

Mutations and pathogenesis of myeloproliferative neoplasms: Review

Abstract

Chronic myeloproliferative neoplasms (MPNs), clonal disorders of hematopoietic system resulting in abnormal proliferation of mature blood elements, share different clinical, hematologic and biological features. Majority of Philadelphia-negative (Ph-negative) classical MPNs- polycythemia vera (PV), essential thrombocythemia (ET), and primary myelofibrosis (PMF)- display alterations in JAK2 or MPL, both of which play pivotal roles in cytokine signaling in hematopoiesis. Both LNK or CBL, negative regulators of signaling pathways and mutated or deleted transcription factors such as IKZF1, EZH2, Tp53 and RUNX1 are infrequently targeted genes in Ph-negative MPNs. IKZF1 and Tp53 were associated with transformation to acute myeloid leukemia (AML), yet with unclear prognostic impact. Mutations of CHEK2, the tumor suppressor gene against uncontrolled cell growth, were reported to cause susceptibility to ET. Mutations of NRAS gene, a member of MAPK signaling pathway frequently mutated in MPNs, seem to affect expression of JAK2 target genes and are primarily associated with transformation to AML. Mutations in epigenetic regulators, such as, TET2, DNMT3A, ASXL1, EZH2, and IDH1/2 were described in MPNs and other myeloid malignancies at variable frequencies. Alterations in DNMT3A, ASXL1, EZH2, and IDH1/2 are more frequent in PMF than PV and ET. Recent studies suggest in Ph-negative MPNs, mutations affecting epigenetic regulation might be prognostically more relevant than mutations affecting JAK-STAT signaling. Mutations in calreticulin (CALR) were newly discovered in a majority of JAK2V617F- and MPL-negative ET and PMF patients. Further studies are needed to address importance of CALR mutations in MPN. This review summarizes pathogenesis and molecular events of MPNs.

Key Words: Chronic myeloproliferative neoplasms, Epigenetic regulators, Essential thrombocythemia, Molecular events, Polycythemia vera, Primary myelofibrosis

Introduction

Chronic myeloproliferative neoplasms (MPN) are a group of heterogeneous disorders characterized by excessive production of clonal myeloid cells and a frequent tendency to transform to acute myeloid leukemia (AML).¹ Defective hematopoiesis is the main contributing factor in the pathogenesis of MPN. Hematopoiesis is a dynamic and strictly controlled process in which cell maturation and proliferation continue. Acquired mutations (point mutations or chromosomal rearrangements) may disrupt this process and result in defects affecting the myeloid series. It is acknowledged that most of the somatic mutations that occur during the division of the pluripotent hematopoietic stem cell have no effect on phenotype. Yet, some mutations are reported to cause proliferation. The malignant clone gradually ensues as the mutated stem cell leading to proliferation enters cell cycle more frequently. The resulting monoclonal hematopoiesis is a characteristic feature of all myeloid malignancies. In humans, development of MPN is the most common phenotypic expression of clonal hematopoiesis. This review focuses on Philadelphia-negative (Ph-negative) classic MPN, which is one of the 9 disorders included in the current classification of MPN by World Health Organization (WHO).¹ Classic MPN comprises of polycythemia vera (PV), essential thrombocythemia (ET) and primary myelofibrosis (PMF). PV is characterized by excess production of red blood cells and ET is caused by overproduction of platelets. PMF, on the other hand, is characterized by accumulation of excess fibrotic tissue in the bone marrow. In the last decades, genomic studies have identified various genes relevant to the pathogenesis of MPN, which imply that many different cellular pathways may be responsible for the clonal evolution of the hematopoietic stem cells. MPN related somatic mutations have been divided into two main functional categories: 1) mutations that affect the cytokine receptor signalling and 2) mutations related to regulation of gene expression. This review focuses on the molecular basis and developments in the clonal evolution of disease as well as the contribution of various lesions to disease progression and their use as prognostic markers.

JAK2V617F mutation

For more than two decades, researchers have investigated the underlying genetic disturbances in PV. Defects in the signalling pathways of the erythropoietin receptor (EPOR) was proposed as the underlying pathology. These pathways include transcription activators such as tyrosine kinase JAK2, transcriptional signal transducers, transcription 3 signal mediator and activator molecule (STAT3) and transcription 5 signal mediator and activator

