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Molecular pathophysiology of Philadelphia-negative myeloproliferative disorders: beyond *JAK2* and *MPL* mutations

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The classic Philadelphia-chromosome negative chronic myeloproliferative disorders (MPDs), recently renamed as myeloproliferative neoplasms, that include polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF), had been largely neglected among hematologic neoplasms until early 2005 when the first recurrent molecular abnormality was described, consisting of a G>T point mutation at nt1849 in *JAK2* and resulting in a valine to phenylalanine substitution at residue 617 (V617F).[1-4] Then, the discovery of mutations in *MPL*, represented by a W>L or W>K shift at codon 515,[5] and of variable molecular abnormalities (point mutation, insertion, deletion) in *JAK2* exon 12[6] was also reported. Almost all patients with PV have a somatic genetic defect in *JAK2* that is represented by the V617F allele in 90-95% of cases and by abnormalities in exon 12 in roughly 2%, while they are spared by *MPL* mutations; on the other hand, only 60% of patients with ET or PMF harbor the *JAK2*V617F mutation and 3-7% exhibit the *MPL*W515L/K mutation. While other infrequent mutations in *MPL* can also occur,[7] exon 12 abnormalities have not yet been reported in PMF or ET patients. The presence of any of these molecular abnormalities, that point to a clonal myeloproliferation, stands as a major diagnostic criterion in the revised classification of myeloid neoplasms of the World Health Organization.[8] They are *gain-of-function* abnormalities that conferred growth-factor independence to cells transduced with mutant allele and induced a myeloproliferative disease when expressed in murine transplant models.[9]

It was unexpected to find a single mutated allele associated with more than one disease, notwithstanding the fact that the different MPDs are strictly related to each other and show substantial phenotypic mimicry. There are possible explanations for this. One is that the unique clinical phenotypes mirror the stem/progenitor cell level at which the mutational event occurred. However, this does not seem to be the case, since the V617F allele has been found in myelo-lymphoid progenitors in patients with PV or PMF, as well as in flow cytometry purified populations of hematopoietic stem cells and committed progenitors. Similar observations have been reported for *MPL*W515L/K mutations.[10] In only a minority of ET cases a restriction of V617F allele to megakaryocytic lineage was described, although it was unclear whether a very low burden of mutated granulocytes might have meant the abnormal genotype went undetected. Another possibility is that different diseases are caused by variable *dosage* of mutant V617F allele (information about *MPL*W515L/K mutation in this regard is scarse) that in turn influences the level of activation of the JAK/STAT signaling pathway. Indeed, there are substantial differences in the median burden of V617F allele in peripheral blood granulocytes, with the highest level being found in PV and the lowest in ET patients.[11,12] Furthermore, homozygosity for the *JAK2*V617F mutation, that originates from mitotic recombination of the short arm of chromosome 9, is present in approximately 30% of PV or PMF patients as opposed to 2-4% of ET. Variable proportions of wild-type, heterozygous and homozygous progenitors are present in most patients with PV, while homozygous progenitors are reported as being rare in ET; mutated erythroid progenitors are more sensitive to erythropoietin than normal ones, and most erythropoietin independent erythroid colonies (EEC) are made up of homozygous progenitors. Conceivably, duplication of mutant allele is expected to result in a higher level of JAK2/STAT activation than in cells harboring one mutant and one wild-type allele, possibly because of the loss of competition between normal and mutated allele and/or impaired interaction of mutant JAK2 with cellular regulators such as the suppressor of cytokine signaling-3 (SOCS3).[13] A correlation between

