources, supervision, validation, and writing/review and editing. Stefan N. Constantinescu, senior author, involved in conceptualization, funding acquisition, methodology, resources, supervision, validation, and writing/review and editing.

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CONFLICT OF INTEREST STATEMENT

PG is employed by Cytogenomic Medical Laboratory, Bucharest, Romania. SNC is cofounder of MyeloPro Diagnostics and Research GmbH, Vienna, Austria. The other authors have no financial or nonfinancial interests to disclose.

DATA AVAILABILITY STATEMENT

Data are available at the Department of Cellular and Molecular Pathology, Stefan S. Nicolau Institute of Virology, Bucharest, and are accessible from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

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