molecule (STAT5). The first mutation was described in 2005 by Vainchenker et al. in France.² It was determined that in PV, the inhibition of JAK2 by a small molecule (AG490) or siRNA led to a decrease in erythropoietin (EPO) independent colony production by the bone marrow mononuclear cells. This observation led the researchers to directly sequence JAK2 in the hematopoietic cells of PV patients, which resulted in the discovery of repetitive point mutations. The guanine-to-thymine mutation was shown to result in a substitution of valine to phenylalanine codon 617 within the pseudokinase domain of the JAK2. These findings were rapidly confirmed by other groups. In another study including PV patients, researchers identified loss of heterozygosity (LOH) on chromosome 9p and a commonly shared 6.2 Mbp region.³ Since the JAK2 gene plays a role in erythropoiesis, this specifically identified region was screened for mutations and similarly was shown to harbor the JAK2V617F mutation. DNA content analysis demonstrated that JAK2V617F mutation was only present in hematopoietic cells and that in all PV patients, erythroid precursor cells were homozygous for the mutation. Based on the quantification of the allele burden of JAK2V617F mutation in the granulocytes by polymerase chain reaction (PCR), patients were stratified into two groups: those with low allele burden of JAK2V617F mutations (<50%) and those with high allele burden (>50%). The mitotic recombination and duplication of the mutant allele were shown to account for the homozygosity for the JAK2V617F mutation. Also, it has been documented that during the clinical course, the heterozygote mutation transforms to homozygous state as a result of mitotic recombination. The median disease duration for patients with high allele burden and low allele burden was 48 months and 23 months, respectively. These findings support the notion that low allele burden disease transforms to high allele burden disease during follow up. Furtheron, the aforementioned results suggest that the pathogenesis of PV is a multi-step process. In the first run, the acquired mutation in the JAK2V617F gene results in low allele burden. Later on, homologous recombination leads to the appearance of precursor cells and granulocytes showing homozygosity for JAK2V617F in the presence of a high allele burden. The JAK2V617F mutation was reported to be present in more than 95% of the patients with PV. Table 1 summarizes the frequency of the JAK2V617F mutation in MPN reported by different groups.⁴

The JAK2V617F mutation is present in 40-60% of ET patients.⁴ JAK2 plays an important role in the intracellular signaling pathway of a group of growth factors such as IL-3, EPO, G-CSF and thrombopoietin (TPO). The JAK2V617F allele burden was found to be higher in ET patients showing clonal hematopoiesis compared to those showing polyclonal hematopoiesis (26% and 16%, respectively). It is reported that JAK2V617F clone remains stable in the presence of both clonal and polyclonal hematopoiesis. 70% of PV patients harbor the homozygote

JAK2V617F mutation with the high allele burden (>50%) where as it is less common in ET patients. JAK2V617F mutation is homozygous in all PV patients, including those with low allele burden. On the contrary, JAK2V617F homozygosity is rarely expressed in the hematopoietic colonies of ET patients. .It was reported in ET patients that a higher JAK2V617F allele burden existed in thrombocytes compared to granulocytes where as the allele burden was higher in the granulocytes of PV and PMF patients. Furthermore, the expression of JAK2V617F mutations was demonstrated in the erythroblasts of ET patients. In one study that used mutant single nucleotide polymorphism (SNP) genotyping for the mutant alleles of JAK2V617F, the presence of interdependent multiple abnormal clones was demonstrated in ET.⁵ In the same study, it was reported that the real prevalence of biallelic JAK2 mutations in ET was 5-10%.

Approximately 50% of PMF patients carry the JAK2V617F mutation and nearly 13% carry it in the homozygous state.⁶ It is believed that the homozygosity of this mutation in PMF is associated with the presence of cytogenetic anomalies with poor prognosis.

One of the questions to be addressed regarding the biology of the JAK2V617F in MPNs is how different phenotypes are constituted in the presence of this common mutation. In PV, the amount of phosphorylated STAT3 and STAT5 increase in the bone marrow cells. In ET patients, an increase in the amount of phosphorylated STAT3 but a decrease in the amount of phosphorylated STAT5 are observed. In PMF patients, both the expression of STAT3 and STAT5 decrease. These observations imply that alternative or additional molecular events may play a role in the pathogenesis of PV, ET and PMF. Moreover, it is known that clonal hematopoiesis exists in a major proportion of ET and PMF patients not harboring the JAK2V617F mutation. Consequently, it may be considered that JAK2V617F mutation emerges as a result of different genetic events.

Table 1

The frequency of JAK2V617F mutation in polycythemia vera, essential thrombocythemia, and primary myelofibrosis

	Method	PV:mutation (homozygote)	ET:mutation (homozygote)	PMF:mutation (homozygote)
James et al.	Sequencing	89 (30)	43	43
Levine et al.	Sequencing	74 (25)	32 (3)	35 (9)
Kravolics et al.	Sequencing	65 (27)	23 (3)	57 (22)
Baxter et al.	Sequencing	97 (26)	57 (0)	50 (19)

Jones et al.	ARMS/pyrosequencing	81 (33)	41 (7)	43 (29)
Levine et al.	Allele specific/Taqman	99	72	39

JAK2 exon 12 mutations

Other mutations of JAK2 are related to erythrocytosis and misdirect the autoinhibitor domain activating the kinase activity. A few functional mutations affecting the JAK2 exon 12 are lie in the vicinity of JAK2V617F mutations. JAK2 exon 12 mutations are present in 2.5-3.4% of all PV patients, but are present in 30% of JAK2V617F-negative PV patients.^{7, 8} Up to date, 17 different mutations of the JAK2 exon 12 have been reported.³ 2/3 of the patients with the JAK2 exon 12 mutations, have isolated erythrocytosis, a distinguishing bone marrow morphology and decreased serum EPO levels. In the remaining cases, either leukocytosis or thrombocytosis or both accompany the erythrocytosis. Thus far, no JAK2 exon 12 mutations have been reported in ET or PMF. However, it has been rarely demonstrated in patents with refractory anemia and refractory anemia with ring sideroblasts (RARS-T). It is believed that JAK2 exon 12 mutation results from long duration activation of JAK2 related intracellular signal pathway. Thrombotic events may be encountered in patients with JAK2 exon 12 mutations. Moreover, myelofibrosis or transformation to acute leukemia may be encountered in PV.⁹ BaF3 cells that express the mice EPOR and carry the JAK2 exon 12 mutation may proliferate without the aid of IL-3 added to the medium. This mutation also increases the phosphorylation of JAK2 and ERK. As a result, the myeloproliferative disease phenotype that involves erythrocytosis is constituted. In some series, approximately 2.7% of the patients that have a clinical syndrome mimicking PV do not carry JAK2 mutations. The low sensitivity of the methods used for genotyping, the presence of hereditary genetic diseases related to erythrocytosis, procedures such as the use of interferon that have the potential to eliminate JAK2 mutated clones may be accounted for the aferomentioned finding. The presence of additional hereditary phenomena not yet described may as well be considered responsible for disease phenotype.