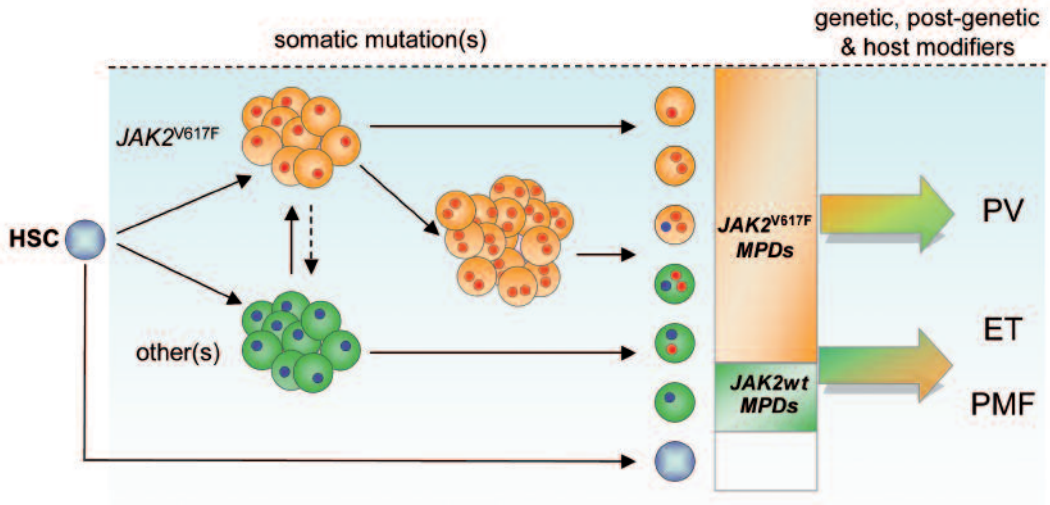

Figure 1. The figure describes a working model of genetic events and other mechanisms possibly involved in the pathophysiology of myeloproliferative disorders. A hematopoietic pluripotent stem cell (HSC) is the target of *JAK2*V617F mutation (other abnormalities in *JAK2* exon 12 or *MPL* may have similar effects; for clarity, the *JAK2*V617F mutation only is presented as a model, and the mutated allele is indicated by a red dot). However, a pre-*JAK2* mutated HSC cell (green) might also exist as the result of a still unknown molecular lesion, indicated by a single blue dot; this cell might subsequently acquire the *JAK2*V617F mutation, although also the inverse process could happen (dashed arrow). A *JAK2*V617F heterozygous cell may then undergo a mitotic recombination process at 9p leading to *JAK2*V617F homozygosity, which is likely associated with growth advantage. As a consequence, the clonal population at the basis of a myeloproliferative disorder may be comprised of a variable proportion of cells harboring isolated or multiple molecular defects (please note that the existence of some of the cells depicted has not been formally proven). *JAK2*V617F mutation is probably necessary for PV to occur, while other still undiscovered genetic abnormalities may be at the basis of *JAK2* un-mutated ET or PMF. Finally, normal HSC persist in many patients for length of time and in variable amount. All these cell-heritable and irreversible genetic events may be complemented by the effects of post-genetic regulatory mechanisms, including miRNAs or epigenetic modifications, and/or of host genetic modifiers, such as gender, SNPs and possibly others, including also non-genetic modifiers (ie, ferritin iron availability). The phenotypic pleiotropy of a myeloproliferative disorder would then be the result of a combination of somatic mutation(s), inherited genetic variability, post-genetic regulation and host modifiers.

mutant allele burden and disease phenotype is indirectly supported also by findings in animal models. The adoptive transfer of the V617F allele caused a rapid appearance of polycythemia, leukocytosis and splenomegaly in mice, pointing to a PV-like disease that eventually recapitulated the evolution to a post-PV myelofibrosis with time. The fact that thrombocytosis was not observed in these mice was ascribed to the exceedingly high levels of mutant *JAK2* mRNA that were produced in hematopoietic cells with the retroviral infection. This hypothesis has recently received experimental support thanks to the successful development of conditional transgenic mice that expressed variable levels of *JAK2*V617F,[14] in which the phenotype closely reflected the ratio between normal and mutated *JAK2* mRNA. Likewise, the V617F allele burden correlates with hematologic characteristics and clinical endpoints in MPD patients. However, owing to the wide distribution of individual values, it seems quite unlikely that load of mutant V617F allele alone represents the key mechanism at the basis of MPD pleiotropy, although it is likely that changes in V617F allele burden correlate with disease progression.[12]