MPL mutation

ET and PMF patients mostly do not carry JAK2V617F mutations. One study reported that JAK-STAT pathway may be activated by the stimulation of the hematopoietic specific cytokine receptors including EPOR or thrombopoietin receptor (MPL).¹⁰ Sequencing of the MPL gene encoding the TPO receptor has yielded the

discovery of a new molecular abnormality in JAK-2 mutation negative MPN. MPL mutations are localized in the juxtamembrane region of the receptor. 60% of MPL mutations are W515L and the remaining 40% are W515K mutations.¹⁰⁻¹² In different series, the frequency of MPL mutation was reported to be between 0-10% in PMF and 0-6% in ET.¹³⁻¹⁶ In 50 % of the patients with the W515K mutation and in 17% of those with the W515L mutation, the allele burden is more than 50%. MPL mutations result in cytokine independent proliferation in cell lines and increased susceptibility to thrombopoietin (TPO). As a result, the JAK-STAT signal pathway is activated. It has been demonstrated that MPL W515L mutation induces myeloproliferation characterized by splenomegaly, leukocytosis, marked thrombocytosis, extramedullary hematopoiesis and myelofibrosis. Moreover, it was shown that JAK kinase inhibition halts the proliferation of MPL W515L-transformed cells. Consequently, it can be deduced that inhibition of the JAK-STAT signal may be effective in treatment of JAK2V617F and MPL W515L mutation positive MPN.¹⁰ MPL mutation is present in granulocytes and it has not been detected in non-hematopoietic DNA. These findings indicate that MPL W515L mutation is a somatic mutation of clonal hematopoietic cells. Also, the absence of MPL W515L allele in normal individuals points out that it is not a common nucleotide polymorphism. These data support the notion that MPL W515L is a pathogenic mutation for JAK2V617F negative MPN.¹⁰

Over 50 % of ET patients that carry the MPL mutant allele also harbor the JAK2V617F mutation. In ET, a correlation between W515K mutation and low hemoglobin and high platelet counts has been reported. Also, the bone marrow examination in W515K mutation positive patients shows an increase in megakaryocytes instead of erythroid precursors.

Among PMF patients, 30% of those positive for the MPL mutant allele also carry the JAK2V617F mutation.¹² The mutational load of MPL W515L, MPL W515K and JAK2V617F remains stable during the clinical course of PMF patients. In a mouse model, it was demonstrated that MPL W515L mutation induced a lethal and rapidly progressive form of MPN characterized by severe thrombocytosis, leukocytosis, splenomegaly, hepatomegaly, megakaryocytic hyperplasia in the bone marrow and bone marrow fibrosis. Erythrocytosis was not observed in the presence of MPL W515L supporting that MPL mutation causes thrombocytosis while JAK2V617F mutation induces erythrocytosis. PMF patients positive for the MPL W515L/K mutation are older patients with severe anemia and require more frequent transfusions.¹⁰ Approximately 50% of PMF patients exhibit clonal hematopoiesis yet do not harbor JAK2 and MPL mutations. Therefore, it is not possible to explain the pathogenesis

in PMF solely by the presence of these mutations. The genetic basis of PMF likely stems from the combination of several genetic and epigenetic phenomena.

LNK mutation

Lymphocyte specific adaptor protein (LNK), is a JAK-STAT inhibitor adaptor protein. It is considered that LNK plays a role in the pathogenesis of myeloproliferative disease. It consists of three regions including proline rich amino region, PH domain required for insertion into the cell membrane and the SH2 domain which binds to MPL-JAK2. LNK is a negative regulator of TPO and MPL mediated JAK2 activation.¹⁷ In LNK knock out mice; an increase in megakaryocytes and erythroid precursor cells as well as enhanced hematopoietic stem cell regeneration was documented.¹⁷ Some studies demonstrated that LNK in vitro suppresses the signals generated by MPL W515L and JAK2V617F.^{18, 19} In a mouse model, LNK deficiency facilitated the development of myeloproliferative disease induced by TEL-JAK2 and JAK2V617F. This model showed that an interaction between JAK2V617F and LNK is required to suppress the PV/PMF expression and that the SH2 domain of LNK is needed to suppress the myeloid expansion induced by TEL-JAK2. The presence of LNK mutation in chronic phase MPN was reported as a rare finding. Yet, a higher frequency (9.8 %) was reported for MPN with blastic transformation.²⁰ LNK binds to the phosphorylated tyrosine kinases and inhibits the major cytokine receptor signal that comprises c-KIT, JAK2 and MPL-JAK2.²¹ In conclusion, LNK as a negative regulator of cytokine signalling plays an important role in hematopoiesis.

Casitas B-cell Lymphoma mutation

Casitas B-cell lymphoma (CBL) gene located on chromosome 11q23.3 contains three related but distinct mammalian protooncogenes: c-CBL, CBL-b and CBL-c.^{22, 23} It is thought to play a role in the maintenance of the hematopoietic stem cell homeostasis. CBL functions as an adaptor protein and also increases the E3 ubiquitin-protein ligase activity thus regulating tyrosine kinase activity. In MPN patients, CBL mutations were first analyzed during screening for acquired 11q mutation. To date, 27 CBL variants associated with MPNs have been reported.²⁴

In a cohort of 579 MPN patients, CBL mutations were reported in 3 (6%) patients. Most CBL mutations were localized in the RING region causing a disruption in ligase activity.

CHEK2 gene mutation

CHEK2 is a tumor suppressor gene that regulates cell division and hence protects cells against uncontrolled and rapid division.²⁵ CHEK2 is a key mediator of cellular response to various types of DNA damage. CHEK2 encodes the kinase at the G2 checkpoint control in the cell cycle. CHEK2 kinase is activated on ATM dependent pathways. In addition to DNA repair, it phosphorylates substrates including p53, BRCA1, CDC25A and CDC25C which are involved in cell cycle checkpoint control through coordination of DNA repair, cell cycle progression and apoptosis.²⁶ It is reported that germline CHEK2 mutations (ie.1100delC, I157T) were detected in Li-Fraumeni syndrome, some lymphoid malignancies and in myeloid malignancies such as myelodysplastic syndrome (MDS) and AML.²⁶⁻³⁰ In one recent study, the frequency of CHEK2 mutations in ET was reported as 15.1%.²⁵ Thus, the authors proposed that CHEK2 gene mutations contribute to the susceptibility to ET. The same study also pointed out that germline inactivation of CHEK2 has no direct effect on the development of ET, yet it may result in the disruption of cell cycle checkpoints and trigger the ET cancerogenic process at an earlier age.

IKZF Deletion mutation

Ikaros Family Zinc Finger Protein (IKZF1) gene localized on 7p.12 encodes the Ikaros transcription factor which is of functional significance in normal hematopoiesis. The impact of IKZF1 mutations was studied in mouse models. It was demonstrated that RNA mediated IKZF1 deficiency led to an increase in cytokine sensitivity and expression of p-STAT5. In one study, IKZF1 deletions were detected in 6 out of 29 (21%) post-MPN leukemia patients, yet none among the 526 MPN patients who did not develop leukemic transformation.³¹ In conclusion in two independent cohort studies, the presence of a significant relationship was demonstrated between IKZF1 deletions and post-MPN leukemia. In the aforementioned studies, IKZF1 deficient patients showed complex karyotypes and del 7p was reported as a late occurring event in the genetic evolution of the MPN clone. As a result, it may be deduced that loss of IKZF1 may play an important role in the leukemic transformation in some MPN patients.

EZH2 mutation

Polycomb-group (Pc-G) genes encode proteins that regulate the gene expression pattern during development of multicellular organisms.³² PcG proteins function via three key multiprotein complexes: Polycomb Repressive complex 1 (PRC1), Polycomb Repressive complex 2 (PRC2) and PhoRC. Enhancer of Zeste Homolog 2 (EZH2) proteins are one of the four components of PRC2. EZH2 is the multiprotein enzyme complex responsible for the trimethylation of lysine 27 on histone H3 (EZH2, SUZ12, EED ve YY1). Also, PRC2 makes the chromatine more compact by attracting other polycomb complexes to the gene region.³³ EZH2 shows oncogenic activity. Its increased expression prevents differentiation in epithelial tumors.³⁴ In MPNs, EZH2 gene expression is up-regulated. EZH2 mutations are most commonly reported in PMF. It may be suggested that EZH2 acts as a tumor suppressor in myeloid malignancies. In one study including 614 patients with myeloid malignancies, a total of 49 EZH2 mutations was detected in 42 patients.³³ Of the 30 PMF patients included in this cohort study, 13% carried the EZH2 mutation. It is hard to determine the real prognostic significance of the EZH2 mutation due to the limited number of PMF patients and short follow up period. Finally, EZH2 mutations are not reported to be present in ET and reported at a ratio of 3 % in PV.³³

RAS mutations

Ras proteins are members of the small guanosine triphosphatase (GTPase) super- family. RAS mutations that abnormally activate the RAF/MEK/ERK, PI3K/AKT and other pathways are commonly encountered in all human cancers.^{35, 36} Three oncogenic mutations attributed to the RAS gene are HRAS, NRAS and KRAS mutations. Although KRAS and NRAS mutations are common in myeloid diseases, HRAS mutation are rare.³⁷ RAS mutations are usually single amino acid substitutions such as G12V and G13R. This mutation decreases the sensitivity of RAS for the guanine triphosphate (GTP) activating protein thus enabling high RAS-GTP levels.^{35, 38} NRAS activating mutations of the RAS family are present in approximately 30 % of the hematopoietic malignancies including AML, MDS and MPN.^{39, 40} NRAS/KRAS mutations are rare in PV, yet they have been associated with transformation from PV to acute leukemia.⁴⁰