There is evidence to support the possibility that disease alleles other than *JAK2*V617F or *MPL*W5151L/K are involved in the pathogenesis of MPDs,[15] in addition to the fact that almost 40% of ET or PMF patients still lack a molecular marker. First, in females with *JAK2*V617F-negative ET substantiated proof of the existence of clonal hematopoiesis has been provided through the analysis of X-chromosome inactivation pattern (X-CIP).[16,17] Furthermore, the percentage of granulocytes and platelets with *JAK2*V617F was often lower than expected by the analysis of X-CIP. In addition, in two *JAK2*V617F-mutated patients with a del(20q), discrepancies were found in the size of del(20)q and *JAK2*V617F-mutated clones, leading to the suggestion that V617F allele had been acquired by a pre-existing del(20q) clone.[18] Similarly, some patients contemporarily harbor *JAK2*V617F and *MPL*W515L/K mutation,[19, 20] and clonal dominance by the *MPL* mutated cells has been demonstrated.[21] Second, presence of the V617F allele is robustly associated with the growth of EEC, that is a hallmark of PV and MPDs in general. However, EEC wild-type for *JAK2* have been detected at low frequency in PV patients[22] which would indicate the *JAK2* mutation is a secondary, not a disease-initiating, genetic event. A similar interpretation could be advocated also to explain why exon 12 abnormalities are almost exclusively associated with an erythroid phenotype in the setting of a clear myeloproliferative disorder. Third, the incidence of MPDs among relatives is greater than expected in the normal population, but neither the diagnosis of MPD nor the distinct clinical phenotype encountered in these families were invariably associated with the presence of *JAK2* or *MPL* mutation, suggesting that the *JAK2*V617F mutation might occur as a secondary genetic event on the background of an inherited genetic predisposition.[23] Finally, blast cells of acute myeloid leukemia that developed in patients with a preceding *JAK2*V617F-positive MPD were often

*JAK2*V617F-negative, indicating that they might derive from the transformation of a pre-*JAK2*V617F mutated hematopoietic stem cell that was originally at the basis of the MPD itself.[24] However, that these *JAK2*V617F negative blasts represented the *de-novo* transformation of a residual normal progenitor cannot be excluded on the basis of available data. It is more unlikely that the *JAK2* un-mutated blasts originated from a single cell heterozygous for the V617F mutation that underwent a reverse mitotic recombination process leading to an unmutated *JAK2* genotype. On the contrary, the possibility that the *JAK2*V617F and *MPL* mutation alone might be sufficient for causing an MPD is compellingly supported by the results of animal models, including the recently described transgenic mice that developed phenotypes closely resembling human ET and PV and presented MF-like symptoms as they aged.[25]

There is also the non-mutually exclusive possibility that, on the grounds of the *JAK2* or *MPL* mutation and/or of any other pre-existing or additional molecular abnormality, individual-related characteristics contribute to the phenotypic pleiotropy of the MPDs. This was initially suggested by results in animal models. Depending on the genetic background of mice transplanted with *JAK2*V617F transfected cells, the characteristics of their myeloproliferative disease were somewhat different. In fact, in addition to polycythemia, the *JAK2*V617F allele induced marked leukocytosis and marrow reticulin fibrosis in Balb/c mice, while leukocytosis was minimal and fibrosis was limited to the spleen in case of C56Bl/6 strain.[26] Compelling support to the existence of genetic modifiers in humans has been recently provided by Pardanani *et al.*,[27] who genotyped a number of single nucleotide polymorphism (SNP) loci in *JAK2* and *EPO-R* of patients with MPDs. They found that 3 *JAK2* SNPs were inversely associated with PV or ET, but not PMF, while 3 additional SNPs in *JAK2* and one in *EPO-R* were significantly associated with PV only.[27] Due to the design of the study, these should not be strictly considered as genetic abnormalities leading or predisposing to the disease, but rather as genetic markers of potential interest due to their significant association with the disease, similar to polygenic predisposition to many common diseases. Gender is another genetic-based host-modifier that may have relevance,[28] exemplified by the fact that PV is more common in men while ET is definitely more frequent in women. An example of a non-genetic modifier might be the availability of iron stores. It was by taking into an account these possible variables that an intriguing hypothesis of a *biological continuum* of ET and PV was formulated.[29]