241 Tp53 mutation

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243 p53 mutations are the most commonly observed genetic alterations in human cancers including lymphomas
244 and leukemias.⁴¹ p53 transduced mouse hematopoietic stem cells showed increased in vitro proliferative and
245 differentiation capacity. Also, increased expression of the mutant p53 triggered the transformation of immature mice
246 hematopoietic cells in vivo. As a result, two types of hematopoietic diseases, MPN and MDS, were induced.⁴¹
247 Although there is a genetic tendency for leukemic transformation in MPNs, the underlying mechanisms leading to
248 this transformation are still unclear. Of a total of 22 patients who transformed to AML from MPN, 6 patients
249 (27.3%) were found to carry somatic mutations in Tp53 gene.⁴² Thus, it is considered that Tp53 plays an important
250 role in leukemic transformation. Tp53 mutations although rare in PV have been associated with transformation from
251 PV to acute leukemia.⁴²

253 RUNX1 mutation

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255 Runt transcription factor 1 (RUNX1) point mutations in MDS and AML were first identified in 1999.⁴³
256 Some mutants of RUNX1 exhibit a negative effect on transactivation activity. These mutations cause loss of function
257 and on the other hand carry some oncogenic potentials as well. Some MPN patients develop leukemic transformation
258 shortly after a few years of initial diagnosis. The mechanism of the leukemic transformation is not clear because the
259 underlying molecular pathogenesis has not been elucidated. In one study, the frequency of RUNX1 mutation was
260 reported to increase during leukemic transformation from MPN, but RUNX1 mutation was not detected in the
261 chronic phase.⁴⁴ In the aforementioned study, 5 out of 18 patients (27.7%) with MPN who had developed acute
262 leukemia were shown to carry a RUNX1 mutation.

264 TET2 mutation

265 Ten-Eleven Translocation (TET) gene family is made up of TET oncogene family member 1 (TET1), TET
266 oncogene family member 2 (TET2) and TET oncogene family member 3 (TET3).⁴⁵⁻⁴⁷ TET2 gene localized on
267 chromosome 4q24 encodes the hydroxylase enzyme that enables enables the hydroxylation of 5'-methylcytosine
268 (5mC).⁴⁵ TET2 mutations are loss-of-function mutations, which increase DNA methylation and result in decrease of

hydroxymethyl cytosine levels. TET2 mutations may occur before or after the acquisition of JAK2V617F or MPL mutations and they are detected in approximately 15% of myeloid malignancies.⁴⁸⁻⁵² IDH mutations exert functionally similar effects as TET2 mutations on DNA methylation. Acquired somatic mutations in the TET2 gene suppress the catalytic activity of the α -ketoglutarate dependent enzyme that is responsible for the oxidation of 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) in DNA. Low 5hmC levels lead to hypermethylation of CpG islands in various DNA promoter regions. TET2 mRNA is highly expressed in the Lin⁻, Sca-1⁺ and c-Kit^{hi} multipotent progenitor cells isolated from the bone marrow and thymus of C57BL/6 mice. This pattern of expression is maintained in myeloid precursor cells, yet is low in mature granulocytes. Furtheron, in one study comparison of patient samples with a control group showed that low 5hmC levels resulted in significant decrease in DNA hypermethylation. Currently, it is assumed that TET2 mutations lead to loss-of-function in TET2. As a result, these mutations cause accumulation of 5hmC in DNA and result in hypermethylated DNA.⁵³ DNA hypermethylation inhibits cell differentiation. In vitro studies demonstrated that TET2 deficiency prevents the normal differentiation process of hematopoietic cells. In transgenic animal models using conditional knock-out systems increase in the monocytic cell line and a disease phenotype mimicking chronic myelomonocytic leukemia (CMML) were demonstrated. TET2 mutations include frameshift mutations due to insertions or deletions, splice-site mutations and base substitutions. These mutations occur more frequently at advanced age. In familial MPNs, TET2 mutations are almost always acquired. The incidence of TET2 mutations in familial MPNs are similar to the incidence in sporadic PMF. In clonal analysis studies held in patients with MPNs, no certain chronological line of events were observed between acquisition of TET2 and JAK2 mutations. TET2 mutations in MPNs may appear later at advanced disease stage. These two genetic events, TET2 and JAK2V617F mutations in PMF patients, seem to be independent of each other. TET2 mutations have not been shown to influence the frequency of thrombosis, leukemic transformation or overall survival (OS). It is considered that TET2 mutation status is not a new prognostic marker in PMF patients. In multivariable analyses in in PMF patients, no correlation was detected between the presence of TET2 mutation and anemia (hemoglobin<10 g/dl). The frequency of TET2 mutation was reported as 17% in PMF, 11% in ET and 7-16% in PV.⁵⁴

DNMT3a mutation

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298 DNA methyl transferase (DNMT) family consists of 3 genes: DNMT1, DNMT3A and DNMT3B. DNMT

299 gene family encodes the DNA methyl transferase-the enzyme that catalyzes the addition of a methyl group to the

300 cytosine remnants of CpG dinucleotides.⁵⁵ The frequency of DNMT3A mutations in AML was reported as nearly

301 20%.⁵⁵⁻⁵⁷ DNMT3A mutations are believed to be loss-of-function mutations. DNMT3A mutations are usually

302 expressed in heterozygous form and are predominantly located in the methyl transferase domain. They often present

303 as repetitive mutations of the R882 codon. DNMT3A mutations were reported to show poor prognosis in AML

304 patients.⁵⁵ In one study, DNMT3A mutations were detected in 3 of 62 (4.8%) patients with excessive blast refractory

305 anemia (RAEB) while in another study including MDS patients, its frequency was given as 8%.^{58, 59} DNMT3a

306 mutations are very rare in MPN. In study population of 135 MPN patients, only 2 (1.5%) were found to carry

307 DNMT3A mutations.⁶⁰ DNMT3A mutations were also investigated in PMF as well. Studies involving a larger

308 patient population are needed to better assess the clinical and prognostic significance of the mutation in MPNs.⁶¹

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310 ASXL1 mutation

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312 Additional Sex Combs-Like 1 (ASXL1) gene, the human homologue of 'Drosophila Additional sex combs'

313 (Asx), is located on chromosome 20q11.1. Asx deletion results in the dysregulation of the genes encoding the Pc-G

314 and Trithorax group (Tx-G) proteins.⁶² It is hypothesized that Asx influences the suppression and activation of

315 homeotic gene expression. Moreover, functional studies in Drosophila have demonstrated that Asx encodes chromatin

316 related protein similar to Pc-G proteins.⁶³ It is not clearly known how ASXL1 mutations promote myeloid

317 transformation. In vitro studies held on non-hematopoietic cells showed the physical interaction of ASXL1 with

318 heterochromatin protein 1a (HP1a) and lysine-specific demethylase 1 (LSD1) to suppress retinoic acid receptor

319 activity and with peroxysome proliferator activated receptor gamma to suppress lipogenesis.^{64, 65}

320 The three members of the human ASXL gene family (ASXL1, ASXL2 and ASXL3) consist of an amino-

321 terminal homologous region (ASXH domain) and a C-terminal plant homeodomain (PHD domain).^{64, 66} Analyses

322 regarding the conserved domain structure of mammalian ASXL proteins determined that N-terminal region (10-100

323 aminoacid) of ASXL1 may contain a specific DNA binding site, namely HARE-HTH region (HB1, ASXL1,

324 restriction endonuclease helix-turn helix domain).⁶⁷ The results of the aforementioned study indicates that the PHD

domain is specific for ASXL. It is proposed that PHD domain prefers the methylated lysine on the tail of the histone H3 over the lysine on the N-terminal tail of histone H3. Additional functional studies are required to understand the effects of ASXL1 domains. ASXL1 mutations on exon 12 are frameshift mutations that lead to truncated truncation of the protein-coding sequence.

Drosophila Asx forms a complex with the 'chromatin deubiquitinase Kalipso' that forms the Polycomb-repressor deubiquitinase complex (PR-DUB). PR-DUB complex monoubiquitinates nucleosomal histone H2A at lysine 119. The mammalian homologue of Kalipso, BAP1, interacts directly with ASXL1. In vitro deubiquitinase activity of the mammalian BAP-1 - ASXL1 complex has been demonstrated as well.⁶⁸

The exact role of ASXL1 function in hematopoiesis has not been clearly identified. In a mouse model, ASXL1 was shown to cause perinatal death. A small number of surviving mice were analyzed for an overt, short-latency hematopoietic phenotype. In the model of Fisher and colleagues, the sequence in the PGK promoter region expressing neomycin was transferred to the 5' UTR of the ASXL1 exon to disrupt the ASXL1 reading frame (this allele is named as *Asxl1tm1BC*).⁶⁶ As a result, the expression of proteins without nuclear contact sites were enabled in the vicinity of the PHD domain. There were no obvious hematological malignancies in the first months of life for mice that were. During follow up of the surviving mice, no overt hematological malignancies developed for the first few months of life in those homozygous for *Asxl1tm1BC/tm1BC* allele and there was no decrease in the number of multipotent progenitors. In the same study, homozygote *Asxl1tm1BC/tm1BC* allele was the cause of perinatal death in 75% of the cases. Moreover, embryonic lethality was observed in mice totally hybridized to C57BL/6J. In conclusion, the function of ASXL1 on hematopoiesis needs to be assessed using knockout models specific to postnatal and hematopoietic deletions.

Mutations in the epigenetic regulator region ASXL1 were identified in the same year TET2 mutations were discovered in myeloid cancers. Mutations in ASXL1 were determined by comparative genomic hybridization (aCGH) performed on MDS patients.⁶⁹ ASXL1 mutation was first described in MDS by Gelsi-Boyer V. et al, who identified a deletion on chromosome 20q11.⁶⁹ ASXL1 mutations were detected in 4 of the 35 (11%) MDS patients. Further sequence analysis of ASXL1 enabled the identification of the frequency of ASXL1 mutations in MPN and other myeloid diseases. ASXL1 mutations were reported at a frequency of 10%, 17% and 49% for MPN, AML and CMML, respectively.⁶⁹⁻⁷²