All together, these observations support the idea that additional factors, possibly represented by yet unknown disease-causing allele(s) or by host modifying characteristics, or both in variable combinations, concur with *JAK2* or *MPL* mutation to the pathophysiology of MPDs (Figure 1). Two papers published in this issue of Haematologica deal with the *beyond JAK2 and MPL mutation* perspective by two different approaches. With the aim of identifying novel disease-associated molecular markers, Ioana Borze and colleagues employed comparative genomic hybridization (CGH) to discover cytogenetic abnormalities not otherwise detectable with conventional cytogenetics.[30] CGH is a method designed for the identification of genomic regions characterized by copy number alteration. More recently, array-based CGH platforms (aCGH) have been developed where arrays of oligonucleotides covering up to more than 200,000 genes and spaced 150 kb across the genome have replaced metaphase chromosomes as hybridization targets, resulting in higher resolution and facilitating direct mapping of copy number changes to relatively narrow genome regions. Ideally, CGH analysis represents the basis for subsequent molecular genetic strategies aimed at identifying altered genes involved in tumorigenesis. As a matter of fact, a number of regions gained or lost in solid and hematologic malignancies have been identified, which should result in variegated cellular mRNA expression level. Among the 36 patients (21 ET and 14 PV) evaluated by Borze *et al.* only one abnormal case was identified, which presented duplication at 1q and deletion at 13q in relatively large regions including approximately 1,100 and 300 genes respectively. Similar negative findings have been presented in a preliminary report on 20 patients with ET (Espinet B *et al.*, 11th EHA meeting, 2006; abstract 098). On the contrary, the Mayo Clinic group recently reported that the percentage of patients showing copy number changes was 17% of 12 patients with ET, 32% of 22 patients with PV, and 52% of 27 PMF patients (49th ASH meeting, 2007; abstract n.1550). Although the number of patients included in each of these series was quite small and different aCGH platforms were employed, possible explanations for these discrepancies are not obvious, and this approach certainly deserves further investigation. However, a preliminary suggestion from these studies may be that chromosomal defects within the analytical resolution of aCGH approach are unlikely to be a common occurence in MPDs.

The second paper is from Hana Bruchova and colleagues, who performed a gene expression profiling of micro-RNAs (miRNAs) in granulocytes from PV patients.[31] miRNAs constitute a large family of small non-coding RNAs functioning as regulators of post-transcriptional processes; they have been implicated in many physiological and pathological processes, including cancer. The miRNA signature observed by Bruchova *et al*. in PV granulocytes resulted clearly different from that of normal granulocytes, and allowed the identification of a number of miRNAs (at present, 40 out of a total of 326) whose abnormal expression was either correlated or independent from the *JAK2*V617F mutation. It is of interest, and probably not unexpected, that at least some of the abnormally regulated miRNAs found in PV had been detected in PMF granulocytes in another study,[32] and that they were also detected in ET or PMF patients by Bruchova *et al*. Thus, a complex pattern of aberrant miRNA expression might contribute to the molecular complexity of MPDs through transcriptional regulation, although the mechanisms for the abnormal expression of those miRNAs remain completely unknown. As a matter of fact, the loss of expression of miR-15 and miR-16 in chronic lymphocytic leukemia is due to deletion of a region included in the 13q14,[33] while no somatic

molecular lesion of a miRNA gene has yet been reported in MPDs. It is still premature to conclude whether and how aberrant expression of miRNAs concurs with genetic lesions to modify disease presentation, and also if it is related to genetic abnormalities. One limitation of this kind of study is the difficulty of correlating deregulated expression of miRNAs with that of target genes, since there is a pleiotropy of putative target genes. Furthermore, the final regulatory activity of a given miRNA may be that of inhibiting transcription in most instances but also of facilitating it in other cases. Accordingly, the demonstration that expression level of some genes, selected by in silico analysis as possible targets of the deregulated miRNAs, was modulated in the granulocytes of PV patients does not represent a formal proof of a cause-effect relationship.[31] In fact, clarification of the role of abnormally expressed miRNAs in PV and other MPDs will require manipulation of endogenous miRNA levels in hematopoietic stem cells and/or their descendants obtained from patients with MPDs; these experiments are technically demanding, and unlikely to be easily accomplished. Finally, it should also be underlined that in these studies[31, 32] the number of miRNAs included in the analysis represents only a proportion of the total number of miRNAs that are currently known (>600 according to the v.11 release of miRBase at http://microrna.sanger.ac.uk/sequences), raising the suspicion that additional miRNA abnormalities could be found once more comprehensive array platforms are eventually used.

The discovery of the *JAK2* and *MPL* mutations has represented a tremendous improvement in the diagnosis and characterization of the MPDs, and has opened new perspectives for the clarification of disease pathogenetic mechanisms and hopefully for targeted therapy. However, it was also soon realized that a simple model incorporating only *JAK2* and *MPL* mutation is inadequate to explain the pathophysiology and pleiotropy of the MPDs, and in this perspective the two papers in this issue of Haematologica represent valuable contributions to help decipher this intriguing story.

Key words: myeloproliferative disorders, polycythemia vera, essential thrombocythemia, primari myelofibrosis, JAK2, MPL, microRNA

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Molecular characterization of acute myeloid leukemia

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Acute myeloid leukemia (AML) is a genetically heterogeneous clonal disorder characterized by the accumulation of acquired somatic genetic alterations in hematopoietic progenitor cells that alter normal mechanisms of self-renewal, proliferation and differentiation.[1] Non-random clonal chromosome aberrations (i.e., balanced translocations, inversions, deletions, monosomies, and trisomies) are detectable in the leukemic blasts of approximately 55% of adults with AML. These chromosome changes have contributed to the classification of the disease and in the past they have been recognized as the most important prognostic factor for achievement of complete remission, risk of relapse, and overall survival.[2,3] In recent years, a number of gene mutations as well as deregulated expression of genes have been identified, illustrating the enormous heterogeneity of cytogenetically defined AML subsets, in particular in the large subset of AML exhibiting a normal karyotype (Table 1).[4,5] The characterization of the gene mutations has provided insights into the mechanisms of leukemogenesis. From a clinical point of view there are two important aspects. First, some of these gene mutations have emerged as important prognostic and predictive markers. Second, novel therapies are now being developed that target these molecular changes. Based on these findings, it is now recommended to include molecular genetic diagnosis of several of these markers in the initial work-up of a patient with newly diagnosed AML. The importance of a comprehensive genetic diagnostic work-up is highlighted in a study by Lo-Coco *et al.* from the Gruppo Italiano Malattie Ematologiche dell'Adulto (GIMEMA) which appears in this issue of the journal.

Multi-step leukemogenesis

There are several lines of evidence showing that different genetic changes cooperate in leukemogenesis.[1,6] First, data from murine models suggest that a single mutation is not sufficient to cause AML. For example, the *RUNX-RUNX1T1* and *CBFB-MYH11* fusion genes, resulting from t(8;21) and inv(16)t(16;16) respectively, impair myeloid differentiation in these models but they do not cause an overt leukemic phenotype. Second, rare germline mutations have been described in *RUNX1* and *CEBPA* that predispose affected individuals to the development of AML. Constitutional heterozygous loss-of-function mutations in the transcription factor RUNX1 have been associated with familial platelet disorder with propensity to AML. In these individuals, overt leukemia likely develops upon the somatic acquisition of further mutations in hematopoietic progenitor cells. Finally, evidence comes from human disease, since in the majority of AML cases more than one genetic change can be detected (Figures 1 and 2).

There are different types of mutations which appear to fall into broadly defined complementation groups and cooperate in leukemogenesis.[6] One group (class I) comprises mutations which activate signal transduction pathways resulting in enhanced proliferation and/or survival of leukemic progenitor cells such as mutations leading to activation of the receptor tyrosine kinase FLT3 or the RAS signaling pathway. The second complementation group (class II) comprises mutations that affect transcription factors or components of the transcriptional co-activation complex, resulting in impaired differentiation and/or aberrant acquisition of self renewal properties by hematopoietic progenitors. Prominent examples are the recurring gene fusions resulting from t(8;21), inv(16)/t(16;16), t(15;17), as well as mutations in *CEBPA*, *MLL*, and possibly also *NPM1*.

Molecular alterations in cytogenetically normal acute myeloid leukemia

In the last decade, somatically acquired mutations have been identified in several genes in cytogenetically normal (CN) AML: the nucleophosmin 1 (*NPM1*) gene, the fms-related tyrosine kinase 3 (*FLT3*) gene, the CCAAT/enhancer binding protein alpha (*CEBPA*) gene, the myeloid-lymphoid or mixed-lineage leukemia (*MLL*) gene, the neuroblastoma RAS viral