Contrary to the presence of detailed clinical studies assessing the impact of ASXL1 mutations on lifespan and response to therapy in AML and MDS, there have been few studies including limited number of patients as regards to ASXL1 in MPNs. Although ASXL1 mutations are seen in advanced ages in MPN, it is relatively more prevalent in PMF and post-PV/ET myelofibrosis compared to PV or ET.^{70, 73, 74} ASXL1 mutation was reported at a frequency of 2-5% in PV, 5-10% in ET, 13-26% in PMF and 22-38.5% in post-PV/ET myelofibrosis. A recent study documented that OS in PMF patients with ASXL1 mutations was significantly shorter than those without the mutation. However, this study had a limited number of subjects including only 9 ASXL1 mutation-positive patients and 35 ASXL1-negative patients.⁷⁵ The study by Vannucchi AM et al is the single study that assessed the impact of ASXL1 mutations in PMF in a relatively large study population.⁷⁶ Of the 279 PMF patients from the Mayo Clinic enrolled in this study, ASXL1 mutation was detected in 85 (31%) patients. Vannucchi AM et al. reported that ASXL1 mutations did not predict with leukemia free survival (LFS), but correlated with short OS. In the European cohort of the same study including 483 PMF patients, ASXL1 mutation frequency was reported as 21.7%. The presence of ASXL1 mutation in PMF patients of the European cohort showed correlation with short OS and leukemic transformation.⁷⁶ In one study that compared ASXL1 mutations in chronic and blastic phase MPN, it was reported that ASXL1 mutations were neither more frequent in blastic phase MPN nor this mutation resulted in an increase in the risk of leukemic transformation in chronic phase MPN.⁷³ Thus, it may be hypothesized that the ASXL1 mutation may play a critical role in the early phase of MPN development and the clonal evolution of MPNs.

IDH1/IDH2 mutations

Isocitrate dehydrogenase 1 (IDH1) and isocitrate dehydrogenase 2 (IDH2) are the two mutated oncogenes common in gliomas and secondary glioblastomas.⁷⁷ In the assessment of approximately 500 non-central nervous system solid tumor tissue samples, no IDH1 and IDH2 mutations were detected, which supported the notion that these genes are tissue specific oncogenes. Thus, discovery of repetitive IDH1 mutations in 16 of the 188 AML patients who had whole exome sequencing was unexpected.⁷⁸ IDH1 and IDH2 mutations were identified in AML patients, rarely in MDS patients and MPN with leukemic transformation.^{73, 79-82}

There are 3 main IDH isoenzymes in mammalian cells namely IDH1, IDH2 and IDH3.⁸³ IDH1 and IDH2 are nicotinamide adenine dinucleotide phosphatase (NADP⁺) dependent homodimeric enzymes that convert isocitrate to α -KG. IDH1 is found in the cytoplasm and peroxisomes and IDH2 in the mitochondria. The gene encoding IDH3 is yet to be discovered and the data regarding IDH3 mutations are inadequate. Identified IDH1 and IDH2 mutations are specific missense mutations involving conserved codons. Frameshift and nonsense mutations have not been identified and all mutations are heterozygote for the wild-type allele. It is proposed that the mutant protein inhibits the wild-type enzyme and that IDH mutations cause a dominant negative phenotype. Also, it is considered that the mutant protein acts differently from the wild type and leads to a gain of function. High levels of 2-hydroxyglutarate (2-HG) were demonstrated in cells expressing the mutant IDH1.⁸⁴ 2-HG is also present in primary leukemia cells that carry IDH1 or IDH2 mutations.⁸⁵ Mutant IDH enzymes alter the specificity of the substrate. Mutant IDH catalyzes the conversion of α -KG to 2-HG and depletes NADPH instead of producing NADPH molecule by conversion of isocitrate to α -KG. It is not yet known whether 2-HG has a direct oncogenic effect or decrease in NADPH and α -KG correlates with leukemogenesis.⁸⁶

IDH1 is located on chromosome 2q33.3 and IDH2 is on chromosome 15q26.1. IDH mutations are on exon 4 and involve a change of a single amino acid in the arginine residue. These mutations affect 3 specific arginine residues: R132 (IDH1), R172 (IDH2) and R140 (IDH2).⁷⁹ It is known that mutant IDH has decreased affinity for isocitrate and possesses catalytic activity to convert α -KG to 2-HG.^{85, 87-89} The oncogenic features of mutant IDH are attributed to the decrease in α -KG or the accumulation of 2-HG.^{87, 90} IDH mutations were identified in a rate of 70% in low grade gliomas and secondary glioblastomas, 10-20% in AML, 3-5% in MDS, 1-4% in MPN, 9% in MDS/MPN that includes CMML, 15% in AML transformed from MDS, 22% in AML transformed from MPN, 10% in AML transformed from MPN/MDS, 22% in high risk MDS or AML associated with del(5q) and 4% in blastic phase chronic myeloid leukemia (CML).^{78, 79, 81, 82, 91-96} Most studies regarding the phenotypic and prognostic implications of IDH1 and IDH2 mutations in AML reported a consistent relationship between NPM1 mutations and normal or intermediate risk karyotype.^{92, 97-100} Mutant IDH1 was identified as a poor prognostic marker in NPM1⁺/FLT3⁻ patients with normal karyotype where as it was associated with good prognosis in FLT3⁺ AML.^{80, 93, 100, 101} Some studies reported mutant IDH2 as a poor prognostic factor in normal karyotype AML. In another study, the prognosis in patients harboring mutant R140 (IDH2) was found to be more favorable than those carrying mutant R172 (IDH2).^{93, 102, 103} In comparison to AML, data regarding the prognostic importance of IDH mutations in

chronic myeloid neoplasias involving MDS and MPN, are limited.^{79, 81} In one such study examining the frequency of IDH1 and IDH2 mutation in 1473 patients with Ph-negative MPN patients, the frequency of IDH mutations were reported as 0.8% for ET, 1.9% for PV, 4.1% for PMF, 1% for post-ET/PV myelofibrosis, 25% for blastic phase PV, 25% for blastic phase PMF and none for blastic phase ET.⁷⁹ Since this study included only 111 chronic phase PMF with complete clinical data available, detailed prognostic analysis could not be performed. In another study examining the impact of IDH1 and IDH2 mutations on phenotypic and prognosis in 301 chronic phase PMF patients, IDH mutation was detected in 12 patients (4%). 7 patients carried IDH2 mutations (5 R140Q, 1 R140W and 1 R172G) and 5 carried IDH1 mutations (3 R132S and 2 R132C). 6 of these 12 patients (50%) harbored JAK2V617F mutations, as well. These findings indicate that IDH mutations are independent risk factors for leukemic transformation in PMF and that increase the leukemogenic risk in the copresence of JAK2V617F mutation.¹⁰⁴ The last comprehensive study which focused on the correlation of IDH mutations with prognosis in PMF was by Vannucchi AM et al.⁷⁶ In this study which enrolled 374 PMF patients from the Mayo Clinics, IDH1 mutations were identified in 10 (3%) patients and IDH2 mutations in 7 (2%). IDH mutations showed correlation with OS and leukemic transformation.⁷⁶ In the same study which included the 483 PMF patients from the European Cohort, the IDH1/IDH2 mutation was reported as 2.6%. Another finding in this study was that IDH1/IDH2 mutations had no impact on OS yet but increased the risk of leukemic transformation.⁷⁶ It is known that IDH mutations may accompany by JAK2V617F, TET2 and MPL mutations. However, whether or not different IDH mutational variants portend different biological and prognostic features in PMF is not yet known.

Somatic CALR mutations

Calreticulin (CALR) is a multifunctional protein best known for its role as a Ca²⁺ binding chaperone in the endoplasmic reticulum (ER) lumen.¹⁰⁵ The CALR-1 gene is located on human chromosome 19 and mouse chromosome 8, and contains nine exons which are highly similar between human and mouse at a sequence level. Mature CALR is a 46-kDa protein composed of three structurally and functionally distinct domains. The globular N-terminal domain is lectin binding and consists of a signal sequence targetted to the ER. The middle proline-rich or P-domain contains high affinity, low capacity, binding sites for Ca²⁺, and the highly acidic C-terminal domain contains a number of high capacity, low-affinity Ca²⁺ binding sites. The acidic C-terminal domain is involved in cellular

calcium homeostasis—cells that are deficient in CALR have diminished calcium storage capacity in the ER and overexpression of CALR augments ER calcium uptake. The C-domain terminates in a KDEL sequence; KDEL receptors function in the retrieval of KDEL-containing proteins from the cis-Golgi back to the ER.^{106, 107}

The discovery of mutations in the gene CALR was reported independently by two distinct groups.^{106, 107} CALR mutations are all located in exon 9. The two most prevalent mutations accounting for about 80% of cases are del52 (CALRdel52/Type I;c.1092_1143del; L367fs*46) and ins5 (CALRins5/Type II;c.1154_1155insTTGTC; K385fs*47).¹⁰⁸ The CALR mutations were reported to be present in about 15-25% of patients with ET, PMF and post-ET myelofibrosis, who are negative for the JAK2V617F and MPL exon 10 mutations.^{106, 107} Thus, CALR mutations represent an exclusion criterion of PV, that is JAK2 mutated by definition. The screening of other myeloid disorders revealed CALR mutations in 8% of MDS. CALR mutations have not been identified in healthy controls, AML, CML, lymphoproliferative disorders and a variety of solid tumors.^{106, 107}

In summary, CALR mutations occur in the majority of JAK2V617F- and MPL-negative ET and PMF patients. Therefore, CALR genotyping is proposed as a first line laboratory test in patients with a suspicion of MPN. Finally, identification of CALR mutations was suggested as a novel major criteria for the upcoming revision of 2008 WHO diagnostic criteria for ET and PMF.^{109, 110} Its importance for prognosis remains to be addressed in future studies.

Conclusions

In MPNs, somatic lesions have an impact on a wide variety of cellular functions, from cytokine signaling to histone modifications. JAK2 and MPL mutations are now regarded as highly specific gene mutations for MPN and were demonstrated to develop myeloproliferative phenotype in animals. Most other MPN-related genes are expressed in other myeloid malignancies suggesting that these genes are not specific to MPN. Further studies with mouse models expressing single or combinations of mutations will reveal how these mutations target hematopoietic stem cells and their effect on MPN development. Taking into account the clonal evolution of the disease, it is evident that MPN has a very complex genotypical and phenotypical structure. Individualized diagnostic and therapeutic approaches are warranted to successfully treat this complex disease.

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